Biodegradation of Petroleum Oil Sludge by Bioaugmentation of Indigenously Isolated Microflora

A DISSERTATION THESIS SUBMITTED TO
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THE DEGREE OF
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SUBMITTED BY,

Zeel Bhatia (17MMB003)
Neeti Singhal (17MMB014)
Hemangini Gohil (17MBT013)

UNDER THE GUIDANCE OF

Dr. NASREEN MUNSHI

NIRMA UNIVERSITY
INSTITUTE OF SCIENCE
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INSTITUTE OF SCIENCE
NIRMA UNIVERSITY
SARKHEJ GANDHINAGAR HIGHWAY,
AHMEDABAD – 382481

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DECLARATION

We hereby kindly declare that the work titled **"Biodegradation of petroleum oil sludge by bioaugmentation of indigenously isolated microflora**" is my original work. I have not copied from any other student's work or from any other sources except where due references or acknowledgement is made explicitly in the text, nor has any part been written for us by another person.

Date:

Zeel Bhatia **Neeti Singhal** **Hemangini Gohil**

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"Books are my favourite friends and I consider my home library, with many thousand books , to be my greatest wealth. Every new book, based on some new idea inspires me and gives me a new thought to ponder."

~DR. A. P. J. Abdul Kalam

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(ZEEL BHATIA) **(NEETI SINGHAL)** **(HEMANGINI GOHIL)**

INDEX

ABBREVIATION

LB :- Luria Bertani

MM2 :- Minimal salt media

NSO :- Nitrogen, Sulphur, Oxygen

O.D. :- Optical density

ONGC :- Oil and natural gas corporation

PAH :- Polycyclic aromatic hydrocarbon

QS :- Quorum sensing

TCA :- Tricarboxylic acid cycle

TPH :- Total petroleum hydrocarbon

USEPA :- United states environment protection agency

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Introduction

1. INTRODUCTION

Petroleum energy has been a source of fuel which drives towards growth and development. Accounting for 35 percent of the global energy consumption, India is said to be the third largest energy consumer in the world. Gujarat has been considered as the Petrochemical hub of India and has experienced raise in industrial growth rate of 12.5% from 2002-2007 (http://www.gspcgroup.com/). Moreover, there are 800 large industries and 3,20,000 small diligences. Gujarat has been ranked 1st in production of crude oil onshore (55.10%). The state has topmost number of oil and gas fields onshore (31.3%) and offshore (36.6%) of Nation's installed refineries. ONGC and RELIANCE has been considered to be the most prestigious petroleum production companies and many more companies has been on the list. ONGC Ankleshwar is one such benchmark which has the capacity to produce huge amounts of Petroleum and its products.

There are number of localities in Gujarat where petroleum refineries are situated as represented in Figure 1. Apart from that, there are many other petroleum product producing industries in Gujarat. Figure 1 represents major units which fulfil the energy needs of the state.

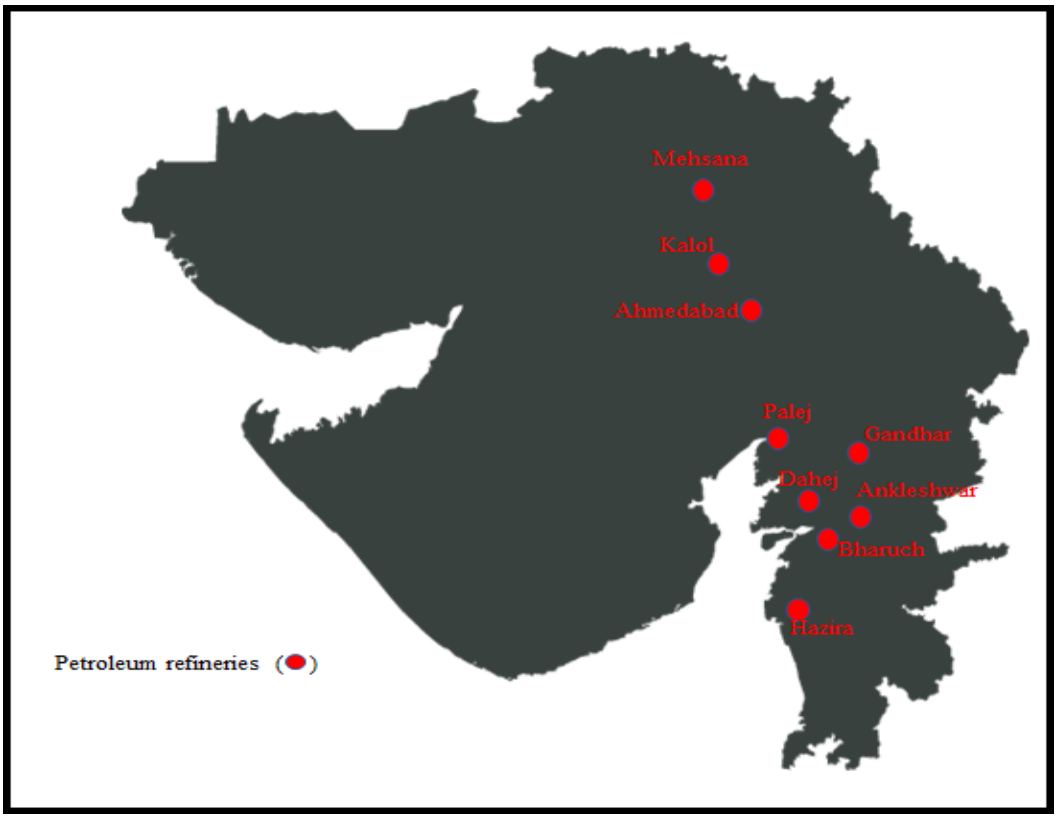


Figure 1: Petroleum refineries in Gujarat

Simultaneously, huge amount of oil sludge is produced during various processes like production of petroleum products, and transportation of petroleum oil. Such oil sludge contributes to soil and water pollution.

Unavoidable exploitation of petroleum and crude oil also results into formation of oily sludge. Processing of one kilogram of crude oil can generate 10-20 grams of oily sludge. (http://www.globalgujarat.com/images/oil-gas-details.pdf).

Oil sludge produced through processing activities can be collected from different points like dissolved air flotation units, tank bottom cleaning, oil/water separator, heat exchanger cleanings, etc (Mishra et al.,2001). Oil sludge is generated at larger scales after petroleum refining, processing of crude oil and disposal of the same becomes a problem as shown in Figure 2.

Figure 2: Petroleum oil sludge generated from petrochemical industries

Environmental pollution is caused due to improper disposal of oily sludge and it poses a serious threat to groundwater. As the demands of the growing population, the use of cleaner and greener energy source has become the need.

The petroleum oil sludge contains total petroleum hydrocarbons (TPH) which has several chemical compounds, originally obtained from crude oil, sediments and water (Dibble *et al*., 1979). Petroleum oil sludge contains organic matter, and hydrocarbons which vary within the sample. Water content varies from 30%-90%, hydrocarbons and organic matter constitute 30.39 ± 3.27 % and 2.28 ± 0.67 % respectively (Bilo'o *et al*., 2012). Environment gets contaminated due to inappropriate disposal of crude oil. TPH is a complex mixture of alkanes, alkenes, aromatic hydrocarbons, nitrogen, sulfur, and oxygen containing compounds (NSO) and asphaltene fractions as shown in Figure 3 (Bhattacharya et al., 2003)

Alkanes form a major portion in petroleum oil sludge while polycyclic aromatic hydrocarbons (PAH) form minor portion. Although PAH forms minor portion, but

these are toxic in nature. Studies suggests that PAH may have carcinogenic and mutagenic effects, and therefore this part of petroleum sludge has to be removed before it comes in contact of any human or animal. PAH like Naphthalene, Phenanthrene, Anthracene etc. are in minor proportions in crude oil (Lu *et al*., 2012). They all differ in their aromaticity, phenanthrene and anthracene represent 19.17% and 17.65% of total identified PAHs respectively (Philemon *et al*., 2012).

As shown in Figure 3 petroleum hydrocarbons can be divided into two classes, cyclic and acyclic, acyclic is further divided into saturated and unsaturated where saturated group consists of alkanes and unsaturated consists alkenes and alkynes. Acyclic and aromatic hydrocarbon together sums up the cyclic hydrocarbon.

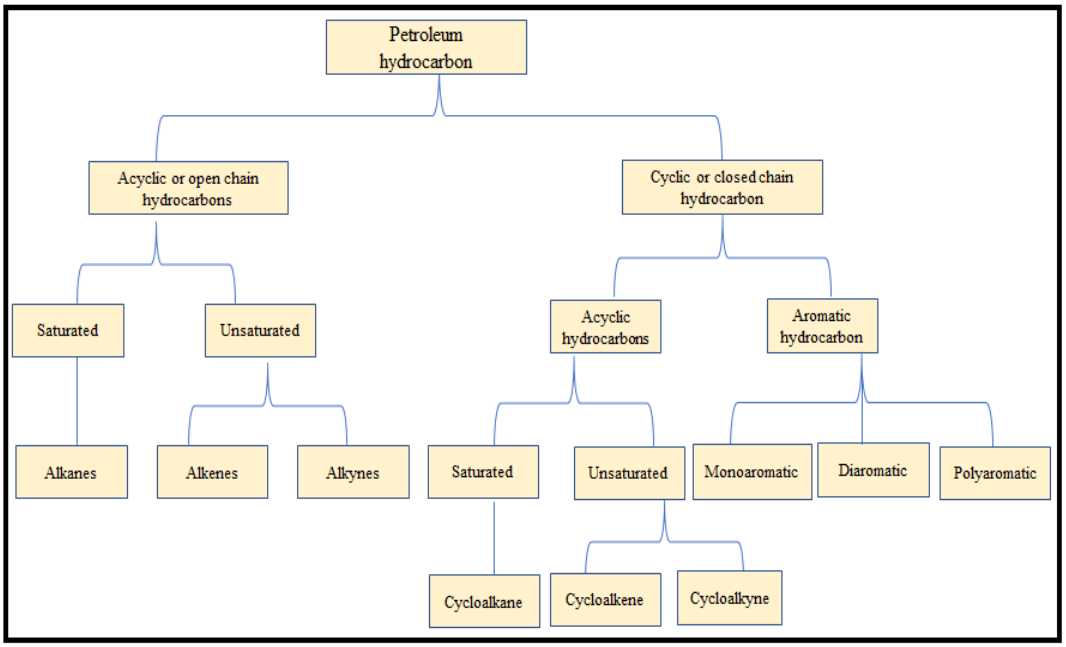


Figure 3: Classification of petroleum hydrocarbon

PAHs are coloured crystalline solids possessing high melting and boiling points, low vapour pressures and low water solubilities. PAHs are hydrophobic which makes them associated with organic as well as inorganic matter. PAH causes depression, nervous disorders, mutations, liver disease, birth defects, cancers, irregular heartbeats etc. Inhalation of PAH compounds leads to headache, respiratory irritation, nausea, dizziness. Oil contamination has adversely affected the flora and fauna as well as human health (Mandal *et al*., 2007). Soil contaminated with oil sludge loses its fertility which may have a bad impact on seed germination (Yoshida et al., 2006 and Gong et al., 2001). Soil polluted due to oil sludge has been a growing concern, because it can be a source of groundwater (drinking water) contamination. Crude oil exposure may cause damage to internal organs like - kidneys, lungs, intestines, liver (Gomer *et al*., 1980; Knafla*et al*., 2006; Zhang *et al*., 1992; Lee *et al*., 2006). Improper disposal leads to these kinds of several hazards (Yustle*et al*., 2000).

There are stringent regulations and norms for the industrial waste disposal in any government body. United States Environmental Protection Agency (USEPA) has

designed the elementary arrangement to monitor the release of pollutants into water bodies. A list of priority pollutants has been developed which are hazardous to environment and needs proper treatment before its disposal. Disposal of untreated oil sludge containing these pollutants affects the environment adversely. USEPA has defined 16 major priority pollutant list as shown in Figure 4 which are hazardous to environment and have to be prevented from reaching the environment.

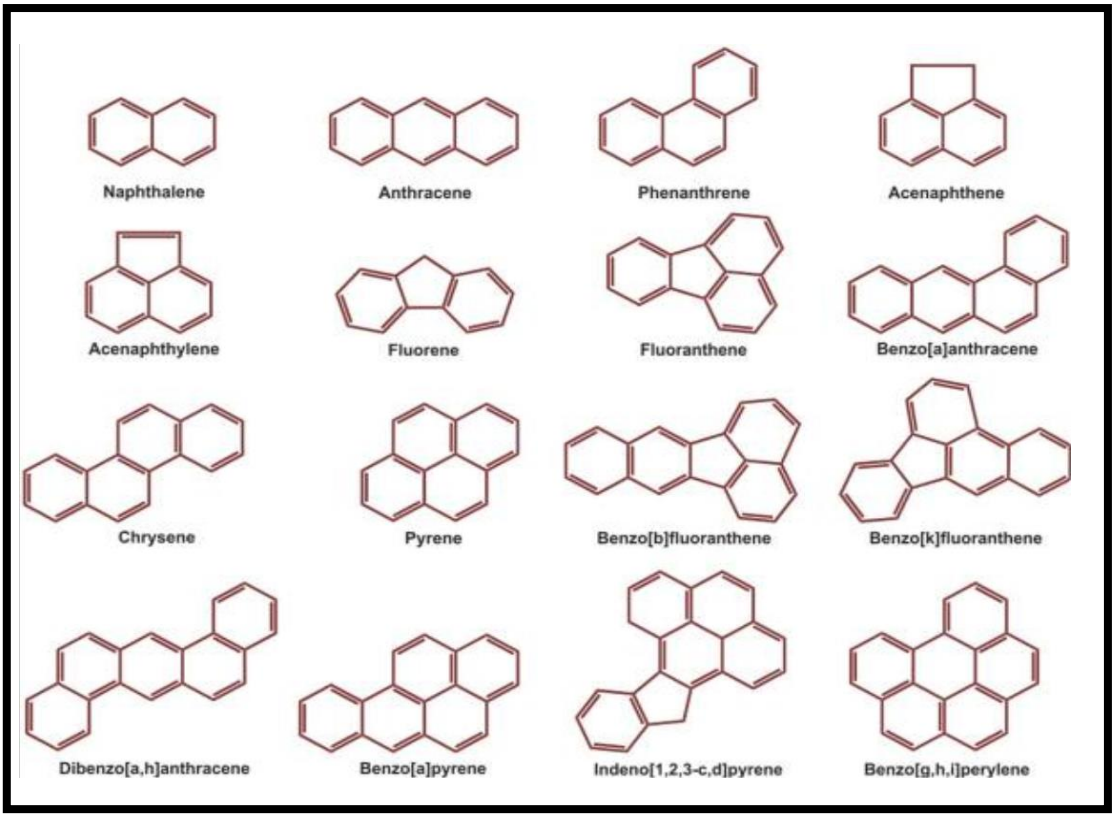


Figure 4: USEPA enlisted 16 priority pollutants which are hazardous in nature

Petroleum hydrocarbons can be removed from immediate environment chemically or biologically. Chemically it can be removed through volatilization, photooxidation, chemical oxidation etc. Chemical methods like volatilization, leaching, photochemical (U.V. light) and oxidation-Reduction reactions (Gupte *et al*., 2016) are rapid at removing hydrocarbons from the environment but it has a major drawback such as it is not able to degrade the product completely and results in incomplete degradation releasing toxic by-products which are in turn harmful to the environment.

