**Int. Journal of Universal Science and Technology**
ISSN: 2454-7263, Volume: 05 No: 02 Published: Jan., 2019
Paper ID: IJUP3119, Web: www.universalprint.org
Title Key: Isolation, Identification and Diversity…

# Isolation, Identification and diversity studies of Microbial Flora near Petrol Stations

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## Abstract

Microbes resistant to petroleum products are isolated from two different sources. The resistant microbial flora are isolated by collecting soil sample nearby to petrol stations and we found that the flora of sample 1 having two 15 microbes while flora of sample 2 having 9 microbes. These microbes isolated and maintained in the laboratory conditions and tried to identify with the manual process such as microscopic observation, differential staining and biochemical identifications. In the microscopic studies we found that these floras are diversified in their size and shape in the forms of cocci, streptococci, staphylococci and rod shaped. These size and shape variations observed in both the sample. In the soil sample which were taken under consideration found that contains from soil sample 2, it was found that, SN-1 was *Salmonella typhi*, SN-2 was *Klebsiella pneumonie*, SN-5 was *Klebsiella pneumonie*, SN-6 was *Shigellis boyaii*, SN-7 and SN-9 were *Salmonella typhi*. From sample 1, it was found that NS-1 was *Klebsiella pneumoniee*, NS-3 and NS-15 were *Shigellis boyaii*, and NS-4 was *Enterobacter cloacae.* Diversity indices for both the samples were measured and found that Sample 1 having; Berger-Parker index 62.5%, Shannon Entropy 1.0408, True Diversity 2.8, Simpson dominance 44.5% and true diversity (order 2) 2.2, while for sample 2 having Berger-Parker index 33.3, Shannon Entropy 1.3108, True diversity 3.7, Simpson dominance 28.4% and true diversity (order 2) 3.5.

**Keywords: Bacteria, Petrol stations, biochemical test, diversity**

## Introduction

Modern industrializations come with increasing soil, water and air pollutions. Several attempts had been made and techniques of bioremediation developed to mitigate and clean up hazardous chemicals (Romantschuk *et. al*., 2000). It includes; Biostimulation by the addition of nutrients, Bioaugmentations by the addition of microbes, aeration by oxidation and compatible combinations of all of these techniques (Alexander, 1994). Community composition of soil greatly affected by heavy pollution and also altered by environmental microbial contamination (Lynch *et. al*., 2004). Major bacterial species are resistant to petroleum products and able to degrade hydrocarbons as they are also naturally synthesize by plants and microbes (Sylvia *et. al.*, 2005). Several microbial species are reported for the degradations of oil hydrocarbons which includes *Psuedomanas* in cold climates (Zhang *et. al*., 2010), *Rhodococcus*, *Sphingomonas* (Aislabie *et. al*., 2006), etc. Temperature variations enhance biodegradations of hydrocarbons and availability of contaminants increases by solubility and mass transfer (Atlas and Bartha, 1997; Pignatello and Xing, 1996). Oil spills are most accidentally happens and cause serious problems environments as contaminants accumulate. Spills occur during manufacturing, transportations and storage by pipeline,

tanker or storage tank accidents. Often contamination and exposure of hydrocarbons to microbial community creates adaptive changes (Saul *et. al*., 2005; Grant *et. al*., 2007), which leads to competitions of bacterial community within and between species results in degradations of contaminants. Contaminations of hydrocarbons and oil spills are worldwide problems and best way to cope-up development of sophisticated techniques of decontaminations. Soil pore size is the important factor responsible for hydrocarbon degradation by allowing bacterial species to colonize on soil surface with least exposure to contaminants (Oluremi et. al., 2015). Soil with smaller pore size (less than 2 μm) bears more microbes for degradation in comparison with larger pore size. Fungi are the important potential scavengers of soil pollutions because of their special growth pattern of spreading mode and symbiosis with plants which converts polymer type of contaminants into simpler forms and extract energy (Sarand *et. al*., 2000; Hosokawa *et. al*., 2009). However, bacteria are supposed to be better scanvenger in comparison with fungi as they are potential colonizers of contaminated soil than fungi (Aislabie *et. al*., 2000).
Resistant microbes colonized on hydrocarbon contaminated soil responsible for hydrocarbon degradation are taken into considerations for this study and their diversity indices were analyzed by cultivating and non-cultivating methods (Vogel. 1996).

