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# Efficiency of consortium for *in-situ* bioremediation and $CO_2$ evolution method of refines petroleum oil in microcosms study

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## ABSTRACT

An *in-situ* bioremediation study was conducted in a laboratory by using mixed microbial consortium. An indigenous microbial consortium was developed by assemble of two *Pseudomonas* spp. and two *Aspergillus* spp. which were isolated from various oil contaminated sites of India. The laboratory feasibility study was conducted in a 225 $m^2$ block. Six treatment options: Oil alone, Oil+Best remediater, Oil+Bacterial consortium, Oil+Fungal consortium, Oil+Mixed microbial consortium, Oil+Indigenous microflora. Out of five treatments, the mixed microbial consortium (Block 5) degraded 55.12% refine petroleum oil compare to degradation of bacterial (Block 3) and Fungal consortium (Block 4) (i.e, degradation rate were 19.88% and 18.07% correspondingly) after the end of treatment (60 days). Bioremediation ability of this consortium was confirmed by $CO_2$ evolution method. The result showed that 136.36 % $CO_2$ release after 12 days incubation. 16r DNA sequencing showed that two bacterial species were *Pseudomonas aeruginosa* and morph taxonomical examination of fungus were *Aspergillus terrus* (At) and *Aspergillus flavus* (Af).The ability of degradation of synthetic mixture of refine petroleum oils makes the consortium potentially useful for bioremediation and microbial enhanced oil recovery.

**Keywords:** *In-situ* bioremediation; $CO_2$ evolution method; Microbial consortium; *Pseudomonas aeruginosa*; *Aspergillus terrus*; *Aspergillus flavus*.

## 1. INTRODUCTION

Large amounts of hydrocarbon contaminants are released into environment as a result of human activities. While release like industrial emission can be controlled and carefully regulated, catastrophic release like major spillage from tankers, pipelines and storage tanks are largely accidental and unavoidable and occur frequently in present times [1]. Oil sludge is carcinogenic and a potent immune-toxicant. Due to industrialization and over use of petroleum hydrocarbon based refinery products are one of the most prevalent pollutants. Oil contamination is a severe threat for our environment and therefore invites general concern. Consequently, the remediation of oil-polluted sites has become an important issue worldwide [2] Bioremediation, the degradation or stabilization of contaminants by microorganisms, is claimed as a safe, effective and economic alternative method of environmental clean-up [3]. In biological treatments it is always necessary to perform laboratory

feasibility tests to determine the microbial potential to degrade the pollutants and to evaluate strategies to optimize the degradation rates before the design of real scale *in-situ* or *ex-situ* (bioreactors, land farming and others) treatments [4, 5]. Thus, the purpose of the present study was to investigate possible methods to enhance the rate of aerobic biodegradation (*ex-situ* treatments) of refines petroleum oil. In this work, the bioremediation processes were applied to a sandy loam soil of Haridwar region, India contaminated by Synthetic Mixture of Refine Petroleum Oil (SMRP Oil) and biodegradation was performed by bioaugmentation (treatment with inoculation of mixed microbial consortium) and study the efficiency of that consortium.

## 2. MATERIAL AND METHOD

### 2.1. Source of soil sample

Soil samples were obtained from depths of 0.5 and 1 m as well as form ground surface in a contaminated area close to the storage and Distribution Centre of Oily Products in Indian Oil Refinary, Haldia. (West Bengal) and local garages, refinery waste, petrol pumps, service stations of Kolkata, West Bengal as well as Haridwar city, Uttrakhjand.The petroleum contaminated soil samples were collected in duly labelled sterile container from the depth of 0.5 to 1.0 cm surface and subsurface. Then all samples were transported in ice to the laboratory and stored at $4^0$C for further analysis.