Then there are conventional methods including landfarming, air sparging, incineration etc. These have been used since early times but the problem with these methods are that the solution is not permanent to the environment (Gupte *et al*., 2016). So a permanent, eco-friendly, cost effective method has to be developed which can remediate the environment such that the land or water is not wasted but can be utilized for the betterment.

Aim of the present study was to remove pollutants biologically, where bioremediation can be an efficient method to solve this problem (Habib *et al*., 2017).

Bioremediation is one of the most effective method for treatment of oil contamination (Fulekar, 2017; Hahn and Loehr, 1992). Bioremediation is carried out by bacteria or fungi, as shown in the Figure 5. Complete mineralization is achieved through bioremediation rendering the environment safe.

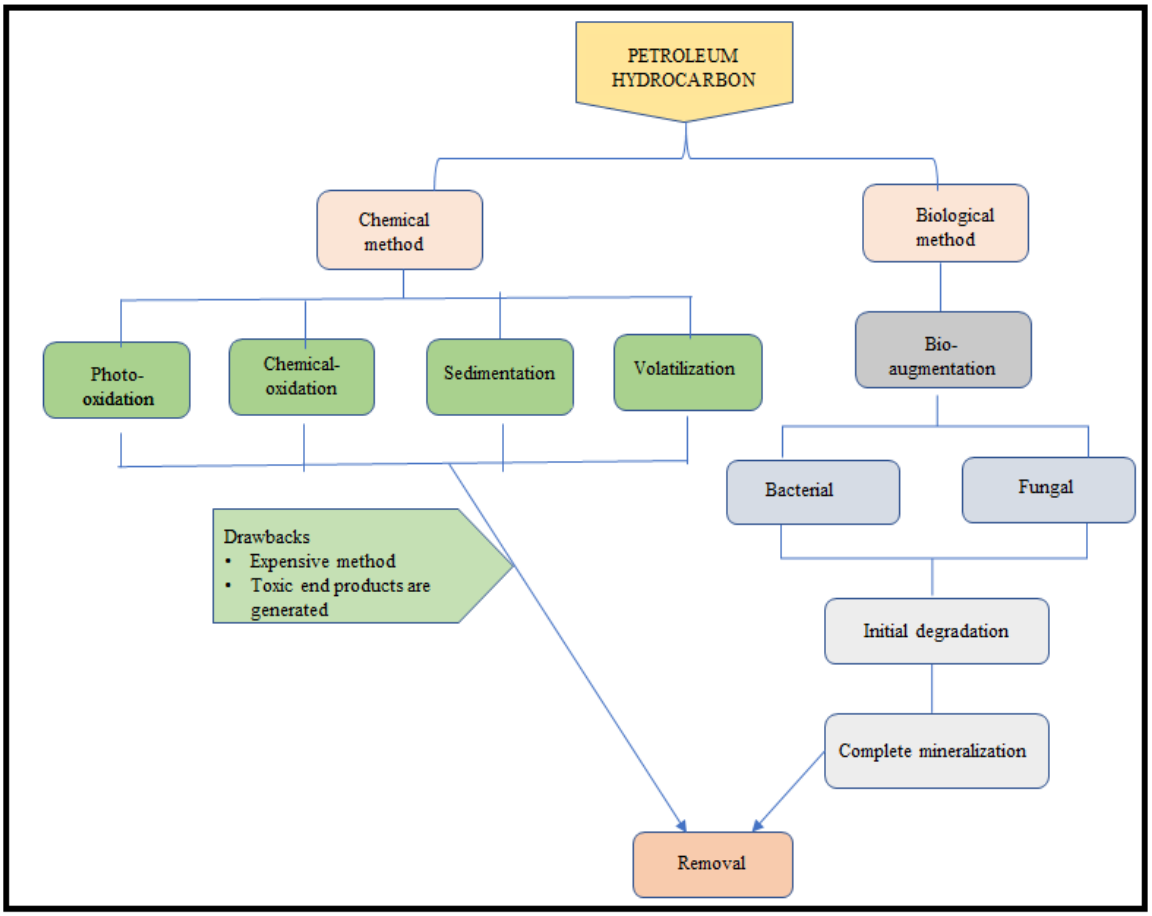


Figure 5: Methods of PAH degradation

In order to breakdown the contaminants present on the site, bioremediation can be an effective method where there is use of microorganisms which are added deliberately or are naturally present at the site which helps in reducing the pollutants.

Strategies to achieve bioremediation are,

- **Bioaugmentation** - Pre-grown bacterial cultures are supplemented to the site of contamination where it enhances the bacterial population present and helps in degradation of contaminants. It reduces clean up cost and time (Nzila *et al*., 2016).

- **Biostimulation** – It involves addition of nutrients in the environment to stimulate existing bacteria capable of bioremediation (Calvin *et al*., 2010).

- **Biosparging** – It is done to increase the biological activity of indigenous microorganisms, where air or oxygen are injected into the saturated zone (Jing *et al*., 2012).

Biosparging, though cheap but requires high quantity of energy to inject air under pressure. Also the air injected may be lost through the surface and so requires an air extraction system and further its purification which becomes a tedious work. Whereas biostimulation may even give rise to some pathogenic strains or unwanted microorganisms from community that may affect the health of those working on site for the process. Hence Bioaugmentation is found to be a suitable method for degradation of total petroleum hydrocarbon (Morzik et al., 2003).

1.1 BIOAUGMENTATION OF INDIGENOUSLY ISOLATED MICROFLORA

Often there are observations that the number of exogenous microorganisms decreases shortly after addition to a site, due to following parameters: nutrient limitations, temperature changes, insufficient substrates, pH, competition between introduced and indigenous microorganisms, grazing by protozoa, shock of pollutant load, and factors associated with quorum sensing (QS) (Nzila *et al*., 2016).

Therefore an effective method to conquer this problem is to isolate already adapted indigenous bacterial population from the same environment. Advantage of adding enriched indigenously isolated bacterial culture is that, it will further enhance the biodegradation at the original site with them being greater in population and favourably adapted to environment.

In bioaugmentation, known oil-degrading bacteria are supplemented to the existing microbial population at the contaminated site, petroleum hydrocarbons bind to soil components, where these bacteria help in effective biodegradation of petroleum hydrocarbon.

However, hydrocarbons differ in their susceptibility to microbial attack. The susceptibility of hydrocarbons to microbial degradation can be generally ranked as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes. High molecular weight polycyclic aromatic hydrocarbons (PAHs) may be degraded slowly and take much longer time for complete mineralization.

Hydrocarbons in the environment are biodegraded primarily by bacteria, yeast, and fungi. The reported efficiency of biodegradation ranged from 6% to 82% for soil fungi, 0.13%to 50% for soil bacteria, and 0.003% to 100% for marine bacteria. Bacteria are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil in environment (Das, Chandran 2011).

There are various microorganisms which degrades total petroleum hydrocarbon by aerobic degradation pathway, few examples of microbial strains are -*Pseudomonas putida, Micrococcus varians, Rhodococcus* spp., *Corynebacterium, Acinetobacter* sp., *Alcaligenes faecalis, Mycobacterium vanbaalenii,* (Darmawan *et al,*. 2015), *Burkholderia, Enterobacter, Arthrobacter, Achromobacter, P. aeruginosa, Pseudomonas fluorescens, Bacillus subtilis, Bacillus* sp., *Flavobacterium sp., Alcaligenes* sp., *Acinetobacter lwoffi, Corynebacterium* sp , *Micrococcus roseus* (Das, Chandran 2011).

As shown in Figure 6 in biodegradation mechanisms, molecular oxygen commences the enzymatic attack on alkane and rings of PAH. Initially, dioxygenase catalyses arene oxidation by aerobic bacterial systems to yield vicinal *cis*-dihydrodiols. These dehydroxylated intermediates are cleaved by ring-cleaving dioxygenases through either an *ortho*-cleavage pathway or a *meta*-cleavage pathway, leading to central intermediates such as catechol that are further converted to tricarboxylic acid (TCA) cycle intermediates (Peng *et al*., 2008).

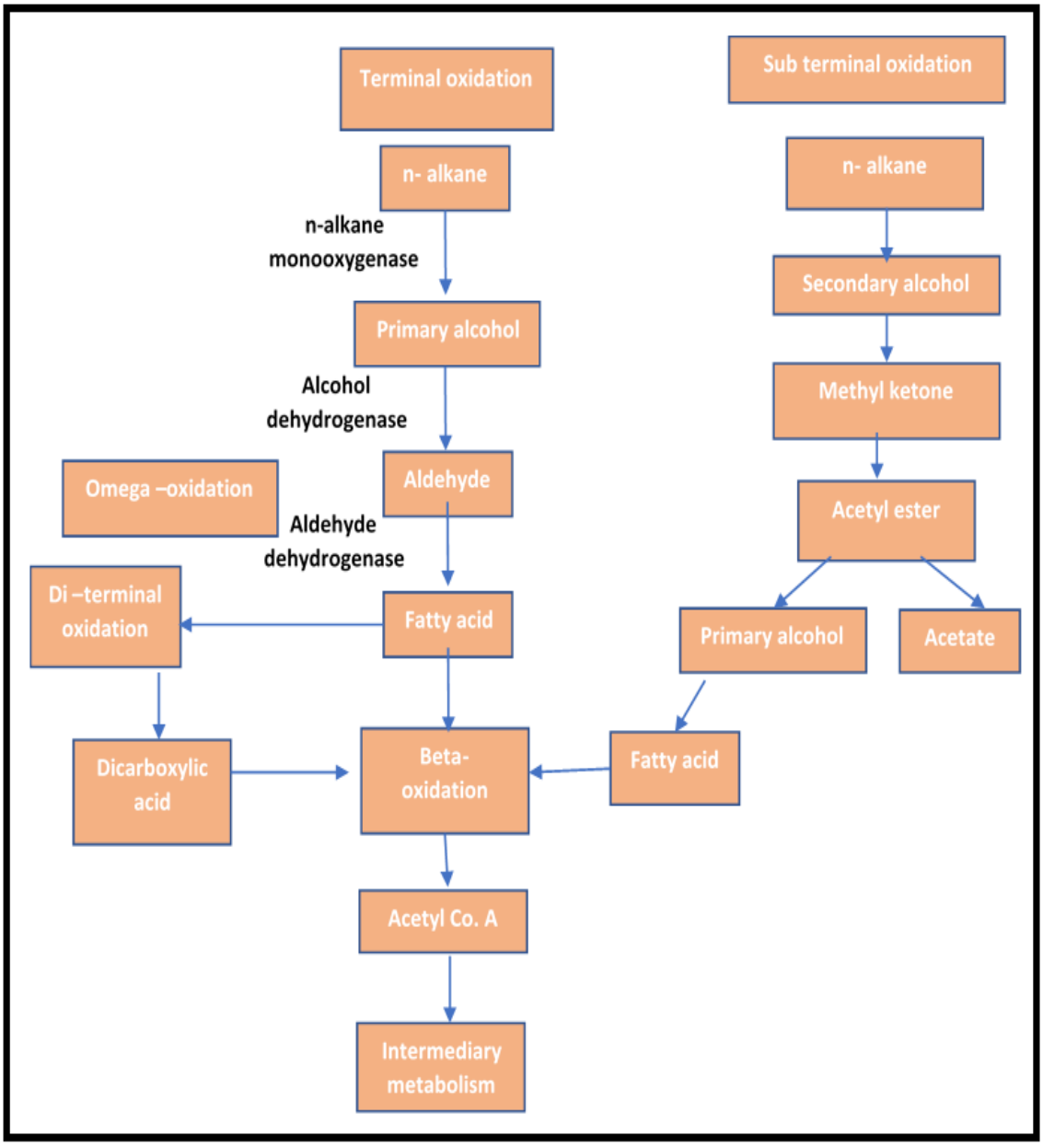


Figure 6: Biodegradation pathway of total petroleum hydrocabon

Rationale of study

2. RATIONALE OF STUDY

Industries and petrochemical processing at ports leads to the generation of large scale oil sludge. Moreover oil drilling and refining also leads to generation of huge amount of oil sludge, which contributes to pollution of environment wherever it is disposed off. Cleaning of petroleum compounds released by their activity is serious environmental issue for industries and government body.

Drilling, mining, processing, and transportation of crude oil leads to the spillage and clogging of oil sludge into the environment, which adversely affects the mother Earth, as it destroys the clean land and water as shown in Figure 7.

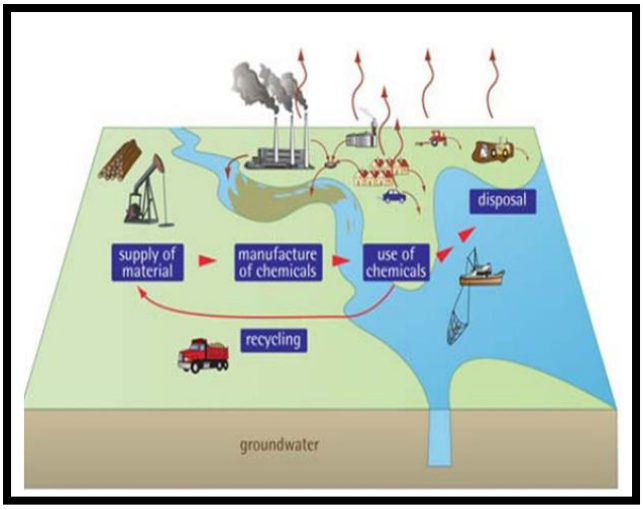


Figure 7: Release of total petroleum hydrocarbon in the environment

It is assumed that in oil sludge waste, there are harnessing microorganisms adapted into it due to the long term hydrocarbon exposure , which have the capacity to degrade the pollutants, but because of their limited number they fail to completely degrade the pollutants. Therefore a better strategy is to isolate those adapted microorganisms, enhance their growth and then incorporate the bacteria which are having highest potential to degrade the PAH in the oil sludge spilled soil to bioremediate a particular micro or macro-environment.

Hence, aim of this study was to investigate biodegradation potential of such bacterial isolates to degrade petroleum oil. So initially screening was done with PAH compounds and further the bacterial isolates were screened for their degradation efficiency on actual crude oil sample obtained from ONGC Ankleshwar site, Gujarat.

