# Effective scale-up of oily sludge bioremediation from a culture-based medium to a two-phase composting system using an isolated hydrocarbon-degrading bacterium: Effect of two-step bioaugmentation

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## Abstract

The scale-up feasibility of oily sludge (OS) biodegradation from a culture-based medium to a new two-phase composting process bioaugmentated with an indigenous isolated strain was surveyed. First, the bacterial strain (*Enterobacter hormaechei* strain KA6) was isolated from OS, and then its ability in biomass production and oil degradation in culture-based medium was

evaluated. Finally, it was added twice to the composting reactors included four in-vessel experiments with the total petroleum hydrocarbons (TPHs) concentrations of 10-30 g kg$^{-1}$. The composting lasted 16 weeks divided into the primary composting stage (PCS) and secondary composting stage (SCS). It was observed that the strain degraded 58.67, 74.79, 45.33, 10.66, and 5.92% of 1, 2, 3, 4, and 5% oil concentrations, respectively, in culture-based medium during 7 days. A total TPHs removal rate of 65.83-81.50% was also reached after a two-phase duration of 16 weeks. Due to the second bioaugmentation, the SCS had higher efficiency than the PCS. The study confirmed the effective scale-up of a culture-based medium to a composting process for treating OS.

**Keywords:** Oily sludge; Composting scale-up; Bioaugmentation; Crude oil; Bacterial isolation; Bacterial inoculation


## Introduction

Although hydrocarbon products are the main sources of energy consumption, they may highly contaminate the environment. Oil refinery plants produce huge amounts of oily sludge (OS) comprising of wide ranges of complex and toxic petroleum substances [1, 2]. Therefore, OS should be treated well before being disposed, or otherwise the compounds may threaten the environment or human health. Although there are multiple chemical and physical technologies for OS detoxification, bioremediation has been introduced as a green and environmentally method for treatment of OS-polluted sites [1, 3].

In bioaugmentation, as a promising strategy for performing bioremediation, native microorganisms able to decompose petroleum compounds are added to contaminated sites [4, 5]. On the other hand, the application of allochthonous strains in new environments has some

limitations resulted mainly from low adaptability. Therefore, the isolation and purification of indigenous strains from OS, which are entirely adapted to that environment and can consume petroleum hydrocarbon as their carbon source, is of great importance [6, 7]. However, most of hydrocarbon-degrading strains present in OS can only degrade a limited range of petroleum hydrocarbon concentration. Since, few isolated strains have strong potential for decomposing target pollutants in a wide range of concentrations and environmental conditions, the utilization of metabolically superior indigenous strains as a bioaugmentation factor is preferred [4, 8].

Considering the abovementioned background, it can be concluded that the most important problem with bioaugmentation is the lack of effective translation and scale-up of culture-based experiments [9]. In this relation, composting process, as an inexpensive, green, and efficient method, can be considered for remediation of different kinds of OS [10]. It has been stated that bioremediation of petroleum compounds occurs in two approximately specific phases: fast degradation of easily-biodegradable materials and then slow decomposition of the remaining compounds. As the later phase of bioremediation suffers from the lack of enough microbial population and activity, decreased removal rates of the petroleum pollutants are expected [10, 11]. Hence, to improve bioremediation efficacy, a two-phase composting system can be considered as a suitable option, because petroleum-degrading microorganisms are amplified via two-step inoculation of specialized isolated strains [1, 12].

Accordingly, it is of importance to isolate petroleum-degrading strains exhibiting high removal ability so as to gather experimental evidences and determine their degradation potential both in culture-based medium and in a full-scale bioremediation process. Hence, this study was performed to scale-up OS treatment from a culture-based medium to a two-stage composting method. The impact of two-step bioaugmentation of an isolated strain as well as the general

efficacy of the two-phase composting system for total petroleum hydrocarbons (TPHs) removal from OS was also examined.

