



Research article

Biodegradation of high concentrations of petroleum compounds by using indigenous bacteria isolated from petroleum hydrocarbons-rich sludge: Effective scale-up from liquid medium to composting process

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ABSTRACT

The scale-up of petroleum hydrocarbons-rich sludge (PHRS) bioremediation from liquid medium to a composting method bioaugmented with two indigenous bacteria, capable of degrading high levels of crude oil, was surveyed. After isolating the strains (*Sphingomonas olei* strain KA1 and *Acinetobacter radioresistens* strain KA2) and determining their biomass production, emulsification index (E_{24}), bacterial adhesion to hydrocarbon (BATH), and crude oil degradation in liquid medium, they were inoculated into the composting experiments. In liquid medium, the removal rate of crude oil were 67.25, 70.86, 61.77, 42.13, and 27.92%, respectively for the initial oil levels of 1, 2, 3, 4, and 5% after 7 days. Degradation of 10, 20, 30, 40 and 50 g kg⁻¹ concentrations of total petroleum hydrocarbons (TPH) were also calculated to be 91.24, 87.23, 84.69, 74.08, and 60.14%, respectively after a composting duration of 12 weeks. The values of the rate constants (k) and half-lives ($t_{1/2}$) of petroleum hydrocarbons degradation were 0.083–0.212 day⁻¹ and 3.27–8.35 days for the first-order and 0.003–0.089 g kg⁻¹ day⁻¹ and 1.12–6.67 days for the second-order model, respectively. This study verified the suitability of the isolated strains for PHRS bioremediation. Successful scale-up of PHRS bioremediation from a liquid medium to a composting process for degrading high amounts of TPH was also confirmed.

1. Introduction

Rapid expansion of crude oil industries has led to the generation of large volumes of hazardous petroleum hydrocarbons that warrants immediate attention due to their environmental and health impacts. Sustainable treatment and disposal of petroleum hydrocarbons-rich sludge (PHRS) is thus necessary to avoid the related environmental pollution (Suganthi et al., 2018; Varjani, 2017). Due to the high cost and secondary pollution associated with physical and chemical treatment methods, widespread application of them has been limited. Therefore, microbial bioremediation, as an environment-friendly and cost-effective technology, is the most feasible solution to attain complete pollutant degradation (Das and Kumar, 2016; Xu et al., 2019).

Bioremediation methods are performed by either adding specialized organisms (bioaugmentation) or by supporting the growth of indigenous microorganisms (biostimulation) (Muangchinda et al., 2018; Roy et al., 2018). Microbial stimulation through addition of nitrogen and phosphorus to oily sludge containing low levels of these nutrients can accelerate cell growth and hence increase the rate of hydrocarbon degradation (Roy et al., 2018; Silva-Castro et al., 2015). From another point of view, bioaugmentation through inoculating special microbiota, is a promising and low-cost strategy for bioremediation of various oily pollutants (Muangchinda et al., 2018). However, addition of allochthonous bacteria into a new environment is not highly advantageous due to the lack of their adaptability. Therefore, introduction of metabolically superior bacteria isolated from the same environment

Abbreviations: BATH, bacterial adhesion to hydrocarbon; BH, Bushnell-Hus; FC, final compost; OC, organic carbon; OD, optical density; PHRS, petroleum hydrocarbons-rich sludge; TPH, total petroleum hydrocarbons

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and their application as bioaugmentation agent is preferred (Rabodonirina et al., 2019; Tao et al., 2017). It is of note that one of the major issues regarding bioaugmentation is the lack of effective scale-up and translation of liquid-based experiments (Li et al., 2019). In this sense, composting process has been widely employed to treat various types of organic compounds such as petroleum hydrocarbons (Chen et al., 2015; Zhang and Sun, 2016).

In kinetic terms, microorganisms have different sensitivities to a wide range of pollutants concentrations present in the contaminated environments. There is also a threshold level below which the contaminant is not detected by organisms and, on the other hand, above a particular level, it is toxic to microorganisms. Moreover, in a limited range, the increase in the pollutant concentration results in decrease in the rate of degradation. Thus, it is of importance to determine the amount of a contaminant that can be decomposed by indigenous microbial community (Vaidya et al., 2017). Most of petroleum hydrocarbon degrading microorganisms present in oily sludge which consume crude oil as their carbon source can only degrade low levels of TPH. Although isolation of microbial populations from diverse petroleum-rich environments has been relatively well studied, there is a paucity of data on indigenous strains that can degrade high levels of petroleum hydrocarbons (Chen et al., 2017; Sarkar et al., 2017).