**Materials and Methods**

Soil sample were collected from nearby petrol stations, and bacterial flora present in it were isolated. The collected soil sample around 2.5 gm directly dissolved in the 47.5 ml of distill water. From these 10 ml soil sample treated as the master tube and further it is to be serially diluted from $10^{-1}$ to $10^{-10}$ by using sterile saline water.

**Purification of microbes**

Isolated microbes were purified with the serial dilutions and continuous pouring, streaking and spreading methods.

**Maintenance of Pure culture/Slant**

The purified microbial isolates are streaked on nutrient agar slant and allow it grow overnight in laboratory condition and stored in a freezing conditions for further use and applications.

**Study of colony morphology**

The purified isolates of bacteria directly streaked on the in the agar plates, so that we can have isolated colony. Colony morphology was studied after getting isolated colony in the agar plates. The characteristics features of each and every colony were noted with Shape, Size, Elevations, Surface, Edges, Color, Structure, and Degree of growth and Nature. Colony morphology with this parameters helps to identify bacteria and biochemical identifications makes easier.

**Differential staining of Microbes**

Simple technique used identifies microbes by using dyes or stain was basic techniques. To study colony morphology and different parameters of it, stain is very helpful. Basic dyes used to study morphology of microbes were methylene blue or basic fuschin. By using Gram staining with standard protocol, bacterial colonies were categorized into Gm +ve and Gm –ve.

**Biochemical reactions:**

To identify purified unknown bacteria with rough identification by colony morphology, analyzed through several biochemical reactions. Amongs all first biochemical reactions studies were **IMViC test** which includes Indole test, Methyl red test, Voges Proskauer test and Citrate test; collectively known as IMViC series of reactions. **The triple sugar- iron agar test (TSI)** designed to distinguish genera of Enterobacteriaceae, having all Gram negative bacilli fermenting glucose with acid productions. **Urease test** is useful for the identification of bacteria producing nitrogen containing compounds such as amino acid (arginine). By aiming at sulphur production, indole reduction and motility, bacteria were cultured in a **SIM medium** (Sulphide Indole Motility Medium). **Getatin hydrolysis test** were conducted for the bacteria that are able to degrade animal protein i.e. Gelatin derived from collagen. **Nitrate reduction tests** were peroformed to identify bacteria, able to reduce nitrate to nitrite or nitrogenous gases in provided growth medium. **Catalase test** were performed to identify catalase producing bacteria. Bacteria unable to produce such enzymes, becomes victim to oxygen poisoning. **Coagulase test** helps to find bacteria capable of clotting blood plasma. **Oxidase test** performed for rapid indentification of bacteria able to produce indophenol blue from the oxidation of dimethyl-p-phenylenediamine and α-naphthol. **Starch hydrolysis test** performed to identify bacteria capable of digesting glycosidic linkage found in starches. **Lipid hydrolysis test** performed for identification of lipolytic microbes, commonly observed in food contaminations.

**Growth on selective and differential media:**

Growth of bacteria is selective for different types of media. Colony morphology on Selective media is peculiar and restricts the growth of particular microbes only. Hence, several selective media were used such as Mannitol salt agar, Hektoen enteric agar (HE), Phenylethyl alcohol agar. Closely related bacteria can be distinguished on selective media such as MacConkey (MCK) agar, Eosin Methylene Blue (EMB) agar. To identify fastidious microbes, enrichment media such blood agar, Chocolated agar, Mannitol salt agar were used.

