Bioremediation studies of petroleum contaminated soil by vermicompost for sustainable agriculture in *Hibiscus esculentus* (L) Moench.

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ABSTRACT

Bioremediation of petroleum contaminated soil by amending vermicompost with earthworms were compared with cowdung on the growth and biochemical parameters of ladys finger, Hibiscus esculentus. Seed germination, growth of plants, flowering and yield of vegetable plant lady's finger, H. esculentus revealed that the onset of flowering and fruiting was earlier with fleshy fruits in pots with vermicompost+Earthworm+ petrol (S4) than that of petrol concentrated soil (S6-S9).Maximum height of 32.1cm was observed in S4 (Soil+vermicompost+ Petrolsoil), much difference was not met with S2 (soil +vermicompost). The lowest height in plant growth was recorded in sample 6, petrol concentration. The seedling perished after 20 days. The total protein content, amino acids and carbohydrates was found to be maximum in S4 (petrol soil + Earthworm+ vermicompost). Available EC (µs/cm), Phospherous% and Potassium % were significantly higher in S4 sample than any other combination, might be the reason for high yield of ladys finger. Petrol Soil+ vermicompost treatment exhibited significant increase in all the parameters compared to normal control and Soil+cow dung combination. Pure control soil and different concentrations of petrol combinations were acidic whereas vermicompost and cowdung soil mixtures are slightly alkaline. EC (µs/cm) was higher in petrol soil + vermicompost. Available Nitrogen (TOC) (%), TOM (%), Phosphorus (%) and Potassium (%) showed significance increase when mixed with vermicompost and cowdung compared to petrol soil concentrations. An Electropherogram and chromatogram pattern strengthens the fact that the free aminoacid and proteins were protected in the S4 treatment than Petrol concentrations. Amending petrol contaminated soil with vermicompost with earthworms altered the soil characteristics, which helps in bioremediation of recalcitrant hydrocarbons and suits the growth of plants where as the crude combinations are not supporting plant growth. This pilot study under Indian condition is an eye-opener to the farmers to utilize the petrol/diesel contaminated soil for agricultural practice provided it should be amended with vermicompost for sustainable agriculture.

Keywords: Vermicompost, bioremediation, Eudrilus eugeniae, Hibiscus esculentus.

INTRODUCTION

Contamination with hydrocarbons occurs frequently as petroleum spills occur often during extraction, transport or when petroleum is refined. These spills can have a profound effect on ground water, vegetation, fauna and humans. Most hydrocarbons are quickly removed from soil as they are volatile or are readily degraded by indigenous microorganisms [1,2]. However, some hydrocarbons, such as polycyclic aromatic hydrocarbons (PAHs), are more resistant to degradation and could, therefore, affect human health. PAHs are ubiquitous, nonpolar, and highly hydrophobic and due to their affinity for fatty tissues tend to accumulate in food chains. Although several hundred PAHs exist, most studies focus on a limited number of them, namely the 16 listed by the US Environmental Protection Agency and the European Community as pollutants. Remediation of contaminated soil has traditionally focused on chemical treatments or physical removal, but recently biostimulation, bioaugmentation and phytoremediation have been promoted as these approaches have a far less destructive effect on the environment [3]. During bioaugmentation microorganisms capable of degrading the targeted contaminant are added to the soil [4] and biostimulation tries to augment indigenous soil microbial activity by adding a nutrient-rich, carbon substrate[4], while in phytoremediation plants that are cultivated in the contaminated area lead to increased microbial activity in the rhizosphere, which accelerates removal of hydrocarbons [5]. However, bioavailability and/or bioaccessibility of contaminants [6] and [7], i.e. the accessibility or availability of contaminants for soil microorganisms, is often limited. Earthworms burrow through the soil, mixing it constantly in their gut. As such, earthworms facilitate and increase the contact between contaminants and soil microorganisms. This suggests that adding earthworms to contaminated soil could increase the removal of the contaminant. The effects of earthworms on the removal of PAHs, which serve as a model for soil contaminated with petroleum or as a remediation test, have been reported by several authors [8,9].