### 2.2. Isolation and screening of indigenous microorganisms

Soil sample were sieved moist using a 2 mm mesh screen and thoroughly mixed. 10 g of soil was added to 95 ml deionised water containing 2 drops of Tween 80 and then was incubated and shake (150 rpm) for 30 min at room temperature. The mixture prepared was called soil solution. A 100 ml Erlenmeyer flask (flask 1) was prepared containing 2.5 ml soil solution and 95 ml of MSM (Mineral Salt Medium) and 2.5 ml of synthetic mixture of refine petroleum hydrocarbons (SMRP) (Petrol, Diesel and Kerosene; 1:1:1) as a sole source of carbon. The flask was incubated at $37^0$C. After 15 days, 2.5 ml of Flask 1 was transferred to a second flask (flask 2) with same condition as flask 1. The incubating-transferring were repeated 4 times and at final stage (fourth period) pure hydrocarbon degrading strains were isolated on petroleum agarose plates. In the preparation of petroleum agarose plates 1-2 drops of sterile SMRP oil was evenly spread with glass spreader, so that a film of SMRP oil got absorb over the entire agarose surface of mineral medium in the petriplate and then inoculums was spread on the medium. The plate was incubated at $37^0$ C for one week in an incubator. Pure and representative colonies were transferred to slant for preservation.

### 2.3. Preservation and subculture of the strains

The isolated strains were preserved in 25% v/v glycerol solution at $-70^0$C. For day-to-day experimentation strains were maintained on nutrient agar slants at $4^0$ C in refrigerator and sub-cultured at an interval of 30 days.

### 2.4. Preparation of consortia inoculum

The bacterial isolates were grown separately in NB and processed to yield separate suspension with an absorbance reading of 0.5 at 550nm. Specific aliquots of bacterial inoculums were then separately added into normal saline solution to give final combined inoculums concentration of 10% (v/v) according to Mukred et al. [6] and used as bacterium consortium. The fungal isolates were cultivated in slant tubes at $30^0$C for 6-7 days. The conidia of each strain were suspended in sterile distilled water according to Lemos et al. [7], and produced fungal consortium. All combined bacterial inoculums and conidia-suspension of fungal isolate were thoroughly mixed to prepare final combined inoculums concentration was 10% v/v according to Malik et al. [8].

### 2.5. *In-situ* microcosms studies

#### *2.5.1. Bioremediation setup*

The total area (225 $m^2$) of the feasibility study was divided into 25 Blocks (four replicate Block

for each treatment and one block was remain undisturbed to check the physic-chemical properties of soil) of a tin vessel. 5 kg of soil (sieved with 2 mm mesh size) from Kanya Gurukula Campus was taken as a normal soil without oil and added 200 g soil in each block separately. In each block 2% SMRP oil was added, thoroughly mixed and left undisturbed for 24 hours to allow the volatilization of the oil. The initial soil pH, temperature, moisture level, organic carbon were also determined over a period of 60 days. The soil used for microcosm study had a pH of 7.78, which was well within the range of optimal degradation. Therefore, no treatment for pH control was needed. The experiment was conducted in the premises of Kanya Gurukul Campus, Gurukul kangri University, Haridwar. The experiment was conducted during March to May months, in the year 2014 and followed the method of Pritchard & Bourquin [9] and Mittal & Singh [10].

### *2.5.2. Experimental design*

The experimental design chosen was a completely randomized block design. The treatment were as follows: (i) oil alone; control where no treatment was done, (ii) Oil+Bacterial consortium, (iv) Oil+Fungal consortium, (v) Oil+Developed consortium, (vi) Oil+Indigenous micro flora.

### *2.5.3. Extraction of SMRP oil sample*

For extraction of SMRP oil broth culture was first taken out and the culture activities were stopped by adding 1% 1N HCl and then the extracted SMRP oil broth was mixed with 50 mL petroleum ether: acetone (1:1) in a separating funnel and was shaken vigorously to get a single emulsified layer. Acetone was then added and shaken gently to break the emulsification, which resulted in three layers. Top layer was a mixture of petroleum ether, SMRP oil and acetone; clumping cells make the middle layer and the bottom aqueous layer contains acetone, water in soluble form. The lower two layers were separated out while top layer containing petroleum ether mixed with SMRP oil and acetone was taken out in a clean bottle.

### *2.5.4. To assess the hydrocarbon degradation*

To assess the rate at which the SMRP oil was being degraded, sample were collected at time zero (just before initiating the bioremediation), 15 days later, 30 days later, 45 days later and at the end of the study (60 days after initiating the process). After evaporation the residual oil content was determined by the absorbance of the extract at 420 nm in a spectrophotometer.

