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Bioremediation of Petroleum Sludge through Phytoremediation, Land Farming and Microbial Enhanced Oil Separation

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Joseph P J

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DECLARATION

I hereby declare that the thesis entitled "Bioremediation of petroleum sludge through Phytoremediation, Land farming and Microbial enhanced oil separation" is the bonafide report of the original work carried out by me under the guidance of Dr. Ammini Joseph, Professor, School of Environmental Studies, Cochin University of Science and Technology, and no part thereof has been included in any other thesis submitted previously for the award of any degree.

Cochin -22
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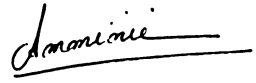
CERTIFICATE

This is to certify that the research work presented in the thesis entitled "Bioremediation of petroleum sludge through Phytoremediation, Land farming and Microbial enhanced oil separation" is an authentic record of research work carried out by Mr. Joseph P J under my guidance and supervision in the School of Environmental studies, Cochin University of Science and Technology in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in Environmental Technology and that no part thereof has been included for the award of any other degree.



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PREFACE

Crude oil is an important energy source as well as feed stock of oil refineries. During the processing of crude oil, various kinds of waste are generated; of this, oily sludge, chemical sludge and bio sludge are of special environmental concern because many of the constituents of this sludge are of hazardous nature. Among these sludges, oily sludge is generated in much higher quantity compared to other sludges. It is estimated that more that 20,000 tons of oily sludge is being generated annually in India.

Uncontrolled disposal practices of this oily sludge cause serious environmental degradation as well as depreciation of aesthetic quality. Oily sludge from the crude oil tank and the dried sludges from treatment lagoons are often disposed off in low-lying areas. Sludge treatment facility is available in a few refineries.

The objective of this research is to study the feasibility of bioremediating the oily sludge from a refinery site. Three different methods of waste treatment were tried i.e. phytoremediation, land farming and microbial enhanced oil separation in laboratory scale treatment systems. A multi-process approach by combination of phytoremediation, biostimulation and microbial enhanced oil separation is also presented. The methods of analysis, experimental procedure, and results are incorporated into five chapters of this thesis entitled "Bioremediation of petroleum sludge through phytoremediation, land farming and microbial enhanced oil separation".

CONTENTS

Abbreviations

ANOVA	analysis of variance
API	American Petroleum Institute
ASTM	American Society for Testing and Materials
BTEX	benzene, toluene, ethylbenzene, xylenes
CEC	cation exchange capacity
CFU	colony- forming unit
CHNS	carbon, hydrogen, nitrogen, sulfur.
CNP	carbon, nitrogen, phosphorus
EPA	Environment Protection Agency
ETP	effluent treatment plant
FID	flame ionization detector
GC	gas chromatography
LSD	least significant difference
MEOS	microbial enhanced oil separation
MPN	most probable number
MS	mass spectrometer
m/z	mass/charge ratio
NSO	nitrogen sulfur oxygen
PAH	polyaromatic hydrocarbon
PHCs	petroleum hydrocarbons
ppm	parts per million
RCRA	resource conservation and recovery act
rpm	revolutions per minute
SD	standard deviation
SGI	speed germination index
TPH	total petroleum hydrocarbon
TPI	tilted plate interceptor
VI	vigour index

Chapter 1

GENERAL INTRODUCTION

Abstract

Hazardous waste is generated in significant amount in refineries world wide. In India, oil refineries generate approximately 20,000 tonnes of oily sludge (a mixture of hazardous hydrocarbon waste) per annum. One of the major problems faced by oil refineries is the safe disposal of this oily sludge. Uncontrolled handling of these sludges often leads to environmental pollution and also affects the aesthetic quality. Recent legislation desires environment friendly sludge management system in the industries. Recycling of sludge in an environment friendly manner is one of the appropriate solutions of sludge management problem. When sludge cannot be recycled or incinerated, the only option left is secure landfilling. The treatment technologies developed can be grouped as physical remediation, chemical remediation and biological remediation.

The objective of this research is to study the feasibility of bioremediating the oily sludge from a refinery site. The strategy adopted is a multiple approach of phytoremediation, land farming and microbial enhanced oil separation in laboratory scale treatment systems.

1.1 Definition and characteristics of hazardous waste

The generation of solid and hazardous waste is increasing day by day with the rapid development of industrial growth worldwide. Hazardous wastes have been variously defined in different countries.

The Hazardous Wastes (Management and Handling Act) 1989 of Government of India categorizes waste oil and emulsions including tank bottom from petroleum refinery industry, slop oil emulsion solid from refinery, and waste water ETP sludges within its purview of hazardous wastes.

According to US Environment Protection Agency (EPA), a waste is considered to be hazardous if it:

1. Exhibits characteristics of ignitability, corrosivity, reactivity and/or toxicity.
2. Is a non specific source waste (generic waste from industrial processes)
3. Is a specific commercial protector intermediate.
4. Is a mixture containing a listed hazardous waste
5. Is a substance that is not excluded from regulation under RCRA, Subtitle C-Hazardous waste management (Wentz, 1989).

According to La Grega (1994) there are four main characteristics for hazardous wastes i.e. ignitability, corrosivity, reactivity, and extraction potential toxicity.

Ignitability

Ignitable wastes are liquids with a flashpoint below 600°C, or solids capable of causing fire under standard temperature and pressure.

Corrosivity

Corrosive wastes are aqueous wastes with a pH below 2 or above 12.5, or which corrode steel at a rate in excess of 0.25 inches per year.

Reactivity

Reactive wastes are normally unstable, react violently with air or water, or form potentially explosive mixture with water. This category also includes waste that emits toxic fumes when mixed with water and material capable of detonation.

Toxicity

The objective of this parameter is to determine whether toxic constituent in a solid waste sample will leach into ground water, if the waste is placed in a municipal solid waste landfill. If this is the case, then the waste will be declared hazardous.

According to the Hazardous Wastes (Management and Handling Act) 1989 of Government of India, hazardous wastes are characterised into eighteen categories (Trivedy, 2004) as given below

Category One : Cyanide waste
Category Two : Metal Finishing wastes
Category Three : Bearing Heavy Metal Salts
Category Four : Bearing Mercury, Arsenic, Cadmium
Category Five : Non-Halogenated Hydrocarbons
Category Six Halogenated Hydrocarbons.
Category Seven: Paint, Glue Industry
Category eight : Waste from Dyes and Dye intermediates containing inorganic chemical compounds.
Category nine : Waste from Dyes and Dye intermediates containing organic chemical compounds
Category Ten : Waste Oil & Oil Emulsions

- Tank bottom from petroleum refining industry
- Slop oil emulsion solid from refinery

Category Eleven	:	Tarry Waste
Category Twelve	:	Waste Water ETP Sludges
Category Thirteen	:	Phenols
Category fourteen	:	Asbestos
Category Fifteen	:	Pesticides
Category Sixteen		Acid/Alkali Slurry
Category Seventeen	:	Off-Specification and Discarded
Category Eighteen	:	Discarded containers and container liners of hazardous and toxic waste

1.2 Management of hazardous waste

The potential damage for public health and to the environment from the mismanagement of hazardous waste justifies the need for implementation of effective hazardous waste management programme (Dawson *et al.,* 1986, Freeman *et al.,* 1988).

A hazardous waste management programme includes:-

- *Minimisation of waste*

  The first step towards waste minimisation is inventory management and second step is equipment modification.

- *Waste recycling*

  Recycling is an important step in evolving cleaner approaches to chemical processing. Adopting recycling techniques would serve to increase productivity by proper utilization of feed components, avoidance of emission, unitisation of process heat for preheating, optimisation of operating parameters and re-utilisation of costlier catalysts and solvents.

- *Treatment of hazardous waste*

  Waste may be made less hazardous by physical, chemical, or biological treatment. Treatment of hazardous waste can serve to prepare the material for recycling or for ultimate disposal in a manner safer than disposal without treatment.

- *Disposal of hazardous waste*

  Landfilling: A landfill is defined as that system designed and constructed to contain discarded waste so as to minimize release of contaminants to the environment. Landfills are necessary because hazardous waste minimization technologies cannot totally eliminate the waste generated, and treatment technologies produce residue.

  Incineration: It is used for complete destruction of the contaminants. Incineration is one of the most effective treatments available and usually adopted for those wastes that cannot be recycled, reused, or safely deposited in a land fill site. It destroys organic chemicals by converting them to carbon dioxide, water and other gases that are removed by scrubbers.

  Deep well injection: It is a process by which waste fluids are injected deep below the surface of the earth. Only certain kind of geologic formation can be used for disposal by deep well injection. The formation must be deep, porous, enough to provide storage space and sandwiched between impermeable layers of rock.

Hazardous waste management in India is governed by the following two acts

1. Hazardous Wastes (Management and Handling Act) 1989.
2. Manufacture, Storage and Import of Hazardous Chemicals Rules, 1989.

Based on further suggestions received and considering the various new methodologies, Government of India has notified the new amendments as the HW (M&H) rules, 2000 and suggested modification in Schedule -1 with list of process generating hazardous wastes and Schedule-2 with list of waste substances with concentration limits.

1.3 Treatment of hazardous waste

Growing public awareness and concern about environmental degradation has resulted in evolving various treatment technologies which can serve to prepare the material for recycling, or for ultimate disposal in a manner safer than disposal without treatment. The treatment technologies developed can be grouped as physical remediation, chemical remediation and biological remediation

Physical remediation

Physical treatment methods are conducted in order to reduce the volume of the wastes and facilitate the solid-liquid separation. Several physical processes including sedimentation, clarification, centrifugation, flotation, filtration, evaporation, distillation, reverse osmosis etc. are used in hazardous waste management. The various physical treatment technologies available for different applications are carbon adsorption, air stripping, filtration, centrifuging, distillation, evaporation, solidification and encapsulation. (Riser-Roberts, 1998).

Chemical remediation.

Chemical treatment methods either destroy contaminants or convert them to different, less toxic form. Various chemical treatment technologies available are hydrolysis, neutralization, oxidation/reduction, precipitation, fixation, ion exchange and coal agglomeration.

Biological remediation.

Bioremediation, is the biological process transformation or mineralization of organic compounds introduced into the environment to less toxic or innocuous forms(Hazen, 1997, Brigmon *et al.*,2002). Bioremediation describes several technologies and practices that take advantage of natural systems and processes to clean up pollution.

Bioremediation technologies can be broadly classified as *ex-situ* or *in-situ* (Iwamoto, 2001). Ex-situ technologies are those treatment modalities which involve the physical removal of the contaminant material to another area for treatment. Bioreactors, landfarming, composting, and some forms of solid–phase treatment are all examples of *ex-situ* treatment techniques. In contrast, *in-situ* techniques involve treatment of the contaminated material in place. Bioventing for the treatment of contaminated soils, and biostimulation of indigenous aquifer microorganism are examples of these treatment techniques. If biological treatment of a hazardous waste is contemplated, care is required to ensure that the other components in the waste neither poisons the organism nor render the residue unfit for landfill disposal. The different types of bioremediation practices are biostimulation, bioaugmentation, intrinsic treatment and phytoremediation.

Biostimulation

Biostimulation aims at enhancing the activities of indigenous microorganisms that are capable of degrading the offending contaminant. It is applicable to oil contaminated sites, an extension of the natural remediation of soil. In many cases the additions of inorganic nutrient act as a fertilizer to stimulate biodegradation by autochthonous microorganism (Atlas and Philp, 2005).

Bioaugmentation

Bioaugmentation involves the inoculation of contaminated soil or water with specific strains or consortia of microorganism to improve the biodegradation capacity of the system for a specific pollutant organic compound. Bioaugmentation often is considered for bioremediation of compounds that appear to be recalcitrant i.e., contaminants that persist in the environment and appear to be resistant to microbial degradation. (Atlas and Philp, 2005).

Intrinsic treatment

The lack of intervention to the bioremediation is considered intrinsic bioremediation or natural attenuation. (Hart, 1996) Intrinsic remediation results from several natural processes, such as biodegradation, abiotic transformation, mechanical dispersion, sorption, and dilution that reduce contaminant concentrations in the environment. (Morin, 1997).

Phytoremediation

Phytoremediation may be defined as the use of plants to remove, destroy or sequester hazardous substances from the environment. The method may offer some solution for dealing with mixed wastes. Phytoremediation technologies exploit various biochemical processes in the rhizosphere including extraction, immobilization, and degradation of contaminants (Glick, 2003). The diverse processes in phytoremediation include phytodegradation, phytoextraction, phytostabilization, phytovolatilization and rhizofiltration. The area adjacent to a plant root, referred to as the rhizosphere, is a continuum extending from the root surface with maximum microbial activity as compared to the bulk soil, which has far less activity. The rhizosphere has nutrients and water exuded from the plant roots, resulting in enhanced microbial activity (Walton and Anderson, 1990; Hou *et al.*, 2001; Hutchinson *et al.*, 2001). The organic substrate produced from the decay of dead root hairs serves as an

important carbon source for rhizosphere microorganisms that have the potential to degrade organic pollutants (Heinonsalo *et al.*, 2000).

1.4 Management of petroleum refinery wastes

The petroleum industry is a major contributor of hazardous materials releasing petroleum hydrocarbons to the environment in a number of ways. Severe subsurface pollution of oils and water can occur via the leakage of underground storage tanks and pipelines, spills at production wells and distribution terminals, and seepage from gasworks sites during coke production. Seepage of gasoline from underground storage tanks has caused widespread soil and aquifer contamination, threatening the safety of the various potable water supplies. The complex and diverse range of petroleum–derived organic compounds released form spillages is of major environmental concern. These consists of aliphatic, BTEX, and PAHs. BTEX and PAHs are of major concern because of their toxicity and carcinogenicity. (Atlas and Philp, 2005).

Oily sludge is generated in significant amount in the refineries during crude oil processing. Crude oil is usually stored in storage tanks. Impurities present in the oil are deposited at bottom of the tank. During cleaning of the tank, the sludge is recovered, and is treated as waste. Oily sludge is also generated from the treatment plant of oily waste water. The sources of oily sludge are API separator and TPI unit (Bhattacharya and Shekdar, 2003).

One of the major problems faced by oil refineries is the safe disposal of this oily sludge. Many of the constituents of the sludge are carcinogenic and potent immunotoxicants (Propst *et al.*, 1999). Improper disposal of this leads to environmental pollution, particularly soil contamination, and posses serious threat to ground water (Chakradhar, 2002). Sludge characteristic differ from product to product depending upon raw material used and manufacturing process involved. Oily sludge which is generated in massive quantity from refineries, is highly viscous in

consistency. Sludge contains sufficient amount of grease and waxy material. The calorific value for the sludge is also high (4000-6000cal/g). It has been observed that heavy metals like chromium, cadmium, copper, nickel, lead, zinc etc. are commonly present in majority of the oil sludge. (Roberts, 1998).

Bioremediation of petroleum in contaminated soil using indigenous microorganism has proven effective (Fiorenza *et al.*, 2000); however the biodegradation rate of more recalcitrant and potentially toxic petroleum contaminants, such a polycyclic aromatic hydrocarbons (PAHs), is rapid at first but declines quickly. Biodegradation of such compounds is limited by their strong adsorption potential and low solubility. Vegetation may play an important role in the biodegradation of complex organic chemicals in soil. For petroleum compounds, the presence of rhizosphere microflora may accelerate biodegradation of the contaminants (Fiorenza *et al.*, 2000). Current research on land farming using oily sludge is expected to open a pathway for better management of oily sludge. Land farming involves the decomposition of oily sludge by microbial action in cultivated soil. The limitation of the method is the probable soil and groundwater contamination due to migration of leachates (Huddleston *et al.*, 1986).

1.5 Scope and objectives of the present study

In India, oil refineries generate approximately 20,000 tonnes of oily sludge (a mixture of hazardous hydrocarbon waste) per annum (Bhattacharyya and Shekdar, 2002). This waste residue is dumped into specially constructed sludge pit, consisting of a leachate collection system and polymer lining system to prevent the percolation of contaminants into ground water (Bhattacharyya and Shekdar, 2003). However these pits face the draw backs of being rather expensive to construct and maintain, and increasingly more and more land is required for this purpose.

The objective of this research is to study the feasibility of bioremediating the oily sludge from a refinery site. The strategy adopted is a multiple approach of phytoremediation, land farming, and microbial enhanced oil separation in laboratory scale treatment systems.

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Chapter 2

PHYTOREMEDIATION OF OIL REFINERY SLUDGE

Abstract

Phytoremediation is an attractive treatment technology for removing contaminants from the environment due to its cost effectiveness and public acceptance. Plants can be used for pollutant stabilization, extraction, degradation, or volatilization. The goal of this study was to evaluate the phytoremediation potential of paddy varieties to remediate an oily sludge generated by a local refinery and to optimize the nutrient and sludge concentration for phytoremediation; also to evaluate the relationship between plant growth and reduction in petroleum hydrocarbon and accumulation of metals. Analysis of physical and chemical properties of sludge were carried out. As the plants did not grow in the raw sludge, sand was added at various proportions. The plant growth was monitored as change in biomass, number of leaves and height of plants till harvest. The grain yield and % sterility was computed. The reduction in TPH was measured. A maximum of 51.4% removal of total petroleum hydrocarbons of oily sludge have been achieved within 90 days. The degradation rate is saturate > aromatic> NSO> asphaltenes. Qualitative variation of PHCs following phytoremediation was elucidated through GC-FID and GC-MS analysis. The accumulation of metals was computed as accumulation factor. The highest accumulation factor of 0.65 was observed for aluminium. Germination test revealed significant drop in percent germination at >10% sludge in the sludge–soil substratum. Similarly seed sterility also was observed to increase significantly at >10% sludge level. So it is assumed that phytoremediation can be used effectively only for soils of low PHC contamination. Among the paddy varieties tested, Pokkali variety proved to be superior to others in effecting phytoremediation.

2.1 Introduction

Phytoremediation is an emerging technology that uses plants to clean up pollutants in soils. This is most appropriate for large areas of low and moderately contaminated soils where the application of conventional remediation technologies would be prohibitively expensive (Salt *et al.*, 1998).

The obvious advantages of remediating contaminated soils with vegetation are: 1) the process is solar-energy driven, requiring little or no inputs; 2) a high potential for public acceptance, having minimum disturbance of the soil surface; and 3) avoidance of the need to transfer contaminants from one phase to another (Cunningham *et al.*, 1996). Investigations on the influence of different plant varieties on phytoremediation are rare. A limited number of studies have directly compared different plant species for their potential to enhance bioremediation (Shann and Boyle, 1994; Schwab and Banks, 1994; Adam and Duncan, 1999). The use of plants was found to improve bioremediation efficiency for both herbicides (Anhalt *et al.*, 2000; Coleman *et al.*, 2002) and PAHs (Banks *et al.*, 1999; Olson *et al.*, 2003). Still phytoremediation is not widely applied. There is little regulatory experience with phytoremediation and it has to be considered on a site by site basis. Further more, the intrinsic characteristics of phytoremediation limit its application (Pilon-Smits, 2005).

Some of the limitations are

- It is generally slower than most other treatments and is climate dependent.
- In most cases, the contamination to be treated must be shallow.
- It usually requires nutrient addition and mass transfer is limited.
- High metal and other contaminant concentrations can be toxic to some plants

- Access to the site must be controlled as contaminants being treated by phytoremediation may be transferred across media (i.e. may enter groundwater or bioaccumulate in animals).
- For mixed contaminant site (i.e. organic and inorganic) more than one phytoremediation method may be required.
- The site must be large enough to utilize agricultural machinery for planting and harvesting.

Although phytoremediation as 'clean up technique' is not yet widely applied, momentum for its use is expected to build; particularly in application niches where other technologies are less suitable or do not exist. It could also be combined application of bioremediation and phytoremediation.

The use of plants for remediation may be especially suited for soils contaminated by organic chemicals to depths of less than 2m (Bell, 1992).Plants can interact with hazardous organic compounds through degradation or accumulation (Finlayson and MacCarthy, 1973). Uptake of the contaminant by the root is a direct function of the pollutant concentration in the soil solution and usually involves chemical partitioning on the root surfaces followed by movement across the cortex to the plants vascular system (Fiorenza *et al.*, 2000). The contaminant may be bound or metabolized at any point during transport. Contaminants may be found in plants as freely extractable residue, extractable conjugate bound to plant material and unextractable residues incorporated in plant tissue (Bell and Failey, 1991). Within a plant, the contaminant may be adsorbed on a cell surface or accumulated in the cell. Many contaminants become bound on the root surface and are not translocated (Bell, 1992).

Plants may indirectly contribute to the dissipation of contaminants in vegetated soil. Soil adjacent to the root contains increased microbial numbers and populations (Paul and Clark, 1989). An extensive root system could increase the plant–microbe association and encourage contaminant degradation (Aprill and Sims, 1990). Many

plants establish a synergistic relationship between their roots and specialized soil fungi (mycorrhizae) for the exchange of nutrients and water. Sometimes this relationship is essential for plant growth, but it may also promote degradation of contaminants. Root debris and sloughed hyphae will increase soil organic matter and distribute microorganisms for maximum contact with contaminants (Heinonsalo *et al.*, 2000;Banks *et al.*, 2004). Plants are generally incapable of assimilating highly adsorbed contaminants such as polycyclic aromatic hydrocarbons (Anderson *et al.*, 1994; Pichtel and Liskanen, 2001). As a result, the greatest research emphasis for phytoremediation of petroleum contaminants has been placed on microbial degradation because of environmental limitations of contaminant transport, and the physiological diversity of the relevant rhizosphere microorganisms.

Several reported studies have evaluated the effect of plants and the associated rhizosphere on the fate of petroleum contaminants (Aprill and Sims, 1990; Schwab and Banks, 1994; Reilley *et al.*, 1996). For the most part, the presence of plants enhanced the dissipation of the contaminants. In the studies using ^{14}C labeled contaminants in closed plant chambers, mineralization was greater in rhizosphere soils than in unvegetated soil, indicating that the bioavailability of the contaminant was increased in the rhizosphere (Ferro *et al.*, 1994).

In phytoremediation the degradation of contaminants is effected with the aid of five processes: phytotransformation, rhizosphere bioremediation, phytostabilization, rhizofiltration and phytoextraction (Salt *et al.*, 1995).

➢ *Phytotransformation*:

Phytotransformation is essentially the absorption and transformation of organic contaminants and nutrients. The contaminants can be degraded to nontoxic or less toxic compounds. Generally, complete mineralization does not take place. The

metabolites accumulate in the plants. The absorption is essentially limited to hydrophilic and moderately hydrophobic organic chemicals.

➢ *Rhizosphere bioremediation*

Rhizosphere bioremediation involves the installation of appropriate plants in areas in which near-surface bioremediation is being conducted. Organic contaminants, which are easily bioavailable and microbially metabolizable, are degraded in the plant root. The plants assist the microbial decontamination in the rhizosphere in different ways.

- Fungi and bacteria that are associated with plant roots metabolize the organic contaminants.
- Plant exudates stimulate the bacterial transformation(enzyme induction)
- Plant improves the conditions of microbial populations and their activities.
- Oxygen is released actively and passively to the rhizosphere by the plants and promotes aerobic transformation.

➢ *Phytostabilization*

Phytostabilization is used to absorb and precipitate contaminants, generally metal, with the aid of certain plants, reducing their bioavailability and so reducing the potential for human exposure to these contaminants. The processes prevent migration of contaminants through erosion and reduce the contamination of ground water. The plants that are used for phytostabilization are characterized by high tolerance for heavy metals. They must possess a large root biomass and the capacity to immobilize heavy metal contaminants through absorption, precipitation, or reduction.

➢ *Rhizofiltration.*

Rhizofiltration refers to the use of plant roots to sorb, concentrate, and precipitate metal contaminants from surface or ground waters (Dushenkov *et al.*, 1995). These waters can be treated in natural, shallow lagoons or constructed wetlands. In addition to the removal of heavy metals, the use of this system for the reduction of organic contaminants through sorption in the roots as well as its possible application for the remediation of surface water that is contaminated by radionuclides have been validated (Dushenkov, 2003). It has also been used for the purification of acid mine water that is severely contaminated with heavy metals (EPA, 2000).

➢ *Phytoextraction*

Phytoextraction is to be considered in close connection with the aforementioned applications of phytostabilization and rhizofiltration. Phytoextraction refers to the use of metal accumulating plants that translocate and concentrate metals from the soil in their roots (Kumar *et al.*, 1995). It has also been proposed for the extraction of radionuclides from sites with mixed wastes. Plants generally employed for phytoremediation have the ability to accumulate and tolerate high concentrations of metals in harvestable tissue, rapid growth rate, and high biomass production.

In the present investigation, a series of experiments were conducted to phytoremediate the oil sludge of a petroleum refinery. The objectives were

1. to evaluate the phytoremediation potential of paddy varieties.
2. to optimize the nutrient and sludge concentration for phytoremediation
3. to evaluate the relationship between plant growth and reduction in petroleum hydrocarbons and accumulation of metals.

2.2 Materials and methods

2.2.1 Characterization of sludge.

The petroleum sludge used in this study was generated during the refining processes at Kochi Oil Refinery, India. The source of the material are crude tank bottom sludge, product tank bottom sludge, American Petroleum Institute (API) separator unit and Tilted Plate Interceptor (TPI) unit of effluent treatment plants. The sludge was collected from the disposal site of the factory. Random samples were collected over a period of six months. A composite of each collection was air dried to a moisture content of ≈10% and stored at 4°C in sealed glass containers.

➢ ***Sludge analysis***

The sludge was analysed using standard procedure (Table 2.1).

Table 2.1 Methods used to characterize the sludge

Sl. No	Property	Method/instrument	Reference
1	Carbon, Nitrogen, Sulphur	CHNS analyzer	
2	Calorific value	Bomb calorimeter	ASTM method D 3286-17 (1977)
3	Total phosphorus	Spectrophotometry	Radojevic and Bashkin (1999)
4.	Total petroleum Hydrocarbon (TPH)	Gravimetric	EPA SW 846 Method 3540 C (2003)
5	Total petroleum Hydrocarbon	Gas chromatography (GC-FID),(GC- MS)	EPA SW 846 Method 8260B (2003)
6	Metals (K, Zn, Mg, Ca, Fe, Cu, Mn, Na, Ni, V, Pb, Li, Al, As, Cd, Cr, Cu, Se, Sn, Tl, Hg)	ICP-AES	EPA Method SW 846 3031 (2003)
7	Ash content	Muffle furnace	ASTM-IP method D482-80 (1990)
8	pH determination	pH meter	EPA method SW 846 9045 (1992)

a) **Carbon, Nitrogen and Sulphur estimation.**

Carbon , Nitrogen, and Sulfur content of the sludge was determined using CHNS Analyzer (Model EL III CHNS analyzer).The determination in CHNS is based on isotope ratio mass spectrometry (IRMS). Quantitative combustion is carried out by oxygen jet injection directly at the sample. Exactly 5 mg of the sludge sample was fed to the digestion chamber of the instrument. The gases pre-separated in the elementar analyzer were injected into mass spectrometer by continuous flow procedure. Digestion temperature was kept at 950 ° C. Injection of reference gases was also performed automatically.

b) **Estimation of calorific value**

Calorific value of sludge was determined by ASTM method D 3286-17(1977) using a bomb calorimeter (model LECO AC-350). Heat of combustion was determined in this method by burning a known weight of the sample in an oxygen bomb calorimeter under controlled conditions. The heat of combustion compared from the temperature observation, before, during, and after combustion was calculated with proper allowance for thermo chemical and heat transfer corrections. Standardization was done using benzoic acid.

c) **Total Phosphorus**

Total Phosphorus was determined by Spectrophotometry. Exactly 1.0 g portions of a sludge sample was weighed and transferred to a porcelain crucible and placed in the muffle furnace; ignited at 550°C for one hour and ash was transferred into a100ml polypropylene bottle. Phosphorous was extracted into 0.5M H_2SO_4 by shaking in a rotary shaker for 16 hours. The extract was filtered. A 10 ml of aliquot of the extract was transferred into a 50ml volumetric flask. Five drops of 0.25% nitrocresol was added and neutralized. Diluted the sample just under 40ml and added 8ml of color

developing reagent; made up to the mark, and determined the concentration by comparing with the standard.

d) **Total Petroleum Hydrocarbon (TPH)**

- ***As Gravimetric (TPH) by EPA Method 3540 C***

  Sludge samples were consecutively soxhlet- extracted with n-hexane, dichloro methane and chloroform (100ml each). The sample was mixed with anhydrous sodium sulphate prior to extraction and quantitatively transferred to extraction thimble. All the three extracts were pooled and evaporated in a rotary vacuum evaporator to about 2 ml. The distilling head was removed, and dried in vacuum, cooled, and weighed. The concentration of TPH in the original sample was calculated as.

  TPH (mg/kg dry weight) = (Gain weight of the flask (mg)/weight of solid (g))*1000

- ***Fractionation of petroleum hydrocarbons***

  After gravimetric quantification, the residual TPH was fractionated into alkane, aromatic, asphaltenes and NSO fractions on a silica gel column (Mishra *et al.*, 2001). The TPH (300mg) was dissolved in n-pentane and separated into soluble and insoluble fractions (Asphaltenes). The weight of asphaltenes was determined gravimetrically. The soluble fraction was loaded on a silica gel (activated at 110°C) column. The alkane fraction was eluted with 100 ml of hexane, aromatic fraction was eluted with 100 ml benzene, and finally NSO fraction was eluted with methanol and chloroform (100 ml each).The methanol and chloroform fractions were combined, evaporated and weighed to get the weight of NSO compounds.