Considering the above background, more potential degrading strains should be isolated and more experimental evidences should be gathered to interpret their role in hydrocarbons degradation both in mineral-based medium and in the composting process. To the best of our knowledge, application of composting process for bioremediation of PHRS by using the mixed culture of isolated indigenous bacteria, capable of degrading high levels of crude oil in liquid medium, has not been previously studied. Thus, the present study was designed with the following objectives: (i) to isolate and identify bacterial exhibiting potential for degradation high amount of crude oil in liquid medium, and (ii) to evaluate usefulness of the selected strains for application in the composting process in order to remove high amounts of TPH from PHRS. The entire study depicts the suitability of the strains for bioremediation of PHRS containing high levels of TPH.

2. Materials and methods

2.1. Oil-degrading bacteria isolation

PHRS were prepared from an oil refinery plant located in Shazand, Iran and used as a source of the indigenous bacterial strains. The physical and chemical characteristics of PHRS have been provided in supplementary material as Table S1. Collected samples were transported to the laboratory within 24 h and isolation of cultivable populations was immediately performed. Isolation of hydrocarbon degrading bacteria was done in Bushnell-Haas (BH) media. First, 100 ml of (BH) medium was added to 5 g of PHRS and then, incubated at the temperature of 30 °C for 7 days. Five ml of this medium was again blended with BH containing crude oil of 1% concentration. After repeating this procedure 3 times, the medium of 100 µl was transferred to Mueller-Hinton agar and incubated and finally, the grown colonies were again spread to the Mueller-Hinton agar surface. So as to assure the capabilities of the isolates in oil degradation, they were individually added to BH containing crude oil of 1% concentration, and incubated for 12 days. In the liquid medium, the biomass production was monitored by determining cell turbidity and optical density at 600 nm ($OD_{600\text{ nm}}$). Out of 24 strains detected, two strains indicating the highest values of $OD_{600\text{ nm}}$ and degradation rate of high levels of oil were used for further examination and introduction into the biological composting experiments. The colonies were subcultured thrice to verify culture purity. The strains were stored at -70 °C for the future analyses.

Table 1

Biochemical identifications of the two bacterial strains isolated from PHRS.

Tests	Strain KA1	Strain KA2
Gram stain	Gram negative	Gram negative
Oxidase	+	-
Catalase	+	+
Nitrate reduction	-	-
Citrate	-	-
Urease	-	-
H ₂ S production	-	-
Indole production	-	-
TSI	Alkaline/Alkaline	Alkaline/Alkaline

2.2. Bacterial identification

Different tests including gram stain, morphology, motility, oxidase, catalase, nitrate reduction, citrate, urease, H₂S production, indole production, and triple sugar iron (TSI) were done in order to identify the isolated strains (Table 1). Molecular identification of bacterial strains was carried out by analyzing the 16S rRNA gene from respective genomes. Genomic DNA was extracted from each strain and 16S rRNA gene was PCR amplified and sequenced following standard procedure. Details of the methods and conditions of primers and PCR are the same as explained in our previous study (Koolivand et al., 2017). The data were analyzed using the Chromas software (<http://www.technelysium.com.au/chromas.html>) and aligned with the CLUSTAL X 2.0 program. Comparisons of the sequences with NCBI nucleotide database were done using BLAST and then submitted to GenBank. The phylogenetic tree was obtained using MEGA 7 software (Chen et al., 2017; Kumar et al., 2016).

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

Determination of E_{24} (%), as a measure of biosurfactant production by the strains was performed based on the procedure published previously (Bayat et al., 2016; Patowary et al., 2017). Briefly, the isolates were mixed with Nutrient Broth and incubated. The mixture containing crude oil and free cell supernatant was vortexed for 2 min and then kept standing for 24 h. E_{24} was calculated through dividing the emulsified height by the total liquid height.

2.4. Calculation of bacterial adhesion to hydrocarbon (BATH)

Cell surface hydrophobicity was used for determination of BATH as described previously (Chen et al., 2018) with slight modifications. Briefly, the isolate was added to Nutrient Agar and incubated for 24 h. Then, one colony was blended with a buffer solution and the primary OD (OD_1) was measured. After adding Hexadecane of 200 µl and shaking for 2 min, it was maintained (30 min) for separating hydrocarbon. The secondary OD (OD_2) was determined and the BATH was calculated according to the following equation:

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

2.5. Dependency of crude oil biodegradation on pH in liquid medium

Different values of pH including 4, 5, 6, 7, 8, and 9 were tested in order to investigate the impact of pH on the removal of crude oil in BH medium. The adjustment of medium pH at the abovementioned values was done by using HCl and NaOH. The crude oil of 1% concentration and the isolates were added to BH and incubated for 7 days. After this incubation period, the OD of the medium and the percentage of crude oil removal were calculated (Das and Kazy, 2014).

