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ORIGINAL ARTICLE

Crude oil degrading efficiency of formulated consortium of bacterial strains isolated from petroleum-contaminated sludge

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Abstract

Crude oil contamination has been widely recognized as a major environmental issue due to its various adverse effects. The use of inhabitant microorganisms (native to the contaminated sites) to detoxify/remove pollutants owing to their diverse metabolic capabilities is an evolving method for the removal/degradation of petroleum industry contaminants. The present study deals with the exploitation of native resident bacteria from crude oil contaminated site (oil exploration field) for bioremediation procedures. Fifteen (out of forty-four) bioremediation-relevant aerobic bacterial strains, belonging to the genera of *Bacillus*, *Stenotrophomonas*, *Pseudomonas*, *Paenibacillus*, *Rhizobium*, *Burkholderia,* and *Franconibacter,* isolated from crude oil containing sludge, have been selected for the present bioremediation study. Crude oil bioremediation performance of the selected bacterial consortium was assessed using microcosm-based studies. Stimulation of the microbial consortium with nitrogen or phosphorous led to the degradation of 60–70% of total petroleum hydrocarbon (TPH) in 0.25% and 0.5% crude oil experimental sets. CO_2 evolution, indicative of crude oil mineralization, was evident with the highest evolution being 28.6 mg mL^{-1}. Ecotoxicity of treated crude oil-containing media was assessed using plant seed germination assay, in which most of the 0.25% and 0.5% treated crude oil sets gave positive results thereby suggesting a reduction in crude oil toxicity.

Keywords Bioremediation · Total petroleum hydrocarbon (TPH) · Microbial consortium · Microcosms · CO_2 evolution · Ecotoxicity

Introduction

Exploration of crude oil is an age-old process that has been leading to severe contamination of land masses, water bodies and groundwater reserves through accidental spillage, storing, refining, production and distribution and sometimes drilling site abandonment (Holliger et al. 1997; Ulrich et al. 2009; Roy et al. 2014). Oily sludge is a waste product that is generated in various stages of petroleum industries, including extraction, storage, transportation, and refining of petroleum crude oil. The practical and effective disposal of this oily sludge has become a global issue due to its toxic nature and large quantity produced every year. Indian refineries produce more than 28,220 tons of sludge every year (Singh and Kumar 2020). Crude oil, extracted from this oily sludge, remains in the environment for a long time due to its persistent nature which rigorously affects the quality of soil by altering physical, physiological, biochemical properties and intrinsic heterogeneous microbial diversity (Margesin et al. 2003; Head et al. 2006). Oil exposure being phytotoxic in nature, has affected plant growth by limiting the nutrient availability for plants (de Jong 1980; Udo and Fayemi 1975; Odukoya et al. 2019). Although oil provides a rich source of carbon and energy, it contains no significant amounts of biologically available nitrogen or phosphorus essential for microbial growth (Prince et al. 2013). These inherent complications led to the development of ecofriendly strategies which gained considerable importance as it relies on the metabolic potential of inhabitant microorganisms

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for reclamation of crude oil contaminated sites (Megharaj et al. 2011). Bioaugmentation and biostimulation-based bioremediation strategies have been undertaken by many investigators to remediate crude oil or other petroleum hydrocarbon-associated environments (Tahhan et al. 2011; Sun et al. 2012; Suja et al. 2014; Jasmine and Mukherji 2015; Wu et al. 2016; Mukjang et al. 2022; Muthukumar et al. 2023; Rondon-Afanador et al. 2023; Nong et al. 2023; Omenna et al.2024). Biostimulation (addition of appropriate nutrients like N and/or P) have been observed to improve metabolic activity of indigenous microorganisms thereby accelerating the hydrocarbon degradation process (Suja et al. 2014; Smith et al. 2015; Wu et al. 2019). Nitrogen and phosphorous-containing water-soluble salts can be supplemented to compensate for the required amount nitrogen and phosphorous in the initial phases of microbial cell growth during hydrocarbon metabolism (Thavasi et al. 2011). The efficacy of this process may get reduced due to the scarcity of efficient microorganisms in highly contaminated areas (Almeida et al. 2013). The availability of suitable microorganisms, especially those possessing the capacity of hydrocarbon degradation influences the process of degrading hazardous petroleum waste (Jasmine and Mukherji 2015; Poorsoleiman et al. 2020). Bioaugmentation strategy that uses specialist microbial isolates in contaminated sites has faced strong competition and predation by autochthonous microorganisms. This has led to the preferred use of native microbes (for bioremediation procedures), a concept known as ‘‘autochthonous bioaugmentation’’ (ABA), proposed by Ueno et al. (2007), for their easy adaptation and acclimatization in the familiar environment thereby increasing the bioremediation efficiency (that is mainly governed by nature and climatic condition of the contaminated sites, Gogoi et al. 2003; Suja et al. 2014; Jasmine and Mukherji 2015; Ambust et al. 2021). Study reports suggest that there are a limited number of microbial strains that are individually capable of biodegrading all the constituents of crude oil. Therefore, it is important to use a combination of strains to achieve proper bioremediation thereby utilizing their broad enzymatic capacities and their synergistic actions (Shetaia et al. 2016; Talukdar et al. 2023; Tripathi et al. 2024). Various studies have thus explored the effectiveness of using combined biostimulation and bioaugmentation as means of restoring petroleum-contaminated habitats (Sun et al. 2012; Roy et al. 2014, 2018; Wu et al. 2017). Microorganisms capable of hydrocarbon degradation as well as beneficial for plant growth have potentially been used in recovering the contaminated sites and improving plant health (Glick 2010; Gkorezis et al. 2016). Rhizosphere associated or free-living rhizobacteria show both plant growth-promoting (PGP) activity as well as degradation of persistent petroleum hydrocarbon (Yenn et al. 2014). Several reports are available on crude oil-degrading bacteria from diverse environments belonging to the genera of *Pseudomonas, Bacillus, Marinobacter, Alcanivorax, Rhodococcus, Mycobacterium, Cycloclasticus, Enterobacter, Dietzia*, *Alcaligenes,* etc. (Kasai et al. 2002; Wang et al. 2011; Yan et al. 2013; Fathepure 2014; Das et al. 2015; Kim et al. 2015; Zhang et al. 2015; Chen et al. 2017; Parthipan et al. 2017; Pi et al. 2017; Mohammed et al. 2023; Talukdar et al. 2023; Tripathi et al. 2024). These bacteria have been extensively used in preparing consortium because of their inherent potential of hydrocarbon degradation and their ability to produce biosurfactant. However, the use of oil-degrading consortium has not yielded satisfactory results all the time and this calls for the need of employing bacterial consortia from a petro-chemically important or extensively crude oil contaminated regions, which is unfortunately very limitedly explored (Zhao et al. 2011; Patowary et al. 2016).