➢ ***Analysis of hydrocarbon constituents by GC-FID***

The hexane and benzene fractions were fed into Varian 3800 gas chromatograph equipped with an FID, split injector(Split ratio was 100:1) and an open tabular column 100 m X 0.25mm ID, fused silica coated with 0.5 micron bonded methyl silicone (Petrocol(TM)DH). Helium carrier gas linear flow was 48cm/s. Injector temperatures was 300 °C and FID temperature was 300 °C, Hydrogen fuel was used at the rate of 29-30 cc/min and zero air @ 300 cc/min. The column oven temperature was programmed as 35 °C held for 15 minutes initially, and further raised by 1 °C /min to 60 °C and held for 20 minutes at 60 °C followed by , 2 °C/min rise up to 200 °C to a total run time of 130 minutes. Injection volume was 1μL. FID signal was recorded and processed on Star work station software for Detailed Hydrocarbon Analysis (DHA) of compounds up to carbon number 15.

➢ ***Analysis of hydrocarbon constituents by GC-MS***

The samples were simultaneously analyzed using gas chromatography coupled with mass spectrometry (GC-MS) for the identification of components above C_{15}. Analyses were performed using a MS 1200 L Single Quadrupole bench top mass spectrograph attached to a Varian 3800 gas chromatograph. The GC was equipped with a split injector and a 30 m X 0.25 mm ID, Low Bleed 5% Phenyl, 95% dimethylpolysiloxane open tabular column 0.25 μm film thickness, helium carrier linear gas flow was 40cm/s. Injector temperature was 280 °C and split ratio was 100:1. Transfer line temperature was 279.6 °C. MS source temperature was 279.7 °C. The column oven temperature programme was initial temperature 65 ° C, ramp 10 °C /min to 300 °C hold for 5 min. The MS

was operated in centroid scan, mass range 40-800, with unit mass resolution.

e) **Metals**

Metal contents were estimated using ICP AES (Inductively coupled plasma and atomic emission spectroscope model: Thermo Electron IRIS Intrepid II XSP DUO). Sample preparation was done as per EPA method SW 846 3031. A representative 0.5 g sample was mixed with 0.5g of finely ground potassium permanganate, and then 1 ml of concentrated sulfuric acid was added while stirring. The sample was then treated with 2 ml concentrated nitric acid. When the reaction was complete 10ml of concentrated HCl was added and the sample heated until there was no gas evolution. The digestate was filtered. The filtrate was collected. The filter paper was washed down to the filtrate once with 5ml of hot concentrated HCl. Excess, manganese was precipitated out as manganese ammonium phosphate and the sample filtered. The filtrate was quantitatively transferred to volumetric flask, made up to volume, and analyzed in ICP-AES.

f). **Ash content**

Ash content was estimated by ASTM-IP method D482-80(1990).The dry sludge contained in a crucible was ignited and allowed to burn until only ash and carbon remained. The carbonaceous residue was reduced to ash by heating in a muffle furnace at 775 °C, cooled and weighed. The ash content of the n-pentane insoluble fraction was also determined similarly.

g). **pH determination**

The pH of the sludge was determined according to EPA SW 846 9045 (1992). The sludge was stirred with water in a 1:1 solution and allowed to settle. The sample was filtered and pH of the filtrate determined using pH meter.

2.2.2 Germination test

Germination test was carried out by keeping 25 seeds each of paddy *Oryza Sativa* (variety pokkali) in Petri dishes containing sludge, and sludge mixed with river sand at proportions 2.5, 5, 10, 20, 40, and 80%. A control set was maintained by placing the seeds in river sand. Each set had three replicates (Plate I). The petriplates were irrigated uniformly. Germination was recorded daily for seven days and the Speed Germination Index was calculated (Carley *et al.*, 1986).For assessing Vigour index (VI) the length of radical and hypocotyls were measured on the 7^{th} day. . The mean values from the replicates of each treatment and control were recorded. The Vigor Index was calculated using the relation,

VI = (Radical length + Plumule length) * Germination percentage. (Abdulbaki *et al.*, 1973).The germination percentage was analyzed by one way analysis of variance followed by Dunnetts test. Significant results are reported at 0.05 probability level. The data analysis was done using Toxstat software.

2.2.3 Phytoremediation of sludge using paddy (variety pokkali)

The paddy variety pokkali was opted for phytoremediation of petroleum sludge for the reason that it is a crop of the coastal wetlands prone to salinity incursion and is observed to be resistant to flooding and salinity. The cultural practice of the variety is transplanting seedlings to the fields. Therefore seedlings were raised in the laboratory for the phytoremediation of the petroleum sludge.

The seedlings were transplanted to pots containing sludge, and sludge mixed with sand at proportions 2.5%, 5%, 10%, 20%, 40% and 80%. Seedlings were also grown in sand to serve as control. The experiment was set up with six replications per

treatment with nutrient addition (Plate II). Fertilizer was applied every three weeks to provide C: N: P ratio of 100:5:1. The fertilizers added were urea and potassium hydrogen phosphate. Five seedlings were planted in each pot, exposed to sunlight and watered uniformly.

PLATE 1 Germination test

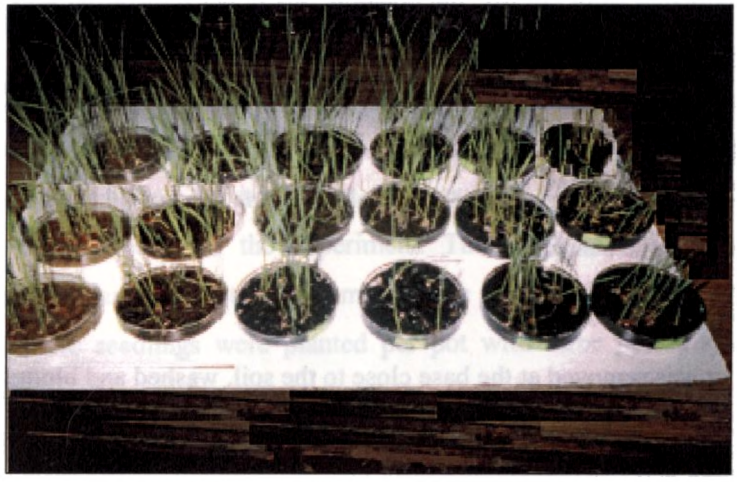

PLATE II Phytoremediation with different sludge concentration

The growth of the plants was measured as plant height and number of leaves on 30, 60, 90 and 104 (harvest) days of growth. The shoot biomass was determined upon harvest. The number of grains per panicle, and the percentage of sterility were determined at the time of harvest. The sludge-soil substrate was sampled after 30 days, and at the time of harvest to determine the TPH. For determination of TPH, the shoots of the plants were cut off; the contents of the pot transferred into a porcelain tray, and the roots and stalk were separated. It was air dried and sifted in a 2 mm sieve. The degradation of TPH was measured as percentage of gravimetric reduction in TPH. The uptake of metals Al, Cu, V, Cr, Ni, Zn, and Fe by the plants was analyzed in terms of accumulation factor calculated as ratio of metal concentration in biomass to that in the substrate.

- ***Plant biomass analysis***

The shoot was removed at the base close to the soil, washed and blotted dry. A portion of 5 g was dried in oven at 105°C to constant weight. Dry weight of the biomass per pot was calculated.

- ***Metal analysis***

Dried plant samples are extracted into an acid solution using wet ashing procedure in a mixture of acids to estimate the metal accumulation. The dried shoots were ground, and sieved. 0.5 g of the sample was taken into a 50 ml Kjeldahl flask. Added 1 ml of $HClO_4$, 5 ml HNO_3 and 0.5 ml H_2SO_4. Swirled gently and digested for about 15 minutes after the appearance of white fumes. The flask was cooled and diluted with 10 ml of water and boiled for a few minutes. This was filtered into a 50 ml volumetric flask and made up to the mark with water. Metal analysis was done by ICP-AES technique.

➢ ***Data analysis***

The shoot length and the number of leaves were graphically plotted. The shoot biomass was evaluated by one-way analysis of variance. Significant F value was compared to control by Dunnetts test. The grain yield and percentage of sterility was computed. The accumulation of metals and reduction of TPH with increasing sludge concentration were plotted graphically.

2.2.4 Optimization of nutrient enrichment

The objective was to study the optimum nutrient level sufficient for plant growth to effect phytoremediation of petroleum hydrocarbons. Four levels of nutrient combinations were chosen for the experiment. The seedlings of paddy (variety pokkali) were transplanted to pots sludge mixed with river sand at proportions 2.5%, 5% and 10%. Five seedlings were planted per pot with three pots per treatment. Fertilizer was applied every three weeks to provide C: N: P ratio of 100:5:1, 100:10:1, 100:15:1 and 100:20:1. The fertilizers added were urea and potassium hydrogen phosphate. They were exposed to sunlight and watered uniformly. The experimental set up is represented in Table 2.2.

Table 2.2 Design of nutrient optimization experiment

	Sl= 2.5%	Sl=5%	Sl=10%
N0	R1 R2 R3	R1 R2 R3	R1 R2 R3
N1	R1 R2 R3	R1 R2 R3	R1 R2 R3
N2	R1 R2 R3	R1 R2 R3	R1 R2 R3
N3	R1 R2 R3	R1 R2 R3	R1 R2 R3
N4	R1 R2 R3	R1 R2 R3	R1 R2 R3

Sl= Sludge concentration
N0-N4 = C: N: P levels
N0= Without nutrient enrichment
N1=100:5:1
N2=100:10:1
N3=100:15:1
N4=100:20:1
R1, R2 and R3 are replicates.

The effect of nutrients was assessed in terms of plant height & number of leaves, rhizosphere microbial count, biomass, and TPH reduction. Plant height & number of leaves were measured on 90th day. The rhizosphere microbial count was enumerated on the 90th day of cultivation. The biomass was estimated as dry weight on 30th, 60th and 90th day of cultivation. The sludge-soil substrate was sampled after 30 days, 60 days, and 90 days to determine the TPH. The degradation of TPH was measured as percentage of gravimetric reduction in TPH. Qualitative analysis of hydrocarbon degradation of 2.5% sludge at 100:20:1 was done using GC-FID and GC-MS on 90th day.

Enumeration of bacterial population

Total heterotrophic bacteria were enumerated using pour plate method (Pepper *et al.*, 1995). 1gm (dry weight) of soil-sludge were diluted in 99mL of 0.2% tetra sodium pyro phosphate($Na_4P_2O_7$) for soil dispersal (Alef and Nannipieri, 1995), shaken on a rotary shaker at 150 rpm for 30 min, and allowed to settle for 10 min. The solute was serially diluted in 0.85% sodium chloride (NaCl), transferring 1 mL of solution into 9mL of NaCl each time. Two dilutions were selected for the plating procedure to obtain a concentration of microorganism that had from 30 – 300 CFU/Plate. Petri plates of nutrient agar were inoculated with an aliquot of 1 mL. The plates were well spread, and incubated at 28 °C for 96 h.

2.2.5 Effectiveness of different varieties of paddy in phytoremediation

Four local varieties of paddy were selected for the experiment. The varieties selected were D1, 1285, Matta and Manikyam. The seedlings of these varieties along with pokkali seedlings were transplanted to pots containing sludge mixed with river sand at proportions 2.5%, 5%, 10%. The experiment was set up with three replications per treatment as before. Fertilizer was applied every three weeks to provide C: N: P

ratio of 100:20:1 for 2.5% sludge, 100:10:1 for 5%sludge and 100:5:1 for 10% sludge based on the observation of the previous experiment. The fertilizers added were urea and potassium hydrogen phosphate. Five seedlings were planted in each pot. They were exposed to sunlight and watered uniformly.

The growth of the plant was measured as shoot biomass on 30, 60 and 90 days. The sludge-soil substrate was sampled after 30 days, 60 days and on 90th day to determine the TPH. The degradation of TPH was measured as percentage of gravimetric reduction in TPH. The results were analyzed statistically by analysis of variance in Toxstat software.

2.2.6 Surfactant enhanced phytoremediation

The objective of the experiment was to enhance the degradation rate of petroleum hydrocarbons through addition of surfactant. The pokkali seedling were transplanted to pots containing sludge mixed with river sand at proportions 2.5%, 5%, 10%. The experiment was set up with three replications per treatment as before. Fertilizer was applied every three weeks to provide C: N: P ratio of 100:20:1 for 2.5% sludge, 100:10:1 for 5% sludge and 100:5:1 for 10% sludge with the addition of Tween 80 at 0.1, 0.5 and 1 % of sludge. The growth of the plant was measured as plant height and shoot biomass determined on the 90th day. The sludge-soil substrate was sampled on day 90 to determine the TPH. The degradation of TPH was measured as percentage of gravimetric reduction in TPH.

2.3 Results

2.3.1 Properties of sludge

The sludge collected from the disposal site was colored black, nearly of solid consistency, and sticky in nature .The chemical composition of the sludge is given in Table 2.3. The sludge had a mean pH of 5.8 and ash content 4.11 %. The N:P ratio was 28:1 and the carbon content significantly higher. The oil sludge had a TPH of 850 g/kg .

Table 2.3 Composition of the sludge collected for the study

Sl. No	Property	Value [a]
1	Ash Weight, %	4.11 ± 0.25
2	Calorific value of oil sludge, cal/g	7663 ± 359
3	pH	5.8 ± 0.8
4	Carbon, %	74.29 ± 4.4
5	Total P, %	0.0098 ± 0.001
6	Total N, %	0.279 ± 0.052
7	Sulphur, %	2.544 ± 0.2
8	TPH, g/kg	850.3 ± 150

[a] Mean ± standard deviation for 6 samples

The calorific value was 7663 cal/kg. The petroleum hydrocarbon was fractionated into four fractions (Table 2.4). Among the four fractions of petroleum hydrocarbons (Saturate, Aromatic, NSO, Asphaltenes), the one present in the highest proportion was the saturate (40.38%) and the lowest was the NSO fraction (5.33%).The ash content of the asphaltene fraction was 15.28%.The hexane and benzene fractions were analyzed in GC-FID.

Table 2.4 Fractional composition of TPH of sludge collected from refinery site (On dry weight basis)

TPH	Composition % [wt/wt] [a]
Saturate (hexane extract)	40.38 ± 2.22
Aromatic (benzene extract)	27.89 ± 1.24
NSO	5.33 ± 0.48
Asphaltenes	26.4 ± 1.26
n-pentane insoluble ash content (%)of asphaltenes	15.28 ± 1.18

[a] Mean ± standard deviation for 6 samples

The hexane fraction contained compounds other than saturated hydrocarbons (Fig 2.1) and benzene fraction had compounds other than aromatics (Fig 2.2). The classes of compounds present were Aromatics, Iso-paraffins, Naphthenes, Olefins, and Paraffins (Table 2.5). The components ranged from C_6 to C_{14}.

Table 2.5 Identified components (below carbon number 15) in the sludge upon GC-FID analysis

Class of compounds (Hexane and benzene elute)	Weight % of (Hexane and benzene elute)	Components	
		Carbon number	Name of components
Aromatics	2.0125	14 12 10 14	n-Octylbenzene t-1-Butyl-3,5-Dimethylbenzene 1Methyl-4-n-propyl-benzene. n-Nonylbenzene
Iso-Paraffins	2.217	7 8 8	2,3-Dimethypentane. 2,2,4-Trimethylpentane, 3,4-Dimethylhexane, 3-Methylheptane
Naphthenes	78.3805	6 7 8 8 10	Methyl cyclo pentane, Cyclohexane Cis-1,2 dimethylcyclopentane Ctc-1,2,3 trimethylcyclopentane. Cis-1,3 dimethylcyclohexane Sec-butyle cyclohexane
Olefins	8.9775	6 8 14	1-Hexene trans-2-Octene, 1-Tetradecene
Paraffins	8.412	7 8 14	n-Heptane 3,3-Dimethylhexane, n-Tetradecane

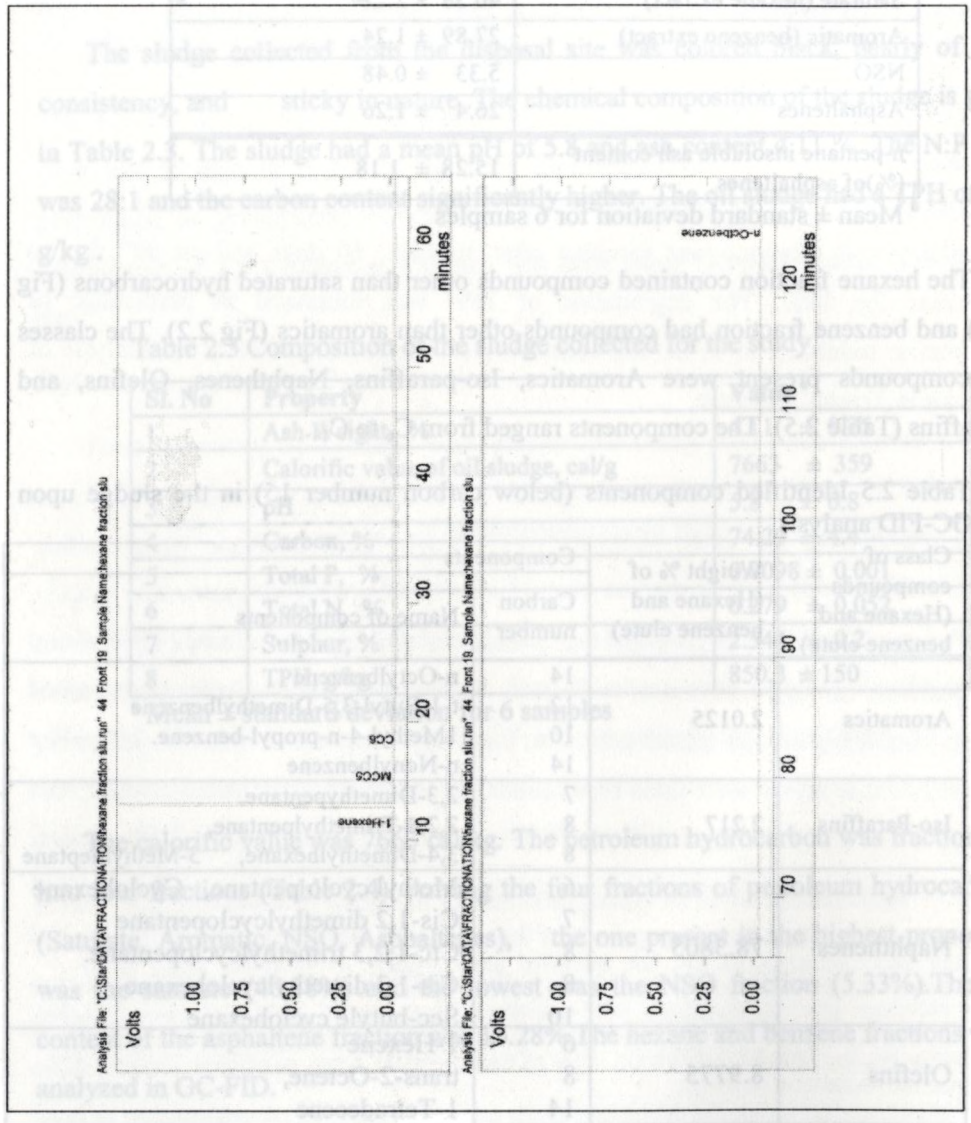

Fig. 2.1 Chromatogram of hexane fraction of TPH in GC-FID analysis

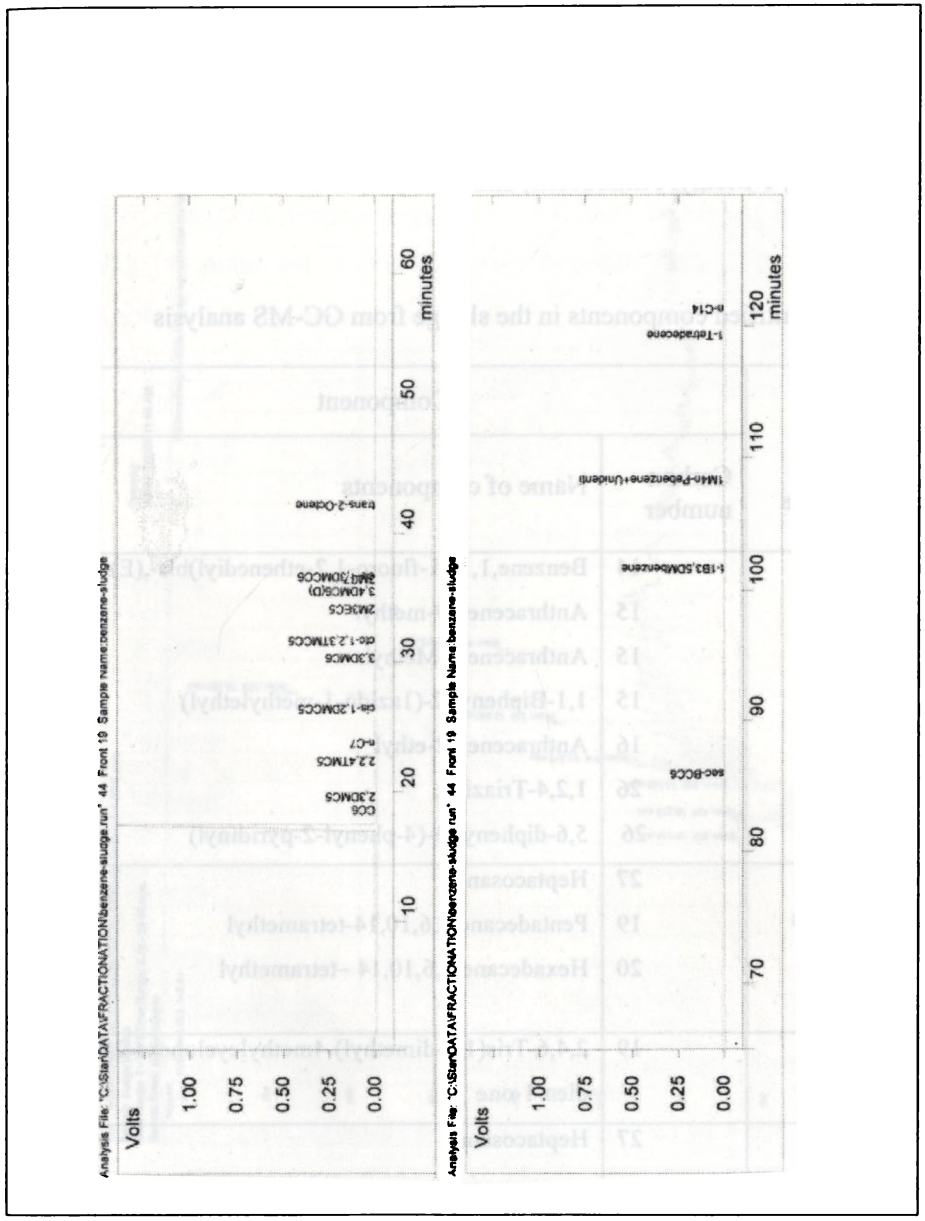

Fig. 2.2 Chromatogram of benzene fraction of TPH in GC-FID analysis

The GC-MS analysis showed that the sludge contained a range of hydrocarbons from C_6 to C_{27} (Fig 2.3 and 2.4). The components identified with >50% probability are given in Table 2.6. Among the aromatics the PAH compounds identified are 9-methyl Anthracene, 1-Methyl Anthracene, and 2-ethyl Anthracene.

Table 2.6 Identified components in the sludge from GC-MS analysis

Class of compounds (Hexane and benzene elute	Component	
	Carbon number	Name of components
Aromatics	14	Benzene,1,1-(1-fluoro-1,2-ethenediyl)bis-,(E)
	15	Anthracene, 9-methyl
	15	Anthracene,1-Methyl
	15	1,1-Biphenyl,2-(1azido-1-methylethyl)
	16	Anthracene, 2-ethyl
	26	1,2,4-Triazine,
	26	5,6-diphenyl-3-(4-phenyl-2-pyridinyl)
Iso-Paraffins	27	Heptacosane
	19	Pentadecane,2,6,10,14-tetramethyl
	20	Hexadecane,2,6,10,14 –tetramethyl
Naphthenes	19	2,4,6-Tris(1,1-dimethyl)-4methylcyclohexa-2,5-dien-1-one
Paraffins	27	Heptacosane

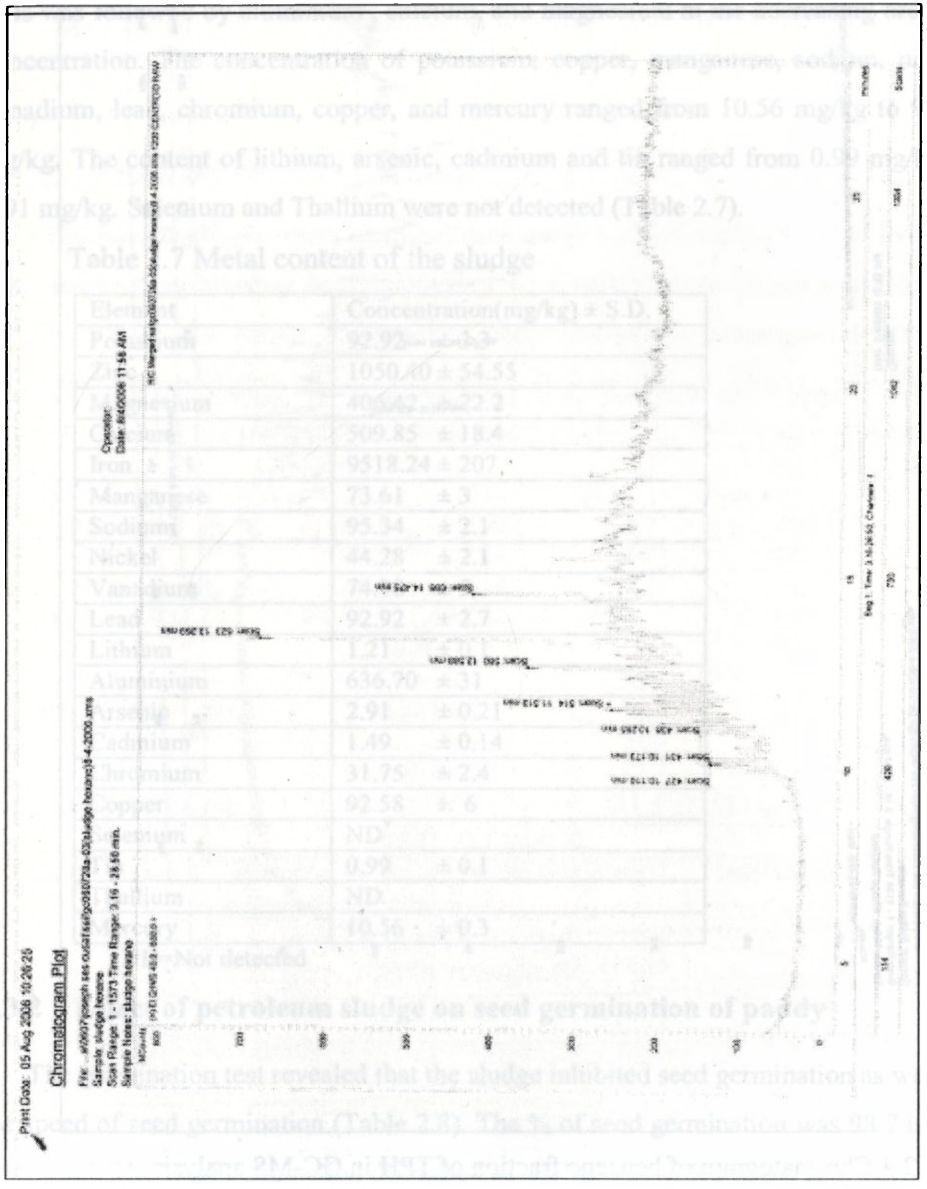

Fig. 2.3 Chromatogram of hexane fraction of TPH in GC-MS analysis

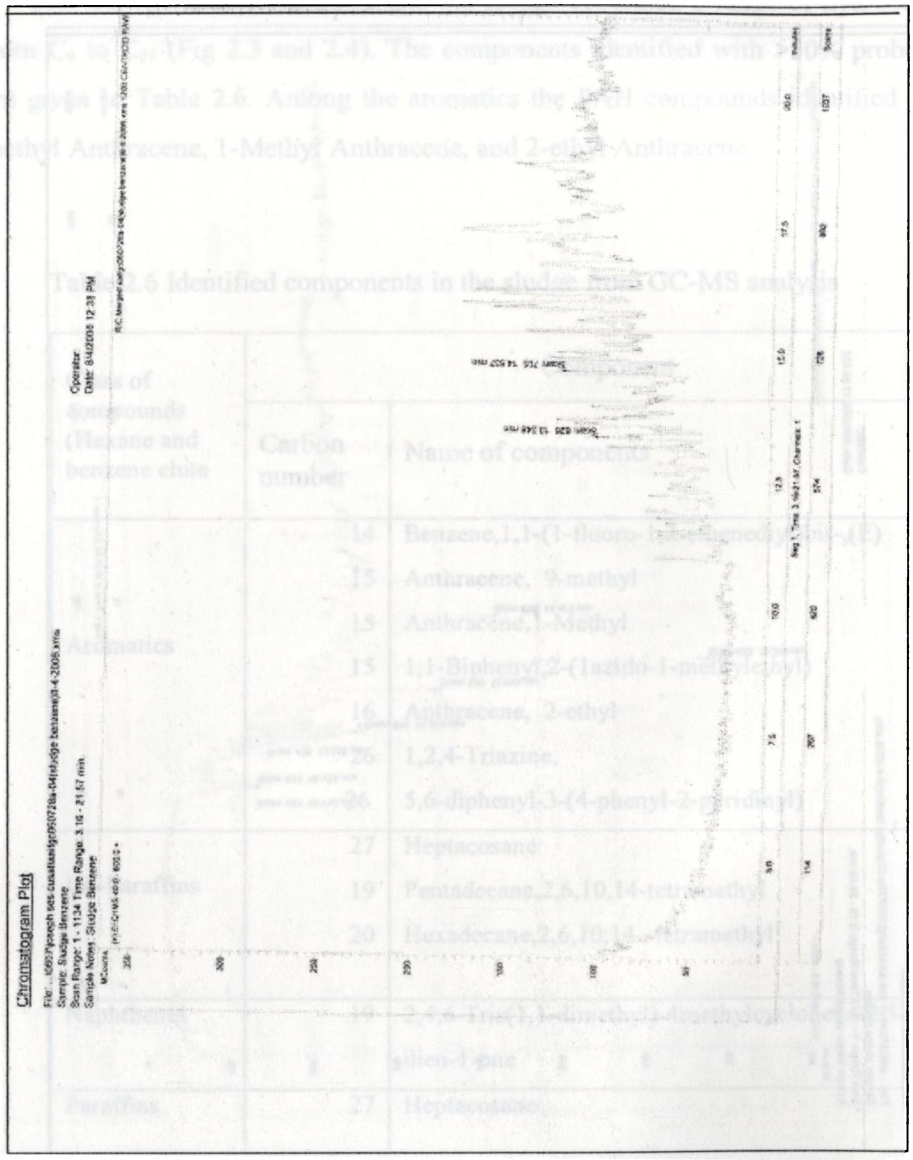

Fig 2.4 Chromatogram of benzene fraction of TPH in GC-MS analysis

Among the fifteen elements analyzed, iron was the most abundant followed by zinc. The concentration of iron was 9518.24 mg/kg and that of zinc 1050.4 mg/kg. This was followed by aluminium , calcium, and magnesium in the decreasing order of concentration. The concentration of potassium, copper, manganese, sodium, nickel, vanadium, lead, chromium, copper, and mercury ranged from 10.56 mg/kg to 95.34 mg/kg. The content of lithium, arsenic, cadmium and tin ranged from 0.99 mg/kg to 2.91 mg/kg. Selenium and Thallium were not detected (Table 2.7).

