

Biodegradation of oily waste sludge using vermiremediation and composting process bioaugmented with isolated hydrocarbon-degrading bacteria: Performance and ecotoxicity assessment

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Abstract: Degradation of petroleum hydrocarbons (PHs) contents of oily waste sludge (OWS) is necessary in order to prevent the related environmental pollution. The present study aimed to investigate the degradation of total petroleum hydrocarbons (TPHs) from OWS using bioaugmented composting (BC) with hydrocarbon-degrading bacterial consortium (HDBC) as pre-treatment followed by vermicomposting (VC) by *Eisenia fetida*. After isolating two indigenous bacterial strains from OWS, the ability of their consortium in degradation of crude oil was tested in Bushnell-Haas medium (BHM). Then, biodegradation of OWS was measured in the VC alone, BC alone, simultaneous BC and VC (BCVC), and BC followed by VC (BCFVC) containing high levels (30 g/kg) of TPHs. Toxicity tests including the mortality of mature earthworms and the numbers of juveniles were conducted at the TPHs of 0-40 g/kg. The obtained results indicated that the HDBC removed 18-64% of TPHs of crude oil (1-5%) in BHM after 7 days of incubation. After a period of 12 weeks, the removal rates of TPHs in the VC, BC, BCVC, and BCFVC experiments were 23.7, 79.5, 85.2, and 91.8%, respectively, verifying the efficacy of simultaneous application of HDBC and worms in bioremediation of OWS. The TPHs contents of OWS exhibited toxic effects on *E. fetida* at some concentrations and the median lethal concentration (LC₅₀) of TPHs was computed to be 14.5 g/kg after 28 days. This study demonstrated the effectiveness of composting bioaugmented with HDBC as a pre-treatment step followed by vermicomposting in bioremediation of OWS.

Key words: Bioaugmented composting. Total petroleum hydrocarbons. Hydrocarbon-degrading bacterial consortium. Oily waste sludge. Pre-treatment. Vermicomposting

Abbreviations list¹

¹BC, bioaugmented composting; HDBC, hydrocarbon-degrading bacterial consortium; IC, immature compost; LC₅₀, median lethal concentration; BHM, Bushnell-Haas medium; OD, optical density; PHs, petroleum hydrocarbons; OWS, oily waste sludge; TPHs, total petroleum hydrocarbons; VC, vermicomposting

1. Introduction

Exploration, drilling, refining, and transportation of oil and its associated products could lead to petroleum hydrocarbons (PHs) pollution in aquatic and terrestrial ecosystems (Behera et al. 2021, Rodriguez-Campos et al. 2019). Various chemical or physical remediation technologies with different degrees of success have been applied to treat PHs-polluted sites (Rondon-Afanador et al. 2023, Zhang et al. 2023). However, bioremediation methods which rely on the activity of microorganisms with specific degradative abilities to breakdown PHs, have generally been reported to be more eco-friendly and cost-effective than non-biological ones (Behera et al. 2022, Jabbar et al. 2022).

Land farming and composting/biopiling are among the bioremediation technologies that have been applied and gained approval for the treatment of hydrocarbons. The ability of earthworms to survive in hostile matrix has resulted in the development of vermiremediation as an emerging bioremediation technology where they can synergistically act with other organisms towards cleaning of contaminated environments. Earthworms provide aerobic conditions for microbial populations through the continuous mixing of the matrix (Rodriguez-Campos et al. 2019, Rorat et al. 2017). They improve the enzymatic, microbiological, and biochemical activities of microbial communities and therefore enhance the efficiency of bioremediation. Earthworms can ingest organic substrate and expel stabilized products and surfactants and thereby increase the availability of contaminants for proliferation of the autochthonous organisms (Martinkosky et al. 2017, Rorat et al. 2017). Due to the ability of earthworms to degrade organic pollutants, vermiremediation has been applied in bioremediation of PHs contaminated environments. It offers many advantages including low energy requirement, ease of operation, and low cost (Kuppusamy et al. 2017, Rodriguez-Campos et al. 2019).