**Diversity study**

Collected samples were purified and identified by biochemical characterization, further these sample were analyzed for diversity indices. Standard index for species richness, evenness and dominance were calculated manually.

**Results and Discussion**

**Sample collection and Maintenance of Pure culture**

Soil sample are collected from nearby petrol pump stations (See Fig. 1). These samples were further serially diluted and microbial flora from these two petrol stations was isolated. Viable cells from contaminate soil samples can only forms colony by the dilution plate count method. Viable cells forms colony on agar plates were isolated and purified and maintained on slant for further studies (Fig. 2 & Fig. 3).

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Figure 1: Source of Soil collection for the isolation of petroleum resistant microbial flora A: sample 1 (Parandekar Petrol Pump, Nanded Road, Latur) and B: Sample 2 (KGN Petrol pump, Babhalgaon Road, Latur).

Figure 2: Isolated microbial flora from sample 1, maintained in a slant

Figure 3: Isolated microbial flora from sample 2, maintained in a slant

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**Study of colony morphology**

Isolated and purified microbes are observed under microscopes with differential staining (Fig. 4). It was found that collected sample both 1 and 2 contains majority of cocci and staphylocci type of bacteria and rod shaped bacteria rarely observed (Table 3-6). Gram staining and colony morphology gives broad category of group of bacteria present in given soil sample (Table 1 & Table 2). For further confirmation of identity of bacteria several biochemical test and differential media were used.

