Performance Evaluation Of Bacterial Consortium For Biodegradation Of Total Petroleum Hydrocarbon: A Comparative Strategic Biostimulation Study

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Research Article

Keywords: Petroleum sludge, Total petroleum hydrocarbon (TPH), Bacterial consortium, Biodegradation, Amendment.

Posted Date: February 10th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1227394/v1

Performance evaluation of bacterial consortium for biodegradation of total petroleum hydrocarbon: A comparative strategic biostimulation study

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Abstract

This research work demonstrated the comparative study of the efficacy of poultry litter extract (PLE) and inorganic fertilizer (NPK) as biostimulating agents for enhancing total petroleum hydrocarbon (TPH) degradation of petroleum refinery sludge (PRS) with bioaugmentation of an indigenously developed bacterial consortium. In this study, six sets of treatments such as natural attenuation, bioaugmentation with the indigenously developed bacterial consortium, and various biostimulation strategies with (MSM100, PLE100, NPK100, MSM50+PLE50, and MSM50+NPK50) were performed to meet 100% nutrient source for bacterial growth to enhance TPH degradation. Among all, the combined sources of MSM50+PLE50 showed the best performance by degrading the TPH up to 91.3 ± 4.1% within 28 d of the incubation period. The GC-FID analysis confirmed the efficacy of TPH degradation of PRS when PLE amendment with MSM. Further, the removal of maltene and asphaltene was also achieved 92 ± 3.7% and 52 ± 2.2% during this treatment. TPH degradation fitted to first-order kinetics with a rate constant 0.09 d^{-1} and half-life period of 7.7 d for MSM50+PLE50 amendment treatment along with bacterial consortium as bioaugmentation. This study revealed the implementation of the PLE amendment not only preferred as a nutrient source for bacterial growth but also enhanced TPH biodegradation in an eco-friendly strategic way by dropping the practice of inorganic salts.

Keywords Petroleum sludge. Total petroleum hydrocarbon (TPH). Bacterial consortium. Biodegradation. Amendment.

Introduction

The rapid evolution in industrial sector has instigated in generation of a huge quantity of pollutants to the environment (Gaur et al. 2021). Amongst these, petroleum hydrocarbon pollutants from the petroleum industries need a special attention due to its carcinogenic and toxic effects towards health and environment. The release of hydrocarbon contaminants during the crude oil processing, extraction and transportation causes a serious environmental concern due to its hazardous nature. A significance amount of oily sludge generates from the petroleum industries in which total petroleum hydrocarbon (TPH) is a major concern. TPH refers to petroleum-based hydrocarbons and composed of complex compounds originate from crude oil and considered to be hazardous towards the human as well as habitats (Almeida et al. 2013; Zeneli et al. 2019; Gaur et al. 2021). An eco-friendly, sustainable, and affordable technique is required for safe disposal of these petroleum hydrocarbon pollutants (Varjani 2017; Suganthi et al. 2018; Koolivand et al. 2019). Till date, a series of physical and chemical treatments have been implemented to achieve this goal, but generation of secondary pollutants and high cost are the major concerns of these methods (Taiwo and Otolorin 2009; Qin et al. 2015; Hu et al. 2017; Sivagami et al. 2019). In this regard, the role of microorganisms plays a major character in the process of biodegradation to achieve the safe disposal of petroleum hydrocarbons (PHCs) (Jasmine et al. 2015; Hamidi et al. 2021).

Biodegradation is defined as the complete removal of pollutants from the environment in presence of living microorganisms (Das et al. 2008; Gaur et al. 2021). But under natural circumstances, the availability of hydrocarbon degraders is not sufficient to achieve the complete degradation of hydrocarbons (Szulc et al. 2014). This results low degradation rate of PHCs. To overcome this issue, bioaugmentation strategy can introduce by addition of selected microbes to the polluted sites. Several studies have been documented the successful degradation of PHCs by involving bioaugmentation strategy (Mishra et al. 2001; Sarkar et al. 2017). In a similar manner, adequate amount of nutrients is required for biomolecule synthesis which boost potential hydrocarbon degradation (Fallgren and Jin 2008). The hydrocarbon degradation has been successful with biostimulation strategy by inorganic nitrogen and phosphorus (Almeida et al. 2013; Sarkar et al. 2016; Zeneli et al. 2019). The combinational approach of bioaugmentation and biostimulation result better biodegradation performance than the individual approaches (Wu et al. 2016; Sarkar et al. 2017).