However, despite the success of vermiremediation in PHs treatment, their application are limited as earthworms are reported to be sensitive to high concentrations of hydrocarbons (Almutairi 2019, Rodriguez-Campos et al. 2019). Martinkosky et al. (2017) reported that contaminated soil containing more than 30 g/kg of PHs resulted in a significant decrease in *E. fetida* capabilities for degrading PHs. In this regard, Kuppusamy et al. (2017) suggested to integrate vermicomposting with other methods as either combined or used after being pre-treated. Some studies reported further enhancement by combining vermiremediation with bioaugmentation (Guarino et al. 2017, Rodriguez-Campos et al. 2019). Inoculation of hydrocarbon-degrading bacterial consortium (HDBC) shows the improved activity of earthworms and thereby improves the overall efficiency of the PHs degradation process (Chachina et al. 2015). Since some inoculated bacteria may not be efficient in the new environment containing PHs, application of HDBC isolated from oily waste sludge (OWS) as a bioaugmentation agent is of advantage (Rabodonirina et al. 2019, Tao et al. 2017).

It is clear that earthworms can enhance the removal of low to medium levels of PHs. However, their biodegradation capacities have not been frequently reported for high levels of PHs. Hence, the application of a biological pre-treatment step for reducing PHs concentrations is of importance (Rorat et al. 2017). In this regard, composting process bioaugmented with HDBC isolated from OWS can be used as a pre-treatment for decomposing and reducing high levels of PHs (Huang et al. 2019, Poorsoleiman et al. 2019, Ren et al. 2018). To the best of the authors' knowledge, the application of composting process bioaugmented with HDBC as a pre-treatment step followed by vermicomposting (VC) has not been studied. The current study was undertaken to investigate the abilities of two bacterial strains isolated from OWS for degradation of high levels of total

petroleum hydrocarbons (TPHs), both in Bushnell-Haas medium (BHM) and in the composting process. Moreover, the impact of bioaugmentation as pre-treatment on the degradation of TPHs from OWS was also determined, both separately and in combination with VC. Another aim of the present work was to evaluate the ecotoxicity of various concentrations of TPHs on *Eisenia fetida*.

2. Materials and methods

2.1. Preparation of HDBC and earthworm

The HDBC was isolated from OWS collected from an oil refinery plant situated in Iran. Isolation of the HDBC was immediately initiated after transporting the OWS to the laboratory. An amount of 5 g of the OWS and 100 ml of BHM was blended well. Following a 7-day incubation time, 5 ml of the culture was transferred to fresh BHM. The abovementioned enrichment procedure was performed thrice. Then, the culture was grown on the surface of nutrient agar and the appeared colonies were identified. For identifying the isolates, several biochemical tests (triple sugar iron, catalase, urease, oxidase, gram staining, indole production, H₂S production, citrate, and nitrate reduction) were conducted. Detailed description of molecular identification by amplification and sequencing of 16S rRNA is available in the published papers (Koolivand et al. 2019a, Koolivand et al. 2019b, Koolivand et al. 2019c).

Eisenia fetida, a common composting worm, was used in the current study since it reproduces quickly and is tolerant of composting conditions (Martinkosky et al. 2017). The earthworms with a mean weight of 0.3-0.4 g were manually separated from a vermicomposting plant located in Iran. After preparing the earthworms and checking their health conditions in terms of body status, activity, moving, etc, they were maintained to be adapted to the composting experiments in order to minimize their mortality due to environmental changes.

2.2. Degradation of crude oil in BHM

The ability of HDBC to grow and degrade hydrocarbons was checked by culturing in BHM containing different levels (1-5% v/v) of crude oil. In order to quantify cell growth in BHM, optical density (OD) was measured by determining cell turbidity at the wavelength of 600 nm over the various intervals (2-12 days). The impact of pH (4-9) on the HDBC growth and crude oil degradation was evaluated by adding the HDBC and crude oil (1%) to BHM and incubating (at 30 °C) for 7 days. Biodegradation of crude oil at different initial concentrations (1-5% v v⁻¹) by the HDBC was also determined by batch experiments in BHM. In all the BHM experiments, the biodegradation of crude oil was computed as TPHs removal against the control treatments containing no HDBC.