In a country like India, with its increasing population, it puts forward the need for more food production. For this purpose people depend on chemical fertilizers for more production in less time. Constant use of fertilizers leads to loss of soil fertility as well as accumulation of toxic substances in the soil which gets into living organisms through the food chain. So, there is a need for shifting from ‘resource degrading chemical agriculture’ to ‘resource protective organic agriculture’. More over due to setting up of petroleum pumps near cultivating areas, has led to contamination of nearby soil leading to low crop production. An alternative method to cope with this problem is bioremediation.

Bioremediation involves the transformation of complex or simple chemical compounds into non hazardous forms by biological agents. It is a relatively cheap and effective means of cleaning the environment and involves the application of organisms and nutrients such as inorganic or organic phosphate and nitrogen to the contaminated soil [10]. The heavy metal content of soil is an important factor when bioremediation is considered for oil contamination cleanup [11]. Due to their biological, chemical and physical actions, earthworms can be directly employed within bioremediation strategies to promote biodegradation of organic contaminants. Earthworms have been shown to aerate and bioturbate soils and improve their nutritional status and fertility, which are variables known to limit bioremediation. Earthworms have also been shown to retard the binding of organic contaminants to soils, release previously soil-bound contaminants for subsequent degradation, and promote and disperse organic contaminant degrading microorganisms [12]. Schaefer and Juliane [13]studied the bioremediation of crude oil contaminated soil using different earthworm species and concluded that earthworms may trigger the degradation process and might therefore, be applied in the remediation of oil contaminated soil with moderate total petroleum hydrocarbon (TPH) values. Ameh et al. [14] reported the survival of earthworms in used engine oil (from automobiles) contaminated soil. They reported that used engine oil concentration level tolerable to earthworms was dependent on the source of the used engine oil [15]. Selective use of vermicompost in contaminated soil have proved effective in influencing soil properties [16]. Addition of vermicompost facilitates humus forms and prevents leaching of nutrients from the soil by their slow release compared to conventional farming using chemical fertilizers [16].

The objective of this project is to assess the bioremediation potential of vermicompost in petroleum contaminated soil. The impact of vermicompost in reducing the toxicity of petroleum contaminated soil were determined by estimating biochemical parameters like total protein, total aminoacd, total carbohydrate concentration, free aminoacids and soluble protein during different growth periods of *Hibiscus esculentus* (L.) Moench and com pared the result with Normal soil and soil amended with cow dung. Polyacrylamide gel electophoresis and paper chromatography were carried out to compare the soluble protein and aminoacids in different treatment periods. Soil pH, electrical conductivity, total organic carbon, total organic matter Nitrogen, Phospherous content of the different treatment procedures were analysed in the pot soil after the completion of experiment to validate the remediation effect.

MATERIALS AND METHODS

Collection of *H. esculentus* seeds, Petrol soil, vermicompost and Cowdung

Hibiscus esculentus (L.) seeds were purchased from KrishiBhavan, Cherpulassery, Palakkad, Kerala, India. Adult earthworms Eudrilus eugeniae, ranging in weight from 0.3 to 0.5 mg each, were collected from Vermicompost Unit of Mercy College Campus. The virgin soil samples were collected from Mercy college campus (soil samples were taken from five randomly distributed points per site (1 ha) at two different depths (0–10 and 10–30 cm and mixed).Petrol mixed soil from Bharath petroleum pump near Mercy College Campus. Dry cow dung purchased from Mercy College Cattle farm. Vermicompost was collected from Vermicompost Unit of Mercy College Campus.

