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Ex situ bioremediation of soil contaminated with crude oil by use of actinomycetes consortia for process bioaugmentation

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ABSTRACT

The potential effects of sawdust, and mixture of cow and sheep dung to biostimulant autochthonous microflora and augmentation for hydrocarbon bioremediation were investigated in test biopile, made of soil polluted with petroleum waste 100kg. The soil was fluffed by 1.5% sawdust, then supplemented with the necessary minerals and watered to provide conditions favoring microorganism growth. industrial aeration was provided in pile by drainage-pip network to simulate bioremediation treatments through a 90- day period. During this period, we monitored total petroleum hydrocarbons and changes in bacterial communities. The (TPHs) had been reduced from 52 to 10.6 g.kg^{-1}. In soil, the dominant microorganism population comprised Gram-positive bacteria from actinomycete group and autochthonous microorganisms which decompose hydrocarbons reached highest level 1.6 x 10^{7} cfu.g^{-1} at 45 days. Based on these data, we conclude that is ex situ (Biopile) experiment the best strategy, inexpensive, efficient, and environmentally friendly and may thus offer a viable choice for petroleum hydrocarbons-contaminated soil remediation.

Keywords: Bioremediation, soil contaminated, bioaugmentation

INTRODUCTION

The technology commonly used for soil remediation includes physical and chemical, evaporation, burying and dispersion. However, these technologies are expensive and can lead to convert contaminants from state to another [1]. For this reason, recent researches are applying new strategies that are more environmental-friendly technologies for the remediation of hydrocarbons contaminated soil. Among these, bioremediation technology which involves the use of microorganisms to degrade hazardous substances into less toxic in the environment through the mechanisms of biodegradation [2,3]. Bioremediation is a natural process that takes advantage of nature's recycling and self-purification capabilities for polluted ecosystems [4,5]. oil bioremediation in soil can be promoted by three types including natural attenuation, biostimulation and bioaugmentation[6]. Biostimulation is the process that promoted by stimulation of the indigenous microbial population, by introducing nutrients and oxygen into the soil, bioaugmentation can be occur through inoculation of an enriched microbial consortium into soil [7,8].

A consortium of microbial community enhance the level of degradation, while a single microorganism can degrade limited types of petroleum compounds. Moreover, some substances can be decomposed only by co-metabolism. In natural conditions, the presence of microorganisms that use the products of primary degradation is of particular importance [9,10]. The efficiency of bioremediation of soil contaminated with crude oil depends on the number of hydrocarbon-degrading microorganisms in the soil. In order to achieve hydrocarbon utilization by bacteria, a number of limiting nutritional requirements need to be provided. The most important limiting factors for population

growth are molecular oxygen for the oxygenases, temperature, pH, content of nitrogen and phosphorus, nitrogen, and metals like K+ and Na+ [11,6,12]. The aim of study is application *ex situ* bioremediation by use biopile technique with consortium of actinomycetes strains.

MATERIALS AND METHODS

Collection of soil samples used for bioremediation study:
The soil used for the experiment in this study was collected from a disposing site adjacent to the South Refinery Company (SRC) in Basrah city southern Iraq; the soil of the site chronically polluted with crude refined oil waste discharge.

Soil samples were collected in sterile plastic container at a depth of 10- 20 cm. The soil found to be a sandy loam (64% sand, 27% silt and 9% clay) with pH 7.6, initial chemical analysis of the soil showed that the total petroleum hydrocarbons (TPHs) was 52g.Kg^{-1} which used for the bioremediation.

Bacterial consortium used for bioaugmentation:
A specialized microbial consortium was constructed at the Biotechnology laboratory, of the Basrah University, Science College in Iraq, to form a stable, biologically balanced and capable of biodegrading environmentally organic polluted. The community consisted of actinomycetes (6 strains) derived from naturally occurring, autochthonous microorganisms, selected and isolated from crude oil contaminated soil long-term polluted with petroleum-derived hydrocarbons in the studied area.

In this study, the presence of indigenous microflora was identified in the contaminated soil to biodegradation in a test piles identification by using 16s rRNA gene belong to *Streptomyces* genus [13]. After the isolation and identification of the *Streptomyces* spp. the strains were cultivated under controlled conditions in the yeast malt extract broth to enrich the original microbial community. Community of the soil used for the biopile experiment were bioaugmentation of the *ex situ* soil a dense suspension (approx. 1 x 10^8 cfu.ml^{-1}) of the resultant bacterial was used as an inoculum [14].