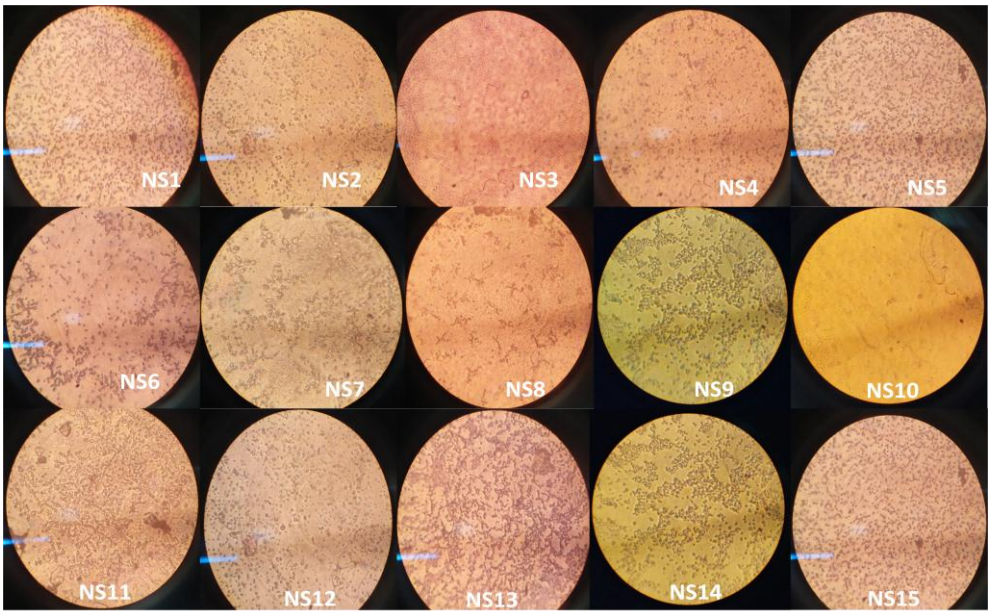


Figure 4: Microscopic observation of colony morpholology of sample 1

Table 1: Colony morphology, size and shape of bacterial isolates from sample 1

| Sr. No. | Bacteria | Color | Margin | Shape | Surface | Size | Tope of Colony |
|---|---|---|---|---|---|---|---|
| 1. | NS-1 | White | Irregular | Circular | Rough | Small | Planar |
| 2. | NS-2 | White | Irregular | Circular | Rough | Small | Planar |
| 3. | NS-3 | White | Irregular | Circular | Smooth | Small | Planar |
| 4. | NS-4 | White | Irregular | Oval | Rough | Small | Convex |
| 5. | NS-5 | white | regular | Circular | Rough | Small | Convex |
| 6. | NS-6 | Creamy | Regular | Circular | Smooth | Small | Cone |
| 7. | NS-7 | White | Regular | Circular | Smooth | Very Small | Flat |
| 8. | NS-8 | Creamish white | irrgular | Circular | Rough | Small | Flat |
| 9. | NS-9 | Creamy | Entire | Circular | Sticky | Small | Flat |
| 10. | NS-10 | Creamish white | Regular | Circular | Sticky | Small | Convex |
| 11. | NS-11 | White | Regular | Circular | Rough | Small | Convex |
| 12. | NS-12 | Creamy | Regular | Circular | Sticky | Small | Flat |
| 13. | NS-13 | Creamy | Regular | Circular | Sticky | Small | Flat |
| 14. | NS-14 | White | Irregular | Circular | Rough | Small | Cone |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 15. | NS-15 | white | Irregular | Circular | Sticky | Small | Flat |

Table 2: Colony morphology, size and shape of bacterial isolates from sample 2

| Sr. No. | Bacteria | Color | Margin | Shape | Surface | Size | Tope of Colony |
|---|---|---|---|---|---|---|---|
| 1. | SN-1 | Yellowish White | Irregular | Circular | Rough | Small | Planar |
| 2. | SN-2 | White | Entire | Circular | Rough | Small | Planar |
| 3. | SN-3 | Yellowish White | Entire | Circular | Smooth | Small | Planar |
| 4. | SN-4 | Creamy yellowish White | Irregular | Oval | Rough | Large | Planar |
| 5. | SN-5 | Yellowish white | irregular | Circular | Rough | Small | Planar |
| 6. | SN-6 | White | irregular | Circular | Smooth | Small | Planar |
| 7. | SN-7 | White | irregular | Circular | Smooth | Very Small | Planar |
| 8. | SN-8 | Yellowish white | Entire | Circular | Rough | Small | Cone |
| 9. | SN-9 | Yellowish white | irrgular | Circular | Sticky | Small | Cone |

Table 3: Diversity of bacterial flora in their shape from sample 1

| Sr. No. | Shape | Numbers |
|---|---|---|
| 1. | Cocci | 10 |
| 2. | Streptococci | 03 |
| 3. | Staphylococci | 01 |
| 4. | Rods | 01 |

Table 4: Diversity of bacterial flora in their shape from sample 2

| Sr. No. | Shape | Numbers |
|---|---|---|
| 1. | Cocci | 03 |
| 2. | Streptococci | 03 |
| 3. | Staphylococci | 02 |
| 4. | Rods | 01 |

Collected samples are diverse in their colony morphology with respect to shape of bacteria. Major group of bacteria dominating on contaminated region of oil spill were Cocci in shapes while streptococci and staphylococci were moderate and rod shaped bacteria was rarely observed (See Table 3 & 4). Diversity indices were calculated by taking in consideration shape and type of bacteria present over contaminated regions.

**Biochemical characterizations**

Biochemical characterization of isolated and purified bacteria was performed, to identify unknown bacteria present in it (Fig. 5-10). From soil sample 2, it was found that, SN-1 was *Salmonella typhi*, SN-2 & SN-5 were *Klebsiella pneumonie*, SN-6 was *Shigellis boyaii*,