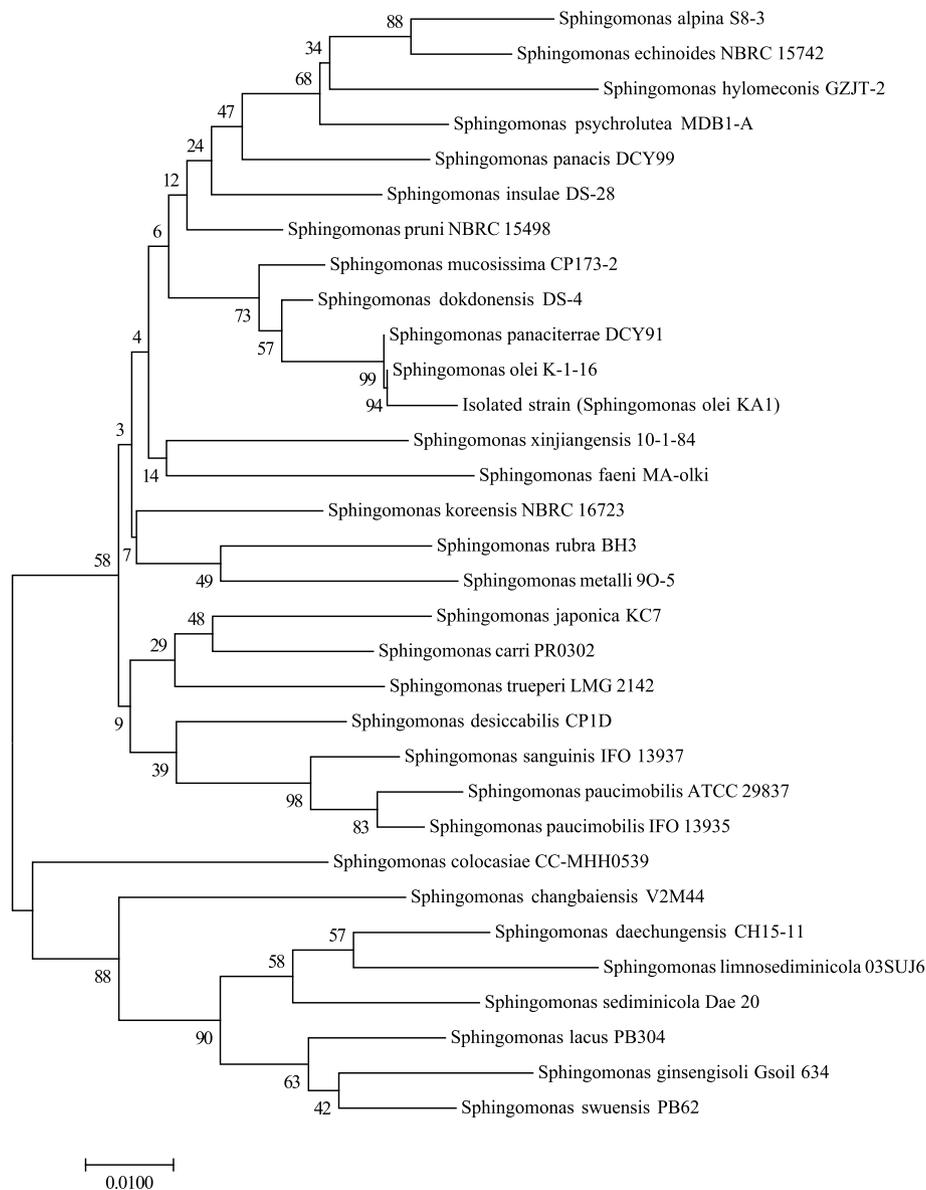


Fig. 1. Phylogenetic tree of the strain KA1 isolated from PHRS.

2.6. Calculation of crude oil removal in liquid medium

The abilities of the strains for biodegradation of different concentrations (1, 2, 3, 4, and 5% v v⁻¹) of oil were tested in BH medium before they were inoculated into the composting bioreactors. The experiments were performed in the Erlenmeyer flasks at neutral pH. The medium was shaken at 120 rpm for 7 days and the amount of oil degradation was calculated as the TPH biodegradation against the control runs (Muthukamalam et al., 2017). The control runs did not experience the inoculation of the strains.