Table 2.7 Metal content of the sludge

Element	Concentration(mg/kg) ± S.D.
Potassium	92.92 ± 3.3
Zinc	1050.40 ± 54.55
Magnesium	406.42 ± 22.2
Calcium	509.85 ± 18.4
Iron	9518.24 ± 207
Manganese	73.61 ± 3
Sodium	95.34 ± 2.1
Nickel	44.28 ± 2.1
Vanadium	74.98 ± 5
Lead	92.92 ± 2.7
Lithium	1.21 ± 0.1
Aluminium	636.70 ± 31
Arsenic	2.91 ± 0.21
Cadmium	1.49 ± 0.14
Chromium	31.75 ± 2.4
Copper	92.58 ± 6
Selenium	ND*
Tin	0.99 ± 0.1
Thallium	ND
Mercury	10.56 ± 0.3

*ND=Not detected

2.3.2 Effect of petroleum sludge on seed germination of paddy

The germination test revealed that the sludge inhibited seed germination as well as the speed of seed germination (Table 2.8). The % of seed germination was 98.7 in the river sand. The % of germination decreased as sludge was added to the soil. Seeds did not germinate at ≥ 80% sludge.

Table 2.8 Effect of petroleum sludge on seed germination, Vigor Index and Speed of Germination (%SGI)

Parameter	Control river sand	% sludge						
		2.5	5.0	10.0	20	40	80	100
Germination (%)	98.7	89.3	86.7	77.3	63.3	37.3	Nil	Nil
Vigour Index	2240.5	1821.7	1673.3	1368.2	1019.9	395.4	Nil	Nil
% SGI	1616.0	1217.0	1278.7	1097.3	1105.3	730.7	Nil	Nil

Analysis of variance (Table 2.9) showed that there was a significant reduction in germination at sludge concentration ≥ 10% .The vigor and speed of germination was also lower in the presence of sludge.

Table2.9 Analysis of variance and Dunnetts test of germination vs. sludge concentration

Replications	Control (river sand)	Sludge (%)				
		2.5%	5.0%	10.0%	20%	40%
R1	100	92	80	88	60	24
R2	100	88	88	68	59	40
R3	96	88	92	76	71	48
Mean± SD	98.7± 2.3	89.3± 2.3	86.7± 6.1	77.3 ± 10	63.3± 6.6	37.3±12.2

ANOVA TABLE

Source	DF	SS	MS	F
Between	5	7382.444	1476.489	25.828
Within(Error)	12	686	57.167	
Total	17	8068.444		

Critical F value = 3.11 (0.05, 5, 12) Since F > Critical F REJECT Ho: All groups equal

DUNNETTS TEST

Group	Identification	Transformed mean	Mean calculated in original units	T stat	Sig
1	C	98.667	98.667		
2	2.5	89.333	89.333	1.512	.
3	5	86.667	86.667	1.944	.
4	10	77.333	77.333	3.456	*
5	20	63.333	63.333	5.723	*
6	40	37.333	37.333	9.935	*

Dunnetts table value =2.50 (1 Tailed value, P = 0.05, df =12, 5)
* = Significant difference. Ho: Control <Treatment. . = no significant difference

2.3.3 Phytoremediation of sludge using pokkali

Three important criteria were identified as relevant to phytoremediation. They were growth and survival of the plant, degradation of petroleum hydrocarbons, and accumulation of metals.

➢ Growth and survival of the plant

The seedlings of *Oryza sativa* transplanted to the sludge at 2.5 to 40 % concentration survived to harvest stage. The seedlings did not survive at 80% and 100% sludge. The height of the plant decreased as the concentration of sludge increased. The inhibitory effect on growth was evident from 30 days of growth till harvest (Fig. 2.5).There was drastic height reduction from 10% sludge concentration upwards.

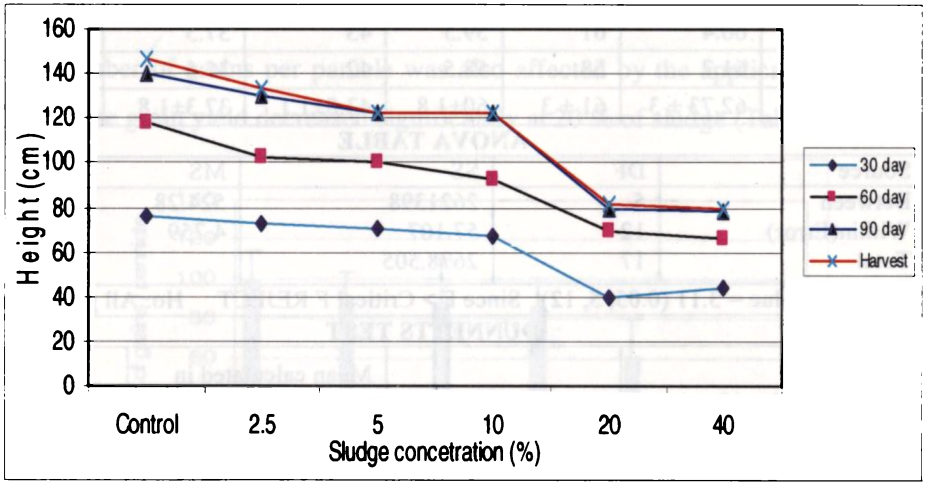

Fig. 2.5 Plant height at different sludge concentrations up to harvest

The number of leaves decreased gradually up to 10% and then the number increased at higher levels but the leaf area was reduced, and tillers were observed. (Fig. 2.6)

The shoot biomass decreased in the presence of sludge. The mean shoot biomass of the plants grown in control soil was 62.73 g/pot. The presence of sludge in the substrate decreased the production of dry matter (Table 2.10). Biomass production at ≥ 10% sludge concentration was significantly less than the control.

Table 2.10 Production of dry matter (g/pot) and analysis of variance

Replications	Sludge concentration (%)					
	Control	2.5	5	10	20	40
R1	66.1	64	62	44.2	39	35
R2	60.4	61	59.5	43	37.5	33
R3	61.7	58	58.5	42	35.5	31.5
Mean ± SD	62.73 ± 3	61 ± 3	60±1.8	43.0±1.1	37.3±1.8	33.1±1.8

ANOVA TABLE

Source	DF	SS	MS	F
Between	5	2621398	524.28	110.
Within(Error)	12	57.107	4.759	166
Total	17	2678.505		

Critical F value = 3.11 (0.05, 5, 12) Since F > Critical F REJECT Ho: All groups equal

DUNNETTS TEST

Group	Identification	Transformed mean	Mean calculated in original units	T stat	Sig
1	C	62.733	62.733		
2	2.5	61	61	0.973	.
3	5	60	60	1.535	.
4	10	43.067	43.067	11.041	*
5	20	37.333	37.333	14.26	*
6	40	33.167	33.167	16.599	*

Dennett table value =2.5 (1 Tailed value, P = 0.05, df =12,5)
* = Significant difference. Ho: Control <Treatment . = no significant difference

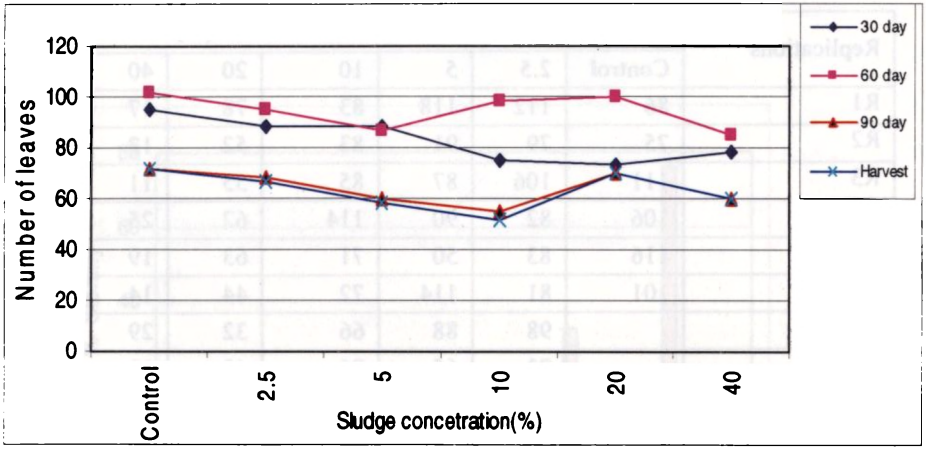

Fig. 2.6 Number of leaves per pot at different sludge concentrations up to harvest

The number of grains per panicle was also affected by the application of sludge (Fig. 2.7). The grain yield decreased significantly at 20 % of sludge (Table 2.11).

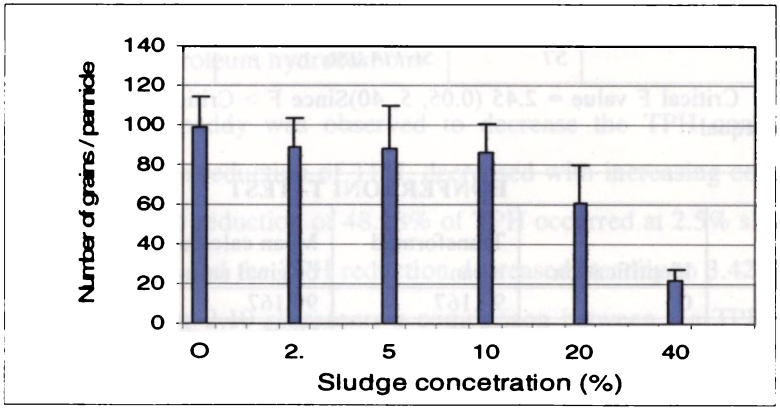

Fig. 2.7 Number of grains per panicle upon growth in sludge

Table 2.11 Analysis of variance of grain number per panicle at different sludge concentrations

Replications	Sludge concentration (%)					
	Control	2.5	5	10	20	40
R1	86	112	118	83	78	17
R2	75	79	91	83	52	12
R3	111	106	87	85	55	11
R4	106	82	90	114	62	25
R5	116	83	50	71	63	19
R6	101	81	114	72	44	14
R7		98	88	66	32	29
R8		73	65	96	43	18
R9				110	56	16
R10				68	85	25
R11				96	63	24
R12				80	100	
R13				94		
Mean ± SD	99.2 ±	89.3±	87.9	86±15.3	61.1±	19±5.9

ANOVA TABLE

Source	DF	SS	MS	F
Between	5	43413.052	8682.61	33.792
Within(Error)	52	13361.034	256.943	
Total	57	56774.086		

Critical F value = 2.45 (0.05, 5, 40)Since F > Critical F REJECT Ho: All groups equal

BONFERRONI T- TEST

Group	Identification	Transformed mean	Mean calculated in original units	T stat	Sig
1	C	99.167	99.167		
2	2.5	89.25	89.25	1.146	.
3	5	87.875	87.875	1.304	.
4	10	86	86	1.664	.
5	20	61.083	61.083	4.752	*
6	40	19.091	19.091	9.843	*

Bonferroni t- table value =2.5 (1 Tailed value, P = 0.05, df =50, 5)
* = Significant difference. Ho: Control <Treatment. .=no significant difference

The seed sterility increased with the concentration of sludge (Fig 2.8). The proportion of sterile seeds was 4 % at 2.5% sludge, 15.34 at 10% sludge, 32 at 20% sludge and 76% at 40% sludge.

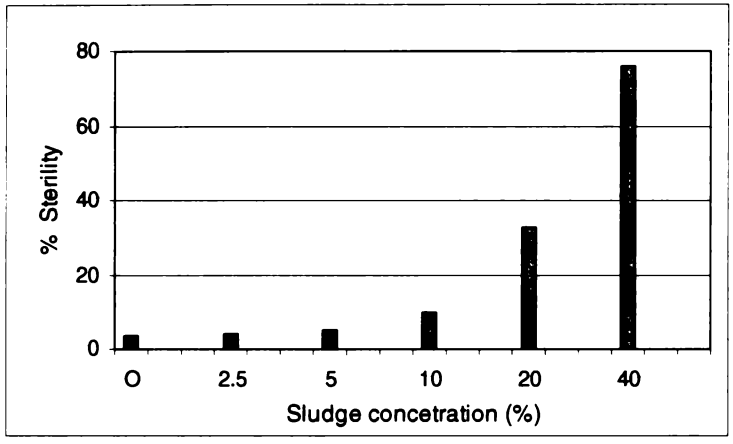

Fig. 2.8 Sterile grains per panicle (%) upon growth in sludge

➢ Degradation of petroleum hydrocarbons

The cultivation of paddy was observed to decrease the TPH content of the substrate. The percentage reduction of TPH, decreased with increasing concentration of sludge. The maximum reduction of 48.18% of TPH occurred at 2.5% sludge at the time of harvest (Fig.2.9) and the TPH reduction decreased steadily to 3.42 % at 40 % sludge concentration. Fig 2.10 represents a comparison between the TPH reduction and biomass accumulation. As the sludge concentration increased, the TPH reduction as well as the biomass production decreased. Correlation coefficient between biomass and TPH reduction was 0.98.

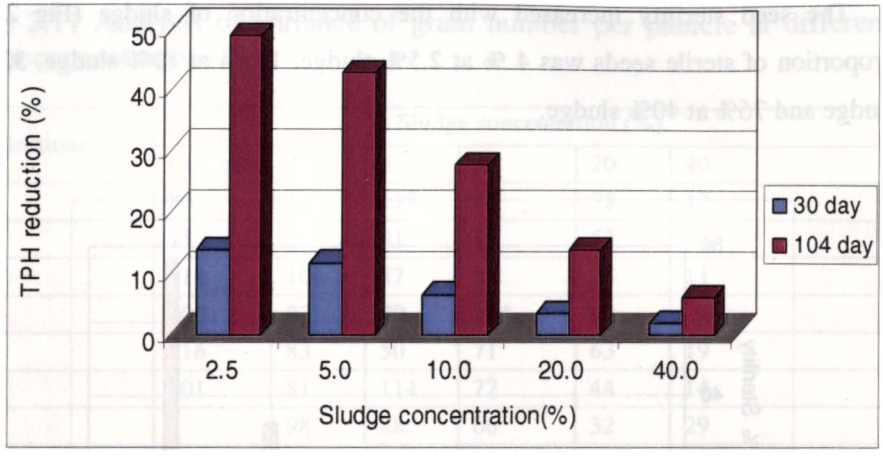

Fig. 2.9 Reduction of petroleum hydrocarbon upon growth of paddy

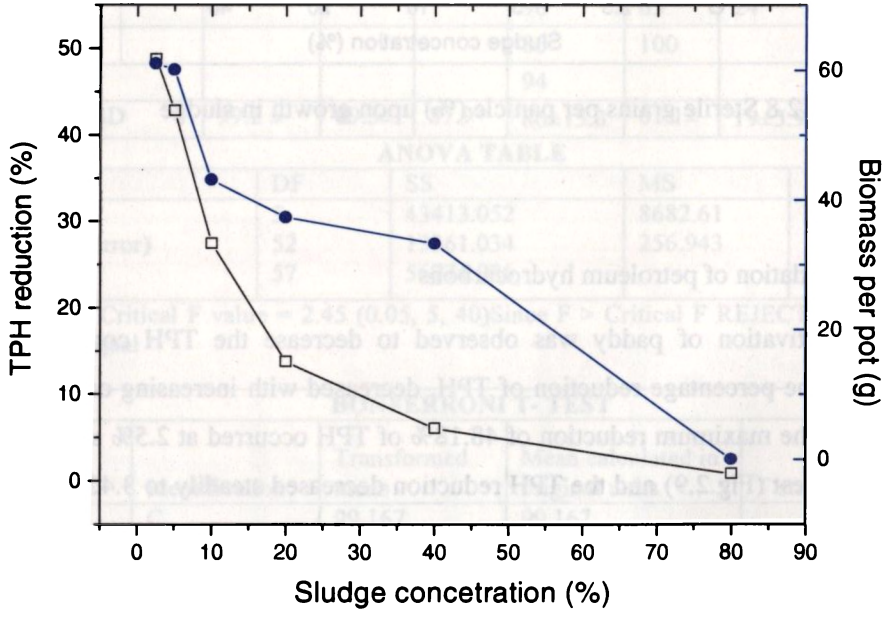

Fig. 2.10 Biomass and TPH reduction upon phytoremediation of petroleum sludge

➢ Accumulation of metals

The uptake of metals by the plants was determined in terms of accumulation factor calculated as the ratio of element's concentration accumulated in biomass upon harvest to that in the sludge. Aluminium and copper were the metals to be accumulated most by the plants. The highest accumulation factor was 0.65 for Aluminium at 5% sludge concentration and 0.64 for copper at 10% sludge. Accumulation decreased beyond 10% sludge (Fig. 2.11).

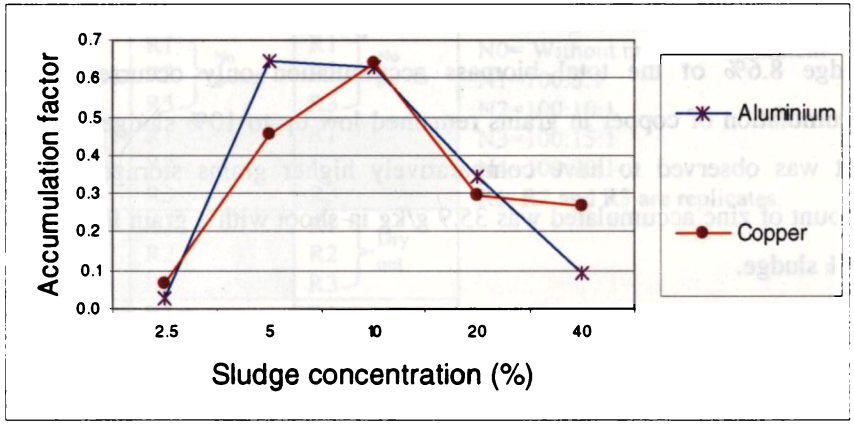

Fig. 2.11 Accumulation of metals by *Oryza sativa* variety pokkali upon growth in petroleum sludge

The accumulation factor for vanadium, chromium, nickel, zinc and iron was low in the range 0.007 to 0.02 (Fig. 2.12). The amount of Al accumulated was 408.7 mg/kg biomass and that of copper 29.5 mg/kg biomass.

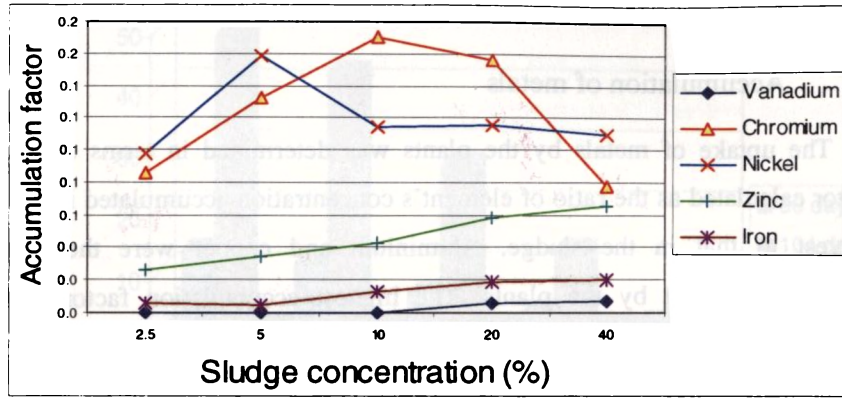

Fig. 2.12 Accumulation of metals by *Oryza sativa* variety pokkali upon growth in petroleum sludge.

The translocation of aluminium into grains was comparatively low. At 40% sludge 8.6% of the total biomass accumulation only occurred in grains. The accumulation of copper in grains remained low up to 10% sludge. Zinc is the metal that was observed to have comparatively higher grains storage (Table2.12). The amount of zinc accumulated was 35.9 g/kg in shoot with a grain load of 9.46 g/kg at 10% sludge.

Table 2.12 Percentage accumulation of metals in grain with respect to total shoot biomass

Element	Sludge concentration (%)				
	2.5	5	10	20	40
Vanadium	ND	ND	ND	ND	ND
Chromium	ND	ND	ND	ND	ND
Nickel	3.0	4.1	5.2	6.1	6.2
Aluminium	1.6	0.8	0.5	2.3	8.6
Iron	3.6	4.3	2.0	9.2	11.0
Zinc	16.3	17.7	20.8	30.4	46.8
Copper	2.4	2.3	5.2	28.9	42.2

*ND= not detected

2.3.4 Optimization of nutrient enrichment

The nutrient enrichment experiments showed that the proportion of nitrogen and phosphorous was significant in the growth of the plants and consequently for phytoremediation. The unenriched sludge did not promote growth of the plant. The growth ceased in a couple of weeks and plants remained stunted. The plants grown in

Table 2.13 Outcome of nutrient enrichment at different N: P levels

	Sl= 2.5%	Sl=5%	Sl=10%
N0	R1, R2, R3 — No growth	R1, R2, R3 — No growth	R1, R2, R3 — No growth
N1	R1, R2, R3	R1, R2, R3	R1, R2, R3
N2	R1, R2, R3	R1, R2, R3	R1, R2, R3 — Dry out
N3	R1, R2, R3	R1, R2, R3 — Dry out	R1, R2, R3 — Dry out
N4	R1, R2, R3	R1, R2, R3 — Dry out	R1, R2, R3 — Dry out

Sl= Sludge concentration
N0= Without nutrient enrichment
N1=100:5:1
N2=100:10:1
N3=100:15:1
N4=100:20:1
R1, R2 and R3 are replicates.

2.5%sludge in all the four levels of nutrient selected were healthy and green. In 5% sludge, nutrient levels (N:P) 5:1 and 10:1 produced green and healthy plants while at 100:15:1 and 100:20:1 the growth ceased and plants dried out within fourteen days. At 10 % sludge concentration, plant growth was limited to 5:1 N: P ratio (Table 2.13).

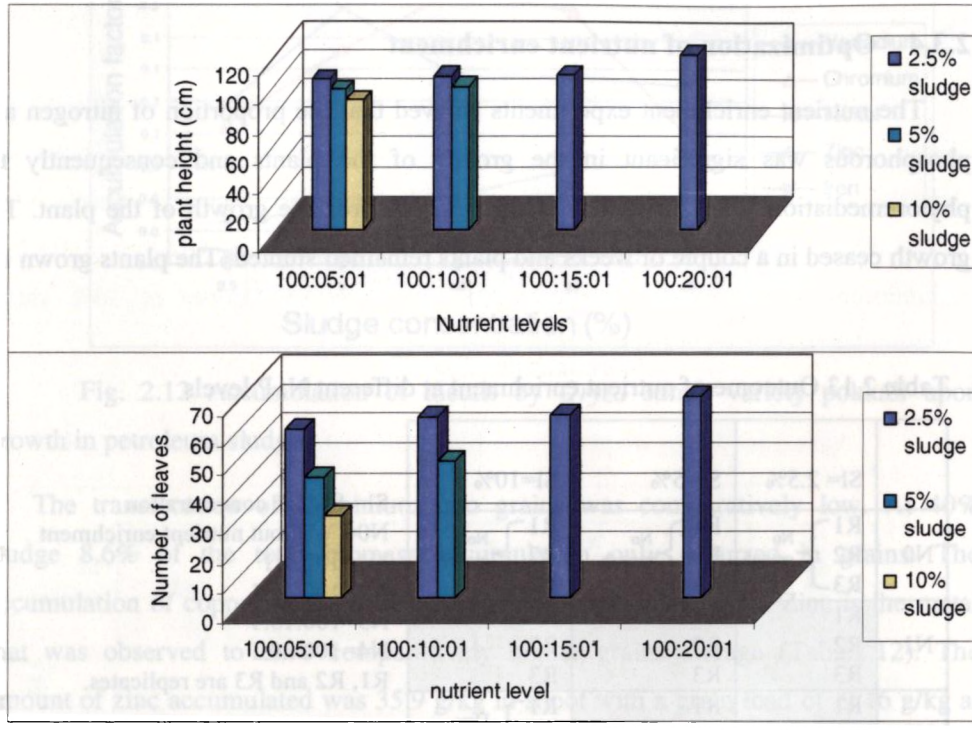

Fig. 2.13 Plant height and number of leaves at different nutrient level and sludge concentrations

The plant height and leaf production was affected by the proportion of sludge. At 2.5% sludge level, the plant height attained was highest (120cm) for nutrient level 100:20:1 i.e. increased rate of fertilization promoted the growth of plants (Fig. 2.13); but as the proportion of sludge increased, the plant height and number of leaves decreased.

The microbial population increased with the fertility of the soil-sludge at 2.5% sludge from $5.5*10^7$ to $1.2*10^9$ CFU/g soil. With increasing proportion of sludge the microbial population decreased (Fig.2.14).

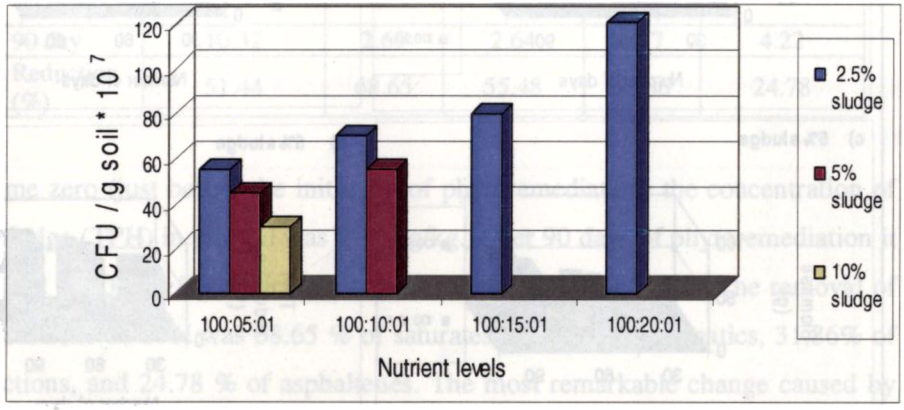

Fig. 2.14 Rhizosphere microbial count on 90th day of phytoremediation

For 2.5 % sludge, biomass production increased from 100:5:1 to 100:20:1nutrient level (Fig. 2.15 a). At 100:5:1, the biomass production per pot was found to be 53.22 g and it was increased up to 70.65 g at 100:20:1 nutrient level at 90 day. TPH reduction also was found to be correspondingly increased from 46.84 to 51.44 % with increase in fertilizer (Fig 2.15 b).Biomass production of 5% sludge at 100:5:1was 55 g and in 100:10:1 it was 65.5 g per pot corresponding to TPH reduction of 32.84 % and 33.25% respectively (Fig.2.15c and 2.15d). For 10% sludge, biomass production was 51.2 g per pot, and TPH reduction was 27.46 % (Fig.2.15e and Fig.2.15f).