Crude oil being a complex mixture has been the major source of petroleum hydrocarbon contamination of different habitats. Majority of the reports have focused on the fate of oil degradation by isolated microbial strains related to marine environments and contaminated soil in the aerobic shake flask technique (Nikolopoulou et al. 2013; Roy et al. 2014; Suja et al. 2014; Kristensen et al. 2015; Varjani et al. 2015; Ma et al. 2021). This study was undertaken with an attempt to investigate the crude oil biodegradation potential of a microbial consortium consisting of fifteen strains isolated from oil-containing sludge of Duliajan oil field, Assam, India in the presence/absence of exogenous nutrient availability where the TPH reduction from different concentrations of crude oil was monitored. Seed germination assay was also performed to assess the relative lowering of TPH toxicity during the course of the biodegradation study.

Materials and methods

Study area and sample collection

Samples were collected from Duliajan oil field, Assam, India (27.3667° N, 95.3167° E). Duliajan is an industrial town of Dibrugarh district in the Indian state of Assam. It is particularly known for its oil-related industry, Oil India Limited, one of the country's largest oil and gas companies. Crude oil-contaminated waste sludge was collected in pre-sterilized DEPC-treated screw-capped bottles (1 L capacity) from oil exploration sites, brought and stored in the lab under aseptic conditions until further processing.

Formulation of hydrocarbon utilizing *bacteria*

The consortium used in this study was formulated by mixing fifteen bacterial isolates (DJ5, DJ25, DJ26, DJ27, DJ29, DJ30, DJ31, DJ32, DJ33, DJ34, DJ-E1, DJ-E2, DJ-E4, DJ-E8 and DJ-E9) obtained after screening of forty-four isolates

obtained from oil containing sludge of Duliajan oil fields following the method of Das and Kazy (2014). Selection of these particular isolates was done based on their physiological, metabolic and phylogenetic characterization to ensure the microcosm study represented a considerable proportion of the culturable bacterial diversity present in the oily sludge. The potential of the isolates to utilize various hydrocarbons as the sole carbon source was evaluated in MSM. The cultures were incubated at 30 °C for 3 days in the presence of Benzene (B), Toluene (T), Ethyl Benzene (E) or Xylene (X) at a concentration of 50 mg L^{-1} and BTEX mixture at 200 mg L^{-1}. Utilization of alkanes as a sole source of carbon by the bacterium was carried out during growth in 100 mL Erlenmeyer flasks containing 20 mL MSM supplemented with pentadecane (C15) or hexadecane (C16) at a concentration of 250 mg L^{-1}. Cell numbers were determined after every 7 days and the residual n-alkane was analysed by gas chromatography. The residual n-alkane from the medium was extracted by adding an equal volume of n-hexane and deep freezing the lower water layer to collect the upper organic phase. Remaining water was absorbed by adding Na_2SO_4. The residual concentrations of the alkanes were analyzed using a GC (Agilent 7820A) equipped with a split/splitless injector, FID detector and an HP-5 column (30 m × 0.32 mm and i.d. 0.25 µm thickness). Nitrogen was used as carrier gas (flow rate 25 mL min^{-1}). The oven program was set initially at 80 °C for 2 min, followed by increasing to 210 °C at 10 °C rise per minute. Utilization of crude oil containing sludge (1%) was analyzed gravimetrically (Mishra et al. 2001) whereas utilization of crude oil was estimated by CFU count. The strains were also tested for their ability to grow in the presence of various temperatures (4–50 °C), at different NaCl concentrations (0–10%) and at different pH range (1.0–11.0), tolerate various heavy metals (Pb, Ni and Cd) at different concentrations (0.1–5 mM). Surfactant production ability of the isolates were examined following the method of Pal et al. (2017). Extraction of genomic DNA and molecular identification by amplification of 16S rRNA gene was performed by following the method as described by Das and Kazy (2014). As amplification and identification of partial 16S rRNA gene was only performed and no polyphasic approached were employed, so the isolates could not be identified to the species level.