As PHCs are rich in carbon content, so nitrogen plays a major role among the nutrients. Low-cost chemical fertilizer, organic waste, compost and agro-waste can be use as alternative source of nutrients for

microbial growth. Till date, use of chemical fertilizer as a nutrient source for microbial growth has been documented for biodegradation of PHCs (Machín-Ramírez et al. 2008). Even so, fertilizers are not sufficient for agronomy for the emergent nations that's why it is necessary to pursuit an inexpensive and environmentally approachable selection to replace the fertilizer as nutrient source for biodegradation of PHCs. In considering to this issue, use of organic wastes (animal manure, compost, biochar, plat residue) for degradation of PHCs has been reported (Medina et al. 2015; Ren et al. 2018a; 2018b; Guo et al. 2020; Olawale et al. 2020). The presence of humic and pulvic substances in animal manures enhance the bioavailability of hydrocarbon for the microbial growth (Ezenne et al. 2014). Till date, use of Poultry waste (PW) as biostimulating agent has been limited for degradation of PHCs. The PW can replace the inorganic nutrients, as it contains high source of N, P and K along with important micronutrients. With the due course of the time, during the biodegradation process, the uric acid and different proteins present in the PW gradually breakdown into nitrogen and phosphorous and easily available by the microorganisms (Fallgren and Jin 2008).

This article focuses on the performance of TPH degradation by the indigenous bacterial consortium with individual/amendment of inorganic chemical fertilizer and poultry litter extract (PLE) in mineral salt media (MSM). The impact of bacterial population was also studied at different nutrient amendments. The GC-FID analysis was conducted to ensure the degradation of TPH in different amendment strategies. Further, the residual TPH were represented in different hydrocarbon fractions (asphaltene and maltene).

Materials and methods

Materials, chemicals, sample characterization, and media preparation

In this study, the petroleum refinery sludge (PRS) was collected from Indian oil corporation limited Haldia, West Bengal, India. The PRS sample was stored in a closed container at 4 °C to avoid volatilization and to avoid any light exposure (Behera et al. 2020). The process of PRS collection and the physicochemical properties of the sample has been reported in the earlier study (Behera et al. 2020). The PW were collected in a ziplock plastic bags from a poultry farm nearby Kharagpur city. The chemical fertilizers such as urea, single super phosphate (SSP), and muriate of potash (MOP) were purchased from agrochemical store from Kharagpur. All the chemicals and solvents (dichloromethane, n-hexane, n-heptane, chloroform, and methanol) used for research work were analytical grade and purchased from Merk India Ltd, Mumbai, India. The PW was oven dried and crushed and passed through 2 mm sieve to remove the solid particles and stored at 4 °C for further use. The

presence of nutrient content, moisture, pH, electrical conductivity was determined as per the standard protocol (manuscript submitted). The C, H, N and S analysis was performed by CHNS analyzer (ELVario MICRO Cube, Elemental TM) (Table S1).

The MSM used for degradation study composed (g L^{-1}) KH_2PO_4 (0.17), K_2HPO_4 (0.435), $Na_2HPO_4.7H_2O$ (0.668), NH_4Cl (0.850), $MgSO4.7H_2O$ (0.0225), $CaCl_2.2H_2O$ (0.0275) and $FeCl_3$ (0.00025) (Jasmine and Mukherji 2015). The inorganic fertilizer (NPK) media was prepared by (g L^{-1}) 0.482, 0.960, and 0.241 for N, P, and K in the form of urea, SSP, and MOP in distilled water. The poultry litter extract (PLE) was used as a substitute of MSM when used as single source of nutrient. A mixture of dried poultry litter and distilled water (25 g L^{-1}) was prepared and sterilized for required media for bacterial growth. Different strategic media were prepared by adding PLE and NPK with MSM for biodegradation study of TPH.

Bacterial inoculum and maintenance

The hydrocarbon degrading bacterial cultures used in this study were previously isolated from the same PRS sample (Behera et al. 2021). The four indigenous bacterial strains used for consortium are *Dietzia lutea* IRB191, *Dietzia lutea* IRB192, *Staphylococcus warneri* BSM19 and *Stenotrophomonas pavanii* IRB19 (Behera et al. 2021). Equal volume of individual strains was mixed together at the end of the log phase to get the effective consortium (Behera et al. 2021). The indigenous isolated strains were preserved at -80 ℃ in sterile glycerol solution (20%, v/v). The optimum degradation strategy for efficient hydrocarbon degradation were pH, temperature, and percentage of oily sludge concentration were 7, 34 ℃, 2% (w/v) respectively (Behera et al. 2021).