2.3. Composting experiments

The composting mixtures were distributed into chemical resistant polypropylene food grade boxes (length×width×height = 20×15×10 cm) with perforated lids to provide aeration. Each box was filled with 2 kg of immature compost (IC) and OWS. According to the results obtained from toxicity tests and BHM experiments, the initial TPHs concentration in the composting experiments was adjusted to be 30 g/kg through mixing the OWS with the initial TPHs level of 255 g/kg (Parhamfar et al. 2020) with the IC. The degradation of OWS was determined by means of various

composting reactors including VC alone, bioaugmented composting (BC) alone, simultaneous BC and VC (BCVC), and BC followed by VC (BCFVC) as follows:

BC: HDBC addition only at week 0

VC: worm addition only at week 0

BCVC: both HDBC and worm addition at week 0

BCFVC: HDBC addition at week 0 followed by worms' addition at week 6

Control: no HDBC and worm addition

The control experiment was designed to determine TPHs removal due to abiotic processes. For each experiment, two sets of boxes were provided. The microbial populations of the OWS and IC were previously destroyed through three cycles of autoclave sterilization at 121 °C with 24-h intervals. At the beginning of the process, the HDBC (1.5×10^8 CFU/mL) was added to the BC and BCFVC experiments at 5% v/v. Ten adult earthworms were weighed and added to the VC and BCFVC experiments at the initiation (week 0) and middle (week 6) of the process, respectively. A mixture of potato and carrot (1:1 w/w) was added into the reactors as food supply just at the initiation of the process to prevent TPHs dilution during the process. The experiments protected from light were operated and monitored for 12 weeks. Moisture content in the reactors was adjusted twice a week to maintain it at 50%. The ratio of C/N/P in the composting mixture including IC and OWS was set at 100/5/1 through addition of KH_2PO_4 and NH_4Cl solutions (Farzadkia et al. 2019, Koolivand et al. 2014, Koolivand et al. 2018).

2.4. Toxicity tests

Earthworm toxicity studies were performed using *E. fetida* as the model species since it has been suggested as an ideal tool for evaluating the impact of PHs contamination (OECD 2004, Ramadass et al. 2017). Ten mature earthworms with well-developed clitellum were hand-picked, and randomly placed in each toxicity experiment. The feed mixture was added in the containers on a weekly basis to provide suitable conditions for growth and cocoon production. After 28 days of exposure, the earthworms were separated and placed on a moist tissue and mortality was assessed by enumerating the number of surviving worms. The experiments were incubated for an additional 4 weeks with similar conditions and then the number of juveniles was recorded (Ramadass et al. 2016, Thavamani et al. 2015). All the abovementioned tests were conducted in two replicates.

2.5. Sampling procedures and analytical methods

All sub-samples were randomly selected from the particular points of the reactors and mixed to prepare one composite-sample. Seven composite-samples were obtained at weeks 0, 2, 4, 6, 8, 10, and 12. The samples were immediately analyzed for TPHs in triplicates. The PHs contents in the samples were extracted with n-pentane and quantified by a GC-FID (Shimadzu, Japan) according to the TNRCC method (TNRCC 2001). The thermal conditions and other operating parameters of the GC have been described in the previous articles (Koolivand et al. 2013a, Koolivand et al. 2013b).

2.6. Statistical analysis

All results are the means of determinations made on two replicates. Statistical significances of differences between the experiments were determined via one-way ANOVA using SPSS 19.0 statistical software ($p < 0.05$). The median lethal concentration (LC_{50}) of PHs was computed using

Probit analysis in SPSS. The sequences of 16S rRNA were analyzed using Chromas and aligned by means of CLUSTAL. The similarity searches for the sequences in NCBI database were conducted by BLAST.

3. Results and discussion

3.1. Biochemical and taxonomic characteristics of the HDBC

The similarity search in the NCBI Genbank database showed that the isolated HDBC were *Staphylococcus equorum* strain KA4 and *Acinetobacter radioresistens* strain KA5. The accession numbers for the nucleotide sequences of these two strains deposited in NCBI were MK127546 and MK127547, respectively. The findings of the biochemical tests performed for the isolated strains have been shown in Table 1.

