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Laboratory Scale Experiment on the Bioremediation of Refined Petroleum Hydrocarbon by using CO_2 Evolution Method

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Abstract: **The purpose of the present study was to evaluate suitable method which enhances the bioremediation of petroleum hydrocarbon. Direct application of microorganisms originating from the remediation site was helpful in situ treatment for effective removal of petroleum hydrocarbon from polluted site. Laboratory scale experiments were used to assessment of refine petroleum oil such as petrol, diesel, kerosene and their synthetic mixture. The present work was to isolate and identify hydrocarbons degradation bacterial genera from contaminated site. In this study MSM (minimal salt media), BHB (bacto Bushnell Haas Broth) were used as a basic culture media. Using DCPIP (2,6 dichlorophenol indophenols sodium salt) indicator we were selected best two isolate. Upon morphological and biochemical test, it was determined that these strains belong to bacterial genera: *Pseudomonas spp. and Arthobacter spp*. Finally on the basis of turbidity *Arthobacter spp*. was selected for biodegradation purpose. Plackett-burman design was applied to screen culture condition. On the basis of this study nitrogen, potassium and phosphorus were selected which enhance the bacterial growth in presence of synthetic mixture oil as a single carbon source. Efficiency of biodegradation of *Arthobacter spp*. was measured by respirometric (microbial CO_2 production) method. The result showed that CO_2 production was higher in synthetic mixture oil (60%) and petrol (56.4%) under optimum condition, moderate in kerosene (53%) and low in diesel (17.45%) but very low in without optimum condition of synthetic mixture (13.12%).**

Keywords: ***Bioremediation, Bioaugmentation, DCPIP, Pseudomonas spp. and Arthobacter spp., plackett-burman design, respirometric method.***

1. INTRODUCTION

Uncontrolled and catastrophic release of petroleum poses ecological and environmental repercussions as a lot of hydrocarbon components are toxic and persistent in terrestrial and aquatic environments. Several physic-chemical methods of decontaminating the environment have been established and employed. Biological degradation a safe, effective and an economic alternative method, is a process of decay initiated by biological agents, specifically in this case by microorganisms. [1] Microbial decomposition of petroleum and petroleum products are of considerable important. Petroleum is a rich, source of carbon and the hydrocarbons within it are really oxidized aerobically with the release of carbon-dioxide by a verity of microorganisms in soil. [2] In biological treatment it is always necessary to perform laboratory feasibility test 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) treatment. [3]

Thus, the aims of the study were to investigate possible method to enhance the rate of aerobic bioremediation of refine petroleum oil as well as their mixture.

2. MATERIAL AND METHOD

Sources of soil sample: The petroleum contaminated soil samples were collected from Indian oil refinery (Haldia,W.B) and garages, filling stations and auto-mechanic workshops; petrol pump etc(from Haridwar) in a sterile containers transported in ice to the laboratory and duly labeled which were stored at -4^0C for further analysis.

Isolation, screening and identification of indigenous bacteria: Petroleum degradable bacteria were isolated by serial dilution method. Sterile physiological saline, i.e. 0.85% (w/v) sodium chloride was used as diluents.1 g of homogenized, 2mm sieved soil sample was aseptically transferred into a sterile test tube containing 9 mL of the diluents. Subsequently, three fold (10^{-3}) serial dilutions were prepared. 1mL aliquot of each dilution was aseptically spread onto oil-MSM (Minimal salt media) agar plate. Here Synthetic oil mixture (petrol, diesel and kerosene; 1:1:1) were used. Hanson et al (1993) [4] method was used for screening hydrocarbon degrading isolates. This method consisted of incorporating into the medium an electron acceptor dye such as 2, 6-dichlorophenol-indophenol (DCPIP) to test the ability of microorganisms to utilize the hydrocarbon substrate by

observing the color change of DCPIP from blue (oxidized) to colorless(reduced). This is one of the quick and reliable method for the screening of petroleum oil degrading bacteria. Identification was carried according on their morphology, colony physiological and biochemical characteristics, following the criteria described in theBergey's Manual of Determination Bacteriology [5].