Hibiscus esculentus (L.) Moench growth studies

This investigation was carried out at Mercy College, Palakkad, Kerala. The experiment was carried out from October 2011 to February 2012 to assess the integrated effect of vermicompost with earthworm and cowdung in the bioremediation potential of petroleum contaminated soil by investigating the quantity and quality of *H. esculentus* growth patterns and biochemical parameters. The experiment was conducted by using pot culture method with three replications for each sample. Polythene bags were used for raising crops with each sample having 5 kg of the contents. Different Experimental combinations are as follows: S1= 5kgsoil +5Earthworms; S2=5kg soil +30 g vermicompost+5 Earthworms; S3=5kg soil+ 30g cow dung +5Earthworms; S4=5kg Soil+ 30g vermicompost+5 Earthworms+ petrol(5g/Kg); S5 =5kg Soil+ petrol (5g/Kg) + 30 g cow dung +5Earthworms; S6= 5kg Soil+Petrol(20g/Kg) +5Earthworms; S7=5kg Soil+ Petrol (15g/Kg) +5Earthworms; S8= 5kg Soil+ Petrol (10gl/Kg) +5Earthworms; S9= 5kg Soil+ Petrol (5g/Kg) +5Earthworms. The average day and night temperatures were recorded as $37^0C\pm2^0C$ and $23^0C\pm1^0C$. Eight seeds were sown at a depth of 1.5 cm in each bag. The weeds were removed regularly and watering was done daily. Plants from each sample were drawn randomly for various analyses and its dry weight was recorded. They were then processed for different analysis. Parameters such as growth, total free amino acids, total protein and total carbohydrates were analyzed at three different growth stages such as 30 days, 40 days and 50 days intervals.

Estimation of protein in the leaf sample of *H. esculentus*

The total soluble protein content in the leaf samples were estimated according to Lowery *et al* [17] method using BSA as standard. The optical density (O.D) was measured at 660nm on spectrophotometer. Total proteins were calculated with the following formula.

$$\text{Total Soluble Protein} = \frac{O.Dofsamplexconc.ofs\tan dard}{O.D.ofs\tan dardxVol.ofsample} \text{ X 1}$$

Estimation of amino acids

Total free amino acids were determined by [18] Hamilton and Van Slyke method. For the estimation of total free amino acids 1 ml of standard glycine (1mg/1ml) was taken in two test tubes. To the rest of the test tubes except blank, 1ml of each sample was added. To this 1ml of 10% Pyridine (10 ml pyridine dissolved in distilled water and made up to 100ml) &1ml of 2% ninhydrin (2g ninhydrin dissolved in methanol and made up to 100ml) was added. Blank contained 1ml of pyridine and 1ml of ninhydrin. The tubes were heated in boiling water bath for 30 minutes. The contents in each tube were made up to 50 ml with distilled water. The optical density of these colored solutions was measured at 570 nm using spectrophotometer. Total free amino acids were calculated by the formula.

$$\text{Total free amino acids} = \frac{\mathrm{Re}\,adingofsamplexvol.ofsample}{Weightoffreshtissuex1000} \text{ X Dilution factor}$$

Estimation of carbohydrates

Total carbohydrates were determined by [19] Malik and Srivastava method 0.0.4ml of leaf extract was taken in 9 test tubes. Into 2 other test tubes 0.4 ml of glucose standard (0.1g in 100ml distilled water) was taken. To all the 12 test tubes including blank, 4 ml of Anthrone reagent was added (100 ml anthrone reagent : 0.2g in 100 ml conc. H_2S0_4).The test tube contents are made up to 2 ml . All tubes were heated in boiling water for 10 minutes. They are then cooled and optical density was measured at 620 nm using spectrophotometer. Total carbohydrates were calculated by the following formula.

$$\text{Total Carbohydrate} = \frac{Con.ofGlu\cos esolutionXO.D.ofsample}{O.D.ofGlu\cos e} \text{ X Dilution factor}$$

Polyacrylamide gel electrophoresis and Paper chromatography

Polyacrylamide gel electrophoresis was carried out by following the method of Chrambach andRodbard [20]. 10% resolving gel was prepared for separating the native protein bands agaist a wide molecular weight marker. The gel was stained with coomasie brilliant blue R 250), and cleared the bands with destaining solution (methanol 145 ml : 10 ml acetic acid : 45 ml distilled water and the bands were visualized in white light transilluminator. One way paper chromatography was done using whatman.No.1 fiter paper with Butanol: acetic acid: water in the ratio 3:1:1 as developing solution. The samples S1-S9 were spoted on the whatman No.1 paper along a mixture of amino acid standards. The chromatogram was developed using ninhydrin. RF values were calculated for each sample. The separated amino acids were identified from the chromatogram by comparing RF values with those of standard. Rf value were calculated by the following formula:

$$R_f = \frac{\text{Distance from start to center of substance spot}}{\text{Distance from start to solvent front}}$$