Preparation of crude oil degrading microcosms

Estimation of the total organic carbon (TOC) of crude oil was done following the method of Walkley and Black (1934). Briefly, 0.125 ml of crude oil was gently mixed with 5 ml of 1N $K_2Cr_2O_7$ till maximum dispersion of crude oil has occurred. To this mixture, concentrated H_2SO_4 (7.5 ml) was added which was kept in boiling water bath for 30 min. The extract was collected and measured spectrophotometrically at 600 nm. Nitrate was estimated by following the method of Cataldo et al. (1975). Briefly, aliquots of 1 ml of extracted samples were pipette out into 50 ml of Erlenmeyer flasks, and mixed thoroughly with 0.8 ml of 5% (*w*/*v*) salicylic acid in concentrated sulphuric acid (SA- H_2SO_4). The flasks were kept at room temperature and after 20 min, 19 ml of 2N NaOH were added slowly to raise the pH above 12. The samples were then incubated for 10 min at room temperature and absorbance was measured at 410 nm. Phosphate in crude oil was determined according to the method of Murphy and Riley (1962). The concentration of phosphate was determined spectrophotometrically at 880 nm. Microcosms were prepared in butyl rubber capped serum vials (27 ml; Sigma-Aldrich, USA) with 4 ml normal saline (0.85%) and different concentrations of crude oil (0.25%, 0.5%, 1% and 2%). N and P amendments were done to maintain a final C: N: P ratio of 100:10:1 within each set (Roy et al. 2018).

Fifteen bacterial strains were selected for assessing their crude oil biodegradation potential by forming a consortium (MC setup). Microcosm with MC setup was supplemented with nitrate (MC + N), phosphate (MC + P) and a combination of nitrate and phosphate (MC + NP) for assessing the possible differences in bioremediation potential when the microbial strains were amended with nutrients. However, GC–MS analysis of the final formulation was not carried out. Details of various microcosm setups have been illustrated in Table 1. Nitrate (N) amendment was done with the addition of $NaNO_3$ and phosphate (P) by adding K_2HPO_4. Initial inoculum was used in between 10^8 and 10^{10} cells mL^{-1} for each isolate. An uninoculated microcosm setup (Abiotic control) was prepared to evaluate the possible abiotic losses. Microcosm setups were prepared in triplicates for each time point for different treatments and incubated at 30 °C in static conditions. The time points were set at 0, 15, 30, 60, 90 and

Table 1 Details of microcosm setups for degradation of 0.25%, 0.5%, 1% and 2% crude oil

Microcosm designation	$NaNO_3$ as N source	K_2HPO_4 as P source	Treatment
MC	–	–	Bacterial strains
MC + NP	+	+	Bacterial strains with both N and P amendment
MC + N	+	–	Bacterial strains with N amendment
MC + P	–	+	Bacterial strains with P amendment
Abiotic control	+	+	Uninoculated control to account for abiotic loses

120 days for each sacrificial vial. The amendments of nutrients made in various microcosm setups have been illustrated in Table 2.

Assessment of heterotrophic cell viability and crude oil TPH degradation within microcosms

Aerobic heterotrophic cell count was enumerated by serial dilution plating technique. 100 µl of the diluted sample (10^{-9}–10^{-10}) from each microcosm setup (Abiotic control, MC, MC + N, MC + P and MC + NP of 0.25%, 0.5%, 1% and 2% crude oil) was plated on Nutrient agar (HIMEDIA, India), incubated overnight at 30 °C to determine the cell numbers. This was performed to delineate any relationship between the reduction in TPH and cell viability. Residual TPH of crude oil at different time points was extracted by mixing an equal volume of *n*-hexane with a microcosm mixture followed by vigorous vortexing for 1 h. Particulates present were separated by collecting the supernatant in a fresh tube after centrifuging the whole mixture for 10 min at 10,000 rpm. The extracted TPH content and reduction percentage were determined by gas chromatographic (GC-FID) analysis as described by Roy et al. (2018).