## Materials and methods

### Bacterial isolation

Ten g of the OS sample, taken from an oil refinery located in Shazand, Iran, was added to 100 ml of carbon-free mineral medium containing crude oil (1%) as the sole energy and carbon source. Then, it was placed in a shaking incubator (150 rpm) for 1 week at 35 °C and a fresh medium was contaminated with 5 ml of the incubated culture. This enrichment was repeated for several times. Afterwards, 100 µl of the culture was spread on nutrient agar and incubated for 48 h at 35 °C. Single-grown colonies were recultured on nutrient agar plates. Besides, so as to make sure that crude oil-degrading bacteria are isolated, each colony was cultured in Bushnell-Hus (BH) solution containing crude oil (1%) as the sole energy and carbon source. So as to attain the strain that had the highest growth rate and, in turn, ability in degradation of the pollution, the standard amount of 0.5 McFarland ($1.5 \times 10^8$ CFU ml$^{-1}$) of the isolated strains was prepared and added to BH solution containing 1% of crude oil and then optical densities at the wavelength of 600 nm (OD$_{600\ nm}$) were monitored at various intervals (2, 4, 7, 10, and 12 days) at 35 °C.

### Bacterial identification and characterization

The bacterial strain selected in this research was identified and characterized by the selective media and procedure expressed in the references [13, 14]. Also, the strain was confirmed molecularly through PCR amplification (by the universal bacterial primers) of the 16S rARN gene. The PCR mix contained 1 µL (10 pmol) of the primer, 2 µL DNA, 12.5 µL PCR Master Mix in a final 25 µL reaction volume. Moreover, the thermocycling program was as follows: initial denaturation at 93 °C for 5 min, 34 cycles of denaturation at 94 °C (1 min), an annealing

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temperature at 56 °C (1 min), an extension at 73 °C (1 min), and a final extension at 70 °C (5 min). The National Center for Biotechnology Information (NCBI) database-BLAST was used for sequencing the amplified gene. The strain was identified according to the similarity (%) of the sequences with those in the NCBI database.

**Optimization of culture-based tests for composting experiments**

In order to determine the highest concentrations that can be biodegraded by the strain, the contents of 1, 2, 3, 4 and 5% of the crude oil were tested. It should be noted that the optimization of TPHs level is of importance because this value can be considered for composting experiments. Therefore, the 0.5 McFarland concentration of the strain was prepared and added to the Erlenmeyer flasks containing 500 ml of BH solution with the mentioned concentrations of crude oil. Then, the flasks were incubated for 7 days at the temperature of 35 °C (rpm of 120 and initial pH of 7) and the rates of TPHs degradation were determined. It should be pointed out that over these experiments the control tests were run at the same conditions with no bacterial addition. In addition, so as to study the effect of pH on TPHs bioremediation, different values (4, 5, 6, 7, 8, and 9) were tested using HCl and NaOH. Therefore, BH solutions with 1% of crude oil and the strain (0.5 McFarland) with the mentioned pH values were incubated for 1 week at 35 °C. After the incubation, both the growth rate and TPHs removal efficiency were determined.

**Assessment of OS bioremediation in the in-vessel composting experiments**

In this stage, a 16-week bioremediation process was performed in four 2-L bioreactors filled with the composting materials. Hence, OS and mature compost (MC), both of which had been autoclaved, were mixed at different ratios to attain the TPHs contents of 30, 20, and 10 g kg$^{-1}$, respectively in the composting reactors $E_1$, $E_2$, and $E_3$. A very important point to make here is that these concentrations of TPHs in composting reactors were chosen based on the findings

attained by the optimization of crude oil concentration for bioremediation tests described in section 2.3. The ratio of C/N/P in all experiments was set at 100/5/1 by using $NH_4Cl$ and $KH_2PO_4$ [15, 16]. The inoculation was performed through adding the strain (0.5 McFarland) to the reactors (10% (v/w)). After the primary composting stage (PCS), which lasted 8 weeks, the secondary composting stage (SCS) was initiated via the reinoculation by using the addition of the strain (1 McFarland) to the reactors (10% (v/w)). In order to evaluate the effectiveness of the two-stage inoculation method, the reactors $E_4$ was considered as the control experiment. It should be noted that initial TPHs content was 20 g $kg^{-1}$ and only the first inoculation was done in this reactor. The moisture was retained at 50-55% during the PCS and SCS. Also, oil-free diaphragm pumps were utilized to supply air in the level of 1 l $min^{-1}$ $kg^{-1}$ [17].

