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# Isolation and Identification of Petroleum degrading organisms from oil contaminated water and soil sample

[1]Nikita Nilkanth, [2]Komal Sonne, [3]Ekata Koyande

Department of Biotechnology
S. S. & L. S. Patkar College of Arts and Science and V. P. Varde college of Commerce and Economics Swami Vivekananda Rd, Piramal Nagar, Goregaon West, Mumbai, Maharashtra 400104.

***Abstract:*** **Oil spill have become a global problem particularly in industrialized and developing countries. Crude oil contamination of the environment is a world-wide threat to environment sustainability and hence a global concern. For this we need to go for the natural solution, i.e. Bioremediation. Organisms were isolated from oil contaminated places i.e. Arabian sea (gateway of India), Gorai creek, Petrol pump, Synthetic soil sample. These isolates were screened using Bushnell Hass agar medium in which diesel oil was used as sole carbon source. The percentage degradation of hydrocarbons estimated using Gravimetric analysis. Time lag and growth pattern of isolates were checked by taking optical density at 530 nm and determining pH at different intervals. 9 different isolates were obtained. The graph of bacterial degradation of oil and the growth curve of isolates was plotted. The bacterial identification was done by performing suitable biochemical test compared with Bergeys Manual. Taxonomic characteristics of these isolates identified them as *Bacillus spp., Clostridium spp., Vibrio spp. , Pseudomonas spp*. These isolates can be used for degradation of toxic compounds spread during oil spill, land farming, to determine benzene toxicity.**

***Keywords:*** **Petroleum, Bioremediation, Gravimetric analysis, Bergey's manual.**

## I. INTRODUCTION

Crude oils are composed of mixture of paraffin, alicyclic and aromatic hydrocarbons. Microbes exposed to hydrocarbons become adapted, and exhibit selective enrichment for growth. Microbial degradation of oil is caused by an attack on the aliphatic or light aromatic components of the oil which is an ability for isolating high numbers of certain oil-degrading micro-organisms. This ability of the organism acts as a commonly taken evidence for them to become active degraders of the environment in which they are surviving. The most rapid and complete degradation of the organic pollutants can be brought under aerobic conditions. The initial intracellular attack of organic pollutants is an oxidative process and the activation and incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidases. Peripheral degradation pathways convert organic pollutants into intermediates, for example, the tricarboxylic acid cycle. Biosynthesis of cell biomass occurs from the central precursor metabolites, for example, pyruvate. Sugars required for various biosynthesis and growth are synthesized by gluconeogenesis. The degradation of petroleum hydrocarbons can be mediated by specific enzyme system. Other mechanisms involved are; (a) Attachment of microbial cells to the substrates (b) Production of biosurfactant

## II. MATERIALS AND METHODS

***Standard plate count:***

Water and soil samples were collected from four different oil contaminated places i.e. A(Arabian sea-Gateway of India), B(Gorai creek), C(Petrol pump), D(synthetic soil) sample. It was serially diluted till $10^{-8}$ using sterile physiological saline and average cfu/ml was calculated. [1][5]

***Preparation of culture suspension:***

Using water and soil samples organisms were isolated on sterile Luria Bertani agar plate. The plate after isolation was incubated at 37 c for 24 hours. After incubation, isolated colonies were observed and were further sub- cultured onto Luria Bertani agar slant. The colonies obtained were dissolved in 5ml sterile physiological saline

***Screening of isolates:***

Loop full of culture suspension was inoculated into 30 ml of Bushnell Hass broth containing 1 ml effluent (1.025gmof diesel oil) sample in side arm flask. These flasks were incubated at room temperature for 7 days on shaking intervals at 130 rpm. Optical density of the culture broth was checked on regular basis for seven days at 530nm and also pH was determined at daily intervals. To check the viability; organisms from the flasks were daily cultured on to Luria Bertani agar plate [1]

***Gravimetric analysis:***

After 7days the flasks were taken out and bacterial activities were stopped by adding 1N HCl. The amount of biodegraded oil was measured using gravimetric analysis. For the extraction of crude oil 30 ml of culture broth was mixed with 30 ml of petroleum ether and acetone in 1:1 ratio in a separating funnel. Acetone was added to the above layer and shaken gently to break the emulsification which results in 3layers.Top layer was mixture of petroleum ether, crude oil and acetone clumping cells make the middle layer and the bottom aqueous layer contains acetone water and bio surfactant in soluble form. The lower two layers were separated out while top layer containing petroleum ether mixed with crude oil and acetone was taken out in clean beaker. The extracted oil was passed through anhydrous sodium sulphate to remove moisture. Petroleum ether and acetone was evaporated on water bath on their respective boiling point. (Petroleum ether $42^0$c – $62^0$ c and acetone$56^0$c).The gravimetric estimation of residual oil left after biodegradation was carried by weighing the quantity of oil. The biodegraded crude oil was further fractioned for its gross and molecular composition. [12][6]

**The % of degradation was calculated as follows;**

Weight of Residual crude oil= Weight of beaker containing extracted crude oil–Weight of empty beaker.