Objectives

3. OBJECTIVES

- To isolate petroleum hydrocarbon degrading bacteria from oil sludge contaminated samples.
- To determine simple aromatic hydrocarbon degradation efficiency of bacterial isolates.
- To check PAH biodegradation efficiency of bacterial isolates.
- To investigate crude oil biodegradation capability of selected bacterial isolates.

Materials

4. MATERIALS AND METHODS

Bacterial strains were isolated earlier in laboratory. These isolates were used in present study. Table 1 describes list of isolated strains which were obtained by providing different carbon sources and different samples collected from ONGC Ankleshwar site, Gujarat.

Table 1: Bacterial cultures isolated in laboratory.

Sr no	Substrate	Inoculum	Isolate
1	INLET SUMP OIL WAX	CRUDE OIL CONTAMINATED WATER SAMPLE	AWIS01
2			AWIS02
3			AWIS03
4			AWIS04
5			AWIS05
6			AWIS06
7	CRUDE OIL	CRUDE OIL CONTAMINATED WATER SAMPLE	AWCO11
8			AWCO12
9			AWCO13
10			AWCO14
11			AWCO15
12			AWCO16
13			AWCO17
14	DEWATERED SLUDGE	CRUDE OIL CONTAMINATED WATER SAMPLE	AWDS21
15			AWDS22
16	ANTHRACENE	CRUDE OIL CONTAMINATED WATER SAMPLE	AWS ant31
17			AWS ant32
18			AWS ant33
19			AWS ant34
20			AWS ant35
21			AWS ant36
22			AWS ant37
23	ANTHRACENE	STORED SLUDGE FROM WATER LOGGED PIT	ASS ant41
24			ASS ant42
25			ASS ant43
26			ASS ant44
27			ASS ant45
28	NAPHTHALENE	STORED SLUDGE FROM WATER LOGGED PIT	ASS nap51
29			ASS nap52
30			ASS nap53
31			ASS nap54
32			ASS nap55

4.1 Materials

- Phenanthrene
- Stored sludge from water logged pit
- Activated bacterial cultures
- DCPIP dye
- Crude oil

Methods

4.2 Methods

4.2.1 Solubility Assay

- Solubility of compound is essential in solvents because it makes substrate bioavailable to the bacteria. It involved dissolution of phenanthrene in solvent and biodegradation assay medium, so solubility of phenanthrene was initially tested in acetone, in which different amounts of phenanthrene was dissolved in 10ml acetone, further acetonic solution of hydrocarbon was tested for their solubility in Mineral salt medium (MM2) media.
- Similarly solubility of naphthalene and anthracene was tested in acetone, further acetonic solution of phenanthrene and naphthalene was investigated for their solubility in MM2 medium.

4.2.2 Enrichment method

- This process was performed with phenanthrene as carbon source and 5% stored sludge from water logged pit as inoculum and incubated at 28º C for a month.
- At the interval of a week, 5% of previously inoculated media was transferred to freshly prepared MM2 media thereby incubating it further in
- After 5 cycles of enrichment, 10^{-5} fold dilutions of cultures were prepared and 0.l ml of sample was spreaded on MM2 agar plates and 0.5 % w/v of acetonic solution of phenanthrene was sprayed on the plates. The plates were incubated at 28º C for 20 days as described in Figure 8.
- After 20 days of incubation, the plates were observed, the colonies possessing unique characteristics were streaked on Nutrient agar plates and were preserved as pure culture at 4º C (Pinyakong *et al*., 2012).

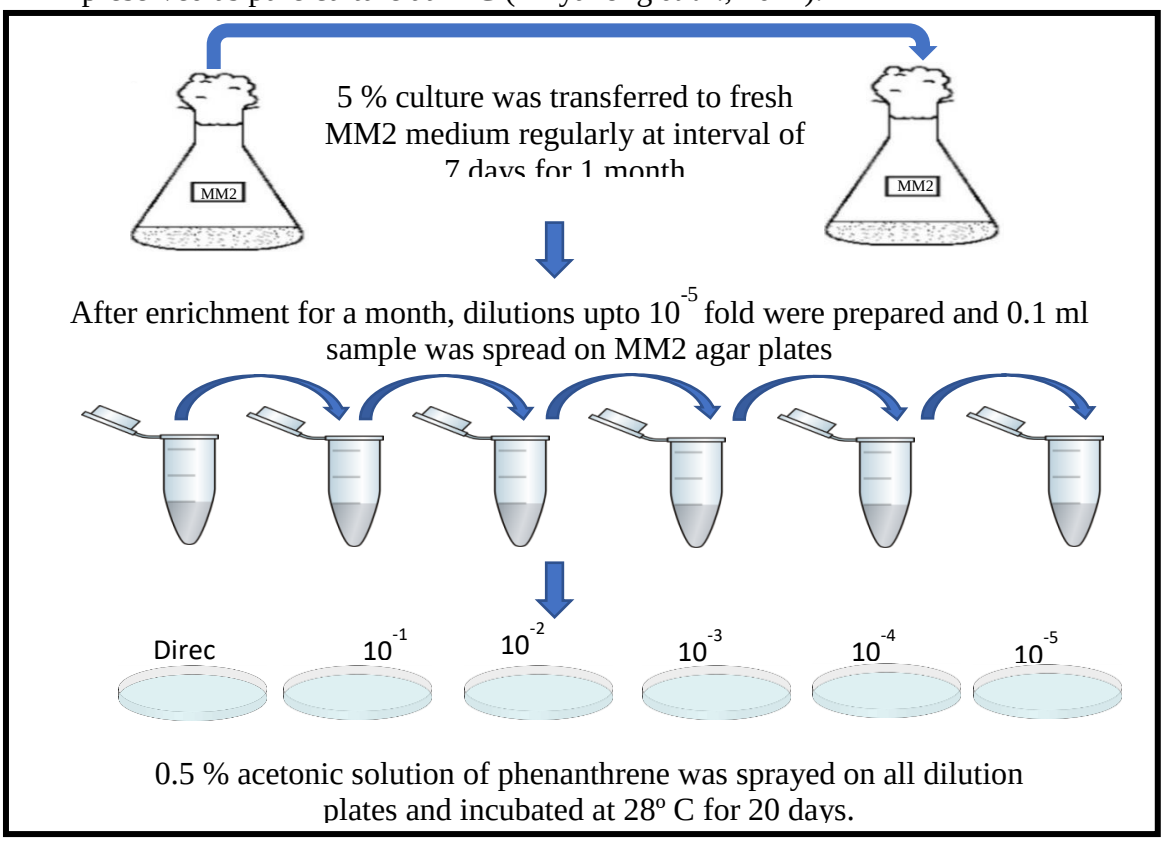


Figure 8: Process of enrichment for phenanthrene utilizing bacteria

4.2.3 DCPIP Assay

Screening of most efficient petroleum hydrocarbon degrading bacteria was done by colorimetric method using DCPIP dye. One of the colorimetric indicator is 2,6-dichlorophenolindophenol (DCPIP). DCPIP is a redox indicator dye which is blue in colour in its oxidized state, and after accepting electron from substrates, gets reduced and turns colourless. The principle of this redox indicator is based on the oxidation of the carbon source (hydrocarbon substrates) in which electrons are transferred to the electron acceptors, the utilisation of substrate can be observed based on the loss of indicator's blue colour. Its chemical formula is $C_{12}H_7Cl_2NO_2$ with maximum absorption spectra of 600 nm (Hanson *et al*., 1993 & Biodia *et al*., 2010).

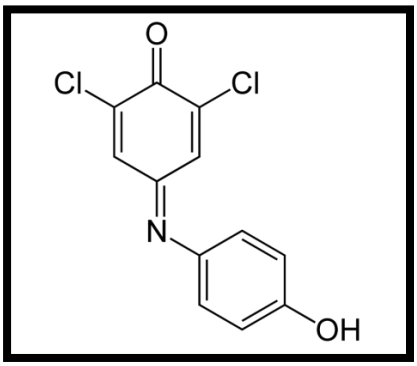


Figure 9: Structure of 2,6 dichlorophenol indophenol

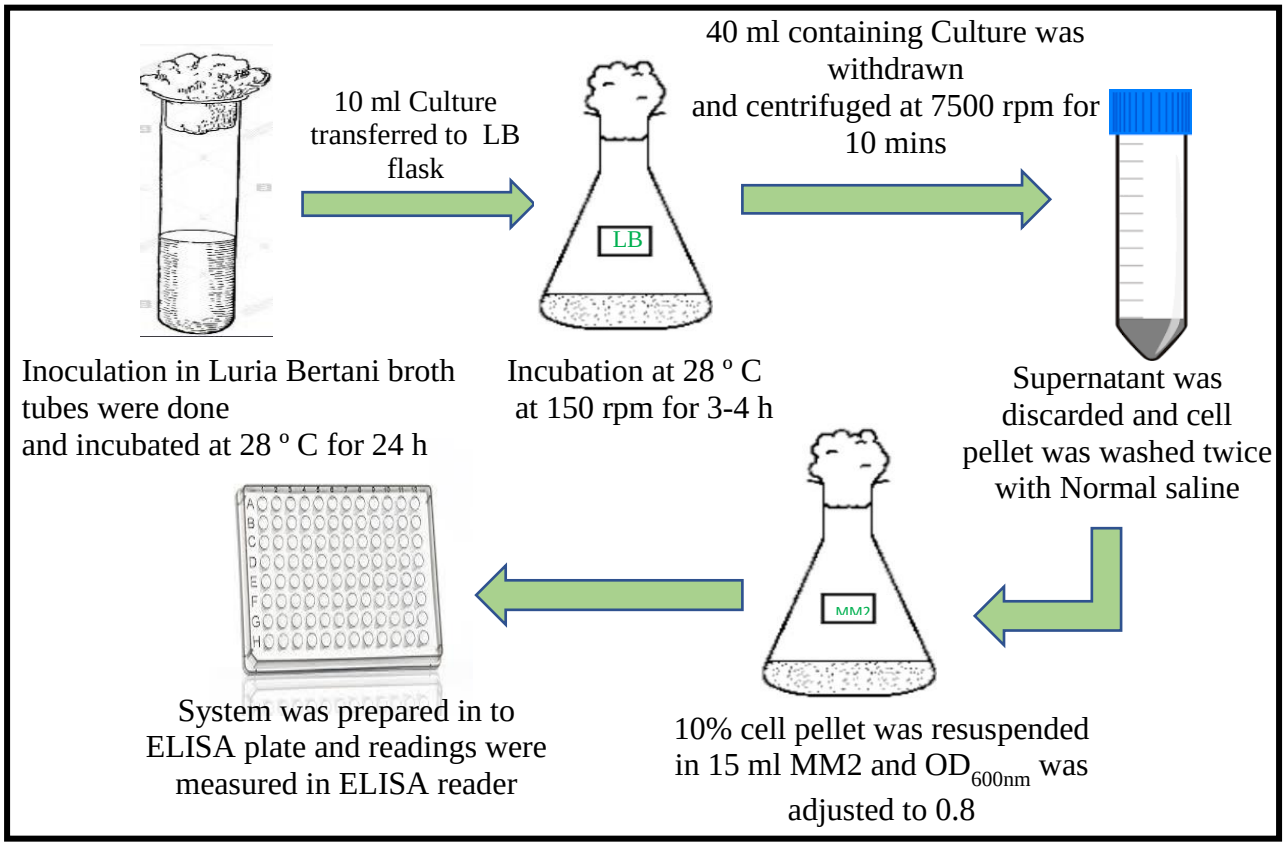


Figure 10: DCPIP assay to rapidly screen hydrocarbon degrading bacteria

- Inoculation of bacterial cultures were done in tubes containing Luria Bertani broth and tubes were incubated in shaking condition at 150rpm at 28º C for overnight.
- After overnight incubation, 5% culture was transferred to flasks containing Luria Bertani broth and was incubated on shaker at 150 rpm and 28º C for 3-4 h for activation of culture.
- Following 4 h of incubation, harvesting of cells were done. 10% media was withdrawn from flask and was centrifuged at 7500 rpm for 10 minutes. Supernatant was discarded and cell pellets were washed twice with Normal saline.
- Cell pellets were resuspended in MM2 and OD was determined at 560nm.
- As shown in table 2, system was prepared in each well of ELISA plate, and plates were incubated at 28º C
- At intervals of 24 h, readings were taken in ELISA plate reader as shown in Figure 10.

Table 2: Details of reaction mixture used for DCPIP assay

	DCPIP Dye (µl)	Substrate (µl)	MM2 (µl)	Distilled water (µl)	Cell inoculum (µl)
Test	150µl	1.3	100	-	3.3
Only dye	255	-		-	-
Only MM2	-	-	255	-	-
Dye+Substrate+MM2	150	1.3	100	3.7	-
Dye+MM2	150	-	100	5	-
Substrate+ Dye	150	1.3	-	104	-

As shown in table, DCPIP dye, MM2, bacterial isolate and substrates were added into individual wells of ELISA plate and were observed for visual decolorization.

4.2.4 Inoculum standardization

- Inoculation of bacterial cultures (AWIS04, AWCO11, AWDS21, ASSnap52, AWSant32) were done in tubes containing Luria Bertani broth and tubes were incubated in shaking condition at 150rpm at 28º C for overnight.
- After overnight incubation, 5% culture transfer was done in flasks containing Luria
  Bertani broth and was incubated on shaker at 150rpm and 28º C for 3-4 h for activation of culture.
- Following 4 h of incubation, harvesting of cells were done. 10% media was withdrawn from flask and was centrifuged at 7500 rpm for 10 minutes. Supernatant
  was discarded and cell pellets were washed with Normal saline twice.

- Cell pellets were resuspended in MM2 medium spiked with phenanthrene and OD for 0^{th} hour was determined at 560 nm.

4.2.5 Sodium Benzoate degradation assay

Culture activation

- Selected isolates were grown overnight in Luria Bertani medium.
- Overnight grown cells were transferred to new Luria broth and incubated at $28^{o} \pm 2^{o}$ C with shaking at 150 rpm. OD for growth of this activated culture was observed at 600nm, when OD reached to 0.6 to 0.8, further process were carried out.

Cell harvesting

- Following 4 h of incubation, harvesting of cells were done. 10% media was withdrawn from flask and was centrifuged at 7500 rpm for 10 minutes. Supernatant was discarded and cell pellets were washed twice with Normal saline.
- Cell pellets were resuspended in MM2 containing Sodium benzoate as source of carbon.

Spectrophotometric analysis of growth

- Optical density (OD.) for growth was measured at 600 nm at regular interval to determine growth of isolates in sodium benzoate containing medium.