### *2.5.5. Fraction of SMRP oil and analysis of fractions*

For isolation of various compound class fractions (saturate, aromatic and NSOs), column chromatographic technique was used. The separation was carried out on packed activated silica-gel columns by successive elution with petroleum ether for saturates, benzene for aromatics and methanol for NSO compounds. Silica gel (60-120 mesh) was activated at $150^0$C for 24 hours and cooled in desiccators. The glass column (Internal diameter 1.1 cm, length 65 cm and reservoir capacity 100 ml) was packed by placing a thin cotton plug at the bottom. The slurry of 20 g activated silica gel was filled. The column was washed with petroleum ether. The extracted oil sample was dissolved in chloroform, absorbed on silica gel. The adsorbed sample was charged at the top and eluted saturates with 10 ml of petroleum ether ($40^0$-$60^0$C), aromatics with 10 ml benzene and NSO with 10 mL methanol, respectively. Each fraction was transferred in air tight bottle and taking O.D at 420 nm in a spectrophotometer [11].

## 2.6. Laboratory scale experiment on the bioremediation of refined petroleum hydrocarbon by using $CO_2$ evolution method (Standardized Biodegradability Tests-ASTM D-5864)

A specially equipped 150 ml Erlenmeyer flask contains 50 ml of optimized BH broth and 5 ml of SMRP oil. A reservoir holding 10 ml of barium hydroxide solution was suspended out of the flask to trap $CO_2$. After inoculation(10% v/v), the test flasks were sparged with $CO_2$ free air (flasks were aerated with compressed air that had been scrubbed free of $CO_2$ by passage through a series of

three 250 ml bottles each containing 200 ml of 5N NaOH) to ensure aerobic conditions and that $CO_2$ was trapped only from microorganism's metabolizing the test substrate. The flasks were sealed and incubated with shaking in a dark room for 0, 4, 8, 12, 16, 20, 24, 28 days under normal temperature (32 to $37^0$C), pH (7) condition because as per ASTM method [12], the test shall continue for at least 28 days or until the $CO_2$ evolution has reached a plateau. Non inoculated flasks were included as control for abiotic losses [13].

### 2.7. Measurement of $CO_2$ evolution

Periodically, the 10 ml of $Ba(OH)_2$ a plus 10 ml rinsing water (DW) was removed for $CO_2$ measurement by titration with 0.1 N HCl to the phenolphthalein end point. All the samples were analyzed at time zero and at least a 28 day time period to allow for a smooth biodegradation plot for test system (ASTM D-5864). 3 ml of 20% $H_2SO_4$, were added on the day prior to terminating periodically.

The percentage $CO_2$ evolution-was based on the following formula:

$$\% \, CO_2 \text{ evolution} = \frac{TF\text{-}CF}{C} \times 100\%$$

Where,

TF = ml of 0.1 N HCI required to titrate Ba $(OH)_2$, samples from the test flask;

CF = ml of 0.1 N HCl required to titrate Ba $(OH)_2$ samples from the control flask;

C = A constant which is equal to the theoretical amount of 0.1N HCl required to titrate the $CO_2$ evolved from metabolizing total carbons in the test substrate by bacteria. For example, for 10 mg carbon: C = 16.67 ml of 0.1N HCl.

### 2.8. Molecular characterization of best petroleum remediating microorganisms

Bacterial strains of the consortium were identified by 16S rDNA sequence structure performed by Royal Life Sciences Pvt. Ltd. (Affiliated to MIDI Sherlock, USA) and fungal cultures were identified as morph taxonomically from Agharkar Research Institute, Pune, India (National Fungal Culture Collection of India).