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SN-7 and SN-9 were *Salmonella typhi* (Table 5,6). Rest of the bacteria we are unable to identify with simple IMViC and biochemical test, further tests are needed to identify it. Hydrocarbon resistant microbes are present often around petroleum industry and used it as the substrate for carbon and energy source. Most commonly observed hydrocarbon resistant bacteria reported by several authors are *Pseudomonas, Bacillus, and Acinetobacter* (Mittal and Singh, 2009); *Flavobacterium, Acinetobacter and Pseudomonas* (Mandri and Lin. 2006); *Yokenella, Alcaligenes, Roseomonas, Stenotrophomonas, Acinetobacter, Flavobacterium, Corynebacterium, Streptococcus, Providencia, Sphingobacterium, Capnocytophaga, Moraxella and Bacillus* (Rusanky *et. al.*, 1987; Antai, 1993; Bhattacharya *et. al.*, 2002); *Pseudomonas, Streptococcus, Escherichia coli, Staphylococcus, Klebsiella, Bacillus, Mycobacterium, Enterobacter aerogenes, Salmonella, and Micrococcus (*Makut and Ishaya, 2010).

On enrichment media, bacteria can produces different effect with respect to their peculiar biochemical characteristics. On blood agar, streptococci, which produce partial destructions of cells around colonies i.e. incomplete hemolysis are called alpha-hemolytic *Streptococci*. While streptococci producing complete destruction of cells around colonies are called beta-hemolytic streptococci. Green coloration has been around hemolytic zone of agar, hence bacteria comes under this category are supposed to be viridians group of bacteria. Those streptococci unable to produce hemolysis on blood agar are categorized as the non-hemolytic gamma hemolytic *Streptococci.*

Difference in bacterial community on soil with same parameters is because of oxygen and acidity variations cause due to spilled crude oil, petroleum products mostly are sulphur containing (Haris, 1962). Oxygen variations mainly depletion results into less growth of green grasses in contaminated areas in comparison with area not contaminated with oil spillage.

Table 5: Biochemical characterization of bacterial isolated from sample 1

| Bacteria | Indole test | Methyl red | Voges-Proskar | Citrate test | Catalase Test | Gram Stain | Shape | Bacteria identified as |
|---|---|---|---|---|---|---|---|---|
| NS-1 | -ve | -ve | +ve | +ve | +ve | -ve | Cocci | *Klebsiella pneumoniee* |
| NS-2 | -ve | +ve | +ve | +ve | +ve | +ve | Cocci | *Staphylococcus* spp. |
| NS-3 | -ve | +ve | -ve | -ve | +ve | +ve | Cocci | *Shigellis boyaii* |
| NS-4 | -ve | -ve | +ve | +ve | +ve | -ve | Rod | *Enterobacter cloacae* |
| NS-5 | -ve | -ve | -ve | +ve | +ve | +ve | Cocci | *Staphylococcus* spp. |
| NS-6 | -ve | +ve | +ve | +ve | -ve | +ve | Cocci | *Aeromonas* spp. |
| NS-7 | -ve | -ve | +ve | -ve | -ve | +ve | Cocci | *Staphylococcus* spp. |
| NS-8 | -ve | +ve | +ve | +ve | +ve | +ve | Staphylococcus | *Staphylococcus* spp. |
| NS-9 | -ve | +ve | +ve | +ve | +ve | -ve | Streptococcus | *Micrococcus* |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | spp. |
| NS-10 | -ve | +ve | +ve | -ve | +ve | +ve | Staphylococcus | *Streptococcus pneumoniae* |
| NS-11 | -ve | -ve | -ve | +ve | +ve | -ve | Streptococcus | *Micrococcus* spp. |
| NS-12 | -ve | -ve | -ve | -ve | +ve | -ve | Cocci | *Staphylococcus* spp. |
| NS-13 | -ve | -ve | -ve | +ve | +ve | +ve | Cocci | *Streptococcus pneumoniae* |
| NS-14 | -ve | +ve | +ve | +ve | +ve | +ve | Streptococci | *Streptococcus pneumoniae* |
| NS-15 | -ve | +ve | -ve | -ve | +ve | +ve | Cocci | *Shigellis boyaii* |

