Bioremediation of Soil Contaminated from Petroleum hydrocarbons leaked at Petrol Station, Kota

ABSTRACT

Soil contaminated with hydrocarbons of petroleum and its products need a very cost effective process of remediation which is known as bioremediation. There are numerous factors which affect the efficiency of bioremediation process which generally includes microbial population, environmental conditions and on composition of hydrocarbon spills. The main motive of present work was to find out possible methods to increase the rate of degradation of hydrocarbons by bacteria aerobically (*ex–situ* treatments). In current work, application of bioremediation process were done on sandy soil collected from petrol stations of Kota which has been contaminated with diesel oil, leaked from underground storage tank. General microbiological laboratory procedures and experiments were used to evaluate the results of biodegradation of the diesel oil contaminated soil. Biostimulation (addition of Tween 80 surfactants and phosphorus-nitrogen solutions) and Bioaugmentation by bacterial consortium were used to enhance the biodegradation process. The present work was to focus on the biological activities and their effect on limiting nutrients in control conditions. Respirometric methods were used to measure the efficiency of biodegradation process. The present investigation results showed that natural bioremediation of diesel contaminated soil can be achieved by the biological agents (especially bacteria). It has been observed by respirometric data indicating that 5% removal of total petroleum hydrocarbons (TPH) in 50 days treatment. Predominantly *Bacillus spp.*,

Staphylococcus spp. and Pseudomonas spp. of bacteria from soil were isolated and identified at the end of the experiment.

Keywords- Bioagumentation, Biostimulation, Bioremediation, Bacterial Consortium, Biodegradation

1. INTRODUCTION

Petrol and diesel are essential compounds for vehicles and motors to run and required in day to day life. But many times these compounds may create problems when get leaked into soil. Petroleum hydrocarbons contaminate the soil by affecting the soil health and life. Petrol and petroleum products can destroy the fertility of soil and can harm the useful microbes present by decreasing their number. Leakage of petrol and diesel in nearby soil at petrol pumps is a very common problem. This can be unnoticeable if leakage is at small level concentrations. But it can be easy to see or smell if leakage of oil occurs at large scale concentrations. The discoloration of soil contaminated with petroleum hydrocarbons as compared to nearby areas and poor or less vegetation growth shows the level of contamination. This problem can be reduce or degrade by using environmental friendly methods, such as bioremediation. Bioremediation is a process by which microorganisms are used to reduce or degrade environmental hazards. Accumulation of toxic chemicals and wastes creates environmental hazards in the nature. Bioremediation is the way to speed up the process of waste degradation naturally and by these naturally occurring microbes can be recycled as fungi, bacteria and yeasts cells are used to degrade hazardous pollutants in the soil, air and water into less toxic or non-toxic substances. Microorganisms digest contaminants like nitrates, carbon tetrachloride and oil into water, carbon dioxide and other byproducts and give off these products.

Biological method creates the conditions to detoxify the contaminants by microorganisms to flourish and perform their metabolic activities. Microorganism uses contaminants as energy source to do metabolic activities during bioremediation process. The main concept of this is to give necessary requirements to microorganisms and to promote organisms for degradation process. Bioremediation methods can be applied on the same site or ex-situ means to increase the growth of micro flora which are locally present on the site or addition of microbial consortium within the desired characteristics. In in-situ remediation treatment of contaminated soil occurs at

the same location where it is found, but in ex-situ remediation contaminated soil treated elsewhere from its original place or excavation of sample for the treatment.

Scientists have worked a lot to identify the best solution to clean up the hydrocarbon contaminated sites. They have researched on specific microbes which can help to reduce the level of contamination. Microorganisms involved in bioremediation are; *Bacillus, Pseudomonas, Flavobacterium, Dechloromonas* etc. Basically two bioremediation approaches used for the treatment of contaminated soil are biostimulation and bioagumentation.

Hydrocarbon compounds are essential elements for human life. Leakage from underground storage oil tanks causes serious problems of contamination to soil, soil microbes and water bodies. Oil spills from tanks and pipelines causes pollution in water and soil which can be severe to human health and can cause cancer.