## 3. RESULT AND DISCUSSION

The first and foremost criterion for designing a bioremediation program was to study the native micro flora of the system and to analyses the physico-chemical composition of soil. The soil sample taken for feasibility study (garden soil of Kanya Gurukula Mahavidyalaya, Haridwar) was analysed for detection of different treatment boxes. The soil contains $6x10^8$/ g of total heterotrophs and $1.2x10^3$/ g of fungi. The pure hydrocarbon utilizing bacteria as well as fungus were identified in presence of SMRP Oil as a carbon source. Two bacterial and fungal strains were identified by using standard procedures. The experimental outcome of cultural, morphological and biochemical characterization proved that both bacterial species were *Pseudomonas aeruginosa* (Ps-I and Ps-II) (Tables 1 and 2, Fig. 1) and two fungal species were identified as *Aspergillus flavus* (Af) and *Aspergillus terrus* (At) (Table 3, Fig. 2). All were used to make the consortium. *In situ* bioremediation approach was adopted in laboratory conditions. Physical and chemical properties of soil sample taken were analysed for pH, temperature, and moisture level, organic carbon (Table 4) in remaining block at zero time intervals i.e, initially and after 60 days of optimal value of pH was 7 and temperature $32^0$C to $35^0$C for maximum degradation.The soil used was sandy loam in texture and its pH was 7.78. Moisture content of soil was 5 %, while water holding capacity was 50. The temperature recorded during the study varied from $32^0$C to $35^0$C.

Since maintenance of temperature in open soil system was not feasible, the bioremediation efforts should be concentrated during such a period of year when the temperature was suitable for treatment. Verstraete et al. [14] reported that a doubling rate of biodegradation of gasoline in an acidic (pH 4.5) soil by adjusting the pH to 7.4. Extremes in pH were shown to have negative influence as well as at low temperature the viscosity of oil was increased, volatilization of alkanes reduced, so the degradation was affected. Rhaman et al. [15] reported $30^0$C to $40^0$C was the normal temperature for petroleum hydrocarbon degradation.

**Table 1.** Cultural and morphological characteristics of selected bacterial isolates.

| Code no of isolate | Size | Shape | Elevation | Margin | Opacity | Texture | Pigment | Gram's reaction | Motility |
|---|---|---|---|---|---|---|---|---|---|
| Ps-I | Big | Irregular | Slightly raised | Irregular | Opaque | Smooth | Deep Green | -ve rod | motile |
| Ps-II | Big | Irregular | Slightly raised | Irregular | Opaque | Smooth | Brownish green | -ve rod | motile |

**Table 2.** Biochemical characterisation of bacteria.

| Name of biochemical test | Code number of isolates | |
|---|---|---|
| | Ps-I | Ps-II |
| Citrate utilization | + | + |
| Urease production | + | + |
| Nitrate reduction | + | + |
| Oxidase | + | + |
| Catalase production | + | + |
| Gelatin Utilization | + | + |
| Starch Hydrolysis | - | - |
| Indole production | - | - |
| M.R test | + | + |
| V.P test | - | - |
| Lipid hydrolysis | + | + |
| Glucose utilization | + | + |
| Sucrose utilization | _ | _ |
| Mannitol utilization | + | + |
| Lactose utilization | + | + |
| Maltose utilization | + | + |

**Table 3**. Cultural and size and shape of spore of fungal isolates.

| Code no of isolate | Colour of the colony | Appearance of the colony |
|---|---|---|
| At | Brown | Brownish in colour and gets darker as it ages on culture media |
| Af | Green | Conidial heads were typically radiate, later splitting to form loose columns, biseriate but hading some heads with phialides borne directly on the vesicle. |

