

## ISOLATION OF POTENT HYDROCARBON DEGRADING MICRO-ORGANISMS AND ITS APPLICATION IN BIOREMEDIATION

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### ABSTRACT

**Objective:** Oil spillage has become a global environmental problem as its constituents are toxic, mutagenic and carcinogenic. Natural bioremediation is the only eco-friendly solution to resist its devastating environmental and economic damage. Microbes are used to change harmful substances to non-toxic substances. The current work focuses on the performance of different bacterial species in degrading the oil components like benzene and polycyclic aromatic hydrocarbons (PAH)s.

**Methods:** Sample was collected from different areas affected by the oil spill in Mumbai that is from the shore of Juhu, Dadar and Manori in form of tar balls and was enriched and isolated on Bushnell and Hass's media containing 1% crude oil as a sole source of carbon. The potent isolates were then identified by standard biochemical tests referring to Bergey's Manual.

**Results:** Two partially identified strains were *Pseudomonas flavescens* and *Bacillus sp.* biofilms of *Pseudomonas spp.* was prepared on glass matrix to determine its oil degrading efficiency. An indigenous consortium was developed by the assembly of seven isolates of oil-degrading bacteria.

**Conclusion:** The developed consortium was able to degrade crude oil completely within 4 d. The obtained isolates seemed to have the potential for bioremediation of oil contaminated soil and tar balls which was justified by setting up of a bioreactor.

**Keywords:** Bioremediation, Tar balls, Consortium, Bioreactor, *Pseudomonas flavescens*, Biofilm

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### INTRODUCTION

Environmental pollution entails any adjustments in the surroundings but it is restricted in use especially to mean any deterioration in the physical, chemical and biological quality of the environment [1]. Hydrocarbons enters into the environs through waste disposal, accidental spills, leakage tankers and losses during transport or storage. The amount of natural crude oil spoilage was estimated to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per years. Biodegradation has at least three important processes: first, they cause a minor change in an organic molecule leaving the main structure still intact; second, degradation can also be thought of as fragmentation, where the original structure of the molecule can still be recognized in the fragments and finally, a complete mineralization of a compound to inorganic form [2]. Outmoded methods of spillage cleanup includes the use of mechanical devices such as skimmers, oil blooms, but, these methods are very expensive and labour intensive process. Therefore bioremediation has become very practical approach to oil spill clean-up efforts in recent years. Bioremediation is defined as the use of microbes to detoxify or remove pollutants owing to their diverse metabolic capabilities [3]. Research and application of bioremediation in the marine environment is still needed to be studied due to more complicated aspects and also difficulties. Therefore comprehensive study in biodegradation processes that involves various factors such as pollutants, physical and chemical conditions, deadliness, competition and metabolites accumulation have to be done. This study focus on isolation of potential microbes capable of degrading crude oil from oil spill affected areas as individual microbial populations can usually metabolize only a limited range of substrate so the performance of mixed population with different degradation capabilities was studied [4]. The study also focuses on biofilm production as they found to have implication in bioremediation of hydrocarbons [5].

### MATERIALS AND METHODS

#### (a) Sample collection

Three tar balls samples from different areas affected by oil spill two years ago in Mumbai was collected from the shore of Juhu, Dadar

and Manori in sterile polyethylene bags [6]. Samples were collected in 5 cm depth from the surface of the soil to avoid surface contamination. Collected samples were transferred into the laboratory under sterile conditions and stored at 4 °C until processed for analysis. Crude oil (color: blackish brown) was collected from a garage in Bandra, Mumbai.

