Bioremediation of oily sludge contaminated soil using bacterial consortium: A sustainable waste management strategy

A Dissertation Thesis Submitted to Institute of Science, Nirma University,

In Partial Fulfilment for the Degree of

MASTER OF SCIENCE

Submitted by,

Brijal Kathrotiya (21MMB010)

Megha Prajapati (21MMB022)

Yosha Thakar (21MMB031)

Milan Gajera (21MMB041)

Under the guidance of

Dr. NASREEN S. MUNSHI

ACKNOWLEGEMENT

Nirma University, Institute of Science is highly acknowledged to provide the best facilities for students for successful completion of dissertation program during the curriculum of the Master's degree. First of all, we wholeheartedly express our gratitude to Prof. Sarat Dalai, Director, Institute of Science, Nirma University, who inspired us at every step in accomplishing our work.

After that, from the bottom of our heart, we are much obliged to our guide Dr. Nasreen S. Munshi. She recognized our caliber and guided us throughout our dissertation. Her inclining words always taught us to keep learning new things. Next, we would deeply thank the Ph.D. scholar, Ms. Priya Patel, our mentor who has constantly supported and motivated us during our whole dissertation program and without whom it would have been impossible to complete the work. She was always available whenever we faced difficulty at any point of time. We would also like to acknowledge DST-FIIST for providing infrastructural grant.

Further, we would also like to thank other Ph.D. students, Ms. Vishwata Patel and Ms. Hinal Mehta for encouraging us to work hard. We would thank the faculty members, Dr. Amee Nair, Dr. Vijay Kothari, Dr. Sriram Seshadri, Dr. Sonal Bakshi, Dr. Aarthi Sundararajan, Dr. Ravi Kant and Dr. Shruti Chatterjee and Dr. Sanjib Bhattacharyya for guiding us. We are thankful to Mr. Sachin Prajapati and Ms. Arti Verma who worked endlessly to provide us the materials we required to complete our project. We are grateful to Dr. Svetal Shukla and Mr. Valji Desai for providing us superior library facility.

We are truly blessed for having such encouraging parents, who always showed faith in us and motivated us at each and every stage. Their love and support are unconditional. We also thank our companion for their perception and appreciation. At last, we thank the Almighty God with whose blessings we were able to complete our dissertation program successfully.

DECLARATION

We hereby declare that the thesis submitted is original and is the outcome of the independent investigations carried out by us and contains no plagiarism. The research is leading to the discovery of new conflation of scientific facts already known. This work has not been submitted to any other University or Body in quest of a degree, diploma or any other kind of academic award.

We hereby further declare that the text, diagrams or any other material taken from other sources have been acknowledged, referred and cited to the best of my knowledge and understanding.

Date:

_____	_____	_____	_____
Brijal Kathrotiya	Yosha Thakar	Megha Prajapati	Milan Gajera

INDEX

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ONGC: Oil and Natural Gas Corporation

O.D: Optical Density

TPH: Total Petroleum Hydrocarbon

GC-FID: Gas chromatography- Flame Ionization Detector

CFU: Colony Forming Unit

PAH: Polyaromatic hydrocarbons

PHC: Petroleum hydrocarbons

MM: Minimal media

ELISA: Enzyme Linked Immunosorbent Assay

IF: Inoculating fluid

INTRODUCTION

1. Introduction

Over the decades, to meet the demand and increasing requirements in economic, social or environmental sectors, exploitation of earth and its resources is being done (Cegarra-Navarro *et al.*, 2016). Global economy depends on one of the most important sectors that is, the oil and gas sector. However, the waste generated by this sector has a negative impact on the environment (Arscott, 2004). During the processing of crude oil, it generates oily sludge as one among the other waste end products. Oily sludge is also one of the most hazardous solid wastes generated while periodic cleaning of crude oil and refinery products storage tanks is done (Dotson *et al.*, 1972) (Figure 1). It is reported that 1 ton of oily sludge is produced for every 500 tons of crude oil processed in the refinery (Hu *et al.*, 2013). Petro-chemical industries, waste engine oil, petroleum sludge, oil spills and vehicle exhaust are main sources of soil contamination by petroleum hydrocarbon (Figure 2). The oily sludge is a recalcitrant residue characterized by being a stable water-oil emulsion of water, solids, petroleum hydrocarbons and metals (Mazlova and Meshcheryakov, 1999). As it is hazardous waste, its improper disposal can cause serious threats to the environment and human health (Liu *et al.*, 2009; Zhao *et al.*, 2020). Because of dumping of oily sludge in the environment, the substances which are lighter will volatilize and heavier ones will remain as it is.

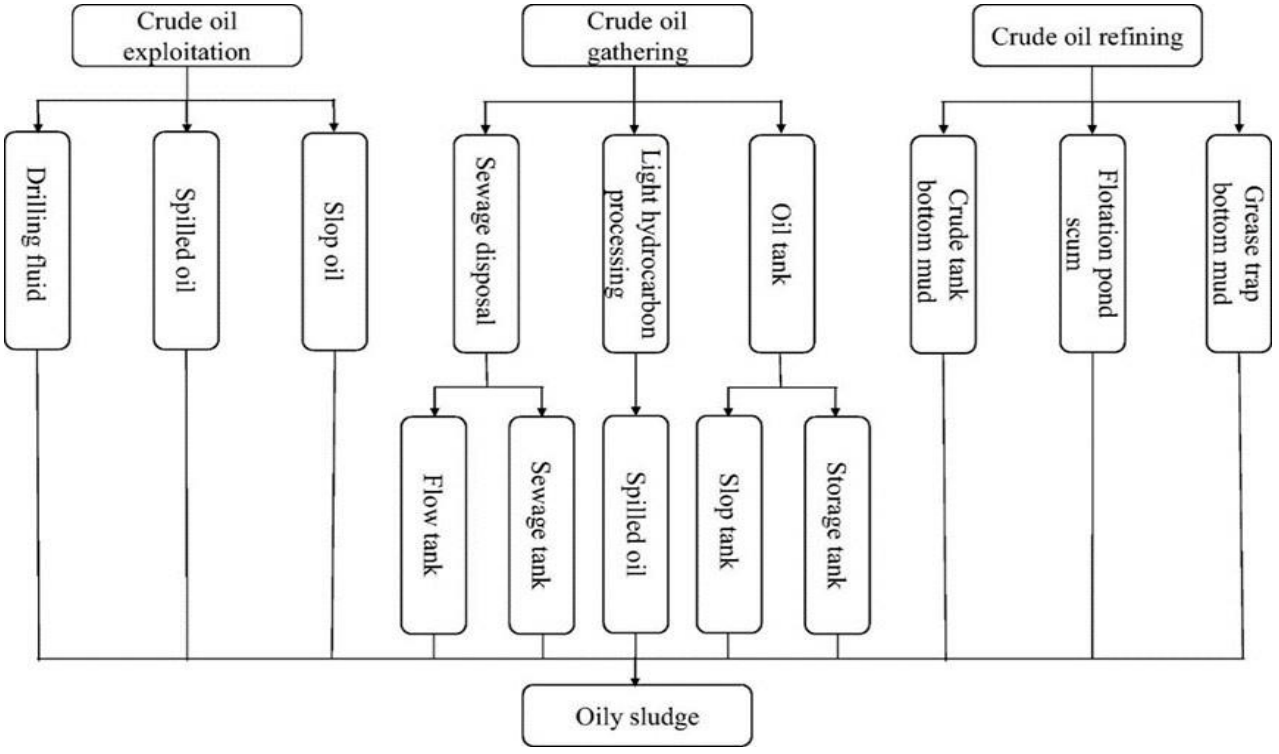


Figure 1: A schematic diagram of the oily sludge production in petroleum refinery (Hui *et al.*, 2020)

Overtime, they accumulate and reside in the fine pores and become inaccessible. Their fate and behavior are affected by factors such as soil type and their physico-chemical properties, their concentration, structures of the components and their solubility, environmental conditions like temperature, pH, moisture content and wind, and the availability of degradative microorganisms. The compounds in oily sludge have different solubility, for example, the polycyclic aromatic hydrocarbons (PAHs) have very low solubility and hence cause a problem in environment as they are less bioavailable (Mahmoud, 2004).

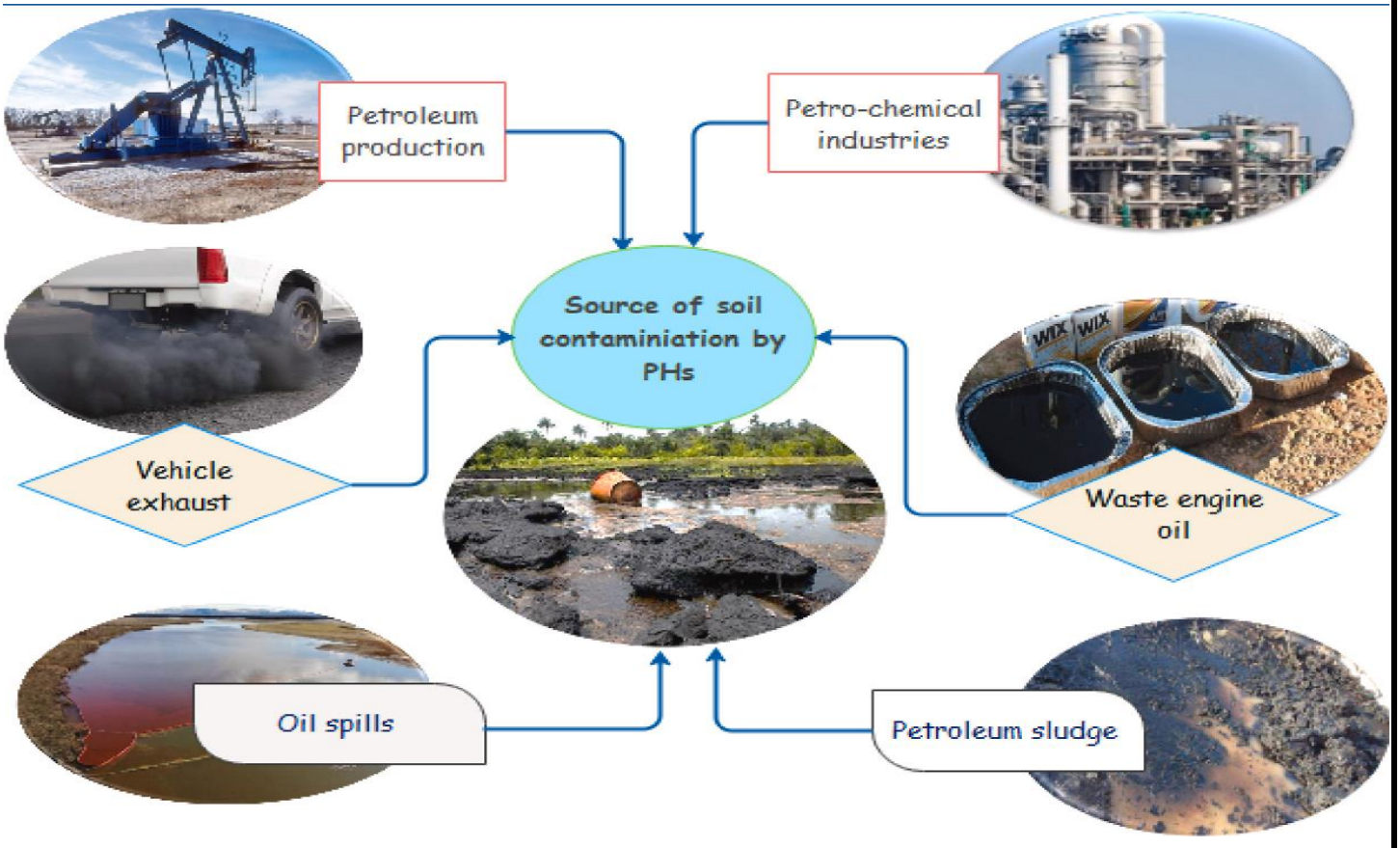


Figure 2: Sources of soil contamination by petroleum hydrocarbons (Ambaye *et al.*, 2022)

a. Composition of oily sludge

As stated, oily sludge is a complex emulsion of several petroleum hydrocarbons (PHCs) mixed with some solid particles and heavy metals (Genouw *et al.*, 1994; Al-Futaisi *et al.*, 2007). Petroleum hydrocarbons (PHCs) are one of the most widespread heterogeneous organic contaminants that affect the soil ecosystem (Hongtao, 2002; Ivshina *et al.*, 2015). The chemical composition of the oily sludge varies greatly, which depends on crude oil source, processing method, equipment and reagents used in the refining process. The total petroleum hydrocarbon (TPH) content in oily sludge usually ranges

from 15-50% w/w, whereas water and sediments are in the range of 30-85% and 5-46% respectively (Liu *et al.*, 2012; Mohan and Chandrasekhar, 2011; Tahhan *et al.*, 2011). Composition and concentration of oily sludge is shown in Table 1.

Table 1: Composition and concentration of oily sludge

Elements	**Concentration**	**Reference**
Water	30-85%	Xuening *et al*., 2020
Sediments	5-46%	
Asphaltene	8-10%	Oknina *et al*., 2015
Resin	7-22.4%	
Wax	48.67%	Mishra *et al*., 1999
Aliphatic hydrocarbon	40-52%	
PAHs	28-31%	
Phenol	90- 100 mg/kg	Bhattacharyya *et al*., 2003
Metals		
Nickel	17-25 mg/kg	Hamme and Odumeru, 2000
Chromium	27- 80 mg/kg	
Zinc	7- 80 mg/kg	
Manganese	19- 24 mg/kg	
Cadmium	0.8-2 mg/kg	
Copper	32- 120mg/kg	
Lead	0.001- 0.12 mg/kg	

The potentially dangerous PAHs are present in least amounts in oil sludge but are potentially immune toxicants in nature (Gibson and Subramanian, 1984; Mueller *et al.*, 1991; Field *et al.*, 1992;

Sutherland *et al.,* 1995). Polyaromatic hydrocarbons (PAHs) are class of prevalent mutagenic or carcinogenic environmental contaminant. PAHs have been identified as hazardous chemicals by different State and Central Pollution Control Boards, because of their toxicity along with, carcinogenic and mutagenic effects on air and soil ecosystem (Bisht *et al.,* 2015). Due to anthropogenic activities, PAH are released in environment from natural sources such as open burning, seepage of petroleum or coal deposits, and volcanic activities. Deposition of PAH in soil mainly takes place through atmosphere in the form of aerosols in gaseous phase in air, entering human body. Some crops, such as wheat and lentils may synthesize PAHs or absorb them via water, air, or soil. Human beings come in contact with PAH through inhalation, ingestion, or direct dermal contact i.e. breathing ambient and indoor air, eating food containing PAHs and smoking cigarettes. Long-term exposure of PAHs can lead to health issues like decreased immune function of eyes, kidney damage, liver damage, respiratory problems and abnormalities (asthma). Meanwhile, repeated contact with skin may induce redness and skin inflammation (Diggs *et al.,* 2011; Olsson *et al.,* 2010).

b. Bioremediation of petroleum hydrocarbon

Oily sludge contamination can lead to changes in chemical and physical properties of soil which may create nutrient deficiency and inhibit seed germination leading to lesser survival of plants (Mutairi *et al.,* 2008). To bring an effective solution for treatment and management of this waste that is produced world-wide has become a priority. Elimination of petroleum hydrocarbon pollution can be achieved by physiochemical and biological methods. But, most of the physiochemical methods are costly and generate secondary pollutants. Biological methods such as bioremediation are more suitable as they are affordable and ecofriendly (Gomez *et al.,* 2013). Many projects are going on for the sustainable management of oily sludge which focus mainly on reducing environmental contamination by petroleum hydrocarbons to acceptable levels. Several studies have shown the approaches to remediate the PAH-polluted soils and sediments with different remediation strategies (Kumar *et al.,* 2021). Various strategies for treatment of oily sludge are shown in Figure 3.

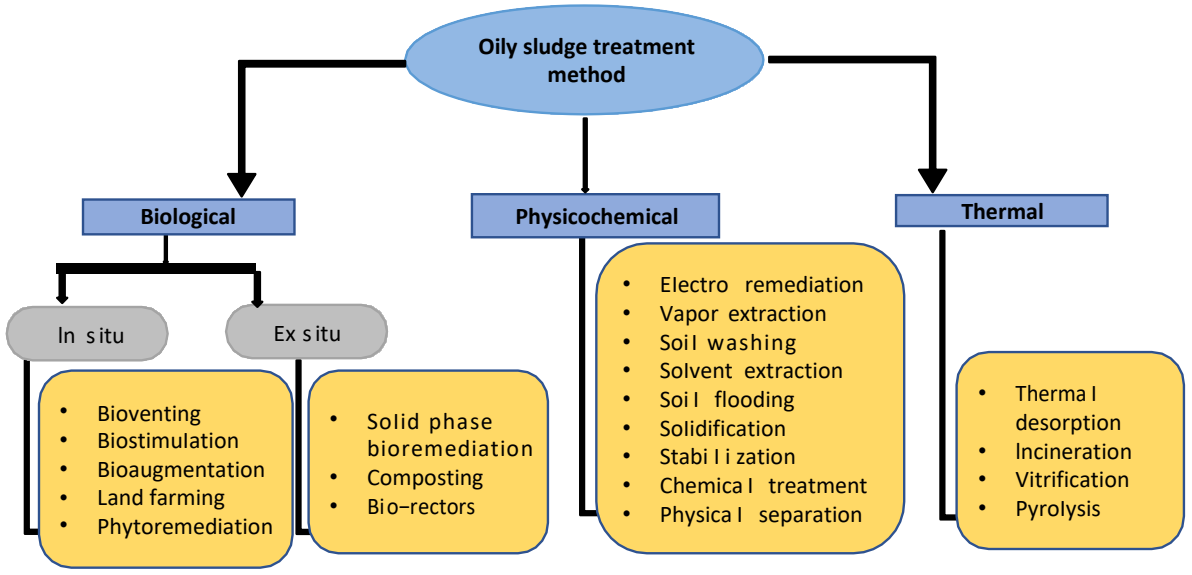


Figure 3: Various methods for treatment of oily sludge (Azubuike *et al.*, 2016; Leung, 2004; Kensa, 2011)

Depending on the degree of saturation and aeration at contaminated area, different strategy can be used. Along with the economic benefits and increasing the recovery rate of oil from oil tanks pyrolysis was used for treatment of oily sludge (Wang *et al.*, 2021). High treatment cost remains a major issue with physical and chemical methods. Bioremediation is a process which mainly uses microorganisms for detoxification of contaminants present in soil and other environments, becoming a best alternative as conventional clean-up technologies (Bento *et al.*, 2005, Patowary *et al.*, 2016). Bioremediation methods are generally categorized into *in-situ* and *ex-situ* techniques. *In-situ* procedures, such as biosparging, bioventing, bioaugmentation, and biostimulation are applied to soil at the site with little disturbance (Vidali, 2001). *Ex situ* techniques such as land farming and composting, are the ones in which soil is excavated and taken away from the site for treatment (Nataraj *et al.*, 2007). *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *Bacillus subtilis* are most frequently used microorganisms for treatment of petroleum-oil contaminated soil (Kishore *et al.*, 2006). Table 2 represents the bacterial strain capable of degrading petroleum hydrocarbon.