Analysis of residual nitrate and phosphate in microcosms

After the extracted TPH (in organic phase) being separated, the aqueous phase is subjected to analysis of residual nitrate and phosphate as mentioned above, to determine its utilization by microorganisms as essential nutrients for their metabolism. MC + NP, MC + N and Abiotic control setups were tested for residual nitrate where 1 ml of extracted samples in test tubes were mixed thoroughly with 0.8 ml of 5% (*w*/*v*) salicylic acid in concentrated sulphuric acid (SA-H_2SO_4). The flasks were kept at room temperature and after 20 min, 19 ml of 2N NaOH was added slowly to raise the pH above 12. The samples were then incubated for 10 min at room temperature and absorbance was measured at 410 nm (Cataldo et al. 1975). Residual phosphate was estimated for MC + NP, MC + P and Abiotic control setups by following the method of Murphy and Riley (1962).

Table 2 Different amendments of nutrients in various setups

Crude oil concentration (%)	TOC (ppm)	Nitrate amendment (M)	Phosphate amendment (M)
0.25	77,250	0.5	0.025
0.5	154,500	1	0.05
1	309,000	2	0.1
2	618,000	4	0.2

Assessment of CO_2 evolution in microcosms

Evolved carbon dioxide (CO_2) was periodically collected every 30 days in alkali (NaOH), which has been back titrated with HCl (Tahhan et al. 2011; de Quadros et al. 2016). Briefly, a CO_2 trapping system was setup for each vial by wrapping rubber balloon containing 2 ml of 1 M NaOH around the neck of the vial and kept for 24 h. This provided a closed expandable system for the containment of CO_2 and necessary contact time for its maximum absorption by NaOH. Vials were sacrificed at different time points for measuring the entrapped CO_2. The resulting mixture of excess NaOH and Na_2CO_3 was titrated with standard HCl (1 M). Excess of NaOH was neutralized when the titration reached the first colorless phenolphthalein endpoint and all the Na_2CO_3 was converted to $NaHCO_3$. Continuing the titration till the second methyl orange endpoint converted $NaHCO_3$ to H_2O and CO_2. The volume difference between the first and second endpoints was used to calculate the CO_2 evolved by microbial activity in microcosms by following the equation as described by Crossno et al. (1996).

Ecotoxicity bioassay by seed germination and plant growth

Plant seed germination assay was performed to assess the bioremediation efficacy of crude oil degrading microcosms with (MC + NP, MC + N, MC + P) or without (MC) various amendments. At the end of 120th day, treated and untreated samples were mixed with garden soil at equal (1:1) and twofold (1:2) proportion (sample:soil, *w*/*v*) in small earthen pots, where seeds of white mustard (*Sinapis alba*) were sown in triplicates. White mustard is a widely and easily grown crop in the Indian scenario. It is a fast-growing plant. The difference in growth of the plant under various conditions (control and treated) was easier to identify. Therefore, white mustard seeds were chosen for the assay. Various ecotoxicity studies have also reported the use of seeds of white mustard (Salanitro et al. 1997; Jiang et al. 2016; Hawrot-Paw et al. 2020; Ambust et al. 2021). Moisture content was maintained by keeping the pot partially immersed in water. Growth of seedlings was monitored till 15 days and growth parameters (germination, shoot height and root length) of the seedlings was recorded at the end of 15 days following the method of Tang et al. (2011).

Results and discussion

Crude oil characteristics; taxonomic assignment, formulation, physiological and metabolic potential of hydrocarbon utilizing bacterial consortium

Total organic carbon (TOC) of crude oil (1%) was estimated to be 3.09×10^5 ppm. Total nitrogen (N) and phosphorus (P) in crude oil was estimated to be 59.34 ppm and 38.03 ppm respectively.

A microbial consortium was formulated to assess the crude oil bioremediation performance of the native microorganisms isolated from crude oil-contaminated sludge. Fifteen bacterial isolates were screened from 44 isolates obtained from the sludge based on their physiological, metabolic and phylogenetic characterization. Seven *Bacillus*, three *Stenotrophomonas* and one each of *Pseudomonas*, *Paenibacillus*, *Franconibacter*, *Rhizobium* and *Burkholderia* strains were selected for constructing the hydrocarbon utilizing bacterial consortium. Phylogenetic affiliation (the assigned taxonomy) and various physiological (growth under different pH, temperature, salinity and heavy metal concentration) and metabolic potential (degradation of various hydrocarbons, biosurfactant production, etc.) of the strains have been briefed in Table 3. The observation suggested their potential of degrading hydrocarbons and tolerating various physicochemical conditions. Most of the strains could utilize crude oil, petroleum sludge and BTEX compounds except DJ-E1, E4, E8, E9. Pentadecane and hexadecane were utilized at different proportions by the strains with the maximum utilization shown by DJ25, DJ30, DJ31, DJ32 and DJ34. All the strains could produce biosurfactant and displayed dominant growth in the presence of 1 mm of Pb and Ni, but Cd proved to be lethal for few strains.

