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Assessment of Bioremediation of Oil and Phenol Contents in Refinery Waste Water via Bacterial Consortium

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Abstract

The present study concerns with the biotreatment of waste water of petroleum refinery. The experiments were performed to assess the degradation potential of microbial species present in the waste water and using microbial consortium to treat the water in a suspension form. The four bacterial isolates were isolated and assessed for the biodegradation of crude oil and phenol in the waste water gravimetrically and by spectrophotometry respectively. The isolated bacterial strains were identified as *Alcaligenes odorans, Bacillus subtilis, Corynebacterium propinquum* and *Pseudomonas aeruginosa* which were inoculated into waste water in the form of bacterial consortium. After inoculation of consortium, it was found that oil and phenol contents in the waste water reduced up to 70% and 85% respectively via bioremediation. The search for cheaper and environment friendly options for enhancing degradation of petroleum contamination was the main research interest and bioremediation is showing a greater effective way of remediating natural ecosystem contaminated with crude oil from a number of decades.

Keywords: Bioremediation; Bacterial consortium; Bacterial isolation; Crude oil; Phenol

Introduction

Environmental pollution implies any alterations 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]. Contamination of the environment with crude oil results in pollution. Wide scale production, transport use and disposal of petroleum globally have made it a major contamination in both prevalence and quantity in the environment. The oil gets mixed with the river or marine water by many ways as accidental spills or discharge of refineries in river or other water bodies. Multiple initiatives have been developed to resolve the problem of petroleum pollution. An array of procedures has been developed including physical, chemical and biological techniques. Among these procedures, bioremediation is currently associated to physicochemical procedures.

The demand of petroleum as a source of energy and as a primary raw material for chemical industries in recent years has resulted in an increase in world production. This dramatic increase in production, refining and distribution of crude oil has brought with it an ever increasing problem of environmental pollution [2]. The persistence of petroleum pollution depends on the quantity and characteristic of hydrocarbon mixture and on the properties of the affected ecosystem. The ability to isolate high numbers of certain oil-degrading microorganisms from oil polluted environment is commonly taken as evidence that these microorganisms are active degraders of that environment [3]. Virtually every refinery, from primary distillation to final treatment, contains various fractions of oils and other hydrocarbon compounds in their waste waters. The oil and grease in this waste water may appear as free oil, dispersed oil, emulsified oil, soluble oil or as a coating or suspended matter.

Other than oil and grease contamination, phenol and its derivatives are also among the most important contaminants present in the environment. Phenol is one of the major organic pollutants encountered in waste water produced by industrial and refinery activities. Phenols have been reported as highly toxic and hazardous to living organisms [1]. Due to the potential toxicity and persistence of this kind of contaminant in the environment, rapid removal and detoxification is the need of the hour.

Many treatment methods are used in the treatment of oil wastewater. Most of the physicochemical and thermal methods are expensive, as they require expensive equipment and machineries and expend good amount of energy. Bioremediation is a promising method, where wastewater adapted consortium of microbial species is used for the degradation of pollutants from water. The present investigation was carried out with primary objective to study oil and phenol degradation using the bacteria isolated from waste water itself in the form of a consortium.

Material and Methods

Collection of sample and isolation of bacteria

The water sample was collected from Mathura Refinery Unit of Indian Oil Corporation Limited in a pre-sterilized glass bottle (5L capacity). The collection site inside refinery was DAF (Dissolved Air Floatation) outlet after physical separation in the refinery itself. The sample was brought to the laboratory at temperature maintained from 2-10°C in an insulated container. 6 samples were collected at the interval of 30 days each from January to June, 2011.

Serial dilution technique was used for the isolation of bacteria. In this technique, sample suspension was prepared by serially diluted upto 10^6. From this solution (10^6 ml^{-1} sample) 1 ml was pipetted on to the plates with nutrient medium-3 (composed of 2 g yeast, 1 g beef

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extract, 5 g NaCl, 5 g peptone and 15 g agar in 1000 ml distilled water) spreaded by glass spreader and incubated at 30˚C temperature for (24 hr) observation. Each single colony that appeared onto the plate was considered as one colony forming unit (c.f.u.). The colonies which grew vigorously and produced clear zone around them, were marked and were re-isolated onto a fresh nutrient agar plate and slants for further experiments.