Table 1

3.2. Crude oil biodegradation in BHM

The OD₆₀₀ values, reflecting the ability of the HDBC in biomass generation in BHM containing crude oil, were calculated to be 0.28, 0.55, 1.53, 1.59, and 1.30, respectively during 2, 4, 7, 10, and 12 days of incubation. The cell growth indicated that the HDBC was capable of consuming crude oil as a sole source of carbon. Based on these OD₆₀₀, the incubation duration of 7 days was selected for all the tests conducted in BHM since the logarithmic phase of growth lasted for 7-10 days. The influence of pH on the HDBC ability for crude oil biodegradation has been provided in Fig. 1. The growth of the HDBC and the biodegradation of crude in pH range 6.0-8.0 were significantly higher than those at pH values 5.0 and 9.0. The optimal pH for cell growth and oil degradation was found to be 7.0. Fig. 2 indicates the degradation efficacy of different concentrations of crude oil by the HDBC. The HDBC could degrade effectively 1-3% crude oil concentration, while the degradation rate dropped dramatically at the levels of 4-5%. This is due to the fact that high concentrations of PHs are inhibitory for the HDBC and can block its growth and metabolism as previously reported (Zhang et al. 2017).

Fig. 1

Fig. 2

3.3. OWS degradation in the composting experiments

Based on the results obtained from the BHM, the maximum concentration of TPHs for HDBC growth was found to be 30 g/kg. Accordingly, the mixing ratio of IC:OWS was adjusted at 7.7:1 to obtain the initial TPHs of 30 g/kg in the composting reactors. As provided in Table 2, the removal rates of TPHs in the BC, VC, BCVC, BCFVC, and control reactors were 79.5, 23.7, 85.2, 91.8, and 6.6%, respectively. The value of TPHs removal in the BC approach showed a high potential of the isolated HDBC in biodegrading the OWS, while the low efficacy of TPHs removal in the VC approach verified that the worms were not capable of degrading high concentrations of TPHs as most of them could not survive at high TPHs concentration. Our findings are not in line with Martinkosky et al. (2017) results reporting a significant decrease (80%) of TPHs by *E. fetida* in oil-contaminated soil containing high level (30 g/kg) of TPHs. There has been also a challenge regarding the effective duration of vermiremediation. For example, Ekperusi and Aigbodion (2015) observed the highest TPHs removal after 60-90 days of vermiremediation. In our case,

results are similar because, after a lag period of approximately two weeks, the effective TPHs removal by VC lasted up to approximately 8 weeks (56 d) and from this time the TPHs removal nearly stopped. On the contrary, Rodriguez-Campos et al. (2019) reported that most of TPHs was degraded within 28 days. Comparison of the results of these different works only would be effective under identical experimental conditions, and accordingly, more investigations should be done regarding various aspects of vermiremediation of TPHs.

The ANOVA statistical analysis indicated that there are no significant differences between BC, BCVC, and BCFVC experiments. However, the highest removal rates of TPHs in the BCFVC and BCVC experiments containing both the HDDB and earthworms suggests the combined synergistic action of bacterial consortium and earthworms in the biodegradation of TPHs. In this combined action, the HDDB could initially break down the high concentrations of TPHs which are toxic to earthworms, while the earthworms could make TPHs more available through a direct degradation and/or an indirect effect by stimulating the HDDB metabolism. The higher efficiency of BCFVC compared to BCVC showed that the bacterial pre-treatment followed by earthworms gives better results than the simultaneous application of them. A significant increase in the degradation of TPHs was reported in other studies due to the simultaneous application of earthworms and bacteria (Chachina et al. 2016, Rodriguez-Campos et al. 2019).

As presented in the Fig.3, in all the BC and VC experiments, the rate of degradation dropped after over half of the TPHs were degraded. This can be explained by the fact that the easily-biodegradable fraction of TPHs is degraded first and hence the remaining compounds are persistent to degradation (Abtahi et al. 2020, Poorsoleiman et al. 2019). The trend of TPHs removal in the BCFVC experiments showed that the bacterial pre-treatment followed by worms' addition can overcome the problem of the final slow degradation.