Design of experiment

Laboratory scale TPH degradation experiments were conducted in shake flask study for 28 days. For this, six different strategies (T1-T6) were performed in 250 ml flask. The details of the media composition using (MSM, PLE, and NPK) in six different treatments were described in Table 1. The treatment T1 was considered as control without bioaugmentation of bacterial consortium.

The biostimulation strategy was maintained via adding different media of MSM, PLE, and NPK. Briefly, in case of T2, T3, and T4 biostimulation was reached via adding MSM, PLE and NPK media respectively. In these cases (T2, T3, and T4), 100% nutrient source was maintained as single media composition. Whereas in T5

and T6, biostimulation was performed by combination of two medium (MSM+PLE) and (MSM+NPK) at a ratio 1:1 respectively. In each set of strategy, the bioaugmentation was performed by inoculating 5% (v/v) of indigenously developed bacterial consortium. For this study, T1 is consider as control, in which biostimulation via MSM nutrients and no bioaugmentation was performed with indigenous developed consortium. However, 2% (w/v) of sludge was supplemented as the primary source of carbon and energy in each treatment flask. The initial pH of each medium was maintained at 7 and incubated at 34 ºC and 120 rpm (Behera et al. 2021).

Table 1

Total hydrocarbon degrading bacteria

To enumerate the total bacterial population (CFU mL^{-1}), in each three days intervals 1 mL of media subsequently diluted with sterile NaCl solution (0.9 w/v). From this proper dilution, 100 µL of solution were plated on nutrient agar. The total bacterial population were counted after 12 to 24 h of incubation at 34 ℃ and express in (Ln N).

Residual TPH extraction and analysis

Hydrocarbon extraction and gravimetric analysis

The residual TPH present in degradation study, was extracted and quantified gravimetrically at various time intervals (7 d, 14 d, 21 d, and 28 d) from each treatment strategy. Further, the TPH was fractionated to maltene (soluble in n-heptane) and asphaltene (insoluble in n-heptane) and weighted gravimetrically. The following equation (1) was used to calculate the percentage of TPH degradation gravimetrically.

$$Degradation\ of\ TPH\ (\%) = \frac{(Initial\ TPH - Final\ TPH)}{Initial\ TPH} \times 100 \quad (1)$$

Gas Chromatographic analysis

The extracted TPH samples were dissolved in dichloromethane and concentrated to a particular volume by evaporation for GC-FID analysis (PerkinElmer GC system/Clarus 480 with flame ionization detector and DB 5, 30 m × 0.32 mm × 0.25 µm, capillary column). The gas chromatography methodology was followed as per our previous study of total run time for 45 min (Behera et al. 2020).

Kinetic of TPH degradation

Kinetic study is essential to (a) measure the biodegradation speed, (b) to understand biodegradation process. Further, kinetic study exemplifies the existence of residual TPH at any time and future prediction of its occurrence during degradation (Kuppusamy et al. 2016). Mostly the biodegradability of hydrocarbons explains the first-order and second order kinetics.

First-order kinetics:

$$C_t = C_i e^{-k_1 t} \quad (2)$$

$$t_{1/2} = \frac{ln_2}{k_1} = \frac{0.693}{k_1} \quad (3)$$

Second-order kinetics:

$$\frac{1}{C_t} = k_2 t + \frac{1}{C_i} \quad (4)$$

$$t_{1/2} = \frac{1}{k_2 C_i} \quad (5)$$

where C_i (g kg^{-1}) and C_t (g kg^{-1}) represents the initial ($t = 0$) and final ($t = t$) TPH concentration respectively, ($t_{1/2}$) is the time (d) required to reduce the initial TPH concentration by one-half. Whereas k_1 (d $^{-1}$) is the first-order rate constant and k_2(kg^{-1} d^{-1}) is the second-order rate constant. The rate constant (k_1 and k_2) were calculated from the slope by plotting $\left(-\ln\frac{C_t}{C_i}\right)$ and $\frac{1}{C_t}$ versus time (t), respectively. The time required to reduce the half of the concentration of TPH during degradation process is known as half-life time ($t_{1/2}$). It is necessary for environmental fate modelling, chemical screening and alteration of pollutants. The corresponding half-life ($t_{1/2}$) value were calculated using equation (3) and (5).

