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Abstract

The impact of two-step inoculation of indigenous strains and their synergistic effect in the scaling-up of petroleum hydrocarbons biodegradation from a mineral-based medium (MBM) to a two-phase composting process were investigated. After isolating the strains KA3 and KA4 from heavy oily sludge (HOS), their emulsification index (E24), bacterial adhesion to hydrocarbon (BATH), and oil degradation efficiency were evaluated in the MBM. Then, they were inoculated twice into the composting bioreactors lasted for the primary 8 weeks as the first phase (FP) and subsequent 8 weeks as the second phase (SP). Based on the results, 57.12, 61.17, 59.87, 32.50, and 16.38% of oil was removed by the consortium of the two strains, respectively, for the concentrations of 1, 2, 3, 4, and 5% in the MBM. In the composting reactors, removals of 20 g kg-1 initial concentration of total petroleum hydrocarbons (TPH) were found to be 63.95, 61.00, and 89.35% for the strains KA3, KA4, and their consortium, respectively. The computed biodegradation constants indicated the synergistic effect of the two strains and the effectiveness of the second-step inoculation. The study demonstrated the successful scaling-up of HOS biodegradation from MBM to the two-phase composting process through two-step inoculation of the isolated strains.

Keywords	Biodegradation scale-up; Heavy oily sludge; Two-step inoculation; Composting process; Synergistic effect
Taxonomy	Biochemical Mechanism, Biochemistry Application
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Corresponding Author	Ali Koolivand
Corresponding Author's Institution	Arak University of Medical Sciences
Order of Authors	Maryam Parhamfar, Hamid Abtahi, Kazem Godini, Reza Saeedi, Majid Sartaj, Jose Villaseñor, Frederic Coulon, Vinod Kumar, Tayebeh Soltanighias, Ehsanollah Ghaznavi-Rad, Ali Koolivand

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Dear editor,

I would like to submit the manuscript entitled “Biodegradation of petroleum hydrocarbons by a two-step inoculation composting process using synergistic effect of indigenous bacteria isolated from heavy oily sludge: field application of a mineral-based medium in a two-phase composting system” for possible evaluation.

The novelty of this study is the investigating of the scaling-up of HOS bioremediation from MBM to a two-phase composting system through two-step inoculation of isolated indigenous strains. Moreover, the synergistic effects of two isolated strains both in MBM and in the composting system and also the impact of two-step inoculation of the strains (both alone and consortium) into the composting process were studied.

I affirm that the manuscript is the original work of the authors and all the authors agree that it should be submitted to this journal. The manuscript has been prepared in accordance with the journal instructions. The contents of this manuscript have not been published in a refereed journal, and it is not being submitted for publication elsewhere.

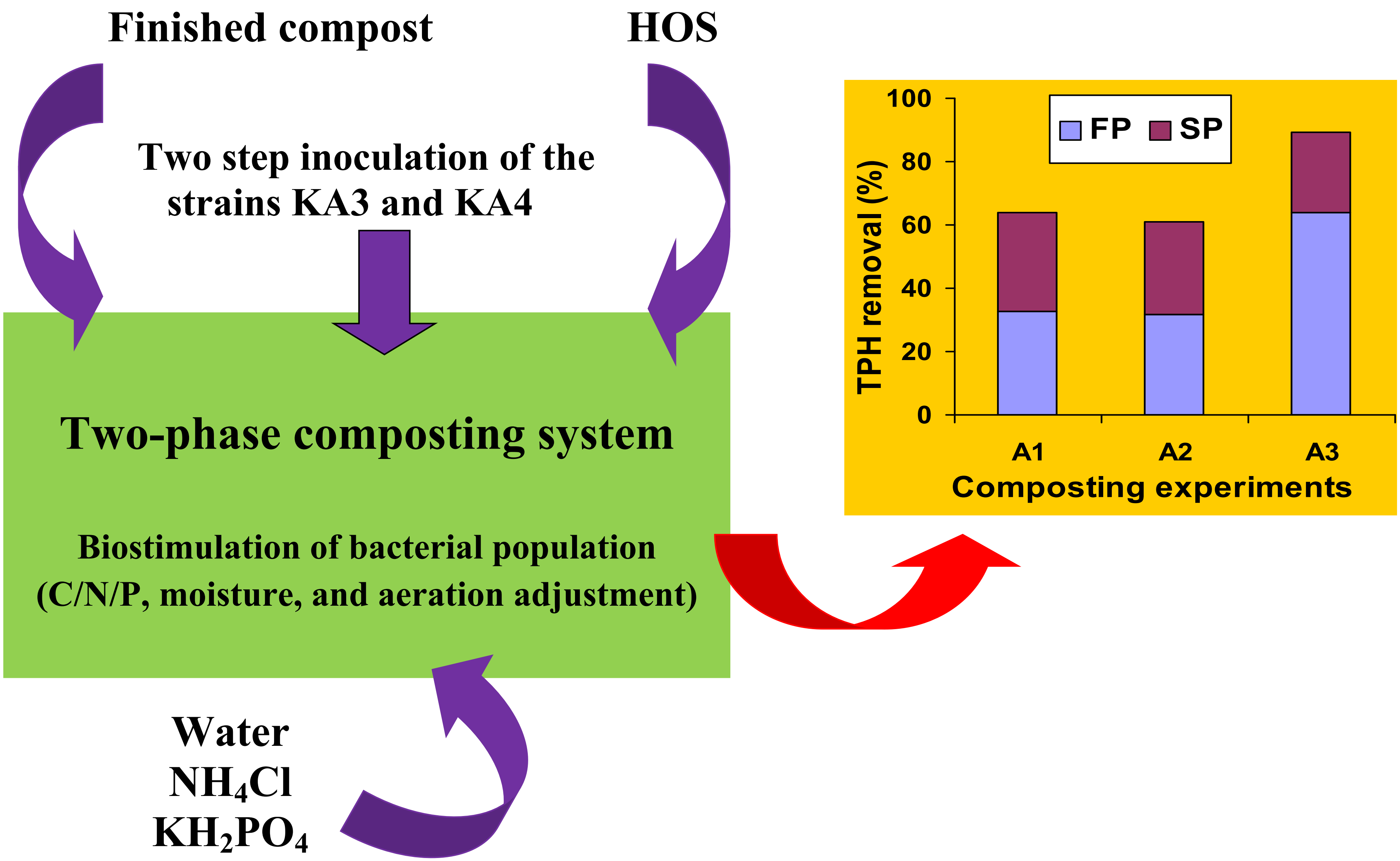
Yours sincerely,

Corresponding author, Ali Koolivand

Email address: alikoulivand@arakmu.ac.ir

Highlights

- The strains KA3 and KA4 were isolated from the HOS sample.
- The strains can degrade TPH both in mineral-based medium and in composting process.
- The two isolated strains exhibited the synergistic effect in oil biodegradation.
- Approximately 90% of TPH was removed through the two-phase composting system.
- Successful scaling-up was achieved from mineral-based medium to composting process.