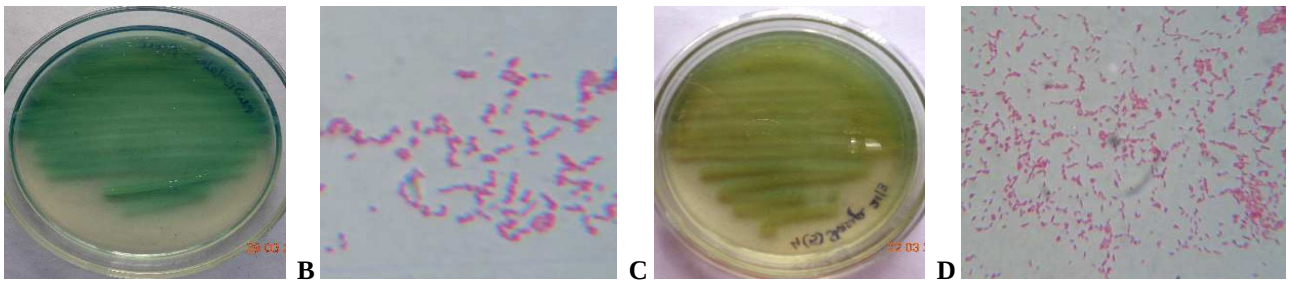

**Figure 1.** Showing colony colour on plates and microscopic view of Ps-I (A and B) and Ps-II (C and D).

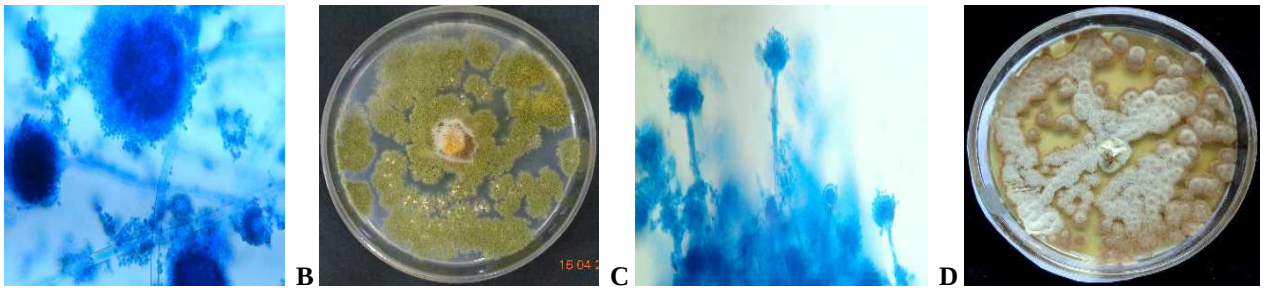

**Figure 2.** Macroscopic and microscopic appearance of At (A and B) and Af (C and D).

**Table 4.** Physico-chemical parameters of soil of *in situ* bioremediation process.

| Days | Time zero* | After 60 days* |
|---|---|---|
| **Temperature** | $32^0$C to $35^0$C | $32^0$C to $35^0$C |
| **pH** | 7.7 | 7.30 |
| **Moisture contain** | 5% | 5.6 |
| **Organic carbon** | 2.35 | 2.43 |

*= Average of triplicates

SMRP Oil was extracted from six treatment boxes- Oil alone, Oil and best remediating microorganisms, Oil+ bacterial consortia, Oil+fungal consortia, Oil+microbial consortium, Oil+indegenious micro-flora and assessment the degradation rate.

### 3.1. Block 1: Oil alone

The hydrocarbon utilizing bacteria count during the study was also found nil, though some heterotrophic bacterial activity in an open environment was not possible. It was because the soil was sterilized and 2% $HgCl_2$ treatment also arrested the soil microbial activity. The minimum loss of SMRP Oil (6.02%) can be attributed to the abiotic losses like evaporation of low volatile fraction of SMRP Oil and photo-oxidation etc. (Table 5).

### 3.2. Block 2: Oil+ best remediater

The Box 2 which shows the bioremediation of best remediater (Ps-I). The absorbance of SMRP Oil decreases from 0.332±0.002 to 0.292±0.004 O.D value which means 12.05% extracted SMRP Oil was degraded within 60 days. Initially within 15 days rapid degradation was occurred (i.e 6.93%), then degradation rate was decreased (Table 5).

### 3.3. Block 3: Oil+Bacterial consortium

The Box 3 containing bacterial species of Ps-I +Ps-II. Both *Pseudomonas* spp. degraded extracted SMRP oil 19.88% within 60 days which means that presence of Ps-II enhanced the degradation rate. After 30 days degradation rate was moderately increased (Table 5).

### 3.4. Block 4: Oil+ Fungal consortium

At and Af were formed fungal consortium; they degrade extracted SMRP Oil near about same as bacteria consortium. They degraded extracted SMRP Oil 18.07% within 60 days. But in case of fungal consortium till 45 days degradation rate was increased equal proportion but after 45 days (12.65) degradation rate was increased rapidly (18.07) (Table 5).

### 3.5. Block 5: Oil+ Mixed consortium

Both fungus and bacteria degraded extracted SMRP Oil 55.12% within 60 days which means mixed microbial consortium degrade more, than individual consortium. (Table 5) In case of mixed microbial consortium rapid degradation occurred within very short period of time (i.e 15 days) (43.07%) but after then the degradation rate moderately increased.