Amount of crude oil degraded = Weight of crude oil added in the media – Weight of Residual crude oil

% degradation = (Amount of crude oil degraded / Amount of crude oil added in the media) ×100

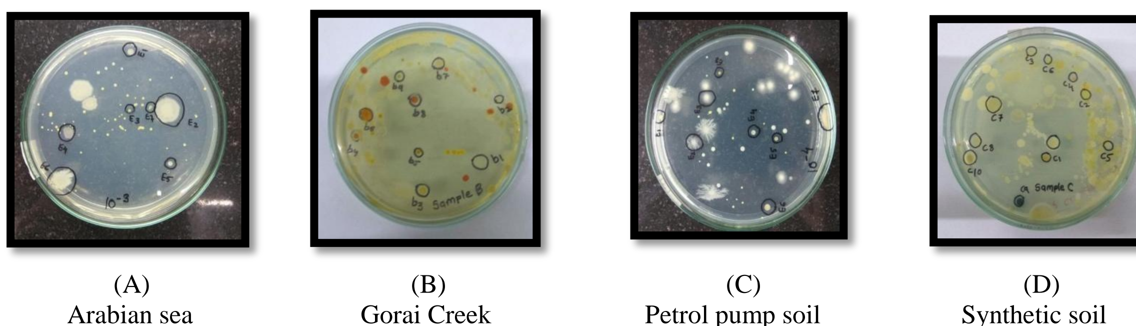
***Bacterial Identification:***

Morphology of the isolates was studied by standard procedure of gram staining. (Oil immersion, 100X) & gram nature (Gram (+) or Gram (-)), Shape of cell (cocci, bacilli, cocco-bacilli) along with their arrangement (scattered, clusters, chain) were studied. Further biochemical tests such as motility, oxidase, Catalase, nitrate reduction, Indole formation, methyl red reduction, Vogus-Proskauer test, citrate utilization, sugar fermentation (glucose, maltose, lactose, fructose ) , triple sugar iron agar test , mannitol salt agar test. [4]

***Characterization of Bacterial Isolate:***

On the basis of results obtained the isolates were identified with the help of Bergey's manual of systematic bacteriology. The isolates were identified till the species level.

## III. RESULTS



(A) Arabian sea | (B) Gorai Creek | (C) Petrol pump soil | (D) Synthetic soil

**Fig. 1:- Standard plate count of sample**

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**Table 1:- Standard Plate Count**

| SAMPLE | DILUTION $10^{-6}$ | DILUTION $10^{-7}$ | DILUTION $10^{-8}$ | AVERAGE cfu/ml |
|---|---|---|---|---|
| A | 8 | 34 | TLTC | $29.13\text{x } 10^{-6}$ |
| B | 7 | 44 | TLTC | $24.8\text{x}10^{-6}$ |
| C | 4 | TLTC | TLTC | $13.67\text{x}10^{-6}$ |
| D | 4 | TLTC | TLTC | $16\text{x}10^{-6}$ |