Physicochemical characterization

pH of the soil samples were determined using pH meter. Total organic carbon of the soil samples were done following the titration method of Walkley and Black [21]. Total Nitrogen in the soil samples were calculated in auto analyzer. Soil phosphorus fractions were measured in Flame photometer using lithium as an internal standard. Physicochemical characterization of soil samples were carried out in the soil science laboratory, IRTC,Mundur, Palakkad, Kerala.

RESULTS AND DISCUSSION

Ladys finger (*H. esculentus*), is a multipurpose vegetable crop low in calories and is a good source of many nutrients including vitamins B and C, fiber, calcium, and folic acid. Seed germination, growth of plants, flowering and yield of vegetable plant lady's finger, *H. esculentus* revealed that the onset of flowering and fruiting was earlier with fleshy fruits in pots with vermicompost+ Earthworms+ petrol soil (S4) than that of petrol concentrations(S6-S9) (Table 1,Fig.1 and plate.1). This might be due to better moisture holding capacity and supply of micronutrients, macronutrients to the soil by the application of earthworm and vermicompost. Maximum height of 32.1cm was observed in S4 which is almost equal to S2 (soil +vermicompost+ Earthworms). The lowest height in plant growth was recorded in sample 6, petrol concentration 1. The plant in S6 survived only for 10 days. This might be due to high concentration of petroleum in the soil. Plant growth has been used to evaluate the suitability of plants for vegetating contaminated soils [22, 23, 24]. Increasing microbial population in the rhizosphere leads to an increase in microbial activity, which may increase metabolic and co metabolic transformations of biodiesel into less toxic products and reduce the time required to remediate soil [25].

Vermicompost is believed to be a material that can enhance soil remediation. Vermicompost is produced through the degradation of organic wastes through the action of earthworms that results in the bio-oxidation and stabilization of the wastes. This is a different process than traditional composting which requires a thermophilic stage, while vermicompost undergoes a mesophilic stage. The resulting vermicompost material is a finely divided peat-like substance with excellent structure, porosity, aeration, drainage, and moisture holding capacity [26,27]. In limited studies and applications, vermicomposts have been shown to be effective in the remediation of soil contaminated with hydrocarbon compounds, such as biodiesel. The fungal and bacterial content of vermicompost has been implicated in the degradation of polycyclic aromatic hydrocarbons (PAHs). When used as a soil inoculant vermicompost may be well suited to promote growth of PAH degrading fungi and bacteria [28]. The protein content, amino acids and carbohydrates was found to be maximum in S4 (petrol soil +vermicompost+ Earthworm). The protein concentration found to increase from 30 days to 60 days (Table 2, and Fig. 2). This might be due to large amounts of hydrocarbons present in the petrol soil. The soluble proteins are kept intact in S1-S5 where as no protein bands were seen in the electropherogram of S6-S9 (Plate-1). Ladys finger after the remediation treatments in S4 retained the free essential aminoacids like tryptophan, cysteine, lysine, methionine, leucine, Isoluceine and Phenyl alanine (Plate-2) but not in S6-S9.

The total carbohydrate content was found to be higher in S3. The integrated effects of vermicompost on different soil samples and its effect on growth, yield, contents of carbohydrates, protein and amino acids of H.esculentus during bioremediation have been found to be highly variable(Table2,3,4). The chemical properties of the soils used in the experiment gave the idea about the fertility of the soil (Table 5). Available EC (μs/cm), Phospherous% and Potassium % were significantly higher in S4 sample than any other combination, might be the reason for high yield of ladys finger. Available Nitrogen (TOC) (%), TOM (%), Phosphorus (%)and Potassium (%) showed significance increase when mixed with vermicompost and cowdung in comparison with petrol soil concentrations .Amending petrol contaminated soil with vermicompost altered the soil characteristics ,which suits the growth of plants. Petrol Soil+ vermicompost treatment exhibited significant increase in all the parameters compared to normal control and Soil+ cow dung combination (Table 1-6).