Results and discussions

Total bacterial population

The sample from each treatment were individually analyzed for total bacterial papulation by plate count method at different time (0th, 3rd, 6th, … and 28th day) and the result were depicted in Fig. 1. The increased biomass with the period of time was represented by CFU mL^{-1} (Fig. 1). The result of this current study displayed maximum populations in the treatments T5. In treatment T5, the bacterial population was increased from 0.97 ± 0.03 × 10^8 CFU mL^{-1} (3rd day) to 2.9 ± 0.08 ×10^8 CFU mL^{-1} (6th day) and reached the maximum 1.1 ± 0.28 ×10^{10} CFU mL^{-1}

at 15th day of incubation. It was observed that the bacterial population rapidly increased during the first 15 days of incubation then reduced to 2.2 ± 0.24 ×10^{9} CFU mL^{-1} on 28th day. The rapid increased in biomass is due to the combined effort of presence of hydrocarbon of the PRS, MSM, and additional source of nutrients in PLE. Similar type of trends was also observed in different treatments, but the increased biomass varies in different strategies. Next to the PLE and MSM amendment, the PLE100 (T3) showed the total bacterial population reached to maximum 9.5 ± 0.23 ×10^{8} CFU mL^{-1} followed by MSM100 (T2) strategy (Fig. 1). In case of Treatment T1(control) the maximum growth was reached up to 1.3 ± 0.04 ×10^{8}. The availability of indigenous culture in treatment T1 utilized the petroleum hydrocarbons for their microbial activities. The increase number of populations with time confirmed the survival of bacterial consortium and utilization of hydrocarbons as sole source of carbon and energy (Tao et al. 2017). In this study, the used bacterial consortium is indigenous in nature so they can easily adapt to PRS. Increase in microbial population corresponded to increase in TPH degradation observed in this study.

Figure 1

Total Petroleum hydrocarbon degradation

The TPH degradation percentage was estimated for each treatment in seven days intervals by solvent extraction followed by gravimetric method and represented in Fig. 2. The TPH degradation percentage was increased in each treatment with respect to incubation time which signified that the survival of bacterial consortium in different amendments and utilization of hydrocarbons as their metabolic activities. The highest TPH degradation was noticed for the treatment T5 and the TPH degradation was achieved 91.3 ± 4.1% after 28 days of incubation (Fig. 2). However, a substantial amount (82.55 ± 3.4%) of TPH degradation was observed in treatment T2. Further the percentage of TPH degradation was 78.5 ± 2.5 and 62.7 ± 3.1% for the treatment T3 and T4 respectively. In this study the NPK amendments showed comparatively lower degradation than PLE amendments but higher than the control. This may be due the bioavailability of the nutrients in PLE amendment is more than the NPK amendment for bacterial activities. However, treatment T5 showed the maximum TPH degradation, where both MSM and PLE were supplied (1:1) (Fig. 2). The presence of humic and fulvic substances in the PLE which enhanced the bioavailability of TPH in the medium for bacterial growth and activities (Ezenne et al. 2014). Further, the addition of organic amendments may increase the microbial activities by enhancing the dissolved organic matter in the medium (Rahman et al. 2002; Wei et al. 2014).

Several researchers have reported the use of organic waste for biodegradation of hydrocarbons (Rahman et al. 2002; Olawale et al. 2020).

Figure 2

The TPH degradation of the PLE amendment of this study represented the better result than the results obtained previously (Gholami-Shiri et al. 2017; Roy et al. 2018; Jasmine and Mukherji 2019; Zeneli et al. 2019; Hamidi et al. 2021) and a comparative result has represented in Table 2.

Table 2

The extracted maltene fractions comprised of aliphatic, aromatics and NSO in the range 52 ± 4%, 39 ± 2%, and 9 ± 1%, respectively (Behera et al. 2020). The heavier asphaltene was found to be 90 ± 3 g kg^{-1}, which is 50% of TPH in this PRS sample. The degradation of maltene and asphaltene fractions for different amendment strategies has been described in Fig 3. It was noticed that both the maltene and asphaltene fractions reduced in each strategy as compared to control (T1) (Fig. 3). At the end of 28^{th} day, the degradation of maltene was highest (92 ± 3.7%) and asphaltene was found to be (52 ± 2.2%) for T5. Presence of particulate matter along with organic matter in the PRS enhanced the degradation of maltene (Jasmine and Mukherji 2015). The degradation of asphaltene is low due to its complex and recalcitrant in nature (Jasmine and Mukherji 2019). But Reddy at al. (2011) reported the degradation of asphaltenes (26%) in slurry phase reactor in 10 days.