Degradation kinetics

- One ml culture was withdrawn in microcentrifuge tube, sample was centrifuged at 7500 rpm for 10 minutes. Further supernatant was separated and diluted for determination of remaining sodium benzoate concentration in medium after biodegradation assay.
- OD was taken at 230 nm. Sodium benzoate degradation percentage was calculated as follows,

Formula for calculating Sodium benzoate degradation :

$$\textbf{Degradation rate (\%)} = \frac{\text{Initial concentration} - \text{Concentration of SB at time "t"}}{\text{Initial concentration of SB}} \times 100$$

4.2.6 Study of PAH utilization as carbon source

- After screening of bacterial isolates to grow and degrade sodium benzoate, best strains were selected to check their ability to grow on PAH. Naphthalene and Phenanthrene were selected as carbon source in this assay (Lakshmi,Velan, 2011; Mohd-Kamil *et al.*, 2013; Romero *et al.*, 1998; Sadighbayan *et al.*, 2016; Nigam *et al.*, 1998).
- Selected isolates were grown overnight in Luria Bertani medium.
- Overnight grown cells were transferred to new Luria broth and incubated at $28^{o} \pm 2^{o}$ C with shaking at 150 rpm. OD for growth of this activated culture was observed at 600 nm, when OD reached to 0.6 to 0.8, further process were carried out.
- Following 4 h of incubation, harvesting of cells were done. 10% media was withdrawn from flask and was centrifuged at 7500 rpm for 10 minutes. Supernatant was discarded and cell pellets were washed with Normal saline twice.
- Cell pellets were resuspended in MM2 containing phenanthrene and naphthalene as source of carbon
- After inoculation, flasks were incubated at $28^{o} \pm 2^{o}$ C for 10 days with shaking at 150rpm.
- Growth of bacterial isolates were measured at OD_{560nm} at regular interval.

4.2.7 Degradation of crude oil

- Bacterial isolates showing good degradation efficiency on sodium benzoate, phenanthrene and naphthalene were selected and their ability to grow and degrade actual crude oil was assessed as depicted in the figure.
- The 5 screened bacterial isolates **(AWIS04, AWCO15, ASS Phn66, AWS ant33, AWDS21)** were further monitored for their efficiency to degrade total petroleum hydrocarbon fraction from petroleum crude oil (Rahman *et al.*,2002).

Culture activation

- Selected isolates were grown overnight in Luria Bertani medium.
- Overnight grown cells were transferred to new Luria broth and incubated at $28^{o} \pm 2^{o}$ C with shaking 150 rpm. OD for growth of this activated culture was observed at 600nm, when OD reached to 0.6 to 0.8, further process were carried out.

Cell harvesting

- Following 4 h of incubation, harvesting of cells were done. 10% media was withdrawn from flask and was centrifuged at 7500 rpm for 10 minutes. Supernatant was discarded and cell pellets were washed with Normal saline twice.
- Cell pellets were resuspended in MM2 containing 1% crude oil as source of carbon.

- After inoculation, flasks were incubated at 28º ± 2º C for 10 days with shaking at 150 rpm..
- Uninoculated controls were kept so as to monitor natural weathering of crude oil.

Crude oil extraction

- After an incubation of 20 days, residual crude oil was extracted with the help of Hexane using separating funnel (Gunstone, Hamilton, 2001; Li et al., 2012) as shown in Figure 11.
- Based on the efficiency to degrade total petroleum hydrocarbon, a bacterial consortium comprising all 5 bacterial isolates was developed and were kept in another flask containing 1% crude oil, to monitor the cumulative oil degradation capacity.

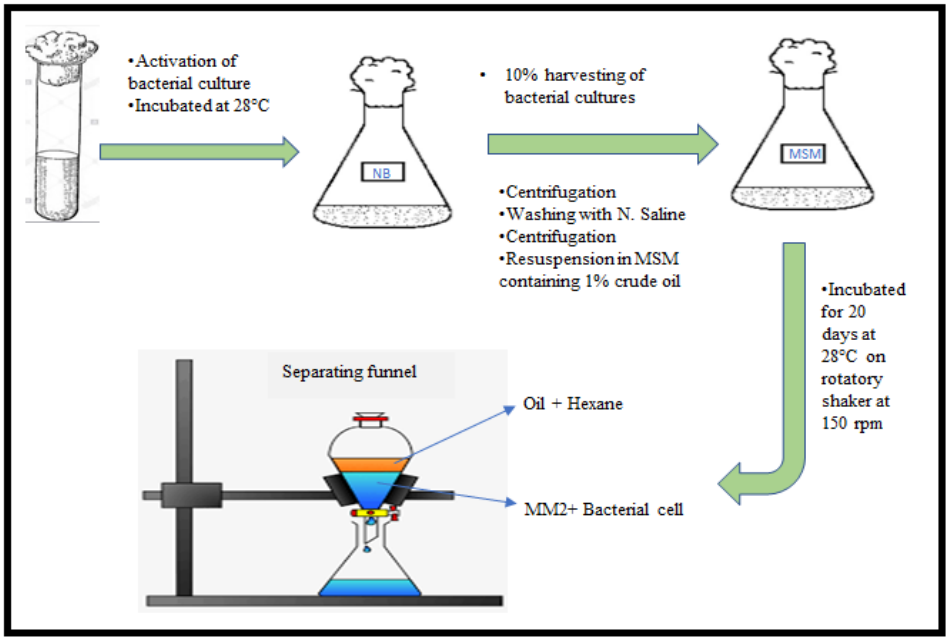


Figure 11: Crude oil biodegradation assay and its extraction process

4.2.8 Molecular identification of crude oil degrading strains

(a) Genomic DNA isolation

- Bacterial cells were harvested in 1.5 ml microcentrifuge tube by centrifugation at 7500 rpm for 10 minutes followed by discarding of supernatant.
- The pellet was resuspended in 180 µl of digestion solution and later, 20 µl Proteinase K solution was added. In order to obtain a uniform suspension, the content was by mixed vortexing.
- The sample was then incubated at 56◦C while vortexing occasionally.
- Further 20 µl RNase A solution was added to the sample and mixed by vortexing. Mixture was incubated at room temperature for 10 minutes.
- 200 µl of Lysis Solution was added to the sample and vortexed for almost 15 s until a homogenous mixture was obtained.

- 400 µl of 50% ethanol was added to the sample and vortexed.
- The prepared lysate was transferred to a GeneJET Genomic DNA Purification Column placed in the collection tube. The column was centrifuged for a minute at 6000x g. The Flow Through solution in the collection tube was discarded and GeneJET Genomic DNA Purification Column was placed into a new 2 ml collection tube.
- 500 µl of Wash Buffer was added to the column and centrifuged for a minute at 8000x g. Flow through solution was discarded and purification column was inserted back into the collection tube.
- Wash Buffer II was added to GeneJET Genomic DNA Purification column and the column was centrifuged for 3 minutes at 14,500 x g. Collection tube with flow through solution was discarded and GeneJET Genomic DNA Purification column was transferred to a sterile 1.5 ml microcentrifuge tube.
- 200 µl of Elution Buffer was added to the centre of GeneJET Genomic DNA Purification column membrane to elute genomic DNA followed by incubation at room temperature for 2 minutes and centrifugation at 8000x g for a minute.
- The purification column was discarded and the purified DNA was immediately used for 16s rDNA amplification purpose.

(b) 16S rRNA gene amplification

- To amplify the 16S rRNA gene for identification of crude oil degrading microorganisms.
- The details of PCR reaction mixture required for amplification of gene are described in Table 3.
- The volume of PCR system was set according to need of the product. The choice of primers varies according to the gene of interest that is to be amplified.
- Here universal primers : F27 and R1492 were used for PCR amplification (Table 4).
- Amplified product was verified by agarose gel electrophoresis.

Table 3: Details of PCR reaction

Component	**Volume**
Master mix	25 µl
Template	2.5 µl
Forward primer	2.5 µl
Reverse primer	2.5 µl
Dnase, Rnase free water	17.5 µl
Final volume	**50 µl**

Table 4: PCR conditions for amplification

Temperature	**Time**
95º C (initial denaturation)	4 min
94º C	30 sec
52º C (annealing)	1 min
72º C (initial extension)	1.5 min
72º C	10 min

Results and discussions

5. RESULTS AND DISCUSSION

5.1 Substrate solubility assay

In order to isolate phenanthrene degrading strains, bioavailability of phenanthrene in media used for isolation becomes important aspect and hence solubility of phenanthrene was estimated in an organic solvent and minimal salt medium (MM2). Solubility of phenanthrene, anthracene and naphthalene was tested in acetone, in which different amounts (0.05 g, 0.5 g, 1 g) were dissolved in 10 ml acetone (Table 5). Then, acetonic solution of substrates obtained on basis of solubility in acetone, its solubility was investigated in MM2 media (Ho *et al.,* 2000) (Table 6)

Table 5: Solubility of phenanthrene in acetone.

Volume of Acetone	Amount of Phenanthrene	Solubility	Molarity
10 ml	0.05 g	Soluble	28 mM
10 ml	0.5 g	Insoluble	-
10 ml	1 g	Insoluble	-

On the basis of phenanthrene solubility in acetone, 28 mM stock solution of phenanthrene was prepared.

Table 6: Solubility of phenanthrene in MM2.

Volume of MM2 medium	Volume of 0.028M stock solution of phenanthrene	Solubility	Molarity
100 ml	50 µl	Soluble	0.014 mM
100 ml	100 µl	Insoluble	-
100 ml	500 µl	Insoluble	-

Further , for rapid screening of hydrocarbon degrading isolates, DCPIP assay was carried out in which five substrate listed below were used (Table 7).

Table 7: Stock solution of substrates used in DCPIP assay

Substrate	Concentration
Sodium benzoate	5 mM
Benzene	1000 mM
Naphthalene	780 mM
Anthracene	14 mM
Phenanthrene	28 mM

Amongst five substrates, naphthalene and anthracene were insoluble in water and hence their solubility were investigated in acetone (Ho *et al.*, 2000) (Table 8).

Table 8: Solubility of naphthalene and anthracene in acetone

Substrate	Amount of substrate (g)	Solubility in 10 ml acetone	Molar solution
Naphthalene	0.1	Soluble	-
	0.5	Soluble	-
	1	Soluble	780 mM
Anthracene	0.025	Soluble	14 mM
	0.1	Insoluble	-
	0.5	Insoluble	-
	1	Insoluble	-

5.2 Enrichment assay for isolation of phenanthrene degrading bacterial strains

Bacteria isolated from the oil contaminated site are adapted to PAH exposure and have capability to utilize and grow on oil contaminants as they have chronic exposed and are present since long. Bioremediation by bacteria is preferred but because of lower abundancy of such strains in soil it is difficult to achieve complete degradation of pollutants. Therefore in order to check their degradation efficiency, bacterial population has to be increased and enrichment is one of the methods (*Lakshmi, Velan et al., 2011).*Enrichment is a common long-used practice, it effectively increases the population of target organism. Enrichment of these indigenous bacteria led to isolation of 12 new bacterial isolates by using phenanthrene as sole source of carbon and energy. Isolated bacterial strains were selected their colony morphology were studied and were preserved as pure cultures (Zhang *et al*., 2010). Phenanthrene is a polycyclic aromatic hydrocarbon composed of three fused benzene rings. Twelve Bacteria were isolated which could grow by utilizing phenanthrene as sole source of carbon, isolates were selected based on their uniqueness in colony morphology as shown in Figure 12 and Table 9. In study of Gran-Scheuch *et al.*,(2017), total bacterial growth in Antarctic soil samples was tested which were supplemented with glucose or phenanthrene and compared with a non-supplemented control. Soil samples were collected from diesel-exposed sites and non-contaminated site. A total of 350 colonies were isolated, 111 originating from non-diesel-exposed sites and 239 originating from diesel-contaminated soils.

Table 9: Designation and source of bacterial isolates.

Sr No.	Substrate	Inoculum	Isolates
1	PHENANTHRENE (0.05% w/v ACETONIC SOLUTION)	STORED SLUDGE FROM WATER LOGGED PIT	ASS Phn61
2			ASS Phn62
3			ASS Phn63
4			ASS Phn64
5			ASS Phn65
6			ASS Phn66
7			ASS Phn67
8			ASS Phn68
9			ASS Phn69
10			ASS Phn610
11			ASS Phn611
12			ASS Phn612

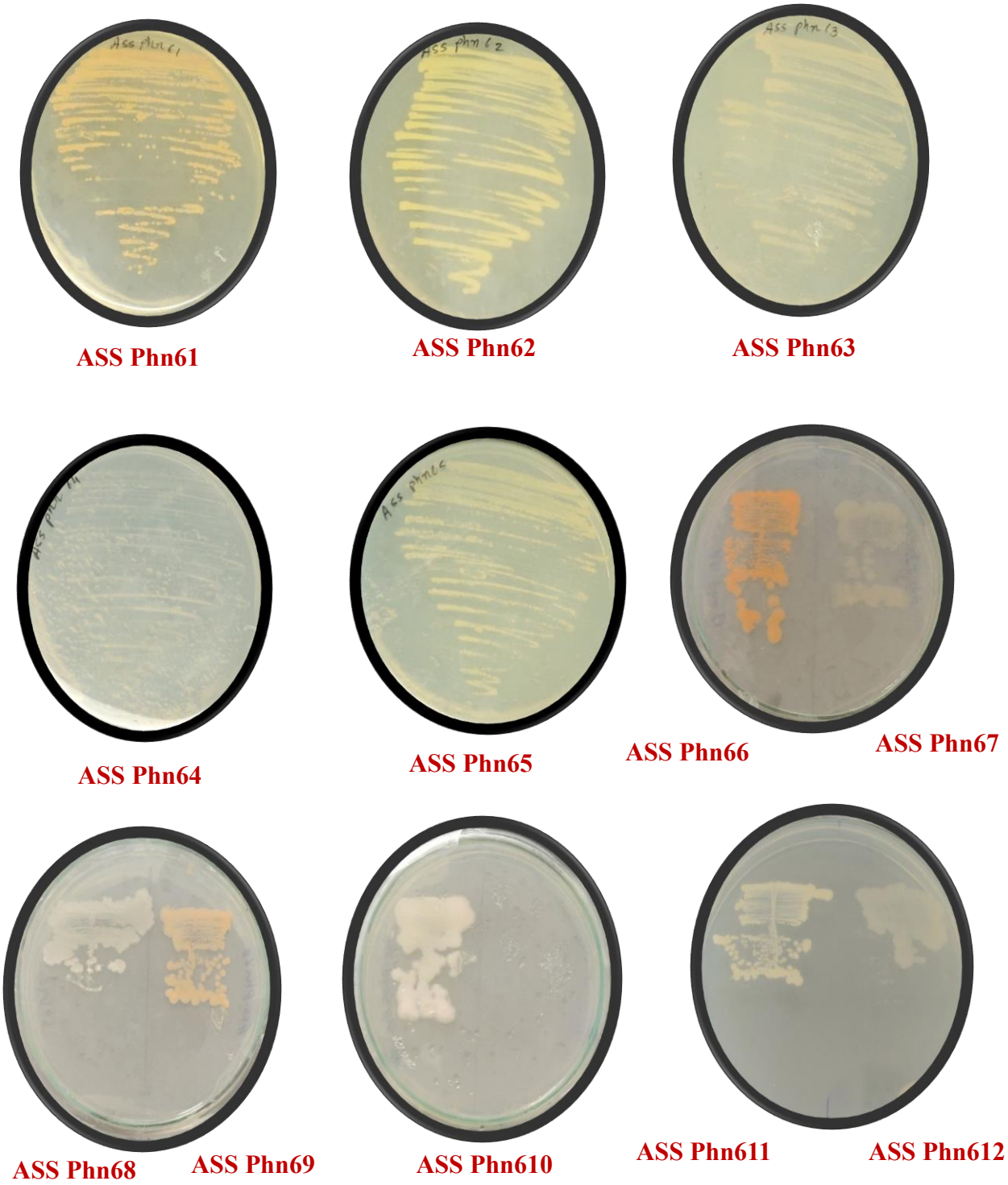
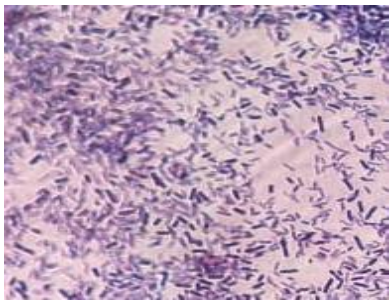


Figure 12: Bacterial isolates obtained from phenanthrene enrichment.