**Sampling**

Before the samples were taken, the mixture of MC and OS were fully blended and some subsamples were gathered from different depths of the composting experiments. Then, the subsamples were completely mixed to reach a representative sample. The frequencies of sampling for organic carbon (OC), TPHs, pH, and Temperature were biweekly over the biological process time composting time.

**Analytical methods**

A portable thermometer was utilized to record daily temperature. By heating the composting samples at 105 °C during 24 h, their water level was measured. The OC content was quantified by loss-on-ignition procedure [18]. In order to detect the TPHs, a gas chromatograph (GC)-flame ionization detector (FID) system was applied after extracting petroleum compounds of the composting samples with n-pentane [19]. The GC thermal program and other operating

conditions have been explained in the previous articles by Koolivand et al. [20, 21]. All the TPHs and OC analyses were performed in duplicate.

**Statistical analysis**

SPSS 19.0 was used to analyze the data obtained; differences among biotreatment runs were performed by means of the one-way ANOVA with a significance level of 0.05. Besides, so as to determine the relationship among the parameters, regression analysis was used via Microsoft Excel.

# Results and discussion

**Metabolic and taxonomic identification of the isolated strain**

Taxonomic characteristics of the strain isolated from OS were surveyed by 16S rRNA gene analysis. The results of search in NCBI Genbank database depicted that the strain had the most similarity to *Enterobacter hormaechei* strain KA6. The accession number of the gene sequence of the strain KA6 in NCBI is MK127548. The findings of biochemical tests performed for the strain are provided in Table S1 (supplementary materials).

**Scale-up of OS bioremediation from culture-based medium to composting process**

*Impact and change of pH*

The potential of the strain in the utilization of crude oil in the culture-based medium was examined to translate and scale-up this ability for biodegrading TPHs in the composting process. The $OD_{600}$ measured after the incubation periods of 2, 4, 7, 10, and 12 days were 0.26, 0.78, 1.39, 1.46, and 1.20, respectively. Accordingly, as the logarithmic phase of growth lasted 7-10 days, the duration of incubation process was 7 days for all the culture-based experiments.

As pH is a basic parameter influencing microbial growth and activity, its effect on the strain growth and crude oil removal was tested to find the best value and operate the composting

experiment in this pH. An increase or decrease in pH may influence the solubility of hydrocarbons due to improved dissociation of acid functional groups. It has been documented that the appropriate level for pH ranged from 6 to 8, and the optimum value is 7 [22, 23]. In this work, the results of culture-based experiments (Table 1) indicated that the isolated strain exhibited high bioremediation rates at pHs of 6-8 to be under 60%. Also, at pHs of 5 and 9, the growth rate of the strain and, in turn, bioremediation rate declined dramatically. The observations reported by Muangchinda et al. [4] accord our results. The results attained from culture-based runs give good information for translation of bioremediation processes. Hence, we operated the composting process at neutral pH and also, monitored the changes of pH values over the composting period. In this study, the pH values of all composting bioreactors ranged between 6 and 8 (Fig. 1). Apart from $E_1$, pH variations followed a same trend over bioremediation process. During the first 6 weeks, a downward trend was seen and then it fluctuated slightly over the following 2 weeks and finally it started to go up. The reason of this change is the fact that organic acids, which cause pH value to decrease, are generated from TPHs breakdown. In contrast, when the generated organic acids are consumed by microorganisms, pH starts to rise [24]. Besides, the lowest pHs occurred in $E_2$ and $E_3$ may be an indirect indication of more degradation of TPHs. It should be noted that MC utilized as a bulking agent may play an effective role in keeping pH in the suitable limit during bioremediation.