The chromatogram of the sample reveals that free aminoacids like glycine .glutamic acid isoleucine etc. were found in experimental and control soil (plate 3). The potassium content of okra helps in maintaining proper balance of the fluids. The proper balance of the fluids helps in transmitting nerve impulses. It is also required for optimum muscle function as well as metabolism. The oil and protein found within the okra seeds is an important direct source of proteins from vegetables. Okra is rich in amino acids as well [29]. The leaf extract electropherogram showed protein bands in all the experimental groups expect petrol concentrations (Plate.2). Amino acid composition and molecular size are the foundations of protein functionality [30]. Pooled data on ladys finger growth and yield presented in (Table.1) revealed that the application of vermicompost and Earthworms recorded significantly higher number of leaves, fruits and maximum shoot length over the control and petrol treatments. These observations were in conformity with those of few workers [31] reports. Since phosphorus is the structural component of cell organelles and also it favours the formation of metabolites required for growth similarly potash application enhances the uptake of and N and hence there is an improvement in growth characters. The application of N in combination with P significantly increased the plant height number of leaves and internodes in chrysanthemum [32]. The application of vermicompost improvement in shoot length, number of leaves and fruits in chilli [33].

Table 1. Height of *H. esculentus* after different treatment periods.

Treatments	Plant height (cm)				
	10 days	20 days	30 days	40 days	50 days
S1(CONTROL)	8.9±0.5	10.44±0.8	12.08±1.2	12.3±1.1	13.5±0.92
S2	0.92±0.2	7.78±1.3	14.65±1.3	16.7±0.98	20.3±1.8
S3	1.4±0.5	5.48±0.4	9.54±0.6	9.9±1.2	10.13±1.09
S4	1.68±0.2	7.83±0.4	14.02±1.9	17.6±1.23	32.10±1.09
S5	1.83±0.5	7.0±0.98	12.11±1.2	16.2±2.0	18.23±1.0
S6	2.3±0.9	4.3±0.7	-	-	-
S7	2.2±0.9	3.4±0.7	6.9±0.3	7.2±0.2	7.9±0.8
S8	1.2±0.1	4.5±0.2	7.8±0.5	10.3±0.8	13.2±0.2
S9	2.5±0.3	6.4±0.2	7.5±0.4	10.5±0.5	13.3±0.9

Note: Values are meam of tree triplicate analysis

Table 2. Estimation of protein concentration in the leaf samples of *H. esculentus* at different treatment groups.

Treatments	Total soluble protein (g%)		
	30 days	40 days	50 days
S1(CONTROL)	1.44	1.732	2.376
S2	2.177	3.790	4.000
S3	1.799	2.458	2.035
S4	2.737	2.620	6.211
S5	2.285	2.195	2.094
S6	0.214	-	-
S7	1.914	0.854	1.788
S8	0.393	1.327	0.835
S9	1.939	1.424	1.976

Note: Values are meam of tree triplicate analysis

Table 3. Estimation of amino acid concentration in the leaf samples of *H. esculentus* at different treatment groups.

Treatments	Total free aminoacid(mg/g)		
	30 days	40 days	50 days
S1	0.29	0.46	0.64
S2	0.61	1.11	2.29
S3	0.8	1.01	2.10
S4	1.47	1.23	2.45
S5	0.69	0.46	1.20
S6	0.001	-	-
S7	0.12	0.20	0.21
S8	0. 25	0.31	0.42
S9	0.89	0.91	1.20

Note: Values are meaN of tree triplicate analysis.

Table 4. Estimation of carbohydrate concentration in the leaf samples of *A. esculentus* (L.) Moench at different treatment groups.