Table 2

Fig. 3

3.4. Toxicity tests

Although earthworms are suitable ecotoxicological tools to measure the toxicity of PHs, information on their sensitivity to contaminated environments is limited (Martinkosky et al. 2017). The results of the toxicity tests conducted on *E. fetida* have been presented in Table 3. Different earthworms' mortality was registered after a 28-day exposure to various concentrations of TPHs ranging from 0 to 40 g/kg. No earthworms' mortality was naturally observed in the control samples, while it was 100% in the experiments containing 40 g/kg of TPHs. The LC_{50} of TPHs was found to be 14.5 g/kg. The number of juveniles was also studied as the end points of reproductive effect. As can be seen from Table 3, exposure of earthworms to TPHs resulted in a decrement in juveniles' number. There was remarkably high statistical difference in the number of juveniles between the control and the TPHs contaminated experiments. The results of the toxicity test obtained in the present research were different to those reported by Ramadass et al. (2017 and 2016). This difference can be attributed to the fact that the findings of the toxicity tests are highly dependent on several parameters such as the physiologic characteristics of earthworms and the type of OWS applied.

Table 3

4. Conclusions

The consortium of the isolated bacterial strains could effectively degrade TPHs contents of crude oil in BHM. It also significantly decreased the TPHs contents of the OWS in the bioaugmented composting process. The earthworms used after the bioaugmented composting process can complete the process of OWS bioremediation by further degrading TPHs. Our observations indicated that *E. fetida* have an ability to cooperate with the isolated bacterial consortium in order to enhance the efficiency of TPHs degradation. The ecotoxicity results showed some toxic effects of the OWS on earthworms depending on the initial levels of TPHs. This research verified the efficacy of the composting process bioaugmented with bacterial consortium as pre-treatment followed by vermiremediation for TPHs removal from OWS.

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Data Availability: All requests for data and materials will be made available upon reasonable academic request.

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Table titles

Table 1 The results of the biochemical tests performed for the isolated bacterial strains

Biochemical tests	Strain KA4	Strain KA5
Gram stain	Gram positive	Gram negative
Triple sugar iron	Acid/Alkaline	Alkaline/Alkaline
Nitrate reduction	+	—
H ₂ S production	—	—
Indole production	—	—
Oxidase	—	—
Catalase	+	+
Urease	+	—
Citrate	—	—

Table 2 Time course profile for TPHs removal in the composting experiments

Process duration (day)	TPHs removal rate (%)				
	BC	VC	BCVC	BCFVC	Control
0.00	0	0.00	0.00	0.00	0.00
2.30	14	9.33	3.83	12.50	9.33
1.50	28	31.40	8.70	30.17	31.40
1.20	42	19.33	5.97	18.03	19.33
0.70	56	10.60	4.23	13.60	17.93
0.60	70	6.67	0.70	7.03	8.17
0.30	84	2.17	0.30	3.90	5.60
6.60	Total	79.50	23.73	85.23	91.77

Table 3 Effect of different initial concentrations of TPHs on mortality of *E. fetida*

Initial TPHs concentration (g/kg)	Percentage of earthworms' mortality ($\pm$ SD)	No of juveniles ($\pm$ SD)
0	0 (0)	19 (1.0)
5	3.3 (5.8)	11.3 (0.6)
10	43.3 (5.8)	6 (1.0)
20	70.0 (10.0)	0 (0)
30	97.0 (5.8)	0 (0)
40	100 (0)	0 (0)