Table 1

Fig. 1

*Impact of initial concentration of TPHs*

Table 1 also indicates that the strain can grow in BH medium with 1-5% concentrations of crude oil. As shown, the TPHs removal rates were 58.67, 74.79, 45.33, 10.66, and 5.92%, respectively,

at the initial amounts of 1, 2, 3, 4, and 5% within 7 days. As can be observed, bioremediation efficiency went down with the increase in initial contaminant concentration. In kinetic terms, a very low level of contaminants may not be detectable and, in contrast, a very high level can be toxic to microorganisms. Hence, in a specific range of concentrations, the rise in pollutant levels leads to reduction in degradation rate [25]. Varjani and Upasani [1] stated that, at very low contents of hydrocarbon compounds, bacterial activity is limited and consequently a decrease is expected to happen in bioremediation rate. Our observations confirmed this as at the concentration of 1% the growth rate was lower than that of 2%. The culture-based tests performed in this study illustrated that 2% of crude oil was the highest content, under which the isolated strain could grow effectively and use it as the sole carbon and energy. Thus, it can be concluded that initial crude oil content is a major factor in the performance of bioremediation in culture-based medium.

The concentrations of 10, 20, and 30 g kg$^{-1}$, respectively, in the bioreactors of $E_3$, $E_2$ and $E_1$ were set according to the data obtained from culture-based medium. Further, the mixing ratios of OS to MC were calculated based on these concentrations. The identifications of OS and MC applied in the current research have been supplied in Table S2 (supplementary materials). As presented in Fig. 2, TPHs was treated 65.83, 75.40, 81.50, and 51.65 %, respectively in the bioreactors $E_1$, $E_2$, $E_3$, and $E_4$. These values demonstrate that initial hydrocarbon concentration is also a basic factor in the composting process applied to purify crude oil. A very important point to make here is the optimization of mixing ratio of OS to MC because it influences both degradation rate and bioremediation cost. The observations of the current research express that the amount of MC utilized in the composting process is an effective variable and are in accordance with those attained in the previous works [15, 26].

Fig. 2

**Impact of two-step bioaugmentation on the composting performance**

In bioremediation processes, easily-biodecomposable materials are remediated in the early stage and intermediate products or metabolites and remaining compounds, which are almost persistent to biological degradation [27], are accumulated in the later stage and, thereby, TPHs degradation declines. As can be seen in Fig. 2, in all reactors except for $E_4$, a downward trend was observed in the concentration of TPHs within the first 14 weeks. Fig. 3 shows the removal efficiencies of TPHs in both the PCS and SCS. The removal percentages reached 33.43, 36.10, and 37.80%, respectively, in $E_1$, $E_2$ and $E_3$ during the PCS; and, the values for the SCS were 32.40, 39.30, and 43.70% (Fig. 3). However, in $E_4$ which was not reinoculated in the end of its PCS, TPHs bioremediation was low during the SCS.

As mentioned above, $E_1$, $E_2$, and $E_3$ had satisfying removal efficiency in SCS that was attributed to the second bioaugmentation with a higher concentration of the strain. In the current research, the bioreactors of $E_2$ and $E_4$ had the same situation in terms of initial TPHs concentration; but $E_4$ was a one-step inoculation bioreactor. Interestingly, the two bioreactors were capable of degrading TPHs equally over the PCS; but $E_2$ exhibited about 24% higher TPHs removal during the SCS. Hence, a new bioaugmentation through the addition of bacterial strains at a higher content than that in the first step can reach hopeful results in TPHs removal over the SCS. Since the shortage of petroleum-degrading strains may limit contaminant decomposition, the utilization of well-adapted and native bacteria can be entirely helpful [28, 29]. Besides, during bioremediation, the population number and metabolic activities of introduced microorganisms may decline; thus, in order to keep the bacterial activity at an appropriate amount, a two-step bacterial bioaugmentation can lead to the more efficient breakdown of

pollutants [30]. In agreement with our study, other researchers reported that the bioremediation rate of a two-stage composting is more than that of the traditional one-stage method [12, 31].