#### (a) Enrichment and Isolation of microorganisms from samples

**Bushnell Hass (BH)** broth medium was used for enrichments. The composition is as follows: MgSO<sub>4</sub>·7H<sub>2</sub>O (0.2g/l), K<sub>2</sub>HPO<sub>4</sub>(1.0g/l), KH<sub>2</sub>PO<sub>4</sub> (1.0g/l), FeCl<sub>3</sub> (0.05g/l), NH<sub>4</sub>NO<sub>3</sub> (1.0g/l), CaCl<sub>2</sub> (0.02g/l) with pH 7.2. For isolation agar (20g/l) was added for BH agar [2]. One gram of tar ball samples was inoculated in 250 ml flasks containing 100 ml of BH broth. The medium was supplemented with 1% crude oil as the only source of carbon. The flasks were kept on a rotary shaker at 100rpm at room temperature until the visible degradation of oil was observed. Second enrichment was done by using 10% seed from the first enrichment, sand particles were added for quick degradation. Third enrichment was done using 10% seed from second enrichment. One loopful enriched samples were streaked on BH agar plates respectively and were incubated at room temperature to observe isolated colonies. To obtain pure culture isolated colonies were streaked and subcultured on BH slants.

#### (a) Identification

The seven colonies obtained were observed for their morphological characters like size, shape etc. Gram's staining was done by preparing smear, heat fixing it. The smear was observed in 100X oil immersion lens.

#### (b) Biochemical analysis of colonies

On the basis of Gram's nature the Gram-negative colonies were selected and partially identified referring to Bergey's Manual [5] using various tests like oxidase test, indole, methyl red test, Voges Prausker test, glucose, nitrate, lactose and citrate utilisation, 6.5% NaCl tolerance, H<sub>2</sub>S and CO<sub>2</sub> production etc [1, 2, 4, 6-8].

**(a) Biofilm production**

*Pseudomonas* cells (isolate IV) the most potent oil degrading group of organism identified using Bergey's Manual was tested for biofilm formation on 18 mm glass cover slip being immersed in 10 ml of BH media with 1% crude oil in sterile saline tubes. The organism was inoculated and incubated at R. T for 3 d. The coverslips were recovered from culture tubes, washed thoroughly with saline solution and stained with crystal violet dye and viewed under 100X oil immersion lens. The formation of biofilm was studied at different intervals of growth at 24, 48 and 72 h [5, 9].

**(c) Consortium development**

3% seed from every sample was mixed and inoculated in 150 ml of Bushnell and Hass broth with 1% crude oil as a sole source of carbon the flask was incubated on a rotary shaker at 100rpm at room temperature and was observed for visible degradation of oil [4].

**Bioreactor setup**

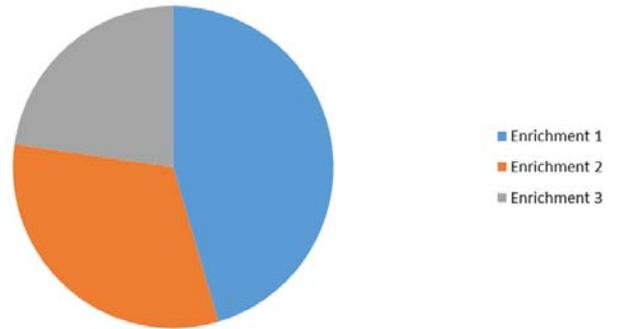
To study the degradation potential of the built consortium bioreactor was set up proposed by Darin Maliji *et al.* [10]. A sterilized glass column measuring 5 litres capacity was used in which 3 times filtered sea water measuring 90 ml was added containing 10% seed from the consortium, 1% crude oil as a carbon source and aeration was provided using an oxygen pump, 2g sand particles were added to enhance the oil degrading capability. The reactor was run and continuous aeration was provided, daily the tank was observed for oil reduction and was continued until total oil was degraded.