2.7. Calculation of PHRS decomposition in the composting process

Six cylindrical composting bioreactors of 2 L capacity were used for 12 weeks. According to the results obtained from crude oil decomposition in liquid medium, the initial amounts of TPH was adjusted by adding multiple amounts of finished compost (FC) to PHRS. The composting experiments B₁, B₂, B₃, B₄, and B₅ contained 10, 20, 30, 40 and 50 g kg⁻¹ of initial TPH, respectively. A 1 McFarland concentration of the two strains was added to each reactor in a volume of 5% (v v⁻¹) at the start of the biological process. The control reactor (B₆) was also

used without the bacterial inoculation at the TPH levels of 20 g kg⁻¹ to investigate the contribution of non-biological processes in TPH removal. The C/N/P ratio was adjusted at 100/5/1 (Koolivand et al., 2017, 2018b) by adding NH₄Cl and KH₂PO₄ and air was supplied by using oil-free pumps (HAILEA Model ACO 5505) at the 1 l min⁻¹ kg⁻¹ of aeration rate (Koolivand et al., 2018a). As the pH values were within the appropriate range required for composting process, it was not adjusted in all the reactors. The amount of moisture was adjusted at a range of 50–55% by adding water over the whole period of the composting process.

2.8. Analytical methods

The TPH and organic carbon (OC) were sampled biweekly during the whole process time. The pH was determined by using a pH meter (JENWAY model 3510) after mixing 1 g of the composting materials into 10 ml of distilled water (TMECC, 2002). Loss-on-ignition method was applied to quantify the OC levels of the samples based on TMECC (2002). The extraction of petroleum compounds was done with n-pentane and then the TPH quantification was carried out by a gas chromatograph (GC) (Shimadzu, Japan) according to TNRC method

(2001). Details of the methods are the same as explained completely in our previous studies (Koolivand et al., 2013a, 2014). All the tests were performed in duplicate.

2.9. Bioremediation kinetic study

Biodegradation of TPH was modeled by the first and second-order kinetics as follows:

$$C_t = C_i e^{-k_1 t} \quad (1)$$

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

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

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

where C_i is the initial levels of petroleum hydrocarbons (g kg^{-1}), C_t is the levels of petroleum hydrocarbons (g kg^{-1}) at time t , $t_{1/2}$ is the time (day) required for decomposing half of the initial TPH, k_1 (day^{-1}), and k_2 ($\text{g kg}^{-1} \text{day}^{-1}$) are respectively, the biodegradation constants for the first- and second-order kinetics.

2.10. Statistical analysis

The differences between the experiments were determined by using one-way ANOVA test with SPSS software (P value ≤ 0.05). The correlations between various variables were investigated by the regression analysis of Microsoft Excel software.

3. Results and discussion

3.1. Metabolic and taxonomic characteristics of the isolates

The analysis of 16S rRNA gene sequence and NCBI Genbank search indicated that the bacteria are *Sphingomonas olei* strain KA1 and *Acinetobacter radioresistens* strain KA2. The phylogenetic analyses for the two strains are provided in Figs. 1 and 2. The strains KA1 and KA2 have the NCBI GenBank nucleotide accession numbers of MK127543 and MK127544, respectively. The results of biochemical test performed for the strains were also provided in Table 1. The biomass production of the mixed strains in BH medium containing crude oil of 1% concentration was measured at the intervals of 2, 4, 7, 10, and 12 days. The corresponding OD_{600} for these intervals were found to be 0.36, 0.91, 1.58, 1.64, and 1.31, respectively. The biomass was rapidly produced over the day 7–10 of the incubation time. Therefore, the logarithmic growth phase of the mixed strains was estimated to be 7–10 days. This period was selected as the incubation duration for all the experiments carried out in BH medium. The values of E_{24} and BATH were found to be 35.00 and 15.25%, respectively, indicating the possibility of bio-surfactant production and the strains affinity to the petroleum hydrocarbons.