Optimization of selected isolates: Plackett-Burman design is useful not only in evaluating the significant of some variables on the bioprocess, but also in comparing between different categories. Hence maintain a comprehensive evolution of the overall process. This design which is based on the linear first order regression model, offers a good and fast screening procedures and gives the effect of change of more than one factors in single experiment.[6] Seven variables such as (i)Tempeterure, (ii) pH, (iii) protein source(Peptone), (iv) Potassiusm source (KH_2PO_4, $K_2HPO_{4)}$,

Table 1. Coded value and actual values of the variables.

Variables	Coded levels	
	-1	**+1**
Temperatur	37 ^{0}C	42^{0}C
pH	5	7
Peptone	.001%(w/v)	1.5% (w/v)
KH_2PO_4	.005%(w/v)	2%(w/v)
K_2HPO_4	.001%(w/v)	2%(w/v)
NaH_2PO_4	.001%(w/v)	1%(w/v)
Na_2HPO_4	.005%(w/v)	1.55%(w/v)

(v) Phosphorous source (NaH_2PO_4, Na_2HPO_4) were selected as the various nutritional parameters for Plackett-Burman design in this study. The concentrations for the different variables were selected according to some preliminary experiments (data not shown). The above said seven variables affecting the growth were screened at two levels, maximum (+) and minimum (-) [Table1]. This said concentrations were mixed in 100 ml distilled water and added 1ml inoculums and 0.1ml petrol. Now experiments were carried out as per the design in Table 5.They were performed in duplicates and the average growth was taken as the response (Y).Growth (i.e O.D) was read at 600 nm after 72 hours.

Selecting best potential isolate by Turbidometry: BH broth(50mL) in each was prepared in three set of each isolate and after autoclave 5mL sterile (sterilized by filtration, Millipore size, 0.25 mm) petrol, diesel and kerosene and 1 mL inoculums (bacterial number was adjusted to give initial cell number 1X 10^8 CFU mi^{-1}) of each isolate was added . Flasks were agitated at 180rpm at 30^{0}C on a rotary shaker for seven days. Optical density of inoculated and un inoculated control were .measured by spectrophotometer at 600 nm

Study on bioremediation by Co_2 evolution method: Laboratory bioremediation studies were carried out under optimum condition for assessing the hydrocarbon degradation potential of best potential strain. BH broth was prepared in five sets for refine petrol, diesel, kerosene, mixture of refine oil (with optimum condition) and another mixture of refine oil for without optimum condition and subjected to treatment for 10, 20, 30 days. Produced CO_2 was trapped in a 10 mL solution of Barium hydroxide [$Ba(OH)_2$] (0.2N), located in a reservoir was suspended in the flask. During this procedure, the flasks were aerated periodically.

Measurement of CO_2 evolution: Periodically, the 10 ml of $Ba(OH)_2$ plus 10 ml rinsing water (DIW) was removed for CO_2 measurement by titration with 0.1 N HCl to the phenolphthalein end point. The percentage CO_2 evolution-was based on the following formula:

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

where,

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

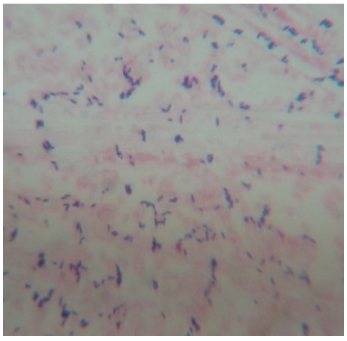
CF = ml of 0.1 N HCl required to titrate Ba(OH)I samples from the control flask;

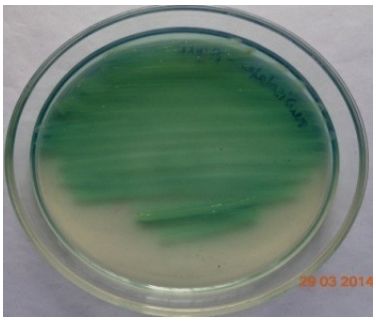
C = A constant which is equal to the theoretical amount of O.lN HCl required to titrate ah the CO2 evolved from metabolizing total carbons in the test substrate by bacteria.