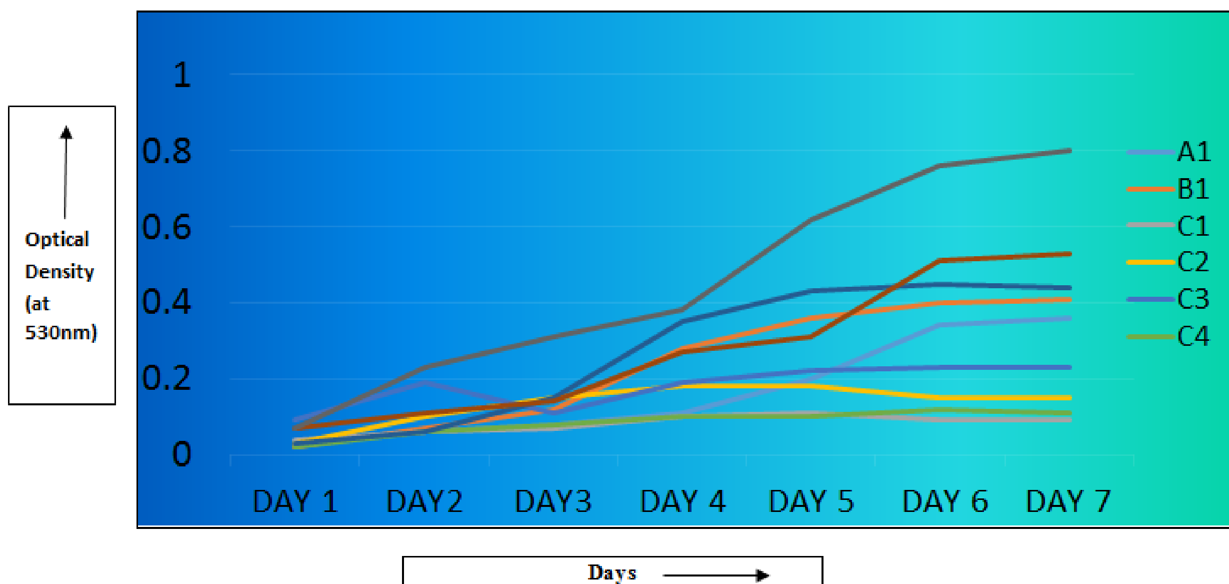
***pH determination of media:***

pH at the time of inoculation (day 0) = 6.5

pH after incubation=4

***Screening of Isolates:***

Optical Density of the media containing isolates and petroleum oil.




***Gravimetric Analysis:***

**Table 2:- Estimation of % degradation**

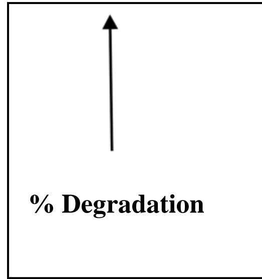
| Sr.No. | Isolates | $X_o$ (g) | $X_s$ (g) | Biodegraded oil (g) | % of degradation |
|---|---|---|---|---|---|
| 1 | A1 | 16.499 | 19.998 | 0.548 | 53 |
| 2 | B1 | 16.523 | 20.523 | 0.511 | 49 |
| 3 | C1 | 16.564 | 20.863 | 0.475 | 46 |
| 4 | C2 | 16.805 | 18.071 | 0.527 | 51 |
| 5 | C3 | 16.577 | 18.928 | 0.424 | 41 |
| 6 | C4 | 16.563 | 21.997 | 0.347 | 33 |
| 7 | D1 | 16.772 | 20.212 | 0.739 | 72 |
| 8 | D2 | 16.623 | 19.323 | 0.799 | 77 |
| 9 | D3 | 16.432 | 19.239 | 0.728 | 71 |

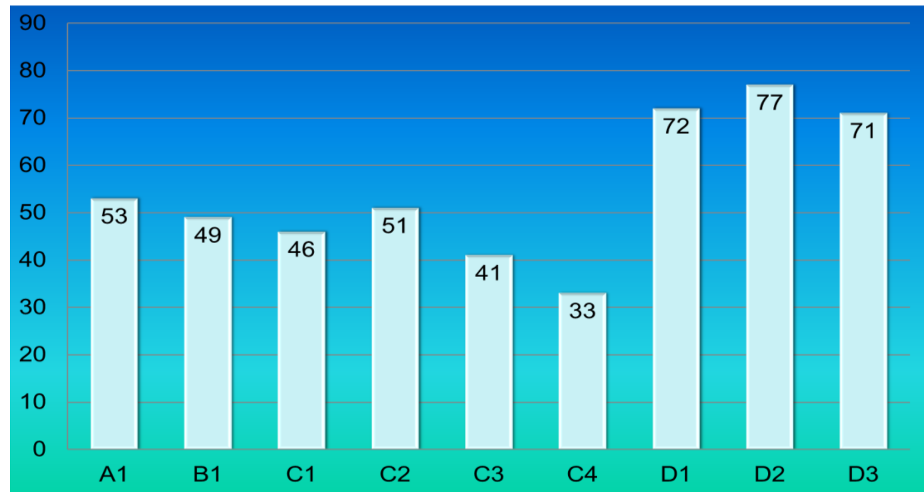
Key;

Xo = initial weight of empty beaker.