Finished compost
HOS
Two step inoculation of the strains KA3 and KA4
Two-phase composting system
Biostimulation of bacterial population
(C/N/P, moisture, and aeration adjustment)
Water
NH_4Cl
KH_2PO_4
FP
SP
TPH removal (%)
100
80
60
40
20
0
A1
A2
A3
Composting experiments

Biodegradation of petroleum hydrocarbons by a two-step inoculation composting process using synergistic effect of indigenous bacteria isolated from heavy oily sludge: field application of a mineral-based medium in a two-phase composting system

Maryam Parhamfar[a], Hamid Abtahi[a], Kazem Godini[b], Reza Saeedi[c,d], Majid Sartaj[e], José Villaseñor[f], Frederic Coulon[g], Vinod Kumar[g], Tayebeh Soltanighias[h], Ehsanollah Ghaznavi-Rad[i], Ali Koolivand[j]*

a Molecular and Medicine Research Center, Arak University of Medical Sciences, Arak, Iran

b Department of Environmental Health Engineering, Faculty of Health, Hamedan University of Medical Sciences, Hamedan, Iran

c Workplace Health Promotion Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

d Department of Health, Safety and Environment (HSE), School of Public Health and Safety, Shahid Beheshti University of Medical Sciences, Tehran, Iran

e University of Ottawa, Department of Civil Engineering, 161 Louis Pasteur, Ottawa, Ontario K1N 6N5, Canada

f Department of Chemical Engineering, Institute of Chemical&Environmental Technologies, University of Castilla-La Mancha, Campus Universitario S/n,13071, Ciudad Real, Spain

g School of Water, Energy and Environment, Cranfield University, Cranfield, United Kingdom

h National Center for Microbial Resource, National Center for Cell Science, Pune, Maharashtra, India

i Molecular Research Center, Faculty of Medicine, Arak University of Medical Sciences, Arak, Iran

j Department of Environmental Health Engineering, Faculty of Health, Arak University of Medical Sciences, Arak, Iran

*Corresponding Author: Ali Koolivand, Email address: alikoulivand@arakmu.ac.ir

Conflict of interest

The authors declare that they have no conflict of interest.

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Abbreviations list[1]

[1] BATH: bacterial adhesion to hydrocarbon, BH: Bushnell-Hus, FP: first phase, FC: finished compost, MBM: mineral-based medium, OC: organic carbon, OD: optical density, HOS: heavy oily sludge, SP: second phase, TPH: total petroleum hydrocarbons

Abstract: The impact of two-step inoculation of indigenous strains and their synergistic effect in the scaling-up of petroleum hydrocarbons biodegradation from a mineral-based medium (MBM) to a two-phase composting process were investigated. After isolating the strains KA3 and KA4 from heavy oily sludge (HOS), their emulsification index (E_{24}), bacterial adhesion to hydrocarbon (BATH), and oil degradation efficiency were evaluated in the MBM. Then, they were inoculated twice into the composting bioreactors lasted for the primary 8 weeks as the first phase (FP) and subsequent 8 weeks as the second phase (SP). Based on the results, 57.12, 61.17, 59.87, 32.50, and 16.38% of oil was removed by the consortium of the two strains, respectively, for the concentrations of 1, 2, 3, 4, and 5% in the MBM. In the composting reactors, removals of 20 g kg^{-1} initial concentration of total petroleum hydrocarbons (TPH) were found to be 63.95, 61.00, and 89.35% for the strains KA3, KA4, and their consortium, respectively. The computed biodegradation constants indicated the synergistic effect of the two strains and the effectiveness of the second-step inoculation. The study demonstrated the successful scaling-up of HOS biodegradation from MBM to the two-phase composting process through two-step inoculation of the isolated strains.

Key words: Biodegradation scale-up; Heavy oily sludge; Two-step inoculation; Composting process; Synergistic effect

1. Introduction

Population growth, rapid industrialization and urbanization have increased the world consumption of energy. Despite the continuing rapid growth in renewable energy, crude oil is still the most important strategic source of material and energy and its total production reached 4474 million tonnes in 2018, which shows an increase of 11.9% compared with 2008 [1]. Processing crude oil in petroleum refinery industries annually produces huge quantities of heavy oily sludge (HOS) [2, 3]. It has been recognized that HOS is mainly composed of petroleum hydrocarbons, water, soil, and heavy metals. Improper disposal of oily sludge can lead to serious environmental and health issues. Hence, there is a need for effective technologies to treat this type of industrial waste before disposal [4, 5]. Until now, multiple physical and chemical methods have been used as treatment strategies for decontamination of petroleum compounds. However, most of these approaches are not economically and/or ecologically viable. As an alternative, bioremediation technologies such as composting process has shown to be an environmentally sound and cost-effective method to treat petroleum compounds [6, 7].

In bioremediation, easily biodegradable compounds are rapidly decomposed and then the rate of biodegradation decreases. This reduction is due to both the persistent nature of the residual hydrocarbons and the limitation in the metabolic activities of microbial population [8, 9]. A two-phase composting process, in which the microbial activities are supported through two-step inoculation, was proposed by some researchers to overcome this limitation [3, 10].

Bioremediation of oily sludge is also limited due to the scarcity of native specialized microbes needed for degrading various fractions of petroleum hydrocarbons. In order to promote bioremediation performance, microbial inoculation and bioaugmentation strategies can be used [11, 12]. However, no single microbial species has the ability to metabolize all classes of compounds typically found in crude oil. A consortium composed of many different species is thus required to take advantage of their synergistic interactions [13, 14]. However, the antagonistic effects such as competition for carbon sources may also influence the growth of bacterial species and thereby decrease the process efficacy, especially in a full-scale bioremediation process. Thus, oily sludge decomposition during bioremediation processes can be complicated by not only biological factors, but also physicochemical parameters [15]. Thion et al. [16] observed antagonistic interactions between the mixed culture of fungus and bacterium in bioremediation of contaminated soils. Hence, one of the most important problems of petroleum hydrocarbons biodegradation has been the lack of effective scale-up of mineral-based medium (MBM) results to full-scale bioremediation methods [17]. For this reason, the appropriate combination of native bacterial species and investigation of their metabolic characteristics and interactions should be performed to optimize and scale-up MBM experiments to full-scale bioremediation processes [18, 19].

To the best of our knowledge, scaling-up of HOS bioremediation from MBM to a two-phase composting system through two-step inoculation of isolated indigenous strains has not been reported before. This study presents the results of the scale-up of a HOS biodegradation process from MBM to a two-phase composting method at full scale. The synergistic effects of two isolated strains both in MBM and in the composting system and also the impact of two-step inoculation of the strains (both alone and consortium) into the composting process were studied. The composting system used in the current research consisted of the first phase (FP), lasted for 8 weeks, followed by the second phase (SP), also lasted for 8 weeks.