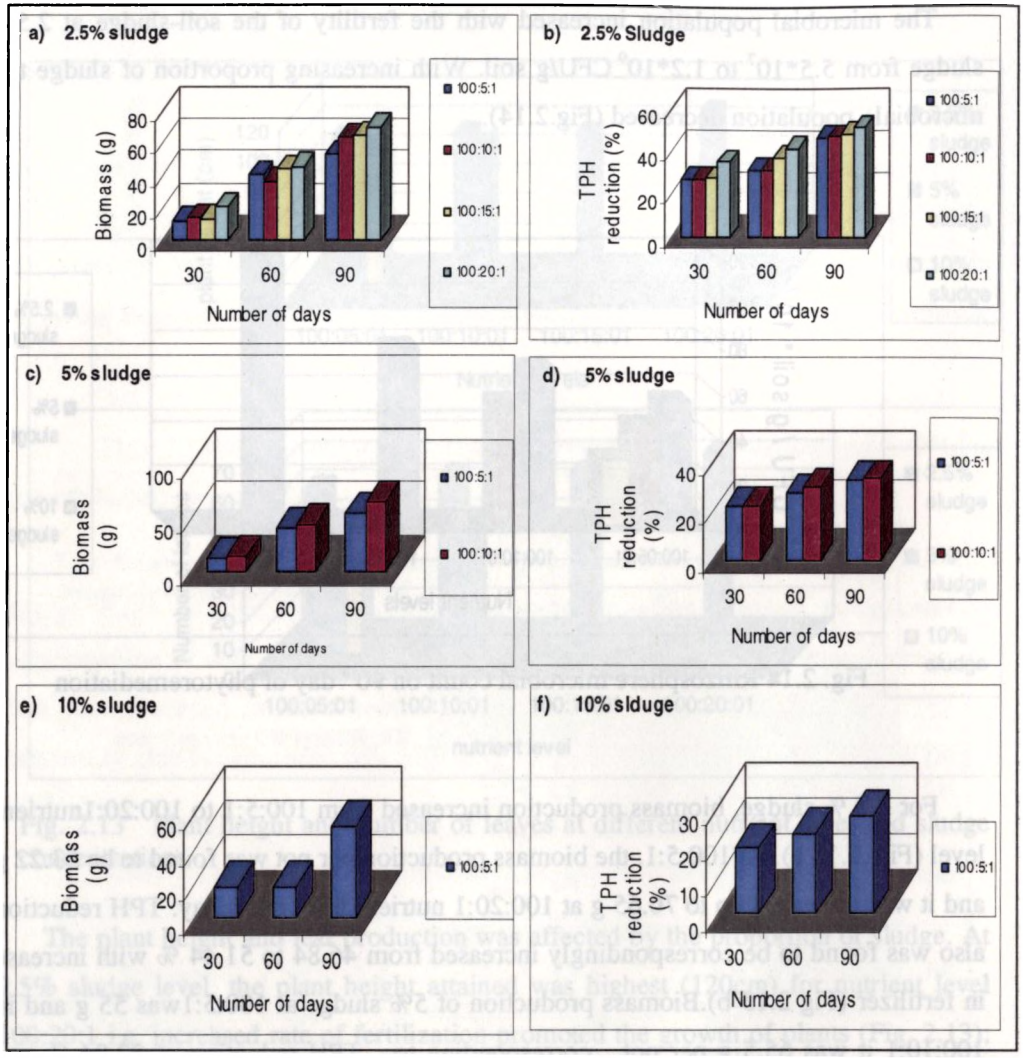

Fig. 2.15 Biomass and TPH reduction upon phytoremediation at different nutrient levels

The maximum reduction of 51.44% occurred in 2.5% sludge enriched with fertilizer at the rate 100:20:1. Solvent extractable fractions of these samples at time zero and at the time of harvest is given in Table 2.14 and Fig.2.16.

Table 2.14 TPH content of soil at time zero and removal of various fractions of TPH at 90 day following phytoremediation of 2.5% sludge at C:N:P 100:20:1

Period	TPH content g/Kg	TPH Fractions g/Kg			
		Saturate	Aromatic	NSO	Asphaltenes
Time zero	21.25	8.58	5.93	1.13	5.61
90 day	10.32	2.69	2.64	0.77	4.22
Reduction (%)	51.44	68.65	55.48	31.86	24.78

At time zero (just before the initiation of phytoremediation) the concentration of the oily sludge (TPH) in the soil was 21.25 g/kg. After 90 days of phytoremediation it was reduced to 10.32 g/kg, indicating a removal of 51.44 % of TPH. The removal of various fractions of TPH was 68.65 % of saturates, 55.48 % of aromatics, 31.86% of NSO fractions, and 24.78 % of asphaltenes. The most remarkable change caused by the phytoremediation was substantial decline in the saturate and aromatic fraction with a contaminant increase in the resin and asphaltene fractions in the system.

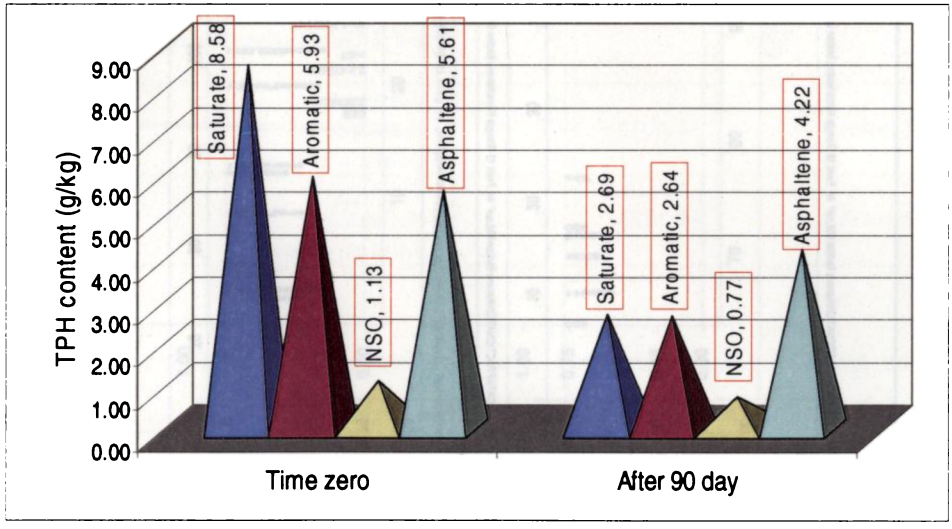

Fig. 2.16 Fractions of TPH at the initial and final stage of phytoremediation

GC- FID results of the hexane and benzene eluted fractions of TPH following phytoremediation is given Fig 2.17 and 2.18. The number of components of the PHCs was observed to have increased; still Naphthenes occupy the larger proportion (Table2.15). Certain components have degraded while new degradation products have appeared. The GC-MS spectrum of the hexane and benzene extract (Table2.16) revealed the presence of C_{17}, C_{19} and C_{20} hydrocarbons. The qualitative change in the spectrum of hydrocarbons in the sludge upon phytoremediation is given in Fig. 2.19 and Fig.2.20.

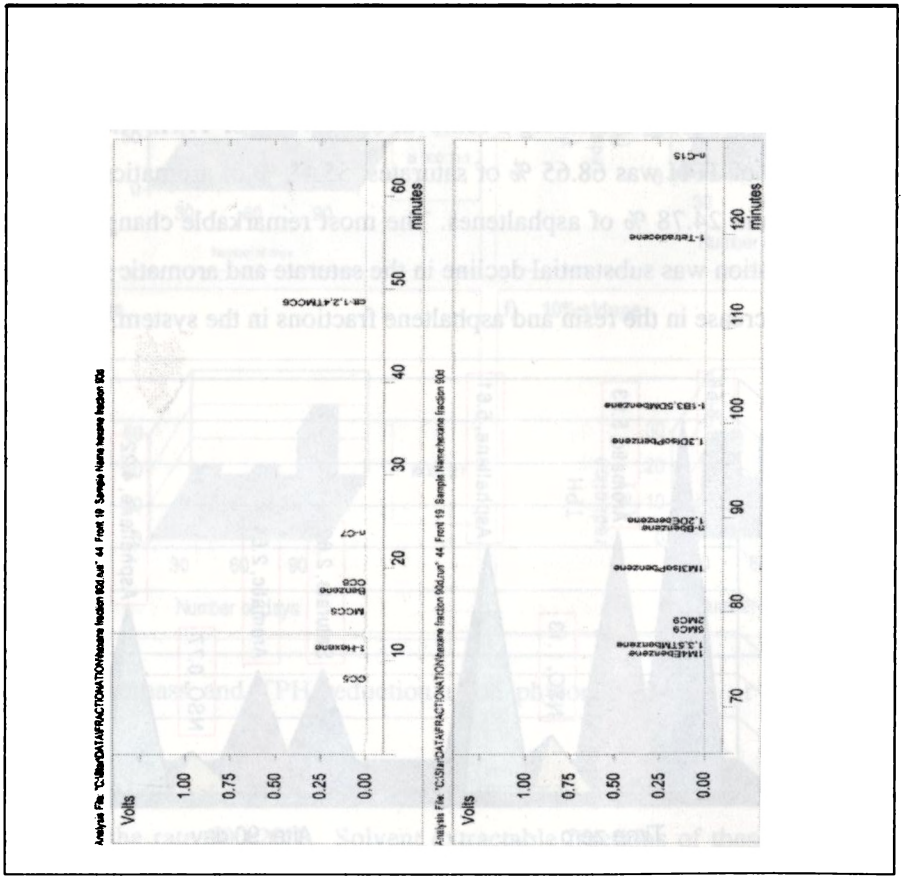

Fig 2.17 Chromatogram of hexane fraction of phytoremediated sludge

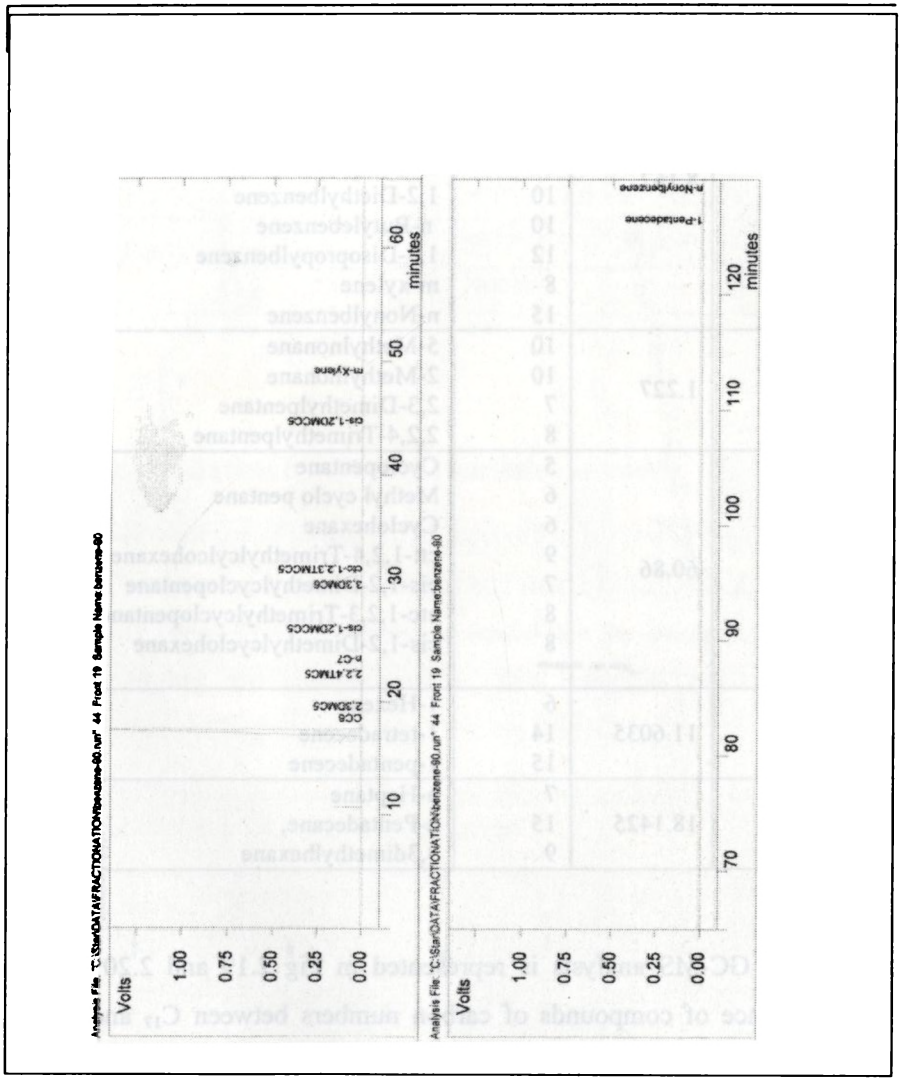

Fig 2.18 Chromatogram of benzene fraction of phytoremediated sludge

Table 2.15 Identified components of in the sludge after 90 day phytoremediation

Class of compounds (Hexane and benzene elute)	Weight %	Components	
		Carbon number	Name of components
Aromatics	8.167	6	Benzene
		9	1,3,5-Trimethylbenzene
		9	1-Methyl -4-Ethylbenzene
		12	t-1-Butyl-3,5-Dimethylbenzene
		10	1-Methyl-3-Isopropylebenzene
		10	1,2-Diethylbenzene
		10	n-Butylebenzene
		12	1,3-Disopropylbenzene
		8	m-xylene
		15	n-Nonylbenzene
Iso-Paraffins	1.227	10	5-Methylnonane
		10	2-Methylnonane
		7	2,3-Dimethylpentane
		8	2,2,4-Trimethylpentane
Naphthenes	60.86	5	Cyclopentane
		6	Methyl cyclo pentane
		6	Cyclohexane
		9	ctt-1,2,4-Trimethylcylcohexane
		7	cis-1,2-Dimethylcyclopentane
		8	ctc-1,2,3-Trimethylcyclopentane
		8	cis-1,2-Dimethylcyclohexane
Olefins	11.6035	6	1-Hexene
		14	1-tetradecene
		15	1-pentadecene
Paraffins	18.1425	7	n-Heptane
		15	n-Pentadecane,
		9	3,3dimethylhexane

The result of GC-MS analysis is represented in Fig 2.19 and 2.20 .The data showed the presence of compounds of carbon numbers between C_{17} and C_{20} only (Table2.16).This clearly shows that the higher molecular weight compounds have degraded and therefore a wider range of compounds were observed in the GC-FID chromatogram. No PAH compounds were found in GC-MS chromatogram.

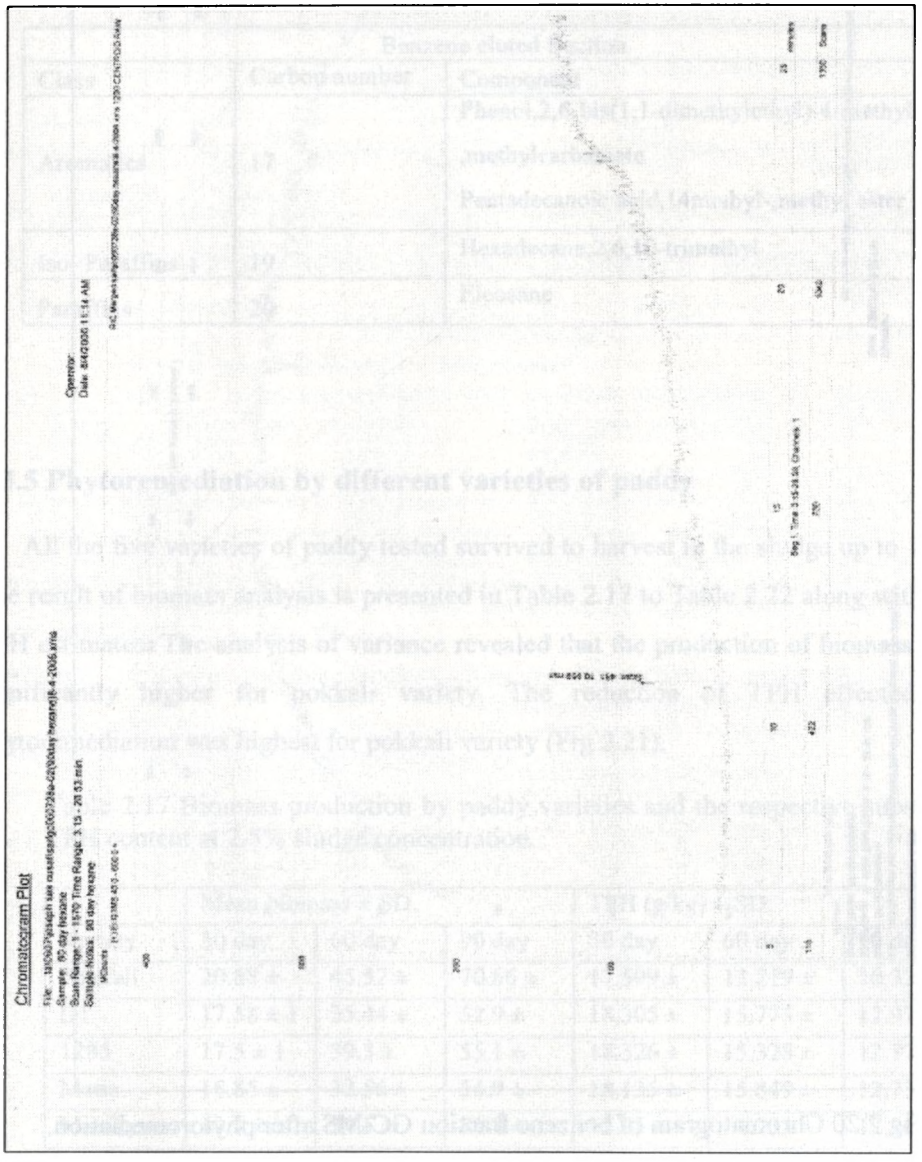

Fig 2.19 Chromatogram of hexane fraction GC-MS after phytoremediation

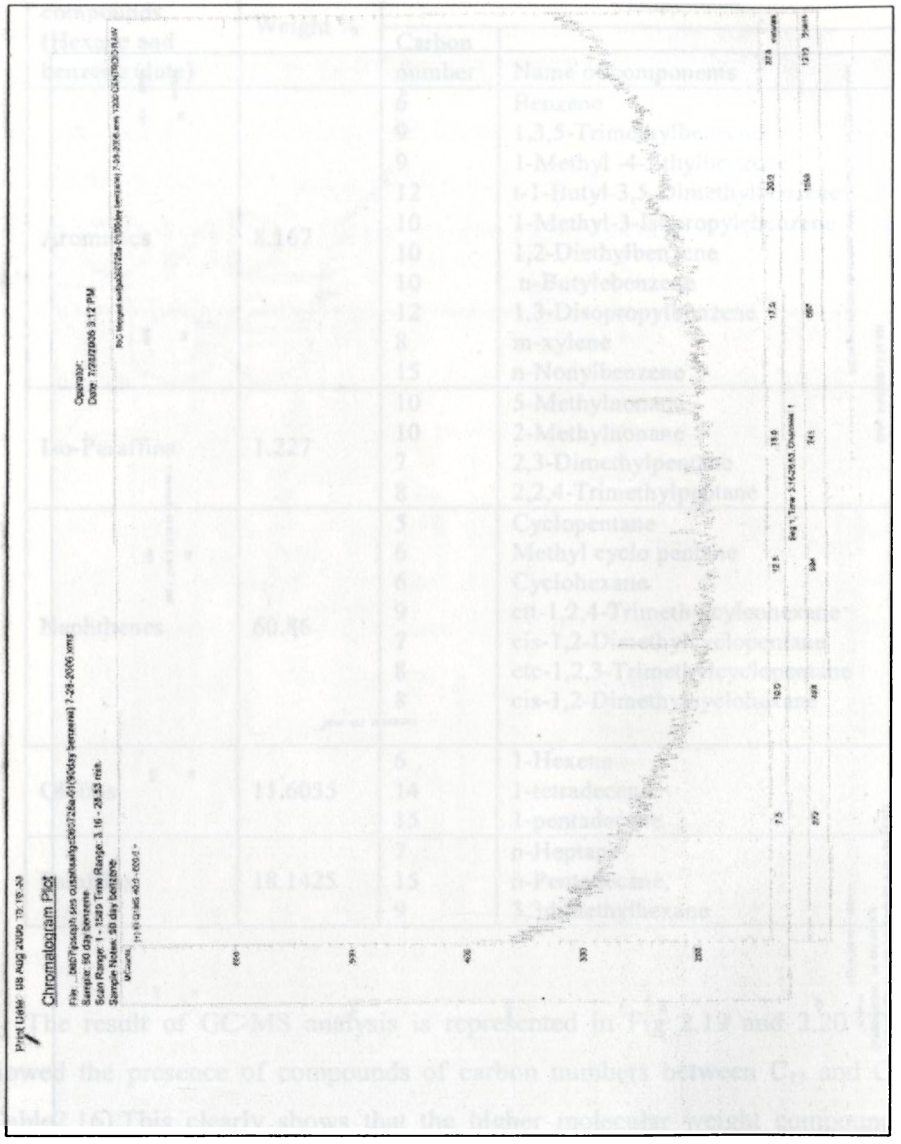

Fig 2.20 Chromatogram of benzene fraction GC-MS after phytoremediation.

Table 2.16 GC-MS data for 90 day phytoremediated soil

Benzene eluted fraction		
Class	Carbon number	Component
Aromatics	17	Phenol,2,6-bis(1,1-dimethylethyl)-4-methyl-,methylcarbamate Pentadecanoic acid,14methyl-,methyl ester
Iso- Paraffins	19	Hexadecane,2,6,10-trimethyl
Paraffins	20	Eicosane

2.3.5 Phytoremediation by different varieties of paddy

All the five varieties of paddy tested survived to harvest in the sludge up to 10%. The result of biomass analysis is presented in Table 2.17 to Table 2.22 along with the TPH estimates. The analysis of variance revealed that the production of biomass was significantly higher for pokkali variety. The reduction of TPH effected by phytoremediation was highest for pokkali variety (Fig 2.21).

Table 2.17 Biomass production by paddy varieties and the respective substrate TPH content at 2.5% sludge concentration.

	Mean biomass ± SD.			TPH (g/kg) ± SD.		
Variety	30 day	60 day	90 day	30 day	60 day	90 day
Pokkali	20.88 ±	45.52 ±	70.66 ±	17.599 ±	13.219 ±	10.32
D1	17.58 ± 1	35.44 ±	52.9 ±	18.305 ±	15.775 ±	12.973 ±
1285	17.5 ± 1	39.3 ±	55.1 ±	18.326 ±	15.328 ±	12.777 ±
Matta	16.85 ±	32.56 ±	54.9 ±	18.135 ±	15.849 ±	12.735 ±
Manikyam	19.9 ±	34.5 ±	56.8 ±	18.05 ±	14.733 ±	12.501 ±

Table 2.18 Analysis of variance of biomass production of different varieties of paddy at 2.5% sludge concentration following 90 days growth.

ANOVA TABLE				
Source	DF	SS	MS	F
Between	4	617.663	154.416	22.846
Within(Error)	10	67.587	6.759	
Total	14	685.249		
Critical F value = 3.48 (0.05, 4, 10) Since F > Critical F REJECT Ho: All groups equal				

DUNNETTS TEST					
Group	Identification	Transformed mean	Mean calculated in original units	T stat	Sig
1	Pokkali	70.667	70.667		
2	D1	52.9	52.9	8.7	*
3	1285	55.1	55.1	7.333	*
4	Matta	54.9	54.9	7.428	*
5	Manikyam	56.8	56.8	6.532	*
Dennett table value =2.47 (1 Tailed value, P = 0.05, df =10,4) * = Significant difference. Ho: Control <Treatment					

Table 2.19 Biomass productions by paddy varieties and the respective substrate TPH content at 5% sludge concentration

	Mean biomass ± standard			TPH (g/kg) ± standard deviation		
Variety	30 day	60 day	90 day	30 day	60 day	90 day
Pokkali	18.24 ±	37.84 ±	43.13± 2.6	19.651 ±	17.975 ±	12.088±.3.00
D1	14.8 ±	30.6 ± 2.5	32.1± 4	19.782 ±	18.216 ±	14.436 ± 2.45
1285	16.5 ±	32.3± 4	31.2 ± 3.6	19.612 ±	18.05 ±	14.839 ± 2.00
Matta	14.6 ±	29.9± 3.2	29.7 ± 4.2	19.725 ±	18.169 ±	14.946 ± 1.40
Manikyam	17.9 ± 3.4	31.75 ±	31.41 ± 1.9	19.776 ±	18.103 ±	14.584 ± 1.50

Table 2.20 Analysis of variance of biomass production of different varieties of paddy at 5% sludge concentration following 90 days growth

ANOVA TABLE				
Source	DF	SS	MS	F
Between	4	786.737	196.684	31.971
Within(Error)	10	61.52	6.152	
Total	14	848.257		

Critical F value = 3.48 (0.05, 4, 10) Since F > Critical F REJECT Ho: All groups equal

DUNNETTS TEST

Group	Identification	Transformed mean	Mean calculated in original units	T stat	Sig
1	Pokkali	65.5	65.5		
2	D1	46	46	9.629	*
3	1285	48.167	48.167	8.559	*
4	Matta	47.133	47.133	9.069	*
5	Manikyam	48.967	48.967	8.164	*

Dennett table value =2.47 (1 Tailed value, P = 0.05, df =10,4) *= Significant difference. Ho: Control <Treatment.

Table 2.21 Biomass productions by paddy varieties and the respective substrate TPH content at 10% sludge concentration

	Mean biomass ± standard			TPH (g/kg) ± standard deviation		
Variety	30 day	60 day	90 day	30 day	60 day	90 day
Pokkali	16.87 ±	33.24 ±	51.16 ±4.4	17.361 ±	16.723 ±	15.422±.600
D1	12.84 ±4.2	24.56 ±	39 ± 4	18.045 ±	17.318 ±	16.681± 445
1285	13.5 ± 4	25.3 ± 2.6	40.16 ±	18.479 ±	17.199 ±	16.553± 345
Matta	11.72 ±	24.65 ±	41.16 ±	17.772 ±	16.923	16.338± 445
Manikyam	16.9 ± 2.2	28.45 ± 3	37.8 ± 4.2	17.692 ±	17.242 ±	16.487± 350

Table 2.22 Analysis of variance of biomass production of different varieties of paddy at 10% sludge concentration following 90 days growth

ANOVA TABLE				
Source	DF	SS	MS	F
Between	4	343.876	85.969	6..304
Within(Error)	10	136.38	13.638	
Total	14	460.256		
Critical F value = 3.48 (0.05, 4, 10) Since F > Critical F REJECT Ho: All groups equal				

DUNNETTS TEST					
Group	Identification	Transformed mean	Mean calculated in original units	T stat	Sig
1	Pokkali	51.167	51.167		
2	D1	39	39	4.035	*
3	1285	40.167	40.167	3.648	*
4	Matta	41.167	41.167	3.316	*
5	Manikyam	37.8	37.8	4.433	*
Dennett table value =2.47 (1 Tailed value, P = 0.05, df =10,4) * = Significant difference. Ho: Control <Treatment.					

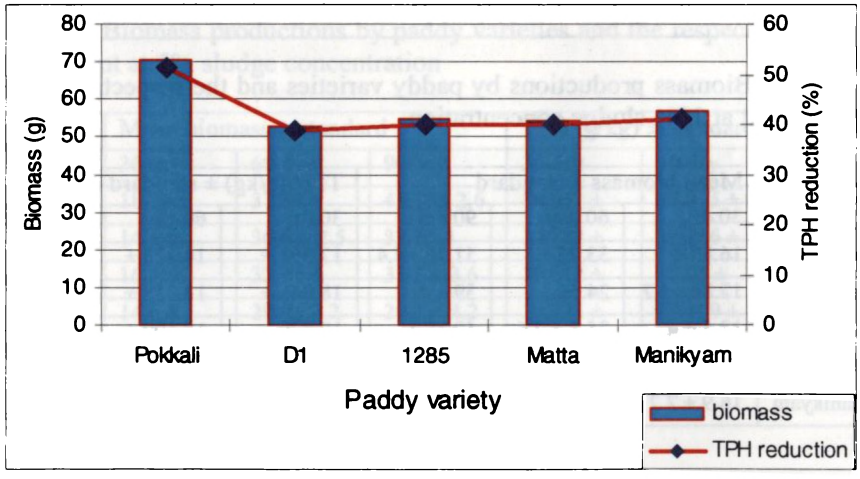

Fig. 2.21 Biomass vs. TPH reduction by different varieties of paddy at 2.5% sludge concentration upon 90 phytoremediation

2.3.6 Effect of surfactant on phytoremediation

The effect of surfactant Tween 80 on the height of the plants is shown in Fig 2.22. Irrespective of the concentration of sludge, the surfactant had inhibitory effect on growth of the plants, but the intensity of inhibition was dependent on the sludge level indicating a synergistic toxicity. The plants did not survive beyond 0.5% and 1% surfactant concentration at 10% and 5% sludge levels respectively. At 2.5% sludge, the plant height decreased with increasing surfactant concentration.