Table 2: List of bacterial strains reported for bioremediation of petroleum hydrocarbon

Sr No.	Bacterial strain	Types of pollutants	Efficiency of degradation (%)	Reference
1	*Pseudomonas aeruginosa*	Phenanthrene	78.7	Obo *et a*l., 2016
2	*Mycobacterium spp.*	Naphthalene	92.7	Hesham *et al*., 2014
3	*Sphingomonas spp.*	Pyrene	98.6	
4	*Bacillus subtilis*	n-octadecane	12	Fathepure *et al*., 2014
5	*Alteromonas spp.*	Hexadecane	100	
		Eicosane	91	
		Phenanthrene	41	
6	*Burkholderia spp.*	Pyrene	59-62	You *et a*l., 2018
7	*Caulobacter spp.*		21-24	
8	*Rhodobacter spp.*	Alkane	87.35-99.89	
9	*Stenotrophomonas acidaminiphila*	PAH	91.7-94	Cerqueira *et al*., 2011
10	*Bacillus cereus*	TPH	88.4	
11	*B. megaterium*		89-92	
12	*B. cibi*		89.7-91	
13	*Acinetobacter spp.*	PHC	37	Verma *et al.*, 2006
14	*Bordetella avium*	Naphthalene	95	Abo-State *et al*., 2017
15	*Achromobacter marplatensis*	PHC	49	Muratova *et al*., 2018

Further, the efficiency at which TPH are bio-degraded depends greatly on different factors such as (Ali *et al.*, 2022)

1) pH

2) Temperature

3) Oxygen

4) Diversity of the microbial community

5) Remediation-friendly environment

6) Degree of adaptability

7) Structural properties of molecules

8) Cellular transport properties

9) Susceptibility to contaminants

Two methods of bioremediation aimed at enhancing and accelerating the process are bioaugmentation and biostimulation (Odokuma *et al.,* 2003; Odu *et al.,* 2005). The process of "bioaugmentation" involves introducing an external microbial population to a polluted area, whereas in order to increase the nutrient and microbial activities of the local microbial flora on a polluted site, biostimulation is performed (Nwadinigwe and Onyeidu, 2011). Oily sludge mineralization rates depend on the survival rate of indigenous microorganism which can be enhanced using biostimulating agents. The purpose of using biostimulating agents is to increase the soil's natural fertility status and speed up the rate of microbial oil degradation (Amadi *et al.,* 1990). Organic nutrients like biogas slurry, manure, composted spent mushrooms, rice straw, and corncobs are commonly used as biostimulating agents (Simarro *et al.,* 2013, Suja *et al.,* 2014; Kauppi *et al.,* 2011). Some of the organic nutrients which have been reported as biostimulating agents have been listed below in the table 3.

Table 3: List of organic nutrients reported as biostimulating agent for bioremediation of petroleum hydrocarbon contamination

Biostimulating agents	**Type of contaminant**	**Remarks**	**References**
Compost made from wood chips and sewage sludge	Crude oil	17% TPH degradation within 19 months	Atagana,2008

Brewery grains, banana skin and mushroom compost	Lubricating oil	92% removal of TPH in 84 days	Abioye *et al.*, 2012
Poultry droppings	Petroleum hydrocarbons contaminated marine sediments	98.92% PAH and 95.35% TPH degradation in a bioreactor containing 20g poultry litter and 1 liter of petroleum hydrocarbon contaminated seawater	Chikere *et al.*, 2012
Cow dung	Hydrocarbon polluted mangrove swamp	49.88% and 69.85% of TPH reduction in 28 days and 70 days respectively.	Orji *et al.*, 2012
Tea leaves, soycake and potatoskin	Diesel fuel	75%TPH degradation within 84 days	Dadrasnia and Agamuthu, 2013
Oil palm emptyfruit bunch andsugar cane bagasse	Crude oil	95% degradation in 20 days	Hamzah *et al.*, 2014

In the present study, tea leaves was used as biostimulating agent. Tea leaves contains essential elements like phosphorus (P), nitrogen (N), potassium (K) which could stimulate the indigenous microflora in oily sludge contaminated soil thus speeding up the degradation process (Chen and Lin, 2016). Tea leaves also contain several polyphenols, including flavanols (in particular catechins and gallic acid), theaflavins (3-6%), and phenolic acids (10-12%) (Sharma & Rao 2009). It also contains carbohydrates (15%), proteins (15%), flavanols (6-8%), minerals (10%), volatiles (<0.1%), amino acids (13-15%), methylxanthines (8-11%), carotenoids (<0.1%), chlorophyll (0.5%) and lipids (7%) (Sharangi 2009).

But only about 10% of the total population of soil microflora is capable of degrading the pollutants naturally and hence take longer time to remove the contaminations. Thus, bioaugmentation process in which specific degraders are introduced into the soil is used along with biostimulation to increase bioremediation efficiency (Pimmata *et al.*, 2013; Simarro *et al.*, 2013). The result of bioaugmentation depends on the interaction between exogenous and indigenous populations of microorganisms because of the competition, mainly for nutrients. The effect of bioaugmentation on crude oil and bacterial community has been studied extensively (Muangchinda, 2020). Comparative studies have also been

done for biostimulation and bioaugmentation and it is reported that it gives more effective results (Hamidi., 2021 and Adams *et al.*, 2015). Bulking materials such as sand, gravel, sugarcane bagasse, coconut husk, sawdust, straw and wood chips has also been added in contaminated soil to improve degradation rate as reported in Armendariz *et al.*, 2004.

c. Hydrocarbon degradation pathways

As described, hydrocarbons are grouped into straight-chain (n-alkanes), branched- chain, cyclic compounds, mono- or polycyclic hydrocarbons (Natalia, 2013). Most hydrocarbons are polar and have low chemical reactivity at ambient temperature. The nature, arrangement and presence of unsaturated bonds have a major role in determining the differences in their reactivities.

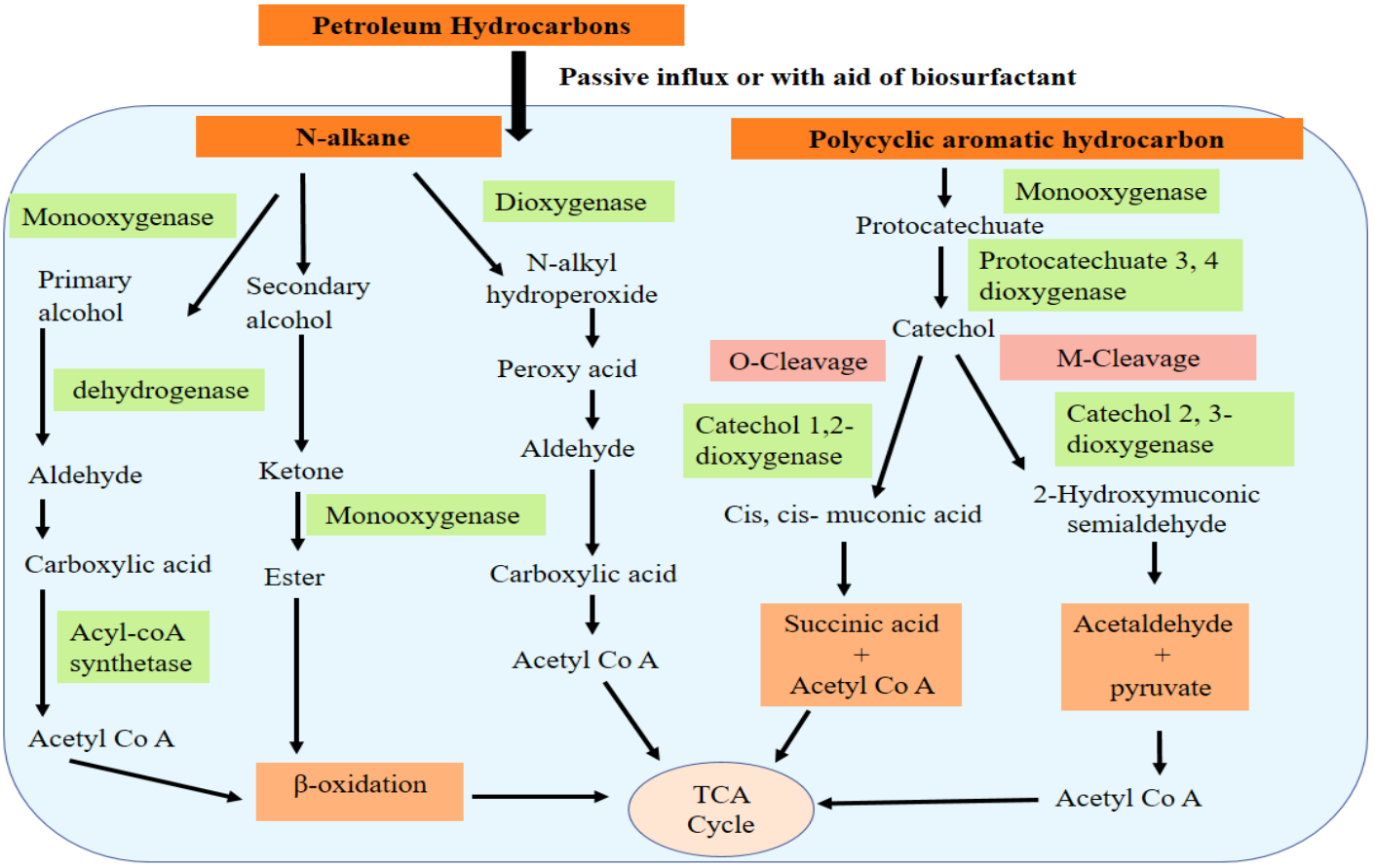


Figure 4: Degradation of petroleum hydrocarbons (Chikere *et al.*, 2011; Varjani, 2016; Hwang *et al.*, 2007*)*

The most important phase in the degradation of petroleum hydrocarbons is the formation of contact between hydrocarbon degrading bacteria and substrates. Microbes degrade petroleum hydrocarbon by the process of absorption in the form of large hydrocarbon molecules or tiny pseudo-soluble molecules. Bacterial adherence and its hydrophobicity play an important role for successful uptake

of substrate (Shi, 2019). Some petroleum hydrocarbons are sorbed into the soil matrix and are not bioavailable to microbes for degradation. Thus, microorganism releases biosurfactant which increases substrate bioavailability by increasing the hydrophobicity of the cell surface, allowing substrates to interact more easily with bacterial population (Pacwa-Płociniczak M. *et al.,* 2011). Microbial surfactants are low-molecular mass compounds such as lipopeptides, glycolipids, and phospholipids (Janek *et al.,* 2010). Petroleum hydrocarbons such as alkanes and polycyclic aromatic hydrocarbons are degraded by enzymes such as monooxygenases and dioxygenases. Figure 4 represents the petroleum hydrocarbon degradation pathway. Generally aerobic degradation begins with the oxidation of a terminal methyl group in the case of n-alkanes with two or more carbon atoms to produce a primary alcohol. To create acetyl-CoA, fatty acids are conjugated to CoA and then processed further by oxidation, which is then further oxidized to the equivalent aldehyde, and lastly transformed into a fatty acid. Oxygen is used by aerobic alkane degraders to activate the alkane molecule. Thus, during degradation of alkane, terminal methyl group is targeted by monooxygenases where a primary alcohol is generated. Similarly, dioxygenase adds two oxygen atoms to the n-terminal methyl group of alkane. As a result, fatty acid is produced which gets metabolized via β oxidation to acetyl CoA. The alkane-activating enzymes, which are monooxygenases, produce reactive oxygen species to compensate for the hydrocarbon poor chemical reactivity (Raju, 2009). Polycyclic aromatic hydrocarbons are degraded to protocatechuate and catechol via monooxygenase and protocatechuate 3,4-dioxygenase which then gets converted into acetyl CoA via enzyme catechol-1,2-dioxygenase and catechol-2,3-dioxygenase. Acetyl Co-A is then further metabolized via TCA cycle to carbon dioxide and water (Atlas and Bartha 1998; Hamme *et al.,* 2003; Elsas *et al.,* 2007). Various hydrocarbon degrading enzymes are enlisted in Table 4.

Table 4: Enzymes involved in hydrocarbon degradation pathway

Sr. no.	**Target**	**Microorganism**	**Degrading enzyme**	**Reference**
	Alkanes			
1	C1-C8 alkanes alkenes and cycloalkanes	*Methylococcus, Methylosinus, Methylocystis, Methylomonas, Methaylocella*	Soluble methane mono-oxygenase	Das *et al.,* (2011)

2	C1-C8 (halogenated) alkenes and cycloalkanes	*Methylobacter, Methylococcus, Methylocystis,*	Particulate methane mono oxygenase	Kothari *et al.*, (2014)
3	C5-C16 alkanes, fatty acid, alkyl benzenes, cycloalkanes and so forth	*Pseudomonas, Burkholderia, Rhodococcus, Mycobacterium*	AlKB related alkane hydroxylases	Margesin *et al.*, (2003)
4	C10-C16 alkanes, fatty acids	*Candida maltose, Candida tropicalis, Yarrowia lipolytica*	Eukaryotic P450	Iida *et al.*, (2000)
5	C5-C16 alkanes, cycloalkanes	*Acinetobacter, Caulobacter, mycobacterium*	Bacterial P450 oxygenase system	Cerniglia *et al.*, (1993)
	PAH			
6	C10-C30 alkanes	*Acinetobacter sp.*	Dioxygenase	Das *et al.*, (2011)
7	Fluorene	*Terrabacter spp.*	Dioxygenase *FlnRB-dbfA1A2-FlnED1-ORF16*, Decarboxylase	Peng *et al.*, (2008)
8	Anthracene	*Mycobacterium, Rhodococcus PYR-1*	Multicomponent monooxygenase	
9	Phenanthrene dihydrodiols	*Rhodococcus spp.* P200-P400 & NCIMB12038	Dioxygenase *narAa, narAb and narB*	
10	Naphthalene	*Pseudomonas putida*	Dioxygenase	

d. Role of carrier matrix in bioremediation

In order to increase survivability of organism during bioremediation process, microorganisms are incorporated onto “carriers” a medium that can hold enough microorganisms and maintain their viability under varying conditions (Marschner, 2011). The following characteristics are necessary for

a good carrier (Bayat *et al.*, 2015):

- ✓ Highly absorptive (water-holding capacity) and easy to process
- ✓ Non-toxic to microorganisms
- ✓ Available in adequate amounts at low-cost
- ✓ Good buffering capacity

Carriers can be classified as natural or synthetic in nature. Natural organic carriers have many functional groups which stabilize microbes. This class of carriers includes: alginate, κ-carrageenan, chitosan, sawdust, straw, charcoal, corncob, bagasse (Wojcieszyńska *et al.*, 2013, Gentili *et al.*,2006, Huang *et al.*, 2006, Ullah, 2010, Mohammadi *et al.*, 2009, Gouda *et al.*, 2007,

Wang *et al.*, 2012). Incorporation of microorganism on carrier medium can be done by methods such as adsorption, covalent binding, entrapment and encapsulation. Here in present study saw dust was used as carrier matrix. Saw dust is most common agro-waste produced which has been successfully used for the immobilization of bacterial cells. *Arthrobacter sp*. immobilized on sawdust did not lose their enzymatic activity after 6 weeks of storage and was still able to degrade similar quantities of crude oil (Obuekwe and Al-Muttawa, 2001). Significant increase in production of biosurfactant has been demonstrated in a study by using bacterial consortium along with carrier matrix (Hazaimeh *et al.*, 2014).

e. Biomass development for field scale application

Selection of appropriate substrate plays a vital role in growth of microorganism (Marchant *et al.*, 2014). Media requirements for producing biomass in higher quantity becomes a challenge therefore, cost effective substrate selection is important. Various industrial wastes such as agricultural, dairy (sugars, molasses, plant oils, starchysubstances, lactic whey), distillery, animal fat and oil have been reported as substrate for growing microorganisms (Makkar *et al.*, 2011). Table 5 represents enlist substrates for growing microbes.

Table 5: List of substrates reported for growing different bacteria

Sr. no.	**Microorganism**	**Substrates**	**Reference**
1	*Bacillus subtilis*	Molasses	Makkar and Cameotra, 1997
2	*Bacillus spp.*	Potato cassava	Noah *et al.*, 2002

3	*Bacillus subtilis*	Potato waste	Thompson *et al.*, 2000
4	*Bacillus spp.*	Wheat bran	Ohno *et al.*, 1993
5	*C. bombicola*	Soy molasses	Solaiman *et al.*, 2007
6	*P. aeruginosa*	Whey	Daniel *et al.*, 1998
7	*Bacillus spp.* *Pseudomonas spp.*	Glucose	Bren *et al.*, 2016
8	*Lactobacillus spp.*	Agro-industrial waste	Nitschke *et al.*, 2004
9	*Klebsiella spp.*	Waste soyabean oil	Lee *et al.*, 2008
10	*P. aeruginosa*	Curd whey and distillery waste	Dubey *et al.*, 2005
11	*Trichosporon montevideense*	Dairy industry effluents	Monteiro *et al.*, 2009

Selection of appropriate substrate according to the requirement and utilization capacity of microorganism to develop biomass is essential as this is one of the important economic factor affecting mass production of biomass for field scale application.

f. Challenges in bioremediation

Along with the advantages of bioremediation, there are many challenges. Temperature, type of microorganisms, treatment duration, nutrients, initial TPH concentration and characteristics of oily sludge are most common challenges faced during the treatment of oily sludge contaminated soils. The major challenge is that all compounds are not biodegradable and this limits the bioremediation process. Lab scale studies are challenging to extrapolate at field scale (Abatenh *et al.*, 2017). Many times, a specific bacterial strain that is effective at one site might not be able to function effectively at other site and thus obtaining the bacterial strain capable of functioning at all site is challenging (Ramırez-Garcıa *et al.*, 2019). Uneven distribution of contaminants also effects the bioremediation process.

2. Rationale of the study

The isolates working efficiently for hydrocarbon degradation in the lab are unable to work appropriately in the actual field due to various stress conditions like change in pH, competition with native microbes, toxicity of hydrocarbons and bioavailability of substrates. So, the aim of present study was to develop an appropriate bioremediation strategy to remediate oily sludge contaminated soil. The consortium was developed using efficient crude oil degrading indigenous bacteria isolated from oily sludge contaminated samples collected from ONGC, Ankleshwar earlier. One of the aims was to determine the appropriate carbon source for growing bacterial consortium as mass production of bacterial strains for field application is one of the economic factors affecting field studies. The member of this consortium were fast growers, able to withstand wide range of pH and few were able to produce biosurfactants as well. The present study also investigated to develop an appropriate bioremediation strategy for treating oily sludge contaminated soil using the selected consortium by incorporating anappropriate carrier matrix. The developed strategy at lab level may prove useful for actual field application.

3. Objectives

To address the research problems, the following objectives were devised,

1. Determining appropriate carbon source for biomass development required for field scale bioremediation studies.
2. To investigate oily sludge bioremediation potential of developed bacterial consortium.

   a) Evaluating TPH removal potential of biostimulating agent and bacterial consortium in soil spiked with oily sludge.

   b) Optimization of carrier matrix for amendment of bacterial consortium in oily sludge contaminated soil.

MATERIALS AND METHODS

4. Materials and methods

4.1 Bacterial strains of consortium

Generally it is challenging to achieve the degradation of pollutants by a single bacterial strain. Hence, the bacteria with different abilities to degrade the contaminants could be mixed together to get a diversified characteristic of each individual strain. The advantages of each strain could be combined in this microbial consortium to effectively degrade the contaminants. Initially, 54 bacteria were isolated and screened, from which 31 were found to be fast-growers. Further, from these, 19 were screened on the basis of more than 80% sodium benzoate degradation capacity. A screened total of 8 isolates were found to degrade more than 80% of 1% crude oil and more than 50% of 5% crude oil. Finally, a consortium of 4 different bacterial strains designated as *Pseudomonas balearica* AWSant37, *Pseudomonas aeruginosa* ASSphn611, *Achromobacter xylosoxidans* AWSant34, *Pannonibacter phragmitetus* AWSant36 was selected which gave the best results. These strains were selected on the basis of different characteristics like 1% and 5% TPH degradation potential, biosurfactant production ability and ability to withstand wide pH range as shown in Table 6.