Treatments	Total carbohydrate (g%)		
	30 days	40 days	50 days
S1	4.640	1.028	0.653
S2	6.73	1.319	0.660
S3	5.3139	0.799	0.422
S4	5.039	0.957	2.073
S5	10.011	1.168	0.285
S6	0.185	-	-
S7	7.049	0.614	0.399
S8	3.727	0.799	0.106
S9	3.307	0.636	0.663

Note: Values are meam of tree triplicate analysis.

Table 5. Physicochemical characterization of 9 Experimental soil samples.

Treatment	pH	EC (µs/cm	Available Nitrogen (TOC) (%)	TOM (%)	Phosphorus (%)	Potassium (%)
S1	6.4	63.28	0.3	0.52	0.2	0.8
S2	7.2	759.5	2.52	4.38	3	7.5
S3	7.2	320.5	2.79	4.85	2.5	6.0
S4	7.5	1446.1	2.64	4.59	2.8	8.5
S5	7.1	543.4	2.73	4.75	1.75	5.0
S6	6.5	555.5	0.18	0.31	0.5	0.4
S7	6.5	175.4	0.03	0.052	0.2	0.7
S8	6.9	160.8	0.21	0.37	0.15	0.2
S9	6.4	216.1	0.3	0.52	0.51	0.7

Note: EC - Electrical Conductivity, TOC - Total Organic Carbon, TOM – Total Organic Matter.

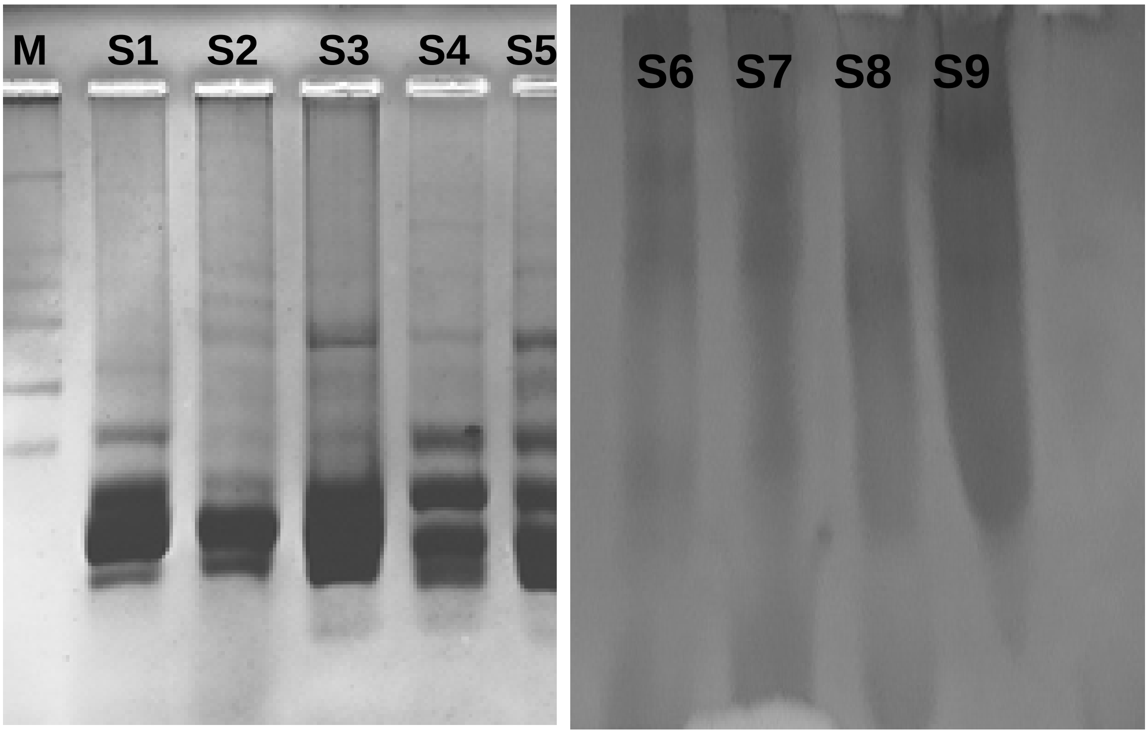
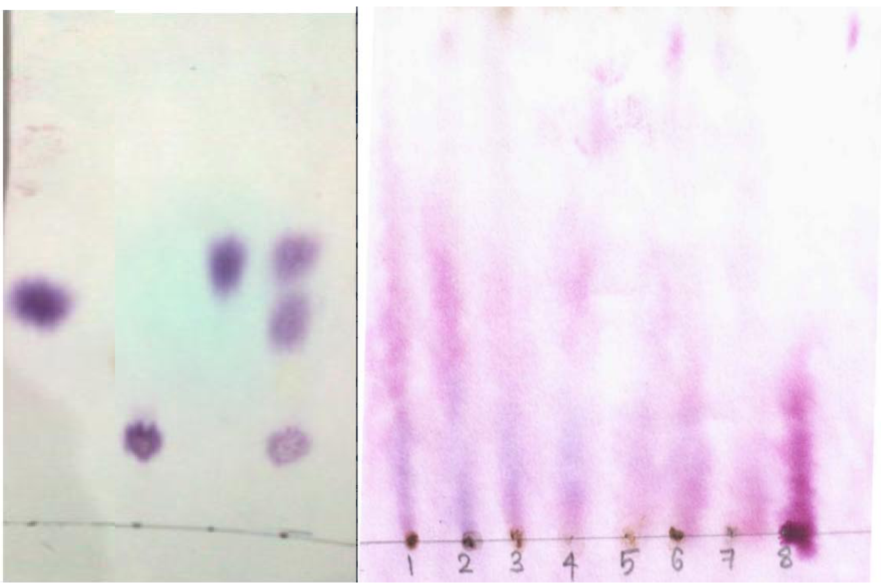