Preparation of contaminated soil:
Hundred kilogram of the original soil samples was air dried, mechanical homogenized by removing any material such as pebbles, plastics and metals and passed through a 2-mm sieve. The prepared soil thoroughly mixed with a hand trowel sanitized with 70% ethanol then used for biopile experiment. The different remediation treatments were applied, fluffed with 1.5% sawdust [15]. For the nutrient amended the soil was fertilized with standard agricultural fertilizers in order to provide the C:N:P ratio of approximately (100:10:10), 2.5 g/Kg) of NPK was added as nitrogen source , 20ml/Kg of nutritious salts solution (NH4Cl 2.5g , KH2PO4 5g Mgso4.7H2O 2.5g and distill water 1000ml) [16], then supplemented with the necessary minerals by the use 5g.Kg^{-1} [17]. The cow and sheep dung was collected from a amendment agents were each sun dried for one week, grinded and sieved to obtain uniform size particles respectively, added 15g/Kg as carbon co-substrate and nitrogen source and was thoroughly and mixed with the contaminated soil to distribute the crude oil and nutrients through the soil particles and also to enhance aeration [15].

***Ex situ* bioremediation in test piles:**
A model biopile was constructed in a separate, insulated outdoor site. The biopile consisted of a 2.25m^2 area of treated soil (as described previously) pile on a heights 40-50cm, contain contaminated soil (100Kg) was placed prismatic hold dug to a depth 0.5m and covered with straw to prevent water from evaporation and the soil input on the concreted layer representing pilling area and aid for collected of leaching water, The oxygenation of the pile was provided with an industrial aeration system through a perforated drainage-pipe network at two different depth of the soil pile. Moisture was maintained at approximately 40-50% . Leaching water from the biopile was collected in a separated reservoir and used for re-watering the biopile. Fresh microbial suspension enriched with biodegradable actinomycetes bacteria 25ml/Kg were surface sprayed and homogenized thoroughly with soil mixture [14]. The soil in biopile was mixed manually once every two week to enhance oxygen and kept moist during the 90 day experiment period. The treated pile and control were protected from direct external influences in a green house.

Bioremediation sampled:
The soils from the treated and control experiment pile were sampled at (zero, 15, 30, 45, 60, 75, and 90) days. Soil were collected at three independent sites, then mixed to obtain ideal sample, collected in sterilize container and transported to laboratory for analysis following procedures of [17].

Bioremediation experiment monitoring:
Bioremediation processes in biopile was monitored by the determined of a viable bacteria count , pH and TPH. The number of microorganisms was determined by the method of the serial dilution on the agar plate (Nutrient agar) incubated at 30°C for the total count of bacteria. CFU on Petri dishes were counted as cfu per one gram of soil [18]. The count of total petroleum hydrocarbon (TPH) in the soil samples was determined gravimetrically after solvent extraction [19] then the percentage of biodegradation was measured [20].

Biostimulant efficiency and level of contaminate:
Evaluation of unamended soil biopile (Natural attenuation) and amended soil (Treated biopile), Biostimulants Efficiency (B.E) was calculated at the end of the 90 days remediation period using The following equation [21].

$$BE\% = \frac{TPH_U\ \% - TPH_T\ \%}{TPH_U\ \%} \times 100$$

Where TPH_U is the removal of crude oil in the untreated soil and TPH_T , the removal of crude oil in the treated soil at different time. To evaluate level of contamination in the treatment soil in biopile, which represents the amount of oil remaining and degrading which calculated from the values of the TPH estimated by gravimetrically: % Degradation = TPH_T / TPH_{U0} x 100,

% Remaining = 100% – % Degradation.
Where TPH_{U0} is the crude oil in the untreated soil at zero time.

Statistical analysis
for significant differences between treatments at the level of $p < 0.05$ using one- way analysis of variance (ANOVA) tests for data analysis which were performed using statistical package for version 20.0 (SPSS)

RESULTS

The examined soil contaminated with petroleum hydrocarbons derivatives originated from the oil refinery in Basra, Iraq. Where pollution is high and chronic, TPH was 4250mg.Kg^{-1}, pH 7.6, and water content 2.5%.

Estimated the effectiveness of monitored natural attenuation bioenrichment and bioaugmentation using a consortium of six with crude oil. pile was used to simulate bioremediation treatments 90-day period. During this period, they monitored TPHs degradation and changes in bacterial communities.

The soil initially contained 52g TPH.kg^{-1} (at 0 time). The percentage reduction in TPH was rapid within the 90 days of the study 10.6g TPH.kg^{-1} the soil amended with activated degradable bacteria when compared to that of the unamended soil pile 37.1g TPH.kg^{-1} as shown in (Table 1).