Table 6: Characteristics of bacterial strains included in consortium

Bacterial strains of consortium	**% TPH degradation in 1% crude oil**	**% TPH degradation in 5% crude oil**	**Biosurfactant production ability**	**Optimum pH**
Achromobacter xylosoxidans AWSant34	93.4	53.2	+	5.5/7/8.5
Pannonibacter phragmitetus AWSant36	80.8	51.9	-	7
Pseudomonas balearica AWSant37	90.3	65.4	-	7/8.5
Pseudomonas aeruginosa ASSphn611	85.4	53.9	-	7

4.2 Sampling site

Oily sludge and soil sample used in the study were collected from ONGC, Ankleshwar, Gujarat (21°54’48.0” N; 72°46’28.4” E). Figure 5 presents the images of sampling site.

A **B**

Figure 5: Sampling site **A**. Soil sampling site and **B**. Oily sludge sampling site

4.3 Determining appropriate carbon source for biomass development

The substrates are the nutrients necessary for microbial cells to grow and function (Bhavsar, 2011). The use of economically feasible and renewable substrates for growth of microorganisms is beneficial as cost associated with biomass development is one of the major factors affecting field application of bioremediation. Hence, to determine appropriate C source which can be used for growing bacteria in mass, following procedure was used (Figure 6).

- The cultures were activated and streaked on nutrient agar plate and incubated at 28^{o} C for 24 h.
- Next day, colony was picked up with the help of swab and inoculated into the IF-A (Inoculating Fluid) tube and 95% turbidity was set using turbidometer.
- 200 µl of IF-A cell suspension was inoculated into the 96-well Biolog GEN III plate containing 71 C sources and 21 chemicals for sensitivity assay using multichannel micropipette.
- The inoculated GEN III plate was incubated at 28°C and analysis of formazan complex indicated by purple/violet colour formed due to respiration of microbes was carried out at 4, 8 and 22 h in ELISA reader at 598 nm.
- Heat map was generated using obtained readings and appropriate carbon source supporting growth of all isolates in consortium was determined.

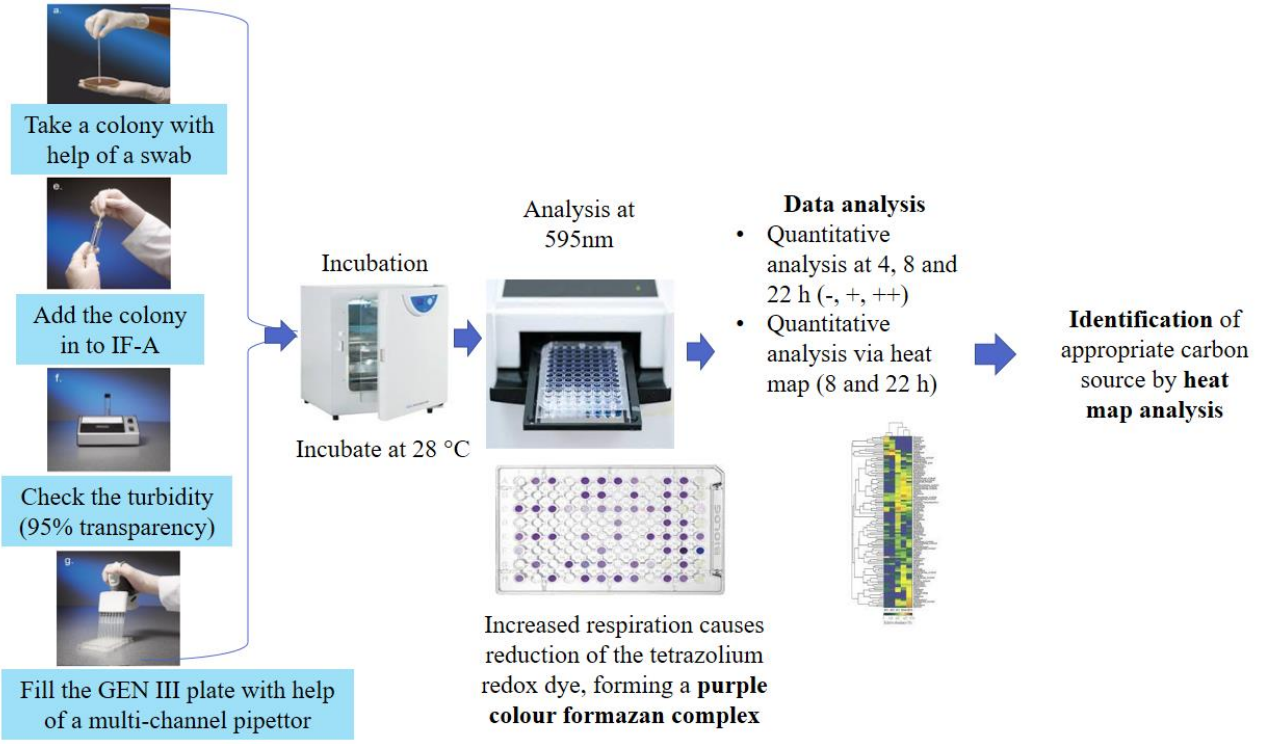


Figure 6: Methodology for determining appropriate carbon source

4.4 Biostimulation using tea leaves for treating oily sludge contaminated soil

a) Experimental design for tea leaves as biostimulating agent

As biostimulating agent to treat the soil contaminated with oily sludge, waste tea leaves collected from the Canteen of Institute of Science, Nirma University, Ahmedabad, Gujarat were used. The tea leaves, soil before amendment with tea leaves and after amendment are shown in Figure 7.

- The collected tea leaves were dried and 30 g of it was added in 1 kg of soil spiked with 10% of oily sludge.
- After 20 days of incubation, %TPH degradation was determined using GC-FID analysis.
- CFU count was also determined to quantify the soil microflora.

a) Waste tea leaves **b) Soil before mixing tea leaves** **c) soil after mixing tea leaves**

Figure 7: Tea leaves as biostimulating agent

b) Determining oily sludge degradation potential using GC-FID analysis

- After incubation period of 0, 20 & 40 days, residual level of petroleum hydrocarbon was determined by solvent extraction method followed by GC-FID analysis. The flow diagram of described experimental set up is shown in Figure 8.

- In 20 g of soil sample collected from experimental sets, 25 ml hexane was added and kept in shaking conditions in shaker for 5 min.

- After shaking, 25 ml hexane was added in it and then sample was transferred to separating funnel.

- The separating funnel was shaken for 2 min and allowed to stand for some time till separated layers could be seen.

- The upper layer of hexane containing hydrocarbons was collected and used for determining residual TPH levels.

- The samples were concentrated to 15 ml for GC-FID analysis.

- GC Condition: MEGA-5 Capillary column; injection volume was 1µl. The oven temperature rises from 50°C-320°C and the run time was 60 min.

- GC chromatograms obtained was used for calculating % TPH degradation on day 20 using following formula:

$$\% \text{ TPH degradation} = \frac{(\text{Area of peak on day 0}) - (\text{Area of peak on day 20})}{(\text{Area of peak on day 0})} \times 100$$

- Similarly, % TPH degradation was calculated at 40 days of incubation as required.

4.5 Investigating TPH degradation potential of consortium from soil spiked with oily sludge

In the present study, experiment was conducted to investigate the performance of consortium in soil contaminated artificially (spiked) with oily sludge. More over the potential of consortium was also investigated to remove TPH from other petroleum contaminated samples. The procedure for bioaugmentation with consortium is displayed in figure 8.

a) Experimental design for bioaugmentation with consortium

- Culture activation was carried out by inoculating loopful of all the 4 pure cultures in nutrient broth.
- Flasks were incubated overnight under shaker conditions (120 rpm) at 28°C.
- The activated culture was centrifuged at 7500 rpm for 10 min and washed twice with normal saline and pellet was resuspended in normal saline.
- 10^8 cells/g of bacterial cells were bioaugmented in 1 kg soil spiked with 10%, 30% and 50% oily sludge respectively and was incubated for 40 days at room temperature.
- At intervals of 20 and 40 days samples were collected and % TPH degradation potential was examined as described ahead.
- CFU count from collected samples was also determined to quantify the soil microflora.

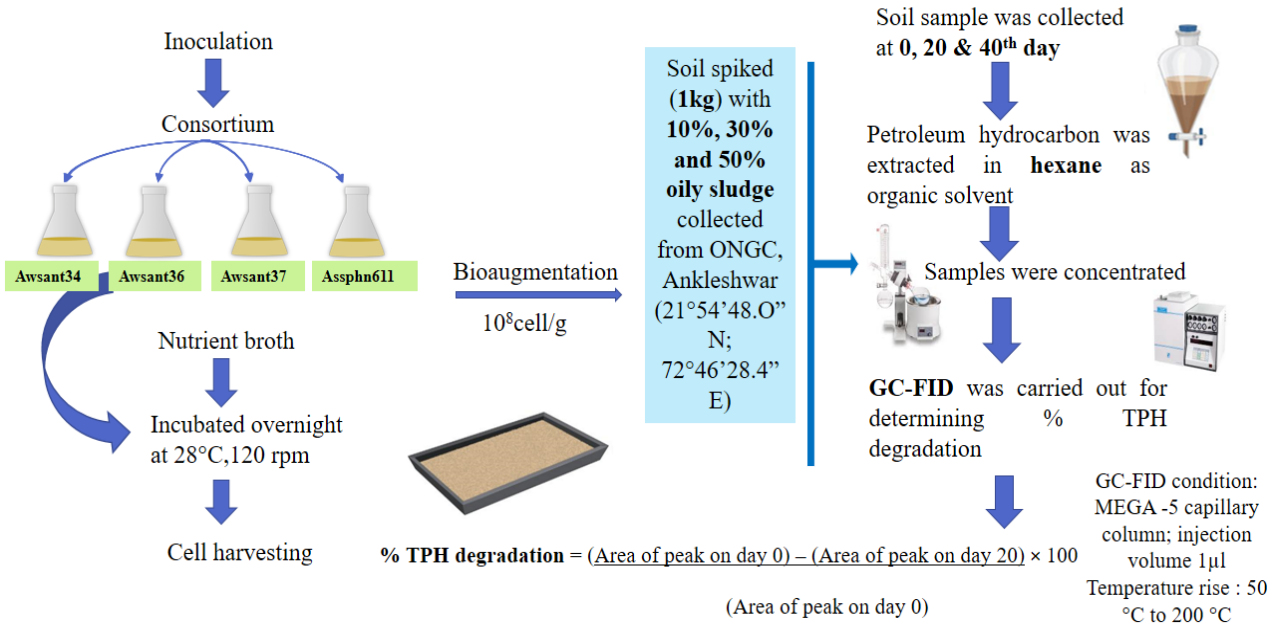


Figure 8: Procedure for bioaugmentation

4.6 Optimization of carrier matrix for field level application of bacterial consortium for oily remediation

Here saw dust was observed to be one of the carrier materials used, which was explored for

the developed consortium. The following experimental design was followed:

- Activated culture equivalent to 10^8 cells/g soil was harvested and mixed with saw dust in the ratio of 3:1 and kept under shaking conditions for 2 h at 28°C and 120 rpm for adsorption of bacterialcells onto saw dust.
- The saw dust containing adsorbed consortium was then mixed with 1 kg soil spiked with 10% oily sludge.
- Soil samples were collected at regular interval of day 0, 20 and 40 days for analyzing residual TPH levels using GC-FID as described ahead and TPH degradation was calculated.
- CFU count was also determined to quantify the soil microflora.

4.7 Treatment of oily sludge contaminated soil by biostimulation and bioaugmentation using carrier matrix

Finally, the treatment method was designed to investigate the bioremediation potential of developed bacterial consortia in combination with biostimulation. The biostimulation was achieved through dried waste tea leaves while bioaugmented culture was immobilized on saw dust as a carrier material. The procedure for this experimental design is as follows:

- Activated culture equivalent to 10^8 cells/g soil was adsorbed on saw dust in the ratio of 3:1as well as 30 g dried tea leaves were mixed in 1 kg soil spiked with 10% of oily sludge.
- The system was kept in plastic trays which were incubated in open environment at 28°C.
- Soil samples were collected at regular interval of 0, 20 and 40 days.
- From the collected samples, oily sludge was extracted in hexane and %TPH levels were analyzed using GC-FID as described ahead.

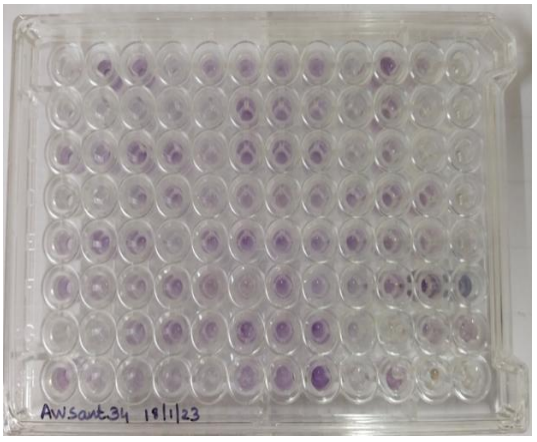
RESULTS AND DISCUSSION

5. Results

The study was conducted to design the appropriate oily sludge bioremediation strategy. Here the efficiency of selected bacterial consortium to utilize up to 50% of oily sludge was determined. Appropriate carbon source supporting consortium growth was determined as this will be essential for mass development of consortium during field studies. Role of tea leaves as biostimulating agent was examined. The importance of saw dust as carrier matrix to adsorb bacterial consortium was also determined.

5.1 Determining appropriate carbon source for biomass development

All microorganism require substrates for growth and metabolism (Bhavsar, 2011). Easily available and cost-effective carbon source for developing biomass for applying it on the field during field studies will play an important role in managing the cost associated with field experiments. Thus, Biolog® GEN III plate was used for finding the carbon source supportingthe growth of bacterial strains used for formulating selected bacterial consortium. Biolog® GEN III plate enables the evaluation of utilization of 71 carbon sources as well as resistance to antimicrobial compounds by individual bacterial strains. Biolog® GEN III plate was inoculated with bacterial consortium and purple colour development in the wells arising because of the utilization of carbon source was detected in the form of O.D (598nm) using ELISA Plate reader (Infinite F50). Utilization of carbon source indicates that organism is metabolically active so in presence of redox dye (tetrazolium) purple colour is produced. Obtained data was used to generate heat map using heat mapper (http://heatmapper.ca/) (Figure 9).



a)

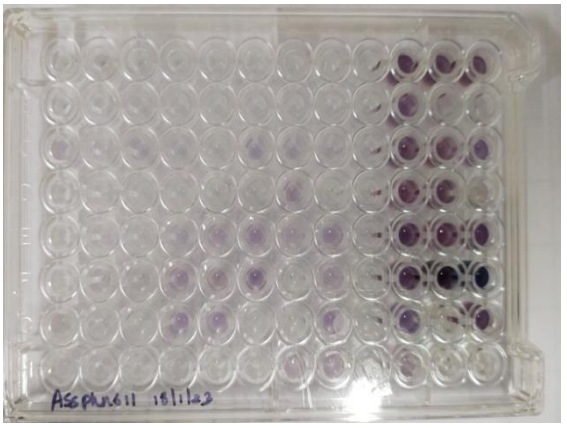
Awsant 34: *Achromobacter xylosoxidans*

b)

Awsant 36: *Pannonibacter phragmitetus*

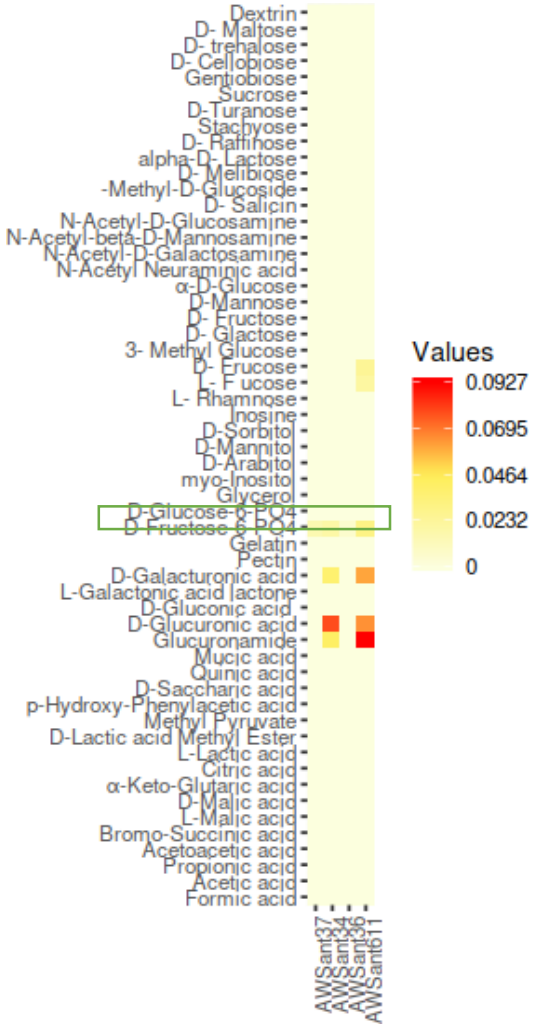
c)

Awsant 37: *Pseudomonas balearica*

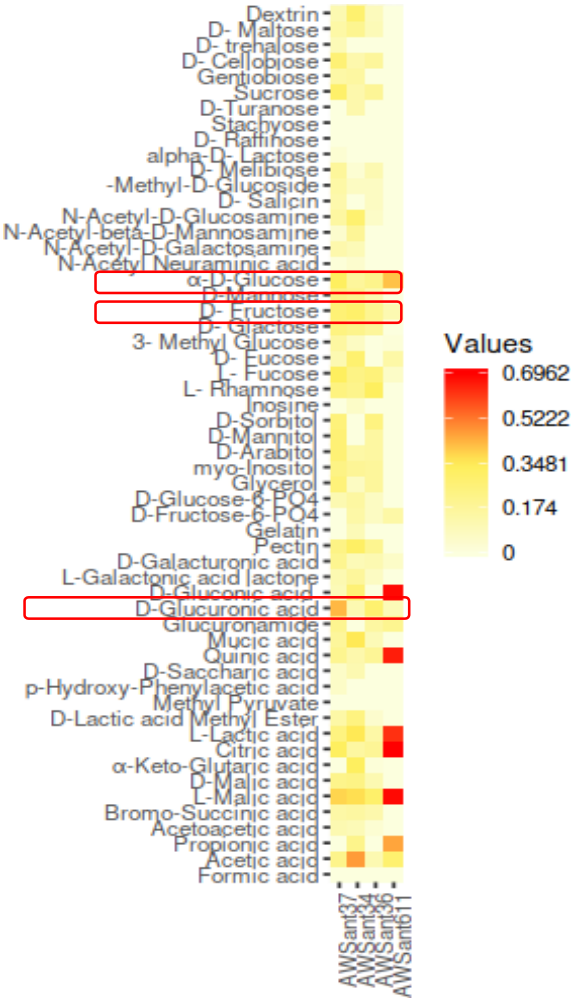


d)

Assphn 611: *Pseudomonas aerugenosa*



e)



f)

Figure 9: Substrate utilization profile of tested bacterial strains of consortium at 22h a*) Achromobacter xylosoxidans* Awsant34; b) *Pannonibacter phragmitetus* Awsant36; c) *Pseudomonas balearica* Awsant37; d) *Pseudomonas aerugenosa* Assphn611; e) and f) heat map of substrate utilization profile at 8h and 22h respectively.