Figure 3

GC analysis

The samples were withdrawn on completion of 28 days for their degradation study. The residual TPH of all treatments after degradation of 28 days were represented by gas chromatograms obtain from GC-FID analysis (Fig. 4). The presence of TPH in the original PRS sample and its GC-FID chromatogram has been reported in our previous report (Behera et al. 2021). From the Fig. 4, it was confirmed that the TPH degradation comparatively higher in case of T5. This depicted the maximum hydrocarbons were utilized by the microorganisms in T5. The obtained chromatograms providing the evidence of gravimetric result of TPH degradation (Fig. 4).

Figure 4

Degradation rate of TPH

Kinetic modelling was practiced to evaluate the rate of TPH degradation in the studied systems. The regression analysis data of TPH degradation fitted to both first and second order kinetics. The corresponding R^2 value, k_1, k_2, and half-life period ($t_{1/2}$) for individual treatment were summarized in Table 3. For the treatment T5 the TPH degradation fitted to first order kinetics with rate constant 0.09 d^{-1}. The biodegradation rate constant for different strategies were in the range 0.09 to 0.03 d^{-1}. The corresponding half-life period for the treatments (T2, T3, T4, T5, and T6) were was found to be in the range 11.4, 17.2, 21, 7.7, and 17.3 d respectively. Agarry et al. (2010) described the suitability of first-order kinetic model for hydrocarbon degradation. The resulted R^2 values of second order kinetics model were higher than those of first order model (Table 3). Therefore, the second order-kinetic described the TPH degradation rate better than the first-order for T1, T2, T3, T4 and T6. Sarkar et al. (2005) in their study effectively described the better fit of second order kinetic model for hydrocarbon degradation. Similarly, Poorsoleman et al. (2020) reported in his study that the TPH degradation fitted to both first and second-order kinetics. The higher degradation constant and lower half-life observed in T5 than other strategy indicating the higher efficiency of TPH degradation.

Table 3

Conclusion

In this current study, the supply of PLE as nutrient amendments minimized the dose of MSM for TPH biodegradation. The nutrient amendment of MSM (50%) with PLE (50%) resulted maximum TPH degradation which is approximately 4 times higher than control when augmented with indigenously developed bacterial consortium. However, the TPH degradation was lowest when NPK used as a single source of nutrient supplement but 2.7 times higher than the control. Based on our kinetic study, we recommended the rate of TPH degradation fitted to the first-order kinetic model for PLE amendment with MSM. The combined utilization of PLE as a nutrient source in MSM, enhanced the TPH degradation and reduced the cast-off inorganic salt, as well as valorising the PL waste management for environmental safety.

Declarations

Ethics approval and consent to participate Not applicable

Consent for publication Not applicable

Availability of data and materials Not applicable

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding This work was supported by DST and the grant number (No. DST/TMD(EWO)/OWUIS-2018/RS-18). The author RS has received the research support from DST.

Author's contributions

Ipsita Dipamitra Behera: Conceptualization, Experimentation, Data curation, writing - original draft, Methodology, validation. Bhim Charan Meikap: Conceptualization, Project administration, Resources, Supervision, Writing - review & editing. Ramkrishna Sen: Project administration, Funding acquisition, Resources, Supervision, Writing- review & editing. All authors read and approved the final manuscript.

Acknowledgments

The authors thankful to Indian Oil Corporation Limited Haldia, West Bengal, India, for providing the petroleum refinery sludge sample. We are grateful to the Department of Biotechnology and Departmental Research facilities of Chemical Engineering, Indian Institute of Technology Kharagpur, for providing necessary facilities for research. RK is thankful to DST for financial supports (No. DST/TMD(EWO)/OWUIS-2018/RS-18).

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393 **Table Captions**

394 Table 1 Details of various amendments for different treatments.

395 Table 2 Comparison of TPH degradation under different biostimulation strategies between present study and
396 previously reported studies.

397 Table 3 Kinetic study of TPH degradation with different treatments.

398 **Table 1**

Treatment No	Amendments	Bioaugmentation	Composition (%)		
			MSM	PLE	NPK
T1	MSM (control)	No	100	0	0
T2	MSM	Yes	100	0	0
T3	PLE	Yes	0	100	0
T4	NPK	Yes	0	0	100
T5	MSM + PLE	Yes	50	0	50
T6	MSM + NPK	Yes	50	0	50