2. Materials and methods

2.1. Isolation of oil-degrading bacteria

HOS was obtained from Shazand oil refinery plant, Iran. The bacterial strains of HOS samples were determined using the serial dilution method. After blending HOS (5 g) with Bushnell-Hus (BH) medium (100 ml) at 160 rpm and incubating at 30 °C for 7 days, 5 ml of the medium was again mixed with BH (100 ml) containing 1% concentration of crude oil as a sole carbon source. The abovementioned method was repeated 3 times to assure that only the bacteria are responsible for the medium turbidity. Then, the medium (100 µl) was spread onto the Muller-Hinton agar and then incubated for 48 h. The formed colonies were again transferred to the surface of Muller-Hinton agar. Each isolated bacterium was mixed with BH consisting of 1% concentration of crude oil, and then incubated for 7 days to verify the colonies capabilities in oil degradation. Cell growth was monitored by measuring cell turbidity determined as optical density at 600 nm ($OD_{600\ nm}$) for the MBM. Six strains showing the highest $OD_{600\ nm}$ and growth in the presence of crude oil were selected. These 6 strains were also exposed to the concentrations of 1, 2, 3, 4, and 5% of crude oil. Finally, the two fastest-growing bacterial strains exhibiting high efficacy of oil degradation were selected for further tests and application in the composting experiments.

2.2. Identification of the isolated bacteria

The isolates were investigated in terms of various characteristics such as morphology, motility, gram stain test, and biochemical tests. Confirmation of the isolates was conducted by the PCR and Bio-Rad Thermal Cycler based on the procedures reported in a previous work [20]. Electrophoresis of the DNA was performed by agarose gel (0.8%) in Tris-Borate-EDTA (TBE) buffer. The PCR product was sequenced by Bioneer Co., Korea mediated by Pishgam Co., Iran.

The sequences were analyzed and aligned by Chromas software and ClustalW program. By using BLAST tools, the sequences were compared with NCBI database. CLUSTAL X 2.0 software was applied to include top hit sequences in alignment analysis. Phylogenetic tree was constructed by MEGA software v 7.0 [21, 22].

2.3. Determination of emulsification index (E_{24})

E_{24} (%) was measured according to the procedure described previously [23, 24]. Briefly, the isolated strains were added to Nutrient Broth and incubated at 30 °C for 48 h. Then, a mixture of the free cell supernatant and oils were vortexed vigorously for 2 min. After keeping the sample at room temperature for 24 h, the E_{24} was determined as follows:

$$E_{24}\ (\%) = (\text{Height of the emulsified layer/total height of liquid column}) \times 100$$

2.4. Determination of bacterial adhesion to hydrocarbon (BATH)

BATH was determined through the method described by [25] with slight modifications. Briefly, the strains were transferred to Nutrient Agar and incubated at 30 °C for 24 h. After adding one colony of the strains to a buffer solution, the primary OD (OD_1) was determined. Then, 200 μl of Hexadecane was added and the mixture was shaken well for 2 min. The hydrocarbon was separated through maintaining at room temperature for 30 min. The BATH was calculated through measuring the secondary OD (OD_2) of the aqueous phase as follows:

$$\text{BATH}\ (\%) = [(OD_1 - OD_2)/OD_1] \times 100$$

2.5. Crude oil biodegradation in MBM

Before inoculation of the isolated strains in the composting rectors, their capabilities in crude oil biodegradation were investigated in the MBM. Multiple concentrations of crude oil including 1, 2, 3, 4, and 5% v v^{-1} were used in the 500-ml Erlenmeyer flasks. The process was conducted at neutral pH and a temperature of 30 °C. After shaking at 120 rpm throughout a 7-day period, oil degradation was calculated. The rates of Total petroleum hydrocarbons (TPH) decrease in MBM were determined as the TPH removal against the control experiments. The control experiments were performed under the same conditions without any inoculation. The oil concentration showing the highest biodegradation was selected to be used in the composting bioreactors.

In order to study the influence of pH on crude oil biodegradation, tests were performed at pH values of 4, 5, 6, 7, 8, and 9. The isolated strains and 1% concentration of crude oil were blended with BH and incubated for 7 days and then the OD and crude oil reduction were calculated. HCl and NaOH were used for pH adjustment of the medium.

2.6. HOS biodegradation in the composting bioreactors

Five cylindrical bioreactors were operated for a period of 16 weeks. In the composting reactors, the sterile finished compost (FC) was blended with sterile HOS in the mixing ratio (FC to HOS) of 12.2:1. The physico-chemical properties of the HOS and FC are presented in the Table S1 (supplementary material). This mixing ratio was selected to reach an initial TPH concentration of 20 g kg^{-1} based on the results of the oil biodegradation in MBM. The composting experiments A_1, A_2, and A_3 contained the strains KA3, KA4, and their consortium, respectively. At the initiation of the process, each reactor was provided with the 0.5 McFarland of the isolates (5% v v^{-1}). At the end of week 8 (FP), the bacterial inoculation was repeated. For reactor A_4, inoculation of the two strains consortium was only performed at the beginning of the process. Comparing the performance of the reactor A_4 with A_3 would allow to investigate the effect of the once and twice inoculation steps on TPH removal. The reactor A_5 was operated as control without any bacterial inoculation to ascertain that it did not have any active microorganisms capable of degrading hydrocarbons. According to the previous papers [26, 27], the ratio of C/N/P in the composting bioreactors were adjusted at 100/5/1 through the addition of NH_4Cl and KH_2PO_4. Aerobic conditions in the reactors were supplied by means of oil-free pumps (HAILEA Model ACO 5505) at the rate of 1 l min^{-1} kg^{-1} [28]. The moisture level of the process was kept constant at 50-55% over the whole composting time.

2.7. Analytical methods

The organic carbon (OC) and TPH were determined bi-weekly over the process time. The value of pH was measured by means of a pH meter (JENWAY model 3510) according to TMECC [29]. The OC was quantified on the basis of loss-on-ignition method described by TMECC [29]. The TPH was extracted with n-pentane and then quantified by means of a gas chromatograph (Shimadzu, Japan) based on TNRCC [30]. The operating conditions of the gas chromatograph have been described in a previous work [31]. All tests were repeated in triplicate.

2.8. Kinetic study

Kinetic study of microbial degradation was also performed to better understand the TPH removal rates during the composting process. Biodegradation of petroleum hydrocarbons was explained by the first and second-order kinetics depicted by the following equations:

$$C_t = C_i\, e^{-kt} \qquad (1)$$

$$t_{1/2} = \ln 2/k_1 = 0.693/k_1 \qquad (2)$$

$$1/C_t = k_2 t + (1/C_t) \qquad (3)$$

$$t_{1/2} = 1/k_2 C_i \qquad (4)$$

Where C_i is the initial concentration of TPH (g kg^{-1}), C_t is TPH concentration (g kg^{-1}) at time t, k_1 (d^{-1}) and k_2 (g $kg^{-1}d^{-1}$) are biodegradation rate for the first-and second-order kinetics, respectively. $t_{1/2}$ is the time (d) needed for removing half of the initial level of TPH.