3.2. Crude oil biodegradation in BH medium

The biodegradation of multiple initial concentrations (1–5%) of crude oil was tested to examine the capabilities of the mixed culture of the two isolated strains for consuming high levels of crude oil. As provided in Table 2, the removal percentages of crude oil were 67.25, 70.86, 61.77, 42.13, and 27.92%, respectively, for the initial concentrations of 1, 2, 3, 4, and 5%. Hence, the mixed culture could degrade a wide range of crude oil concentration. The lower degradation efficacy observed for the concentration of 1% may be attributed to the fact that the concentration of carbon is not enough to support microbial growth and thereby the crude oil decomposition is limited (Varjani and Upasani, 2017). At the initial amounts of 3 and 4%, the mixed strains presented an acceptable degradation efficacy for TPH removal. However, the strains efficacy was very low at the 5% crude oil concentration

due to the toxicity of crude oil (Awasthi et al., 2018). All of the crude oil concentrations tested in liquid medium (BH) were again examined in the composting process so as to evaluate the mixture capacity for TPH degradation by using the two methods.

The biomass production of the mixed isolates and removal of crude oil of 1% concentration was also evaluated at multiple values of pH. As presented (Table 2), 35.24, 55.92, 68.20, 60.84, and 44.06% of TPH were degraded by the mixed isolates at the pHs of 5, 6, 7, 8, and 9, respectively. Therefore, better growth of the mixed strains and higher removal of TPH happened at the neutral pH of 7. The degradation rate decreased slightly at the values of 6 and 8; but reduced greatly at the pH values of 5 and 9. These results accord with other reports (Muangchinda et al., 2018; Wang et al., 2016a) indicating that the petroleum degrading strains exhibited better efficacies at neutral pH. According to these findings, the biological composting experiments were run at the neutral pH at the start of the process.

3.3. Scale up of PHRS biodegradation from liquid-based medium to a composting process

We also surveyed the capabilities of the isolated strains in biodecomposition of petroleum hydrocarbons in the composting process as a real bioremediation technology. In this regard, we simulated the oil degradation in the biological reactors according to the findings of BH medium. The PHRS with an initial TPH of 255.05 g kg^{-1} was added to the FC in the mixing ratio of 26.59:1, 12.23:1, 7.70, 5.48, and 4.17 to obtain an initial TPH of 10, 20, 30, 40, and 50 g kg^{-1} , respectively. These values were selected according to the crude oil concentrations used in BH medium. As presented in Fig. 3, TPH removal were found to be 91.95, 87.20, 84.50, 74.08, and 60.26%, respectively for the reactors B₁, B₂, B₃, B₃, B₄, and B₅ over the composting duration of 12 weeks. Hence, the mixture of the two strains is capable of degrading a wide range of petroleum hydrocarbons concentrations. The difference between the composting experiments is due to the multiple maxing ratios of PHRS to FC resulting in various initial levels of TPH. Correct adjustment of this ratio leads to the promotion of metabolic activities of bacteria and thereby enhancement of petroleum compounds degradation. These findings verified our previous results regarding the dependency of composting efficacy to the amount of bulking agent added (Koolivand et al., 2013b, 2018b).

The very important point about the process performance is the capabilities of the mixed strains in removal of high levels (40 or even 50 g kg^{-1}) of TPH. This is due to the inherent metabolic characteristics of the strains isolated from the PHRS. Moreover, the combination of different strains and thereby the resulted collaboration makes them to be more efficient. Since oily sludge includes various hydrocarbons, and each microorganism has the metabolic ability for consuming specific compounds, bioremediation of petroleum hydrocarbons requires mixed strains. There are various works reporting the higher removal of petroleum pollutants through combination of different strains (Kamyabi et al., 2017; Mnif et al., 2015). The very low TPH removal (3.24%) observed in the reactor B₆ indicated that the biodegradation was the main cause for removal of petroleum hydrocarbons in the composting bioreactors.

3.4. Effect of FC addition on the composting process

Some materials such as FC are used in the composting process in order to provide easily-decomposable carbon and thereby support the bacterial activity. However, when high amounts of these materials are added they can act as a sole source of carbon and therefore limit the target pollutants degradation (Varjani and Upasani, 2017; Zhang and Sun, 2016). So as to understand the positive or negative effect of FC addition on the process performance, the OC and TPH/OC variations were provided in Fig. 4. The decrement trend of TPH/OC verifies the higher biodegradation of petroleum hydrocarbons compared to OC