Table 7: Substrate utilization profile at 4h, 8h and 22h by bacterial strain of developed consortium

Bacterial strain	**4h (+)**	**8h (+ +)**	**22h (+ +)**
Achromobacter xylosoxidans AWSant34	D-Fructose-6-PO_4	D-Fructose-6-PO_4, D-Glucuronic Acid, Glucuronamide, D-Galacturonic acid	Dextrin, N-Acetyl- D-Glucosamine, D-Fructose, D- Fucose,L-Fucose, L- Rhamnose, D-Gluconic acid, Mucic acid, Aceticacid
Pannonibacter phragmitetus AWSant36	D-Fructose-6-PO_4	D-Fructose-6-PO_4	L-Rhamnose, D-Glucuronic Acid, D-Galacturonic acid, L- Malic acid
Pseudomonas balearica AWSant37	D-Fructose-6-PO_4	D-Fructose-6-PO_4	D-Cellobiose, Sucrose, α -D- Glucose, Mannose, D-Galacturonic acid, D-Glucuronic acid,Quinic acid, L-Malic acid, Acetic acid
Pseudomonas aeruginosa Assphn611	D-Fructose-6-PO_4, D-Glucuronic Acid, Glucuronamide	D-Fucose, L-Fucose, D-Fructose- 6-PO_4, D-Glucuronic Acid, Glucuronamide, D-Galacturonic acid	D- Fucose

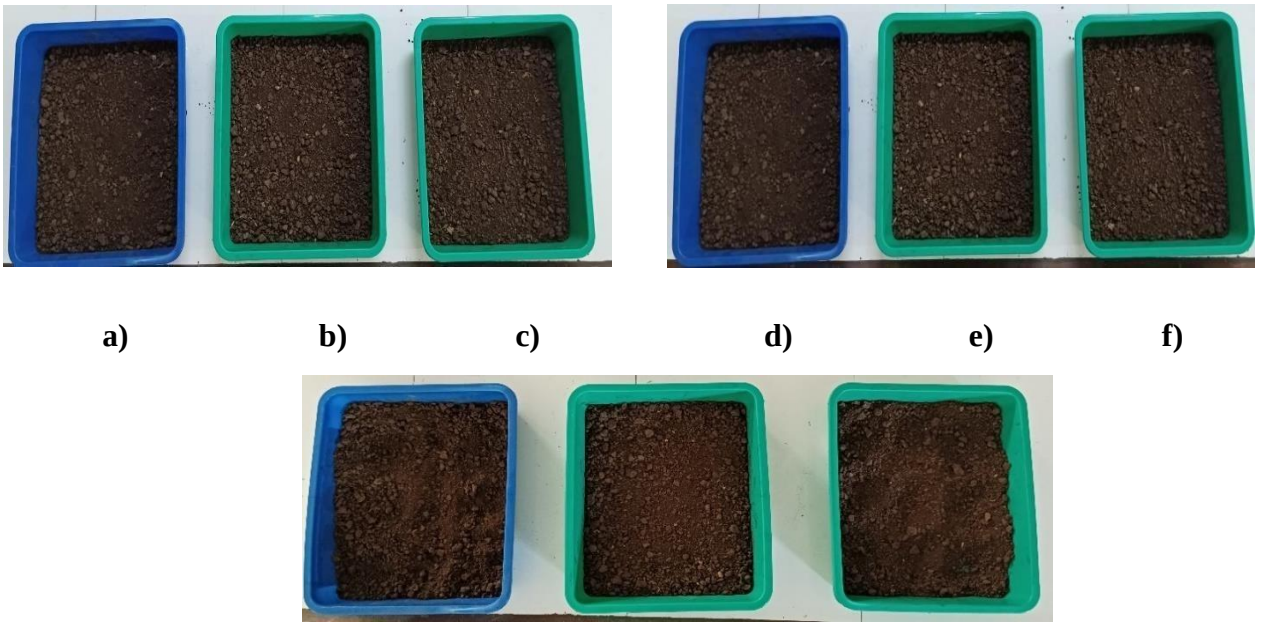
On the basis of observation at 4h, 8h and 22h, organisms have utilized some specific substrate as described in Table 7. The substrate that was utilized maximum such as D-Fructose-6- PO4, α-D-

Glucose, D- Fructose and D- Glucuronic acid by the bacterial strains were taken into consideration. On the basis of this utilized substrate it can be suggested that carbon sources like sugarcane bagasse, molasses and dried citrus fruit peels can be used for biomass development for field scale application.

5.2 Evaluating TPH removal potential of bacterial consortium in soil spiked with oily sludge

5.2.1 Tea leaves as biostimulating agent for treating oily sludge contaminated soil

The activity of indigenous microflora, may be inhibited or affected by certain stress conditions like competition with other microbes, limitation of necessary nutrients or its availability. These factors could be overcome or the activity of indigenous microflora could be accelerated with the help of biostimulating agents. This is because its main aim is to achieve the nutrient balance required for the growth and functioning of the microorganisms. Here, tea leaves were used as boistimulating agent which provides nitrogen (N), phosphorus (P) and potassium (K) that would support the microorganisms for better degradation (Chen and Lin, 2016). Figure 10 shows experimental soil set up for tea leaves as biostimulating agent for treating 10% oily sludge contaminated soil.



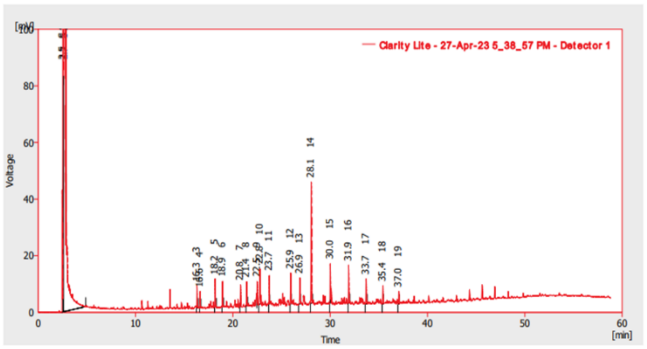
a) **b)** **c)** **d)** **e)** **f)**

g) **h)** **i)**

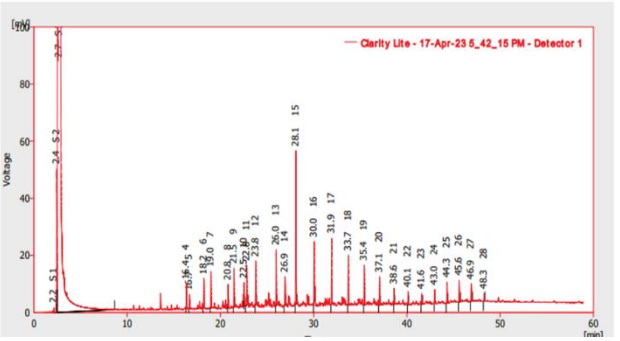
Figure 10: Experimental soil set up for tea leaves as biostimulating agent for treating 10% oily sludge contaminated soil. a), b), c) control and duplicate set- Day 0 respectively; d), e), f) control and duplicate set- Day 20 respectively; g), h), i) control and duplicate set- Day 40 respectively

As stated earlier, the process was carried out with 1 kg of soil spiked with 10% of oily sludge. In

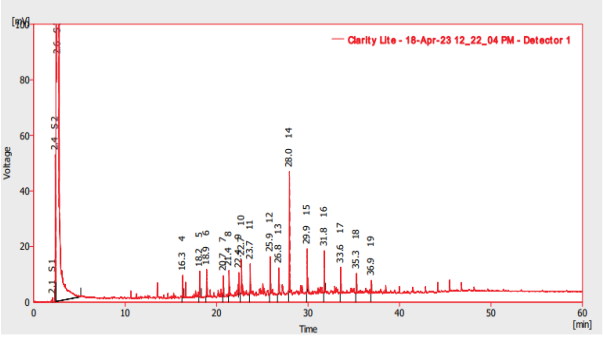
this, 30 g of tea leaves were added and mixed well. Except for the control, in which no biostimulating agent (tea leaves) was added. The dark color of the soil indicates the presence of hydrocarbons in oily sludge which gradually decreases as incubation time exceeds. After 40 days of incubation, visible differences could be observed in the color of the soil setups. On the day 40, the color observed is lighter than that of the day 0, which signifies that the degradation process has been started (Figure 10).The chromatograms of extracted samples were obtained for day 0, 20 and 40 for control and the duplicate sets A and B (Figure 11). By the total area acquired from the obtained chromatograms, % TPH degradation was calculated.



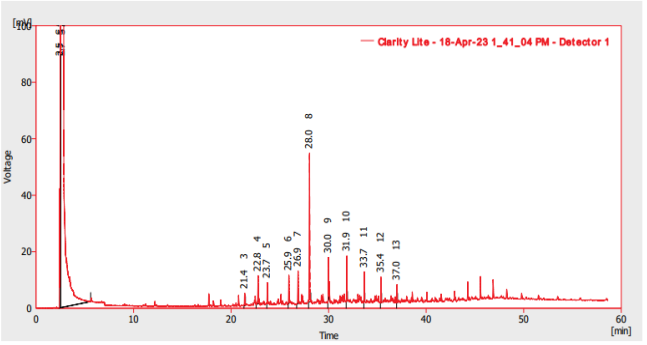
a)



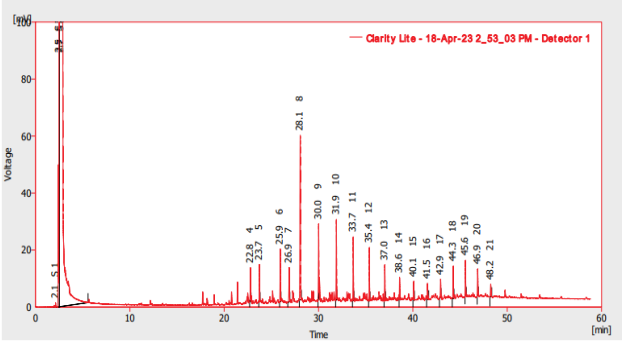
b)



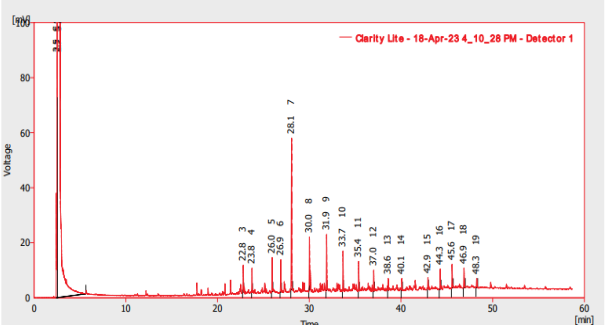
c)



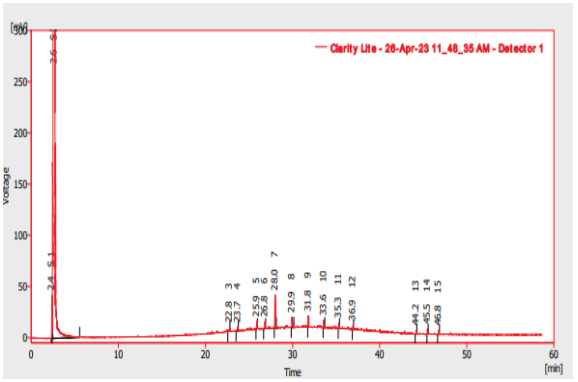
d)



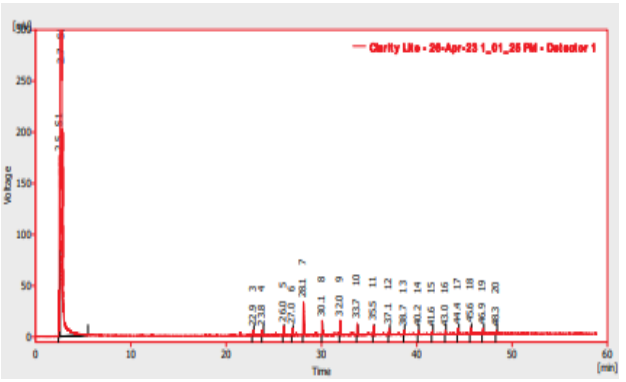
e)



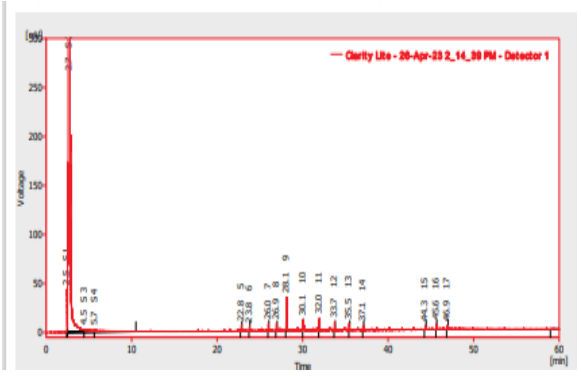
f)



g)



h)



i)

Figure 11: Chromatograms for biostimulation with tea leaves in soil spiked with 10% oily sludge. a), b), c) control and duplicate set- Day 0 respectively; d), e), f) control and duplicate set- Day 20 respectively; g), h), i) control and duplicate set- Day 40 respectively.

TPH degradation was calculated from the obtained total area. Lesser the total area observed, higher is the degradation and similar results were obtained in duplicate set A. But in case of duplicate set B (Figure 11- f), total area (674.307) on day 20 was found to be less as compared to total area (641.396) on day 0 which indicates that coelution phenomenon has taken place. This is because, initially organic compounds when degraded are structurally similar but chemically different compounds termed as isomers are difficult to differentiate by chromatography. This is termed as co-elution phenomena i.e. degrading product formed does not differentiate with the parent compound and is eluting under the main peak which ultimately increases the peak area (Otte *et al.*, 2011). In such case analyzing degradation for longer duration may be helpful. With longer incubation time of 40 days, reduction in total area was observed in the chromatograms. %TPH degradation was found to be higher in soil set up mixed with biostimulating agent (tea leaves) as compared to control (no biostimulating agent) (Figure 12).

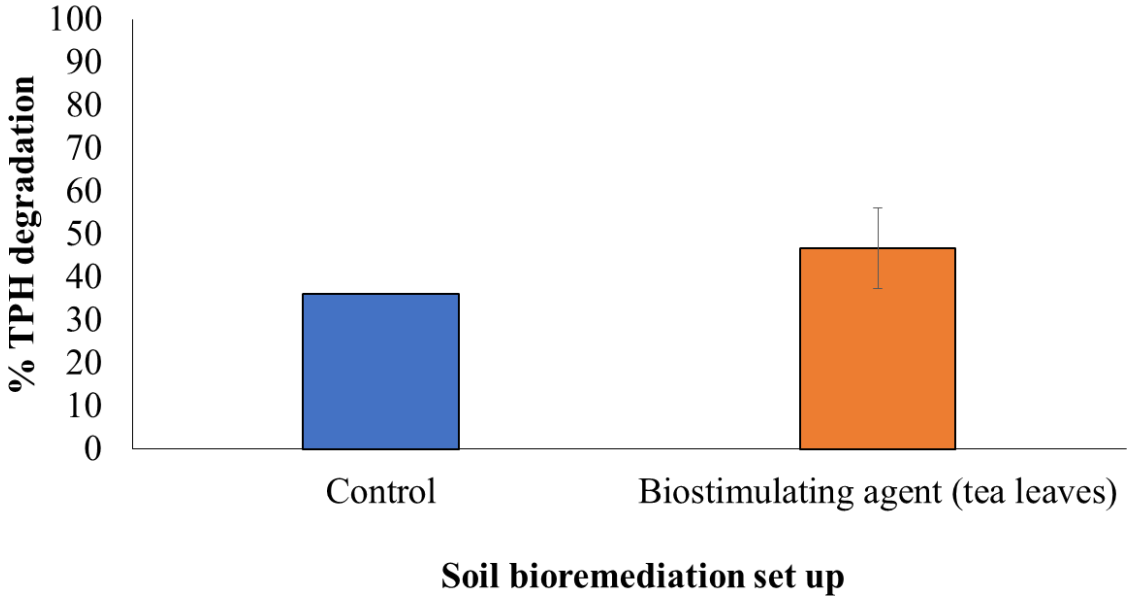
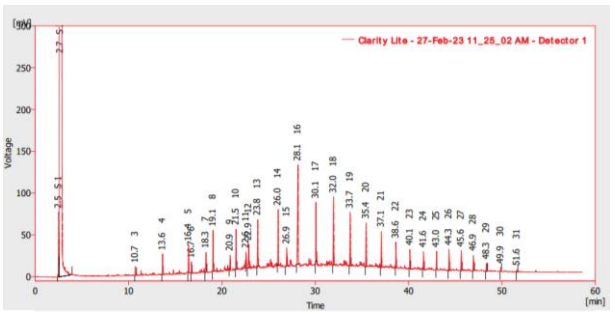


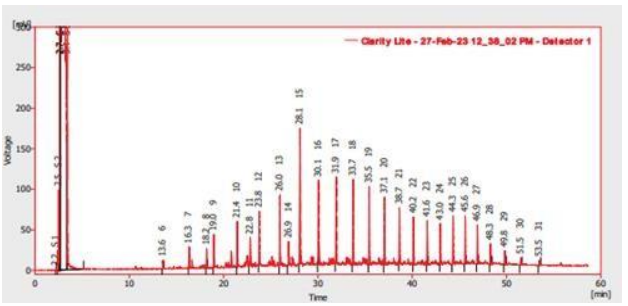
Figure 12: TPH degradation in 10% oily sludge contaminated soil with tea leaves as biostimulating agent in 40 days of incubation. Control soil is inoculated with consortium.

5.2.2 Testing TPH removal potential of selected bacterial consortium

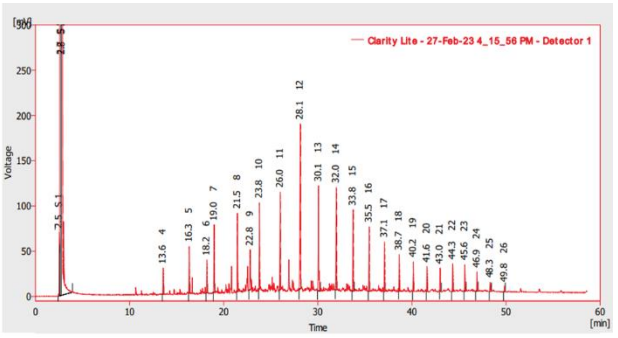
Here, selected bacterial consortium was bioaugmented in soil spiked with 10%, 30% and 50% oily sludge. At Day-0 the color of soil mixed with oily sludge is too dark but with time (Day- 40) the color shade of soil gets lighter. Further, more color change was observed in 10% oily sludge set up as compared to 30% and 50%.TPH content in 0, 20 and 40^{th} day sample was determined using GC-FID analysis. The obtained chromatograms of each sample are represented in figure 13, 14 and 15 respectively.