### 3.6. Block 6: Oil+ Indigenous microflora

Box 6 shows degradation of indigenous microflora from 2.17 to 6.63 % within 60 days which means that all soil contain hydrocarbon degrading microorganisms but a mixed microbial consortium enhanced the degradation rate that why it was essential to make a consortium which was

needed for effective bioremediation (Table 5).

### 3.7. Fraction of SMRP Oil and analysis of fractions

Effect of biodegradation on alkane, aromatics and NSO = asphaltene fractions by best remediator, bacterial consortium and fungal consortium and mixed microbial consortium had been studied for 60 days. Effect was seen at time interval of 15 days (Table 6). These fractions were separated by column chromatography. At the end of 60 days it was observed that mixed microbial consortium metabolized 40.42% alkanes, 36.15% aromatics fraction whereas Ps-I degraded 9.04% alkane and 4.22% aromatic hydrocarbon, Bacterial consortium degraded 12.23% alkane and 8.45% aromatic hydrocarbon and fungal consortium degraded 7.98% alkane and 7.04 % aromatic fraction. From above result it was cleared that fungal consortium degraded near about equal proportion of alkane and aromatic fraction as bacterial consortium.

Assessment of $CO_2$ production by consortium confirmed that the utilization of refine petroleum hydrocarbon fraction as a source of carbon and energy by the microbial community. It was a Standardized biodegradability test (ASTM 5864) which also previously used by various scientists to calculate biodegradation efficiency [5, 13, 16]. Carbon di oxide production in SMRP oil of the control ranged from 0% to 30.30% while % of $CO_2$ evolves in SMRP oil ranged from 0.05% to 136.36% shown in Table 7. There was a progressive increase in the amount of $CO_2$ produced for the first 12 days, after which $CO_2$ production decreased. Large amounts of $CO_2$ were liberated in SMRP oil than in control oil. The progressive increased in the amount of $CO_2$ evolved in the incubated oil in the first 12 days was an indication of the utilization of petroleum hydrocarbon fractions as a source of carbon and energy by the microbial community. Respiration of microbes occurred very rapidly during the initial period of incubation when the lighter and more readily degraded fractions were degraded but slowed down as the residue become more difficult to degrade on account of the increase of the heavier fractions.

**Table 5.** Percent (%) of degradation of SMRP Oil through microcosms study.

| Treatment | SMRP Oil (Synthetic Mixture of Refine Petroleum Oil) | | | | | | | | | | P-value Prob> F$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Initial O.D at 420 n.m | | Final O.D at 420 n.m | | | | | | | | |
| | 0 | | 15* | | 30* | | 45* | | 60* | | |
| | O.D | % of D | O.D | % of D | O.D | % of D | O.D | % of D | O.D | % of D | |
| **Control** | 0.332 ±0.002 | 0 | 0.325 ±0.007 | 2.10 | 0.322 ±0.012 | 3.61 | 0.314 ±0.018 | 5.42 | 0.312 ±0.02 | 6.02 | 0.0029 |
| **Ps-I (Best remediator)** | 0.332 ±0.002 | 0 | 0.309 ±0.002 | 6.93 | 0.302 ±0.003 | 9.04 | 0.299 ±0.002 | 9.94 | 0.292 ±0.004 | 12.05 | 0.0001 |
| **Ps-I+Ps-II (Bacterial Consortium)** | 0.332 ±0.002 | 0 | 0.298 ±0.003 | 10.24 | 0.292 ±0.002 | 12.05 | 0.276 ±0.052 | 16.87 | 0.266 ±0.002 | 19.88 | 0.0005 |
| **At+Af (Fungal consortium)** | 0.332 ±0.002 | 0 | 0.302 ±0.007 | 9.04 | 0.297 ±0.001 | 10.54 | 0.290 ±0.002 | 12.65 | 0.272 ±0.006 | 18.07 | 0.0007 |
| **Ps-I+Ps-II+At+Af (Mixed Microbial Consortium)** | 0.332 ±0.002 | 0 | 0.189 | 43.07 | 0.172 ±0.008 | 48.19 | 0.166 ±0.107 | 50 | 0.149 ±0.002 | 55.12 | 1.0952e-06 |
| **Ind Microbs.** | 0.332 ±0.002 | 0 | 0.323 ±0.004 | 2.71 | 0.260 ±0.005 | 4.22 | 0.25 ±0.10 | 6.02 | 0.242 ±0.002 | 6.63 | 0.0140 |

***=** Average of triplicate, **$=** significant only when the calculated F value was greater than the table F value at P is less than or equal to 0.05.