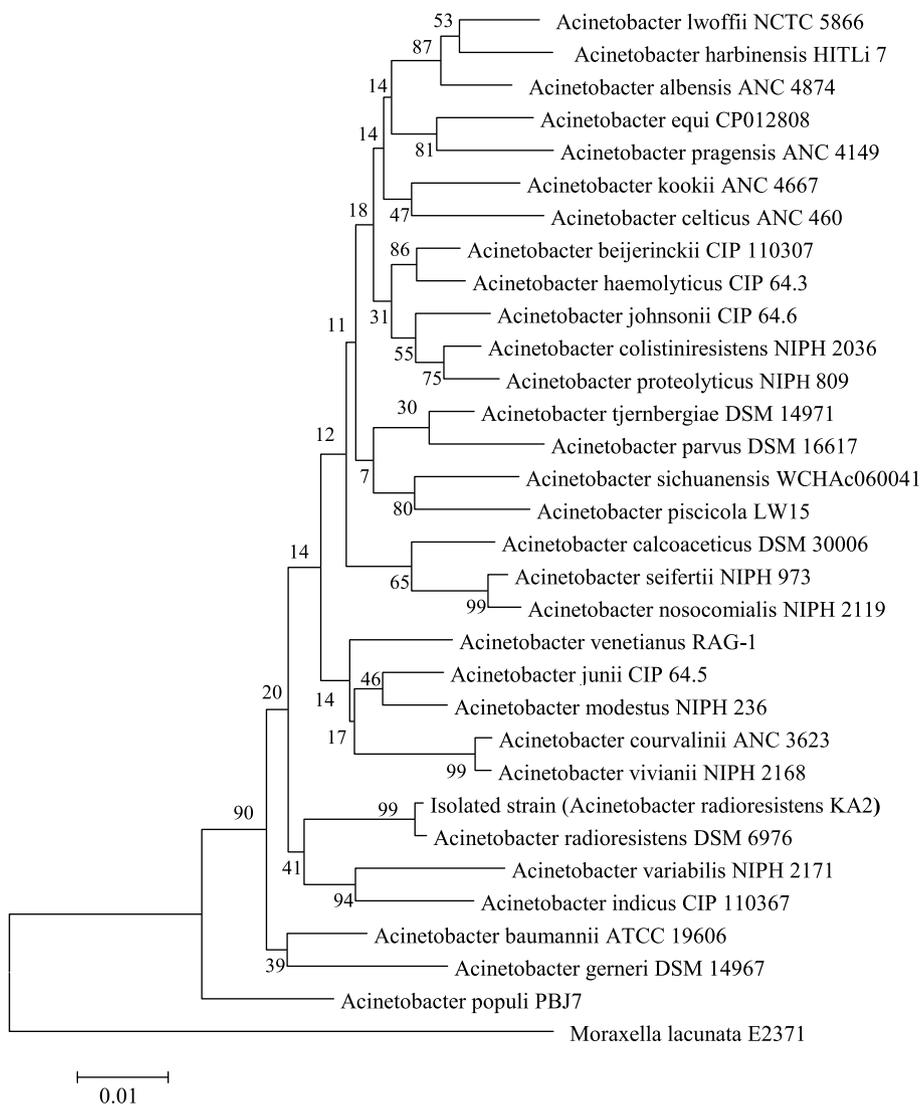


Fig. 2. Phylogenetic tree of the strain KA2 isolated from PHRS.

Table 2

Effect of initial oil concentration (at the pH value of 7) and pH (at crude oil concentration of 1%) on the biodegradation rate of crude oil in BH medium after a period of 7 days.

Variables	Values	OD ₆₀₀	Crude oil degradation (%)
Crude oil concentrations (%)	1	1.58	67.25
	2	1.73	70.86
	3	1.61	61.77
	4	1.02	42.13
	5	0.59	27.92
pH	5	1.02	35.24
	6	1.43	55.92
	7	1.58	68.20
	8	1.36	60.84
	9	0.83	44.06

consumption. This indicated that, in the present study, the organic carbon contents of FC did not compete with the petroleum hydrocarbons and thus FC exhibited a positive effect on the process performance. Bulking agents also promote the water maintaining capacity of the medium, which in turn support bacterial growth. Furthermore, air distribution through the bioreactors is elevated and the higher generated heat lead to rapid removal of TPH (Ma et al., 2016).