Characterization and identification of isolated bacteria

Twelve strains were isolated from the water sample and were characterized by growing on nutrient medium enriched with oil and phenol, grams reaction, physiological properties and microscopic observations [4]. The ability to grow in different media was tested at 37˚C and neutral pH. Results were recorded after 24 hr at OD_{600} by spectrophotometer.

The isolated strains were allowed to grow on the nutrient medium containing 5% crude oil. Out of 12, 4 strains showed clear zone around their colonies showing depletion of oil. These selected 4 strains were also cultured over the MS medium containing phenol as the only carbon and energy source. The bacterial growth was measured spectrophotometrically at 600nm. The selected bacterial isolates which were able to grow in the phenol as well as oil enriched medium. These isolates were identified by BD-BBL™ Crystal Mind Autoreader (an instrument for bacterial identification).

The pure selected strains were transferred to slants, preserved at 4˚C in refrigerator and subcultured at an interval of 30 days.

Composition of media (phenol as carbon and energy source)

Cells were grown at 37˚C in Mineral medium for all phenol degrading bacteria. The compositions were 12.8 g of $Na_2HPO_4.7H_2O$, 3 g of KH_2PO_4, 0.5 g of NaCl, 1g of $(NH_4)_2SO_4$ and 2 g of phenol per litre. The pH was adjusted to 7 with 6N NaOH [5].

Preparation of inoculum

The bacterial cultures were inoculated in nutrient broth medium and kept in the incubator shaker at 200rpm at 37˚C for a period of five days. The growth was recorded depending on the extent of turbidity by nephlometer. Equal volumes of the culture (1 NTU: Nephlometric Turbidity Unit) broths from the above 4 isolates were mixed to prepare mixed bacterial consortium. The inoculum was mixed with the water sample (1:9) and incubated for 10 days at 37˚C for Bioremediation studies.

Biodegradation studies

Laboratory bioremediation studies were carried out at optimized pH (7) and temperature (37˚C) in aerobic condition for assessing the oil and phenol degradation potential of isolated strains. For extraction of crude oil, 50 ml of water sample was mixed with 50 ml petroleum ether: acetone (1:1) in a separating funnels and was shaken vigorously to get a single emulsified layer. Acetone was then added to it and shaken gently to break the emulsification, which resulted in three layers. Top layer was a mixture of petroleum ether, crude oil and acetone; clumping cells make the middle layer and the bottom aqueous layer contains acetone, water and biosurfactant in soluble form. The lower two layers were discarded while top layer was collected in a sterilized beaker. The extracted oil was passed through anhydrous sodium sulphate to remove moisture. The petroleum ether and acetone was evaporated on a water bath. The gravimetric estimation of residual oil left after biodegradation was made by weighing the quantity of oil in a tarred vial.

For estimation of phenol in water samples, chloroform extraction method was employed. The sample was taken in distillation flask. The pH was adjusted to 3 with phosphoric acid and distilled water was added to make up the volume upto 300 ml. then sample was distilled to collect 250 ml of distillate and 5 ml of ammonium chloride solution was added to it to adjust the pH to 10. 1.5 ml of potassium ferricyanide and 4-aminoantipyrene solution each were added to the contents and allowed to react for 3 minutes. Thereafter, the colour was extracted in 10ml chloroform and measured on spectrophotometer at 510nm wavelength. The quantity of phenol was calculated with the calibration curve prepared.

$$Phenol\left(mgL^{-1}\right)=\frac{mg\ of\ phenol\ in\ standard\ curve}{ml\ of\ sample}\times 1000$$

Results and Discussion

Characterization of oil and phenol degrading bacteria

The 4 isolates that were able to utilize oil and phenol as carbon and energy source could potentially degrade oil as well as phenol were studied morphologically and by biochemical characteristics for identification (Table 1). By comparing with those mentioned in Bergey's Manual of Systematic Bacteriology, the bacteria were identified as *Alcaligene sodorans, Bacillus subtilis, Corynebacterium propinquum* and *Pseudomonas aeruginosa*. Further, these were confirmed by BD-BBL™ Crystal Mind Autoreader. The isolated strains were allowed to grow on six different medium i.e., Nutrient Agar Medium (NAM), Trypticase Dextrose Agar (TDA), Trypticase Soy Agar (TSA), Sucrose medium (SM), Mineral Salt medium (MSM) and Yeast Extract medium (YEM). The isolates showed maximum growth in Nutrient Agar medium and Mineral Salt medium, while other media were not found suitable for further studies.