Plate 1. Electropherogram of leaf protein at different treatment periods (M=marker, S1 (Control), S2,S3,S4,S5,S6, S7,S8 and S9).



M Q IL AM S1 S2 S3 S4 S5 S6 S7 S8

Plate 2. Chromatogram of leaf protein at different treatment periods.

Contamination of soil with hydrocarbons occurs frequently. Restoring contaminated soils is costly and time consuming. Earthworms accelerate the removal of hydrocarbons as they burrow through soil by rendering contaminants available for microbial degradation, by feeding on the organic matter that harbour contaminants, and by improving soil structure and aeration. Polycyclic aromatic hydrocarbons (PAHs) are recalcitrant hydrocarbons that can contaminate the environment and can thus serve as models to study the factors that control the removal of petroleum from soil [34]. Vermicompost will also be a 'recipe' to restore the 'degenerated & chemically contaminated soils'of world agricultural ecosystems resulting from the heavy use of agrochemicals in the wake of green revolution. Use of vermicompost would significantly reduce or even replace the use of 'dangerous agrochemicals', reduce the demand of water for irrigation and pest & disease control, thus benefiting the farmers and the economy and ecology of the nation in every way. Lady finger is also known as Okra. It is a very valuable source of nutrition. It can help with the following health conditions: It helps relieve constipation, It helps control blood sugar as it curbs the rate at which sugar is absorbed from the intestinal tract , It helps in healing acid reflux, It contains soluble fibre which helps in controlling the cholesterol level in our bodies, It contains insoluble fiber too which keeps the intestinal track healthy, Lady Finger helps in reducing risks of deadly diseases like cancer, Lady fingers are especially beneficial for the control of colo-rectal cancer, Human body is recommended to have a certain level of vitamin B 6 and folic acid. Half a cup of cooked Lady fingers provide nearly 10% of the recommended levels of vitamin B6 and folic acid. Even the petroleum contaminated soil can be used for the harvest of ladys finger. The vermicompost used for the remediation purpose had completely removed the PAHs thus the plants exhited maximum growth and high yield. The biochemical parameters prove the protection of nutritional quality of ladys finger. This pilot study under Indian condition is an eye opener to the farmers to utilize the petrol/diesel contaminated soil for agricultural practice provided it should be amended with vermicompost for sustainable agriculture.

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