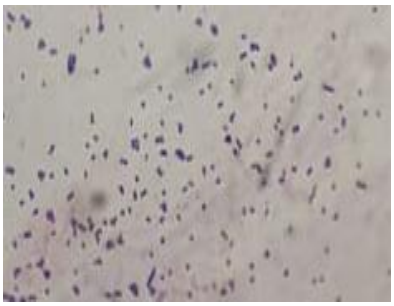
Pure cultures of bacterial isolates which grew utilizing phenanthrene as sole source of carbon, after 20 days of incubation, were streaked on N. Agar plates and were preserved at 4°C as master plates. The colony morphology of bacteria isolated from phenanthrene containing medium is presented in Table 10. Figure 13 and Table 11 show and describe morphology of bacterial isolates respectively.

Table 10: Colony morphological characteristics of bacterial cultures isolated from phenanthrene containing medium

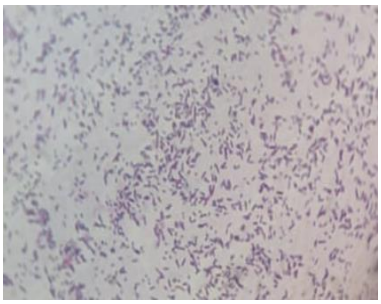
Characteristics	ASS Phn61	ASS Phn62	ASS Phn63	ASS Phn64	ASS Phn65	ASS Phn66	ASS Phn67	ASS Phn68	ASS Phn69	ASS Phn610	ASS Phn611	ASS Phn612
Size	Pin point	Large	Medium	Large	Small	Large	Small	Small	Small	Medium	Small	Small
Shape	Round	Round	Round	Round	Round	Round	Round	Round	Round	Round	Round	Round
Margin	Regular	Regular	Irregular	Regular	Irregular	Irregular	Regular	Regular	Regular	Regular	Regular	Regular
Elevation	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex
Consistency	Moist	Moist	Moist	Moist	Moist	Moist	Moist	Moist	Moist	Moist	Moist	Moist
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Pigmentation	Orange	Yellow	Buff colour	White	Buff colour	Buff colour	Dark orange	Light yellow	White	Buff colour	Buff colour	Buff colour
Morphology	Gram negative rods	Gram negative rods	Gram positive rods	Gram negative rods	Gram negative rods	Gram negative rods	Gram positive rods	Gram positive rods	Gram negative rods	Gram positive rods	Gram negative rods	Gram negative rods