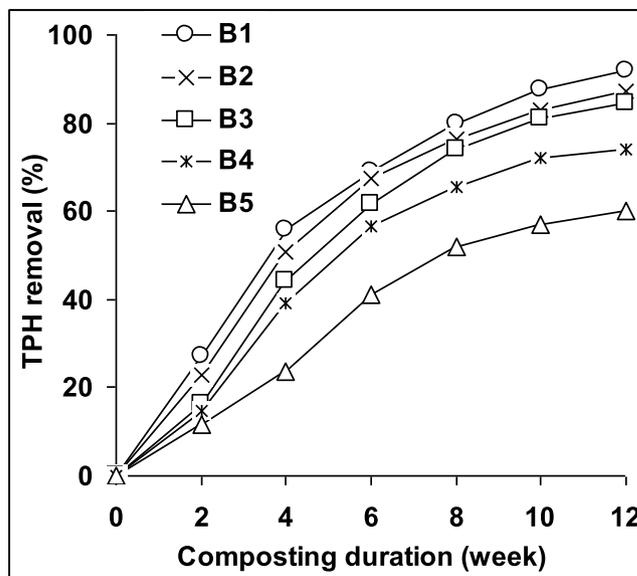


Fig. 3. Trend TPH biodegradation in the composting bioreactors over the process duration.

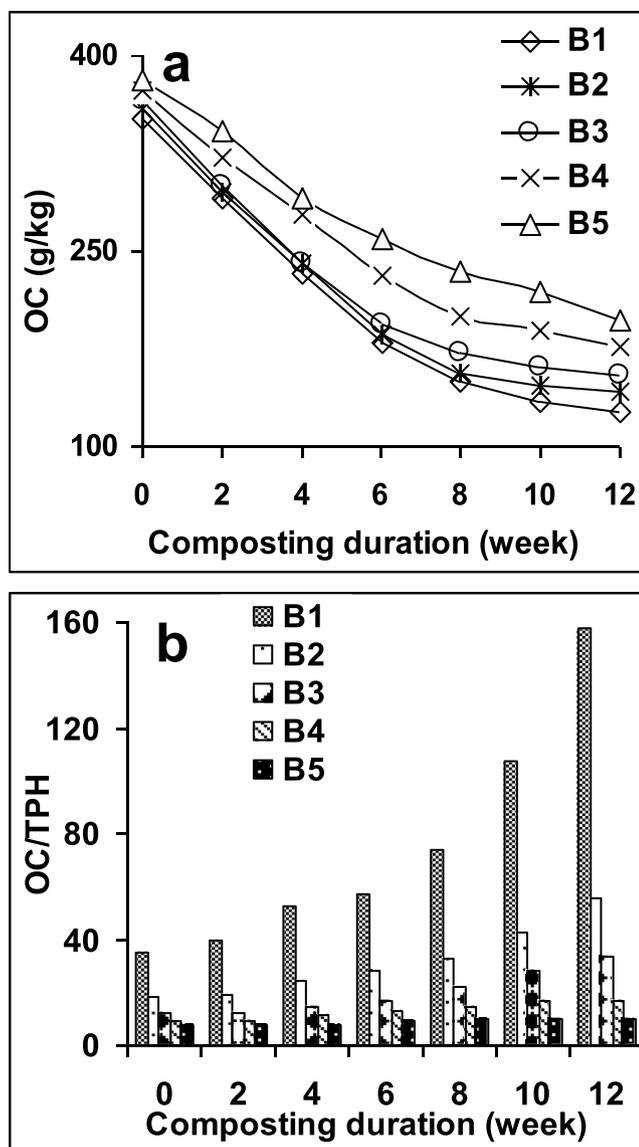


Fig. 4. (a) Trend of OC and (b) OC/TPH variations in the composting bioreactors over the process duration.

Table 3

Kinetic analyses of TPH biodegradation in the composting process.

Composting bioreactors	First-order model			Second-order model		
	k_1 (d ⁻¹)	$t_{1/2}$ (d)	R ²	k_2 (g kg ⁻¹ d ⁻¹)	$t_{1/2}$ (d)	R ²
B ₁	0.212	3.27	0.998	0.089	1.12	0.869
B ₂	0.177	3.92	0.995	0.028	1.79	0.947
B ₃	0.166	4.17	0.991	0.016	2.08	0.953
B ₄	0.122	5.68	0.976	0.007	3.57	0.983
B ₅	0.083	8.35	0.980	0.003	6.67	0.981

3.5. Kinetic study of TPH biodegradation in the composting process

In order to understand the composting process, the kinetic modeling of TPH biodegradation was performed. As can be understood from Table 3, biodecomposition of petroleum hydrocarbons was fitted to the first-and second order model, which is in accordance with other reports on the bioremediation of oily sludge (Nwankwegu et al., 2016; Wang et al., 2016b). The computed values of k and $t_{1/2}$ for the first-order