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409 **Table 2**

Consortium	Amendments	Time of degradation (day)	Percentage of TPH degradation	References
Indigenous culture	Nutrients (Ammonium nitrate and monopotassium phosphate)	80	52.4	Zeneli et al. (2019)
Bacillus cereus BQAR-01d, Bacillus sp. NBRC 101285, and Lysinibacillus fusiformis X−9	Mineral salt medium	30	52.3	Gholami shiri et al. (2017)
Pseudomonas aeruginosa R7-803, pseudomonas fluorescens PSY-11 Citrobacter, and amalonaticus SA01	Mineral salt medium	30	35.4	Gholami shiri et al. (2017)
Pseudomonas aeruginosa (RS1), Microbacterium sp. (RS2), Bacillus sp. (RS3), Acinetobacter bau- mannii (RS4,) and Stenotrophomonas sp. (RS5)	Nitrate and Phosphopous	90	68.4	Jasmine and Mukherji (2019)
Staphylococcus sp. A1(2011), Rhodococcus jostii and Arthrobacter citreus	Mineral salt media	35	67.3	Hamidi et al. (2021)
Indigenous bacterial consortium	Nitrate and Phosphate in microcosm study	120	57-75	Roy et al. (2018)
Dietzia lutea (IRB191), *Dietzia lutea* (IRB192), *Staphylococcus warneri* (BSM19) and *Stenotrophomonas pavanii* (IRB19)	MSM with PLE (50:50)	28	91.3	This study

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Table 3

Treatment No	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
T1	0.009	77	0.92	0.00006	92.3	0.93
T2	0.061	11.4	0.83	0.0009	6.15	0.95
T3	0.04	17.2	0.87	0.0007	7.9	0.98
T4	0.033	21	0.86	0.0003	18.4	0.95
T5	0.091	7.7	0.95	0.0023	2.4	0.93
T6	0.04	17.3	0.87	0.0004	13.8	0.95

Figure captions

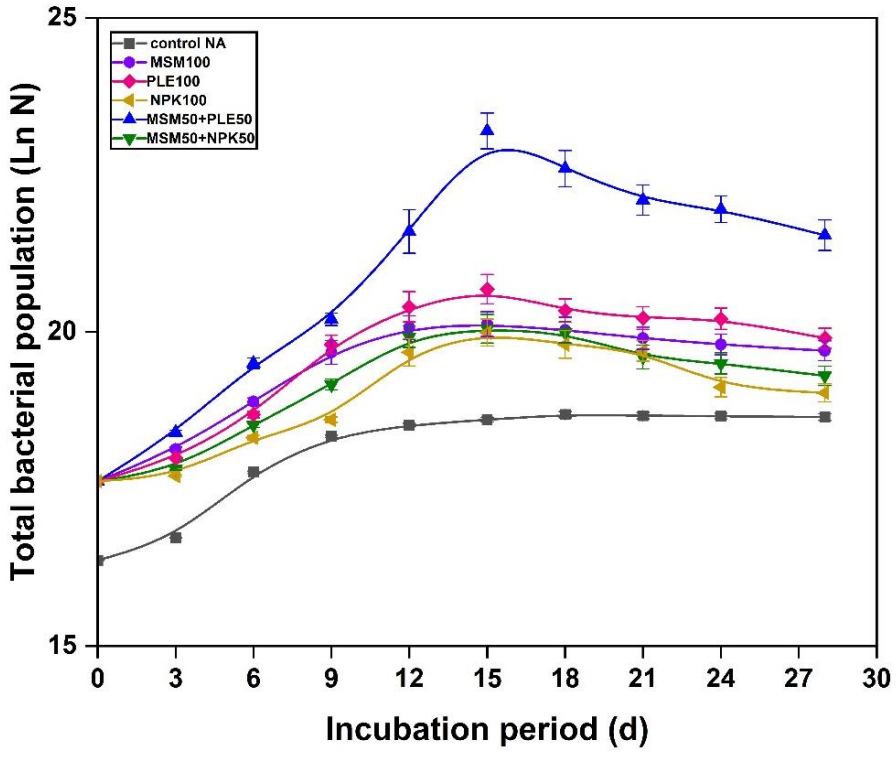
Figure 1 Total bacterial population (Ln N) in various treatments such as only MSM without bioaugmentation (T1 as control NA), MSM100 (T2), PLE100 (T3), NPK100 (T4), MSM50+PLE50 (T5), and MSM50+NPK50 (T6) during incubation period.

Figure 2 Gravimetric analysis of TPH degradation (%) in different treatments such as only MSM without bioaugmentation (T1 as control NA), MSM100 (T2), PLE100 (T3), NPK100 (T4), MSM50+PLE50 (T5), and MSM50+NPK50 (T6) over the incubation period.