Determination of bacterial growth

Bacterial counts of four selected isolates were estimated by plating quadruplicate samples onto Mineral Salt Medium containing oil and phenol for estimation of bacterial strain capable of degrading phenol and oil. A large increase in biomass was observed in first 7 days of the study. After 7 days, count increased very slowly. The optical density was used to monitor the biomass growth. The plot of optical density in relation to time is shown in (Figure 1).

Characteristics	*Alcaligenes odorans*	*Bacillus subtilis*	*Corynebacterium propinquum*	*Pseudomonas aeruginosa*
Gram staining	Negative	Positive	Positive	Negative
Cell shape	Bacilli	Bacilli	Bacilli	Bacilli
Motility	Motile	Motile	Non motile	Motile
Colony	Thin, irregular	Circular	Slimy	Round, raised
Catalase reaction	Positive	Positive	Positive	Positive
Nitrate reduction	Negative	Positive	Positive	Positive
H2S production	Positive	Negative	Negative	Negative
Indole production	Negative	Negative	Negative	Negative
Fermentation Tests	Negative	Negative	Negative	Negative
Dextrose	Negative	Negative	Negative	Negative
Lactose	Negative	Negative	Negative	Negative
Sucrose	Negative	Positive	Negative	Negative
Starch Hydrolysis	Positive	Positive	Negative	Positive
Oxidase Citrate Utilization	Negative	Positive	Negative	Positive

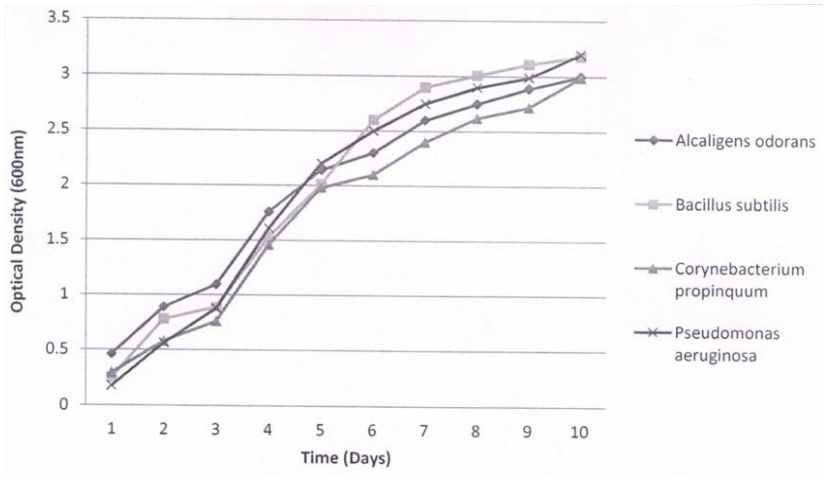
Table 1: Morphological and Biochemical Characteristics of isolates.

Biodegradation study of oil and phenol

The oil and phenol contents in the water samples were determined monthly for six months i.e., 6 samples were evaluated at the time interval of 30 days. In the study, the efficiency of crude oil and phenol degradation of the designed bacterial consortium was tested and results clearly showed that mixed bacterial consortium can carry out 70% degradation of oil and 85% of phenol as well. Rahman et al. [4] also showed degradation of oil upto 78% after incubation of samples for 20 days inoculated with the bacterial consortium. The minimum degradation of oil and phenol was observed to be 35% and 75% respectively. Percentage reduction in the oil and phenol contents is shown in (Figure 2).