Harmful effects of hydrocarbons pollution:

Medical and health issues occur in humans.

Soil characteristics like physiochemical and biochemical properties changes due to petrol and diesel oil contamination. Soil becomes unfit for vegetation growth.

Growth and development of plants become very low due to soil pollution. Due to this pollution soil's nutritive value degrade.

Hydrocarbons show inhibitory effect on microbial biomass and phytotoxic effect on agricultural crops present in soil.

When petroleum products (petrol and diesel) deeply penetrate into the soil causes disturbance in the biogeochemical cycles and shows worse effect on biotic and abiotic components.

Petroleum hydrocarbons are serious concern for many countries, so it becomes very important to bring soil in its original form in natural way (bioremediation). From last few years scientists working on this problem and conducting cost effective techniques to remove contaminants from the soil without much damaging to the environment. The current objectives of our work is to investigate the efficiency of bioremediation on soil contaminated from petrol and diesel oil by respirometric method (carbon dioxide

production by microbes) and isolation and identification of bacterial consortium from the contaminated soil sample. Aim of this research is to find out possible solution for the invention of new processes which reduces time and efforts for bioremediation. Bioremediation process is a process which generally based on microbial metabolic activities. The available technologies to clean up the contaminated soil by these methods are also one of the main factors of bioremediation. Remediation by biological methods has several advantages over chemical methods. These are as follows:

Detoxification of hazardous substances by biological based remediation creates low or minimal toxics and non-transferring from one environment to another.

Bioremediation are cost effective techniques to treat contaminated sites as compare to conventional treatments.

In comparison to excavation based processes, bioremediation are effective and less disruptive to the environment.

2. METHODOLOGY

2.1 SAMPLE COLLECTION:

Sample was collected from petroleum station at Jawahar Nagar, Kota where underground diesel leakage from the tank has been occurred in nearby site. Site for sample collection was identified by comparing the discoloration of soil due to hydrocarbon contamination. Soil sample collected have great amount of oil and leaked to the ground water. Capillary fringe of depth 1-2 feet was used to collect the sample. Sample then stored at 4^0 C at normal refrigerator temperature.

2.2 BIOREMEDIATION ON DIESEL CONTAMINATED SOIL:

This work deals with the process of bioremediation on soil collected from underground storage tank of petrol pump from Jawahar Nagar, Kota.

Microbial CO_2 production was measured by using Flask arrangement to biodegrade diesel oil. 500 mL flask arrangement was used for carrying out biodegradation experiment.

Biodegradation process was enhanced by biostimulation i.e. adding N and P solution or tween 80 surfactants and bioaugmentation i.e. treated with bacterial consortium inoculum (which is known culture of *Bacillus* species).

The impact of three variables (addition of N and P content, tween 80 surfactant and bacterial consortium) on pollutant biodegradation was present in collected soil sample.

Homogenization of soil sample was done by blending and altered solutions were added.

Corrections were performed of Nitrogen by using $(NH_4)2.SO_4$ (1218 mg/200 g of soil) and Phosphorus by using KH_2PO_4 (195 mg/200 g) solutions. Thus C:N:P nutrient ratio was adjusted to 100:15:1.

Addition of tween 80 surfactant and bacterial consortium i.e. *Bacillus* spp. Culture.

Water content of soil changed to 21.8% by considering the inclusion of amendments. 44.5 mL water was added.

Incubation was done in the dark for 40 days at room temperature and shaked regularly.

CO_2 produced was captured in 100 mL KOH solution (0.2N) in the side arm of the Flask arrangement. Measurement of biodegradation efficiency was done by respirometric methods (production of microbial CO_2) by titration.

Periodically withdrawn of KOH solution was done by using syringe after 15 days. Residual KOH (10 mL) was used to titrate the amount of CO_2 absorbed; 1 mL phenolphthalein indicatior (giving pink colour) was added after and titrated it with HCL standard solution (0.1N).

Same method was followed and repeated after 40 days.