Table 6: Biochemical characterization of bacterial isolated from sample 2

| Bacteria | Indole test | Methyl red | Voges-Proskar | Citrate test | Catalase Test | Gram stain | Shape | Bacteria identified as |
|---|---|---|---|---|---|---|---|---|
| SN-1 | -ve | +ve | -ve | +ve | +ve | -ve | Cocci | *Salmonella typhi* |
| SN-2 | -ve | -ve | +ve | +ve | -ve | +ve | Streptococci | *Klebsiella pneumoniee* |
| SN-3 | -ve | +ve | +ve | +ve | -ve | +ve | Streptococci | *Streptococcus pneumoniae* |
| SN-4 | -ve | -ve | +ve | -ve | +ve | -ve | Cocci | *Micrococcus* spp. |
| SN-5 | -ve | -ve | +ve | +ve | +ve | -ve | Cocci | *Klebsiella pneumoniee* |
| SN-6 | -ve | +ve | -ve | -ve | -ve | +ve | Streptococci | *Shigellis boyaii* |
| SN-7 | -ve | +ve | -ve | +ve | +ve | +ve | Streptococci | *Salmonella typhi* |
| SN-8 | -ve | -ve | -ve | +ve | +ve | +ve | Rods | *Bacillus spp.* |
| SN-9 | -ve | -ve | +ve | -ve | +ve | +ve | Staphylococcus | *Salmonella typhi* |

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Figure 5: Citrate test of Bacterial isolates A: Sample 1 and B: Sample 2