2.9. Statistical analysis

One-way ANOVA test (SPSS software) was used to compare the differences (P value ≤ 0.05) between the composting reactors. Regression analysis (Microsoft Excel software) was also applied to determine the possible correlations between the variables.

2.10. Nucleotide sequence accession numbers

The nucleotide sequences from this study were deposited in NCBI GenBank under the accession numbers of MK127545 and MK127546, respectively, for *Enterobacter hormaechei* strain KA3 and *Staphylococcus equorum* strain KA4.

3. Results and discussion

3.1. Taxonomic and metabolic characterization of the isolated bacteria

Taxonomic characteristics of the strains were determined by 16S rRNA gene sequence analysis. The phylogenetic analysis (Fig. 1) and NCBI Genbank database similarity search demonstrated that the bacteria are *Enterobacter hormaechei* strain KA3 and *Staphylococcus equorum* strain KA4. The results of the biochemical tests conducted on the strains have been provided in Table S2 (supplementary material). Table S3 (supplementary material) also presents the metabolic ability of the isolates to grow in the MBM containing 1% of crude oil. As can be inferred, there is a lag in effective bacterial growth during the first 2 days of the incubation period. Then, the biomass was rapidly generated until the day 7-10. From that day onward, the bacterial growth and biomass production continued to increase, but at a lower rate. Thus, the isolates reached to the logarithmic phase in a period of about 7-10 days. This period was selected as the time of incubation for all the tests performed in the MBM. It can be inferred that both the two individual isolates and their consortium can grow well in the presence of crude oil.

Fig. 1

3.2. MBM experiments

3.2.1. Effect of pH on crude oil biodegradation

The effect of pH, as a crucial parameter affecting the bacterial metabolism and petroleum hydrocarbons solubility, on the bacterial growth and decomposition of crude oil (1% concentration) was examined. As can be seen from Table 1, the strains exhibited the highest growth and oil biodegradation at the pH value of 7. At this pH, 48.85 and 46.35% of TPH was removed by the strains KA3 and KA4 during 7 days. At the pHs of 6 and 8, the crude oil degradation decreased slightly and reached to the range of 38.56-41.93%. However, the biodegradation reduced sharply at the pH values of 5 and 9. These findings are in line with other studies [32, 33] reporting that the oil degrading bacteria prefer to grow at neutral pH for TPH removal. The consortium of the two strains also presented the best efficacy and growth in the pH range of 6-8. For this reason, the composting bioreactors were operated at the neutral condition.

Table 1

3.2.2. Effect of initial concentration of crude oil

The effect of initial oil concentrations (1-5%) on the mineralization of petroleum hydrocarbons was examined in this work. The results (Table 2) showed that the strains were more effective to degrade 1-3% concentrations of crude oil as the removal percentage dropped significantly at initial oil concentrations of 4 and 5%. Less effective degradation at these high levels of oil could be due to the bacterial metabolic characteristics and crude oil toxicity. Moreover, high concentration of crude oil can block the aeration, which also affects the bacterial growth [34]. The highest biodegradation occurred at a crude oil concentration of 2% as after 7 days, 53.94 and 50.68% of crude oil was degraded, respectively, by the strains KA3 and KA4. The capacity of the isolates for TPH removal was not high at a very low level (1%) of crude oil. When the carbon source is too low to promote microbial growth, extremely low amount of crude oil would limit TPH removal [3]. Thus, the crude oil amount of 2% was found to be the optimum initial concentration for the isolates to effectively degrade petroleum hydrocarbons. This optimal concentration was the basis for adjusting the mixing ratios of HOS to FC in the composting setups.

Table 2

3.2.3. Synergistic effect of the strains

As can be seen from Tables 1 and 2, the oil degradation by the bacterial consortium was higher than that of the individual strains. Hence, these two strains presented the synergistic effect for TPH biodegradation when they are used in the mixed culture. Several authors [18, 35, 36] already reported that pure single strains were not able to degrade crude oil effectively compared to their consortium. The positive effects of bacterial consortium compared to individual strains will be deeply discussed in section 3.3.1. E_{24} was calculated to investigate the ability of the strain in biosurfactant production. The value of BATH was also measured to determine the affinity of the strains to the petroleum hydrocarbons. The isolates KA3, KA4, and their consortium showed emulsification index of 13, 10, and 18%, respectively. The corresponding values for BATH were found to be 8.62, 16.10, and 21.10%, respectively. These values also verified the better performance of the consortium as compared to each strain.

3.2.4. Relation between crude oil degradation and cell growth

Growth of the individual strains and their consortium in BH medium was also determined (Tables S2, 1, and 2) through measuring the biomass production ($OD_{600\ nm}$). The crude oil concentration decreased in response to increased cell numbers, indicating that the isolated bacteria can utilize crude oil as a sole source of carbon. Regression analysis presented in Fig. 2, also indicated that the oil biodegradation was in direct correlation with biomass formation of the selected strains. These results of the biomass production demonstrated the ability of the isolates to consume petroleum hydrocarbons as a carbon source. The higher optical density observed in the case of the bacterial consortium showed more effective growth of the consortium as compared to the individual strains.

Fig. 2

3.3. Scaling-up of HOS biodegradation from MBM to composting process

3.3.1. TPH removal

Determination of the actual role of microbial community for oil degradation in the aqueous phase is not easy since a large fraction of viscous and sticky oil may attach to the surface of flask instead of dispersing in the liquid medium. Hence, it is of vital importance to evaluate the potential of the isolates in biodegradation of petroleum pollutants in a real bioremediation conditions such as composting process. For this reason, we simulated the TPH removal in the composting bioreactors based on results obtained from the MBM. Accordingly, the HOS containing 255.05 g kg^{-1} of TPH concentration was blended with FC in the mixing ratio of

12.23:1 to reach an initial concentration of 20 g kg^{-1}. The initial TPH concentration is of great importance since the proper adjustment of the mixing ratio greatly affects TPH removal [26, 37].

Fig. 3a indicates the trend of TPH decomposition in the composting treatments. The reduction rates of TPH in the reactors A_1, A_2, A_3, and A_4 were 63.95, 61.00, 89.35%, and 76.20, respectively during 16 weeks. Thus, the biodegradation capacities of the two strains were nearly similar. However, the percentage of TPH degradation significantly increased when their consortium was inoculated to the composting reactors. Hence, application of the two combined isolates resulted in their synergistic effect in terms of TPH removal. As crude oil consists of different hydrocarbons, and each strain can metabolize only a limited range of materials, bioremediation of oily sludge requires a microbial consortium to degrade petroleum hydrocarbons more effectively. A collaboration and synergistic effect between different bacteria makes them act better than a single strain. In recent years, combination of microbial strains for enhancing biodegradation of various types of pollutants has attracted much attention [13, 14].