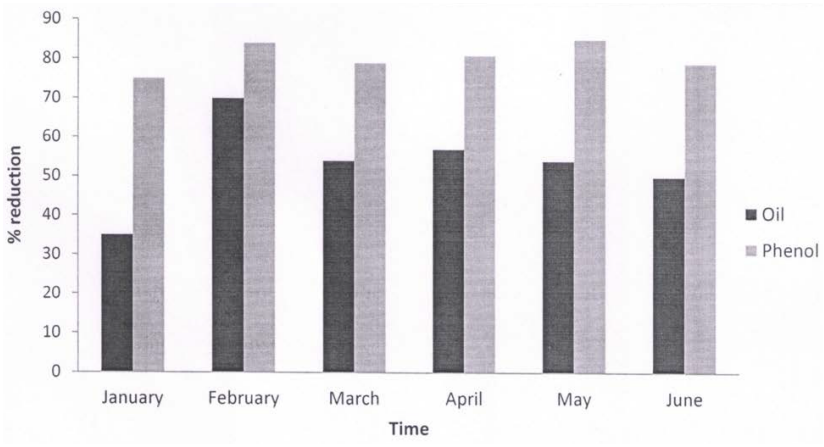
The oil contents were found to be 480-140 mgL^{-1} in the samples before treatment while after treatment, they ranged from 90-267 mgL^{-1}. The phenol concentration in the water samples was present in the range of 3.71-1.2 ppm. The consortium remediated the phenol and remained phenol in the sample was found to be 0.3-0.78 ppm (Table 2), that came under permissible limit according to the Environmental Assessment (EA). Since, all the microbes in the present study exhibited higher biodegradation of oil contents and phenols from the water. Survival of microorganisms in a medium containing petroleum after their inoculation is a key deciding factor in the rate of bioremediation of oil and phenol. They survived and adopted the oil contaminated liquid environment very easily as also reported by many scientists [6,7]. In agreement with the results obtained, some researchers earlier reported better degradation of phenol by the bacterial consortium upto 70% mainly consisting *Pseudomonas* sp., *Bacillus* sp., *Alcaligenes* sp. and *Corynebacterium* sp. [8-10].

Rahman et al. [4] also showed similar results in the case of oil degradation as the efficiency of bacterial consortium could carry out maximum of 78% degradation. This finding correlates with the present study as here is the observation of upto 70% degradation of oil from the waste water after 10 days of incubation. Several other workers [11,12] have described the ability of mixed bacterial consortia to degrade upto 60% crude oil. Apart from this, many other genera have also been reported for phenol degradation: *Acinetobacter, Agrabacterium, Burkholderia, Klebsiella, Ralstonia*and *Rhodococcus* [13]. Also the introduction of mineral medium helped in the process of biodegradation which is earlier supported by other workers [14].



Growth profile of bacterial isolates.

Figure 1: Growth profile of bacterial isolates observed during 10 days at 600nm wavelength.



Percentage (%) reduction of oil and phenol in waste water.

Figure 2: Reduction percentage in the oil and phenol contents of waste water after treatment with bacterial consortium.

Samples	Oil contents (mgL^{-1})		Phenol (ppm)	
	Untreated	Treated	Untreated	Treated
January	140	90	1.20	0.30
February	480	148	2.67	0.42
March	580	267	3.71	0.78
April	260	114	1.73	0.33
May	344	194	2.15	0.32
June	378	186	2.39	0.49

Table 2: Determination of oil and phenol in waste water before and after treatment of refinery waste water via bacterial consortium.

Conclusion

Bioremediation has an edge over other treatment methods because it can efficiently degrade oil and phenol from the waste water and doesn't allow the contaminant to accumulate. The present study clearly showed that by using the mixed bacterial consortium which can efficiently degrade the crude oil components and phenols as well, temperature of 37˚C and pH 7, maximum percentage of degradation can be achieved. The bacterial cells were able to utilize phenol and oil as the carbon and energy source. Furthermore, these strains show promise for possible utilization for waste water treatment. Hence we suggest use of the above optimized conditions and the mixed bacterial consortium for bioremediation of crude oil contamination.

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