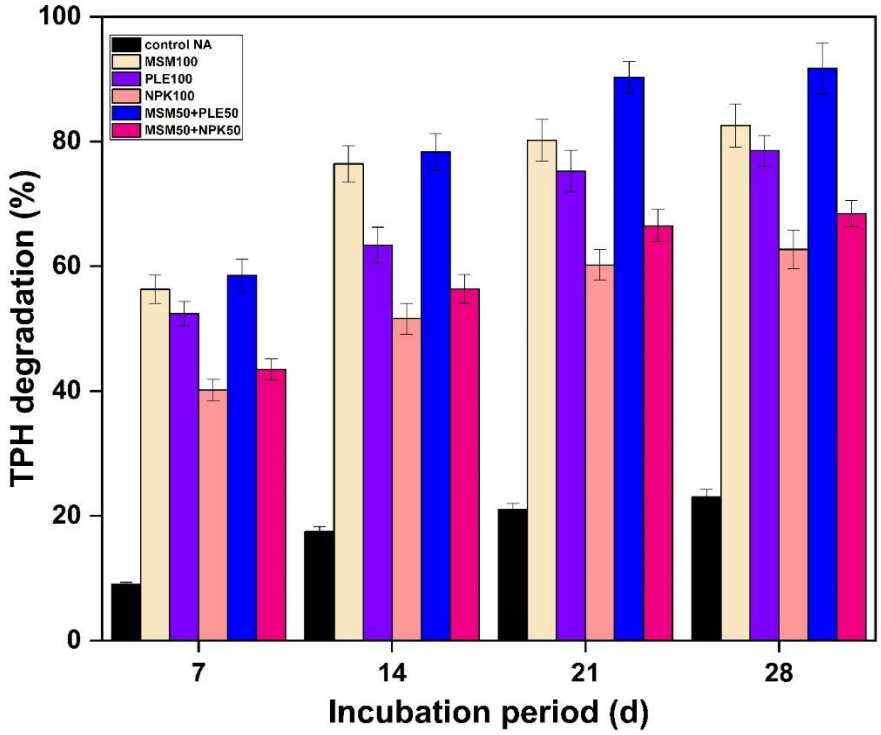
Figure 3 Biodegradation of maltene and asphaltene fraction (%) for different biostimulation strategies after 28 days of incubation

Figure 4 GC-FID chromatograms were representing the residual hydrocarbon peaks with different treatments after 28 days of incubation. (a) MSM100 without bioaugmentation as control (T1), (b) MSM100, (c) PLE100 (T3), (d) NPK100 (T4), (e) PLE50+MSM50 (T5), and (f) PLE50+NPK50 (T6) with bioaugmentation of 5% (v/v) of bacterial consortium.

Figure 1



448 **Figure 2**



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460 **Figure 3**

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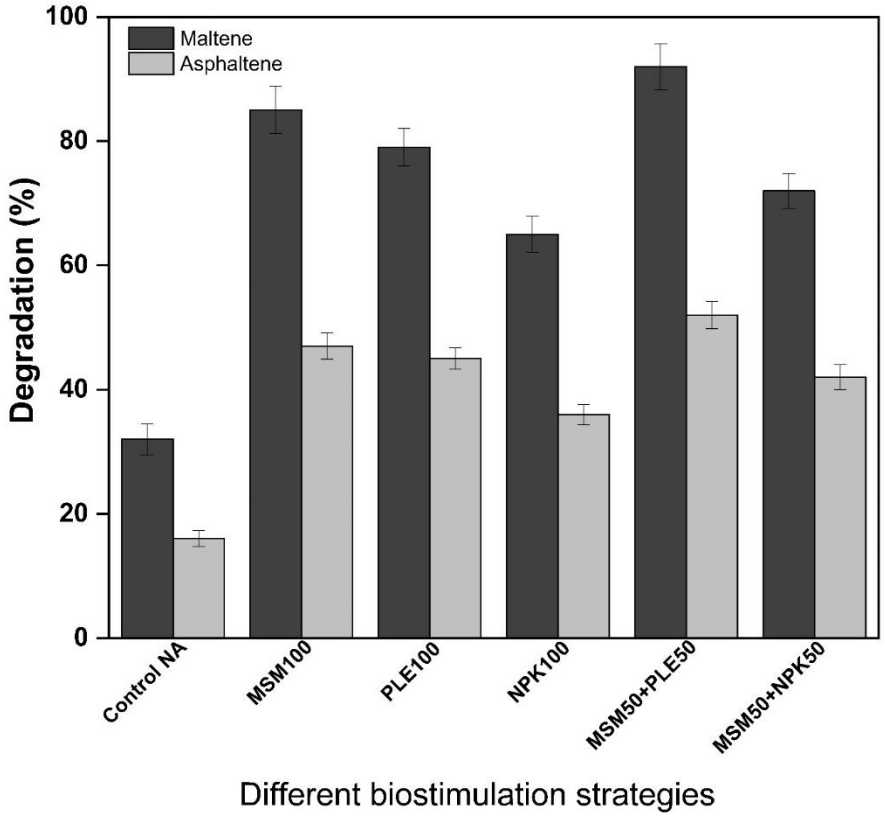