The results of the present study showed that TPH removal by the consortium were 25.40 and 28.35% higher than the individual cultures of KA3 and KA4, respectively. Kamyabi et al. [13] also reported that an additional 20% of pyrene removal was achieved by combined cultures in comparison to individual cultures. Other studies have also described the higher ability of consortium to degrade petroleum pollutants [18, 38]. The negligible TPH removal (3.8%) observed in the control reactor (A_5) indicated that the bacterial populations were responsible for hydrocarbon degradation in the reactors A_1-A_4.

Fig. 3

3.3.2. Effect of two-step inoculation on TPH reduction

In the case of one-step inoculation (the reactor A_4), high degradation of petroleum compounds and thereby TPH removal were initiated until the end of week 6, and then, the biodegradation rate lowered to the end of the process. It has been reported in previous works [39, 40] that the biodegradation of petroleum materials proceeds rapidly in the beginning weeks of the composting process and slows down in the later. This pattern is due to the fact that the type and composition of petroleum hydrocarbons present in crude oil determine their susceptibility to microbial degradation. Accordingly, easily-biodegradable hydrocarbons are consumed first and the remained fractions are resistant to biodegradation [3, 34]. Naturally, the number or metabolic activity of the oil-degrading bacteria declines. As the bioremediation efficacy is a function of the extent to which microbes are maintained in the system, microbial deficiency limits the effectiveness of the process [14]. The inoculation of native and specialist bacterial strains is helpful because of their high adaptation abilities to crude oil containing environments [41, 42]. For this reason, a two-phase composting, in which bacterial degradation of TPH was kept at an acceptable rate through two-step inoculation, was designed in the current work.

Removal percentages of TPH in various durations of the process were shown in Fig. 3b. In the reactors A_1 and A_2, 32.50 and 31.90% of TPH were removed over the FP. The corresponding values over the SP were 31.45 and 29.10%, respectively. As a result, a suitable efficacy of TPH degradation was yielded over both the FP and SP in these two experiments. The higher removal percentage observed at the weeks 10 and 12 supported the positive effect of reinoculation in promoting the process efficacy. These results are in line with other studies reporting the higher efficiency of two-phase composting compared to conventional one-phase system [10, 43]. It is interesting to note that although easily-biodegradable hydrocarbons were consumed over the FP, petroleum hydrocarbons continued to decompose during the SP, mainly as a result of bacterial reinoculation at a high concentration. The effective role of microbial inoculation in hydrocarbon removal has also been indicated previously [20, 41].

The biodegradation rates of TPH in the reactor A_3 were 64.05 and 25.30% over the FP and SP, respectively. Hence, the second-step inoculation did not enhance the process performance during the SP. Comparing the TPH biodegradation in the experiments A_3 and A_4 is also helpful in terms of the effectiveness of the two-phase composting when the bacterial consortium is used. These two reactors were thoroughly similar in terms of initial TPH concentration and bacterial strains. However, unlike other reactors, A_4 was a conventional composting process experiencing a one-step inoculation. Naturally, TPH reductions in these two reactors were similar over the FP of the process duration. The overall removal rate in the A_3 was only 13.15% higher than that A_4. This also demonstrated that the application of two-step inoculation of bacterial consortium is not justifiable. Accordingly, the composting process can perform well in the form of conventional one phase when the microbial consortium is used. Thus, the strains combination and the positive synergistic effect would compensate the requirements of periodic inoculations when using individual strains.

3.3.3. Effect of bulking agent addition on the bioreactors performance

Since microbes prefer to consume less recalcitrant organic carbons, the presence of easily-decomposable materials can help maintain the bacterial activity in the system. On the other hand, the organic materials used must not be preferred over the target contaminant. Furthermore, the bulking agent should not add at high concentrations in which they act as a sole carbon source [3, 10]. In this point of view, the type and level of bulking agent used in the composting process significantly influence microbial growth.

In order to survey the effect of addition of FC (as a bulking agent) on the TPH degradation, the change of OC and TPH/OC was plotted in Fig. 4. The decrement in the ratio of TPH/OC showed that TPH biodegradation was higher than that of OC. Therefore, the bulking agent added to the composting reactors was not a competing carbon source for petroleum hydrocarbons. Hence, the addition of FC influenced positively the process and supported the TPH removal.

Bulking agents such as FC promote the capacity of the composting mixture in maintaining water contents, which can help the bacterial growth. In addition, they facilitate air diffusion through the composting medium resulting in the higher heat generation and rapid TPH removal [44]. The regression analysis (Fig. 5) indicated linear correlation between the biodegradation of TPH and OC. In the large-scale composting facilities, prediction of TPH removal on the basis of OC consumption can be done using these correlations and computed equations.

Fig. 4

Fig. 5

3.3.4. Bioremediation kinetic study

According to the computed values presented in Table 3, TPH removal fitted to the first and second-order model over the FP and SP, respectively. This result is in accordance with other studies reporting that biodegradation of petroleum hydrocarbons proceeds according to the first- and second-order kinetics [45, 46]. The values of $t_{1/2}$ and k_1 for the first-order kinetic over the FP were in the range of 5.17-13.59 d and 0.051-0.134 d^{-1}, respectively. The corresponding values for the second-order kinetic over the SP were respectively, 1.11-5.56 d and 0.009-0.045 g $kg^{-1}d^{-1}$. All the values in the table demonstrated the better performance of the reactor A_3 containing the bacterial consortium compared to the reactors A_1 and A_2. Moreover, the higher values of k_1 and k_2 during SP verified the effectiveness of the second inoculation. The values of k obtained in the present research were different to those computed by Gomez and Sartaj [47]. The reason is the highly dependence of the kinetic values on multiple parameters like the nature of oily sludge, the method of bioremediation, and operational conditions of the system [48, 49].

Table 3

4. Conclusions

The impact of two-step inoculation of native strains and their synergistic effect in the scaling-up of HOS bioremediation from MBM to the two-phase composting system were studied. The strains were effectively able to remove TPH both in MBM and in composting process. The results revealed the synergistic potential of the consortium of the strains KA3 and KA4 as compared to their individual cultures. The second-step inoculation of each strain alone greatly enhanced TPH removal rate. However, the efficacy of the composting process did not significantly increased as a result of the second-step inoculation of the consortium. This research indicated the successful scaling-up of HOS treatment from MBM to the used composting method through two-step inoculation of the isolated strains.