Xs = weight of beaker containing left residual oil.

Estimation of Percentage Degradation by Gravimetric Analysis




Isolates

***Biochemical Tests:***

Sugar
TSI
Nitrate
Mortility
Indole
Methyl Red
Voges Proskauer
Citrate
Oxidase
Catalase
MSA

***Result of Biochemical test:***

| Sr. No | Tests | A1 | B1 | C1 | C2 | C3 | C4 | D1 | D2 | D3 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Sugar fermentation | | | | | | | | | |
| | (i) Maltose | + | + | + | + | - | - | + | + | + |
| | (ii)Glucose | + | + | + | + | + | + | + | + | + |
| | (iii)Lactose | + | + | + | - | - | - | - | + | + |
| | (iv)Fructose | + | + | + | - | - | - | + | + | + |
| 2 | TSI | - | - | + | + | + | - | - | - | - |
| 3 | Mannitol salt agar | + | + | - | - | - | + | + | + | + |
| 4 | Nitrate reduction | + | + | + | + | + | + | - | + | - |
| 5 | Motility | + | + | + | + | + | + | + | + | + |
| 6 | IMVC | | | | | | | | | |
| | (i)Indole | + | - | + | - | - | - | - | - | + |
| | (ii)Methyl red | - | - | - | - | + | - | - | - | + |
| | (iii)Vogues proscus | + | - | + | - | - | - | - | + | - |
| | (iv)Citrate | + | - | + | + | + | - | - | + | - |
| 7 | Oxidase | - | - | + | + | + | + | - | - | - |
| 8 | Catalase | + | + | + | + | + | + | - | - | - |

***Bacterial Identification:***

| Isolates | Identified Organisms |
|---|---|
| A1 | *Bacillus barvis, Bacillus lentus* |
| B1 | *Bacillus endoptyticus* |
| C1 | *Vibrio funissi, Vibrio mytili, Vibrio portersiae* |
| C2 | *Psuedomonassagetis* |
| C3 | *Psuedomonasxantom, Pseudomonas panoic* |
| C4 | *Clostridium aminoxalevicm* |
| D1 | *Clostridium novyl-A, Clostridium novyl-B* |
| D2 | *Bacillus megaterium* |
| D3 | *Clostridium limosum* |

## IV. CONCLUSION

A better understanding of the mechanism of biodegradation has a high ecological significance which depends on the indigenous microorganisms to transform or mineralize the organic contaminants. Microbial degradation process aids the elimination of spilled oil from the environment after removal of large amounts of oil by various physical and chemical methods. This is possible because microorganisms have an enzyme system to degrade and utilize different hydrocarbons as a source of carbon and energy.Isolation of Crude Oil Degrading bacteria was done, bacterial identification and taxonomic characteristics of the isolates showed them belonging to *Bacillus* spp. , *Clostridium* spp. , *Vibrio* spp. and *Pseudomonas* spp. when compared with Bergey;s manual. In future we can even find the genus of these isolates by using 16r DNA technology. From the results obtained *Bacillus* spp. has shown highest percentage degradation i.e. 77% when compared to the other species. The isolates from sample A, B, C showed less than 55% of degradation. We can conclude from this; that the isolates from the synthetic soil sample has the highest percentage of degradation. These species individually or in consortium can be used as Bio surfactant for doing further studies. These species can be used for land farming process also. These organisms can also be used to reduce toxicity of benzene and other compounds polluting the environment. This can be considered as a major aspect of the project.

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## APPENDIX

**Composition of Media:**

BUSHNELL HASS

| COMPOSITION | g//L |
|---|---|
| Magnesium sulphate | 0.200g |
| Monopotassium phosphate | 1.000g |
| Calcium chloride | 0.020g |
| Dipotassium phosphate | 1.000g |
| Ammonium nitrate | 1.000g |
| Ferric chloride | 0.050g |
| Final pH at( 25°C) | 7.0±0.2 |

NUTRIENT AGAR

| COMPOSITION | g/L |
|---|---|
| Peptone | 10 g |
| NaCl | 5 g |
| Meat extract | 10 g |
| Agar | 15 g |
| D/w | 1000 ml |
| pH | 7.2 |

LURIA BERTANI BROTH:

| COMPOSITION | g/L |
|---|---|
| Tryptone | 10 g |
| Yeast extract | 5 g |
| NaCl | 10 g |
| Agar | 15g |
| D/w | 1000 ml |
| pH | 7.2 |