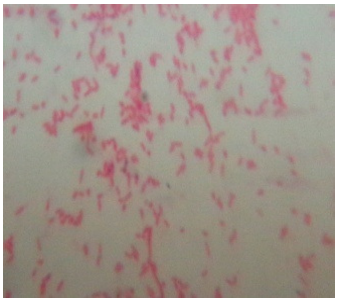
3. RESULT AND DISCUSSION

Isolation, screening and identification for biodegradation potentials: Two bacterial isolates were selected on the basis of growth on oil MSM media as well as screening test and characterized on the basis of morphological (Table 2), cultural (Table 3) and biochemical characteristic(Table 4) according to bergey's manual of Determinative Bacteriology (9th Edition). The selected strains were *Arthobacter spp.* [Code no: 4(9)] and *Pseudomonas spp.* [Code no:5(1)]

(A)







(B) **(C)** **(D)**

Fig 1: Colony of *Arthobacter spp.* (A & B) *and Pseudomonas spp.*(C & D) on plate and microscopic view respectively.

Table 2: Morphological characteristics of selected bacterial isolates

Name of isolate	Size	Shape	Gram's reaction	Motility
4(9)	Very Small	Rods	Gram positive	Motile
5(1)	Small	Rods	Gram negative	Motile

Table 3: Cultural characteristics of selected isolates

Name of isolate	Size	Shape	Elevation	Margin	Opacity	Texture	Pigment
4(9)	Pin point	Circular	Raised	Entire	Opaque	Smooth	White
5(1)	Big	Irregular	Slightly raised	Irregular	Opaque	Smooth	Bluish green

Table 4: Biochemical characteristics of selected isolates

Name of isolate	Citrate test	Urease test	Nitrate test	Oxidase test	Gelatin test	Starch Hydrolysis	Indole test	M.R test	V.P test
4(9)	+ ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve
5(1)	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve

Table 5: Two-level Plackett-Burman design for microbial growth

Exp	Tem	pH	Pep^{1*}	K^{2*}	$K_2{}^{3*}$	Na^{4*}	$Na_2{}^{5*}$	O.D	
								5(1)	4(9)
1	+	+	+	-	+	-	-	.054	.070
2	-	+	+	+	-	+	-	.063	.075
3	-	-	+	+	+	-	+	.056	.087
4	+	-	-	+	+	+	-	.028	.034
5	-	+	-	-	+	+	+	.044	.026
6	+	-	+	-	-	+	+	.036	.059
7	+	+	-	+	-	-	+	.042	.020
8	-	-	-	-	-	-	-	.034	.020

1*=Peptone, 2*=Patassium dihydrogen phosphate, 3*= Dipotassium hydrogen phosphate, 4*=Sodium dihydrogen phosphate, 5*-Disodium hydrogen phosphate.

Optimization of selected isolates: Based on bacterial growth by taking O.D at 600 nm, peptone, potassium di-hydrogen phosphate promoted the growth of both the strains but Temperature, pH, sodium di-hydrogen enhance the growth of *Pseudomonas Spp.* whereas di potassium hydrogen phosphate promotes the growth of *Arthobacter Spp.* (which were shown through ANOVA Table 6). The regression coefficient R^2 (0.99) of both the species ensure the adequate integrity of the model (Table7).

Table 6: ANOVA Results

Paremeters	p-value	Prob > F
	5(1)	4(9)
Model	.020	.027
A-TEMP	.017	.076
B-pH	.013	.204
C- peptone	.010	.010
D-KH_2PO_4	.030	.046
E-K_2HPO4	.090	.044
F-NaH_2PO4	.042	.102
G-Na_2HPO4	.957	.257

Selecting best potential isolate by turbidometry: Out of two strains obtained indigenously from the soil, *Arthobacter spp.*was found to be more efficient refine oil utilize as observed by the turbidity (Table 8) So this strain was used for degradation study purpose.

Table 7: Regression analysis

Paremeters		
	5(1)	4(9)
R^2	.999	.999
Adjusted R^2	.991	.998
Predicted R^2	.992	.985
Adequate Precision	105.83	67.53

Study on bioremediation by Co_2 evolution method: The present accumulated CO2 evolution of three refine oil (petrol, diesel and kerosene) and their mixture in presence of optimum condition and without optimum condition was summarized in Table 9. The maximum % of CO2 evolution from the flasks of synthetic oil mixture and petrol with optimum condition were 60% to 56.4% in 10 days. The maximum % of CO_2 evolution of kerosene was 53.28% which was slightly lower than their mixture. Yet, the CO_2 evolution from the diesel flask was only 17.45% and without optimum condition *Arthobacter Spp.* was unable to degrade oil mixture that's why the % of CO_2 evolution in mixture oil without optimum condition was shown only 13.12%. The increase of the % of accumulated CO_2 evolution with time was graphically shown in Fig2.