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Table titles

Table 1. Effect of pH on the efficacy of the isolated strains in biodegradation of 1% concentrations of crude oil in the MBM after a period of 7 days

Table 2. Efficacy of the isolated strains in biodegradation of various concentrations of crude oil in the MBM after a period of 7 days at an initial pH of 7

Table 3. Kinetic data of TPH biodegradation in the composting bioreactors over the FP and SP

Figure captions

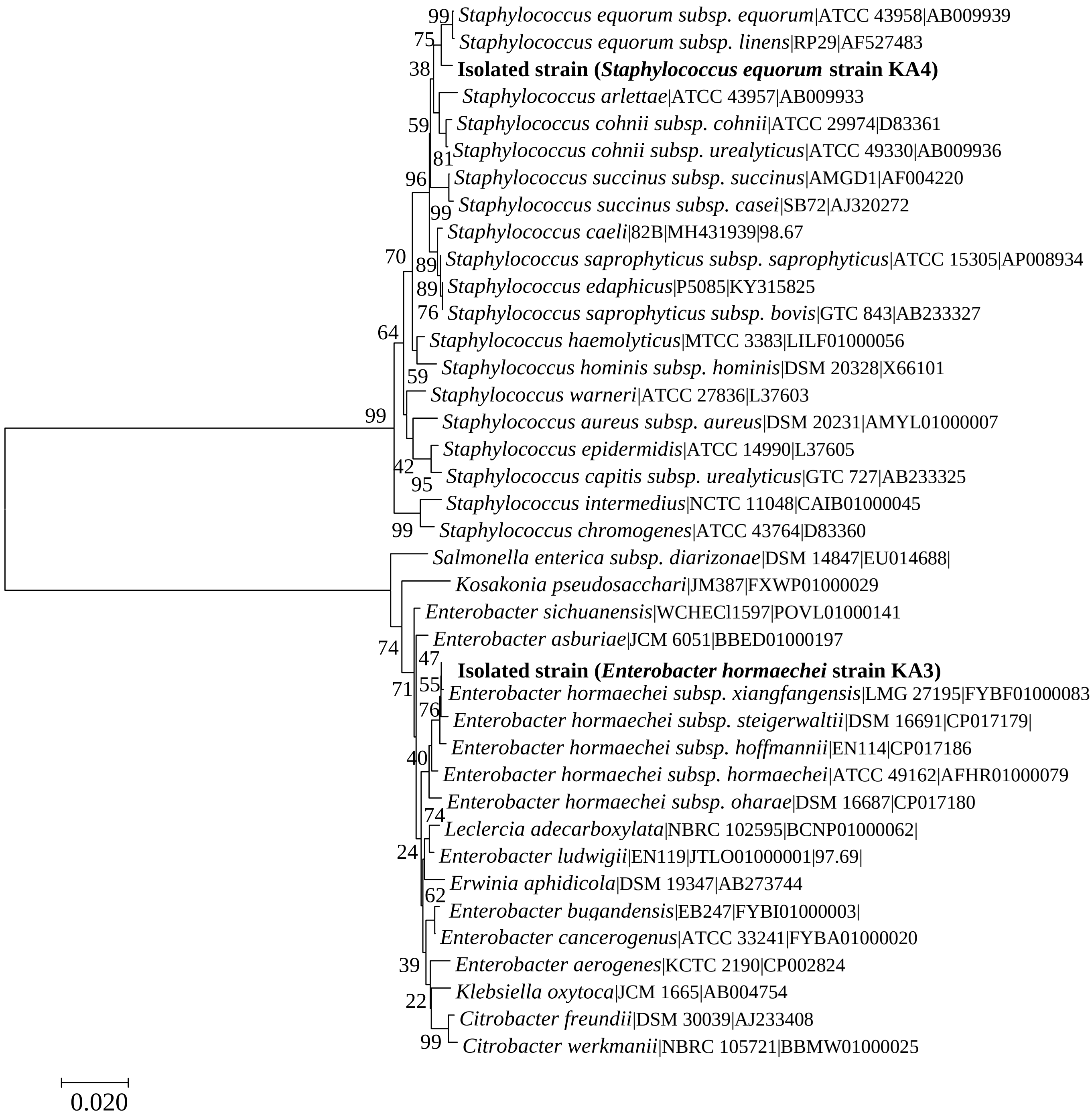
Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences of the two bacterial strains isolated from HOS

Fig. 2. Correlation between biomass generation (OD_{600}) and oil degradation in the MBM

Fig. 3. (a) Residual TPH over the process duration in the composting bioreactors; and (b) percentages of TPH removal over the FP and SP duration in the composting bioreactors

Fig. 4. (a) Trend of OC and (b) TPH/OC changes in the composting bioreactors over the process duration

Fig. 5. Regression analysis of OC and TPH correlation in the composting bioreactors over the (a) FP and (b) SP