Table 1: Remaining of TPH after bioremediation treatment through 90 days

Treatment /days	TPH g.kg^{-1}	
	Biopile	Control
0	52	52
15	34.9	50.6
30	30.2	45
45	26	42
60	21.1	39.6
75	12.4	37.7
90	10.6	37.1

The degradation rate biopile was increase when that period time exposure increase too. After the first month of remediation observed the degradation rate in the soil was 42% in biopile, second month was 59.5%. At the end of remediation period (90 days) crude oil contaminated soil amended with bulking agent showed the highest degradation rate 79.6%. The result suggests that the augmented and amendment agents had a statistically significant effect on the biodegradation of crude oil in soil at the 5% probability level ($P = 0.001$), The result of biodegradation and remaining oil through remediation time shown in (Figures 1).

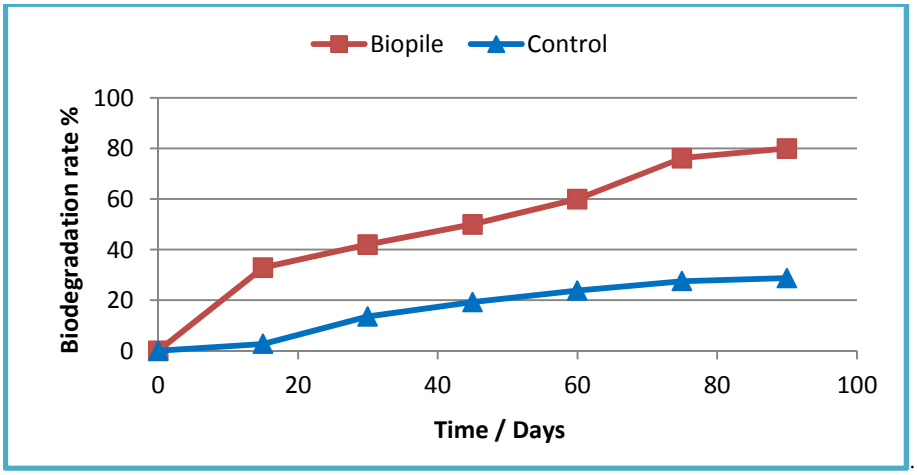

Figure 1: Biodegradation rate during 90 days of treatment

Results determine crude oil pollution levels in the soil treatment and non-treatment, showed the amount of crude oil degraded more than amount of remaining. After 90 days of treatment was removed 79.6% and 21.4% remaining this evident (Figure 2) compared with the natural attenuation in control soil (Figure 3).

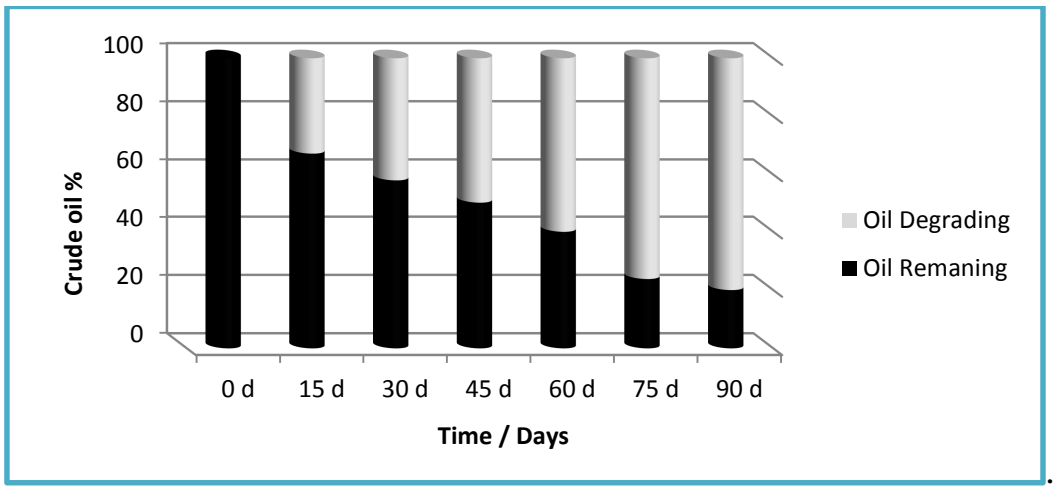

Figure 2: Oil degrading and removing from soil in biopile during the treatment periods