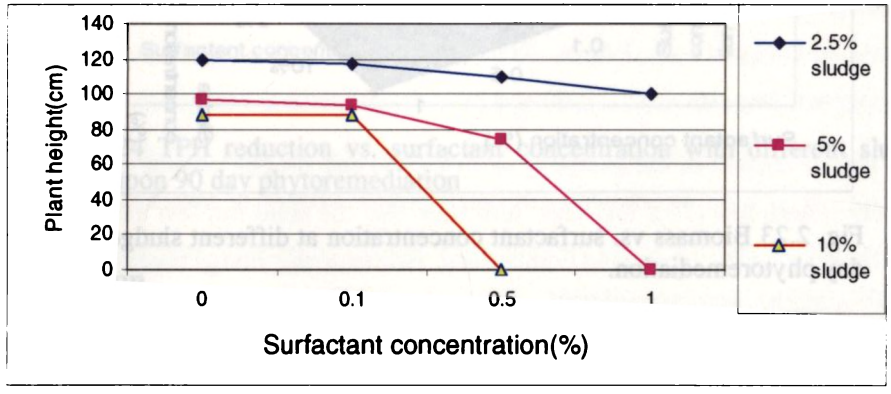

Fig. 2.22 Plant height vs. surfactant concentration at different sludge levels upon 90 day phytoremediation.

Fig. 2.23 illustrates biomass of paddy at 90 day with increasing surfactant concentrations. At 90 day for 2.5% sludge, biomass produced was 70.65 g / pot in the control. It decreased to 57.7 g at 1% surfactant concentration. For 5% sludge concentration with no surfactant, biomass was found to be 65.5 g / pot. The plants survived only up to 0.5% surfactant concentration. For 10 % sludge concentration, plant survived only up to 0.1% surfactant concentration and biomass attained was 24.4 g /pot; 52.34 %less when compared to the control.

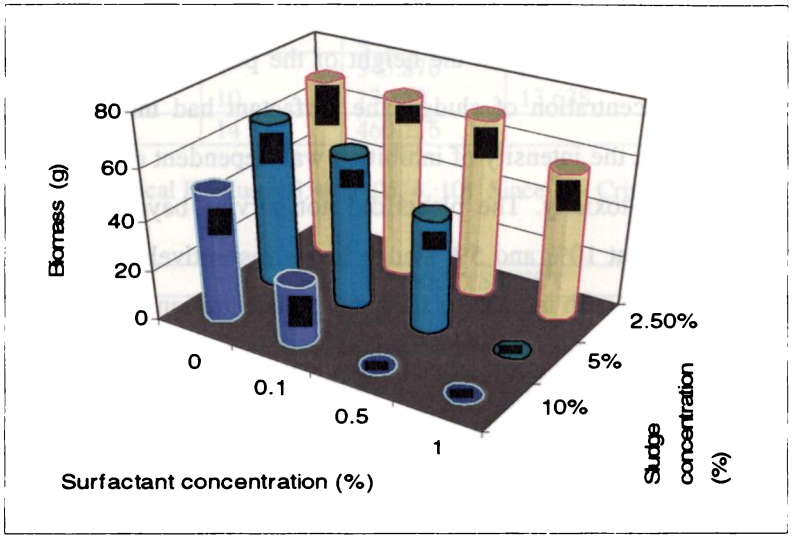

Fig. 2.23 Biomass vs. surfactant concentration at different sludge levels upon 90 day phytoremediation.

Fig.2.24 illustrates TPH reduction at 90 day with increasing surfactant concentrations. At 90 day for 2.5% sludge, TPH reduction was found to be 51.44% in control. It was decreased to 37.82 at 1% surfactant. For 5% sludge concentration with no surfactant, reduction was 33.25 %, and it decreased to 21.5% at 0.5% surfactant concentration. For 10 % sludge concentration, reduction was found to be 22.1 % at 0.1% surfactant concentration. It is obvious that addition of Tween -80 inhibited the growth of the plants as well as the degradation of petroleum hydrocarbons.

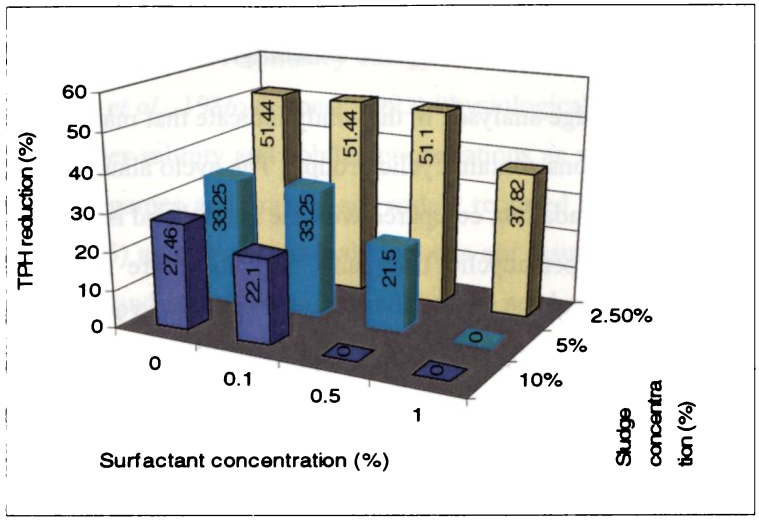

Fig. 2.24 TPH reduction vs. surfactant concentration with different sludge levels upon 90 day phytoremediation

2.4 Discussion.

The oily sludge exhibits high content of petroleum hydrocarbons i.e. about 85 % of which 40% is saturated hydrocarbons. Aromatics and asphaltenes content are similar i.e., 27 %. NSO fraction is 5.33%. In the broadest sense crude oil can be classified in to four organic compound classes: saturates, aromatics, resins, and asphaltenes (Tissot and Welte, 1978). Typically, about 15% of crude oil is comprised of molecules containing hetero atom such as oxygen, sulfur and nitrogen. This fraction includes compounds known by variety of names, including polar, asphaltenes, resins, and NSO (Tisssot and Welte, 1978; Hunt, 1996). Tissot and Welte indicate that the average composition of 527 crude oil samples is 58.2% saturates, 28.6% aromatics, and 14.2% polar compounds; although the absolute values vary widely for different oils. On average, there is rough parity between paraffins, naphthenes and

aromatics. The asphaltenes and resins tend toward high molecular weight (Boduszynski, 1987).This greatly reduces environmental mobility and bioavailability and are described as being environmentally and toxicologically inert (API, 1994).

GC-FID data of the sludge analysed in this study indicate that major portion of the saturate and aromatic fractions contain cyclic group. The cyclo alkanes are known to be more resistant to biodegradation, compared with the normal and isoalkanes, and the tetracyclic (steranes) and pentacyclic triterpenes (hopanes) are among the most resistant compounds of an oil mixture. (Peters and Moldowan, 1993). GC-MS data indicate that higher fractions i.e. above C_{15} contain cyclic groups. Among the components detected in the sludge, 9-methyl Anthracene, 1-Methyl Anthracene and 2-ethyl Anthracene are PAHs. The PAHs are well known as environmental carcinogens (Haeseler *et al.,* 1999) and have the property to bioaccumulate.

The ash content of the sludge is low (4.11%) and the ash content of asphaltenes fraction is also low (15.25%).This indicates that the solid fraction of the sludge is composed primarily of organic compounds. However the sludge contains a variety of metals. It can be reasoned that the metals are not all within the solid phases as the n-pentane insoluble ash is low. This implies that it may not be possible to remove the metals from the sludge by filtering or clarifying the solids. The C: N: P ratio of the sludge was found to be 7500:28:1. Therefore nutrient enrichment was done to provide the nitrogen and phosphorus needed for plant and microbial growth as has also been reported previously. (Fiorenza *et al.*, 2000; Hutchinson *et al.*, 2001).

The germination test showed that paddy seeds could not germinate in the raw sludge. However, upon amending with soil, germination occurred; but the speed of germination and percentage of seed germination decreased $\geq$ 10% of sludge. It may be noted that the basic requirements for seed germination are air, water, and optimum temperature. The seeds embedded in the thick slurry of sludge could have been subject to asphyxiation and toxicity. The higher physico-chemical constituents of the

sludge can disturb the osmotic relationship (Cramer *et al.*, 1987; Cripps *et al.*, 1991). Presence of dissolved constituents with increased BOD, COD and with less O_2, seem to affect the availability of respiratory energy to the early growth of the radical and plumule. (Saxena *et al.*, 1986; Sannasi, 1992).Physiological process of germination is prevented by higher salinity and solute concentrations as well. Reduced plant height and biomass of legumes and grasses are widely reported from oil contaminated site (Merkl *et al.*, 2005) and petroleum hydrocarbons are assumed to interfere with plant life cycle. Debojit and Das (1994) reported that the seed germination of French bean *Phaseolus vulgaris* was affected by the presence of crude oil (0.1%) in the soil.

The seedlings transplanted to the unamended sludge dried out in nearly seven days; but through soil amendment the pokkali paddy variety could be made to grow in soil-sludge mixture up to 60:40 by weight. The growth of the plants in height, and biomass was gradually reduced with increasing sludge concentration, the decrease being highly significant at $\geq$ 10% sludge. This was reflected in seed production as well. Seed sterility was induced by sludge. Phytotoxicity could account for part of the observed poor growth (Anderson *et al.*, 1993; Vouillamoz and Milke, 2001). Phytotoxicity of petroleum hydrocarbon is reported to be highest for low molecular weight and aromatic hydrocarbons (Chaineau *et al.*,1997).The sludge used in this experiment does contain a variety of low and high molecular weight aromatics .

Petroleum hydrocarbon contamination may affect plants by retarding seed germination and reducing plant height, stem density, photosynthetic rate, and biomass, or resulting in complete mortality. (DeLaune *et al.*, 1979; Ferrell *et al.*, 1984, Li *et al.*, 1990; Lin and Mendelssohn, 1996; Pezeshki *et al.*, 2000). In a study conducted by Kulakow *et al.*, (2000) all the legumes tested, died within 60 days when grown on weathered hydrocarbon contaminated sediment. Oil dosage and oil type are two of the most important variables that determine the degree of damage to plants.(Alexander and Webb, 1985).In a study with two fuel oils, the resistance of seeds to oil

contamination followed the decreasing order: sunflower > bean> wheat> clover> maize> barley> lettuce (Chaineau *et al.*, 1997).

The effectiveness of bioremediation of petroleum hydrocarbons has usually been evaluated by measurements of the degradation of total oil (Sandvik *et al.*, 1986; Song *et al.*, 1990; Wang *et al.*, 1990) and a limited number of individual compounds (Douglas *et al.*, 1992).Quantitative changes in oil composition has also been determined as changes in different structurally related fraction of the oils, i.e. that is the fractions of saturated hydrocarbons, aromatic hydrocarbons, resins, and asphaltenes (Oudot, 1984; Fusey and Oudot, 1984).The estimation of biodegradation based on total oil measurements or determination of the structurally related fractions is , however difficult to interpret because the composition of oil changes as the oil degrades, unlike measurements of individual contaminants. However, insight into the compositional changes among different structure related compounds during bioremediation may serve to optimize the remediation scheme and evaluate the soil quality with respect to long term deposition (Jensen *et al.*, 2000).

The cultivation of paddy in the sludge was observed to decrease the TPH content. The % of reduction of TPH decreased with increasing concentration of sludge. Correlation coefficient between biomass production and % reduction of TPH is 0.98, which indicates that plant growth is responsible for the degradation of petroleum hydrocarbon. According to Merkl *et al.* (2005) phytoremediation of petroleum hydrocarbon is effected through stimulated microbial growth, through effective root penetration, and optimal fertilization; the rhizosphere condition created by the plant could degrade PHCs to nearly 50% within 3-4 months in amended sludge. The maximum reduction of 51.4% of PHC occurred at 2.5% sludge at the time of harvest in this investigation. The reduction of TPH decreased with increasing concentration of sludge. Probably the consistency and concentration of the sludge could have reduced

aeration and development of rhizosphere bacteria (Glick , 1995; Siciliano *et al.*, 1997; Burd *et al.*, 1998).

The biomass grown on soil amended sludge showed the tendency to accumulate metals in shoot. Among the metals analyzed Al and Cu recorded the highest accumulation factor of 0.65 and 0.64 respectively. The extent of accumulation in grains was low. In soil, metals are associated with several fractions: 1). In soil solution, as free metal ions and soluble metal complexes, 2) adsorbed to inorganic soil constituents at ion exchange sites, 3) bound to soil organic matter ,4) precipitated such as oxides, hydroxides, carbonates, and 5) embedded in structure of the silicate minerals (Pilon-Smits ,2005). For phytoextraction to occur, contaminants must be bioavailable. Bioavailability depends on metal solubility in soil solution (Farina *et al.*, 1980). It is already clear from the sludge analysis that the metals in this sludge are not all associated with the solid phases. The selective accumulation of metals only reflects their respective behavior of bioavailability. Bioavailability of metals may be enhanced by metal chelators that are released by plants and bacteria. Chelators such as siderophores, organic acids, and phenolics can release metal cations from soil particles. This usually makes the metals more available for plant uptake (Taiz and Zeiger , 2002). Furthermore, plants extrude H+ via ATPases, which replace cations at soil CEC sites, making metal cations more bioavailable. Some plant roots release oxygen, which can lead to the oxidation of metals to insoluble forms that precipitate on the root surface (Horne, 2000). Uptake of inorganic pollutants by plant root is by biological process via membrane transporter proteins. These transporters occur naturally because inorganic pollutants are either nutrients themselves or are chemically similar to nutrients and are taken up inadvertently (Abedin *et al.*,2002).

A variety of environmental factors affect or alter the mechanisms of phytoremediation. Soil type and organic matter content can limit the bioavailability of petroleum contaminants. Water content in soil and the availability of oxygen required

for aerobic respiration, temperature, and nutrient availability can influence the rate and extent of degradation in oil-contaminated soil (Huddleston *et al.*, 1986).Adequate soil nutrients are required to support the growth of plants and their associated microorganisms. This may be especially true during phytoremediation efforts, when the plant/microbe community is already under stress from the contaminant. Xu and Johnson (1997) have shown that petroleum hydrocarbons can significantly reduce the availability of plant nutrients in soil. Low nutrient availability results from the fact that petroleum hydrocarbons have high carbon contents, but are poor suppliers of nitrogen and phosphorus. As soil microorganisms degrade the hydrocarbons, they use up or immobilize available nutrients creating nutrient deficiencies in contaminated soil. Biederbeck *et al.* (1993) found that, following initial applications of an oily waste sludge to sandy soil, the soil had very low nitrate levels due to immobilization of nitrogen by rapidly growing populations of oil degrading bacteria as well as suppression of nitrogen-fixing bacteria. Two years following oil application, however, sludge treated plots contained more nitrate than untreated controls, presumably due to the gradual remineralization of the previously immobilized nitrogen. The trend was similar for phosphorus, which was initially low following incorporation due to immobilization by an expanding microbial biomass, but became more available one year later.

Petroleum hydrocarbons also may limit the accessibility of nutrients to plants and microorganisms by reducing the availability of water in which the nutrients are dissolved (Schwendinger, 1968). Nutrient deficiencies in soil caused by petroleum hydrocarbons may be offset by the application of fertilizer or green manure to the soil. For pokkali rice, at 2.5% sludge concentration, optimum nutrient level (C:N:P) was found to be 100:20:1. As the sludge concentration increased, the plant survived at low nutrient level i.e. at 5% sludge concentration 100:10:1 C:N:P and at 10% sludge concentration, 100:5:1 nutrient level.

It is reported that when the population of indigenous microorganisms capable of degrading the target contaminant is less than 10^5 CFU/g of soil, bioremediation will not occur at a significant rate (Forsyth *et al.*,1995). Addition of nutrient, mineral fertilizers, different agricultural byproducts, and molasses along with bacterial inoculation has been reported to enhance the degradation process (Al-Hadhrammi *et al.,* 1997; Calvo *et al.*,1997; Olivera *et al.,* 1997).

An initial bacterial population of about 10^3 CFU/g was observed at the initiation of the phytoremediation. An increase of microbial populations was encountered in all amended soil samples particularly with 2.5% sludge upon plant growth. 10^9 CFU/g bacterial populations were observed in soil samples amended with 2.5% sludge at 90 days of phytoremediation. Several researchers have described an increase in microbial activity and rate of biodegradation following addition of inorganic nutrients (Radwan *et al.*, 2000; Vasudevan and Rajaram, 2001). At sludge concentration >10 %, microbial population was found to be decreasing. Brown *et al.* (1983) reported that the population of total soil bacteria is greatest when 1% of these sludges is added to the soil ; whereas, 5 and 10% sludge additions results in slightly lower microbial populations. This may be due to the toxic effect of sludge which prevents the bacterial proliferation, exceeding the soil-binding capacity and resultant waste migration, oxygen starvation caused by demand exceeding transfer rate and water exclusion by hydrophobic components (Huddleston *et al.*, 1986). It may be assumed that at fertilization rate of 100:20:1, there is high biomass production of paddy which facilitates high rhizosphere microflora and thereby high degradation of TPH at 2.5% sludge.

The effect of biodegradation on the molecular composition of petroleum hydrocarbon has been studied extensively. (Fedorak and Westlake,1981; Oudot 1984; Bossert and Bartha 1986; Peters and Moldowan,1993) and as a result general agreement exists about the relative rates of aerobic biodegradation i.e. linear alkanes >

branched chained alkanes > aromatic hydrocarbons. Within the aromatic fraction the degradation rates decrease with increasing number of rings and according to the alkylation of the molecule (Fedorak and Westlake, 1981). In the present investigation, TPH (fractionated) content at time zero and at the time of harvest are compared. It could be seen that saturate fraction degraded more compared to other classes. Asphaltenes was the least degraded class. The degradation was in the order 68.65 % for saturates, 55.48% aromatics, 31.86 % NSO and 24.78% asphaltenes. A comparison of the composition of the saturate and aromatic fraction before and after phytoremediation is given below. Among the aromatics prior to phytoremediation compounds C_{10} to C_{26} were present. After phytoremediation compounds C_6 to C_{17} were only were found. Similarly in iso-paraffins, compounds C_7 to C_{27} present. After phytoremediation compounds only up to C_{19} were present in the substrate. In the case of Naphthenes also before phytoremediation, C_6 to C_{19} were present; but after phytoremediation compounds C_5 to C_9 were detected. Olefin and paraffin components have come to acquire C_{15} compound; probably this has arisen by degradation of a higher molecular weight compound.

Many of the concerns about the adverse environmental impact of petroleum hydrocarbon are related to the bioavailability of individual hydrocarbons, particularly those on the U. S. Environmental Protection Agency (EPA) list of Priority Pollutants (Keith *et al.*,1979). The sludge was detected to contain 9-methyl Anthracene, 1-Methyl Anthracene, 2-ethyl Anthracene that are PAH compounds. PAHs with log K_{ow} values above 4 are not considered to be mobile within the environment, however, those with a value less than 4 (for example naphthalene and phenanthrene) readily enter into food chain, concentrating from the primary levels to the top of the food chain because of the slowness of their degradation in biota . In phytoremediated soil, there was no PAH compound detected compared to the non-phytoremediated sample. It is reported that PAH dissipation was faster in rhizosphere soils compared with

Table 2.23 Comparison between TPH content at time zero and after 90 day of phytoremediation

Time zero			After 90 days of phytoremediation.	
Class	Carbon Number	Component	Carbon Number	Component
Aromatics	12	t-1-Butyl-3,5-	6	Benzene
	14	Dimethylbenzene	8	m-xylene
	14	n-Octylbenzene	9	1,3,5-Trimethylbenzene
			10	1-Methyl -4-Ethylbenzene
	15	Benzene,1,1-(1-fluoro-		t-1-Butyl-3,5-
		1,2-ethenediyl)bis-,(E)	10	Dimethylbenzene
	15	1Methyl-4-n-propyl-	10	
		benzene.		1-Methyl-3-
	15	n-Nonylbenzene	10	Isopropylebenzene
				1,2-Diethylbenzene
	15	Anthracene, 9-methyl	12	n-Butylebenzene
	15	Anthracene,1-Methyl	12	1,3-Disopropylbenzene
	16	1,1-Biphenyl,2-(1azido-		
		1-methylethyl)-		
			15	n-Nonylbenzene
	16	Anthracene, 2-ethyl		
	26	1,2,4-Triazine,5,6-		
		diphenyl-3-(4-phenyl-2-		
		pyridinyl)-		
Iso-Paraffins	7	2,3-Dimethypentane.	7	2,3-Dimethylpentane
	8	2,2,4-Trimethylpentane	8	2,2,4-Trimethylpentane
	8	3,4-Dimethylhexane	10	5-Methylnonane
		(D)	10	2-Methylnonane
	8			
		3-Methylheptane		
	19			
		Pentadecane,2,6,10,14-		
		tetramethyl		
	20			
		Hexadecane,2,6,10,14 –		
		tetramethyl		
	27			
		Heptacosane		

Naphthenes	6 6 7 8 8 10 19	Methyl cyclo pentane. Cyclohexane Cis-1,2 dimethylcyclopentane Ctc-1,2,3 trimethylcyclopentane Cis-1,3 dimethylcyclohexane Sec-butyle cyclohexane 2,4,6-Tris(1,1-dimethyl)-4methylcyclohexa-2,5-dien-1-one $C_{19}H_{32}O$	5 6 6 7 8 8 9	Cyclopentane Methyl cyclo pentane Cyclohexane cis-1,2-Dimethylcyclopentane cis-1,2-Dimethylcyclohexane ctc-1,2,3-Trimethylcyclopentane ctt-1,2,4-Trimethylcylcohexane
Olefins	6 8 14	1-Hexene trans-2-Octene 1-Tetradecene	6 14 15	1-Hexene 1-tetradecene 1-pentadecane
Paraffins.	7 8 14	n-Heptane 3,3-Dimethylhexane n-Tetradecane	7 8 15	n-Heptane 3,3dimethylhexane n-Pentadecane

unplanted bulk soil (Harvey *et al.*, 2001).Degradation of phenanthrene was faster in rhizosphere soils planted with slender oat compared with unplanted bulk soil controls and correlated with an increased number of degraders in the rhizosphere soil (Miya and Firestone,2000).The same has been reported for the degradation of several organic chemicals in the rhizosphere of alfalfa and alpine bluegrass suggesting the potential stimulation of bioremediation around plant roots (Nichols *et al.*, 1997).

Previous research has shown that phytoremediation is not always an efficient way to remove persistent organic contaminants. (Huang *et al.*, 2004). For phytoremediation to become an effective method for removal of persistent contaminants, many limitations of the existing technology (US EPA, 2000) must be overcome. One serious limitation is that many plant species are sensitive to contaminants (Rock, 1997; US EPA, 2000). Therefore they grow slowly and do not accumulate sufficient biomass, in heavily contaminated soil for effective bioremediation. It may be possible to facilitate phytoremediation through selection and acclimation of more contaminant tolerant varieties/species. The present observation is that all the five paddy variety could grow well in the sludge upto 10%; but pokkali was superior in accumulating biomass as well as reducing the TPH. The variety is derived from saline habitat growing in muddy substratum and this obviously should give it the capacity to grow in the sludge-soil substrate.

Adding surfactant to soil contaminated with hydrophobic contaminants may increase the bioavailability of these compounds to hydrocarbon degrading microorganisms (Banat *et al.*, 1991; Banat, 1995).Surfactant-substrate interaction, such as emulsification, solubilization, and partitioning of hydrocarbons between phases, can influence accessibility of substrates to microorganisms(Rouse *et al.*, 1994). The present result indicates that chemical surfactant such as tween-80 does not have a role in enhancing the biodegradation of petroleum hydrocarbons, neither they are promoting plant growth. This is substantiated by previous studies (Rouse *et al.*, 1994). Both enhancement and inhibition of biodegradation of organic compounds have been reported in the presence of surfactants. The surfactant may have interfered with the direct interaction between cells and substrate or it might be used as a growth substrate by microorganisms in preference to the contaminant (Deschenes *et al.*, 1995; Ripper et al 1992).

2.5 Conclusion

It is concluded from the study that the paddy variety pokkali can be effectively used for the phytoremediation of petroleum contaminated soils in an ex-situ treatment facility. A maximum of 51.4% removal of total petroleum hydrocarbons of oily sludge have been achieved within 90 days for 2.5% sludge and 33.25 % for 5% sludge level. The degradation pattern is saturate > aromatic> NSO> asphaltenes. Paddy is not cited as ideal plant for phytoremediation of unamended sludge as the latter has proved to be toxic to dry off the plant within a week of transplantation.

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CHAPTER 3

LAND FARMING OF OIL REFINERY SLUGE

Abstract

Bioremediation is a cost effective means of remediating soils contaminated with petroleum hydrocarbons by the use of indigenous or selected microbial flora. Various factors affecting the efficiency of the process are aeration, nutrients, the type of microbial species, and composition of hydrocarbons. Experiments were conducted for the bioremediation of oily sludge with soil amendments in the presence of a bacterial consortium, inorganic nutrients and a bulking agent (rice straw). Experiments were undertaken in lab conditions for 13 weeks. The results of the present study indicated that the use of bulking agent played an important role in the bioremediation of oil-contaminated soil. A bulking agent such as paddy straw could improve the inherent microbial growth thereby enhancing the degradation of PHCs. Bulked soil showed 45.42% of TPH degradation with corresponding increase in microbial populations compared to abiotic control. Inoculation of microflora into the system did not produce a significant reduction of TPH contrary to many previous results. Qualitative analysis of the spectrum of components has shown that biostimulation does degrade the PHCs fractions selectively in the order saturates> aromatics> asphaltenes >NSO.

3.1 Introduction

Petroleum refining results in the production of large quantities of oil sludge consisting of hydrophobic substances and substances resistant to biodegradation. Clean-up technologies such as incineration and burial of sludge in secure landfills are expensive. Land spreading and land farming are the traditional methods of petroleum sludge disposal (Bartha, 1986, Persson and Welander., 1994). Controlled land treatment i.e. **land farming**, is cheaper and also environmentally safe (Bonnier *et al.*, 1980; El-Nawawy *et al.*, 1987).In land spreading, the sludge is evenly dispersed over a plot of land where it can be degraded by native microbial flora over a period of months or years. In land farming, the sludge is blended into the soil with tilling equipment, often with the addition of fertilizer to increase the rate of degradation. The primary mechanisms involved in the disappearance of hydrocarbons in land spreading and land farming are biodegradation, vaporization, oxidation, and to some extent degradation by sunlight and leaching. When the sludge has been substantially degraded, the plot of land can be used again for further sludge treatment.

Aerobic conditions and appropriate microorganism are necessary for an optimal rate of bioremediation of soils contaminated with petroleum hydrocarbon. (Vasudevan and Rajaram, 2001). In soils, the oxygen content depends on microbial activity, soil texture, water content and depth. Low oxygen content in soil has been shown to limit bioremediation of soils contaminated with petroleum hydrocarbons. (von Wedel *et al.*, 1988). Tillage is a mechanical manipulation of soil to improve soil conditions and alters physical and chemical properties of soil and improves microbial activity (Hillel, 1980; Melope *et al.*, 1987). Tillage redistributes carbon, nitrogen, water, and reduces spatial distribution within the soil (Rhykerd *et al.*, 1999).

Composting by way of biostimulation and bioaugmentation is a well studied bioremediation technique (Haug, 1994). Biostimulation along with bulking agents enhances biodegradation. Bulking agents are materials of low density that lower soil bulk density, increase porosity and oxygen diffusion, and can help to form water-stable aggregates. These activities increase aeration and microbial activity (Hillel, 1980, Dickinson and Rutherford, .2006).Compost and sewage sludge were quite useful in remediating diesel-contaminated soil. (Namkoong *et al.*, 2001).

Fyock *et al.* (1991) composted petroleum sludge on large concrete pad using sawmill waste as the bulking agent, with one part sludge to two parts wood. They reported 98.8% drop in linear alkanes after 40days. The total petroleum hydrocarbon content of the compost material dropped from 10% initially to a final value of 1%. Nordrum *et al.* (1992) reported on the same test and indicated that this sludge was from a light crude oil refinery. The TPH dropped from an initial value of 60,000 ppm to 20,000 ppm in 2 weeks. Jack *et al.*(1994), used heat-treated peat moss as the bulking agent for composting oil bottoms at a refinery. They reported that adding sludge at a ratio of 1:2 to the peat gave 78% degradation in eight months. For sludge from a heavy oil refinery they reported only 50% degradation in the same time frame.

The dynamics of compost degradation require the soil waste to be first hydrolyzed, usually by enzymes or acids released by the microbial cells, dissolution of the hydrolyzed solids into the aqueous phase; and then consumption of the dissolved solids by microbial cells. This underlines the importance of adequate moisture content in the compost, as the solubilization process is usually the rate controlling step and adequate contact between water and the solid substrate is imperative(US EPA 1998; Headley *et al.*, 2000).

Bioaugmentation involves the supplementation of microorganism to degrade the pollutants involved (Baud-Grasset and Vogel, 1995). One way to enhance biodegradation of organic compounds is to inoculate the environment with

microorganisms that are known to metabolize these chemicals readily (Sepic *et al.*, 1995). There is considerable evidence that bioaugmentation enhances degradation of a variety of organic contaminants in soils and surface biological waste treatment processes. For this approach to be successful in subsurface environments (Goldstein *et al.*,1985) the added microorganisms

- must be able to survive in what to them is a foreign, hostile environment and compete for nutrients with indigenous organisms.
- must be able to move from a point of injection to the location of the contaminant at what are very often low concentrations, in a medium where bacterial transport is normally very slight, especially in fine – grained materials.
- must be able to retain their selectivity for metabolizing compounds for which they were initially adapted.