Fig. 3

**Impact of bulking agents on the composting performance**

The growth rate and activity of microorganisms are influenced by the kind and content of carbon source applied in composting methods [1, 32]. Usually, bacteria target the compounds that are not very complicated and are easily biodegradable, and hence the existence of an easily-biodegradable carbon source plays an important role in initiating and maintaining the activity and growth of microbial communities. Of course, it should be taken into account that these organic materials must be utilized in their suitable level, otherwise they may be consumed as the sole source energy of and carbon by microorganisms leading to a decrease in bioremediation of TPHs [12]. Therefore, in order to study the impact of MC on TPHs bioremediation, the variations of OC and OC/TPHs were considered in this work (Fig. 4). An increase in the ratio of OC/TPHs illustrated that TPHs was degraded more than that of OC over the composting process. As mentioned above, another feature of MC employment as a bulking material is the adjustment of initial crude oil content. Furthermore, bulking materials can improve the growth rate of microorganisms through enhancing the water-maintaining capacity of composting materials. What's more, oxygen diffusion can be facilitated by using these materials; this leads to an improvement in heat generation and consequently faster hydrocarbon compound biotreatment [22]. Thus, it can be claimed that the MC employed in this research did not have a competing property towards TPHs; and more importantly it strengthened the bioremediation of TPHs.

Fig. 4

**Temperature fluctuations in the composting bioreactors**

In this study, we recorded temperature fortnightly (Fig. 5a) in the three bioreactors $E_1$, $E_2$, and $E_3$. As can be clearly seen, during the composting process, the temperature changed approximately equally in the reactors. The highest levels of temperature were observed during weeks of 8-10, which were 27, 30 and 30 °C, respectively, in $E_1$, $E_2$, and $E_3$. The reactors exhibited the mean increase rates between 0.67 and 0.88 °C week$^{-1}$. The activity and metabolism of microorganisms and, in turn, transformation of petroleum substances cause the temperature to go up during composting reactions [33, 34]. In this research, in $E_2$ and $E_3$, higher temperatures were accompanied with higher TPHs biodegradation rates. Further, $E_1$ showed the minimum temperature and accordingly a decrease in bioremediation efficiency. The rate decreased quickly from 1.25 to 1.33 °C week$^{-1}$ in the bioreactors; this can be on account of the fact that TPHs bioremediation declined after the temperature peaked. Of course, it can be explained that since 2-L composting pilots were applied in this study, the temperature of ambient air affects the reactors temperature, in particular over the last weeks of the composting.

Fig. 5

**Correlation between OC, temperature, and TPHs biodegradation in the composting bioreactors**

The results obtained from the regression analysis (Fig. 6) confirmed a strong correlation between the rate of petroleum decomposition and carbon consumption. Since the base nature of petroleum compound is naturally organic carbon, this correlation is predictable. In order to use the composting process in full-scale applications, the correlation equations calculated for these two parameters can be helpful. Moreover, the findings of regression analysis also confirmed that the bioremediation rate was correlated linearly with temperature increase. This correlation can be attributed to this fact that when temperature increases, the pollutants' viscosity declines, and

thereby their availability for biodegradation enhances. Moreover, it affects the growth and metabolism of microbial communities involved in petroleum breakdown [35, 36].

Fig. 6

## Conclusions

The feasibility of scale-up of a culture-based medium to a composting system experiencing two-step bioaugmentation of an isolated strain for OS treatment was investigated. The strain isolated exhibited a strong ability to breakdown a wide range concentration of crude oil in culture-based medium. This ability was observed to be successfully scaled-up to the composting process. The two-step inoculation of the native strain highly promoted the efficiency of the composting process for OS decomposition. The current study revealed the successful scale-up of a culture-based medium to the utilized composting process for degradation of OS.

## Acknowledgements

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**Table 1** Effect of pH and crude oil concentration on degradation rate in culture-based medium after a 7-day incubation period

**Figure captions**

**Fig. 1** Variations of pH in the composting bioreactors over the process duration

**Fig. 2** Residual TPHs in the composting bioreactors over the process duration

**Fig. 3** Degradation percentages of TPHs over the PCS and SCS of composting duration

**Fig. 4** Variations of OC and OC/TPHs in the composting bioreactors over the process duration

**Fig. 5** Variations of temperature in the composting bioreactors over the process duration

**Fig. 6** Regression correlation of (a) OC and TPHs degradation during PCS, (b) OC and TPHs degradation during SCS, and (c) temperature and TPHs degradation