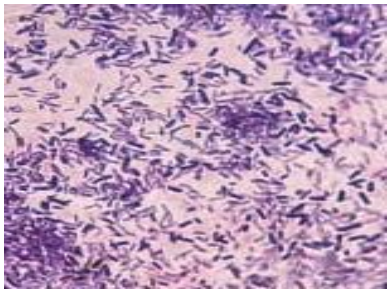
ASS Phn61



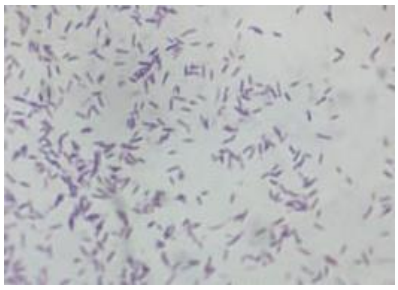
ASS Phn62



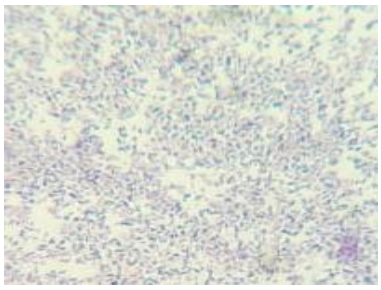
ASS Phn63



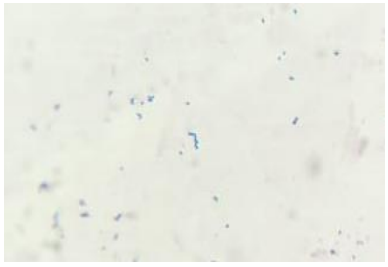
ASS Phn64



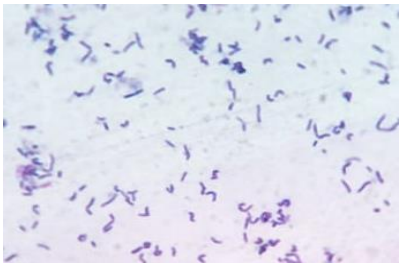
ASS Phn65



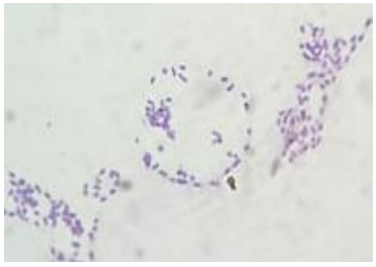
ASS Phn66



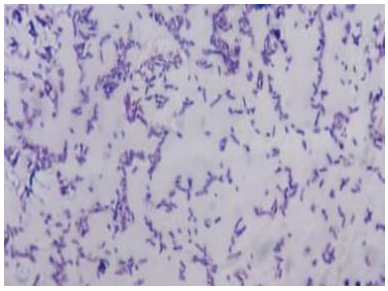
ASS Phn67



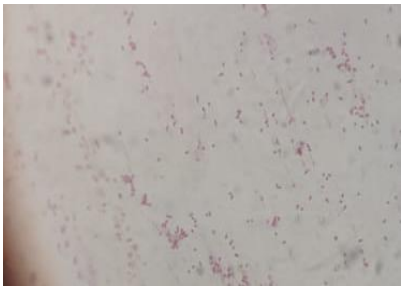
ASS Phn68



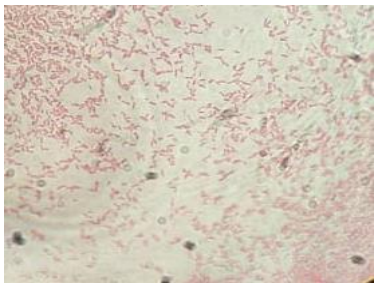
ASS Phn69



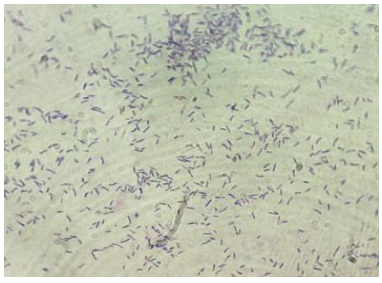
ASS Phn610



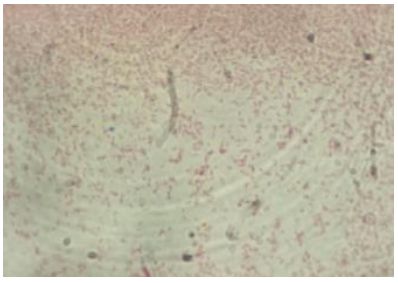
ASS Phn611



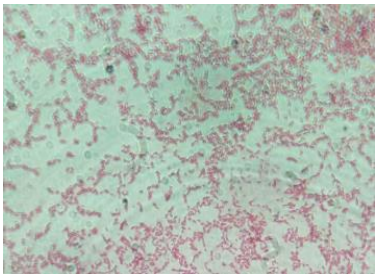
ASS Phn612



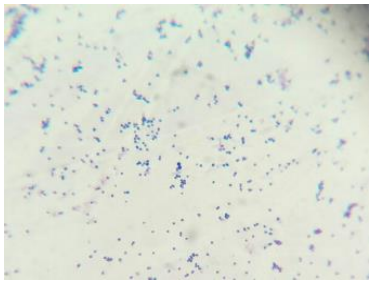
ASS nap51



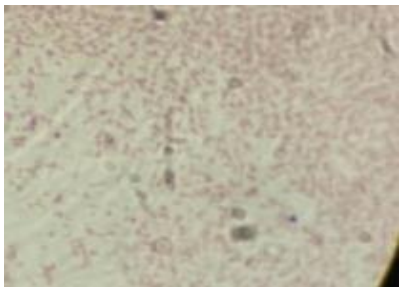
ASS nap52



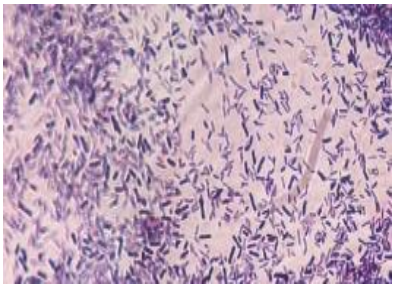
ASS nap53



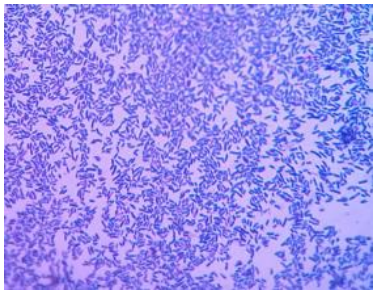
ASS nap54



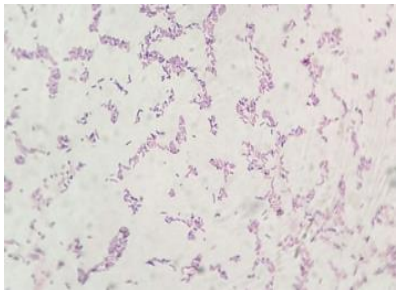
ASS nap55



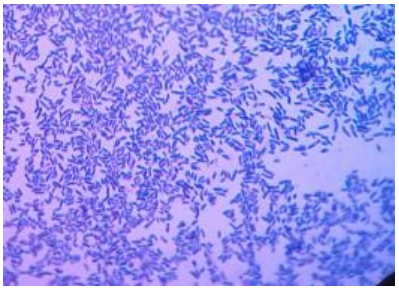
AWS ant31



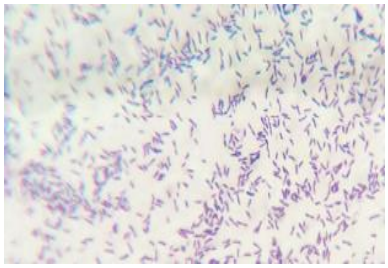
AWS ant32



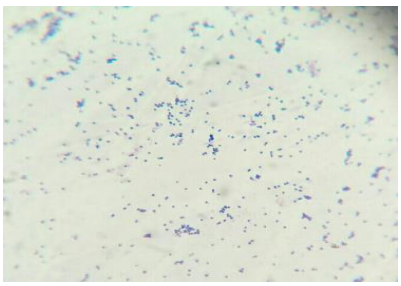
AWS ant33



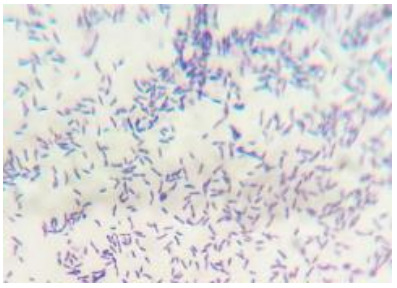
AWS ant34



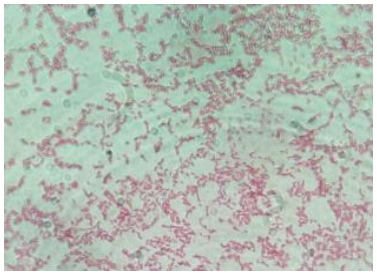
AWS ant35



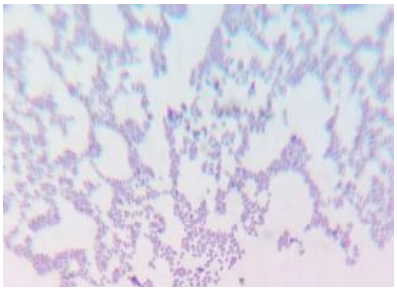
AWS ant36



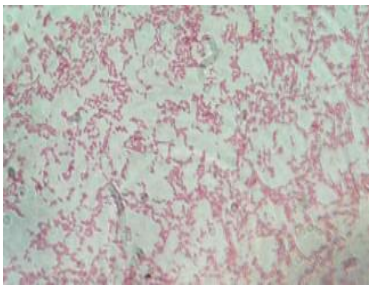
AWS ant37



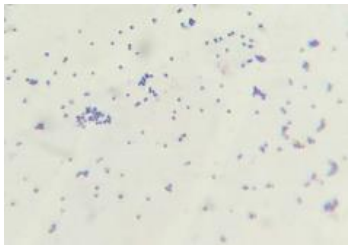
ASS ant41



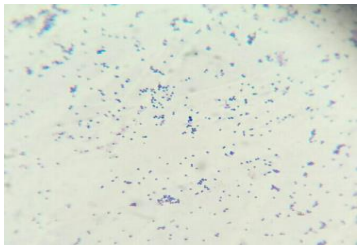
ASS ant42



ASS ant43



ASS ant44



ASS ant45

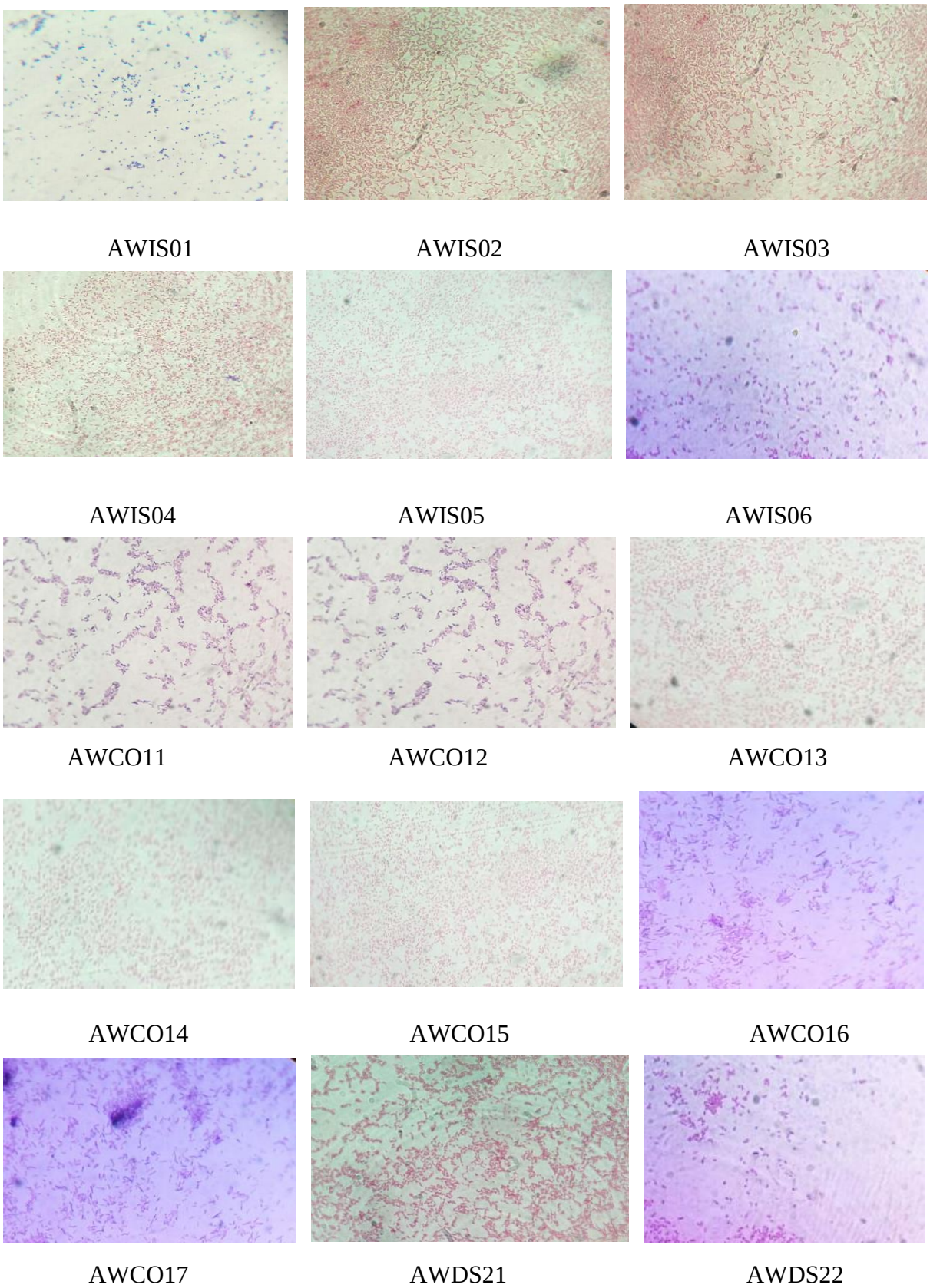


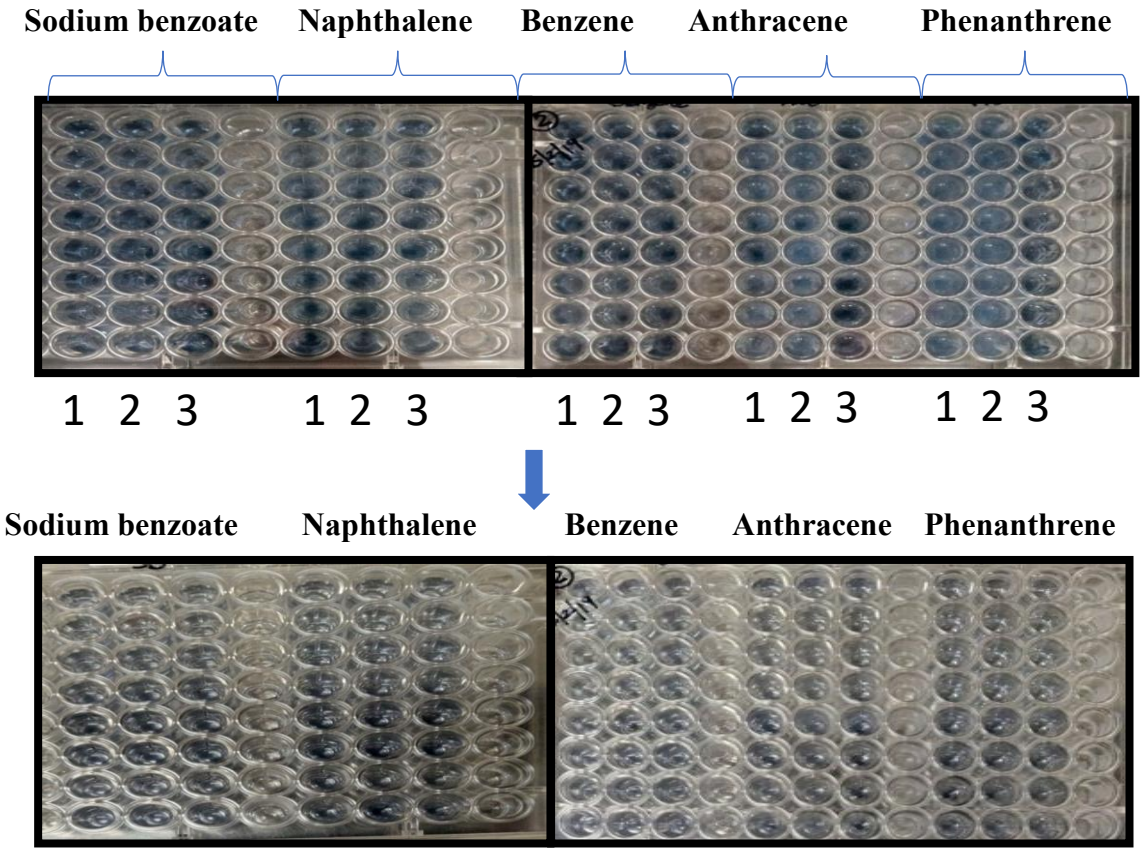
Figure 13: Morphological study of organisms

Table 11: Morphological study of organisms.

Isolate	Morphology	Isolate	Morphology
ASS Phn61	Gram negative rods	AWS ant36	Gram positive rods
ASS Phn62	Gram negative rods	AWS ant37	Gram positive rods
ASS Phn63	Gram positive rods	ASS ant41	Gram negative rods
ASS Phn64	Gram negative rods	ASS ant42	Gram positive cocci
ASS Phn65	Gram negative rods	ASS ant43	Gram negative rods
ASS Phn66	Gram negative rods	ASS ant44	Gram positive cocci
ASS Phn67	Gram positive rods	ASS ant45	Gram positive rods
ASS Phn68	Gram positive rods	AWIS01	Gram positive cocci
ASS Phn69	Gram negative rods	AWIS02	Gram negative rods
ASS Phn610	Gram positive rods	AWIS03	Gram negative rods
ASS Phn611	Gram negative rods	AWIS04	Gram negative rods
ASS Phn612	Gram negative rods	AWIS05	Gram negative rods
ASS nap51	Gram positive rods	AWIS06	Gram negative rods
ASS nap52	Gram negative rods	AWCO11	Gram negative rods
ASS nap53	Gram negative rods	AWCO12	Gram negative rods
ASS nap54	Gram positive cocci	AWCO13	Gram negative rods
ASS nap55	Gram negative rods	AWCO14	Gram negative rods
AWS ant31	Gram positive rods	AWCO15	Gram negative rods
AWS ant32	Gram positive rods	AWCO16	Gram negative rods
AWS ant33	Gram negative rods	AWCO17	Gram negative rods
AWS ant34	Gram positive rods	AWDS21	Gram negative rods
AWS ant35	Gram positive rods	AWDS22	Gram negative rods

5.3 DCPIP ASSAY

The final volume of the well after addition of media, substrate, dye and organism was 255 µl. Initially the color observed was blue. After 13 days of incubation of ELISA plate, decolorization of dye was observed due to bacterial activity. In order to re-evaluate the results, another set was kept comprising dye, organism, substrate and MM2 medium in ELISA plate, decolorization was observed gradually after 3 days of incubation period.



Key : 1. Test 2. Test 3. Control w/o substrate

Figure 14: Decolorization of DCPIP dye after substrate utilization by bacterial isolates

Figure 14 reveals decolourization of DCPIP dye when kept with 5 different substrates namely sodium benzoate, naphthalene, benzene, anthracene and phenanthrene. From 0^{th} to 10^{th}day, there was gradual decolourization in the dye from blue colour to almost colourless. It was reported in study of Hanson *et al.*(1993) in wells containing (Media+ Dye+ Organism) crude oil as substrate, complete decolorization was observed after 12 h. While we got complete decolorization after 15 days. The decolorization was observed in test, as well in control MSM+DYE+SUBSTRATE hence, further optimization of assay may be required and biodegradation ability of isolates were analysed by sodium benzoate degradation assay.

5.4 Inoculum standardization

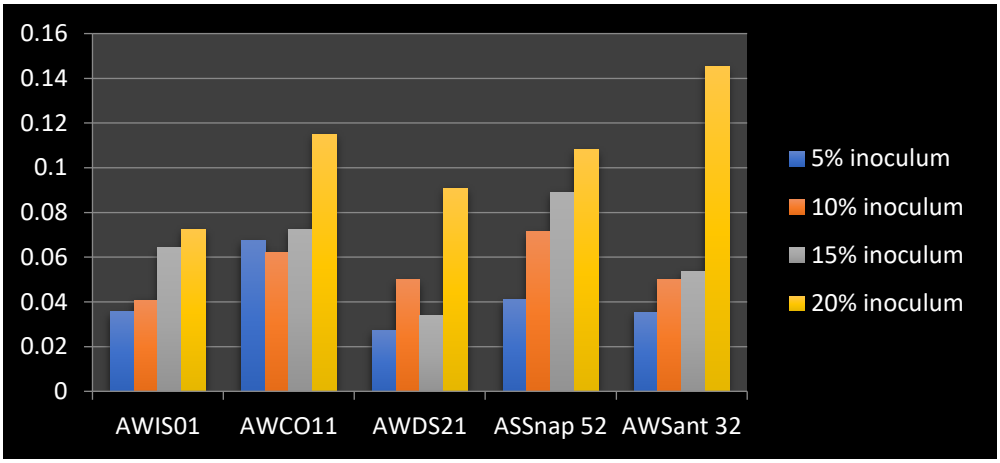


Figure 15: Standardization of inoculum

- As shown in Figure 15, 10 % inoculum was finalized, after observing the growth OD, as the range of OD required was 0.04 – 0.09 and in 10% inoculum, the required OD was obtained.

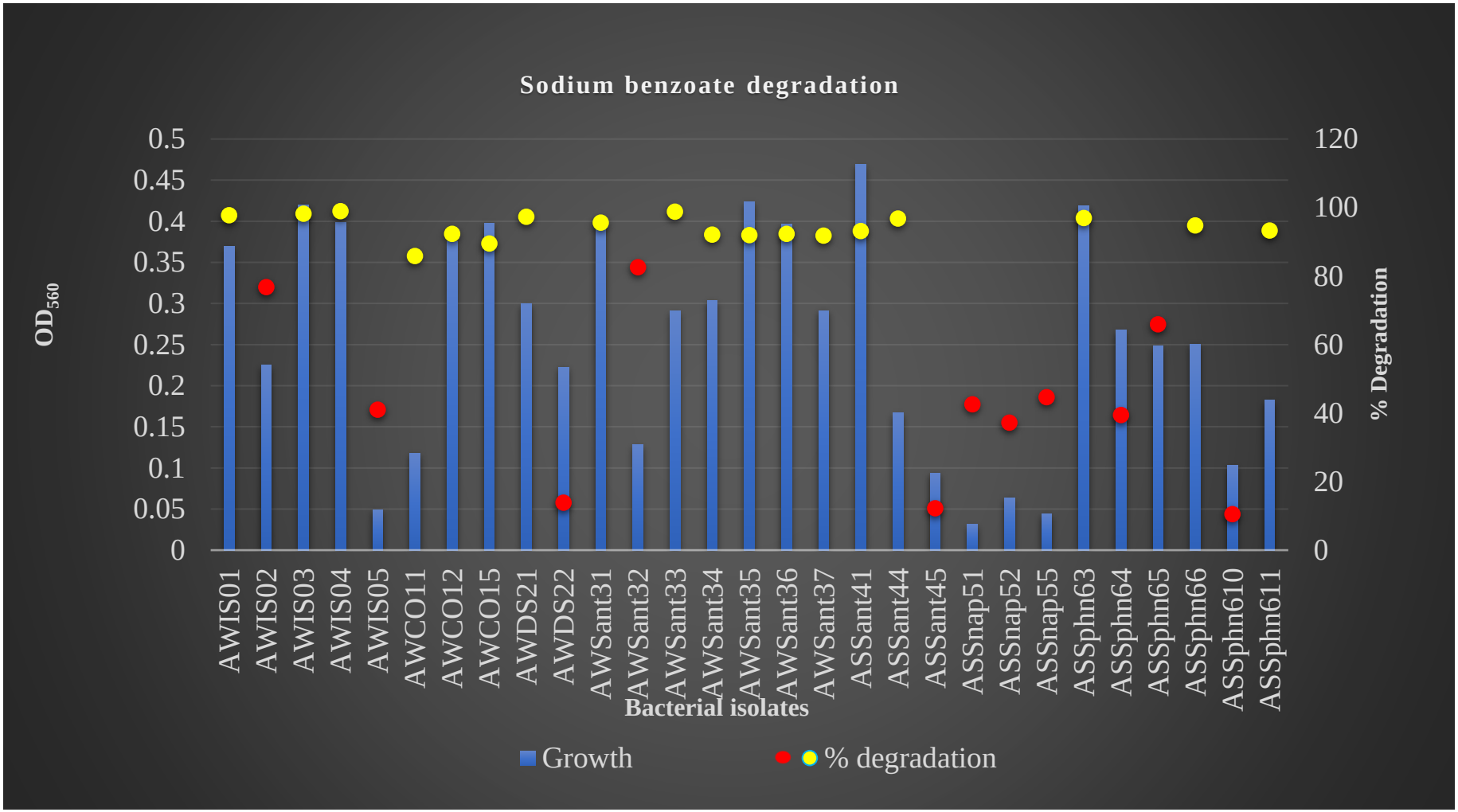
5.5 Sodium benzoate degradation assay

Sodium Benzoate is a single ring hydrocarbon which is much easier to be degraded than any polycyclic aromatic hydrocarbon, therefore ability of bacteria to degrade this simple compound was assessed. Sodium benzoate degradation ability of 27 isolates were assessed. Efficiency of all 27 isolates to degrade sodium benzoate was different and on the basis of sodium benzoate % degradation, they were categorized into two groups,

Isolates showing degradation from 10 to 80 % : AWIS02, AWIS05, AWDS22, ASSant45, ASSnap51, ASS nap52, ASS nap55, ASS phn64

Isolates showing more than 80 % degradation : AWIS01, AWIS03, AWIS04, AWCO11, AWCO12, AWCO15, AWDS21, AWS ant31, AWS ant32, AWS ant33, AWS ant34, AWS ant35, AWS ant36, AWS ant37, ASS ant41, ASS ant44, ASS Phn63, ASS Phn66 (Figure 16).