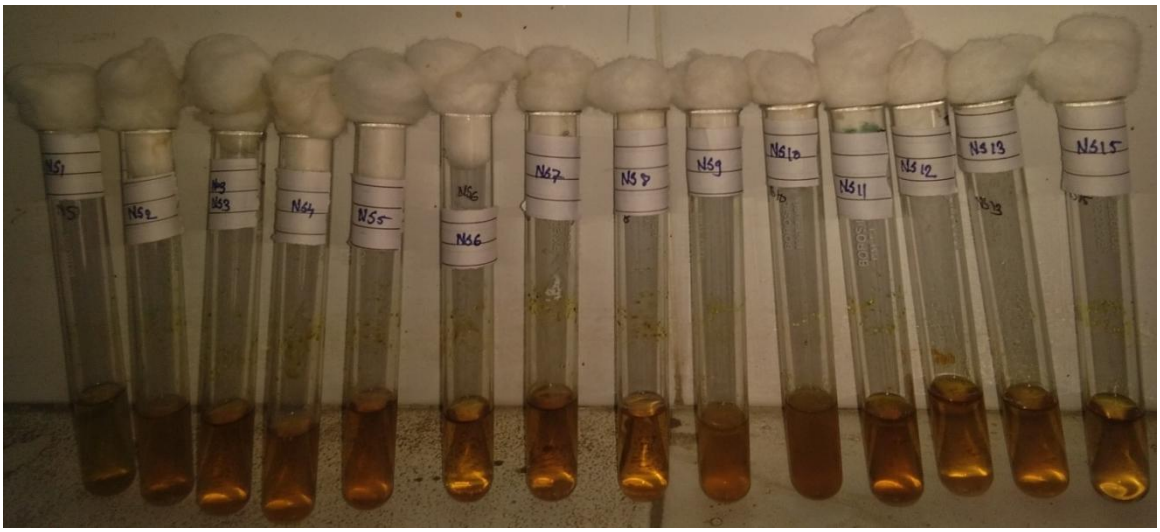

Figure 6: Results of Indole test of sample 1

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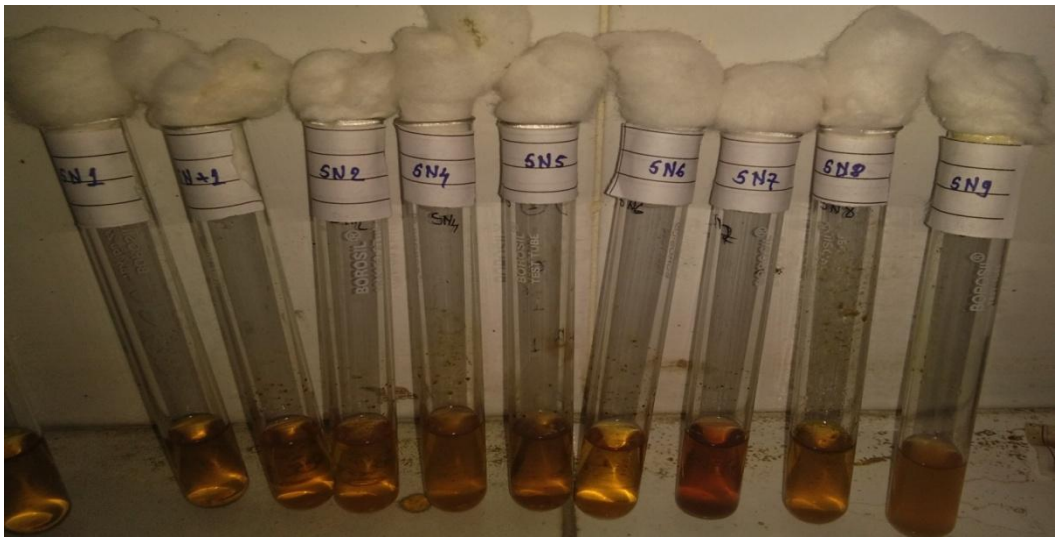