A group of microorganisms were selected because low microbial population and insufficient microbial diversity can affect bioremediation efficacy (Lin et al. 2010). The use of such exogenous/endogenous microorganisms in hydrocarbon-associated habitats and their efficiency in hydrocarbon degradation have been demonstrated in various studies (Cerqueira et al. 2011; Teng et al. 2011; Yang et al. 2016; Yuan et al. 2017; Roy et al. 2018; Muthukumar et al. 2023; Omenna et al. 2024). Biosurfactant production has been an essential property for promoting hydrocarbon uptake and degradation by lowering its interfacial surface tension thereby enhancing its solubility (Pal et al. 2017). Heavy metals are ubiquitous in petroleum contaminated areas. Bacteria possess various transporters for exporting heavy metals out of the cells in order to survive in contaminated habitats. These properties justified their selection for constructing the bacterial consortium to be used in microcosms and evaluate the best possible way for utilization of crude oil.

Cell viability and crude oil TPH degradation within microcosms

The potential of the isolates to bioremediate TPH in microcosms were assessed for its possible adoption as an approach for reclaiming contaminated sites. Almost 10^2 fold increase in cell count was observed in all the setups for different concentrations of crude oil. However, the maximum cell growth occurred at different time points for different concentrations of crude oil used. For 0.25% crude oil concentration, the highest cell number was reached within 15 days after which it gradually dropped. For the rest of the concentrations, the maximum cell yield was obtained within 30–45th day (Fig. 1). No viable cells were observed for abiotic control sets. The increase in cell counts and viability hinted towards the capability of the organisms to degrade the crude oil components and utilize the simpler products, which in turn supported their growth. Chaineau et al. (2005) reported that a sharp increase in viable cell count mostly occurs within the first 15 days of adding nutrients. However, the efficiency of the microorganisms gradually decreased with increasing TPH content. Reports from similar kind of experiments showed the reduction of TPH biodegradation with increasing crude oil concentrations, although the cell viability remained almost same (Rahman et al. 2003; Behera et al. 2021).

TPH degradation of 0.25% crude oil was nearly 67% for the MC + N and MC + P set, which were also recorded for the maximum cell yield. Considerable degradation of more than 60% was also observed in the setup with only microbial consortium (MC setup). Crude oil concentration of 0.5% was also reduced following a similar pattern as MC + N and MC + P resulted in maximum TPH reduction followed by MC and MC + NP setup. However, when 1% and 2% crude oil concentrations were used, MC setup resulted in the maximum degradation although the maximum microbial count was less than that of nutrient-amended setups. The results clearly suggested that the microorganisms used to construct the microcosms (which represented a considerable fraction of the petroleum sludge community) have their inherent capacity for synergistic petroleum hydrocarbon utilization (Fig. 2).

Using a microbial consortium instead of the pure culture of microorganisms could be more advantageous from the application perspective of bioremediation and satisfactory degradation results can be obtained if a mixed bacterial culture is used. Before field application, depending on the requirement, consortium could be defined for providing the necessary metabolic diversity and robustness. Bioremediation process in nature depends on cooperative metabolic activities of mixed microbial populations where the degrading bacteria get benefits from synergistic interactions, thereby increasing the bioremediation efficiency (Gallego et al. 2007; Jacques et al. 2008; Mukred et al. 2008; Cao

Table 3 Biodegradation relevant characteristics of selected bacterial isolates considered for microbial consortium

Parameters	Isolates														
	F. pulveris DJ34	*Bacillus* sp. DJ5	*Bacillus* sp. DJ25	*Bacillus* sp. DJ26	*Bacillus* sp. DJ27	*Steno-tropho-monas* sp. DJ30	*Pseu-domonas* sp. DJ31	*Steno-tropho-monas* sp. DJ33	*Bacil-lus* sp. DJ-E4	*Steno-tropho-monas* sp. DJ29	*Bacil-lus* sp. DJ-E9	*Rhizobium* sp. DJ-E2	*Bacillus* sp. DJ32	*Paeniba-cillus* sp. DJ-E1	*Burkholde-ria* sp. DJ-E8
pH 1	–	–	–	–	–	–	–	–	–	–	–	–	-	-	-
pH 3	+	+	+	+	+	+	+	+	–	+	–	–	-	-	-
pH 5	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
pH 7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 9	–	+	–	–	+	–	+	–	+	–	+	+	-	+	+
pH 11	–	–	–	–	–	–	–	–	+	–	+	+	-	+	+
15˚C	+	+	+	+	–	+	+	+	–	+	–	–	+	-	+
25˚C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
35˚C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
45˚C	+	–	+	+	+	+	+	+	+	+	+	+	+	+	-
Salinity (1–6%)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pb (1 mM)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ni (1 mM)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cd (1 mM)	+	+	+	–	+	+	–	+	+	–	+	+	-	-	+
BTEX (200 ppm)	+	+	+	+	+	+	+	+	–	+	–	+	+	-	+
Crude oil (1%)	+	+	+	+	+	+	+	+	–	+	–	+	+	-	-
Sludge (1%)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C16 (250 ppm)	+	+	+	+	+	+	+	–	+	+	–	+	+	+	-
C15 (250 ppm)	+	+	+	–	–	+	+	+	+	+	+	+	+	+	+
Biosur-factant produc-tion	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T80 hydrol-ysis	–	–	–	+	+	+	–	+	+	+	+	–	-	+	-