Good degradation efficiency and growth were observed in isolates AWIS03, AWIS04 and AWSant33.



Percent degradation 10 to 80% Percent degradation above 80%

Figure 16: Sodium benzoate degradation by different bacterial isolates

5.6 Growth of bacterial isolates by utilizing phenanthrene and naphthalene as carbon source

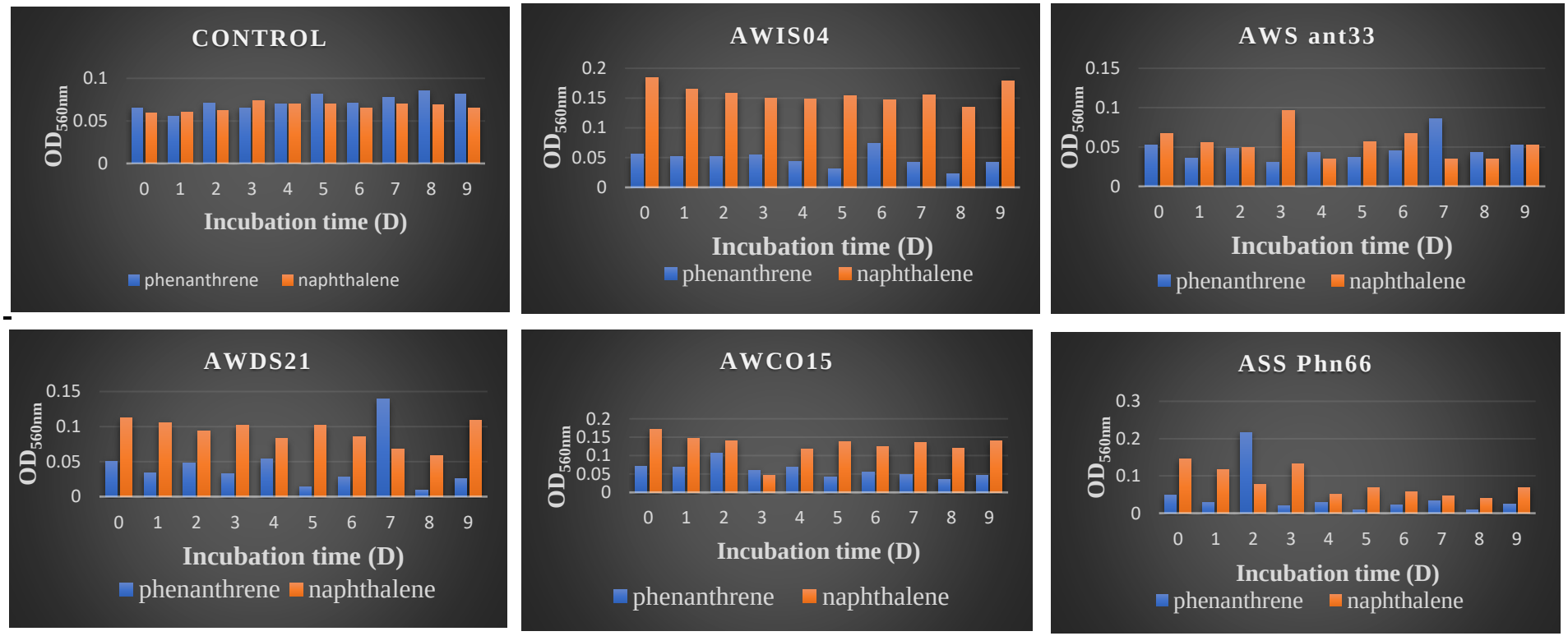


Figure 17: Growth of bacterial isolates utilizing phenanthrene and naphthalene as sole source of carbon

Bacterial isolates which showed agreeable sodium benzoate degradation efficiency were selected further to check their ability to utilize phenanthrene and naphthalene as sole source of carbon and energy. Figure 17 shows the graphs in which the listed isolates namely, AWDS21, AWSI04, AWS ant33, AWCO15, ASS Phn66 were able to survive in a medium containing phenanthrene and naphthalene as carbon sources.

On the basis of SB degradation efficiency, five bacterial cultures were selected as shown in Table 12.

Table 12: List of bacterial isolates obtained after screening with sodium benzoate as sole source of carbon

Sr no.	Isolates
1	AWIS04
2	AWDS21
3	AWCO15
4	ASS Phn66
5	AWS ant33

5.7 Crude oil extraction

Bacterial isolates having best degradation capacity were taken to next level where Crude oil was provided as the sole source of carbon. Cultures were incubated for 20 days with 1% crude oil as substrate and incubated for 20 days for degradation assay. After 20 days of incubation, crude oil was extracted using hexane as solvent, in Separating funnel, two layers were formed (Li *et al*., 2012), one containing hexane + residual Crude oil after degradation, another layer containing media+ cell mass. The layer containing hexane + crude oil was collected and outsourced for Gas chromatography at Biocare research India Pvt. Ltd. Paldi, Ahmedabad, Gujarat. Figure 18 displays crude oil extraction in solvent hexane.

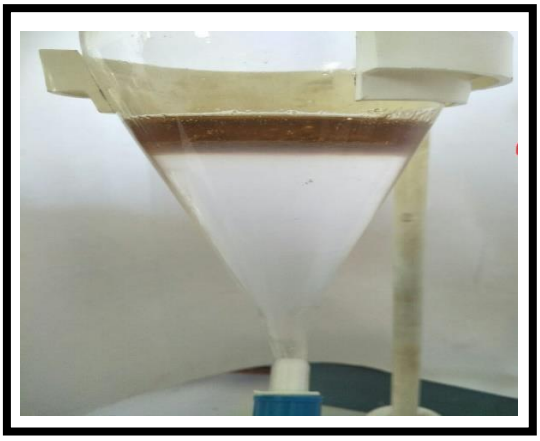
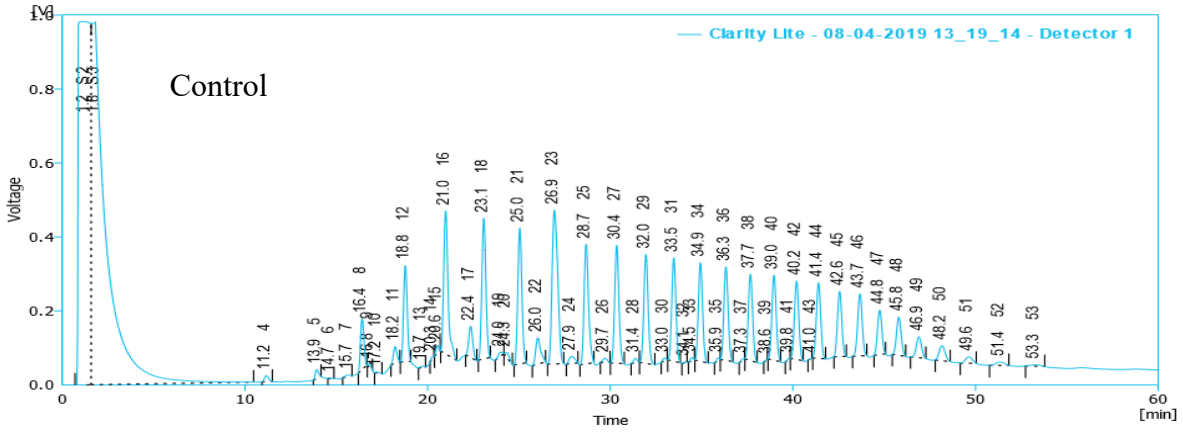


Figure 18: Extraction of crude oil with the help of hexane

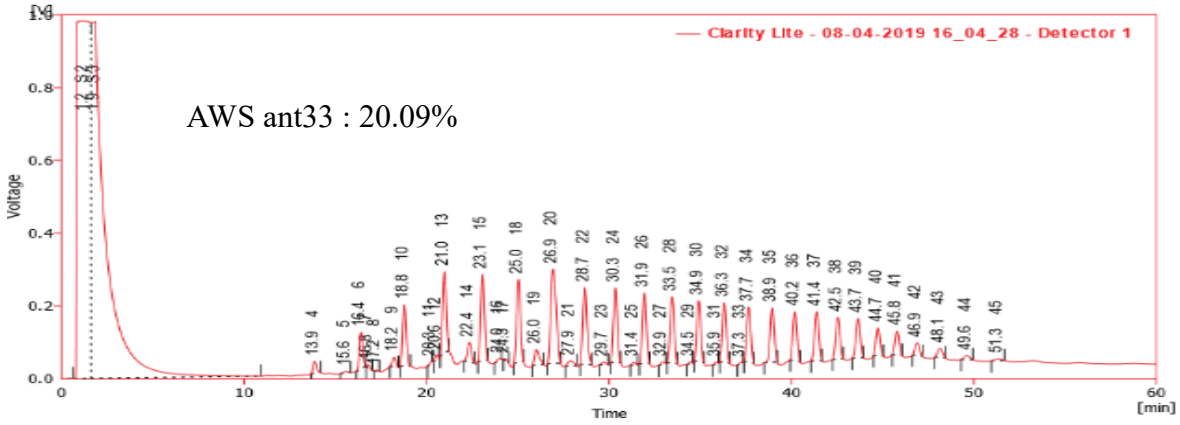
Gas chromatography analysis was done to check the TPH degradation capacity of screened bacterial isolates from crude oil. Crude oil % degradation was calculated by comparing the peak area of control as well as the peaks obtained in the samples containing test organisms after incubation period. Figure 19 represents chromatogram of GC.

TPH % degradation was determined using formula,

$$\text{TPH degradation rate (\%)} = 100 - \left[\frac{\text{Area of test} \times \text{Dilution factor if any}}{\text{Area of control}} \right] \times 100$$

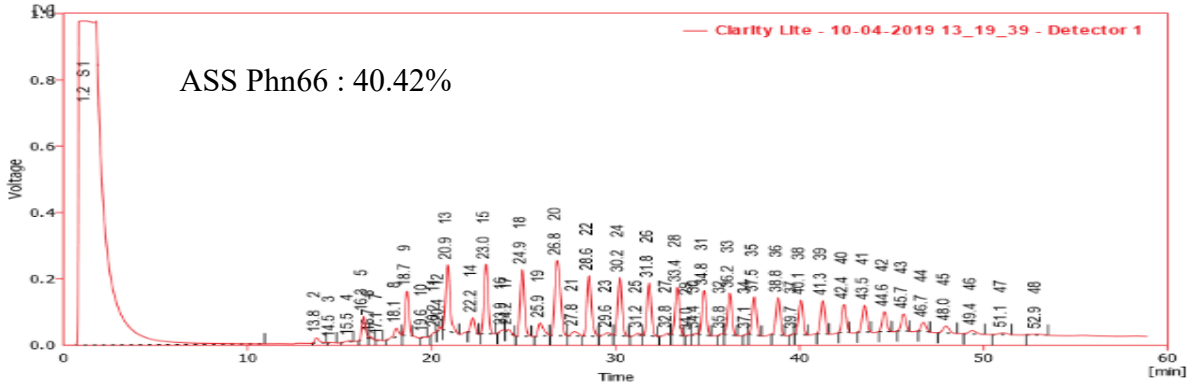


(a) Total area of control (MM2 + 1% crude oil) was 93713.18



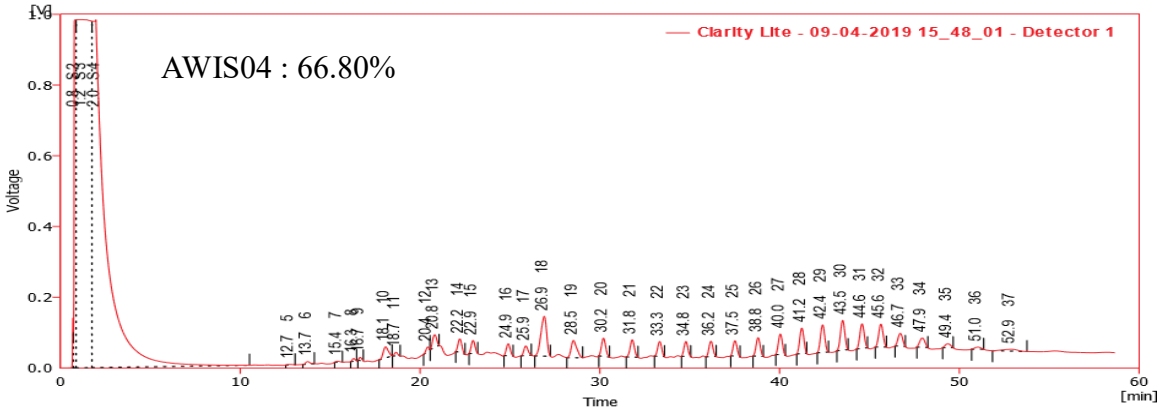
(b) Chromatogram of crude oil treated with AWS ant33

Total area of control was observed as 93713.18 and total area of AWS ant33 was reduced to 56367.95, so % TPH degradation obtained was 20.09%.



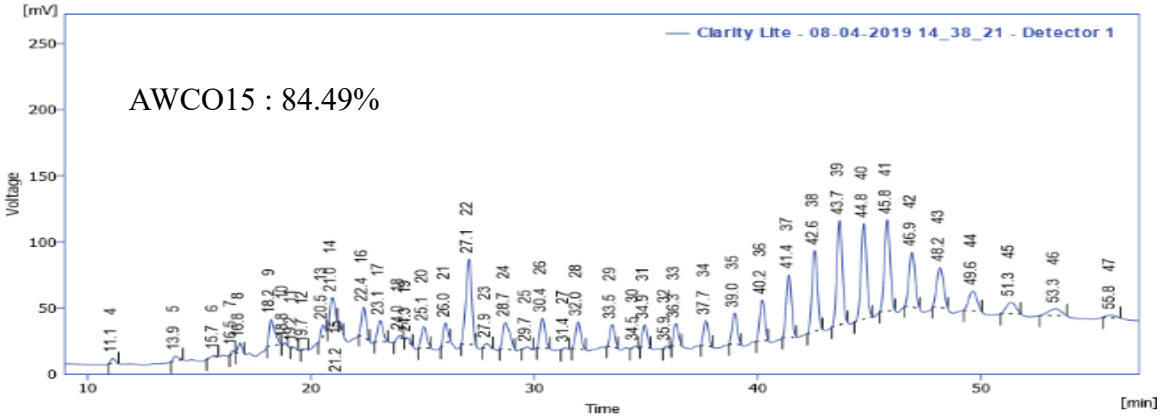
(c) Chromatogram of crude oil treated with ASS Phn66

As compared to control, total area of ASS Phn66 was 49409.205 and 40.42% TPH degradation was observed.