**Table 6.** Effect of microbial degradation on various fraction of SMRP Oil (Synthetic Mixture of Refine Petroleum Oil).*=Average of triplicates.

| Treatment | Incubation Period (Days) | SMRP Oil (Synthetic Mixture of Refine Petroleum Oil) | | | | |
|---|---|---|---|---|---|---|
| | | Alkanes | | Aromatic | | NSO+Asphalt |
| | | R* | D | R* | D | R* |
| ***Pseudomonas* sp.-I** | **0** | 0.188 | 0 | 0.213 | 0 | 0.112 |
| | **15** | 0.180 | 4.25 | 0.209 | 1.88 | 0.109 |
| | **30** | 0.176 | 6.38 | 0.208 | 2.35 | 0.105 |
| | **45** | 0.173 | 7.98 | 0.206 | 3.29 | 0.102 |
| | **60** | 0.171 | 9.04 | 0.204 | 4.22 | 0.101 |
| ***Pseudomonas* sp.-I+*Pseudomonas* sp.-II** | **15** | 0.178 | 5.32 | 0.204 | 4.23 | 0.110 |
| | **30** | 0.172 | 8.51 | 0.200 | 6.10 | 0.111 |
| | **45** | 0.171 | 9.04 | 0.198 | 7.04 | 0.109 |
| | **60** | 0.165 | 12.23 | 0.195 | 8.45 | 0.109 |
| ***Aspergillus terrus* +*Aspergillus flavus*** | **15** | 0.180 | 4.25 | 0.208 | 2.35 | 0.111 |
| | **30** | 0.178 | 5.32 | 0.204 | 4.23 | 0.110 |
| | **45** | 0.174 | 7.45 | 0.200 | 6.10 | 0.110 |
| | **60** | 0.173 | 7.98 | 0.198 | 7.04 | 0.101 |
| **Consortium (Bacteria+ fungus)** | **15** | 0.146 | 22.34 | 0.170 | 20.19 | 0.105 |
| | **30** | 0.133 | 29.25 | 0.159 | 25.35 | 0.098 |
| | **45** | 0.122 | 35.10 | 0.151 | 29.11 | 0.088 |
| | **60** | 0.112 | 40.42 | 0.136 | 36.15 | 0.085 |

*=Average of triplicate

**Table 7.** Percent (%) of $CO_2$ evolves from SMRP (Synthetic Mixture of Refine Petroleum Oil) oil by fungus-bacterium consortium. *=Average of triplicate.

| Treatment period (days) | % of $CO_2$ evolves in Control* | % of $CO_2$ evolves in Consortium* | P value Prob> F[@] |
|---|---|---|---|
| 0 | 0 | 0.05 | 0.0160 |
| 4 | 4.32 | 47.62 | |
| 8 | 6.49 | 106.06 | |
| 12 | 12.98 | 136.36 | |
| 16 | 15.15 | 134.19 | |
| 20 | 30.30 | 32.46 | |
| 24 | 6.49 | 28.13 | |
| 28 | 2.16 | 19.48 | |

The use of autochthonous microorganisms inhabiting hydrocarbon polluted niches for bio-degradation and bioremediation has been widely accepted as a formidable approach due to avalanche of successes recorded by various researchers. The mechanisms of adaptation employed by the autochthonous microorganisms to achieve this feat includes synthesis of inducible enzyme, mutations such as single nucleotide change or DNA-rearrangement that results in degradation of the