'+' Positive; '–' Negative

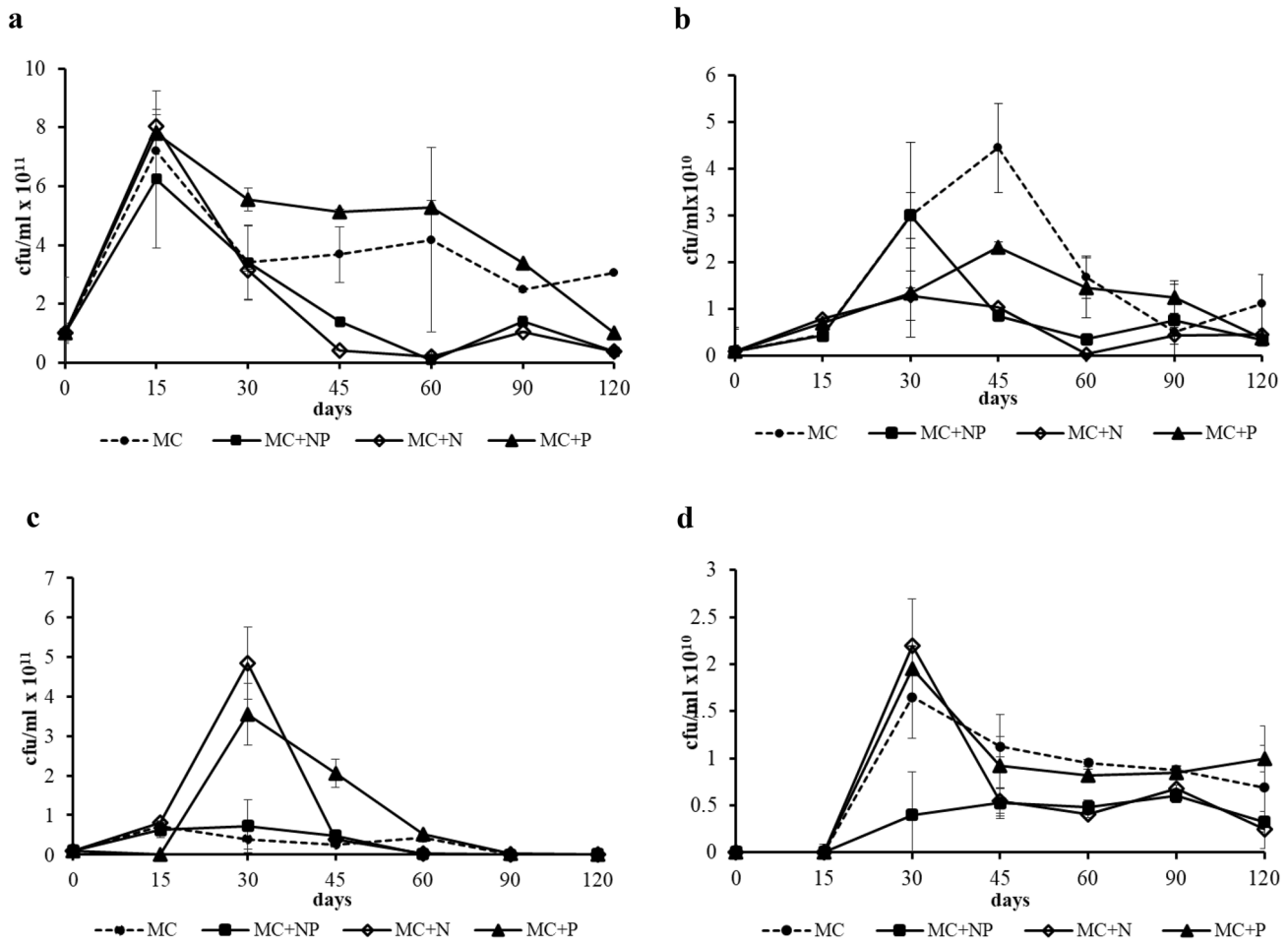

Fig. 1 Cell viability assay: Cell viability in different setups of microcosms with crude oil concentration of **a** 0.25%; **b** 0.5%; **c** 1% and **d** 2%

et al. 2009; Cerqueira et al. 2011; Janbandhu and Fulekar 2011; Tyagi et al. 2011; Morris et al. 2013; Wu et al. 2013; Yenn et al. 2014; Patowary et al. 2016; Dhote et al. 2016; Koolivand et al. 2017; Kumari et al. 2018; Suganthi et al. 2018).