**Degradation of phenol**

Quantitatively residual phenol was estimated via a measure of its absorbance at 510 nm wavelength by using UV-visible Spectrophotometer and 4 amino Antipyrine as a colour indicator. Standard solution of phenol was prepared with known quantity of phenol started from 100mcg, 1 ml of 4 amino antipyrine as indicator until the colour of phenol becomes colourless to red. Noted the absorbance values of phenol at 510 nm. 10% cultures of respective isolates were inoculated in 90 ml Bushnell and Haas medium containing 100mcg phenol in one flask this was considered as 1st enrichment, later 10% seed from 1st enrichment was inoculated in 90 ml Bushnell and Haas medium containing 200mcg. After 1-2 w of incubation, the comparison was done between absorbance values of phenol from supernatant and absorbance values of the standard to estimate the degraded phenol [11].

**RESULTS AND DISCUSSION**

Tarball samples collected were used for isolating oil-degrading microorganisms.



**Fig. 1: Pie chart showing degradation of oil sample by enrichments over no. of d**

Pie chart fig. 1 shows that 1st enrichment took 10 d to degrade oil whereas the best potent cultures when were adapted to that conditions where oil was degraded within 7 d in 2nd enrichment. In 3rd enrichment, the consortia developed from 2nd enrichment showed best results of degrading oil within 5d.



**Fig. 2: Shows degradation of oil after enrichment**

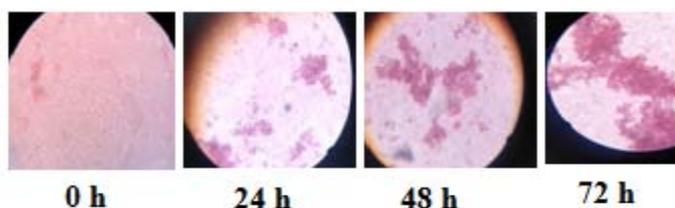
**Identification of bacterial isolates**

From third enrichment, isolates were obtained on Bushnell and Haas medium containing 1% crude oil. A total of 7 oil-degrading bacteria designated as I, II, III, IV, V, VI and VII were isolated. Their morphological characteristics and Gram staining were observed in [table 1].

**Table 1: Morphology and Gram staining of oil-degrading bacterial isolates**

Samples	Morphology	Gram staining	Isolates
Juhu	Cocci	Gram-negative	I
Manori	Pleomorphic	Gram-positive	II
	Sporulating bacilli	Gram-positive	III
Dadar	Rods	Gram-negative	IV
	coccioids	Gram-negative	V
Consortia	Coccobacilli	Gram negative	VI
	bacilli	Gram-positive	VII

Biofilm production Isolate IV showed a special character of forming a biofilm.



**Fig. 3: Shows biofilm production at different intervals (0 h, 24 h, 48 h and 72 h)**

*Pseudomonas* (Isolate IV) obtained from isolation formed biofilms on glass coverslips which helped in degradation of oil. Biofilms formed

on the glass surface were observed after 48 h and at 72 h the cells formed larger biofilms.

### Biochemical results

Table 2(a): Biochemicals of Gram-negative oil-degrading bacteria were performed referring to Bergey's Manual [10]

Isolate	Glucose utilisation	Lactose utilisation	Indole	M R	V P	Citrate utilisation	Cetrimide test	PPA test	Lysine decarboxylase	Ureaase test	Oxidase test	NaCl 6.5 %	Nitrate reductase	H <sub>2</sub> S production	CO <sub>2</sub>	Culture identified
I	+	+	-	+	-	+	-	-	+	-	-	+	+	-	+	not identified
IV	+	-	-	+	-	+	+	-	-	+	+	-	-	-	-	<i>Pseudomonas</i> sp.
V	+	+	-	+	-	+	-	-	-	-	-	+	-	-	+	not identified
VI	-	-	+	+	-	+	-	-	+	-	-	+	+	-	+	not identified

Key: '+' = Positive test, '-' = Negative test.