Clarity Lite - 27-Feb-23 11_25_02 AM - Detector 1
Voltage
Time
[min]

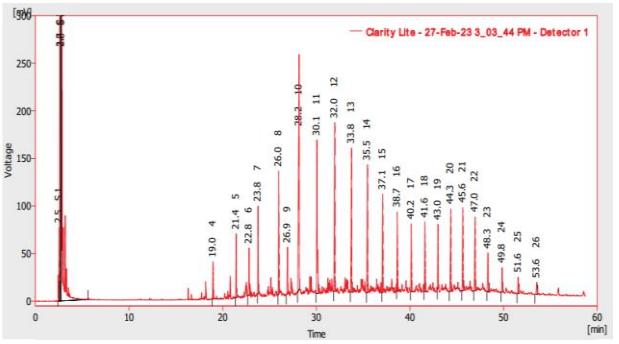
a)


Clarity Lite - 27-Feb-23 12_38_02 PM - Detector 1
Voltage
Time
[min]

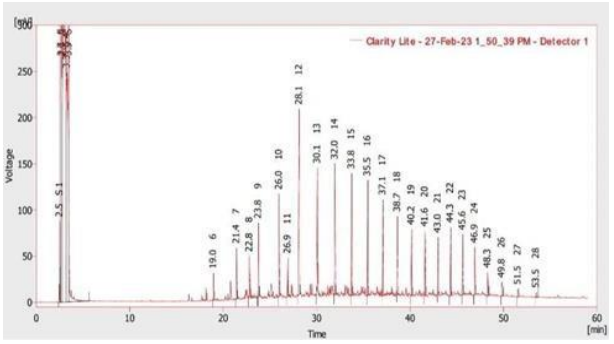
b)


Clarity Lite - 27-Feb-23 4_15_56 PM - Detector 1
Voltage
Time
[min]

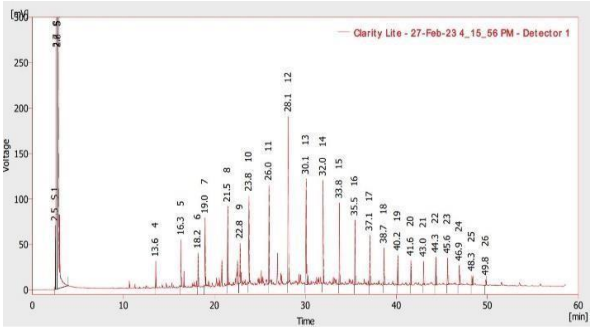
c)


Clarity Lite - 27-Feb-23 3_03_44 PM - Detector 1
Voltage
Time
[min]

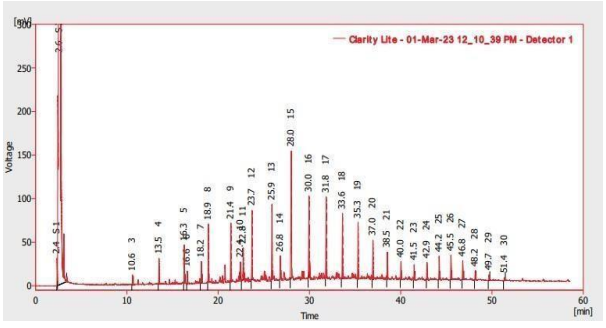
d)


Clarity Lite - 27-Feb-23 1_50_39 PM - Detector 1
Voltage
Time
[min]

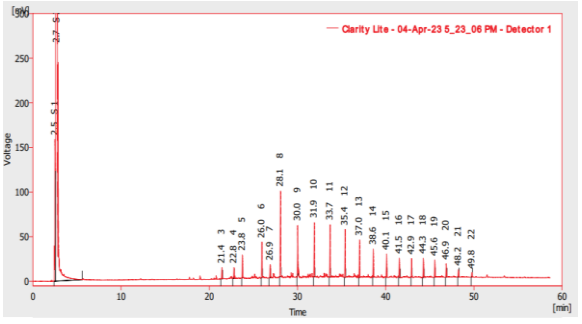
e)


Clarity Lite - 27-Feb-23 4_15_56 PM - Detector 1
Voltage
Time
[min]

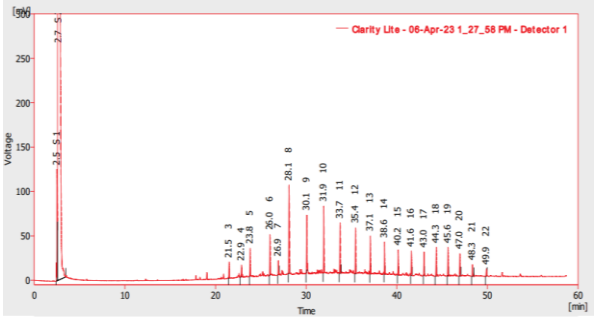
f)


Clarity Lite - 01-Mar-23 12_10_39 PM - Detector 1
Voltage
Time
[min]

g)

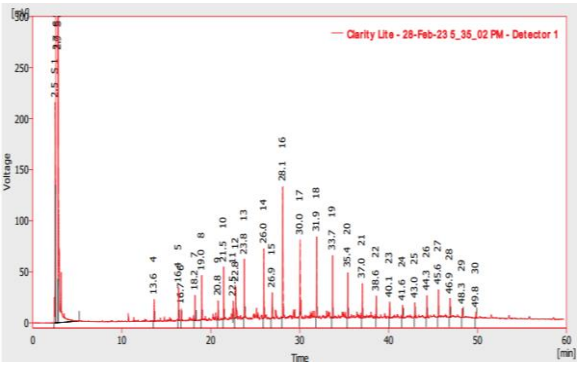

Clarity Lite - 04-Apr-23 5_23_06 PM - Detector 1
Voltage
Time
[min]

h)

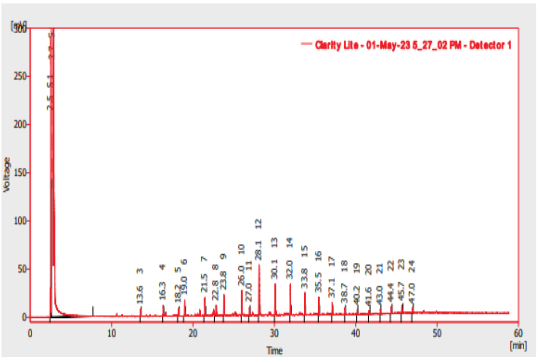

Clarity Lite - 06-Apr-23 1_27_58 PM - Detector 1
Voltage
Time
[min]

i)

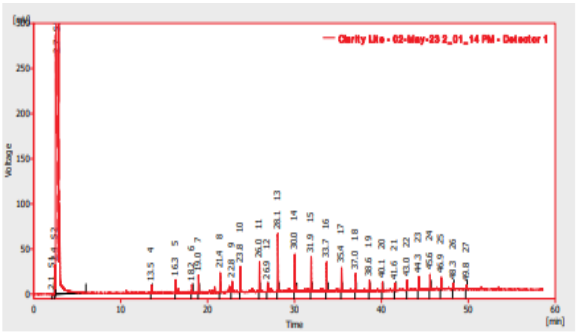
Figure 13: GC-FID chromatogram representing 10% oily sludge degradation by bacterial consortiuma), b), c) control and duplicate set- Day 0 respectively; d), e), f) control and duplicate set- Day 20 respectively; g), h), i) control and duplicate set- Day 40 respectively.



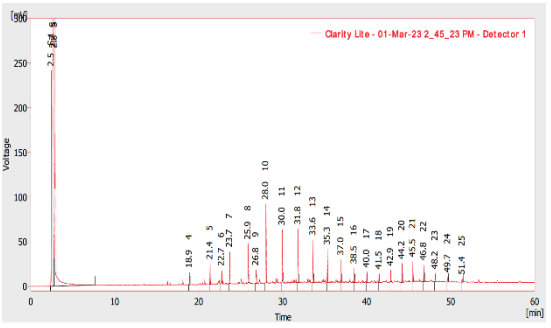
a)



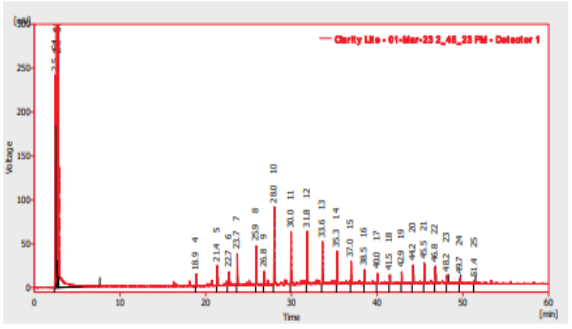
b)



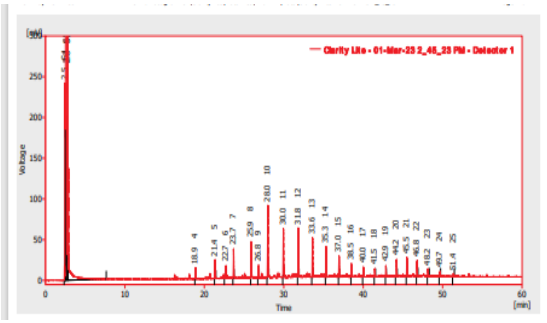
c)



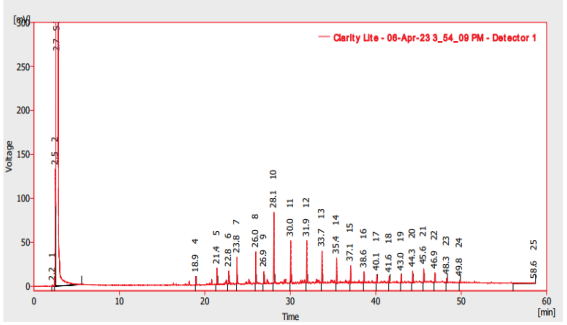
d)



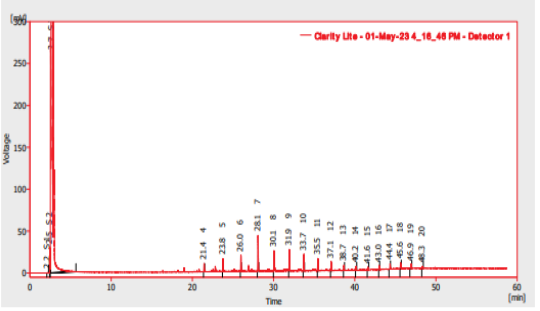
e)



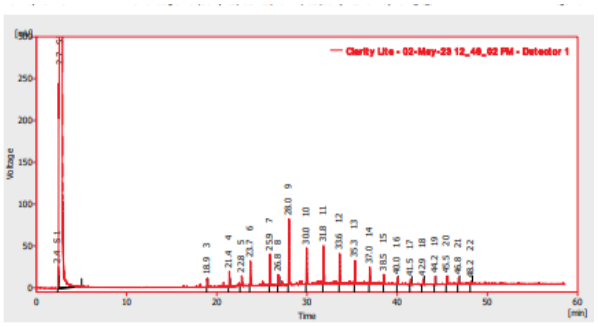
f)



g)

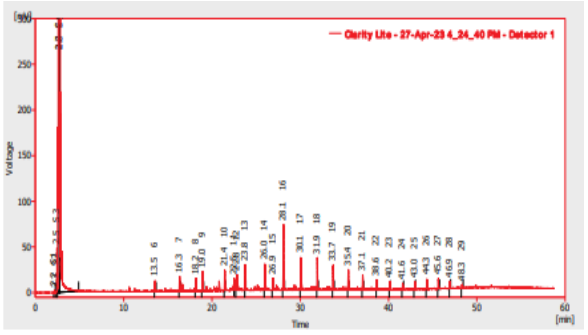


h)

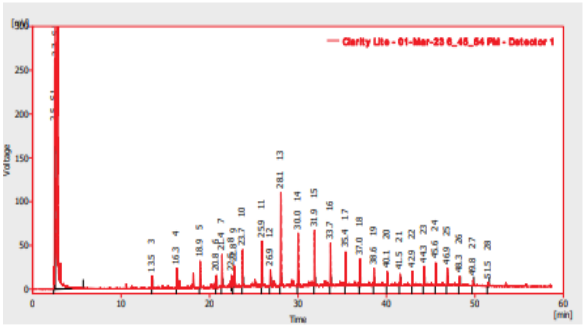


i)

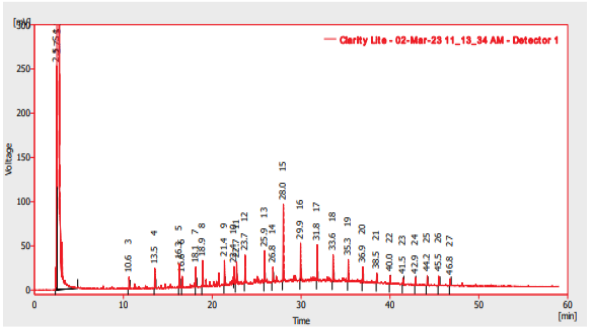
Figure 14: GC-FID chromatogram representing 30% oily sludge degradation by bacterial consortium a), b), c) control and duplicate set- Day 0 respectively; d), e), f) control and duplicate set- Day 20 respectively; g), h), i) control and duplicate set- Day 40 respectively.



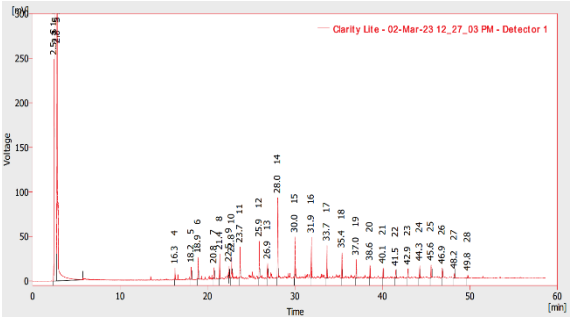
a)



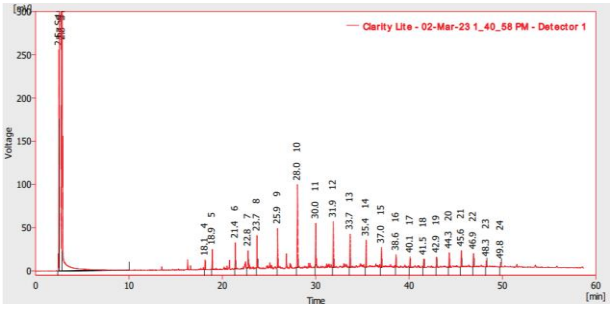
b)



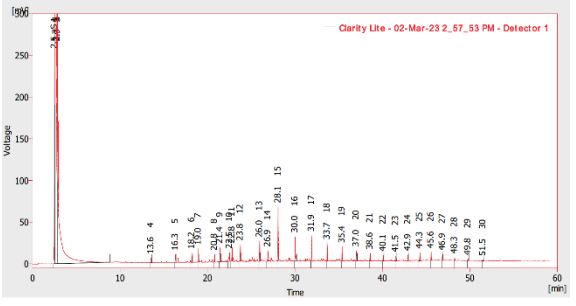
c)



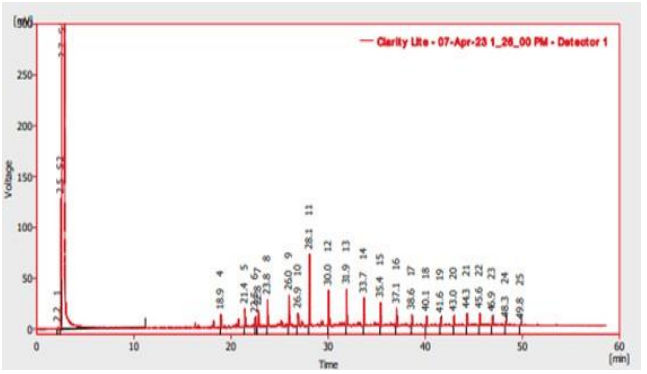
d)



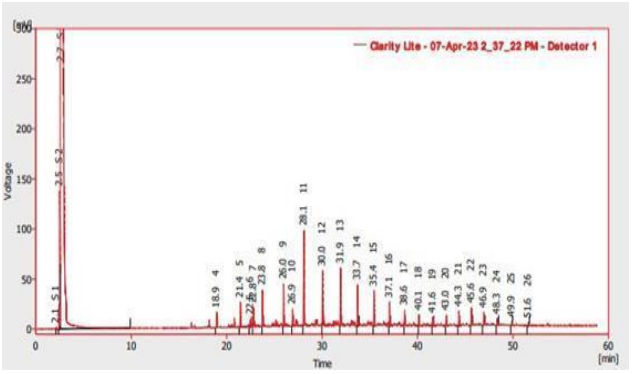
e)



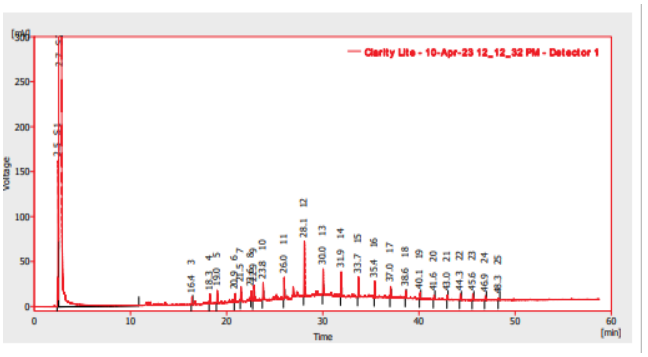
f)



g)



h)



i)

Figure 15: GC-FID chromatogram representing 50% oily sludge degradation by bacterial consortium a), b), c) control and duplicate set- Day 0 respectively; d), e), f) control and duplicate set- Day 20 respectively; g), h), i) control and duplicate set- Day 40 respectively.

As the incubation time increases from 0 to 40 days, reduction in peak area and size was observed in all experimental set up of 10%, 30% and 50% (Figure 13, 14 and 15). Data obtained from chromatograms were then analyzed for %TPH degradation. Each peak in chromatogram represents particular type of hydrocarbon and total area of all the peaks was used to calculate %TPH degradation. Results were plotted in graphical format as shown in Figure 16. Degradation was also seen in control (no bacterial consortium) soil setup which indicates the role of indigenous microflora in bioremediation process. Indigenous microflora can naturally withstand high levels of toxicity and play an important role in bioremediation (Ouyang *et al.*, 2005). In case of bacterial consortium, %TPH degradation was found to be higher as compared to control sets in all experimental set ups (10%, 30% and 50%) (Figure 16). Fold rise in comparison to control was found out to be 1.21> 1.05 >3.37 in case of 10%, 30% and 50% set up respectively. Thus, bioaugmentation of bacterial consortium supports the activity of indigenous microflora. Further, less amount of degradation activity was seen in soil spiked with 50% oily sludge may be because high oily sludge

concentration is found toxic for bacterial consortium.

