

# Bioremediation of hydrocarbon contaminated gasoline station soil by a bacterial consortium

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## Abstract

A study was undertaken to find methods for enhancing rates of hydrocarbon biodegradation in gasoline-contaminated soil by *ex-situ* bioremediation. Garden soil was treated with gasoline-spilled soil from a gasoline station and different combinations of amendments were prepared using mixed bacterial consortium, poultry litter, coir pith and rhamnolipid biosurfactant produced by *Pseudomonas aeruginosa*. Bacterial growth, hydrocarbon degradation and growth parameters of *Phaseolus aureus* RoxB (including seed germination, chlorophyll content, shoot and root length) were measured for a period of 90 days. Approximately 78% of the hydrocarbons were effectively degraded within 60 days in soil samples amended with all additives. Maximum germination rate, shoot length, root length and chlorophyll content in *Phaseolus aureus* were each recorded after 60 days. Further incubation to 90 days did not cause significant improvements. Statistical analysis using Analysis of Variance and Duncan's Multiple Range test revealed that the level of amendments, incubation time and combination of amendments significantly influenced bacterial growth, hydrocarbon degradation, seed germination and chlorophyll content (1% probability level). All tested additives, including rhamnolipid biosurfactant, had significant positive effects on the bioremediation of gasoline-contaminated soils.

**Keywords:** *bioremediation, gasoline spilled soil, amendments, mixed bacterial consortium, poultry litter, coir pith, biosurfactant, Phaseolus aureus RoxB.*

## 1 Introduction

Contamination with petroleum hydrocarbons has caused critical environmental and health defects and increasing attention has been paid to developing and



implementing innovative technology for cleaning up such contamination [1]. During accidental spills, action can be taken to remove, recover or remediate the contaminant immediately, whereas in gasoline stations, the spills due to leakage may be minor but are continuous and prolonged. Because of its persistence, the potential for contamination of groundwater is high. Bioremediation methods are currently receiving favourable publicity as they offer environmentally friendly treatment technologies for the remediation of hydrocarbons [2].

The aim of amendments is to improve the fertility status of such soils and to enhance the rate of oil degradation, thus minimising the potential for contamination of groundwater and improving crop production [3]. The addition of organic waste material such as poultry litter and coir pith to the soil facilitates aeration and increases the water holding capacity of the soil, thus enhancing bioremediation [3,4]. This study was designed to test the use of mixed consortium (MC), poultry litter (PL), coir pith (CP) and biosurfactant (BS) on gasoline spilled soil (GS) and study the bioremediation potential by observing bacterial growth, oil degradation and growth parameters of green gram (*Phaseolus aureus* RoxB).

## 2 Methods

### 2.1 Sample preparation

The Red soil [5] and gasoline contaminated soil samples collected from 10 different gasoline stations were mixed thoroughly and used for the preparation of amendments. Crude oil degrading mixed bacterial consortium containing five strains (*Micrococcus* sp. GS2-22, *Bacillus* sp. DS6-86, *Corynebacterium* sp. GS5-66, *Flavobacterium* sp. DS5-73, *Pseudomonas* sp. DS10-129) previously isolated on a hydrocarbon-containing medium [5] were added to 200 ml of nutrient broth and kept in a shaker for 24 h at room temperature. The members of the mixed consortium were selected by their efficiency of crude oil degradation [5]. For the preparation of amendments, the poultry litter was collected from a Poultry farm; air-dried and sieved (to less than 0.5mm). Composted coir pith used in this study was available locally for soil conditioning. Rhamnolipid biosurfactant was produced by *Pseudomonas* sp. DS10-129 [6,7].

### 2.2 Amendments

The experiment was set up as a factorial design consisting five treatments and a control: 1) RS (Control); 2) RS+GS; 3) RS+GS+MC; 4) RS+GS+MC+PL; 5) RS+GS+MC+PL+CP; 6) RS+GS+MC+PL+CP+BS, five time periods (1, 15, 30, 60 & 90 days) and three replicates per treatment or time period (Table 1).