compound and acquisition of genetic information from closely related or phylogenetically distinct population within the hydrocarbon-challenged community through horizontal gene transfer [17]. Here in this study the mix microbial consortium degrade 40.42% saturated and 36.15% aromatic hydrocarbon present in the refine petroleum oil. Refine petroleum oil contains many kinds of hydrocarbon, resins and asphaltenes. Muthuswamy et al. [18] reported that mixed population had a broad enzymatic capacities which enhanced petroleum degradation. This mixed culture had a metabolic versatility over to pure culture. Due to presence of bacterial and fungal species which synthesize the degradative enzymes for different parts of the decomposition pathway is considered to be well suited to the refine petroleum degradation. Vasudevan [19] and Rahman et al. [15] had illustrated the ability of mixed microbial consortia to degrade 28 to 51% saturated and 0 to 18% of aromatic present in crude oil or upto 78% crude oil. Microorganisms not directly involved in the degradation process also probably play a role by producing micronutrients or surface-active agents for the solubilization of aromatic hydrocarbons [20]. Various organisms had the capability of degrading various forms of hydrocarbons and thus when a consortium of these microbes was applied to degrade various forms of hydrocarbons in a single source like refine oil; the total degradation was more effective This result made it obvious that the metabolic capability of the consortium was not restricted to one type of refine oil. But such type of result could not expect from pure cultures which were substrate specific. Hasanuzzaman et al. [21] observed 75 and 85% degradation of total crude oil by *Pseudomonas aeruginosa* strain at 20 and $30^0$C, respectively. Since in this study all isolates were mesophilic in nature, they all exhibited optimum activity at $32^0$C-$35^0$C. Increase in crude oil concentration decreased the percent degradation but an increase in the quantity of crude oil degradation was noticed. Zhang et al. [22] reported 58 and 60% degradation of crude oil with the initial concentration of 0.7 g/l in mineral salt medium by *P. aeruginosa* in the presence of 1 g/l glycerol and 0.22 g/l rhamnolipids, respectively, used as emulsifiers. Tzarkova and Groudeva [23] reported that compounds such as saturates, aromatics, and polar compounds present in different crude oil samples were degraded to different degrees by the same organisms. The degradability was not solely determined by the chemical structure but other factors as well. The bioavailability of these compounds in different crude oil samples might differ. Saturated compounds with molecular weight larger than 500 might not be degraded by the organisms, because this size corresponds to the exclusion size for passage through the outer membrane of Gram-negative bacteria [24]. Generally, it was believed that microbes preferably degrade/metabolize $C_8$–$C_{15}$ n-alkanes followed by $C_{16}$–$C_{36}$ n-alkanes due to the simplicity of these hydrocarbons. Saturated, cyclic high-molecular weight compounds like hopanes are usually not attacked by the microbes due to their complexity. Although it was not possible to specifically emphasize the metabolic pathway of degradation by individual microbes and microbial consortium without complete characterization of the refine oil before and after degradation, from the percent degradation of the total refine oil content it was conclude that the bacterial consortium and fungal consortium and mixed microbial consortium had the capability of degrading a wide range of hydrocarbons. Due to the highly complex nature of the refine oil, it was very difficult to understand the degradation mechanism especially for aromatics. The effectiveness of bio-augmentation (i.e mixed microbial consortium such as bacterial and fungal) was observed in this study. Before there were very little research [25] was conducted with the consortium that was made with bacteria and fungus. After performing 16sr DNA sequencing, sequence aligned with NCBI database gave 98% similarity of Ps-I and 92.6% similarity with Ps-II with Pseudomonas *aeruginosa* and morphologically Af & At identified as *Aspergillus flavus* and *Aspergillus terrus*, correspondingly. The result presented here will be particularly useful in choosing strains for environmental application involving the implantation of microorganism in the soil matrix (bioaugmentation). As contaminated sites usually contain heterogeneous hydrocarbon, it is promising to use for bioaugmented clean-up strains with broad abilities to grow on different hydrocarbons. For this purpose a model consortium including isolates *Pseudomonas aeruginosa* I and II

and *Aspergillus terrus* and *Aspergillus flavus* were proposed for refine petroleum hydrocarbon waste treatment of soil environment. Hence it was suggested that the use of above mixed microbial consortium would be an effective and eco-friendly technology for degradation of refine hydrocarbons.

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## AUTHORS' CONTRIBUTION

All the authors contributed equally for the success of this research. The final manuscript has been read and approved by all the authors.

## TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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