Table 2(b): Shows standard biochemical results Key: '+' = growth, '-' = no growth Special characters of *Pseudomonas flavescens* was observed that is yellow pigmentation, monotrichous flagella, xylose and sucrose utilisation which was confirmed using bergey's manual [3]

Characteristics	Observations	Substrate utilisation	Observations
Number of flagella	1	L-Asparagine	+
Non fluorescent pigments, color:		L-Arginine	+
Green		L-Tyrosine	-
Orange		D-Alanine	+
Yellow	+	L-Arabinose	-
Blue		Butanol	-
Gelatin liquefaction	-	Cellobiose	+
Denitrification	-	Starch	+
Levan formation	-	L-Threonine	-
Lecithinase	-	Methanol	-
Lipase	-	Creatine	+
Growth at 4 °C	-	Ethanol	-
Growth at 41 °C	-	D-Glucose	+
		Glycine	-
		L-Histidine	+
		m-Inositol	-
		L-Lysine	-
		Mannitol	-
		Naphthalene	+
		Phenol	-
		Sucrose	+
		D-Tryptophan	-

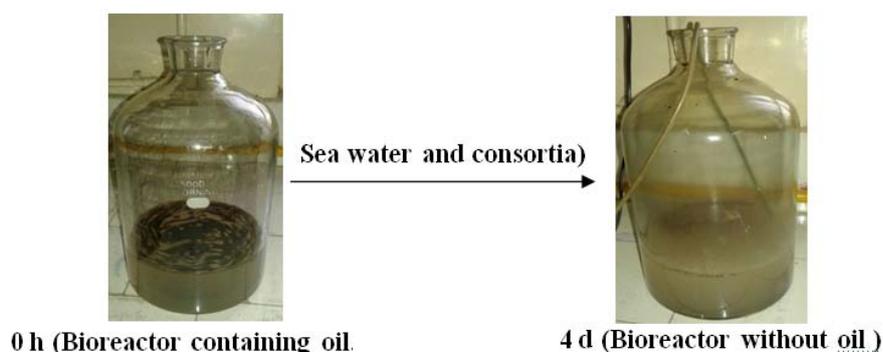


Fig. 4: Shows degradation of oil in lab-scale bioreactor

### Bioreactor setup results

The sea water containing 1% crude oil inoculated with consortium was degraded within 4 d. This can be due to the sequential degradation of oil components using all four isolates.

### Degradation of phenol

Crude oil-degrading microbes could successfully degrade phenol (monocyclic aromatic hydrocarbon) one of the harmful industrial effluent. Amongst which isolate III could not degrade phenol alone.

Consortia developed could degrade the highest amount of phenol 200 mcg/ml which was confirmed by using the 4-aminoantipyrene method.

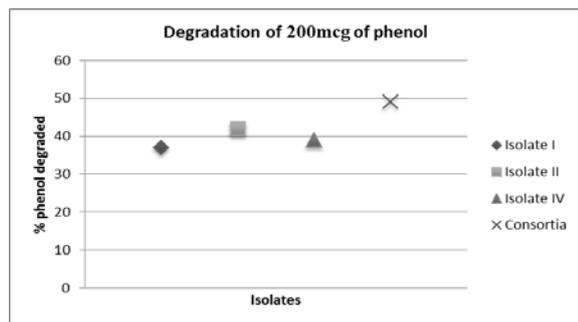


Fig. 5: Shows percentage of phenol degraded after 2<sup>nd</sup> enrichment

Developed consortia degraded the highest amount of phenol that is 49%, isolate I degraded least amount of phenol, 37%, isolate IV degraded 42% of phenol and isolate V degraded 39 % of phenol.

### CONCLUSION

Bioremediation has an edge over other treatment methods because it can efficiently degrade crude oil from the waste water and does not allow the contaminants to accumulate. The developed consortia efficiently degraded oil which can be applied to multiple concrete tanks. The bioreactor setup also gave a better degradation of oil in minimum time. The consortium built can be used to clean up oil spills in affected areas. Isolates from manorri and dadar were showing higher amount of degradation henceforth those areas should be focussed more for oil-degrading bacteria).

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### CONFLICT OF INTERESTS

Declare none

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