Determination of Total hydrocarbon Content:

Initial hydrocarbon content in the soil sample was measured using silica crucible which was heated up to red hot. After this crucible was removed from the burner and allowed to cool at room temperature. Now weight was recorded. Sample kept in oven at 100^0C for

1hour to remove it moisture. Place the sample in the crucible and weight was taken again. Crucible was heated again. Due to heating of the crucible (becomes red hot) oil fumes were produced and later no fumes were produced, this leads to complete evaporation of organic matter from the sample. After complete evaporation crucible were taken out from the burner and weighted again after cooling at room temperature. The difference between previous weight and later weight was calculated and this was the Total Petroleum Hydrocarbon (TPH).

2.3 MICROBIAL ISOLATION:

Isolation of microorganisms was done by general microbiological laboratory methods. Serial dilutions up to 10^{-6} were prepared in distilled water from contaminated soil sample. General bacteriological media i.e. nutrient agar was prepared for inoculation. Soil samples from different dilutions were inoculated on nutrient agar plates and incubated for 24-48 hours at 37^0 C.

2.4 MICROBIAL IDENTIFICATION:

Cells grown on nutrient agar plates were used for identification by Gram staining and endospore staining techniques. Biochemical test by Carbohydrate test, Indole production test, Catalase test, Oxidase test, Urease test, Citrate utilization test and Nitrate reduction test were performed for the identification of microbes.

3. OBSERVATIONS

3.1 Determination by titration of Co_2 absorbed by KOH :

After 20 and 40 days of observation showed that hydrocarbon present was converted into CO^2 which reacts with KOH solution. When titrated with 0.1 N HCl we got the following readings:

Titration days	Volume of KOH(mL)	Used volume of HCl (mL)		
		A	B	C
20	10	2.3	2.0	2.0

40	10	6.9	6.6	6.6

3.2 Bacterial isolation:

Bacterial colonies on agar plates were observed for numbers and colonial distribution which show the following observation:

S.No.	Sample Dilutions	No. of Colonies
1	10^{-3}	248
2	10^{-4}	213
3	10^{-5}	159
4	10^{-6}	131

3.3 Bacterial identification:

24 hours old cultures grown were used for staining and for morphological studies further biochemical tests were performed which shows the following observations:

Name	Type of Bacteria	Shape	Appearance	Carbo-hydrate test	Indole Test	Catal-ase test	Oxi-dase test	Urease test	Citrate test	Nitrate reduction Test	Spore Forming Bacteria
A	Gram -ve	Rod	Brownish Pigment	(-ve)	-	+	+	-	+	+	-
B	Gram +ve	Rod	White	(-ve)	-	-	+	-	-	-	-
C	Gram	Rod	Translu-	(+ve)	-	-	-	-	+	-	-

	+ve		cent								
D	Gram +ve	Rod	Yellow	(-ve)	-	-	-	-	-	+	-
E	Gram +ve	Rod	White	(-ve)	-	-	+	-	-	+	+
F	Gram +ve	Cocci	White	(+ve)	-	+	-	-	-	+	-

4. RESULTS

At the end of the work we got the following results:

4.4 Results of Titration:

Calculation of Total Degraded Hydrocarbon:

After 20 days: Taken initial KOH solution 0.1N = 10mL

Titrated with 0.1 HCl

Volume of HCl were consumed = 2.3mL

Total used KOH in CO_2 = 2.5mL

2.5*0.1N = 0.1N*2.5mL CO_2 (mol. wt. 44)

= 2.2g in 1000mL

= 2.2*2.5/1000g CO_2 in 2.5mL

Total degraded hydrocarbon = 0.0055g CO_2 in 2.5mL

After 40 days: Taken initial KOH solution 0.1N = 10mL

Titrated with 0.1 HCl

Volume of HCl were consumed = 6.9mL

Total used KOH in CO_2 = 3.5mL

3.5*0.1N = 0.1N*3.5mL CO_2 (mol. wt. 44)

= 2.2g in 1000mL

= 2.2*3.5/1000g CO_2 in 3.5mL

Total degraded hydrocarbon = 0.0077g CO_2 in 3.5mL

CALCULATION OF BIODEGRADATION EFFICIENCY:

After 20 days: The biodegradation efficiency can be expressed as:

. BE% = (total biodegraded carbon / initial soil organic carbon content) .100

BE% = (0.0055 / 0.1559). 100

BE% = 3.527%

After 40 days: The biodegradation efficiency can be expressed as:

BE% = (total biodegraded carbon / initial soil organic carbon content) .100

BE% = (0.0077 / 0.1559). 100

BE% = 4.939%

4.2 BACTERIA ISOLATION:

Formula for calculating number of bacterial colonies grown on nutrient agar plates:

Dilution 10^{-3}: No. of cells/mL. = $248/1*10^{-3}$

Dilution 10^{-4}: No. of cells/mL. = $213/1*10^{-4}$

Dilution 10^{-4}: No. of cells/mL. = $159/1*10^{-5}$

Dilution 10^{-4}: No. of cells/mL. = $131/1*10^{-6}$

4.3 BACTERIA IDENTIFICATION:

Microbes grown on differential media were identified and various species of bacteria identified by staining and biochemical tests. *Staphylococcus* spp. were identified as whitish color, gram positive coccus shaped, catalase positive bacteria. *Bacillus* spp. was identified as gram positive, rod shaped and nitrate positive. *Pseudomonas* spp. was identified as gram negative, brownish, rod shaped and citrate positive bacteria.

5. DISCUSSION

Refined products of crude oil are known as petroleum products which include diesel fuels, kerosene etc. crude oil and these products are composed of petroleum hydrocarbons. Their characteristics depend on the amount of carbon present in their molecular structure. Problems can be increase by many of them if present in soil. Petroleum products may cause severe health risk to humans if transported from soil to water resources as these products extremely mobile. Toluene, Benzene, Xylene and Ethylbenzene are some of examples of petroleum hydrocarbons which cause cancer. Contaminated soil also releases the fumes of petroleum hydrocarbons in the atmosphere which generally get inhaled by the humans. Some of the petroleum hydrocarbons are carcinogenic at very low levels. Most of the petroleum hydrocarbons are broken down into less harmful smaller products in human body. Inhalation of fumes of petroleum hydrocarbons or ingestion of contaminated soil creates health issues to adults and children. Therefore reducing the level of petroleum hydrocarbons from soil is a need to be removed on a large scale. The results of our work will help to reduce or remove the level of contamination occurs due to petroleum hydrocarbons in soil of petrol pumps by which we can try to improve the fertility and quality of soil. This is very little effort and done on a small area but if major concern occurs towards this problem we can achieve a big goal.

6. CONCLUSION

Satisfactory results got for the strategies used for bioremediation to increase the biodegradation of diesel oil contaminated soil from petrol pump. When all the amendments were added the efficiency in terms of mineralization showed the doubling process of biodegradation after during 40 days of treatment with removal of Total Petroleum Hydrocarbon (TPH). The main limiting factor was the nutrient shortage. Bacteria helpful in bioremediation process were *Pseudomonas* spp., *Bacillus* spp. and *Staphylococcus* spp. Bioremediation process mainly depends on the effect of bioaugmentation with bacterial species.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Pol J Microbiol. 2019 Jun; 68(2): 193–201.

Published online 2019 Jun 28. doi: 10.33073/pjm-2019-019

PMCID: PMC7256828

PMID: 31250589

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Journal List

Saudi J Biol Sci

v.29(1); 2022 Jan

PMC8717088

Saudi J Biol Sci. 2022 Jan; 29(1): 211–216.

Published online 2021 Aug 26. doi: 10.1016/j.sjbs.2021.08.069

PMCID: PMC8717088

PMID: 35002411

Bioremediation potential of hydrocarbon degrading bacteria: isolation, characterization, and assessment

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Bioremediation of Petroleum hydrocarbon by using Pseudomonas*species isolated from Petroleum contaminated soil*

ISSN : 0970 - 020X, ONLINE ISSN : 2231-5039 30 Dec 2014Volume 30, Number 4

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DOI : http://dx.doi.org/10.13005/ojc/300436