It was shown that an inoculum of *Cellulomonas* sp. and nutrients was able to degrade the hydrocarbon contaminants more effectively than just fertilizer alone (Schwendinger, 1968). Venkateswaran *et al.* (1995) showed that 35% of light crude oil resins that were polycyclic aromatic compounds were degraded by a *Pseudomonas* species, suggesting that perhaps bioremediation may also work on certain fractions of resins and asphaltenes. The seeding of microorganisms has been used in a number of different environments to degrade organics (U.S EPA, 1985).

The aim of this study was to bioremediate oil refinery sludge through a land farming approach. The method of biostimulation and bioaugmentation was applied.

3.2 Materials and methods.

3.2.1 Remediation of petroleum sludge by biostimulation.

The objective of the experiment was to provide congenial condition for microbial growth so as to promote degradation of petroleum hydrocarbons. The petroleum sludge was mixed with soil at 2.5% w/w as done for the previous phytoremediation. These samples of 1.5kg each were taken in replicate plastic trays. The samples in three trays were left as such with regular watering in the ambient laboratory conditions under subdued light to serve as abiotic controls. The rest of the samples were enriched with nutrients at two levels i.e. C:N:P 100:5:1 and 100:10:1 .Powdered paddy straw was added as bulking agent(2.5% w/w) to one set of samples . The treatment combinations were as given below.

1. Soil + Oil sludge (abiotic control).
2. Soil + Oil sludge + Nutrients (100:5:1)
3. Soil + Oil sludge + Nutrients (100:10:1)
4 Soil + Oil sludge + Nutrients (100:5:1) + Straw
5. Soil + Oil sludge + Nutrients (100:10:1) + Straw.

The trays were maintained in the laboratory at ambient temperature (28 ± 4 °C) throughout the investigation period of thirteen weeks. The trays were watered regularly to retain 60-70% moisture. The soils were turned over twice a week to expose a new layer to air. pH of the system was monitored weekly throughout the experiment and adjusted to 6-8 by the addition of calcium hydroxide. The TPH was estimated gravimetrically on 30, 60 and 90th days of observation after extracting in n-hexane, dichloromethane, and chloroform solvent system (100 ml each) successively, and pooled and dried in a rotary evaporator and weighed. Fractionation of TPH was

done on the 90th day and GC-FID analysis carried out. The microbial count (CFU) was determined on weekly basis.

3.2.2 Bioaugmentation Experiment

The sludge was mixed with soil at 2.5% w/w, and enriched with nutrients as done for the biostimulation experiment. The soil samples in three replicate trays were inoculated with 10ml of microbial inoculum of 10^8 CFU/ml. The experiment was set up as in the previous one for biostimulation.

Preparation of microbial inoculum

The source of microbial inoculum was the rhizosphere soil of the phytoremediation trials set up previously. The soil/sludge mixture was dispersed in sterile water suspension and inoculated into mineral medium agar of composition 0.8 g/l K_2HPO_4 , 0.2g/l KH_2PO_4 , 0.05 g/l $CaSO_4.2H_2O$, 0.5 g/l $MgSO_4 H_2O$, 0.09 g/l $FeSO_4.7H_2O$, 1.0 g/l $(NH_4)_2SO_4$, and 15 g/L agar (Bardi *et al.*, 2000) to which a sludge extract was added as carbon source. The petridishes were incubated at 28°C for 5 days. The random pool of colonies developed was transferred to 200 ml of the above mineral medium containing sludge extract and incubated on rotary shaker at 180 rpm for 5 days. This mixed culture was stored at 4°C and used as inoculum. The treatment combinations were as given below.

1. Soil + Oil sludge (abiotic control)
2. Soil + Oil sludge + Nutrients (100:5:1)
3. Soil + Oil sludge + Nutrients (100:10:1)
4 Soil + Oil sludge + Nutrients (100:5:1) + Microbial inoculum
5. Soil + Oil sludge + Nutrients (100:10:1) + Microbial inoculum

Changes in TPH of the soil samples were monitored over time by gravimetric method after extracting in n-hexane, dichloromethane, and chloroform solvent system (100 ml each) successively, and pooled and dried in a rotary evaporator and weighed.

Fractionation of TPH was done on the 90th day to determine the gravimetric proportions of saturate, aromatic, NSO and asphaltenes fractions. The microbial count was determined on weekly basis.

3.3 Results

3.3.1 Effect of biostimulation

The initial TPH of the experimental sample was 21.27 g/kg. It reduced gradually to 19.8 g/kg in the abiotic control within 90 days. (Table3.1). Enrichment of sludge with nutrients increased the rate of degradation of TPH though there was no significant difference between the two nutrients levels. Straw amended treatments recorded lower levels of residual TPH (Table 3.2). The maximum TPH reduction of 45.42% was obtained at N: P 10:1 upon addition of straw in 90 days.

Table 3.1 Changes in total petroleum hydrocarbon (g/kg) content during biostimulation experiment.

Category		TPH concentration g/kg		
		30 day	60 day	90day
1	Abiotic control	21.26	20.85	19.8
2	2.5% sludge + nutrient (100:5:1)	17.43	14.88	13.40
3	2.5% sludge + nutrient (100:5:1) + straw	17.73	14.80	12.48
4	2.5% sludge + nutrient (100:10:1)	16.95	13.06	13.05
5	2.5% sludge + nutrient (100:10:1) + straw	17.13	12.97	11.61

Table 3.2 Test of significance between categories of biostimulation experiment based on 90th day residual TPH value

Category	t-value	Significance
2 & 4	1.652	No significant difference at alpha =0.05
3 & 5	6.9091	Significant difference at alpha =0.05
2 & 3	4.0895	Significant difference at alpha =0.05
4 & 5	12.8557	Significant difference at alpha =0.05

Fig. 3.1 and 3.2 depicts the comparative reduction of TPH after 90 days of biostimulation with respect to abiotic control. Biostimulation certainly has a positive effect on biodegradation of petroleum hydrocarbons.

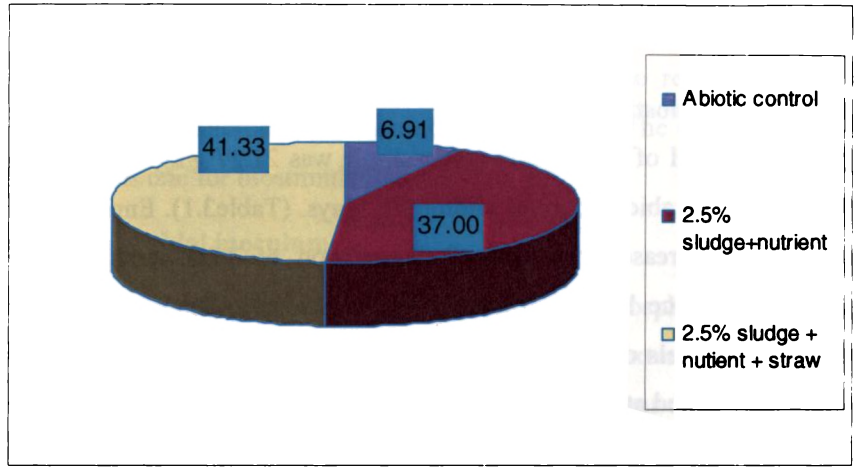


Fig. 3.1 Comparative display of TPH reduction (%)following biostimulation in 100:5:1 nutrient concentration with respect to abiotic control.

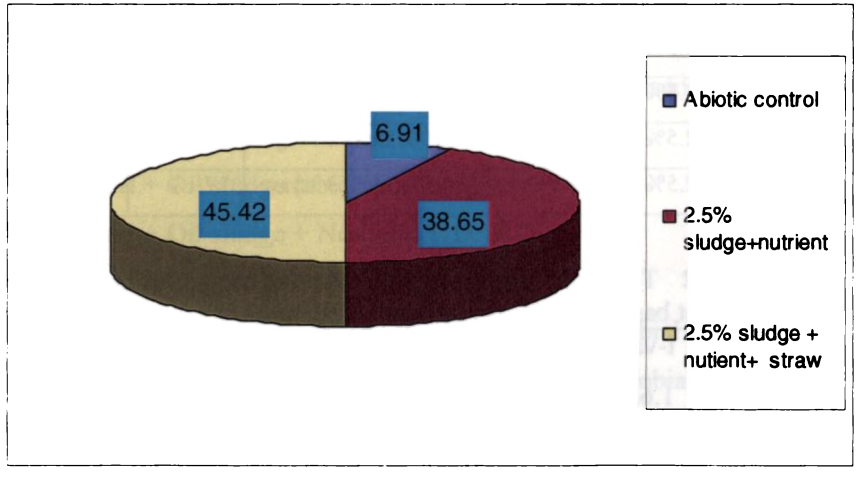
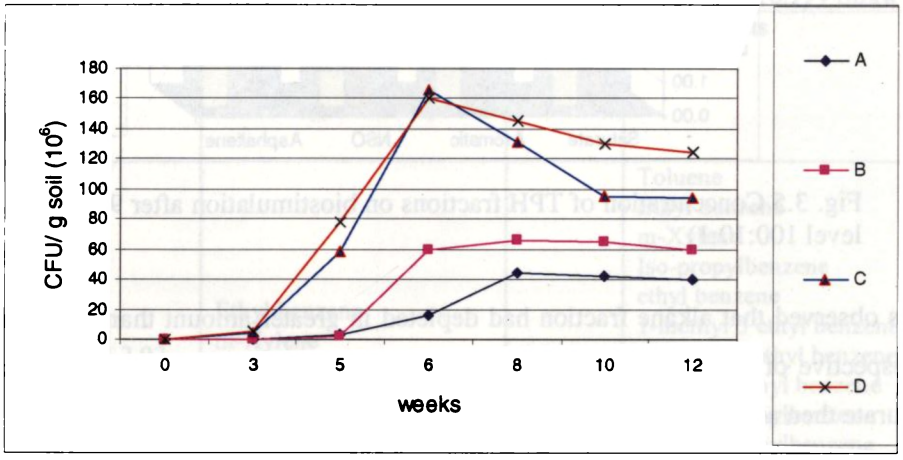


Fig. 3.2 Comparative display of TPH reduction (%) following biostimulation in 100:10:1 nutrient concentration with respect to abiotic control

The bacterial count in abiotic control ranged from 10^3 to 10^4 CFU/g soil, while biostimulation increased the microbial count. The microflora increased exponentially up to 6th week to 10^8 CFU/gin nutrient level 100:10:1 and further stabilized. The microbial population was lower at the low nutrient level (Fig. 3.3).



A=2.5% sludge+ nutrient (100:5:1) ,**B**=2.5% sludge +nutrient (100:5:1) + Straw
C=2.5% sludge +nutrient (100:10:1),**D**=2.5% sludge +nutrient (100:10:1) +Straw

Fig. 3.3 Microbial count during biostimulation.

The treated samples were fractionated for studying the effect of biostimulation on different components of oil sludge. The results are presented in Fig 3.4 and 3.5. It

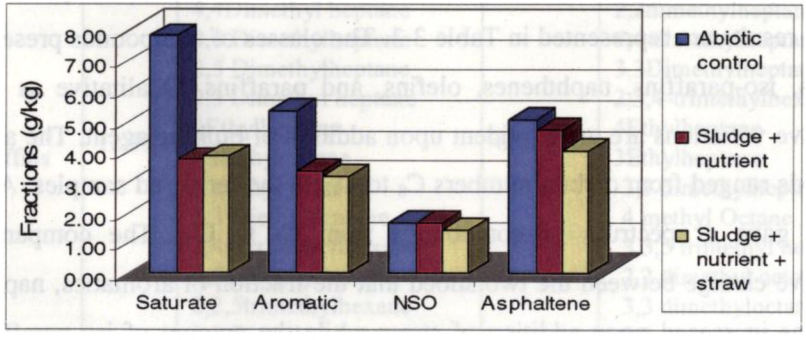


Fig. 3.4 Concentration of TPH fractions on biostimulation after 90 days (nutrient level 100:5:1)

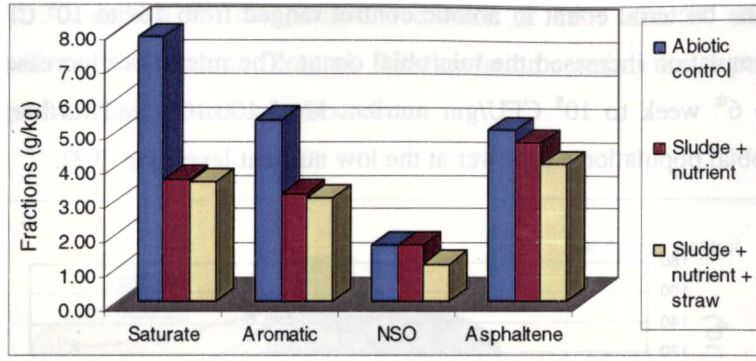


Fig. 3.5 Concentration of TPH fractions on biostimulation after 90 day (nutrient level 100:10:1)

was observed that alkane fraction had depleted in greater amount than other fraction irrespective of the nutrient level. The order of decrease in concentration was firstly saturate then aromatic and asphaltenes, finally least degraded was NSO fraction.

The reduction of saturate was 50.95%, Aromatic 40.14, Asphaltenes, 19.69 and NSO by 13.23 % in 100:05:01 with straw. In nutrient amendment 100:10:01 with straw the reduction of saturate was 55.37%, Aromatic 43.53, Asphaltenes, 34.34 and NSO by 19.77 % .

The degraded fractions extracted in hexane and benzene was analyzed by GC-FID. The results are represented in Table 3.3. The classes of compounds present were aromatics, iso-paraffins, naphthenes, olefins, and paraffins. Qualitative as well as quantitative variations are quite evident upon addition of bulking agent. The aromatic compounds ranged from carbon numbers C_8 to C_{12} in the fertilized samples. Addition of straw gave a spectrum of compounds from C_7 to C_{10}. The comparison of quantitative change between the two shoed that the fraction of aromatics, naphthenes and olefins increased upon addition of straw while the amount of iso-paraffins and paraffins decreased.

Table 3.3 Identified components of petroleum hydrocarbons (below carbon number 15) upon biostimulation

Class of compounds (Hexane and benzene elute)	Sludge +nutrient (100:10:1)		Sludge +nutrient (100:10:1) + Straw	
	Weight %	Components	Weight %	Components
Aromatics	2.02	Ethyl benzene m-Xylene n-pentylbenzene n-hexylbenzene	4.829	Toluene Ethyl benzene m-Xylene Iso-propylbenzene ethyl benzene 1-methyl 3 ethyl benzene 1 methyl 4 ethyl benzene 1,3,5 trimethyl benzene 1,2,3 trimethylbenzene 1,2,4 trimethylbenzene tert-butyl benzene isobutylbenzene sec-butylbenzene
Iso-Paraffins	12.99	3,3 dimethyl pentane 2,3 dimethyl pentane 2,2 Dimethylhexane 2,3,3 -Trimethylpentane 2,3,4-Trimethylhexane 4,4Dimethyl heptane 2,6 Dimethylheptane 2,5 Dimethylheptane 2,3 Dimethyl heptane 4Ethylheptane 4 methyloctane 3 methyl ocatane 3,3 Diethylpentane 2,4,6 trimethylhexane 2,5 dimethylhexane 2,2,5trimethylhexane 2,3 Dimethyl heptane 3,3-diethylpentane 2,4,6 trimethylhexane 2,2dimethyloctane	6.047	3-methyl pentane 2-methyl 3-ethylpentane 2-Methylheptane 2,2,4-Trimethylhexane 2,3,5-trimethylhexane 2,2dimethylheptane 4,4 dimethylheptane 3,3Dimethylheptane 2,3,4-trimethylhexane 4Ethylheptane 3Ethylheptane 3,3 dimethylheptane 4 methyl Octane 3,3,5 trimethyl heptane 2,2 dimethyl octane 3,3 dimethyloctane 2,3,dimethyl octane 3-methyl nonane

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Naphthenes	44.77	Methyl cyclo pentane Cyclohexane Trans-1,3Dimethylcyclopentane Methyl cyclo hexane 1,1,3-Trimethylcylcopentane ctc-1,2,4-Trimethylcyclopentane ccc-1,2,3-Trimethylcylcopentane cis-1,2ethylmethylcycopentane n-Propyl cyclopentane Ethylcyclohexane 1,1,3Trimethylcyclohexene 1,1,4-Trimethylcyclohexane 1,1,2Trimethylcyclohexane trans-1,4ethyl methyl cyclohexane cis-1,2Dimethylcyclopentane ctc-1,2,3-Trimethylcyclopenane	50.994	Methyl cyclo pentane Cyclohexane 3methylcyclopentane Ethylcyclopentane iso-propylecyclopentane n-propylcyclopentane 1,1,3Trimethylcylcohexane ctt-1,2,4-Trimethylcylcohexane Cis-1,2 dimethyl cyclopentane ctc-1,2,3 trimethylcyclopentane cis-1,2 dimethyl cyclo hexane ccc-1,3 ,5 trimethyl cyclohexane 1,1,3 trimethyl cyclohexane Isopropyl cyclohexane n-butyle cyclopentane
Olefins	8.03	1-Hexene Trans-3-Heptene 2,4,4-Trimethyl pentene-2 1-Dodecane	20.616	1-Methyl cyclo pentene 1-Hexene Trans-3-Heptane 2,2,4-Trimethyl pentene-1 cis-2-Octene 1-Nonene trans-4-Nonene trans-2-Nonene 1-Decene
Paraffins	32.18	n-Heptane cis-3-Nonane n-nonane trans-2-Nonane 2,3,4 trimethyl pentane n-Heptane 3,3 dimethyl hexane	17.5055	n-nonane n-pentadecane n-Heptane 3,3 dimethyl hexane n-pentadecane

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3.3.2 Bioaugmentation experiment.

The TPH of the sample decreased upon microbial inoculation (Table 3.4). However addition of microbial inoculum did not produce any statistically significant effect compared to the respective enriched samples (Table 3.5) as concluded by t-test estimate.

Table 3.4 Changes in TPH during bioaugmentation.

Category		TPH reduction g/kg		
		30 day	60 day	90day
1	Abiotic control	21.26	20.85	19.8
2	2.5% sludge + nutrient (100:5:1)	17.43	14.88	13.40
3	2.5% sludge + nutrient (100:5:1)+ Microbial inoculum	16.58	14.46	12.67
4	2.5% sludge + nutrient (100:10:1)	16.95	13.06	13.05
5	2.5% sludge + nutrient (100:10:1)+ Microbial inoculum	16.37	14.24	12.63

Table 3.5 Test of significance between categories of bioaugmentation experiment based on 90th day residual TPH value

Category	t-value	Significance
2 & 4	0.6555	No significant difference at alpha =0.05
3 & 5	0.0796	No significant difference at alpha =0.05
2 & 3	1.2022	No significant difference at alpha =0.05
4 & 5	0.8986	No significant difference at alpha =0.05

Fig 3.6 and 3.7 illustrates the comparative reduction of TPH after 90 day of bioaugmentation with respect to abiotic control

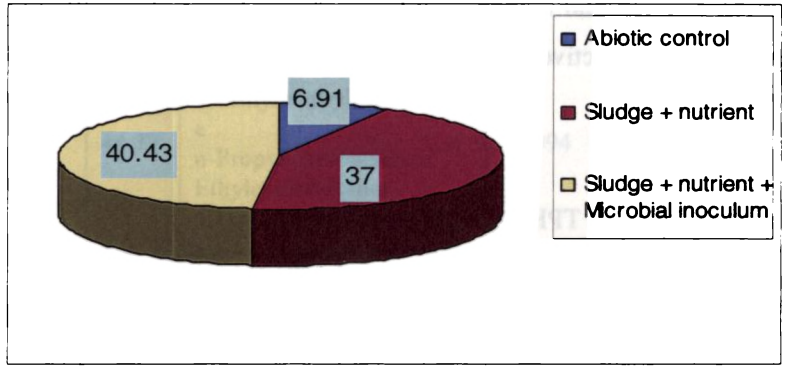


Fig. 3.6 Comparative display of TPH reduction (%) following bioaugmentation in 100:05:1 nutrient concentration with respect to abiotic control

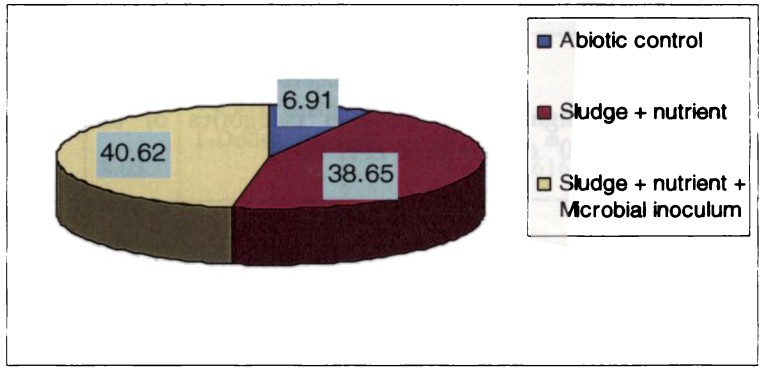
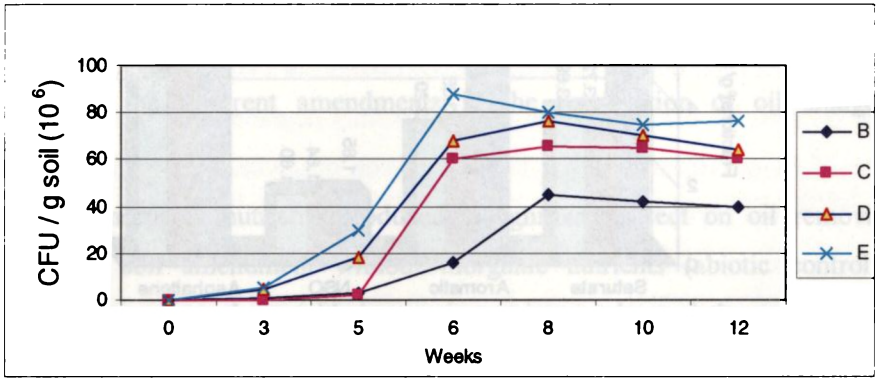


Fig. 3.7 Comparative display of TPH reduction (%) in following bioaugmentation 100:10:1 nutrient concentration with respect to abiotic control

The bacterial count in abiotic control ranged from 10^3 to 10^4 CFU/g soil, while in the enriched and inoculated samples microflora increased to 10^7 CFU /g soil. Data showed increasing trends between three and six weeks. The microbial count nearly stabilized afterwards (Fig.3.8)



B=2.5% sludge+ nutrient (100:5:1), **C**=2.5% sludge +nutrient (100:5:1) + Microbial inoculum
D=2.5% sludge+nutrient(100:10:1),**E**=2.5% sludge +nutrient (100:10:1) + Microbial inoculum

Fig. 3.8 Microbial counts during bioaugmentation

The treated samples were fractionated for studying the effect of bioaugmentation on different components of oil sludge. The results are presented in Fig 3.9 and 3.10. It was observed that alkane fraction had depleted in greater amount than other fractions irrespective of nutrient level. The order of decrease in concentration was firstly saturate then aromatic and asphaltenes, finally least degraded was NSO fraction. The reduction of saturate was 54.08%, Aromatic 40.83, Asphaltenes, 6.95 and NSO by 0.61 % in 100:10:01 without microbial inoculum. In nutrient amendment 100:10:01 with microbial inoculum the reduction of saturate was 55.11%, Aromatic 44.06, Asphaltenes, 8.54 and NSO by 6.06 %.

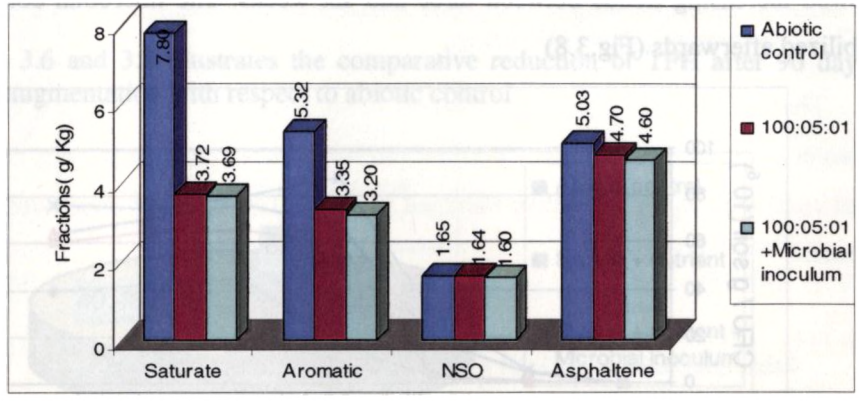


Fig.3.9 Concentration of TPH fractions on bioaugmentation after 90 days (nutrient level 100:5:1)

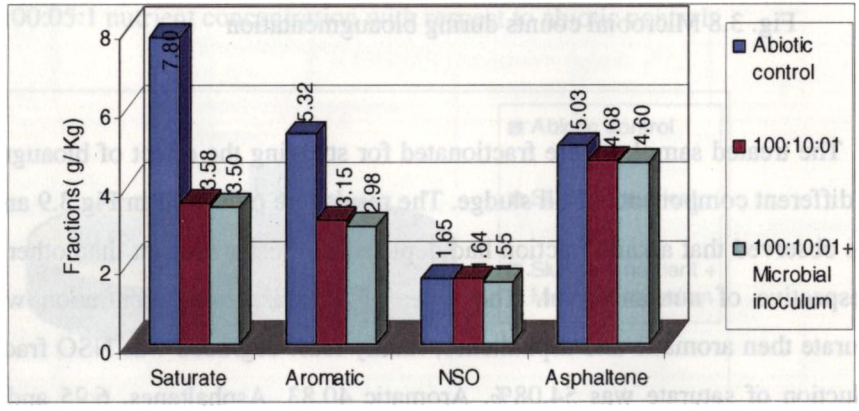


Fig. 3.10 Concentration of TPH fractions on bioaugmentation after 90 days (nutrient level 100:10:1)

3.4 Discussion

In order to investigate the optimum method for the management of oil sludge, soil was supplemented with nutrients, bulking agent and a bacterial consortium as inoculum and set up as separate experiments. Results showed a corresponding influence due to the different amendments in the remediation of oil sludge-contaminated soil.

Addition of inorganic nutrients produced a significant effect on oil removal compared to the soil amendment without inorganic nutrients (abiotic control). Addition of bulking agent along with inorganic nutrients enhanced the removal of petroleum hydrocarbons, indicating development of suitable hydrocarbon degrading microbes in the system.

In the case of the treatments units containing inorganic nutrients, 37 % oil degradation was recorded with 100:5:1 nutrient level; but along with straw the oil degradation was 41.33 % .In the case of treatment unit with 100:10:1 nutrient level with addition of straw 45.42 % oil degradation was recorded. Earlier reports by El-Nawawy *et al.* (1992) indicated upto 71% oil removal from the 5.8% oil sludge amended with fertilizer in 112 days; the rates decreased with the increase in the amount of oil sludge in soil, whereas Sandvik *et al.* (1986) showed 45% oil removal from the oily sludge during bioremediation for 9 months.

Tillage and bulking with wheat bran is reported to influence the disappearance of the hydrocarbons (Rhykerd *et al.*, 1999). The addition of bulking agents tends to have a priming effect on microbial populations. It has also been noted that addition of organic material to soil enhances oil degradation (Chang and Weaver, 1998).

Analysis of compositional changes that occurred during 90 days of biostimulation with and without bulking agent showed that all hydrocarbon classes were subject to

biodegradation in both treatments. It was observed that saturate fraction had depleted in greater amount than other fractions. NSO fraction was the least degraded. The addition of straw was observed to increase the degradation of asphaltenes and NSO fractions.

Within the saturate and aromatic fractions (below C_{15}) of the two treatments the percentage of paraffin components was lower in oil degraded by the bulking agent. The paraffin fraction appears to be more readily degraded with bulking agent. In the case of iso-paraffins the same trend had been observed. Comparing the naphthenes fractions, the percentage of naphthenes had increased in the treatment with bulking agent, indicating that naphthenes are difficult to degrade than other classes.

Data obtained from this biostimulation study indicated that the bioremediation programs for oil impacted soils are highly effective in the elimination of the oil pollutants. In many of the crude petroleum producing countries of the world, natural environments including agricultural soils are badly affected by oil pollution as a result of oil spillage (Atlas,1991).Thus from the result of this study, rehabilitation of such land could be facilitated by providing land farming practices. Those bacteria indigenous to the sludge were found equally good or better at degrading the sludge than the consortia isolated from root zone of pokkali rice contaminated with sludge. Augmentation trials in this study had no measurable effect on the extent or rate of biodegradation ;but it could be seen that bulking agent was more important to the bioremediation process than microbial augmentation. Studies have found that the addition of biomass reduced the percentage of larger soil aggregates, allowing better air and moisture circulation within the soil matrix, as well as improving the exposure of contaminant and nutrients to the microorganism. Soil tilling also become easier with the addition of biomass substance. Addition of biomass also increase the soil pH to near neutral. In general, tillage of soil might enhance the contact between oil and bacterial populations and also moisture retaining capacity of the bulking agent thereby

enhancing the bioremediation process. The amount and thoroughness of aeration determine the rate and extent of the destruction of the waste, since this is, essentially, an aerobic process (Savage *et al.*, 1985). Aeration by the addition of rice, annual plant stems, and wood are suitable cosubstrate for rapid benzo(a)pyrene biodegradation (Field *et al.,* 1995).The result of the present study indicated that the use of bulking agent played an important role in the bioremediation of oil-contaminated soil.