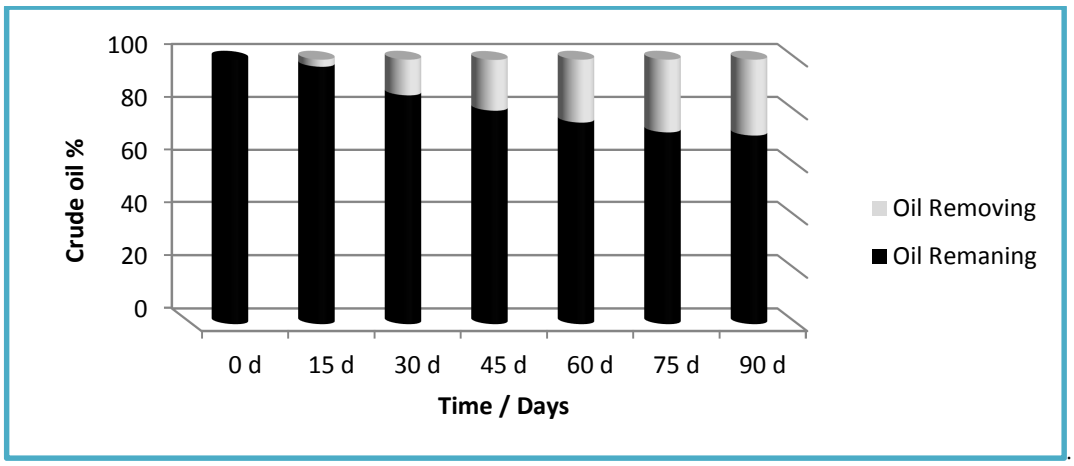

Figure 3: Oil removing from soil in control sample during the treatment periods

The BE shown in (Figure 4) was increase associated with period time, the highest value was 71.4% after 90 days of treated, while the lowest was 31% at 15 days.

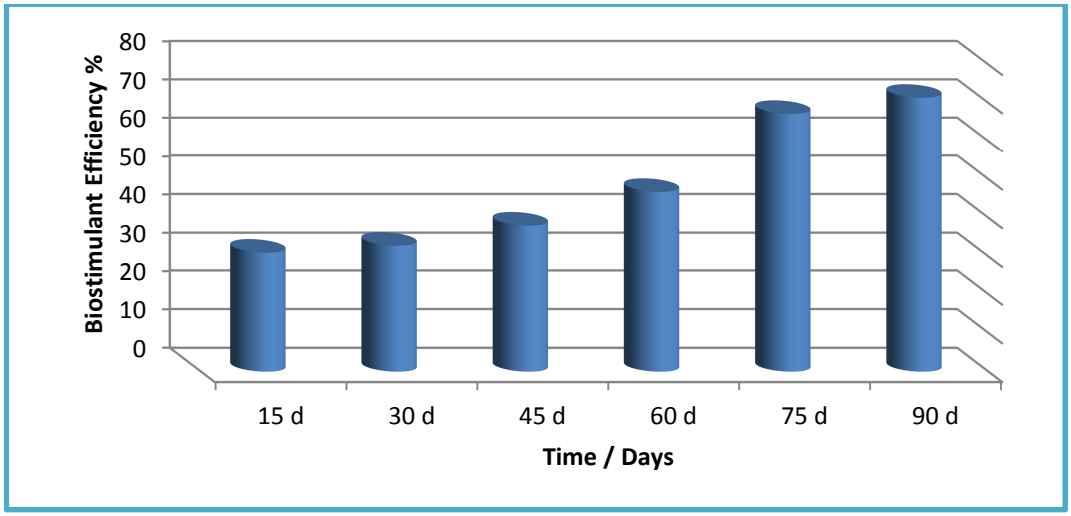

Figure 4: Percent of biostimulants efficiency at different times of treated

The soil sample, which was contaminated with crude oil, contained 1× 10^4 CFU.g^{-1} of indigenous soil microorganisms when plated on nutrient agar. The control soil sample was not amended with the addition of any microbial consortium to study the effects of the natural attenuation on the remediation of the soil. In the adding 25ml of bacterial inoculum onto each Kg of soil and other (fertilizer, water, and dung) resulted in total cell counts generally, it is seen that the microbial (total heterotrophic bacteria counts) increased after 45 days from 5x10^5 to 2 x 10^7 in biopile, while increase from 1x10^4 to 6.4x10^4 in control due to natural attenuation which is illustrated in (Figure 5).