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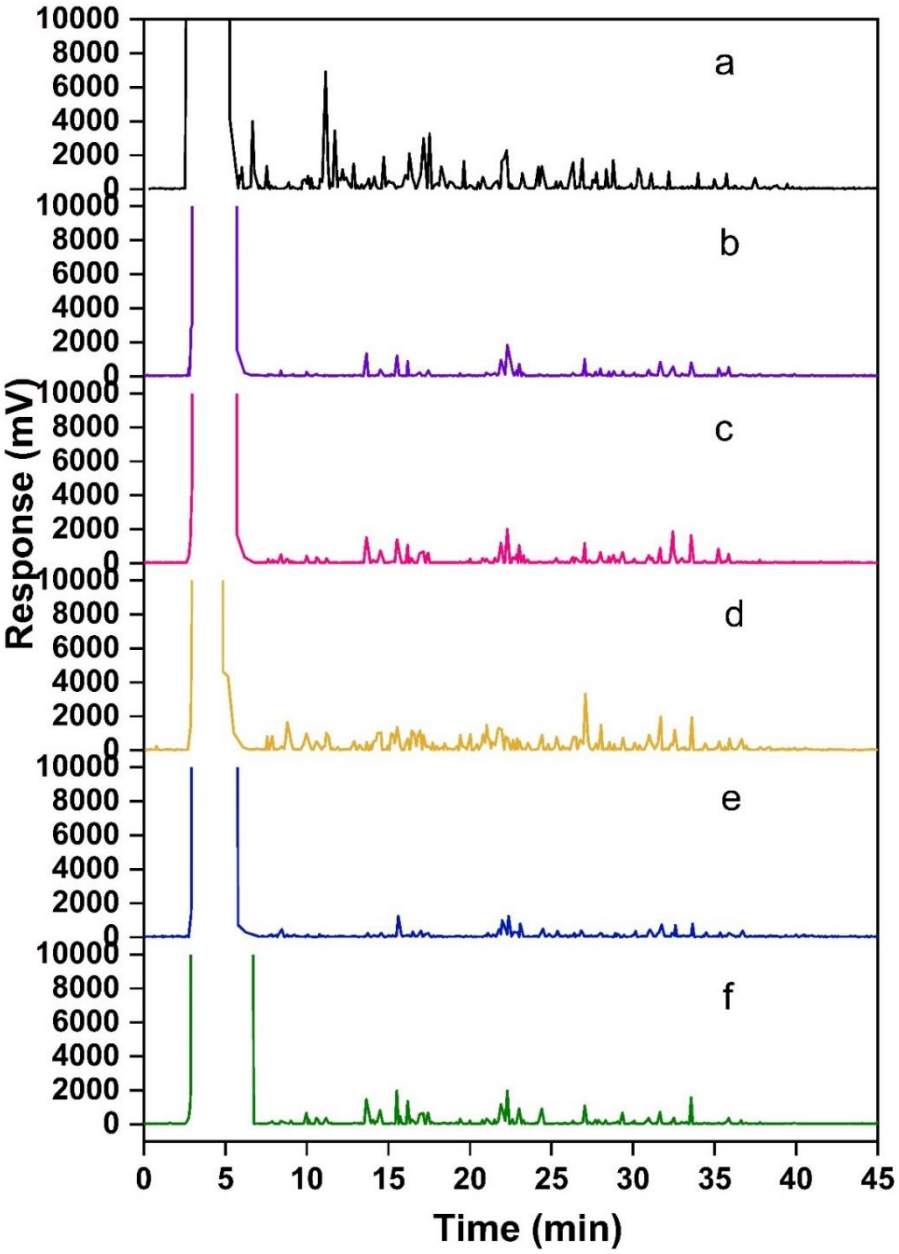
472 **Figure 4**



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