3.5 Conclusion

The results of the present study indicated that the use of bulking agent played an important role in the bioremediation of oil-contaminated soil. A bulking agent such as paddy straw could improve the inherent microbial growth thereby enhancing the degradation of PHCs. Inoculation of microflora into the system did not produce a significant reduction of TPH contrary to many previous results. Qualitative analysis of the spectrum of components has shown that biostimulation does degrade the PHCs fractions selectively in the order saturates > aromatics > asphaltenes > NSO.

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CHAPTER 4

MICROBIAL ENHANCED OIL SEPARATION

Abstract

Oil spills impose a serious damage on the environment. Various processes have been developed to remove oil from contaminated areas. Soil washing using biosurfactant are reported give to promising results. In this study a few strains of microbes and fungi have been isolated and tested for recovery of oil from refinery sludge. Two promising strains of bacteria Bacillus SEB 2 and Bacillus SEB 7 were identified to separate oil from sludge to an efficiency of 93-96%. The separation was completed in 48 h. The process of oil separation is attributed to the production of biosurfactants by the bacteria. The reaction was carried out in stirred bioreactor designated for the purpose. The process of oil separation could be effected only on sludge-soil substrate upto 5% sludge concentration. The limitations of the technique are presented.

A multi-process bioremediation system consisting of phytoremediation, biostimulation and microbial enhanced oil separation system was also attempted. Phytoremediation removed 51.44 % of TPH; biostimulation removed a further 14.11% and microbial bioreactor separated 29.51% with respect to original TPH content. This multi-process system involving phytoremediation, biostimulation, and MEOS could degrade TPH to 95% in 182 days of 2.5% sludge.

4.1 Introduction

Oil is one of the most important resources of energy in the modern industrial world. As long as oil is explored, transported, stored and used there will be the risk of spillage. Oil spills impose a major problem on the environment (Kingston, 2002). Clean up of soil contaminated by oil is among the most urgent ecological problems. The available clean-up technologies are based on bioremediation principles and using physico-chemical treatment by washing the contaminated soil. The above technologies have limits either in starting concentration of pollutants or in residual pollutant concentration level.

Various processes have been developed to remove oil from contaminated areas. Among them mechanical recovery of oil by oil sorbents is one of the most promising counter measures. This process includes the transfer of oil from the contaminated area to some transportable form of temporary storage with the help of oil sorbents (Choi *et al.*, 1993).

There has been a growing interest in surfactant applications in environmental remediation (Abdul *et al.*, 1990; Bognolo, 1999; Bhandari *et al.*, 2000; Barathi and Vasudevan, 2001; McCray *et al.*, 2001). The mechanism behind surfactant enhanced removal of oil from soil have been proposed to occur in two steps: mobilization and solubilization (Vigon and Rubin, 1989;Cheah *et al.*, 1998; Deshpande *et al.*, 1999; Mulligan *et al.*, 2001).The mobilization mechanism occurs at concentration below the surfactant critical micelle concentration (CMC). Phenomena associated with this mechanism include reduction of surface and interfacial tension, reduction of capillary force, wettability and reduction of contact angle. Above the surfactant's CMC, the solubility of oil increases dramatically due to the aggregation of surfactant micelles. The hydrophobic end of the surfactant molecules cluster together inside the micelle structure with the hydrophilic end exposed to the aqueous phase on the exterior.

Consequently, the interior of a micelle constitutes a compatible environment for hydrophobic organic molecules; the process of incorporation of these molecules into a micelle is known as solubilization.

Biosurfactants display excellent surface activity in comparison to synthetic ones despite their bulky molecular structures. They show greater environmental compatibility and also high activity at extreme temperatures, pH, and salinity conditions. Due to their physiochemical characteristics, biosurfactants are better suited to environmental applications than synthetic ones (Harvey *et al.*, 1990; Muller *et al.*, 1993; Lang and Wullbrandt, 1999; Mulligan *et al.*, 2000). The use of biosurfactants would eliminate the need for removing surfactants from effluents during soil washing, as their release will not damage the environment.

Biosurfactants are biodegradable and can also enhance biodegradation of oil by increasing the bioavailability of hydrophobic compounds (Ron and Rosenberg, 2002). Chemically-synthesized surfactants have been used in the oil industry to aid the clean up of oil spills, as well as to enhance oil recovery from oil reservoirs. These compounds are not biodegradable and can be toxic to the environment. Biosurfactants, however, have been shown in many cases to have equivalent emulsification properties and are biodegradable. Thus, there is an increasing interest in the possible use of biosurfactants in mobilizing heavy crude oil, transporting petroleum in pipelines, managing oil spills, oil pollution control, cleaning oil sludge from oil storage facilities, soil/sand bioremediation and microbially enhanced oil recovery (Banat, 1995).

The spontaneous release and function of biosurfactants are often related to hydrocarbon uptake; therefore, they are predominantly synthesized by hydrocarbon degrading microorganisms. Oberbremer and Muller-Hurtig (1989) observed a decrease in surface tension accompanying the biodegradation of spiked petroleum hydrocarbons by indigenous soil microbes. Microbes have been reported to produce

biosurfactants during either active or limited growth or while resting (Desai and Banat.1997). Microorganism must have contact with the hydrocarbon to degrade it. (Rosenberg *et al.*, 1992). For this to occur, they have evolved specific adhesion mechanisms and the ability to emulsify the compounds. Biosurfactants are produced by microorganism during growth on insoluble organic substrates in order to increase their solubility (Falatko and Novak, 1992).Microorganism generally consume only soluble or solubilized (emulsified) organic molecules, and synthesis of an emulsifier may pseudosolubilize target hydrocarbons or enhance direct contact between microorganism and a hydrocarbon substrates (Thibault and Elliott, 1980). These biosurfactants are composed of polysaccharides, polysaccharide-protein complexes, or glycolipids (Rosenberg, 1986). Emulsions formed are stabilized by the polysaccharide polymers secreted extracellularly by the microbes. These can then be absorbed through the lipophilic cell wall to be utilized by the microorganism as a carbon and energy source. These polymers may also act as flocculants (Zajic and Knettig, 1971). Enhancement of microbial degradation of PAHs have been achieved through addition of surfactants in ex-situ treatment of sediments (Abbondanzi *et al.*, 2006).

This chapter describes a study on the microbial aided oil removal from petroleum refinery sludge. The objective of the investigation was to

1. Identify microbes capable of separating out oil from the refinery sludge.
2. Comparative study of the efficiency of different isolates.
3. Optimize the process of oil separation.
4. Measure biosurfactant activity

4.2 Materials and methods

4.2.1 Isolation of bacterial strains

The microbial consortium used for the bioaugumentation experiment was plated on to agar in mineral medium supplemented with oil sludge extract. The colonies developed were transferred individually to mineral medium containing oil sludge extract and incubated as in the previous experiment (section 3.2.2). They were repeatedly plated to agar to select the most actively growing CFU's. Seven isolates were obtained which were named as SEB 1, SEB 2, SEB 3, SEB 4, SEB 5, SEB 6 and SEB 7.

4.2.2 Isolation of fungi

The source material for the isolation of fungi was the rhizosphere soil of the previous phytoremediation experiment. A series of dilution tubes containing 9mL of sterile distilled water was prepared. 1 g sample of soil was placed in a dilution tube, and the tube was agitated for 15 minutes. Serial dilutions were performed; 0.1 mL samples of the final three dilutions were inoculated onto potato dextrose agar plates and spread with a sterile, bent glass rod. The plates were incubated for 3 days at 28°C .The actively growing CFU's were separately removed into liquid medium. After sub-culturing and purification, seven isolates were obtained which were named as SEF 1, SEF 2, SEF 3, SEF 4, SEF 5, SEF 6 and SEF 7.

4.2.3 Culture of Cyanobacteria.

The Cyanobacteria used was *Synechococcus elongatus* available in the laboratory of School of Environmental Studies. The organism was cultured in BG11 medium in the laboratory condition.

4.2.4 Development of bioreactor

The bioreactor used in this study was modified form of jar test apparatus. The stirrer was replaced with spiral type one fabricated for the purpose. The reactor had an adjustable speed regulator and timer. Stirring was programmed as 5 minutes per hour. The test containers were 1 liter borosil glass beakers (Fig. 4.1)

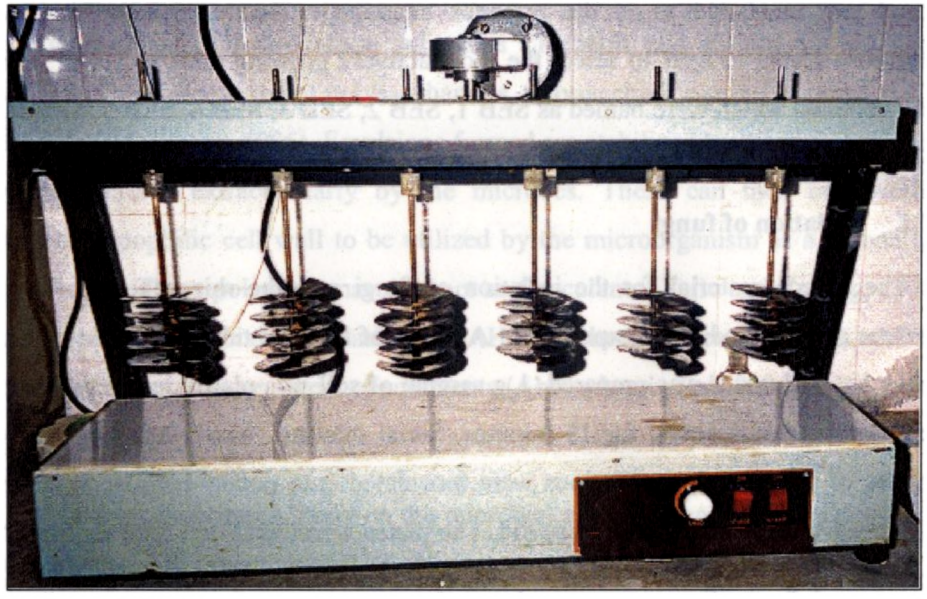

Fig. 4.1 Stirred bioreactor

4.2.5 Screening test

The fourteen isolates of bacteria and fungi, and the blue-green alga *Synechococcus elongatus* were screened in a stirred bioreactor to identify their potential to separate oil from the sludge. The sludge was mixed with river sand as in the pervious experiments to effect sludge concentrations 2.5%, 5%, 10%, 20%, 40%, and 80%.A set of unamended sludge was also taken. 250g of the samples taken in 1

liter borosil beakers was made into slurry with 200mL of distilled water. Nitrogen and phosphorous were added to provide a C: N: P ratio of 100:5:1. 10mL each of the respective inoculum was added to the slurry. The cell concentrations of all bacteria and fungal isolates were 10^8 CFU/mL. The cell density of *S. elongatus* was 2 *10^6 / mL. A control of un-inoculated slurry was also maintained. The slurry was placed on the jar test apparatus and stirred continuously for seven days @ 5 minute per hour. It was then allowed to settle for a few hours. The oil separation was observed visually.

- *Confirmatory test.*

Based on the screening test, seven isolates of bacteria alone were selected and the sludge concentration of the bioreactor study was restricted to 2.5% and 5%.The experiment was repeated as before. The treatment combinations are given in Table 4.1 and the experimental set up shown in Fig 4.2. The parameters of measurement were visual observation of oil separation, and estimation of residual TPH of the sludge.

Table 4.1 The treatment combinations for confirmatory test

Sl. No	Treatments
1	Slurry + Nutrient + SEB 1
2	Slurry + Nutrient + SEB 2
3	Slurry + Nutrient + SEB 3
4	Slurry + Nutrient + SEB 4
5	Slurry + Nutrient + SEB 5
6	Slurry + Nutrient + SEB 6
7	Slurry + Nutrient + SEB 7

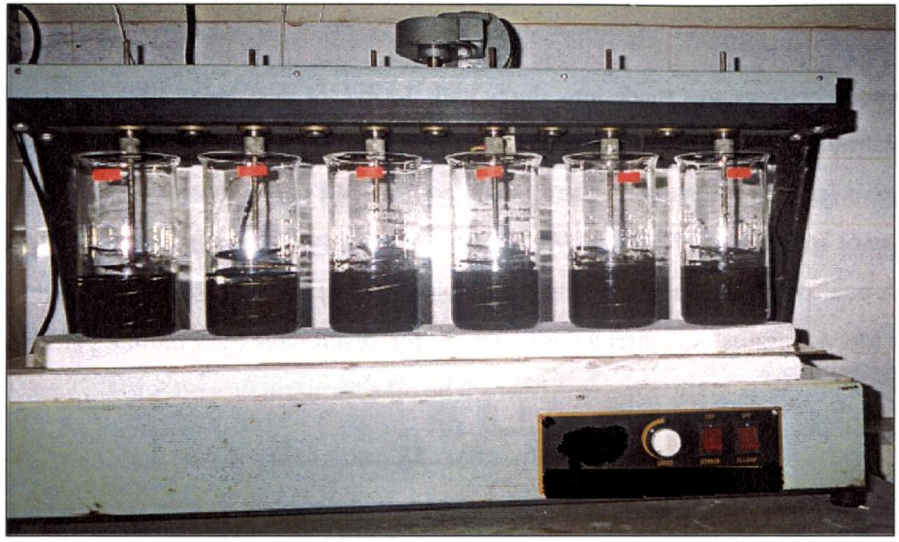

Fig. 4.2 Bioreactor set up

4.2.6 Effect of reaction time on oil separation by the bacterial isolates.

To determine the effect of reaction time on oil separation, the above experiment was repeated using 2.5% and 5% sludge. Visual observation of oil separation and residual TPH in the sludge was determined every 24 hour. The bacterial population was enumerated as done previously (Section 2.2.4) at 24 hour, 48 hour, and 72 hour. Based on the results, two isolates SEB 2 and SEB 7 were selected for further study.

4.2.7 Identification of the bacterial isolates.

The two isolates SEB 2 and SEB 7 were identified by the Environmental Microbiology laboratory of School of Environmental Studies. The identification was based on the following tests.

1. *Colony morphology on nutrient agar*:

   Colony size, Pigmentation, Form, Margins and Elevation

2. *Staining and biochemical tests*

a. Staining reaction

Bacterial cultures 12- 18 h old were taken and smears were prepared on clean glass slides for staining. The primary stain ammonium oxalate –crystal violet was added to the fixed smear and allowed to stand for one minute. Then the slides were rinsed in gentle running water and allowed to dry. The slides were flooded with mordant, Lugol's iodine solution and allowed to stand for one minute. The slides were then rinsed gently in running water. Flooded the slides with the decolorizer (acetone alcohol) and allowed to stand for 30 sec. Rinsed in gentle running water and kept for drying. The counter stain safranin O was added to the smear and allowed to stand for one minute. Then the slides were washed, air dried and observed under an oil immersion microscope. Gram +ve bacteria appear in violet and gram –ve in pink color.

b. Spore staining.

Smears were prepared using 60-70 h old bacterial culture. The slides were flooded with malachite green and allowed to react at room temperature for one minute, and warmed over a Bunsen's burner until rise of steam from the slides was observed. The slide was then waved over the flame without allowing them to boil or dry for about three minutes replenishing malachite green as and when it got evaporated.

The slides were then allowed to cool for five minutes, rinsed with water, and flooded with safranin counter stain. The stain was allowed to react for one minute, washed with water and allowed to air dry, and observed under oil immersion objective. Bacterial endospores appeared green, oval shaped within pink vegetative cell wall.

c. Acid from Mannitol

This characteristic is usually determined by inoculating the organisms into deep agar tubes supplemented with 1% Mannitol in the culture tubes. Cultures were stabbed and streaked with a needle after solidification of agar.

Acidic changes at or near the surface indicates that the substrate is being oxidized by aerobic bacteria, whereas the development of uniform acidity throughout the tube shows that the organism is facultatively anaerobic. Anaerobic bacteria that ferment the substrate, usually produce an acidic reaction in the butt of the tube initially, but acidic materials may diffuse upwards to give an appearance of acid production throughout the tube.

MOF medium was employed for the present work. Transferred 2.2 g of MOF medium to 100 ml of distilled water, added 1.5 g agar and autoclaved at 15 lbs for 15 minutes. Added 1% Mannitol to the sterile basal medium and transferred into 4 mL aliquots aseptically into sterile tubes and autoclaved at 10 lbs for 10 minutes. Converted to slants with a long butt. The tubes were stabbed and streaked and incubated at 28±0.4°C.

The results were recorded as follows:

O. Oxidation (yellow colouration in the butt)

F. Fermentation (yellow coloration through out the tube)

(F) Fermentation with gas production

Alk / N – alkaline reaction (pink or purple coloration in the slant and no reaction in the butt)

d. Voges-Proskaeur reaction

This helps to determine weather the organism ferments carbohydrate with production of non acidic / neutral end products. Glucose is converted to acid which is detected by the formation of Acetoin (2, 3 butaniedion acetyl methyl carbinol) when α-naphthol and KOH were added. To a 48 h old culture, 0.5 mL 6% α-naphthol and 0.5mL 16% KOH solution added, agitated thoroughly and maintained for 2 hours. A positive result was indicated by the development of pale pink color. It appears normally within 5 minutes but may not reach maximum red color intensity for about an hour. Negative tubes were re-examined and confirmed after long period.

Accordingly the results are tabulated in Table 4.2 and 4.3 and the organisms represented in Fig. 4.3 and Fig. 4.4. Both isolates are identified as *Bacillus*. The isolate SEB 2 resembles *Bacillus megaterium* and isolate SEB 7 resembles *Bacillus cereus*. The two isolates are hereafter referred to as *Bacillus* SEB 2 and *Bacillus* SEB 7

Table 4.2 Culture characteristic of the isolate SEB 2

Tests	**Results**
Colony size	Large
Pigmentation	White
Form	Irregular
Margin	Serrate
Elevation	Raised
Gram staining	Gram positive long rods
Spore staining	Gram positive rods with terminal endospores
Acid from Mannitol	Positive
Voges Proskauer	Positive

Fig 4.3 Gram stained cells of the bacterial isolate SEB 2 (*1000 magnification)

Table 4.3 Culture name SEB7

Colony size	Small
Pigmentation	Cream
Form	Round
Margin	Entire
Elevation	Raised
Gram staining	Gram positive short rods
Spore staining	Gram positive rods with terminal endospores
Acid from Mannitol	Negative

Fig 4.4 Gram stained cells of the bacterial isolate SEB 7 (*1000 magnification)

4.2.8 Oil removal by *Bacillus* SEB 2 and SEB 7

The experiment was repeated again as in section 4.2.6 using the two strains and 2.5 and 5% sludge. Residual TPH in the sludge was determined every 24 h for 7 days. Based on the observations, 48 h was selected as reaction time.

4.2.9 Biosurfactant production by *Bacillus* SEB 2 and SEB 7

Many species of bacteria including that of *Bacillus* have been reported to produce biosurfactants, and thereby emulsify oil. Since biosurfactants reduce the surface tension of the medium in which it grows, biosurfactant production was monitored by measuring the reduction in surface tension of the cell free supernatant (Banat *et al.*, 1997 ; Kanga *et al.*, 1997) over time. Therefore in this study surface tension was monitored as surrogate measure of biosurfactant production in the reactor. The experiment was set up as in the previous section (Section 4.2.8), but for 48 hour. 2.5% and 5% sludge was employed and treated with both by *Bacillus* SEB 2 and *Bacillus* SEB 7.

Uninoculated controls were also maintained. After every 4 h, a portion of the slurry was removed, filtered, and surface tension measured using a Tensiometer. pH of the slurry was monitored at intervals of 8 h during the experiment with a pH probe.

Upon completion of the first, 48 hour treatment, a portion of the supernatant (activated slurry) was transferred to a fresh set of reaction vessels containing 2.5 and 5% sludge and the experiment was repeated giving a reaction time of 48 hour. Oil separation was observed visually and residual TPH estimated.

4.2.10 Combined effect of phytoremediation, biostimulation and microbial enhanced oil separation

A Multi-process bioremediation system composed of phytoremediation, biostimulation, and finally the separation of non-biodegradable fraction by biosurfactant activity was adopted. 2.5% sludge subjected to phytoremediation using pokkali variety (Section 2.3.4) was exposed to biostimulation with pokkali biomass (section 3 3.1). The residue was incorporated into the bioreactor as in experiment 4.2.8. The residual TPH of sludge was estimated at each step.

4.3 Results

4.3.1 Screening test

Preliminary experiments on the isolates indicated that fungal isolates and Cyanobacteria *Synechococcus elongatus* did not separate oil from the sludge. The bacterial isolates could effect separation of oil so as to form a floating scum up to the sludge concentration of 5% (Table 4.4). At sludge levels >5%, formation of a homogenous slurry did not occur, probably due to the limitation of the stirrer. There was no separation of oil layer as well. Oil separation did not occur in the uninoculated controls.

Table 4.4 Visual observation of oil separation by the isolates ('+'occurrence of oil separation, '—' no oil separation).

	Isolate	Sludge concentration						
		2.5 %	5 %	10 %	20 %	40 %	80 %	100 %
Control	—	-	-	-	-	-	-	-
Bacteria	SEB1	+	+	-	-	-	-	-
	SEB2	+	+	-	-	-	-	-
	SEB3	+	+	-	-	-	-	-
	SEB4	+	+	-	-	-	-	-
	SEB5	+	+	-	-	-	-	-
	SEB6	+	+	-	-	-	-	-
	SEB7	+	+	-	-	-	-	-
Fungi	SEF1	-	-	-	-	-	-	-
	SEF2	-	-	-	-	-	-	-
	SEF3	-	-	-	-	-	-	-
	SEF4	-	-	-	-	-	-	-
	SEF5	-	-	-	-	-	-	-
	SEF6	-	-	-	-	-	-	-
	SEF7	-	-	-	-	-	-	-
Cyano-bacteria	***S. elong Atus***	-	-	-	-	-	-	-

4.3.2 Confirmatory test

The separation of oil by the bacterial isolates was confirmed. At 2.5% and 5% sludge level, the sludge–soil mixture formed a homogeneous slurry with water and the microbial inoculum and as the reaction proceeded oil separated at the surface as a scum. The estimation of residual TPH showed that the different isolates had removed oil at different rates. The highest reduction of TPH observed was 95% by the isolates SEB 2 and SEB 7 (Table 4.5).

Table 4.5 Oil separation and mean residual TPH (g/kg) after 7 days (Initial TPH for 2.5% sludge =21.26 g/kg and 5% sludge= 42.52 g/kg)

Isolate	Visual observation	TPH (2.5% sludge)		TPH (5% sludge)	
		g/kg	% reduction	g/kg	%
SEB 1	+	2.98	85.98	3.04	85.7
SEB 2	+	1.05	95.06	1.10	94.83
SEB 3	+	1.56	92.66	2.05	90.36
SEB 4	+	2.45	88.48	2.85	86.59
SEB 5	+	3.24	84.76	3.54	83.35
SEB 6	+	2.57	87.91	2.85	86.59
SEB7	+	1.02	95.2	1.08	94.92

'+' occurrence of oil separation.

4.3.3 Effect of reaction time on oil separation by bacterial isolates

The separation of oil as a visible floating layer was observed from 8 h onwards. The results of reaction time vs. residual TPH obtained on various isolates on 2.5% and 5% sludge are shown in Table 4.6 and 4.7. The rate of oil removal was high during the first 48 h following the reaction and then it was almost steady.

Table 4.6 Residual TPH (g/kg) estimated every 24 h in bioreactor fed with 2.5% sludge

Sl. No.	Isolate	Time (h)						
		24	**48**	**72**	**96**	**120**	**144**	**168**
1	SEB 1	11.56	8.35	7.55	6.12	5.11	4.12	2.98
2	SEB 2	7.96	1.10	1.08	1.05	1.05	1.05	1.05
3	SEB 3	8.42	2.32	1.84	1.8	1.61	1.6	1.56
4	SEB 4	8.02	1.99	1.64	1.59	1.54	1.49	1.49
5	SEB 5	8.1	2.4	2.02	1.75	1.54	1.42	1.42
6	SEB 6	8.02	1.99	1.64	1.59	1.43	1.39	1.39
7	SEB 7	8.45	1.08	1.04	1.03	1.02	1.02	1.02

Table 4.7 Residual TPH (g/kg) estimated every 24 h in bioreactor fed with 5% sludge

Sl. No.	Isolate	Time (h)						
		24	**48**	**72**	**96**	**120**	**144**	**168**
1	SEB 1	18.18	3.54	2.79	2.44	2.25	2.14	2.04
2	SEB 2	15.26	1.15	1.12	1.12	1.1	1.1	1.10
3	SEB 3	17.55	2.05	1.78	1.64	1.46	1.31	1.25
4	SEB 4	19.28	2.85	2.35	1.75	1.55	1.40	1.35
5	SEB 5	22.15	3.54	2.75	2.4	2.15	1.64	1.54
6	SEB 6	17.45	2.85	2.45	2.05	1.85	1.45	1.25
7	SEB 7	13.45	1.11	1.08	1.08	1.08	1.08	1.08

Initial bacterial population in the bioreactor was 10^6 CFU/mL; it increased to 10^8 to10^9 within 48 h of reaction. After 48 h there was no further increase in microbial population (Table 4.8).

Table 4.8 Heterotrophic plate count in bioreactor slurry (CFU/mL) of different isolates at 2.5% sludge

		Time (h)		
Sl. No	Isolates	24	48	72
1	SEB1	$37*10^6$	$40*10^9$	$42*10^9$
2	SEB2	$24*10^6$	$18*10^9$	$28*10^9$
3	SEB3	$32*10^6$	$20*10^8$	$6*10^9$
4	SEB4	$24*10^6$	$24*10^8$	$25*10^8$
5	SEB5	$24*10^6$	$24*10^8$	$25*10^8$
6	SEB6	$17*10^6$	$18*10^8$	$30*10^8$
7	SEB7	$11*10^6$	$24*10^9$	$29*10^9$

4.3.4 Oil removal by *Bacillus* SEB 2 and SEB 7

The residual TPH of the bioslurry estimated every 24 h during the reactor operation is represented in Fig. 4.5 and Fig.4.6. The TPH is reduced by 93-96 %

within 48 h by the two strains. Beyond 48 h, the rate of reduction remains nearly constant. The floating scum of oil was clearly visible following bioseparation of 2.5 and 5% sludge (Fig. 4.7 and 4.8).