Figure 7: Results of Indole test of Sample 2

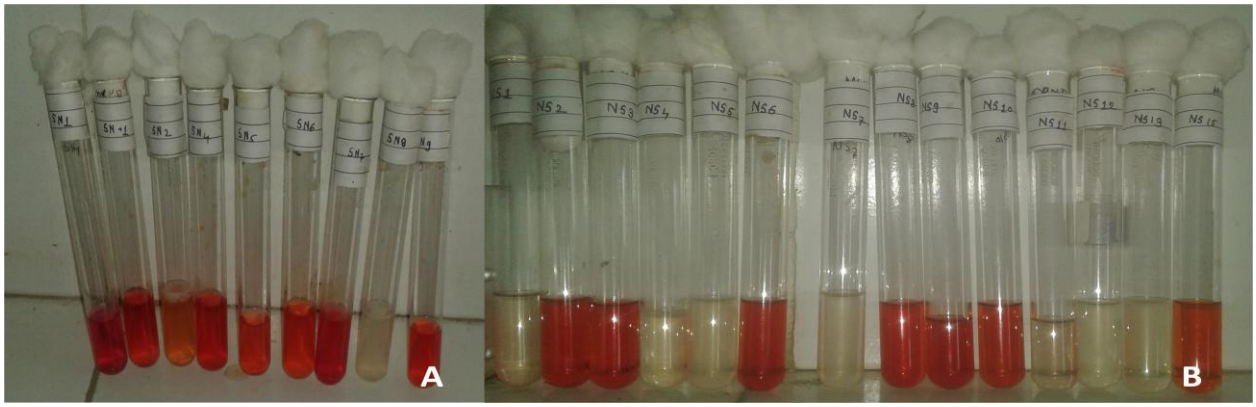


Figure 8: Results of test of Methyl red, A: Sample 1 and B: Sample 2

Figure 9: Results of Voges Proskaur from sample 1

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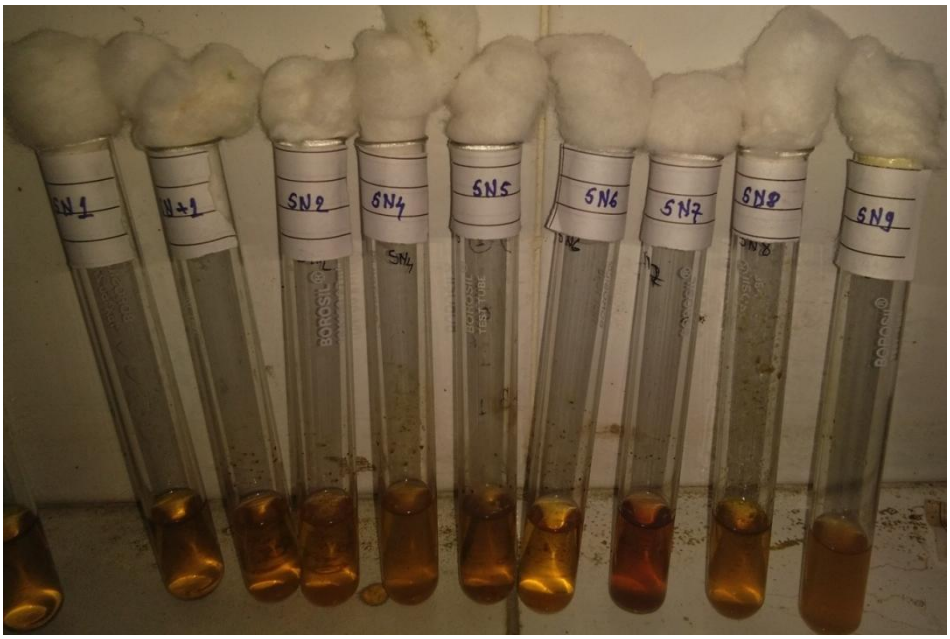


Figure 10: Results of Voges-Proskaur from sample 2

From sample 1, it was found that NS-1 was *Klebsiella pneumoniee*, NS-3 and NS-15 were *Shigellis boyaii*, and NS-4 was *Enterobacter cloacae.*

**Study of Diversity Indices**

Both collected soil sample analyzed for diversity indices, we found that true diversity, Berger-Parker index, Shannon Entropy, Simpson Dominance and True diversity (Order 2) for sample 1 were 2.8, 62.5%, 1.0408, 44.5% and 2.2, respectively. For sample 2; true diversity, Berger-Parker index, Shannon Entropy, Simpson Dominance and true diversity were 3.7, 33.3%, 1.3108, 28.4 and 3.5 respectively (Table 7).

Table 7: Diversity studies of collected sample from contaminated sites.

| Index | Sample 1 (Value) | Sample 2 (Value) |
|---|---|---|
| Number of Classes *N* | 4 | 4 |
| Richness R | 4 | 4 |
| Berger Parker Index $p_{imax}$ | 62.5% | 33.3% |
| Shannon Entropy *H* (nat) | 1.0408 | 1.3108 |
| Shannon Entropy *H* (bit) | 1.5016 | 1.8911 |
| Number Eq. $^{1}D$ (True Diversity) | 2.8 | 3.7 |
| Shannon Equitability H/ln*N* | 75.1% | 94.6% |
| Simpson Dominance SD | 44.5% | 28.4% |
| SD (unbiased – finite samples) | 40.8% | 19.4% |
| True Diversity $^{2}D$ (Order 2) | 2.2 | 3.5 |
| Gini-Simpson Index 1-SD | 55.5% | 71.6% |
| Gini-Simpson Equitability | 74.0% | 95.5% |

**Conclusion and future perspectives**

Hydrocarbon resistant microbes plays key role in biodegradations of petroleum products with active metabolism. Bacteria can colonize contaminated area with oil spilled difference in pore size of soil. Plants are unable survive because of depletion of oxygen in oil

spilled area as the contaminated area were covered with waxy layer. In this study we found that dominance flora of bacteria over contaminated region is of cocci and further identified as *S. typhi*. Flora of sample 1 are more dominant one in contaminated region. By optimizing compatibility of these microbes in a consortium will be effective for bioremediation treatments. Single bacteria specific for certain petroleum products, while consortium of resistant microbes can be used for numerous petroleum products.

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