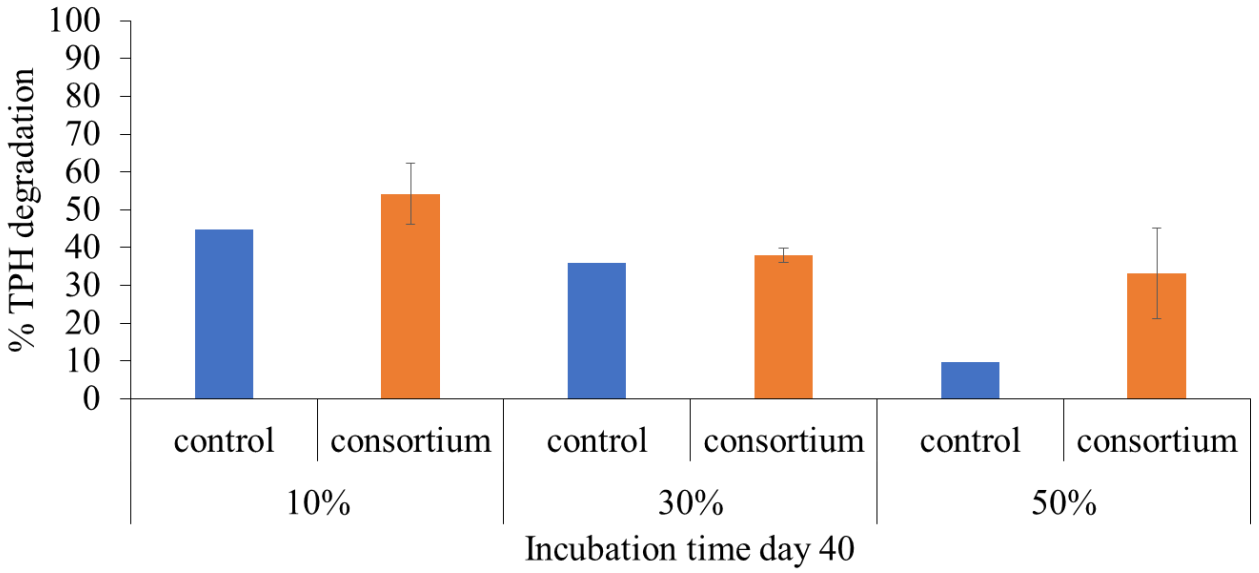


Figure 16: TPH degradation efficiency of inoculated bacterial consortium to degrade 10%, 30% and 50% oily sludge contaminated soil

5.2.3 Optimization of carrier matrix for field level application of bacterial consortium for oily sludge contaminated soil

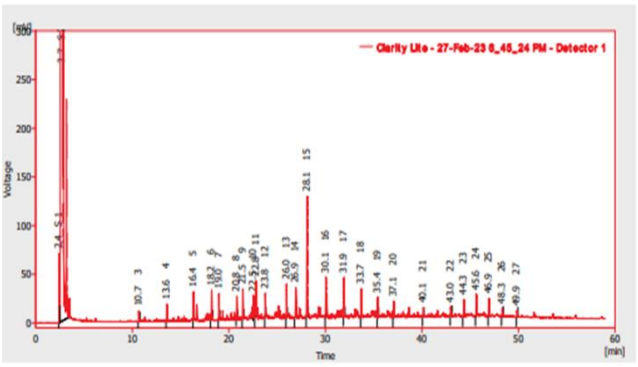
In order to enhance the survivability as well as stability of consortium in oily sludge contaminated soil, carrier matrix was used. Carrier matrix basically entraps the cells and allows its slow diffusion into the soil thus increases cell stability. The bacterial consortium was grown in sufficient amount and embedded onto some carrier matrix or agents like saw dust, peat, wheat bran or sugarcane bagasse by methods like adsorption, covalent bonding, entrapment, or encapsulation.

In this study, saw dust was used as carrier matrix as it is non-toxic to microorganism, easily available, acts as bulking agent and is also cost effective. Bacterial consortium was adsorbed on saw dust and mixed into soil spiked with 10% oily sludge (Figure 17). As observed earlier with tea leaves amendments, dark to light color change could be seen in all the trays of day 0 as compared to trays of day 40 indicating degradation (Figure 17). Sample collection was done at 0, 20th and 40th day. GC-FID was performed to measure % TPH removal efficiency of inoculated consortia. Obtained chromatograms are shown in Figure 18.

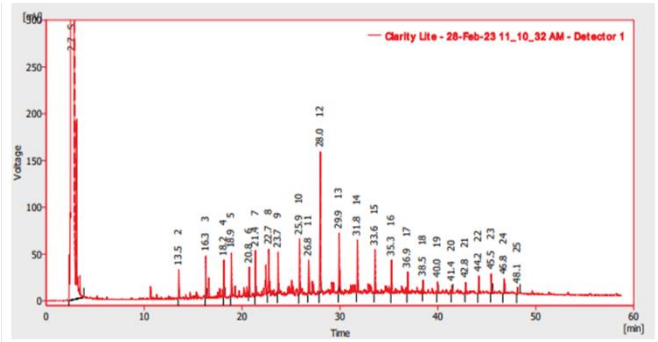
a) **b)** **c)** **d)** **e)** **f)**

g) **h)** **i)**

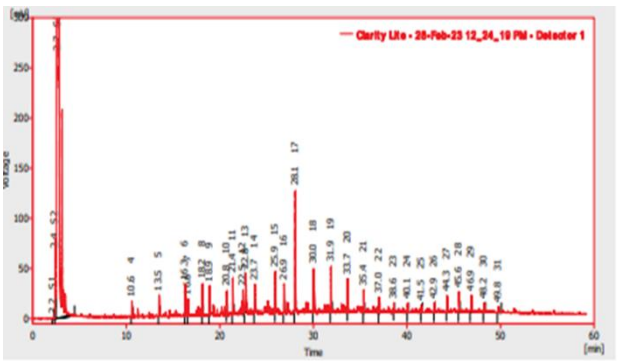
Figure 17: Soil set up of bacterial consortium adsorbed on saw dust for 10% oily sludge degradation.a), b), c) control and duplicate set of tested consortium- Day 0 respectively; d), e), f) control and duplicate set of tested consortium- Day 20 respectively; g), h), i) control and duplicate set of tested consortium- Day 40 respectively



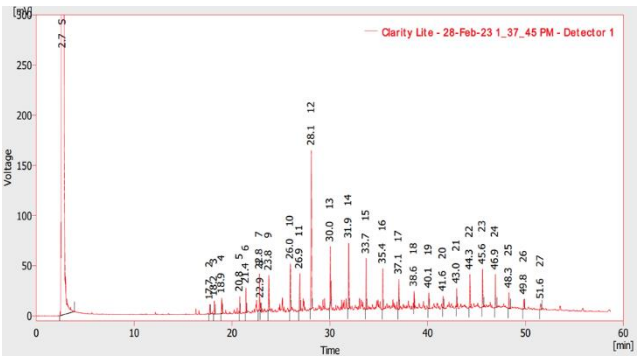
a)



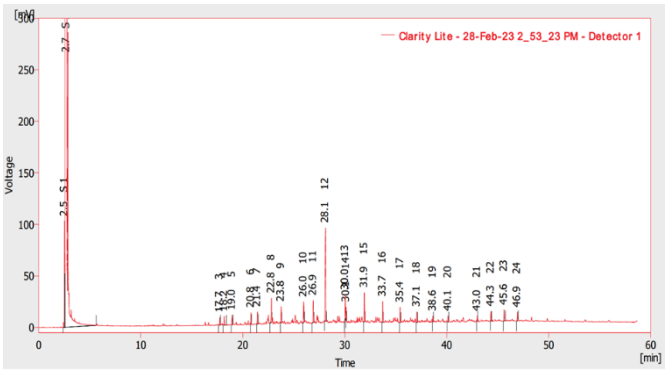
b)



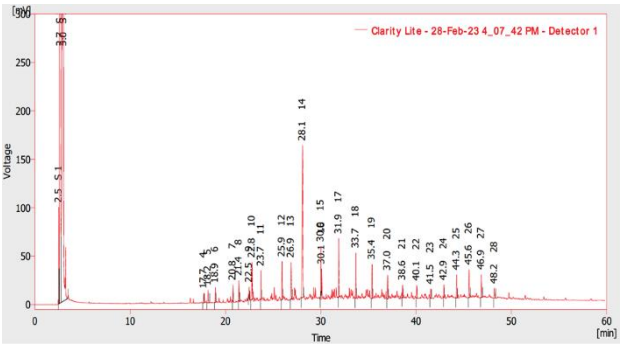
c)



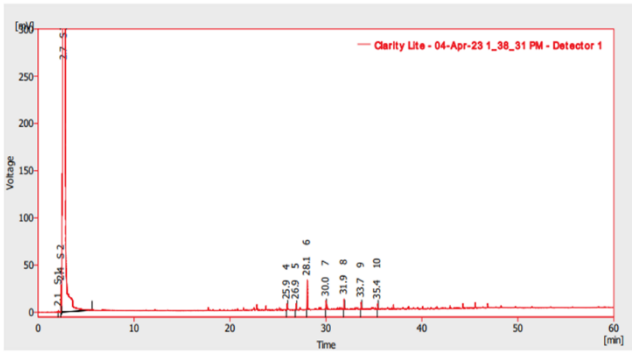
d)



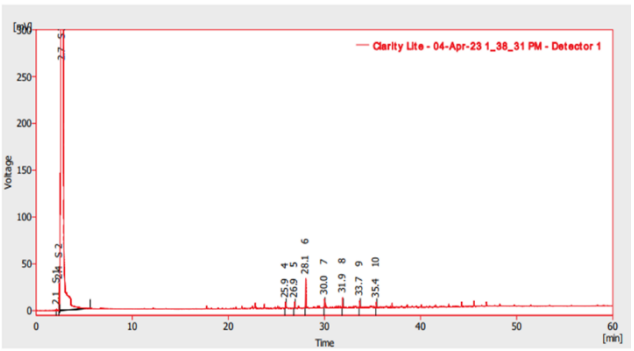
e)



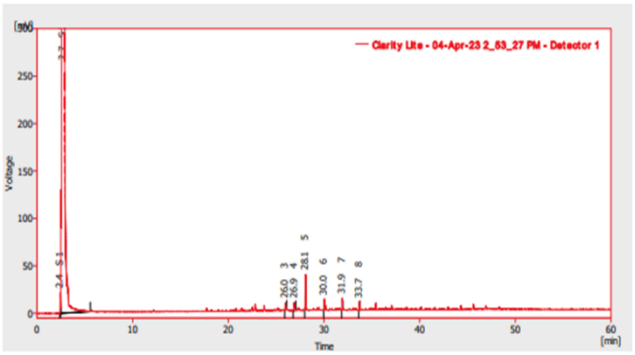
f)



g)



h)



i)

Figure 18: GC chromatograms representing 10% oily sludge degradation by bacterial consortium adsorbed on saw dust. a), b), c) control and duplicate set- Day 0 respectively; d), e), f) control and duplicate set- Day 20 respectively; g), h), i) control and duplicate set- Day 40 respectively.

According, to the data obtained, %TPH degradation was found to be 89.38% when adsorbed on sawdust which was higher in contrast to without (no consortium) adsorption on saw dust i.e. 49.03% in 40 days of incubation (* : $p < 0.05$) (Figure 19).

Figure 19: TPH degradation in 10% oily sludge contaminated soil by bacterial consortium adsorbed on saw dust, after 40 days of incubation

Thus, bacterial consortium was able to degrade oily sludge more efficiently when applied after adsorption on saw dust as compared to without adsorption. Fold rise in comparison to control was found to be 1.13 when bacterial consortium was embedded on saw dust. Considering the obtained result, it can be concluded that adsorbing bacterial consortium on saw dust for treating site contaminated with oily sludge may prove beneficial. Apart from this, degradation could be seen in control tray (no consortium) also in which only sludge and saw dust were mixed with soil. Though the degradation was lower in comparison to the results obtained with consortium but this needs to be resolved for improving the treatment strategy. As reported by Stroud *et al.,* 2007 due to interactions between soil and aliphatic hydrocarbon, adsorption of oily sludge particle takes place on saw dust. Saw dust also adsorbs PAH from surrounding area andincreases its bioavailability for consortium (Leahy and Colwell 1990).

Saw dust is a lignocellulosic material which contains numerous functional groups such as carboxyl, hydroxyl, phenolic, and amide groups in the structure (Sciban *et al.,* 2007). Such functional group aids in binding petroleum hydrocarbon with it. Thus, saw dust can act as an adsorbent. But adsorption of oily sludge onsaw dust does not promote degradation, instead it increases easy accessibility for organism to act. In order to find results of actual degradation by

bacterial consortium, experimental evidence indicating removal of TPH from samples where in bacterial consortium was adsorbed on saw dust and TPH removal only by saw dust needs to be explored. This can be done by performing Total organic carbon (TOC) of 40 day control sample containing only saw dust and 40 day test sample containing saw dust as well as inoculated bacterial consortium. Higher the TOC value lesser will be the degradation. This will provide an idea about extent of degradation which occurred in presence and absence of consortium.

5.2.4 Treatment of oily sludge contaminated soil using tea leaves as biostimulating agent and bacterial consortium adsorbed on saw dust as carrier matrix

Here, bioremediation of oily sludge is accomplished by combining all the tested processes. This strategy was employed to investigate whether the combination of all tested processes would providebetter degradation than applying them individually. Figure 20 represents soil set up images. Color change could be seen from dark to light in all the trays of day 0 (Figure 20 a, b, c) as compared to trays of day 20 (Figure 20 g, h, i) indicating degradation. Figure 21 display the chromatogram of 10% oily sludge contaminated soil amended with tea leaves and bacterial consortium adsorbed onto saw dust.

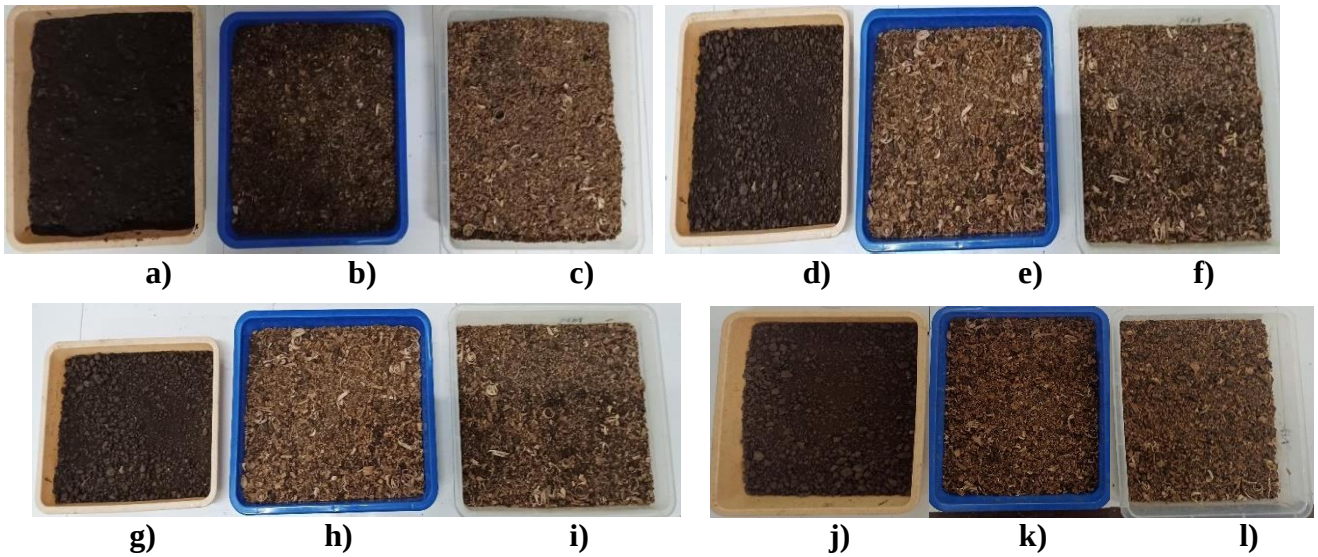
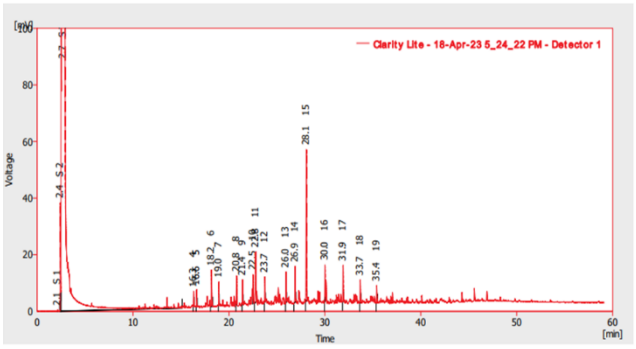
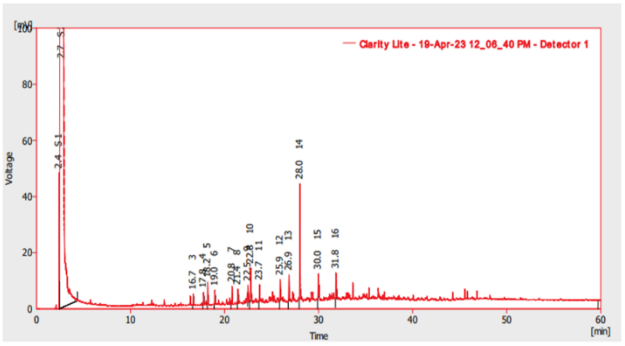


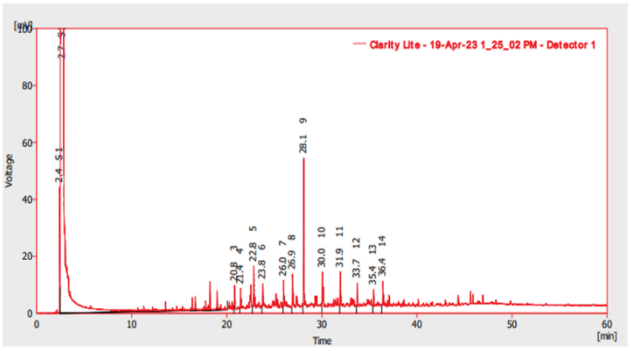
Figure 20: Experimental set up of 10% oily sludge contaminated soil amended with tea leaves andbacterial consortium adsorbed onto saw dust a), b), c) control and duplicate set of tested consortium-Day 0 respectively; d), e), f) control and duplicate set of tested consortium-Day 10 respectively; g), h), i) control and duplicate set of tested consortium- Day 20 respectively; j), k), l) control andduplicate set of tested consortium- Day 30 respectively.