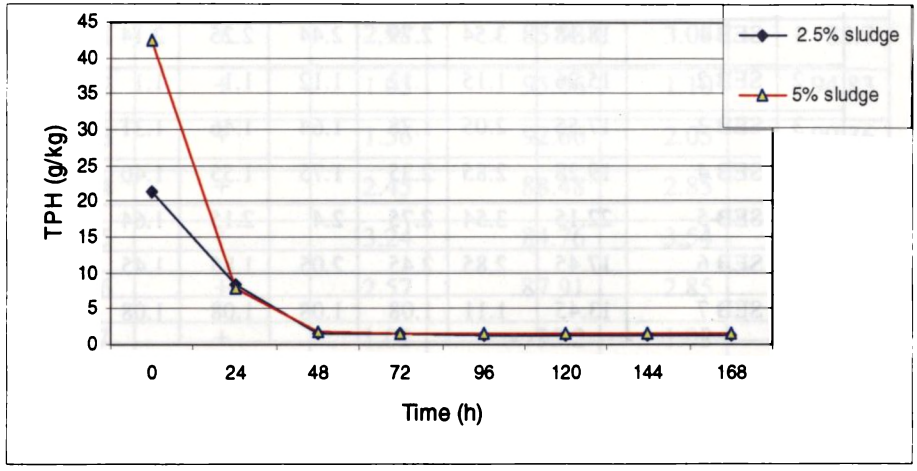

Fig 4.5 Reduction of TPH by *Bacillus* SEB 2

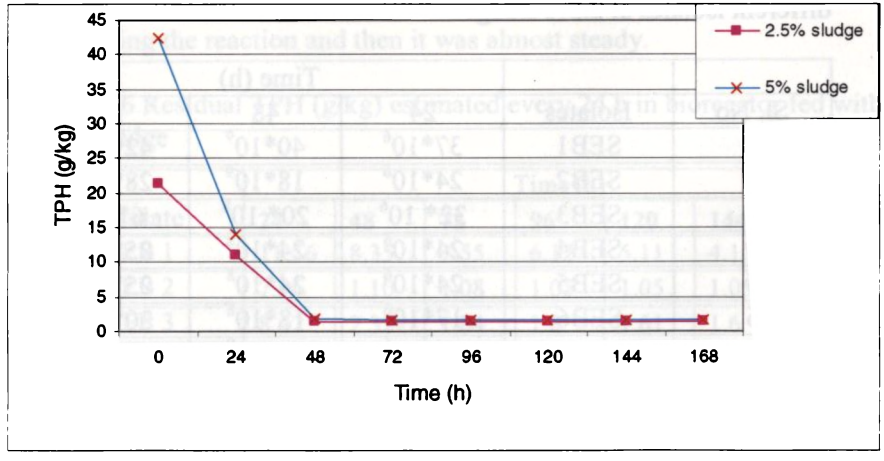

Fig 4.6 Reduction of TPH by *Bacillus* SEB 7 in bioreactor

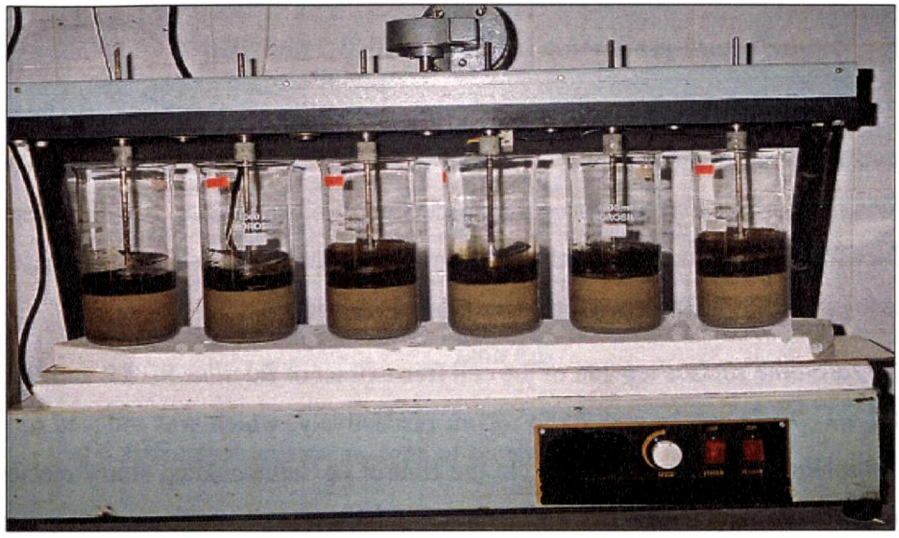

Fig 4.7 Oil separation by *Bacillus* SEB 2 in 2.5% sludge

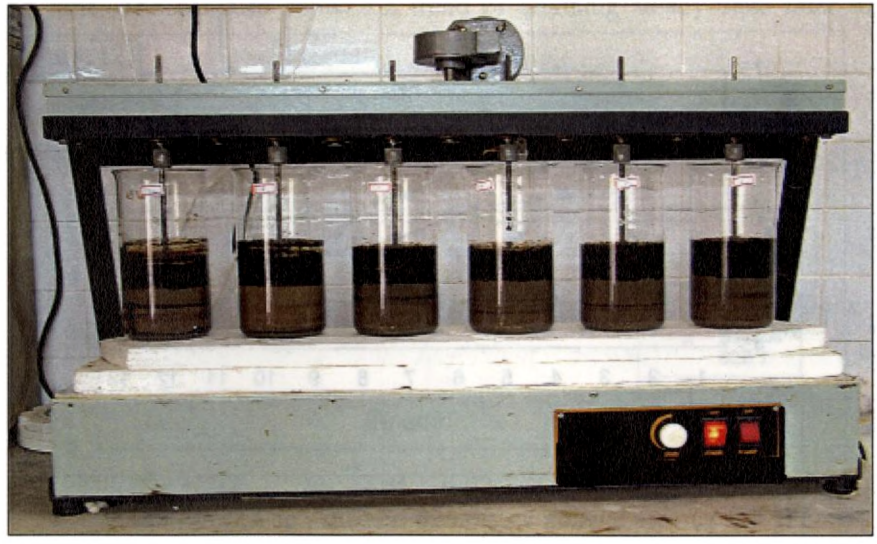

Fig.4.8 Oil separation by *Bacillus* SEB 7 on 5% sludge

4.3.5 Biosurfactant production by *Bacillus* SEB 2 and SEB 7

The result of the measurement of surface tension of the bioslurry from the bioreactor is represented in Fig. 4.9 and 4.10. The surface tension decreased as reaction time progressed indicating the production of biosurfactant. It is likely that the production of biosurfactant reaches maximum in 48 hours and further there is a tendency to level off. Both the species exhibited similar trend.

At zero time surface tension of the cell free extract of *Bacillus* SEB 2 and *Bacillus* SEB 7 was 70.33 mN/m and 70.54 mN/m respectively, which was reduced to 48.5 and 48 mN/m respectively in 48 h. In the control i.e. uninoculated slurry reactor the surface tension ranged from 71 to 62.93 mN/m which was not sufficient to separate oil from the slurry. The pattern of decrease in surface tension was similar in 2.5% and 5% sludge concentration.

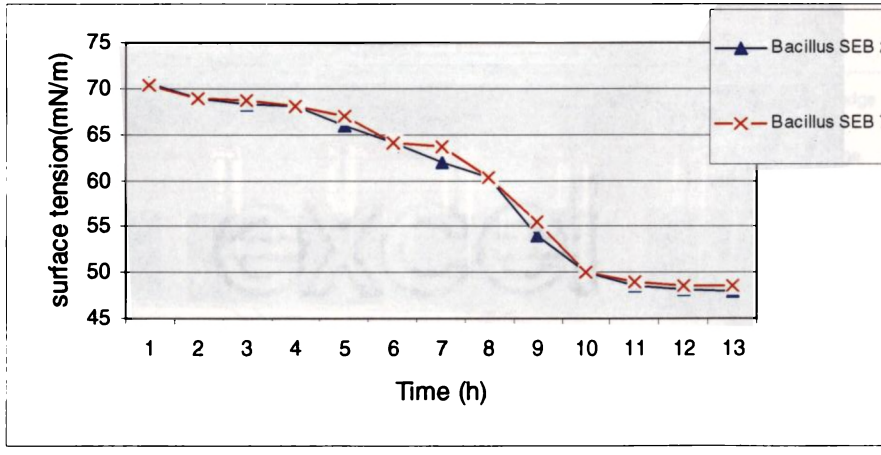

Fig 4.9 Surface tension vs. time of bioslurry at 2.5% sludge

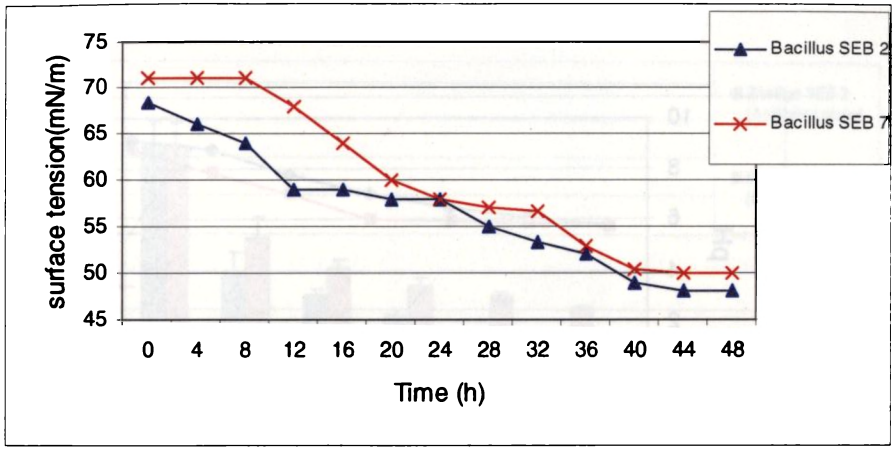

Fig 4.10 Surface tension vs. time of bioslurry at 5% sludge

The initial pH of the bioreactor was 6.08; as time elapsed an increase in pH was observed. It increased to 9.05 for *Bacillus* SEB 2 and 8.86 for *Bacillus* SEB 7 in 48 h of stirring time. The pH also showed a trend of attaining steady state after 48 h (Fig.4.11 and 4.12).

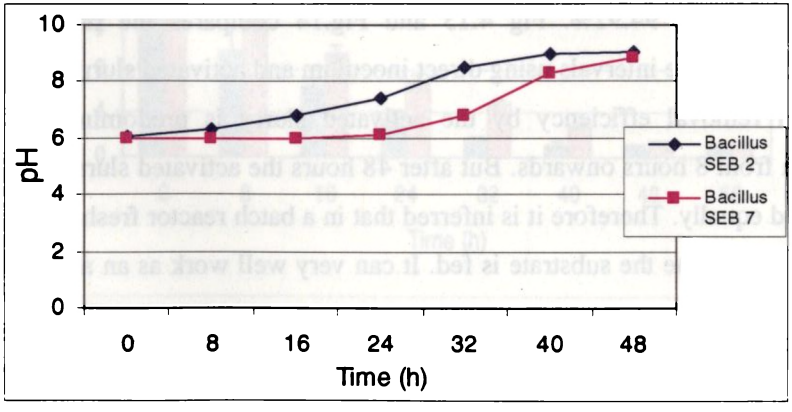

Fig 4.11 pH vs. time of bioslurry at 2.5% sludge

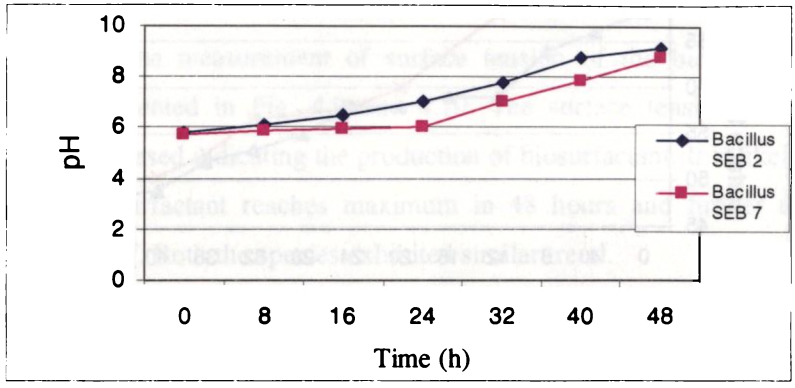

Fig 4.12 pH vs. time of bioslurry at 5% sludge

Upon inoculation of the activated slurry to the bioreactor, the visible separation of oil layer occurred at 8 h of initiation of the process. The residual TPH reached a minimum value of 1.02g/kg in the case of *Bacillus* SEB 2 after 48 h i.e. reduction of 95.2% and in the case of *Bacillus* SEB 7 TPH reached to 1.05 g/kg which correspond to a reduction of 94.92%. Fig 4.13 and Fig.14 compares the progress of TPH reduction over time intervals using direct inoculum and activated slurry. It can be seen that TPH removal efficiency by the activated slurry is predominant over direct inoculum from 8 hours onwards. But after 48 hours the activated slurry and inoculum performed equally. Therefore it is inferred that in a batch reactor fresh inoculum is not required every time the substrate is fed. It can very well work as an activated process reactor.

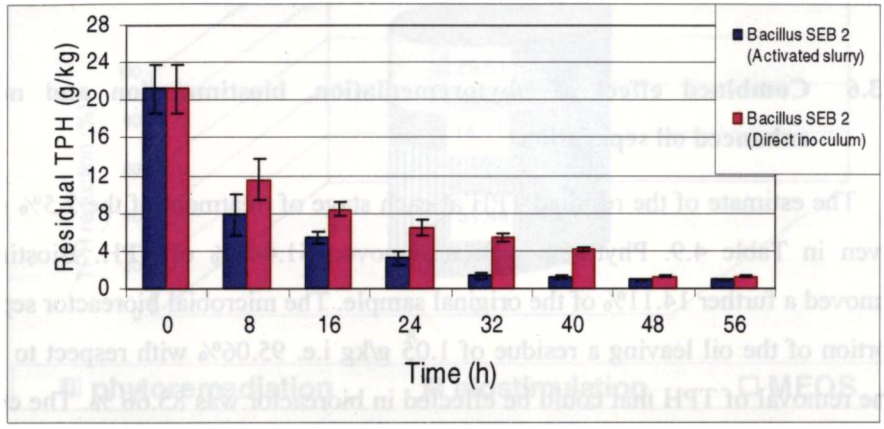

Fig 4.13 Comparison between residual TPH of direct inoculation and addition of activated slurry (2.5% sludge)

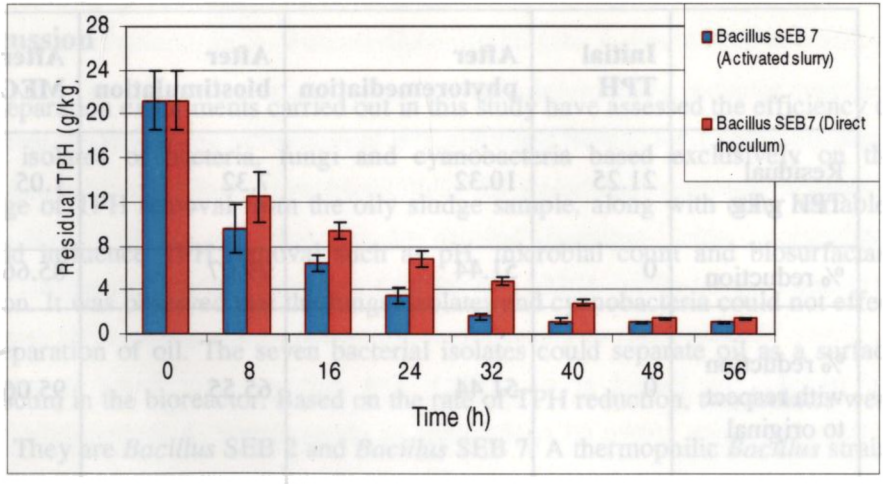

Fig 4.14 Comparison between residual TPH of direct inoculation and addition of activated slurry (2.5% sludge)

4.3.6 Combined effect of phytoremediation, biostimulation and microbial enhanced oil separation

The estimate of the residual TPH at each stage of treatment of the 2.5% sludge is given in Table 4.9. Phytoremediation removed 51.44 % of TPH. Biostimulation removed a further 14.11% of the original sample. The microbial bioreactor separated a portion of the oil leaving a residue of 1.05 g/kg i.e. 95.06% with respect to original. The removal of TPH that could be effected in bioreactor was 85.66 %. The combined efficiency of the three processes to remove PHCs is represented in Fig 4.15.

Table 4.9 Residual TPH at phytoremediation, biostimulation and MEOS

	Initial TPH	**After phytoremediation**	**After biostimulation**	**After MEOS**
Residual TPH g/kg	21.25	10.32	7.32	1.05
% reduction	0	51.44	29.07	85.66
% reduction with respect to original	0	51.44	65.55	95.06

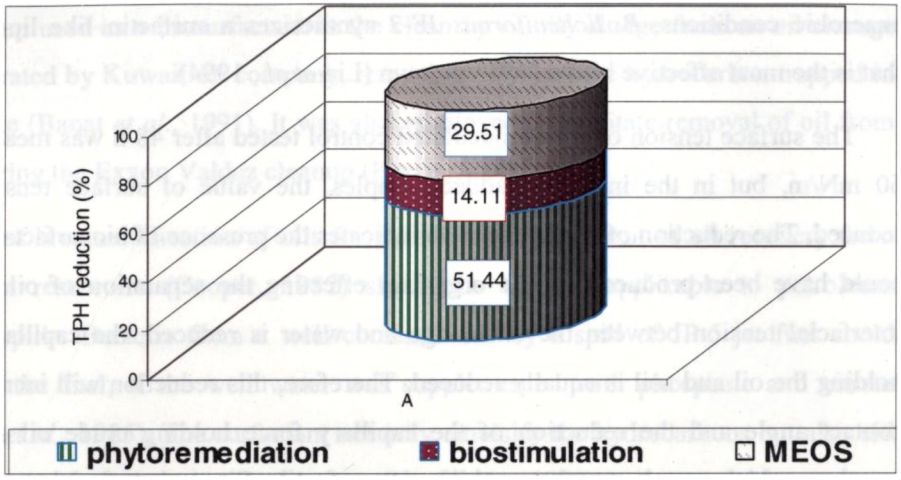

Fig.4.15 Removal of TPH in a multi-process system involving phytoremediation followed by biostimulation and microbial enhanced oil separation

4.4 Discussion

The oil separation experiments carried out in this study have assessed the efficiency of different isolates of bacteria, fungi and cyanobacteria based exclusively on the percentage of TPH removal from the oily sludge sample, along with other variables that could influence TPH removal such as pH, microbial count and biosurfactant production. It was observed that the fungal isolates and cyanobacteria could not effect the bioseparation of oil. The seven bacterial isolates could separate oil as a surface floating scum in the bioreactor. Based on the rate of TPH reduction, two isolates were selected. They are *Bacillus* SEB 2 and *Bacillus* SEB 7. A thermophilic *Bacillus* strain, *B. subtilis and B. licheniformis* have been previously reported to emulsify oil extensively when growing in hydrocarbon. Surfactin, which is produced by *B. subtilis*, has been found to be the most active surfactant. Under both aerobic and

anaerobic conditions, *B. licheniformis* JF-2 synthesizes a surfactin–like lipopeptide that is the most effective biosurfactant known (Lin *et al.*, 1994).

The surface tension of the uninoculated control tested after 48 h was measured as 60 mN/m, but in the inoculum added samples, the value of surface tension was reduced. The reduction of surface tension indicates the presence of biosurfactant whch could have been produced by the organism effecting the separation of oil. As the interfacial tension between the oil sludge and water is reduced, the capillary force holding the oil and soil is equally reduced. Therefore, this reduction will increase the contact angle and the reduction of the capillary force holding crude oil and soil together which result to the mobilization of oil. Since interfacial tension is concentration dependent, as the aqueous solution concentration increases, the interfacial tension also reduces until the surfactant CMC value is reached and remains constant there afterwards.

The work of Abdul *et al.* (1990) and Deshpande *et al.* (1999) have shown that surfactant solution, will not enhance the removal of oil from soil at concentrations greater than their CMC value. Therefore, it is expected that maximum oil removal was obtained at concentrations below the CMC, suggesting that oil removal was due to mobilization, which occurred because of the reduction of interfacial tension. Surfactant may be added during soil washing to mobilize the contaminants (Ellis *et al.*, 1984). The first study of biosurfactant production resulting from PAH metabolism was reported by Deziel *et al.* (1996). Ten cultures were found to produce a biosurfactant when grown on naphthalene or phenanthrene. A thermophilic *Bacillus* strain grows at up to 50°C and produces a biosurfactant with low surface and interfacial tension (Banat, 1993). It emulsified kerosene and other hydrocarbons efficiently and was able to recover >95% of the residual oil from sand pack columns.

A patented oil emulsifying glycolipids from a strain of *P. aeruginosa* has the ability to mobilize oil from solid surfaces and disperse oil slicks (Chakrabarty, 1985).

It has been used as a biosurfactant in the clean up of oily sludge from an oil storage tank operated by Kuwait oil company, removing >90% of the hydrocarbon trapped in the sludge (Banat *et al.*, 1991). It was also employed to facilitate removal of oil from rocks during the Exxon Valdez cleanup (Harvey, 1990).

Use of bioemulsification of oils for microbial enhancement of oil recovery from petroleum reservoirs (Cooper, 1982) should be directly applicable to petroleum product spills. Bacteria from a well contaminated by a spill of JP-5 jet fuel could emulsify the fuel, if the well water was supplemented with phosphate and nitrate (Ehrlich *et al.*, 1985). The surfactant not only emulsify the hydrocarbons but also aid in mobilizing them though soil and water (Vanloocke, 1979).

Results from the soil slurry reactor have shown that biological activity was observed almost within 8 h, as separation of oil layer. Approximately 95% TPH concentration were removed within 48 hours. The oil separation efficiency was reduced beyond 5% sludge. At 10% sludge concentration the separation was almost nil. This could be due to that at sludge level $\geq$ 10%, microbial population could not thrive either due to absence of aeration or toxicity. It had been observed that due to the sticky nature of the sludge a perfect blending of the slurry was not obtained at $\geq$ 10% concentration. In the various experiments in this investigation on the microbial enhanced oil separation an efficiency of 93-95 % of TPH removal was obtained in 48 h.

A multiprocess system involving phytoremediation plus biostimulation, and MEOS could reduce TPH to 95% in 182 days of 2.5% sludge. A previous study by creosote- spiked soil have shown that land-farming , bioremediation and phytoremediation had limited effectiveness in remediation of persistent hydrocarbon from contaminated soils. Studies with an aged oil sludge contaminated soil, also has demonstrated that these technique are limited in their ability to decontaminate TPH contaminated soil. But the combination of multiple processes can overcome the

apparent limitations of the individual method. A multi-process phytoremediation system removed 90% of all fractions of TPH from the soil (Huang *et al.* 2005).

4.5 Conclusion

Novelty of this developed technology on microbial enhanced oil separation rests on separation of two fractions of oil pollutants: migrating fraction that proceeds to separate out oil, and stable fraction which is strongly tied with soil/sludge. *Bacillus* SEB 2 and *Bacillus* SEB 7 can effectively separate migrating fraction of oil from sludge at low concentration in a stirred bioreactor. The mechanism of oil separation is through the activity of biosurfactants. The optimum time required is 48 h. The bioreactor does not require continuous microbial inoculation as it can very well perform as an activated slurry reactor. The limitations of the technology is its incapability to treat sludge of >5%. Refinement of the reactor design to effect thorough blending and mixing of the sludge may improve the functioning of the system. Further isolation and development of microbial consortia is also required.

Reference:

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CHAPTER 5

SUMMARY AND CONCLUSION

Crude oil is an important energy source as well as feed stock of oil refineries. During the processing of crude oil, various kinds of waste are generated; of this, oily sludge and chemical sludge, bio sludge are of special environmental concern because many of the constituents of this sludge are of hazardous nature. Hazardous waste is generated in significant amounts in petroleum refineries world wide. In India, oil refineries generate approximately 20,000 tonnes of oily sludge per annum. One of the major problems faced by oil refineries is the safe disposal of this oily sludge. Uncontrolled handling of these sludges often leads to environmental pollution and also affects the aesthetic quality. Recent legislation desires environment friendly sludge management system in the industries. Recycling of sludges in an environment friendly manner is one of the appropriate solutions of sludge management problem. The treatment technologies developed can be grouped as physical remediation, chemical remediation and biological remediation.

The objective of this research is to study the feasibility of bioremediating the oily sludge from a refinery site. Three different methods of waste treatment were tried i.e. phytoremediation, land farming and microbial enhanced oil separation in laboratory scale treatment systems.

5.1 Characterization of sludge.

The petroleum sludge used in this study was generated during the refining processes of an oil refinery. The source of the material are crude tank bottom sludge, product tank bottom sludge, API separator unit and TPI unit of effluent treatment

plants. The sludge was collected from the disposal site of the factory. Random samples were collected over a period of six months. Sludge was analyzed for Carbon, Nitrogen, Sulphur, Calorific value, Total phosphorus, Total petroleum Hydrocarbon (TPH), Metals and Ash content. The hydrocarbons were fractionated and subject to GC analysis.

The sludge had a mean pH of 5.8 and ash content 4.11 %. The N: P ratio was 28:1 and the carbon content significantly higher. The oil sludge had a TPH of 850 g/kg. The calorific value was 7663 cal/kg. Among the four fractions of petroleum hydrocarbons (Saturate, Aromatic, NSO, Asphaltenes), the one present in the highest proportion was the saturate (40.38%) and the lowest was the NSO fraction (5.33%).The ash content of the asphaltene fraction was 15.28%.The hexane and benzene fractions were analyzed in GC-FID. The classes of compounds present were Aromatics, Iso-paraffins, Naphthenes, Olefins, and Paraffins. The proportion of Naphthenes was highest. In hexane and benzene eluted fraction, Naphthenes reported as highest weight percentage, 78.38 % followed by olefins. The GC-MS analysis showed that the sludge contained a range of hydrocarbons from C_6 to C_{27}. Among the aromatics the PAH compounds identified are 9-methyl Anthracene, 1-Methyl Anthracene, 2-ethyl Anthracene. Among the fifteen elements analyzed, iron was the most abundant followed by zinc. The concentration of iron was 9518.24 mg/kg and that of zinc 1050.4 mg/kg. The presence of potassium, copper, manganese, sodium, nickel, vanadium, lead, chromium copper, and mercury ranged from 10.56 mg/kg to 95.34 mg/kg. The presence of lithium, arsenic, cadmium and tin ranged from 0.99 mg/kg to 2.91 mg/kg.

5.2 Phytoremediation

Phytoremediation is an attractive treatment technology for removing contaminants from the environment due to its cost effectiveness and public acceptance. Plants can be used for pollutant stabilization, extraction, degradation, or volatilization. The goal

of this study was to evaluate the phytoremediation potential of paddy varieties to remediate an oily sludge generated by a local refinery and to optimize the nutrient and sludge concentration for phytoremediation; also to evaluate the relationship between plant growth and reduction in petroleum hydrocarbon and accumulation of metals. As none of the plants tested could grow on the sludge as such, soil amended sludge was used for phytoremediation simulating a condition of low contamination of soils by PHCs. The plants were grown in sludge-soil substrate till harvest under various nutrient enrichment conditions and addition of surfactants. The plant growth was monitored as biomass, height of the plants, number of leaves and grain yield. Rhizosphere microflora was also monitored. Phytoremediation was monitored as gravimetric reduction in TPH, change in gravimetric composition of saturates, aromatic, NSO and asphaltene fractions of PHCs. Qualitative change in the saturate and aromatic fractions were elucidated through GC-FID and GC-MS analysis. The extent of metal accumulation by biomass was computed as accumulation factor.

It was concluded from the study that paddy variety pokkali can be effectively used for the phytoremediation of petroleum contaminated soils in an *ex-situ* treatment facility. A maximum of 51.4% removal of total petroleum hydrocarbons of oily sludge have been achieved within 90 days for 2.5% sludge and 33.25 % for 5% sludge level. The degradation rate is saturate > aromatic> NSO> asphaltenes. The accumulation of metals was computed as accumulation factor. The highest accumulation factor of 0.65 was observed for aluminium. Germination test revealed that significant drop in percent germination at >10% sludge in the sludge–soil substratum. Similarly seed sterility also was observed to increase significantly at >10% sludge level. So it is assumed that phytoremediation can be used effectively only for soils of low PHC contamination. Among the paddy varieties tested, Pokkali variety proved to be superior to others in effecting phytoremediation.

5.3 Land farming

Land farming is controlled land treatment generally done by, blending the sludge into the soil with tilling equipment, often with the addition of fertilizer to increase the rate of degradation. The primary mechanisms involved in the disappearance of hydrocarbons in land farming is biodegradation. The approach used here are biostimulation (stimulation of natural activities by environmental modification) and bioaugmentation (involves the supplementation of microorganism to degrade the pollutants involved).

In the biostimulation trials, the soil amended sludge was bulked with paddy straw and enriched with fertilizers. The mixture was well mixed and kept moist routinely for 13 weeks. In bioaugmentation, the fertilizer enriched soil-sludge mixture was inoculated with a microbial consortia and experiment was done as for biostimulation. The effect was measured as TPH reduction against an abiotic control.

The results of the present study indicated that the use of bulking agent played an important role in the bioremediation of oil-contaminated soil. A bulking agent such as paddy straw could improve the inherent microbial growth thereby enhancing the degradation of PHCs. Biodegradation is strongly dependent on the rate of diffusion of oxygen in soil. One of the methods to promote aeration in soil is to increase the porosity by adding bulking agents. In the present study bulking paddy straw enhanced the degradation of hydrocarbons by promoting microbial activity through increased aeration. Inoculation of microflora into the system did not produce a significant reduction of TPH contrary to many previous results. Qualitative analysis of the spectrum of components has shown that biostimulation does degrade the PHCs fractions selectively in the order saturates> aromatics> asphaltenes >NSO.

5.4 Microbial enhanced oil separation

Oil spill impose a serious damage on the environment. Various processes have been developed to remove oil from contaminated areas. Soil washing using biosurfactant is reported to give promising results. Therefore microbes were isolated from the sludge contaminated soil using standard methods. Out of the seven isolates of bacteria and seven fungi, two isolates of bacteria were observed to effect significant separation of oil from the sludge. These isolates were identified as *Bacillus* SEB 2 and *Bacillus* SEB 7. These were introduced into a stirred bioreactor run for 7 days. The biosurfactant activity and microbial count was monitored. The reaction time was optimized as 48 h for separation of oil from sludge. The process was also successfully repeated as an activated slurry reaction.

Novelty of this developed technology on microbial enhanced oil separation rests on separation of two fractions of oil pollutants: migrating fraction that proceeds to separate out oil, and stable fraction which is strongly tied with soil/sludge. *Bacillus* SEB 2 and *Bacillus* SEB 7 can effectively separate migrating fraction of oil from sludge at low concentration in a stirred bioreactor. The mechanism of oil separation is through the activity of biosurfactants. The bioreactor does not require continuous microbial inoculation as it can very well perform as an activated slurry reactor. The limitations of the technology is its incapability to treat sludge of >5%. Refinement of the reactor design to effect thorough blending and mixing of the sludge may improve the functioning of the system. Further isolation and development of microbial consortia is also required.

5.5 Multi-process approach of phytoremediation, biostimulation and microbial enhanced oil separation

A Multi-process bioremediation system composed of phytoremediation, biostimulation, and finally the separation of non-biodegradable fraction by biosurfactant activity was adopted. 2.5% sludge subjected to phytoremediation using

pokkali variety was exposed to biostimulation with paddy straw. The residue was incorporated into the bioreactor.

The estimate of the residual TPH at each stage of treatment showed that, phytoremediation removed 51.44 % of TPH; biostimulation removed a further 14.11% of the original sample and microbial bioreactor separated a portion of the oil leaving a residue of 4.94 % with respect to original. A multi-process system involving phytoremediation plus biostimulation, and MEOS could reduce TPH to 95% in 182 days of 2.5% sludge. The MEOS alone has proved to be effective in removing 93-96% of oil from 5% sludge within 48 h. Therefore it is this technique that holds promise for future application in bioremediation of petroleum refinery waste.

5.6 Future outlook

The limitation of the technology MEOS is its incapability to treat sludge of > 5%. Refinement of the reactor design to effect thorough blending and mixing of the sludge may improve the functioning of the system. Further isolation and development of microbial consortia is also required.