NITRATE REDUCTION BROTH

| COMPOSITION | g/L |
|---|---|
| $NaNO_3$ | 0.3 g |
| Peptone | 5 g |
| D/w | 1000 ml |
| pH | 7.4 |

TRYPTONE WATER BROTH

| COMPOSITION | g/L |
|---|---|
| Tryptone | 10 g |
| NaCl | 5 g |
| D/w | 1000 ml |
| pH | 7.4 |

MAC CONKEYS AGAR

| COMPOSITION | g/L |
|---|---|
| Peptone | 3 g |
| Pancreatic digest of casein | 17 g |
| Lactose monohydrate | 10 g |
| Bile salts | 1.5 g |
| NaCl | 5 g |
| Crystal violet | 0.001g |
| Neutral red | 0.030g |
| Agar | 13.5 g |
| pH | 7.2 |
| D/w | 1000 ml |

MANNITOL SALT AGAR

| COMPOSITION | g/L |
|---|---|
| Peptone | 10 g |
| Beef extract | 10 g |
| NaCl | 7.5 g |
| D-Mannitol | 10 g |
| Phenol red | 0.025 g |
| Agar | 15 g |
| D/w | 1000 ml |
| pH | 7.4 |

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TRIPLE SUGAR IRON AGAR

| COMPOSITION | g/L |
|---|---|
| Peptone | 20 g |
| Lactose | 10 g |
| Sucrose | 10 g |
| Beef extract | 3 g |
| Yeast extract | 3 g |
| Glucose | 1 g |
| Ferric citrate | 0.3 g |
| $Na_2S_2O_3$ | 0.3 g |
| Phenol red | 0.025 g |
| Agar | 12 g |
| D/w | 1000 ml |
| pH | 7.4 |

GLUCOSE PHOSPHATE BROTH

| COMPOSITION | g/L |
|---|---|
| Glucose | 5 g |
| Peptone | 5 g |
| $K_2HPO_4$ | 5 g |
| D/w | 1000 ml |
| pH | 7.4 |

MOTILITY TEST

| COMPOSITION | g/L |
|---|---|
| Peptone | 5 g |
| Agar | 10 g |
| D/w | 1000 ml |
| pH | 7.4 |

SIMMONS CITRATE AGAR

| COMPOSITION | g/L |
|---|---|
| Sodium citrate | 2 g |
| $K_2HPO_4$ | 1 g |
| Ammonium dihydrogen phosphate | 1 g |
| Magnesium sulphate | 0.5 g |
| NaCl | 5 g |
| Bromothymol blue | 0.08 g |
| Agar | 15 g |
| D/w | 1000 ml |
| pH | 7.2 |

**Composition of Reagents**

GRAM STAINING

Crystal violet

Crystal violet – 1.0 g Absolute alcohol – 10.0 ml Distilled water – 90.0 ml

Grams iodine

Iodine – 1.0 g Potassium iodide – 2.0 g Distilled water – 100 ml

Alcohol – 95 %

Saffranine- 1.0 g

Absolute alcohol – 10.0 ml Distilled water – 90.0 ml

METHYL RED

Methyl red – 0.05 gm Ethanol – 60.0 ml Distilled water – 40.0 ml

OXIDASE TEST

1 % Tetramethylparaphenylenediaminedichloride – 1.0 g Distilled water – 100 ml

α – NAPTHOL

5 % α- napthol – 2.5 g Alcohol – 50.0 ml

KOH

40 % KOH – 20.0 g

Distilled water – 50.0 ml

KOVAC'S REAGENT

Paradimethyl amino benzaldehyde – 5.0 g

Iso -amyl alcohol – 75.0 ml

Conc. HCl – 25.0 ml

1 N HCL

HCl – 8.36 ml of HCl Distilled water – 100 ml

1 N NaOH

NaOH – 4.0 GM

Distilled water – 100 ml

CATALASE TEST REAGENT

3 % hydrogen peroxide – 10.0 ml Distilled water – 10.0 ml

0.2% PHENOL RED

Phenol red – 0.2 g 1N NaOH - 2 ml D/W - 4 ml

0.1 N HCl – 2 Ml D/W - 92 ml

1% BROMOTHYMOL BLUE

Bromothymol blue – 0.1 g D/W - 10 ml

SALINE – 0.85 g of NaCl in 100 ml of distilled water