Various earlier studies have reported the successful utilization of microbial consortium (using microorganisms with crude oil biodegradation potential) for bioremediation of hydrocarbons, that produced better degradation outcomes than their individual counterparts. The use of consortium led to an effective reduction of TPH, thereby decreasing the toxicity of the contaminated environments. Varjani et al. (2015) illustrated the use of a microbial consortium formulated by six indigenous bacterial strains consisting of *Ochrobactrum* sp. (01), *Stenotrophomonas maltophilia* (02) and *Pseudomonas aeruginosa* (03) in removing more than 80% of crude oil TPH. Bacterial consortium consisting of five pure bacterial cultures of *Stenotrophomonas acidaminiphila*, *Bacillus megaterium, Bacillus cibi*, *Pseudomonas aeruginosa* and *Bacillus cereus* obtained from petrochemical oily sludge and soil contaminated by petrochemical waste was reported to biodegrade aliphatic and aromatic hydrocarbons of petrochemical oily sludge in liquid medium (Cerqueira et al. 2012). The consortium was capable of reducing 90.7% of the aliphatic fraction and 51.8% of the aromatic fraction during 40 days of the experiment. Consortium consisting of four bacterial strains namely, *Achromobacter* sp. BAB239, *Pseudomonas* sp. DV-AL2, *Enterobacter* sp. BAB240 and *Pseudomonas* sp. BAB241, showed efficient naphthalene degradation even in the presence of other pollutants as compared to individual bacterial strain (Patel et al. 2012). Tao et al. (2017) investigated the reduction in crude oil TPH by 85% within 7 days when *Bacillus subtilis* strain was exogenously added to an existing bacterial consortium composed of *Betaproteobacteria* and *Gammaproteobacteria* members as compared to 71% when only the indigenous microorganisms were used. *Bacillus* sp. IOS1-7, *Corynebacterium* sp. BPS2-6, *Pseudomonas* sp. HPS2-5 and *Pseudomonas* sp. BPS1-8 were isolated from oil-contaminated soil samples and were considered for constructing an efficient consortium