Triplicate sets of amendments were prepared to enumerate total heterotrophic bacterial counts, percentage of oil degradation and ability to support growth of green gram (seed germination, root and shoot length and chlorophyll content) at each time interval and the mean values computed. Statistical analysis was carried out using Analysis of Variance (ANOVA). Means of the various treatments were



tested for level of significance at 1% and 5% probability by Duncan's multiple range test (DMRT) [8].

Table 1: Preparation of various amendments of soil.

S. No.	Amendment	Constituents *					
		RS	GS	MC	PL	CP	BS
1.	RS	100					
2.	RS+GS	100	10				
3.	RS+GS+MC	100	10	1			
4.	RS+GS+MC+PL	100	10	1	1		
5.	RS+GS+MC+PL+CP	100	10	1	1	1	
6.	RS+GS+MC+PL+CP+BS	100	10	1	1	1	1

RS – Red soil, GS – Gasoline spilled soil, MC – Mixed consortium,

PL – Poultry litter, CP – Coir pith and BS – Biosurfactant solution

\* Units for RS, GS, PL, CP are in grams and for MC and BS in ml

### 2.3 Enumeration of bacteria

Total heterotrophic bacteria (THB) were enumerated in all the treatments by using pour plate technique on plate count agar (Oxoid, UK), which also allowed the growth of all the members of the added MC [9].

### 2.4 Hydrocarbon estimation

Total hydrocarbon contents were determined spectrophotometrically [10] and gas chromatography with flame ionization detector was used to determine the undegraded n-alkane [11].

### 2.5 Growth of green gram *Phaseolus aureus* (RoxB)

The experimental soil samples were set up in 250 ml plastic pots; 10 green gram seeds were placed into each pot at 2 cm depth and all pots were watered regularly. The treated seeds were allowed to germinate and germination percentage was assessed on the 5th day of experiment. At the 10th day of the plantation, the shoot length and root length were measured, mean length calculated and the chlorophyll content estimated colorimetrically as described by Sadasivam & Manickam [12].

## 3 Results and discussion

On the 60<sup>th</sup> day, the bacterial population was highest in RS+GS+MC+PL+CP+BS amended soil indicating the role of the additives in the enhancement of the bacterial population. The untreated red and gasoline spilled soil mixture showed no significant increase in the bacterial populations between day one to day 90 at 1% probability level. Increasing numbers with time in all



the other treatments indicated incremental improvements in soil nutrient availability in soils treated with PL  $\pm$  CP  $\pm$  BS (Figure 1). Our results are similar to the findings of Gian & Jianmei [13] for gasoline-contaminated soil amended with poultry litter.

The hydrocarbon degradation reached a maximum of 78% when all supplements were added to the contaminated soil indicating a recalcitrant hydrocarbon content of 22%. With the addition of each amendment the maximum hydrocarbon degradation increased from approximately 2.0% to 77.3%. Based on our results it appears that addition of biosurfactant to soils contaminated with gasoline was highly effective in increasing hydrocarbon degradation.

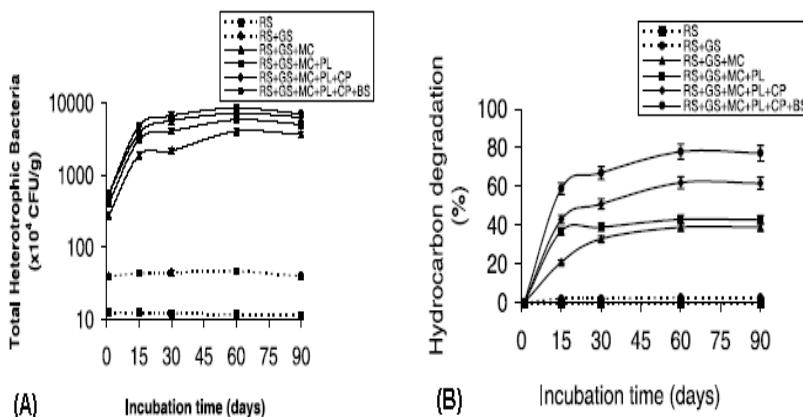


Figure 1: A. Bacterial growth and B. Total Hydrocarbon degradation for each amendment during treatment.