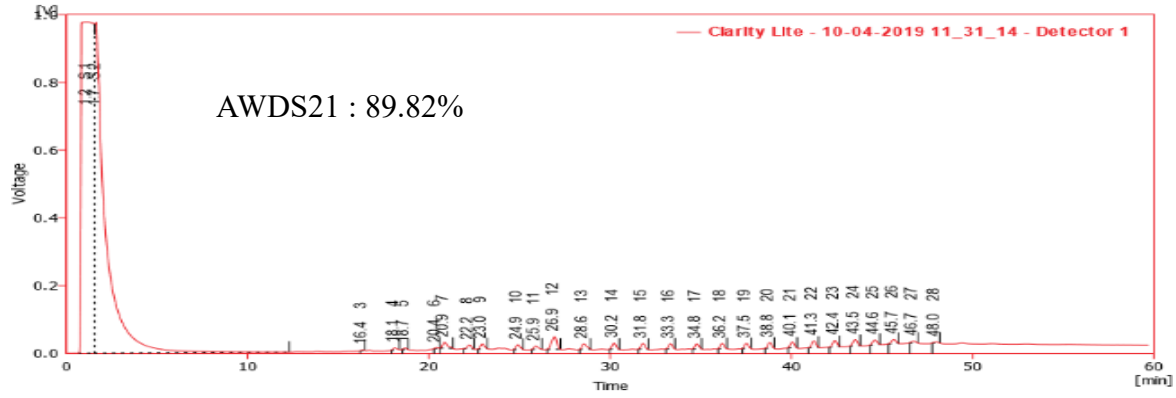
(d) Chromatogram of crude oil treated with AWIS04

As compared to control, total area of AWIS04 was 21308.49 and 66.80% TPH degradation was observed.



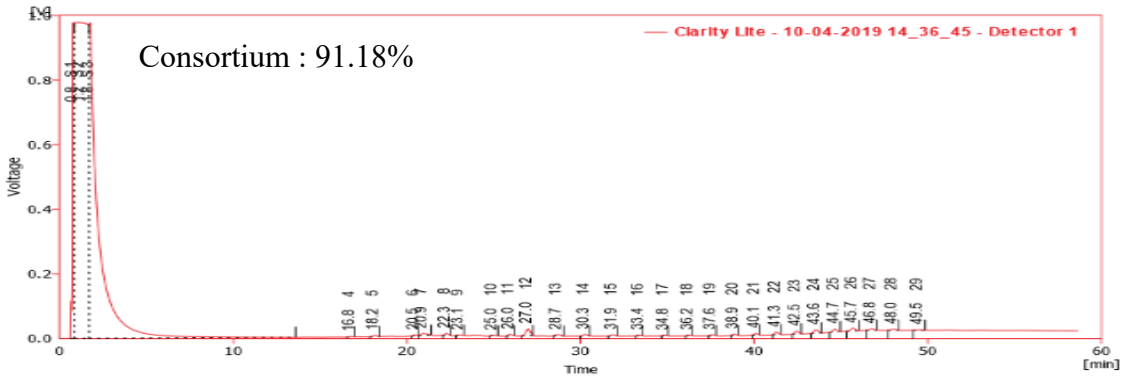
(e) Chromatogram of crude oil treated with AWCO15

As compared to control, total area of AWCO15 was 14527.01 and 84.49% TPH degradation was observed.



(f) Chromatogram of crude oil treated with AWDS21

As compared to control, total area of AWDS21 was 5743.219 and 89.82% TPH degradation was observed.



(g) Chromatogram of crude oil treated with Consortium

As compared to control, total area of consortium was 2256.729 and 91.18% TPH degradation was observed.

Figure 19: Chromatogram of crude oil gas chromatography analysis

Chromatogram obtained from Gas chromatography as represented in Figure 19 indicate control is having more amount of TPH and so, representing highest height in all peaks when compared with test. The decrease in the total petroleum hydrocarbon (TPH) content and its fractions with time represents biodegradation efficiency of isolates. The quantitative values of TPH degradation by bacterial isolates is summarized in Figure 20.

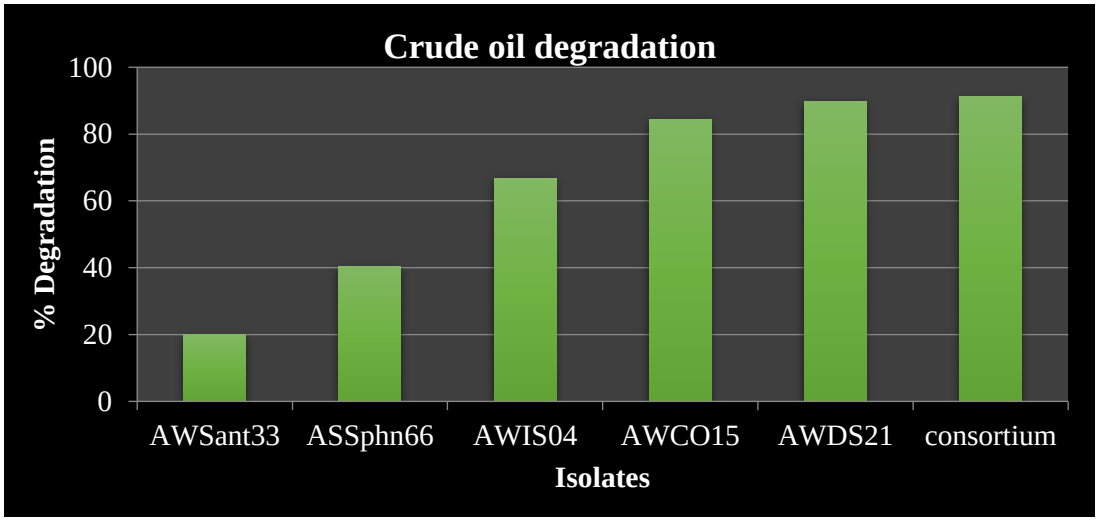


Figure 20: Percent degradation of crude oil by screened bacterial isolates

Isolates AWS ANT 33, ASS Phn66,AWIS04, AWC015, AWDS21 and Consortium of all isolates showed degradation of 20.09%, 40.42%, 66.80%, 84.49%,89.82% and 91.18% respectively. From the above graphs it can be concluded that bacterial isolate AWDS21 was having the best capability to degrade petroleum crude oil. Also, consortium having all the 5 isolates cumulatively showed good degradation efficiency.

5.8 Molecular identification of crude oil degrading strains

Genomic DNA

- Isolated genomic DNA was verified using agarose gel electrophoresis (Figure 21)

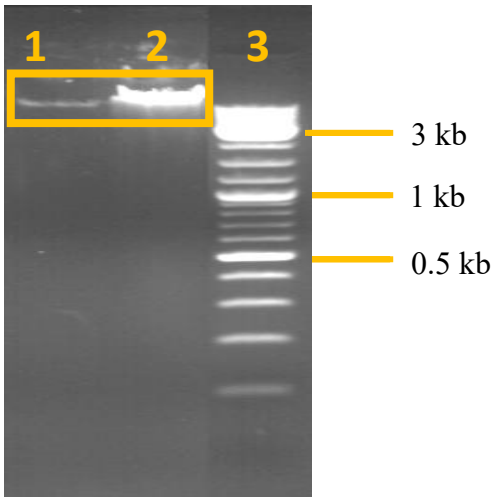


Figure 21: Genomic DNA bands obtained on Agarose gel electrophoresis
Lane : 1 AWCO15 , Lane : 2 ASS Phn66 , Lane : 3 Ladder (mix gene ruler)

16S rRNA gene amplification

16S rRNA was amplified using F27 and R1492 universal primers and the amplified product was verified using agarose gel electrophoresis (Figure 22).

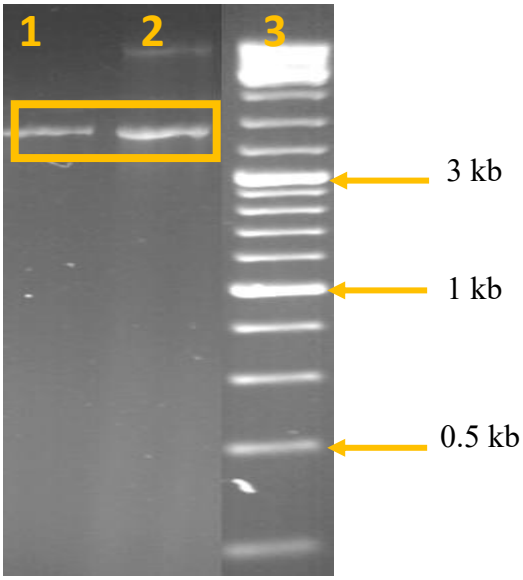


Figure 22:16S RNA bands obtained on agarose gel electrophoresis
Lane : 1 AWCO15 , Lane : 2 ASS Phn66 , Lane : 3 Ladder (mix gene ruler)

Outsourcing for sequencing of amplified product was done and the sequence obtained were further subjected to BLASTn tool at the database of National Center of Biotechnology Information.

From the data obtained after BLAST, the bacteria were identified, as depicted Table 13.

Table 13: Identification of bacterial isolates

Isolate	Identified organism	Query cover	% identity
AWCO15	*Acinetobacter lwoffii*	99%	98.09%
ASS Phn66	*Burkholderia multivorans*	100%	99.05%

Acinetobacter lwofii is a gram negative, non-fermentative bacterium. Crude oil degradation efficiency of this organism was observed to be 40.42%. *Burkholderia multivorans* is a gram negative, non-fermentative. Crude oil degradation efficiency of this organism was observed to be 84.84%.

Conclusion

6. CONCLUSION

- Screening of 40 bacterial isolates was done by investigating their sodium benzoate degradation efficiency, out of which 27 were fast growers.
- 5 isolates had more than 80% sodium benzoate degradation efficiency and hence, those isolates (AWCO15, ASS Phn66, AWS ant33, AWDS21 and AWIS04) were further investigated for their growth and degradation capacity on poly aromatic hydrocarbons and crude oil.
- A consortium of these 5 bacterial isolates were developed and it was observed that, the consortium showed the highest crude oil degradation efficiency i.e. 91.18 %
- Identification of two isolates (AWCO 15 and ASS Phn66) from the consortium was done by genomic DNA isolation and 16S rRNA gene amplification and identified as *Acinetobacter lwoffii and Burkholderia multivorans* respectively. Identification of 3 isolates are still pending and are under progress.

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Appendix

APPENDIX

List of media used in this study

Mineral Salt Medium composition **:**

Ingredients	Concentration
$(NH_4)_2SO_4$	18 mM
$MgSO_4.7H_2O$	1 mM
NaCl	8.5 mM
Na_2HPO_4	10 mM
K_2HPO_4	10 mM
$CaCl_2.2H_2O$	10 mM
$FeSO_4.7H_2O$	10 mM

Nutrient Broth(NB) :

Ingredients	g/l
Peptone	5.0
NaCl	5.0
Meat extract	1.5
Yeast extract	1.5

Nutrient agar:

Ingredients	g/l
Peptone	5.0
HM peptone B	1.5
NaCl	5.0
Yeast extract	1.5
Agar	15

Luria broth:

Ingredients	g/l
Tryptone	10
Yeast extract	5.0
NaCl	5.0

Stock solutions:

DCPIP dye	90 µM
Phenanthrene	0.028 M
Naphthalene	Crystals
Anthracene	10 mM

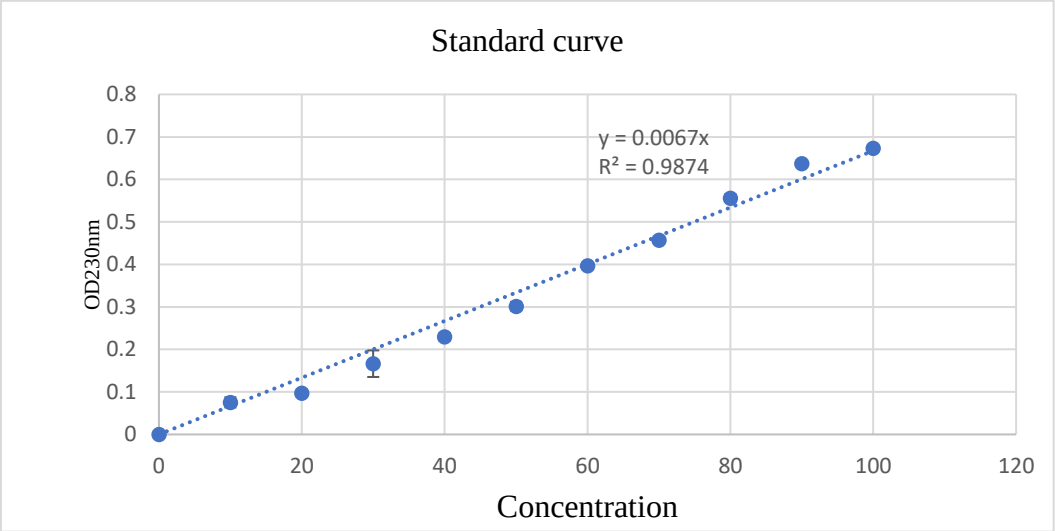
Sodium benzoate standard curve

- Sodium benzoate is a simple compound present as one of the petroleum hydrocarbons in crude oil, the ability of bacteria to grow and degrade a simple compound like this, would help us know the efficiency of bacteria to degrade other Petroleum hydrocarbons,
- Hence, Standard curve was performed as portrayed in table, in order to compare the growth and degradation efficiency of bacteria.

Table : Details of sodium benzoate concentration used for standard curve.

Sr no.	Concentration (µl)	Sodium benzoate (ml)	Distilled water (ml)
1	10	0.1	9.9
2	20	0.2	9.8
3	30	0.3	9.7
4	40	0.4	9.6
5	50	0.5	9.5
6	60	0.6	9.4
7	70	0.7	9.3
8	80	0.8	9.2
9	90	0.9	9.1
10	100	1.0	9.0

Standard curve of sodium benzoate as shown below :-



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