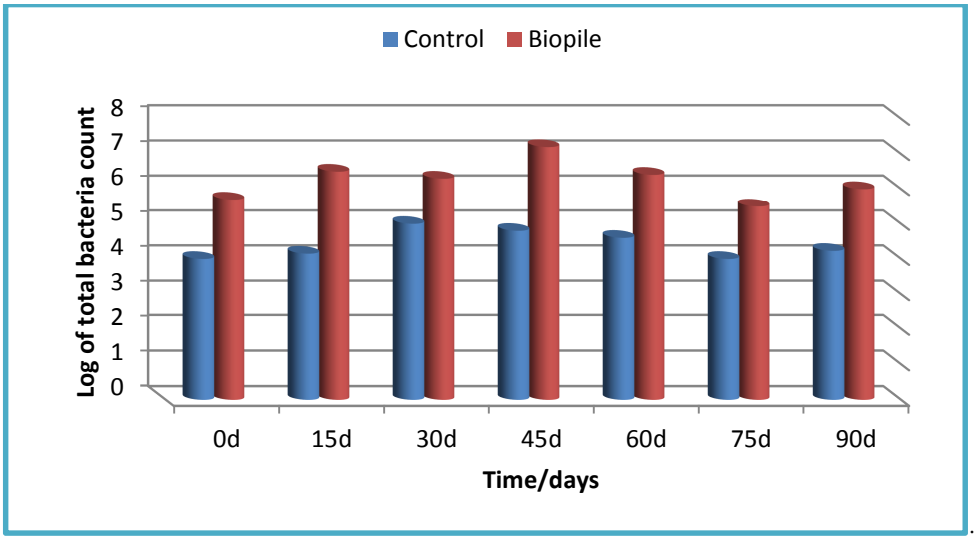

Figure 5: Changes in the number of bacteria through the treatment period

DISCUSSION

In this study a specialized actinomycetes degradable consortium, developed by our group, was used for bioaugmentation. this complex microbial community tended to reveal unique enzymatic activities thus enabling oxidation of heterogeneous organic contaminants.

The biodegradation rate of crude oil in the soil seems to be increase with the time exposure as showed in figure 1. This may be due to the fact that the microorganisms in the soil have efficiency ability in utilizing the crude oil as a source of carbon and energy [22]. used isolated strains belong to actinomycetes as mix or consortium appear higher degrease in TPH compare with untreated soil. The use of mixed cultures an advantage is a broader degradation capacity, synergic effect and co-metabolism [23]. These observations indicate that the sawdust (plant source waste) and the mixture of cow dung and goat dung (animal source waste) used in combination enhanced crude oil biodegradation in soil. Similar observations have been reported for the use of plant and animal-derived organic waste in the bioremediation of soil contaminated with petroleum hydrocarbons [24, 15].

The increase effect of amendment is high when nutrient availability is a limiting factor in the biodegradation of oil. Many studies refer to the use of nutrients such as nitrogen and phosphorus in the bioremediation of soil contaminated with petroleum hydrocarbons [25]. Microorganisms need nutrient to grow. Hence, biodegradation of hydrocarbons in the natural environment is effected by low growth rate of microorganisms due to deficiency of nutrient, especially in nitrogen and phosphorus, therefore when bioremediation is conducted suitable nitrogen and phosphorus are usually applied to the contaminated soil to stimulate biodegradation [26].

Agarry et al., [27]show the bioremediation with different amount of NPK were tested and the results shows that extra amount of NPK (from 2 to 4 g/l) can improve kerosene removal from contaminated soil. The results suggest that high dose nutrient amendment can accelerate the initial oil degradation rate and may shorten the period to clean up contaminated environments and combination of two animal dung wastes and as well as combination of animal dung wastes and plant sawdust residue organic wastes has a relative higher biostimulation efficiency in the biodegradation of petroleum hydrocarbons.

The addition of bulking agents to contaminated soil was increase oxygen diffusion and mineral nutrient availability as well as carbon source quality and physical support surface for bacterial adsorption, and improves soil physicochemical characteristics as to speed up microbial adaptation and selection [28]. Thus, in our system, the results suggested that both plant organic wastes (sawdust and yam peel) and animal dung wastes used alone and/or in combination have also contributed to increased oxygen and nutrient availability for the microorganisms as a result of the increased growth of hydrocarbons degrading bacteria (Figure 5) and the increased TPH reduction that were observed. More also, both the plant and animal organic wastes microbial population supply was also suitable as it may supplied additional hydrocarbon degrading microorganisms [29], which could contribute to metabolize hydrocarbon contaminant together with the soil autochthonous microorganisms.

CONCLUSION

The bioremediation technique for contaminated soil with crude oil and/or other hydrocarbons is applicable in field because of its low cost and because it is environment friendly. Biostimulants and bioaugments in *ex situ* bioremediation for hydrocarbon polluted soil performed under aerobic conditions proved to be a potential method of remediation for most hydrocarbons pollution soils.

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