The addition of the mixed consortium increased the number of hydrocarbon degrading bacteria and showed a proportionate increase in the amount of hydrocarbon degraded. This observation is in general agreement with the earlier reports regarding the use of bioaugmentation, which is best employed in situations of very high (as here) or very low levels of contamination [14]. Furthermore, the soil treated with the mixed consortium only, degraded substantially less hydrocarbon than when the soils contained all the other additives. The organic amendments supplied might also have increased the bacterial population (indigenous and seeded), which so further enhanced the degradation of hydrocarbons. Furthermore the surfactant applied might have played a role in emulsifying the hydrocarbon, which may then have been more readily available for degradation by the bacterial population.

Table 2: Significance level for the different parameters tested within our treatments computed by Duncan's Multiple Range Test (DMRT).

Parameter	Bacteria ( $\times 10^4$ CFU/g)				Hydrocarbon degradation (%)				Seed germination				Shoot length (cm)				Root length (cm)				Chlorophyll content (mg/g)			
Factorial Effect	SE	CD	SL	SE	CD	SL	SE	CD	SL	SE	CD	SL	SE	CD	SL	SE	CD	SL	SE	CD	SL			
Concentration (C)	18.26	35.79	**	0.32	0.63	**	0.75	1.47	**	0.07	0.1	**	0.06	0.11	**	0.08	0.15	**						
Amendment (A)	28.87	56.59	**	0.50	0.99	**	1.19	2.33	**	0.11	0.2	**	0.09	0.18	**	0.01	0.02	**						
Days (D)	31.63	61.99	**	0.55	1.09	**	1.30	2.56	**	0.13	0.2	**	0.10	0.20	**	0.01	0.02	**						
C x A	44.73	87.67	**	0.78	1.54	**	1.84	3.62	**	0.18	0.3	**	0.14	0.28	**	0.02	0.03	**						
C x D	40.83	80.03	**	0.71	1.40	**	1.68	3.30	ns	0.16	0.3	ns	0.13	0.26	ns	0.02	0.03	**						
A x D	70.28	138.6	**	1.24	2.44	**	2.92	5.72	**	0.29	0.5	**	0.23	0.45	**	0.03	0.06	**						
C x A x D	100.0	196.0	**	1.76	3.46	*	4.13	8.09	*	0.41	0.8	ns	0.32	0.64	ns	0.04	0.08	**						

SE - Standard Error, CD - Cumulative Difference, SL - Significant level,

\* Significant at 5% probability level,

\*\* Significant at 1% probability level, ns - not significant at 1% or 5% probability levels



The plant growth study showed that germination efficiency of the *Phaseolus aureus* RoxB seeds in the uncontaminated soil RS was generally above 90% (in all but one treatment). The percentage of seed germination ranged from 20 to 90% in the amendments (data not shown). The effect of oil degradation and possible release of toxic metabolites during degradation may also have lead to the reduction of the growth rate. The addition of MC to the gasoline-contaminated soil did not result in any significant recovery in the shoot length (1% probability level) probably due to the presence of some residual toxic metabolites. Successive further amendments (PL, CP, BS) resulted in an increase in the shoot length with time to values similar or slightly higher than the controls. Root length and chlorophyll content also exhibited similar trends when compared to the shoot length responses, a confirmation of the positive effect of the amendments. Statistical analysis of results (Table 2) revealed that all measured parameters were highly influenced by single factors (concentration (C), amendments (A), number of days (D) treated), two factor combinations (C x A, A x D or C x D) and the three-factor combination (C x A x D) all at 1% probability level.

In general, hydrocarbon contamination reduced both seed germination and plant growth rate. Hydrocarbons may coat plant roots influencing water and nutrient absorption [15]. Hydrocarbon molecules can penetrate into plant tissues and damage the cell membranes causing leakage of cell contents and block intercellular spaces to reduce metabolite transport and respiration rates [16].

However, the severity of the effects of hydrocarbons on plant growth varies with the constituents, amount of the hydrocarbons and with the plant species involved. In this experiment the improvement of the plant growth for green gram with amendments suggests that it could survive or perform better than barley in hydrocarbon-contaminated soils [17]. This is significant for reclamation of hydrocarbon-contaminated soils since such leguminous species of plant would also fix nitrogen and so helps to establish a mantle of vegetation rapidly.

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