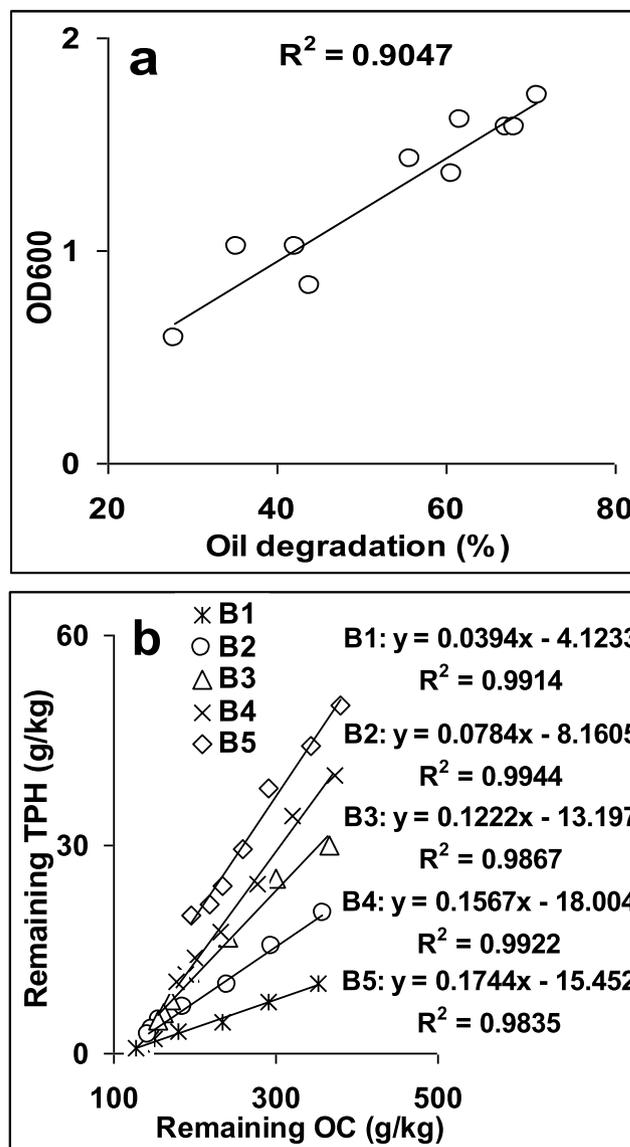


Fig. 5. (a) Correlation between biomass generation (OD₆₀₀) and crude oil biodegradation in BH medium and (b) between OC and TPH variations in the composting bioreactors.

kinetic were computed to be 0.083–0.212 day⁻¹ and 3.27–8.35 days, respectively. The corresponding values for the second-order model were respectively, 0.003–0.089 g kg⁻¹day⁻¹ and 1.12–6.67 days. The values justify the better performance of the experiments B₁, B₂, B₃ containing lower levels of TPH. However, the treatments B₄ and B₅ with high TPH concentrations also exhibited good results in terms of $t_{1/2}$ and k_1 . The values of k computed by Gomez and Sartaj (2013) were not in accordance with our findings mainly because of the fact that many parameters such as sludge composition, type of the microbial strains, bioremediation method, and operational variables of the biological process impact greatly on the kinetic constants (He et al., 2014; Kulikowska, 2016).

3.6. Correlation between different parameters

By measuring the OD_{600 nm} (Table 2), the biomass production of the mixed strains was evaluated in BH medium. Fig. 4a provides the regression analysis verifying the direct correlation between oil degradation and biomass generation. Since the level of crude oil diminished as a

result of cell numbers rise, the bacterial mixture is responsible for consuming crude oil as a source of carbon in BH medium. As the nature of petroleum hydrocarbons is organic carbon, there must have been a correlation between them. For this reason, the regression analysis was performed to obtain the related correlations and equations. As can be seen from Fig. 5b, there is a strong linear correlation between the TPH biodegradation and OC consumption. The computed equations are useful for predicting TPH degradation on the basis of OC removal in the real-scale composting facilities.

4. Conclusions

This study surveyed the effectiveness of mixed culture of two indigenous bacterial strains in bioremediation of PHRS both in BH medium and in the composting process. The mixture of the isolated strains (*Sphingomonas olei* strain KA1 and *Acinetobacter radioresistens* strain KA2) was able to effectively metabolize a wide range of crude oil concentrations (1–5%) in liquid medium. The strains mixture also degraded very high levels of TPH (30–50 g kg⁻¹) in the composting process. The successful scale-up of the liquid-based medium to the composting process for TPH removal from PHRS was observed. The study indicated the suitability of the mixture of the two strains for PHRS bioremediation both in the liquid medium and in the composting process.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jenvman.2019.06.129>.

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