4. CONCLUSION

CO_2 production by microbial activity and respiration confirmed that *Arthobacter spp.* had a potent role in bioremediation of refine oil. Bioremediation strategies to enhance the biodegradation of refine oil showed satisfactory results. The shortage of optimum condition or cultural conditions were the principle limiting factors of the process because in presence of optimum condition synthetic oil mixture as well as in petrol, the CO_2 evolution rate were 60 to 56.4% where as in absence of optimum condition the CO_2 evolution rate was only 13.12%.The effect of nutritional requirement was in agreement with other studies recommend that added of nutritional parameter can enhance the bioremediation.

Table 8: Turbidometry of two bacterial isolate. (Values were the mean + SE of 3 replications)

Name of the strain	Petrol	Diesel	Kerosene
Pseudomonas spp	0.09+(2.26X 10^{-3})	0.10+(1.32X 10^{-3})	0.13+(5X10 $^{-4}$)
Arthobacter spp	0.15+(1.77X 10^{-3})	0.10+0.031	0.15+(1.6X 10^{-3})
Name of the strain	Petrol	Diesel	Kerosene

Table 9: CO_2 evolutions from different refine oil and their mixture

Days	CO_2 evolution (%)				
	Petrol	Diesel	Kerosene	Mixed oil + opt. condition	Mixed oil- opt. condition
0	0	0	0	0	0
10	56.4	17.45	53.28	60.40	13.12
20	55.81	6.68	19.45	52.24	2.5
30	2.23	0.18	0.69	0	0.6

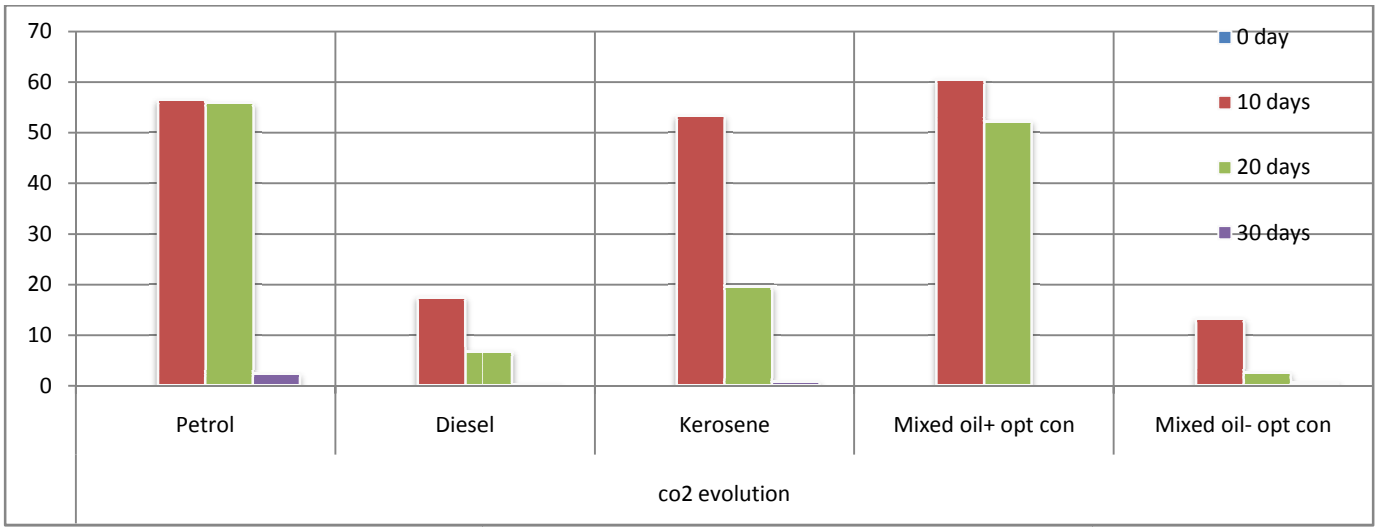


Fig 2: Showing CO2 evolution from different refine oil and their mixture

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