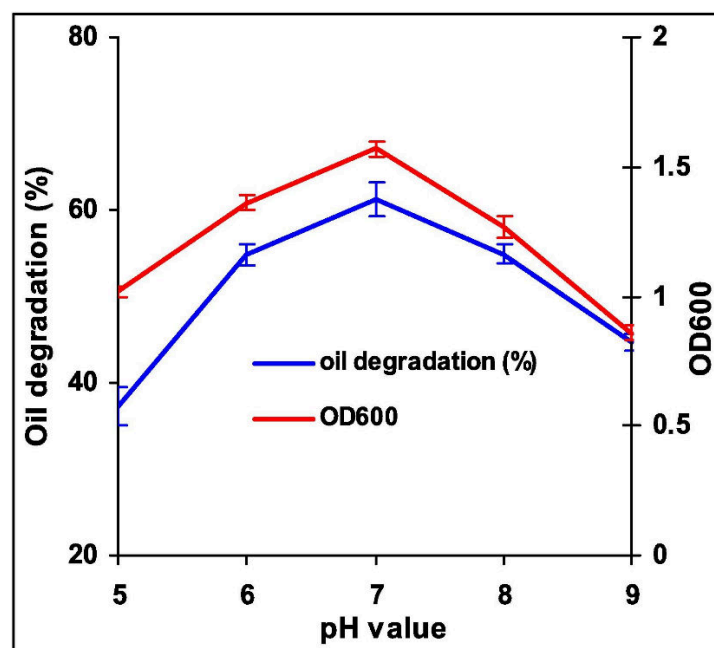


Fig. 1 Influence of pH on the biodegradation of crude oil in Bushnell-Haas medium

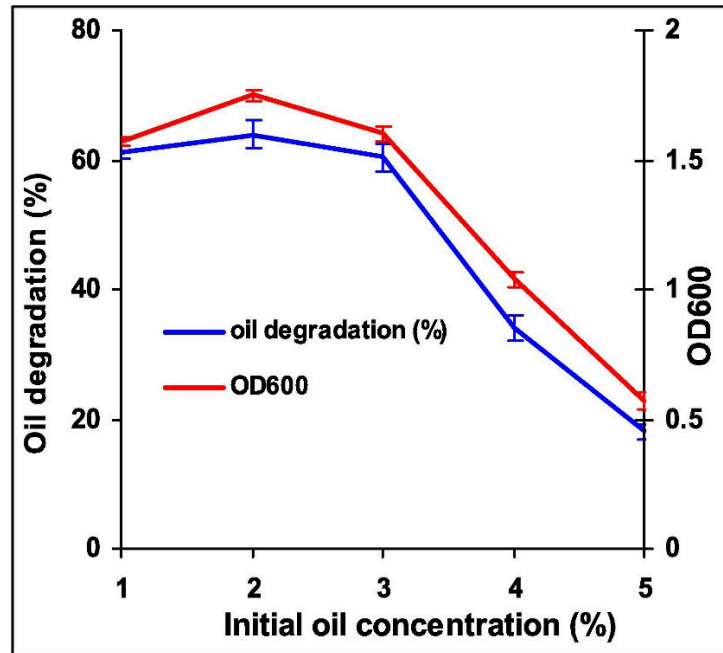


Fig. 2 Impact of crude oil concentration on its biodegradation in Bushnell-Haas medium

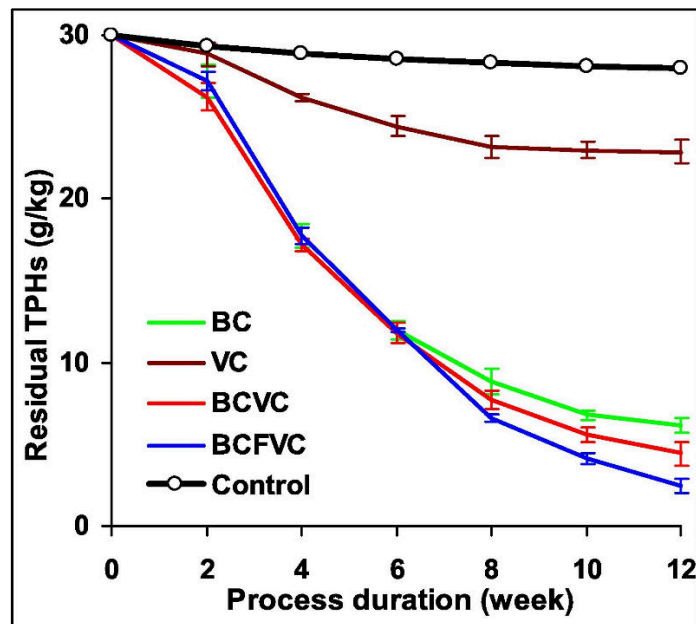


Fig. 3 Trend of TPHs removal in the composting experiments over the process duration



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vermiremediation and composting process
bioaugmented with isolated hydrocarbon-degrading
bacteria: performance and ecotoxicity assessment**

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