Staphylococcus equorum subsp. equorum|ATCC 43958|AB009939
Staphylococcus equorum subsp. linens|RP29|AF527483
Isolated strain (Staphylococcus equorum strain KA4)
Staphylococcus arlettae|ATCC 43957|AB009933
Staphylococcus cohnii subsp. cohnii|ATCC 29974|D83361
Staphylococcus cohnii subsp. urealyticus|ATCC 49330|AB009936
Staphylococcus succinus subsp. succinus|AMGD1|AF004220
Staphylococcus succinus subsp. casei|SB72|AJ320272
Staphylococcus caeli|82B|MH431939|98.67
Staphylococcus saprophyticus subsp. saprophyticus|ATCC 15305|AP008934
Staphylococcus edaphicus|P5085|KY315825
Staphylococcus saprophyticus subsp. bovis|GTC 843|AB233327
Staphylococcus haemolyticus|MTCC 3383|LILF01000056
Staphylococcus hominis subsp. hominis|DSM 20328|X66101
Staphylococcus warneri|ATCC 27836|L37603
Staphylococcus aureus subsp. aureus|DSM 20231|AMYL01000007
Staphylococcus epidermidis|ATCC 14990|L37605
Staphylococcus capitis subsp. urealyticus|GTC 727|AB233325
Staphylococcus intermedius|NCTC 11048|CAIB01000045
Staphylococcus chromogenes|ATCC 43764|D83360
Salmonella enterica subsp. diarizonae|DSM 14847|EU014688|
Kosakonia pseudosacchari|JM387|FXWP01000029
Enterobacter sichuanensis|WCHECl1597|POVL01000141
Enterobacter asburiae|JCM 6051|BBED01000197
Isolated strain (Enterobacter hormaechei strain KA3)
Enterobacter hormaechei subsp. xiangfangensis|LMG 27195|FYBF01000083
Enterobacter hormaechei subsp. steigerwaltii|DSM 16691|CP017179|
Enterobacter hormaechei subsp. hoffmannii|EN114|CP017186
Enterobacter hormaechei subsp. hormaechei|ATCC 49162|AFHR01000079
Enterobacter hormaechei subsp. oharae|DSM 16687|CP017180
Leclercia adecarboxylata|NBRC 102595|BCNP01000062|
Enterobacter ludwigii|EN119|JTLO01000001|97.69|
Erwinia aphidicola|DSM 19347|AB273744
Enterobacter bugandensis|EB247|FYBI01000003|
Enterobacter cancerogenus|ATCC 33241|FYBA01000020
Enterobacter aerogenes|KCTC 2190|CP002824
Klebsiella oxytoca|JCM 1665|AB004754
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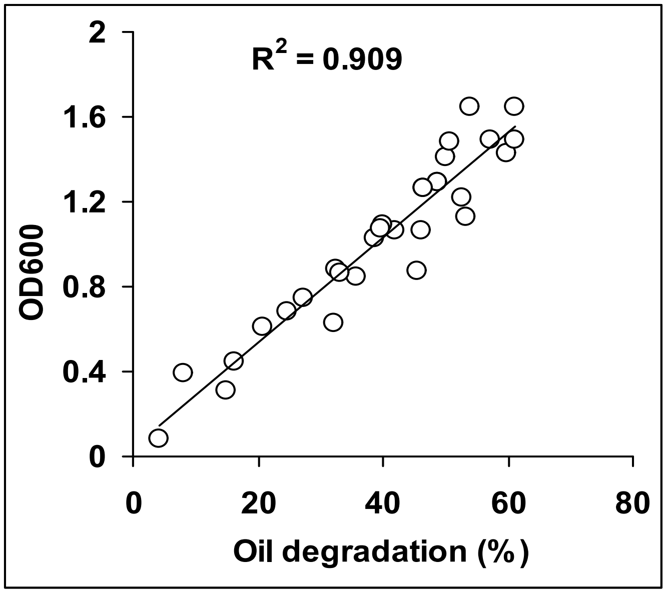
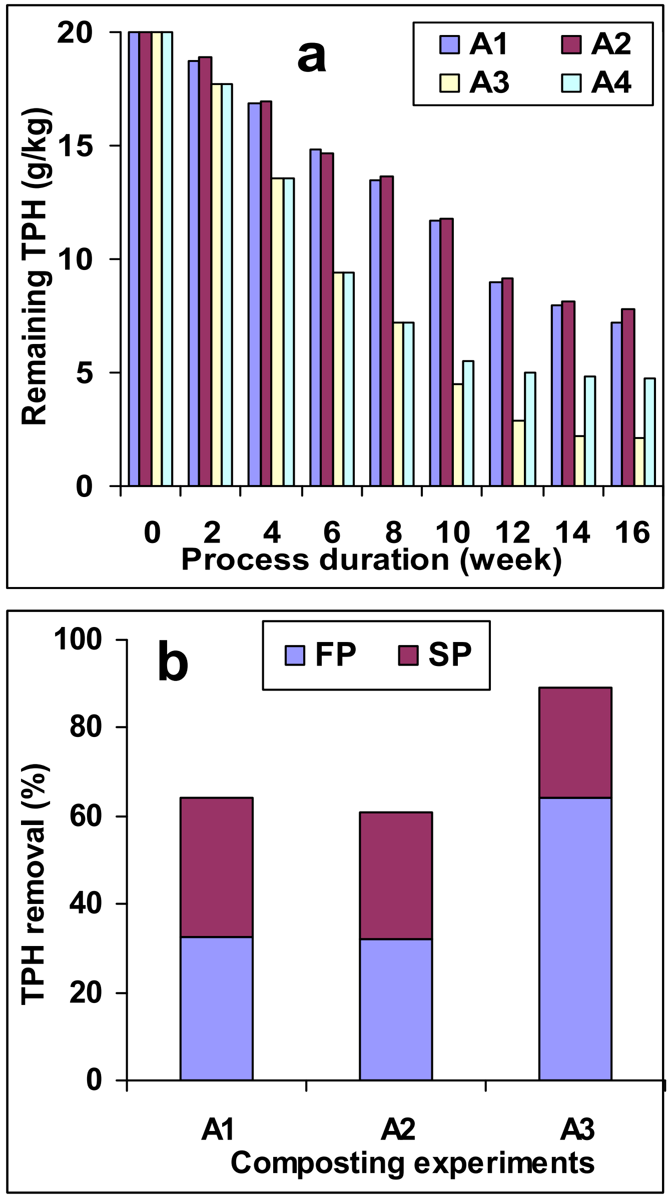
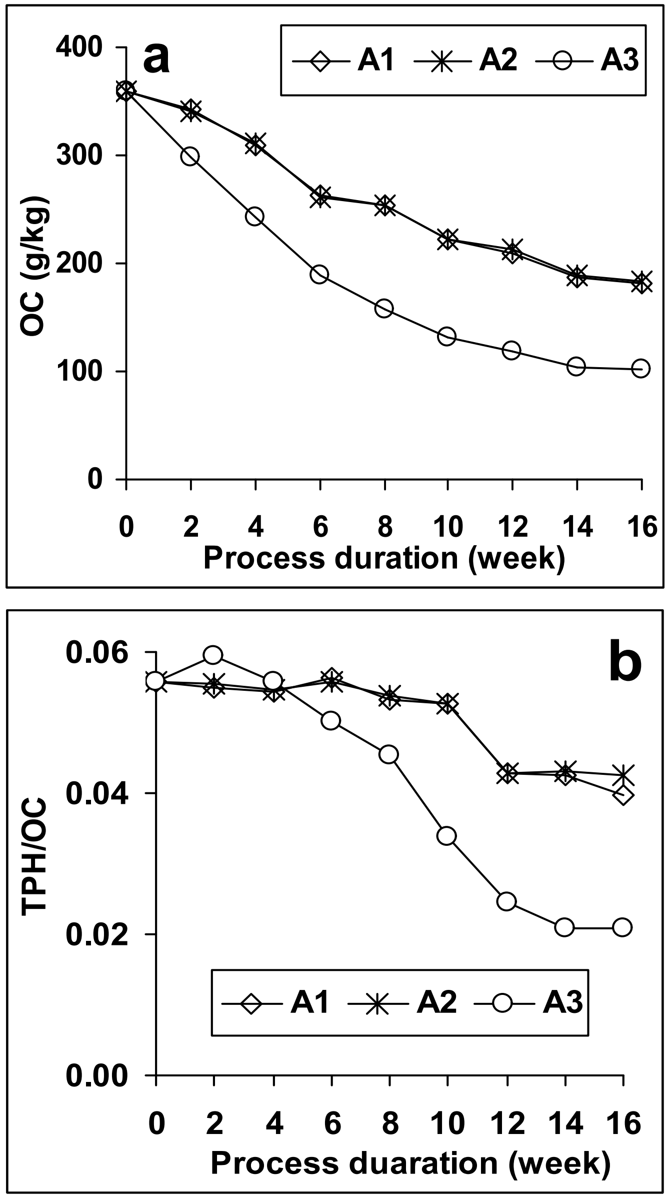
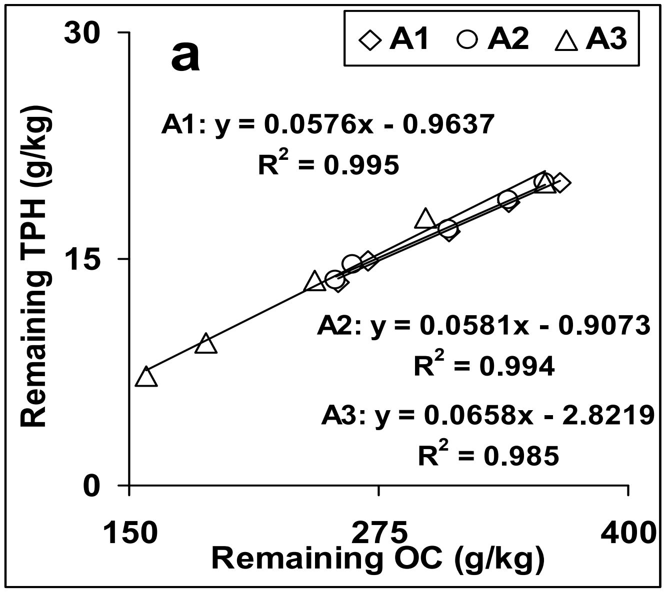