Clarity Lite - 18-Apr-23 5_24_22 PM - Detector 1
Voltage
Time
[min]

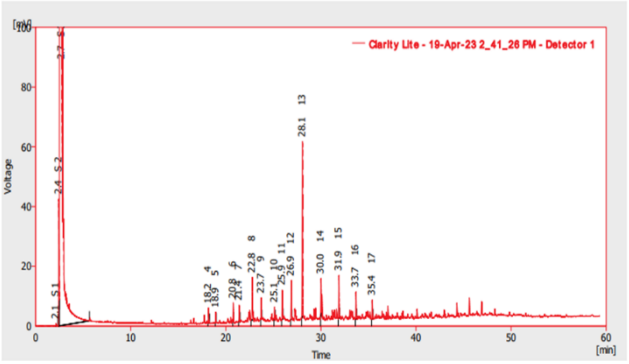
a)


Clarity Lite - 19-Apr-23 12_06_40 PM - Detector 1
Voltage
Time
[min]

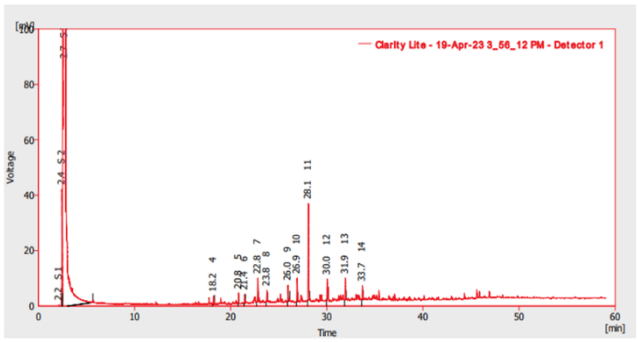
b)


Clarity Lite - 19-Apr-23 1_25_02 PM - Detector 1
Voltage
Time
[min]

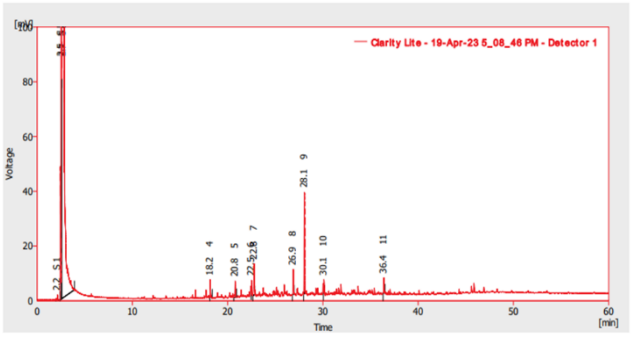
c)


Clarity Lite - 19-Apr-23 2_41_26 PM - Detector 1
Voltage
Time
[min]

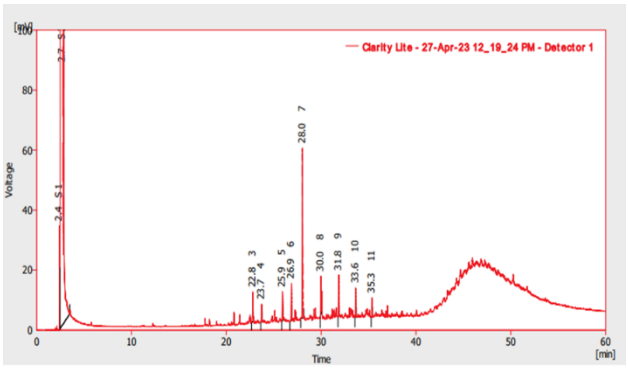
d)


Clarity Lite - 19-Apr-23 3_56_12 PM - Detector 1
Voltage
Time
[min]

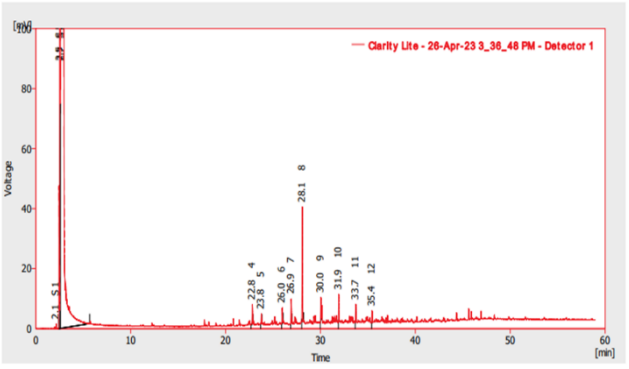
e)


Clarity Lite - 19-Apr-23 5_08_46 PM - Detector 1
Voltage
Time
[min]

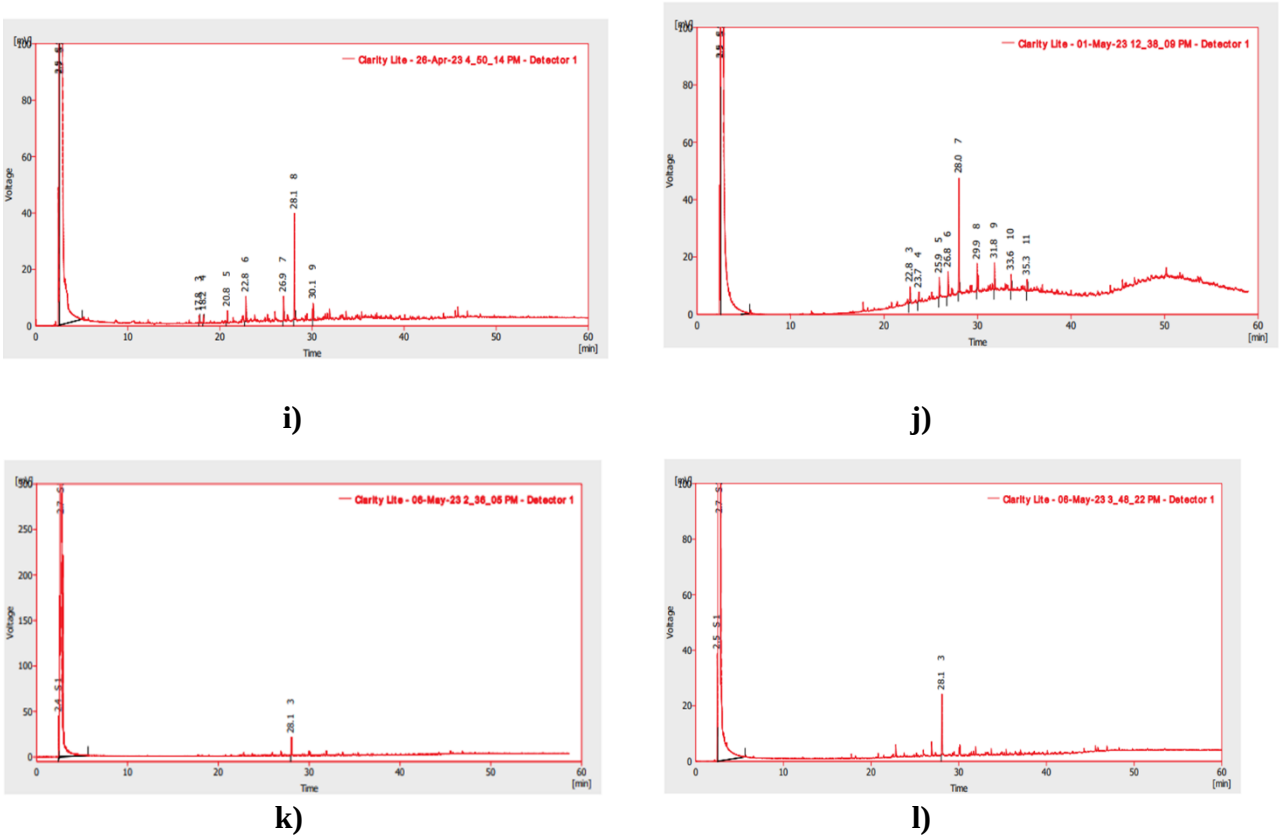
f)


Clarity Lite - 27-Apr-23 12_19_24 PM - Detector 1
Voltage
Time
[min]

g)


Clarity Lite - 26-Apr-23 3_36_48 PM - Detector 1
Voltage
Time
[min]

h)



i) **j)**

k) **l)**

Figure 21: GC chromatograms of 10% oily sludge contaminated soil in presence of tea leaves andbacterial consortium adsorbed onto saw dust a), b), c) control and duplicate set of consortium amendment- Day 0 respectively; d), e), f) control and duplicate set of consortium amendment- Day 10 respectively; g), h), i) control and duplicate set of consortium amendment- Day 20 respectively; j), k), l)control and duplicate set of consortium amendment- Day 30 respectively.

TPH degradation was found to be high at day 30 as compared to day 0 according to total area calculated from GC chromatograms. Figure 22 represents the %TPH degradation efficiency of bacterial consortium adsorbed on the saw dust in presence of tea leaves as biostimulating agent.

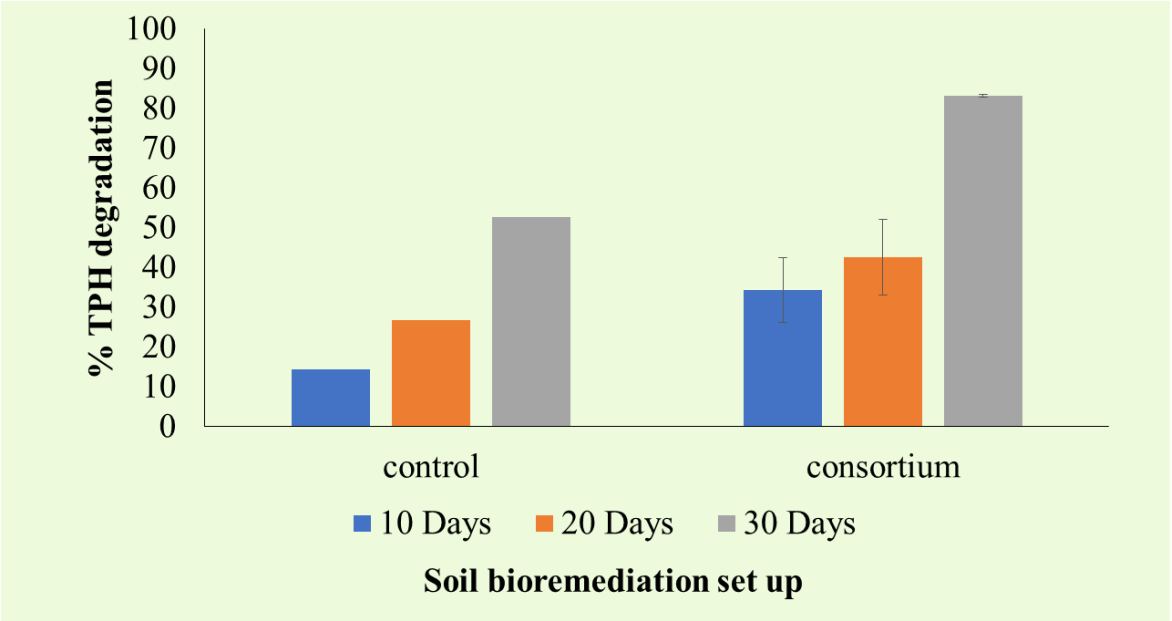


Figure 22: TPH degradation of 10% oily sludge contaminated soil by bacterial consortium using tea leaves as biostimulating agent and saw dust as carrier matrix. Control soil contained only 10% oily sludge.

In comparison to control % TPH degradation was found to be higher in test set up. Fold rise was 2.39, 1.59 and 1.57 at 10, 20 and 30 days respectively in comparison to control. Thus, this strategy enhances the degradation potential of indigenous flora in lesser incubation time as compared to individual experiment (30 days results are ongoing).

5.3. Conclusion

The present study was designed to develop the oily sludge bioremediation strategy.

1. Here, tea leaves serving as source of N, P and K was investigated as biostimulating agent. The results revealed that it enhanced the TPH removal efficiency of indigenous microbiota present in soil contaminated with 10% oily sludge.
2. Further, the efficiency of bacterial consortium to degrade oily sludge was investigated. Studied bacterial consortium included four strains designated as AWSant34: *Achromobacter xylosoxidans*, AWSant36: *Pannonibacter phragmitetus*, AWSant37: *Pseudomonas balearica* and ASSphn611: *Pseudomonas aeruginosa* which showed high TPH degradation efficiency. Among the 4 strains AWSant34: *Achromobacter xylosoxidans* was also a biosurfactant producer. The capability of bacterial consortium to treat 10%, 30% and 50% oily sludge in 40 days was determined. TPH degradation potential of bacterial consortium increased then decreased with higher oily sludge concentration i.e. high degradation was obtained with 10% oily sludge (54.22%), highest was observed in 30% (37.87) and the lowest was obtained in 50 % (33.18%) oily sludge respectively. Further, in all cases (10%, 30% and 50%) bacterial consortium supported the indigenous microflora in removing petroleum hydrocarbon contaminants.
3. Bacterial cell was adsorbed onto saw dust and then bioaugmented in the soil spiked with 10% oily sludge and according to the results obtained adsorbing oily sludge on saw dust proved beneficial (showing 89.38% TPH degradation) in contrast to direct application of bacterial consortium (showing 49.03% TPH degradation).
4. Carbon sources such as D-Fructose-6-phosphate, α-D-Glucose, D- Fructose and D-Glucuronic acid were maximally utilized by the selected strains of bacterial consortium.Carbon sources like sugarcane bagasse, molasses and others providing the above listed substrates can be used for biomass development during field studies.

5.4. Future Aspects

In future, the amendment of bacterial consortium on field scale can be carried out using carrier agent (saw dust) for treating oily sludge contaminated soil. With the help of genomics, proteomics and metabolomics, identification and comparison of gene and protein sequences associated with TPH degradation pathway can be determined. Interaction between added consortium and indigenous flora can be studied via metagenomic studies. Identified cheap substrate can be used for biomass development for application in field.

5.5 References

1. Martínez-Martínez, A., Cegarra-Navarro, J. G., & Garcia-Perez, A. (2022). Sustainability knowledge management and organisational learning in tourism: current approaches and areas for future development. *Journal of Sustainable Tourism*, 1-13.
2. Carlier, J., Arscott, S., Thomy, V., Fourrier, J. C., Caron, F., Camart, J. C., ... & Tabourier, P. (2004). Integrated microfluidics based on multi-layered SU-8 for mass spectrometry analysis. *Journal of micromechanics and Microengineering*, *14*(4), 619.
3. Dotson, W. G. (1972). Fixed points of quasi-nonexpansive mappings. *Journal of the Australian Mathematical Society*, *13*(2), 167-170.
4. Hochberg, S. Y., Tansel, B., & Laha, S. (2022). Materials and energy recovery from oily sludges removed from crude oil storage tanks (tank bottoms): A review of technologies. *Journal of Environmental Management*, *305*, 114428.
5. Mazlova, E. A., & Meshcheryakov, S. V. (1999). Ecological characteristics of oil sludges. *Chemistry and technology of fuels and oils*, *35*(1), 49-53.
6. Xu, Y., Xue, X., Dong, L., Nai, C., Liu, Y., & Huang, Q. (2018). Long-term dynamics of leachate production, leakage from hazardous waste landfill sites and the impact on groundwater quality and human health. *Waste Management*, *82*, 156-166.
7. Xiang, Y., Jiang, L., Zhou, Y., Luo, Z., Zhi, D., Yang, J., & Lam, S. S. (2022). Microplastics and environmental pollutants: Key interaction and toxicology in aquatic and soil environments. *Journal of Hazardous Materials*, *422*, 126843.
8. Hui, K., Tang, J., Lu, H., Xi, B., Qu, C., & Li, J. (2020). Status and prospect of oil recovery from oily sludge: A review. *Arabian Journal of Chemistry*, *13*(8), 6523-6543.
9. Aziz, F., Syed, J. H., Malik, R. N., Katsoyiannis, A., Mahmood, A., Li, J., ... & Jones, K. C. (2014). Occurrence of polycyclic aromatic hydrocarbons in the Soan River, Pakistan: insights into distribution, composition, sources, and ecological risk assessment. *Ecotoxicology and environmental safety*, *109*, 77-84.
10. Ambaye, T. G., Chebbi, A., Formicola, F., Prasad, S., Gomez, F. H., Franzetti, A., & Vaccari, M. (2022). Remediation of soil polluted with petroleum hydrocarbons, and their reuse for agriculture: Recent progress, challenges, and perspectives. *Chemosphere*, 133572.
11. da Silva, L. J., Alves, F. C., & de França, F. P. (2012). A review of the technological solutions for the treatment of oily sludges from petroleum refineries. *Waste Management & Research*, *30*(10), 1016-1030.

12. Sinha, B., Roy, S., & Kumar, K. (2020). Bioremediation of oily sludge: A case base analysis to sustainable supply chain. *Resources, Environment and Sustainability*, *2*, 100008.

13. Jerez, S., Ventura, M., Molina, R., M. I., Martínez, F., & Melero, J. A. (2021). Comprehensive characterization of an oily sludge from a petrol refinery: A step forward for its valorization within the circular economy strategy. *Journal of Environmental Management*, *285*, 112124.

14. Ubani, O., Atagana, H. I., & Thantsha, M. S. (2013). Biological degradation of oil sludge: A review of the current state of development. *African Journal of Biotechnology*, *12*(47), 6544-6567.

15. Field, J. A., De Jong, E., Feijoo Costa, G., & De Bont, J. A. (1992). Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white rot fungi. *Applied and environmental microbiology*, *58*(7), 2219-2226.

16. Abioye, O. P. (2011). Biological remediation of hydrocarbon and heavy metals contaminated soil. *Soil contamination*, *7*, 127-142.

17. Li, X., Zhang, F., Guan, B., Sun, J., & Liao, G. (2020, March). Review on oily sludge treatment technology. In *IOP Conference Series: Earth and Environmental Science* (Vol. 467, No. 1, p. 012173). IOP Publishing.

18. Oknina, N., Kadiev, K., Kadieva, M., Maksimov, A., Batov, A., & Dandaev, A. (2015). Physico-Chemical properties of oil sludges from reservoirs. *Biosci. Biotechnol. Res. Asia*, *12*, 497-505.

19. Bhattacharya, D., Sarma, P. M., Krishnan, S., Mishra, S., & Lal, B. (2003). Evaluation of genetic diversity among Pseudomonas citronellolis strains isolated from oily sludge-contaminated sites. *Applied and Environmental Microbiology*, *69*(3), 1435-1441..

20. Bhattacharya, A., Phukan, D., Baruah, S., Sarkar, A., & Sarkar, P. (2021). Assessing pollution removal efficiencies of some selected parameters by applying different remediation techniques for petroleum oily sludge. *Environmental Challenges*, *5*, 100268

21. Li, C., Zhou, K., Qin, W., Tian, C., Qi, M., Yan, X., & Han, W. (2019). A review on heavy metals contamination in soil: effects, sources, and remediation techniques. *Soil and Sediment Contamination: An International Journal*, *28*(4), 380-394.

22. Bisht, S., Pandey, P., Bhargava, B., Sharma, S., Kumar, V., & Sharma, K. D. (2015). Bioremediation of polyaromatic hydrocarbons (PAHs) using rhizosphere technology. *Brazilian Journal of Microbiology*, *46*, 7-21.

23. Kumar, M., Bolan, N. S., Hoang, S. A., Sawarkar, A. D., Jasemizad, T., Gao, B., ... &

Rinklebe, J. (2021). Remediation of soils and sediments polluted with polycyclic aromatic hydrocarbons: To immobilize, mobilize, or degrade?. *Journal of Hazardous Materials*, *420*, 126534.

24. Liu, S. H., Zeng, G. M., Niu, Q. Y., Liu, Y., Zhou, L., Jiang, L. H., ... & Cheng, M. (2017). Bioremediation mechanisms of combined pollution of PAHs and heavy metals by bacteria and fungi: A mini review. *Bioresource technology*, *224*, 25-33.

25. Johnsen, A. R., Schmidt, S., Hybholt, T. K., Henriksen, S., Jacobsen, C. S., & Andersen, O. (2007). Strong impact on the polycyclic aromatic hydrocarbon (PAH)-degrading community of a PAH-polluted soil but marginal effect on PAH degradation when priming with bioremediated soil dominated by mycobacteria. *Applied and environmental microbiology*, *73*(5), 1474-1480.

26. Rentz, J. A., Alvarez, P. J., & Schnoor, J. L. (2008). Benzo [a] pyrene degradation by Sphingomonas yanoikuyae JAR02. *Environmental pollution*, *151*(3), 669-677.

27. Chen, S., Chang, C., Deng, Y., An, S., Dong, Y. H., Zhou, J., ... & Zhang, L. H. (2014). Fenpropathrin biodegradation pathway in Bacillus sp. DG-02 and its potential for bioremediation of pyrethroid-contaminated soils. *Journal of Agricultural and Food Chemistry*, *62*(10), 2147-2157.

28. Dell'Anno, F., Rastelli, E., Sansone, C., Brunet, C., Ianora, A., & Dell'Anno, A. (2021). Bacteria, fungi and microalgae for the bioremediation of marine sediments contaminated by petroleum hydrocarbons in the omics era. *Microorganisms*, *9*(8), 1695.

29. Das, K., & Mukherjee, A. K. (2007). Crude petroleum-oil biodegradation efficiency of Bacillus subtilis and Pseudomonas aeruginosa strains isolated from a petroleum-oil contaminated soil from North-East India. *Bioresource technology*, *98*(7), 1339-1345.

30. Ali, M., Song, X., Ding, D., Wang, Q., Zhang, Z., & Tang, Z. (2022). Bioremediation of PAHs and heavy metals co-contaminated soils: challenges and enhancement strategies. *Environmental Pollution*, *295*, 118686.

31. Nair, C. I., Jayachandran, K., & Shashidhar, S. (2008). Biodegradation of phenol. *African journal of biotechnology*, *7*(25).

32. Van Hamme, J. D., Singh, A., & Ward, O. P. (2003). Recent advances in petroleum microbiology. *Microbiology and molecular biology reviews*, *67*(4), 503-549.