$R^2 = 0.909$
OD600
2
1.6
1.2
0.8
0.4
0
0
20
40
60
80
Oil degradation (%)

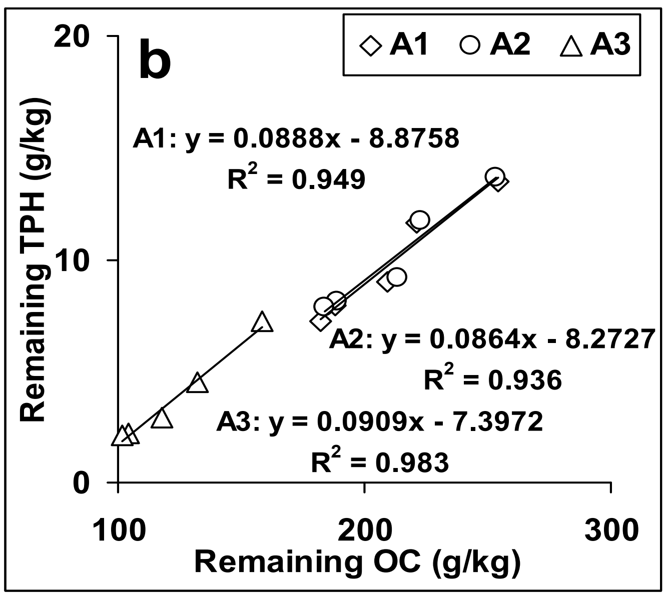

a
A1
A2
A3
A4
Remaining TPH (g/kg)
20
15
10
5
0
0
2
4
6
8
10
12
14
16
Process duration (week)
b
FP
SP
TPH removal (%)
100
80
60
40
20
0
A1
A2
A3
Composting experiments



a
A1
A2
A3
400
300
200
100
0
OC (g/kg)
0
2
4
6
8
10
12
14
16
Process duration (week)
b
0.06
0.04
0.02
0.00
TPH/OC
A1
A2
A3
0
2
4
6
8
10
12
14
16
Process duaration (week)



a
A1 A2 A3
A1: y = 0.0576x - 0.9637
R2 = 0.995
A2: y = 0.0581x - 0.9073
R2 = 0.994
A3: y = 0.0658x - 2.8219
R2 = 0.985
Remaining TPH (g/kg)
Remaining OC (g/kg)
30
15
0
150
275
400



b
A1 A2 A3
A1: y = 0.0888x - 8.8758
R2 = 0.949
A2: y = 0.0864x - 8.2727
R2 = 0.936
A3: y = 0.0909x - 7.3972
R2 = 0.983
Remaining TPH (g/kg)
Remaining OC (g/kg)
20
10
0
100
200
300

Parameter	**pH**	**Strain KA3**	**Strain KA4**	**Consortium**
Percentage of crude oil degradation	5	27.17	24.67	33.27
	6	41.93	38.56	52.78
	7	48.85	46.35	61.09
	8	39.87	39.56	53.38
	9	35.91	32.04	45.50
OD_{600}	5	0.75	0.68	0.86
	6	1.06	1.03	1.22
	7	1.29	1.26	1.49
	8	1.09	1.07	1.13
	9	0.85	0.63	0.87

Parameter	Crude oil concentrations	Strain KA3	Strain KA4	Consortium
Percentage of crude oil degradation	1%	48.85	46.35	57.12
	2%	53.94	50.68	61.17
	3%	50.06	46.12	59.87
	4%	20.76	14.91	32.50
	5%	8.06	4.29	16.38
OD_{600}	1%	1.29	1.26	1.49
	2%	1.65	1.48	1.65
	3%	1.41	1.06	1.43
	4%	0.61	0.31	0.88
	5%	0.39	0.08	0.45

Composting phases	**Composting experiments**	**First-order kinetics**			**Second-order kinetics**		
		k_1 (d^{-1})	**$t_{1/2}$ (d)**	**R^2**	**k_2 (g $kg^{-1}d^{-1}$)**	**$t_{1/2}$ (d)**	**R^2**
FP	A_1	0.051	13.59	0.990	0.003	16.67	0.982
	A_2	0.051	13.59	0.980	0.003	16.67	0.974
	A_3	0.134	5.17	0.977	0.011	4.55	0.938
SP	A_1	0.082	8.45	0.970	0.009	5.56	0.983
	A_2	0.074	9.36	0.945	0.007	7.14	0.960
	A_3	0.157	4.41	0.922	0.045	1.11	0.958

Table S1. Physicochemical characteristics of HOS and FC used in this research.

Parameter	**Unit**	**HOS**	**FC**
TPH	g kg^{-1}	255.05	0.79
OC	g kg^{-1}	528.96	344.85
N	g kg^{-1}	1.75	4.25
P	g kg^{-1}	1.03	2.78
Water content	%	27.63	35.32

Table S2. Biochemical identifications of the two bacterial strains isolated from HOS

Tests	**Strain KA3**	**Strain KA4**
Gram stain	Gram negative	Gram positive
Oxidase	–[a]	–
Catalase	+[b]	+
Nitrate reduction	+	+
Citrate	–	–
Urease	–	+
H_2S production	–	–
Indole production	–	–
TSI	Alk[c]/Alk	A[d]/Alk

a Growth negative, b Growth positive, c Alkaline, d Acid

Table S3. Growth abilities of the isolated strains in the presence of 1% concentration of crude oil in the mineral based medium after a period of 7 days at an initial pH of 7.

OD_{600}	**Incubation period (day)**				
	2	**4**	**7**	**10**	**12**
Strain KA3	0.23	0.75	1.29	1.34	1.07
Strain KA4	0.27	0.64	1.26	1.34	1.05
Consortium	0.30	0.88	1.49	1.55	1.28