33. Fragoso ados Santos, H., Duarte, G. A. S., Rachid, C. T. D. C., Chaloub, R. M., Calderon, E. N., Marangoni, L. F. D. B., ... & Peixoto, R. S. (2015). Impact of oil spills on coral reefs can be reduced by bioremediation using probiotic microbiota. *Scientific reports*, *5*(1), 1-11.

34. Chikere, C. B., & Ekwuabu, C. B. (2014). Culture-dependent characterization of hydrocarbon utilizing bacteria in selected crude oil-impacted sites in Bodo, Ogoniland, Nigeria. *African Journal of Environmental Science and Technology*, *8*(6), 401-406.

35. Varjani, S. J. (2017). Microbial degradation of petroleum hydrocarbons. *Bioresource technology*, *223*, 277-286.

36. Hwang, H. M., Hu, X., & Zhao, X. (2007). Enhanced bioremediation of polycyclic aromatic hydrocarbons by environmentally friendly techniques. Journal of Environmental Science and Health Part C, 25(4), 313-352.

37. Raquel, S., Natalia, G., Luis Fernando, B., & Maria Carmen, M. (2013). Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by a wood-degrading consortium at low temperatures. *FEMS microbiology ecology*, *83*(2), 438-449.

38. Shi, K., Xue, J., Xiao, X., Qiao, Y., Wu, Y., & Gao, Y. (2019). Mechanism of degrading petroleum hydrocarbons by compound marine petroleum-degrading bacteria: surface adsorption, cell uptake, and biodegradation. *Energy & Fuels*, *33*(11), 11373-11379.

39. Supreeth, M., Chandrashekar, M. A., Sachin, N., & Raju, N. S. (2016). Effect of chlorpyrifos on soil microbial diversity and its biotransformation by Streptomyces sp. HP-11. *3 Biotech*, *6*, 1-6.

40. Bihari, Z., Szvetnik, A., Szabó, Z., Blastyák, A., Zombori, Z., Balázs, M., & Kiss, I. (2011). Functional analysis of long-chain n-alkane degradation by Dietzia spp. *FEMS microbiology letters*, *316*(2), 100-107.

41. Ratajczak, A., GEIßDORFER, W. A. L. T. E. R., & Hillen, W. (1998). Alkane hydroxylase from Acinetobacter sp. strain ADP1 is encoded by alkM and belongs to a new family of bacterial integral-membrane hydrocarbon hydroxylases. *Applied and Environmental Microbiology*, *64*(4), 1175-1179.

42. Feng, L., Wang, W., Cheng, J., Ren, Y., Zhao, G., Gao, C., ... & Wang, L. (2007). Genome and proteome of long-chain alkane degrading Geobacillus thermodenitrificans NG80-2 isolated from a deep-subsurface oil reservoir. *Proceedings of the National Academy of Sciences*, *104*(13), 5602-5607.

43. Zhou, R., Huang, C., Zhang, A., Bell, S. G., Zhou, W., & Wong, L. L. (2011). Crystallization and preliminary X-ray analysis of CYP153C1 from Novosphingobium aromaticivorans DSM12444. *Acta Crystallographica Section F: Structural Biology and Crystallization Communications*, *67*(8), 964-967.

44. Tani, A., Ishige, T., Sakai, Y., & Kato, N. (2001). Gene structures and regulation of the alkane hydroxylase complex in Acinetobacter sp. strain M-1. *Journal of*

bacteriology, *183*(5), 1819-1823.

45. Huarte-Bonnet C., Kumar S., Saparrat M.C.N., Girotti J.R., Santana M., Hallsworth J.E., Pedrini N. Insights into hydrocarbon assimilation by eurotialean and hypocrealean fungi: Roles for CYP52 and CYP53 clans of cytochrome P450 genes. *Appl. Biochem. Biotechnol.* 2018;184:1047–1060. doi: 10.1007/s12010-017-2608-z.

46. Huang F.C., Peter A., Schwab W. Expression and characterization of CYP52 genes involved in the biosynthesis of sophorolipid and alkane metabolism from Starmerella bombicola. *Appl. Environ. Microbiol.* 2014;80:766–776. doi: 10.1128/AEM.02886-13.

47. Kobayashi S., Hirakawa K., Horiuchi H., Fukuda R., Ohta A. Phosphatidic acid and phosphoinositides facilitate liposome association of Yas3p and potentiate derepression of ARE1 (alkane-responsive element one)-mediated transcription control. *Fungal. Genet. Biol.* 2013;61:100–110. doi: 10.1016/j.fgb.2013.09.008.

48. Park H.G., Kim V., Kim H., Lee R., Cho M.A., Park S.W., Chun Y.J., Kim D. CYP52A23 from Candida albicans and its Substrate Preference for Fatty Acid Hydroxylation. *Arch. Biochem. Biophys.* 2019;671:27–34. doi: 10.1016/j.abb.2019.06.002.

49. Panasia G., Philipp B. LaoABCR, a novel system for oxidation of long-chain alcohols derived from SDS and alkane degradation in Pseudomonas aeruginosa. *Appl. Environ. Microbiol.* 2018;84:e00626-18. doi: 10.1128/AEM.00626-18.

50. Iwama R., Kobayashi S., Ohta A., Horiuchi H., Fukuda R. Fatty aldehyde dehydrogenase multigene family involved in the assimilation of n-alkanes in Yarrowia lipolytica. *J. Biol. Chem.* 2014;289:33275–33286. doi: 10.1074/jbc.M114.596890.

51. Yin C.F., Xu Y., Li T., Zhou N.Y. Wide distribution of the sad gene cluster for sub-terminal oxidation in alkane utilizers. *Environ. Microbiol.* 2022;24:6307–6319. doi: 10.1111/1462-2920.16124.

52. Tenagy, Tejima K., Chen X., Iwatani S., Kajiwara S. Long-chain acyl-CoA synthetase is associated with the growth of Malassezia spp. *J. Fungi.* 2019;5:88. doi: 10.3390/jof5040088.

53. Wang, M., Ding, M., & Yuan, Y. (2023). Bioengineering for the Microbial Degradation of Petroleum Hydrocarbon Contaminants. *Bioengineering*, *10*(3), 347.

54. Vidali, M. (2001). Bioremediation. an overview. *Pure and applied chemistry*, *73*(7), 1163-1172.

55. Chatterjee, S., Chattopadhyay, P., Roy, S., & Sen, S. K. (2008). Bioremediation: a tool for cleaning polluted environments. *Journal of Applied Biosciences*, *11*(1), 594.

56. Adesipo, Adegbite A., Dirk Freese, and Alfreda O. Nwadinigwe. "Prospects of in-situ remediation of crude oil contaminated lands in Nigeria." *Scientific African* 8 (2020): e00403.

57. Nataraja, S., Chetan, D. M., & Krishnappa, M. (2010). Effect of temperature on cellulose enzyme activity in crude extracts isolated from solid wastes microbes. *International Journal of Microbiology Research*, *2*(2), 44-47.

58. Enwere, C. L., Bjorn, A., Nick, H., & Ebube, O. O. OIL CONTAMINATION OF LAND IN THE NIGER DELTA REGION OF NIGERIA AND THE ASSESSMENT OF BIOREMEDIATION AS AN EFFECTIVE REMEDIAL SOLUTION.

59. Onstott, T. C. (2005). *Enhancement of Bacterial Transport in Aerobic and Anaerobic Environments: Assessing the Effect of Metal Oxide Chemical Heterogeneities* (No. FG02-97ER62472-1). Princeton University, Princeton, NJ.

60. Odokuma, L. O., & Dickson, A. A. (2003). Bioremediation of a crude oil polluted tropical rain forest soil. *Global Journal of Environmental Sciences*, *2*(1), 29-40.

61. Amadi, A., & Bari, Y. U. (1992). Use of poultry manure for amendment of oil-polluted soils in relation to growth of maize (Zea mays L.). *Environment international*, *18*(5), 521-527.

62. Pimmata, P., Reungsang, A., & Plangklang, P. (2013). Comparative bioremediation of carbofuran contaminated soil by natural attenuation, bioaugmentation and biostimulation. *International Biodeterioration & Biodegradation*, *85*, 196-204.

63. Simarro, R., González, N., Bautista, L. F., & Molina, M. C. (2013). Assessment of the efficiency of in situ bioremediation techniques in a creosote polluted soil: change in bacterial community. *Journal of hazardous materials*, *262*, 158-167.

64. Suja, F., Rahim, F., Taha, M. R., Hambali, N., Razali, M. R., Khalid, A., & Hamzah, A. (2014). Effects of local microbial bioaugmentation and biostimulation on the bioremediation of total petroleum hydrocarbons (TPH) in crude oil contaminated soil based on laboratory and field observations. *International Biodeterioration & Biodegradation*, *90*, 115-122.

65. Simarro, R., González, N., Bautista, L. F., & Molina, M. C. (2013). Assessment of the efficiency of in situ bioremediation techniques in a creosote polluted soil: change in bacterial community. *Journal of hazardous materials*, *262*, 158-167.

66. Hamzah, A., Phan, C. W., Yong, P. H., & Mohd Ridzuan, N. H. (2014). Oil palm empty fruit bunch and sugarcane bagasse enhance the bioremediation of soil artificially polluted by crude oil. *Soil and Sediment Contamination: An International Journal*, *23*(7), 751-762.

67. Rosenberg, E., Legmann, R., Kushmaro, A., Taube, R., Adler, E., & Ron, E. Z. (1992). Petroleum bioremediation—a multiphase problem. *Biodegradation*, *3*, 337-350.

68. Kao, C. M., Lin, B. H., Chen, S. C., Wei, S. F., Chen, C. C., Yao, C. L., & Chien, C. C. (2016). Biodegradation of trinitrotoluene (TNT) by indigenous microorganisms from TNT-contaminated soil, and their application in TNT bioremediation. *Bioremediation Journal*, *20*(3), 165-173.

69. Auweter, S. D., Bhavsar, A. P., de Hoog, C. L., Li, Y., Chan, Y. A., van der Heijden, J., ... & Finlay, B. B. (2011). Quantitative mass spectrometry catalogues Salmonella pathogenicity island-2 effectors and identifies their cognate host binding partners. *Journal of Biological Chemistry*, *286*(27), 24023-24035.

70. Bayat, Z., Hassanshahian, M., & Cappello, S. (2015). Immobilization of microbes for bioremediation of crude oil polluted environments: a mini review. *The open microbiology journal*, *9*, 48.

71. Marschner, H. (Ed.). (2011). *Marschner's mineral nutrition of higher plants*. Academic press.

72. Huang, W., Lin, M., Liao, J., Li, A., Tsewang, W., Chen, X., ... & Zheng, P. (2022). Effects of Potassium Deficiency on the Growth of Tea (Camelia sinensis) and Strategies for Optimizing Potassium Levels in Soil: A Critical Review. *Horticulturae*, *8*(7), 660.

73. Kao, C. M., Liao, H. Y., Chien, C. C., Tseng, Y. K., Tang, P., Lin, C. E., & Chen, S. C. (2016). The change of microbial community from chlorinated solvent-contaminated groundwater after biostimulation using the metagenome analysis. *Journal of hazardous materials*, *302*, 144-150.

74. Gao, X., Zhu, S., Zhang, W., Li, D., Dai, W., & He, S. (2016). Analysis of crude oils using gas purge microsyringe extraction coupled to comprehensive two dimensional gas chromatography-time-of-flight mass spectrometry. *Fuel*, *182*, 788-797.

75. Ouyang, W., Liu, H., Murygina, V., Yu, Y., Xiu, Z., & Kalyuzhnyi, S. (2005). Comparison of bio-augmentation and composting for remediation of oily sludge: A field-scale study in China. *Process Biochemistry*, *40*(12), 3763-3768.

76. Stroud, J. L., Paton, G. I., & Semple, K. T. (2007). Microbe-aliphatic hydrocarbon interactions in soil: implications for biodegradation and bioremediation. *Journal of Applied Microbiology*, *102*(5), 1239-1253.

77. Leahy, J. G., & Colwell, R. R. (1990). Microbial degradation of hydrocarbons in the environment. *Microbiological reviews*, *54*(3), 305-315.

78. Šćiban, M., Radetić, B., Kevrešan, Ž., & Klašnja, M. (2007). Adsorption of heavy metals from electroplating wastewater by wood sawdust. *Bioresource technology*, *98*(2), 402-409.

79. Hamamura, N., Olson, S. H., Ward, D. M., & Inskeep, W. P. (2006). Microbial population dynamics associated with crude-oil biodegradation in diverse soils. *Applied and Environmental Microbiology*, *72*(9), 6316-6324.

80. Iyobosa, E., Xianagang, M., Jun, N. H., Fang, S., & Zhennan, W. (2020). Biodegradation of petroleum hydrocarbon polluted soil. *Indian Journal of Microbiology Research*, *7*(2), 104-112.

81. Bhawsar, S., & Cameotra, S. S. (2011). Biodegradation of diesel fuel. *Transylvanian Review of Systematical and Ecological Research*, (12), 169.

82. Silva LJ, Alves FC, de França FP. A review of the technological solutions for the treatment of oily sludges from petroleum refineries. Waste Management & Research. 2012;30(10):1016-1030. doi:10.1177/0734242X12448517

83. Mishra, S., Jyot, J., Kuhad, R. C., & Lal, B. (2001). In situ bioremediation potential of an oily sludge-degrading bacterial consortium. *Current microbiology*, *43*, 328-335.

84. Van Hamme, Jonathan D., Joseph A. Odumeru, and Owen P. Ward. "Community dynamics of a mixed-bacterial culture growing on petroleum hydrocarbons in batch culture." *Canadian journal of microbiology* 46.5 (2000): 441-450.

85. Shen, Lilly, and Dong-Ke Zhang. "An experimental study of oil recovery from sewage sludge by low-temperature pyrolysis in a fluidised-bed☆." *Fuel* 82.4 (2003): 465-472.

86. Mrayyan, Bassam, and Mohammed N. Battikhi. "Biodegradation of total organic carbons (TOC) in Jordanian petroleum sludge." *Journal of hazardous materials* 120.1-3 (2005): 127-134.

87. Ji, L., Fu, X., Wang, M., Xu, C., Chen, G., Song, F., ... & Zhang, Q. (2019). Enzyme cocktail containing NADH regeneration system for efficient bioremediation of oil sludge contamination. *Chemosphere*, *233*, 132-139.

88. Liu, Y., Liu, Y., Liu, Z., & Zhang, A. (2019). Strengthening effects of ammonia nitrogen on the harmless biological treatment of oily sludge. *Chemistry and Ecology*, *35*(1), 20-35.

89. Zare, N., Bonakdarpour, B., Amoozegar, M. A., Shavandi, M., Fallah, N., Darabi, S., & Taromsary, N. B. (2019). Using enriched water and soil-based indigenous halophilic consortia of an oilfield for the biological removal of organic pollutants in hypersaline produced water generated in the same oilfield. *Process Safety and Environmental Protection*, *127*, 151-161.

90. Gao, L. L., Lu, Y. C., Zhang, J. L., Li, J., & Zhang, J. D. (2019). Biotreatment of restaurant wastewater with an oily high concentration by newly isolated bacteria from oily sludge. *World Journal of Microbiology and Biotechnology*, *35*, 1-11.

91. Ghosh, Sayanti, and Saswati Chakraborty. "Influence of inoculum variation on formation and stability of aerobic granules in oily wastewater treatment." *Journal of environmental management* 248 (2019): 109239.

92. Wang, Z., Gong, Z., Wang, Z., Li, X., & Chu, Z. (2021). Application and development of pyrolysis technology in petroleum oily sludge treatment. *Environmental Engineering Research*, *26*(1).

93. Dzionek, Anna, Danuta Wojcieszyńska, and Urszula Guzik. "Natural carriers in bioremediation: A review." *Electronic Journal of Biotechnology* 23 (2016): 28-36.

94. Atagana, Harrison Ifeanyichukwu. "Compost bioremediation of hydrocarbon-contaminated soil inoculated with organic manure." *African Journal of Biotechnology* 7.10 (2008).

95. Abioye, O. P., Pariatamby Agamuthu, and A. R. Abdul Aziz. "Biodegradation of used motor oil in soil using organic waste amendments." *Biotechnology Research International* 2012 (2012).

96. Gupta, Kartikey Kumar, Kamal Rai Aneja, and Deepanshu Rana. "Current status of cow dung as a bioresource for sustainable development." *Bioresources and Bioprocessing* 3 (2016): 1-11.

97. Dadrasnia, A. R. E. Z. O. O., and P. A. R. I. A. T. A. M. B. Y. Agamuthu. "Dynamics of diesel fuel degradation in contaminated soil using organic wastes." *International Journal of Environmental Science and Technology* 10 (2013): 769-778.

98. Hamzah, A., Phan, C. W., Yong, P. H., & Mohd Ridzuan, N. H. (2014). Oil palm empty fruit bunch and sugarcane bagasse enhance the bioremediation of soil artificially polluted by crude oil. *Soil and Sediment Contamination: An International Journal*, *23*(7), 751-762.

99. Azubuike, C. C., Chikere, C. B., & Okpokwasili, G. C. (2016). Bioremediation techniques–classification based on site of application: principles, advantages, limitations and prospects. World Journal of Microbiology and Biotechnology, 32, 1-18.

100. Kensa, V. Mary. "Bioremediation-an overview." *I Control Pollution* 27.2 (2011): 161-168.

101. Leung, M. (2004). Bioremediation: techniques for cleaning up a mess. BioTeach Journal, 2, 18-22.

102. Martínez-Prado, Unzueta-Medina, J., & Pérez-López, M.E. (2014).

ELECTROBIOREMEDIATION AS A HYBRID TECHNOLOGY TO TREAT SOIL CONTAMINATED WITH TOTAL PETROLEUM HYDROCARBONS. Revista Mexicana De Ingenieria Quimica, 13, 113-127.

ANNEXURE

ANNEXURE-1

Composition of Nutrient agar:

Ingredients	**g/L**
Peptone	5
HM peptone B	1.5
Yeast extract	1.5
NaCl	5
Agar	15
pH	7.4+0.2

Composition of Nutrient Broth:

Ingredients	**g/L**
Peptone	5
HM peptone B	1.5
Yeast extract	1.5
NaCl	5
pH	7.4+0.2

Composition of Minimal salt medium (MM2):

Ingredients	**g/100 mL**
Na_2HPO_4	0.141
K_2HPO_4	0.174
$(NH_4)_2SO_4$	0.237
Mg. $SO_4.7H_2O$	0.024
NaCl	0.049

Composition of normal saline:

Ingredients	**g/100 mL**
NaCl	8.5

bioremediation

ORIGINALITY REPORT

8%	6%	8%	%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

	www.scielo.cl	1
	Internet Source	%
1	"Microbial Action on Hydrocarbons", Springer	1
2	Science and Business Media LLC, 2018	%
	Publication	
3	S. Jerez, M. Ventura, R. Molina, M.I. Pariente, F. Martínez, J.A. Melero. "Comprehensive characterization of an oily sludge from a petrol refinery: A step forward for its valorization within the circular economy strategy", Journal of Environmental Management, 2021	1 %

Publication

4 Bhaskar Sinha, Supriyo Roy, Kaushik Kumar. 1 %

"Bioremediation of oily sludge: A case base analysis to sustainable supply chain",

Resources, Environment and Sustainability, 2020

Publication

5 hdl.handle.net 1 %

Internet Source

6 www.researchgate.net

Internet Source 1 %

www.science.gov 1

Internet Source %

7 Handbook of Hydrocarbon and Lipid 1

8 Microbiology, 2010. %

Publication

9

cyberleninka.org 1

Internet Source %

Exclude quotes On Exclude matches< 50 words

Exclude bibliography On