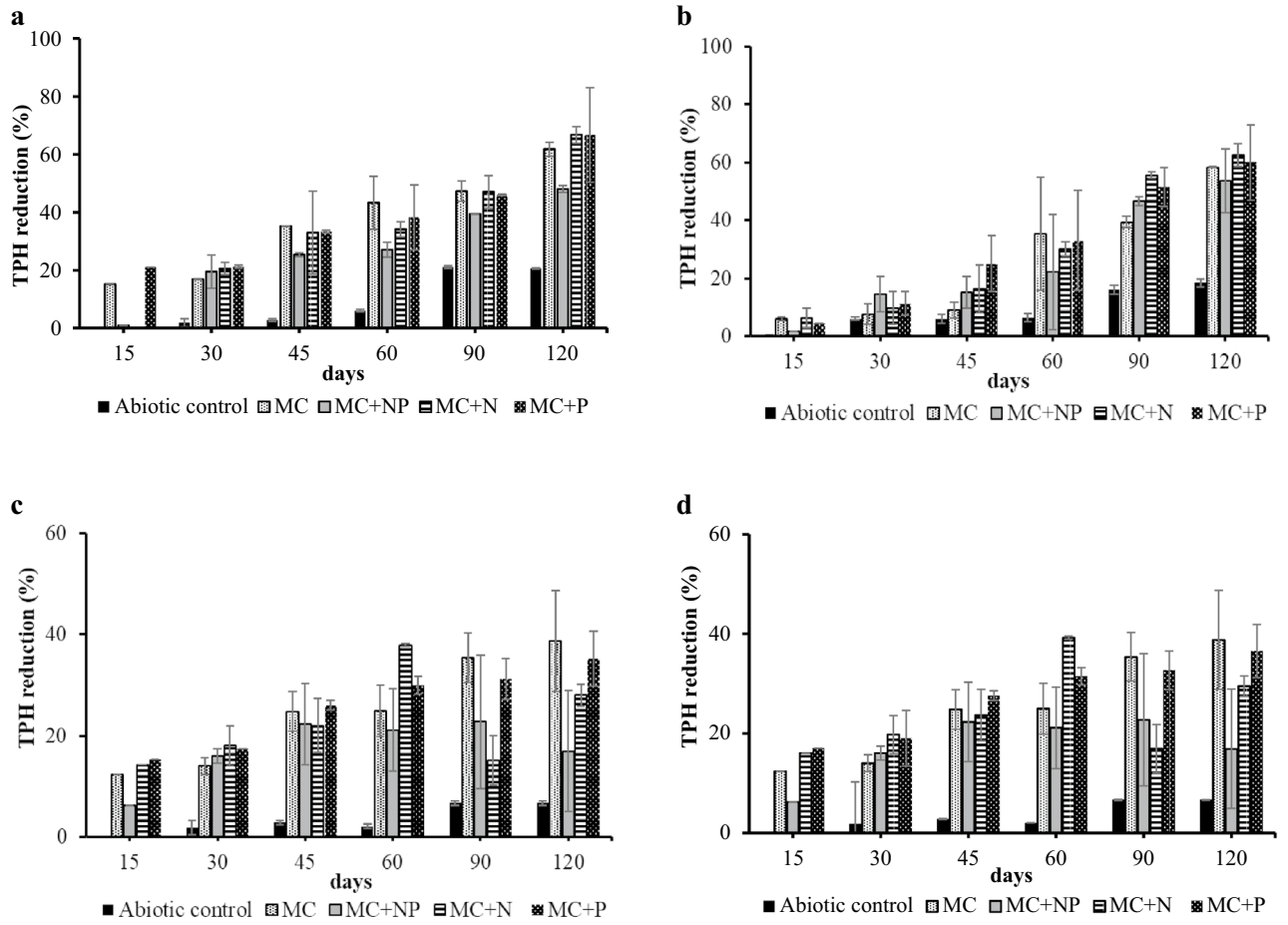

Fig. 2 TPH degradation assay: TPH degradation in different microcosm setups for crude oil concentration of **a** 0.25%; **b** 0.5%; **c** 1% and **d** 2%

capable of utilizing crude oil (Sathishkumar et al. 2008). While utilizing 1% crude oil, this consortium could reduce 77% of crude oil TPH which was the highest when these strains were used individually. Koolivand et al. (2017), reported a two-stage composting treatment of tank bottom sludge with effective removal of TPH by combining different strains of *Pseudomonas* sp., *Bacillus* sp., *Klebsiella* sp., *Staphylococcus* sp., and *Proteus* sp. TPH removal of 96% was also achieved when tank bottom sludge was treated by a bacterial consortium constituting *Shewanalla chilikensis, Bacillus firmus*, and *Halomonas hamiltonii* (Suganthi et al. 2018). Patowary et al. (2016) applied different sets of consortia constituting crude oil degrading strains among which some were biosurfactant producer and some were not. Maximum reduction of crude oil TPH was exhibited by the consortium which had biosurfactant-producing strains. Mnif et al. (2015) also highlighted the fact that co-inoculation of biosurfactant-producing strain could enhance the performance of a consortium in the biodegradation of hydrocarbons. They isolated four strains namely *Lysinibacillus bronitolerans* RI18, *Bacillus thuringiensis* RI16, *Bacillus weihenstephanensis* strain RI12 and a biosurfactant-producing *Acinetobacter radioresistens* RI7 from oil-contaminated soil. Consortium of *L. bronitolerans* RI18, *B. thuringiensis* RI16 and *B. weihenstephanensis* strain RI12 when co-inoculated with *A. radioresistens* RI7 and an exogenous *Bacillus subtilis* SPB1 showed maximum diesel degradation of about 55.4%. Molaei et al. (2022) conducted a pioneering study reporting enhanced biodegradation of TPH and COD removal by a bacterial consortium (removal efficiencies of above 99% and 96%, respectively), that was also capable of in situ generation of biosurfactant. Gojgic-Cvijovic et al. (2012) characterized strains belonging to the genera of *Pseudomonas, Achromobacter, Bacillus* and *Micromonospora* from oil refinery storage fuel tank and polluted soil from its vicinity. Consortium constructed from these strains were used for bioaugmentation of the polluted sample in laboratory condition which was further biostimulated with the addition of nitrogen, phosphorous and surfactant. Over the course of the experiment for twelve weeks, a reduction of 80–90% of TPH was achieved for petroleum sludge and polluted soil. Zhao et al. (2011), formulated a consortium with

Pseudomonas sp., *Rhodococcus* sp., *Bacillus* sp., *Microbacterium* sp., *Roseomonas* sp., *Brucella* sp. and *Rhizobiales* sp. isolated from oil field contaminated soil which exhibited nearly 52% crude oil removal. Bioaugmentation of contaminated soil with this particular consortium in microcosm test confirmed its effectiveness by reducing more than 50% crude oil over a period of 60 days. Microbial consortium consisting of two strains each of *Bacillus* sp. and *Pseudomonas* sp. were also considered for the degradation study of 2% (*w*/*v*) oil sludge (Dhote et al. 2016). The consortium yielded 75% reduction of TPH as compared to the individual strains which were almost 20% lower than the consortium. The result also emphasized the use of biosurfactant-producing strains while constructing the consortium.

However, it is important to stimulate those microorganisms with nutrients that are lacking in the environment to obtain maximum degradation as insufficient N and/or P concentration could retard hydrocarbon biodegradation (Smith et al. 2015). Majority of the studies suggested the use of combined bioaugmentation and biostimulation (especially with N and P) in enhancing microbial growth and hydrocarbon degradation (Sun et al. 2012; Almeida et al. 2013; Ghaly et al. 2013; Suja et al. 2014; Roy et al. 2018). In our study, the combined use of N and P could not help in attaining substantial degradation. Therefore, this study contradicted the use of both N and P in combination for biodegradation.

Estimation of residual nitrate and phosphate in microcosms

To confirm the utilization of N and P during the degradation of crude oil, residual nitrate and phosphate in the medium was determined. Decline in nitrate and phosphate concentration in test samples suggested their consumption during the treatment period. Changes in the concentration of nitrate (plotted on y-axis) in microcosms have been presented in Fig. 3. There was a gradual reduction of nitrate concentration in all the treated setups till 120 days of incubation. It

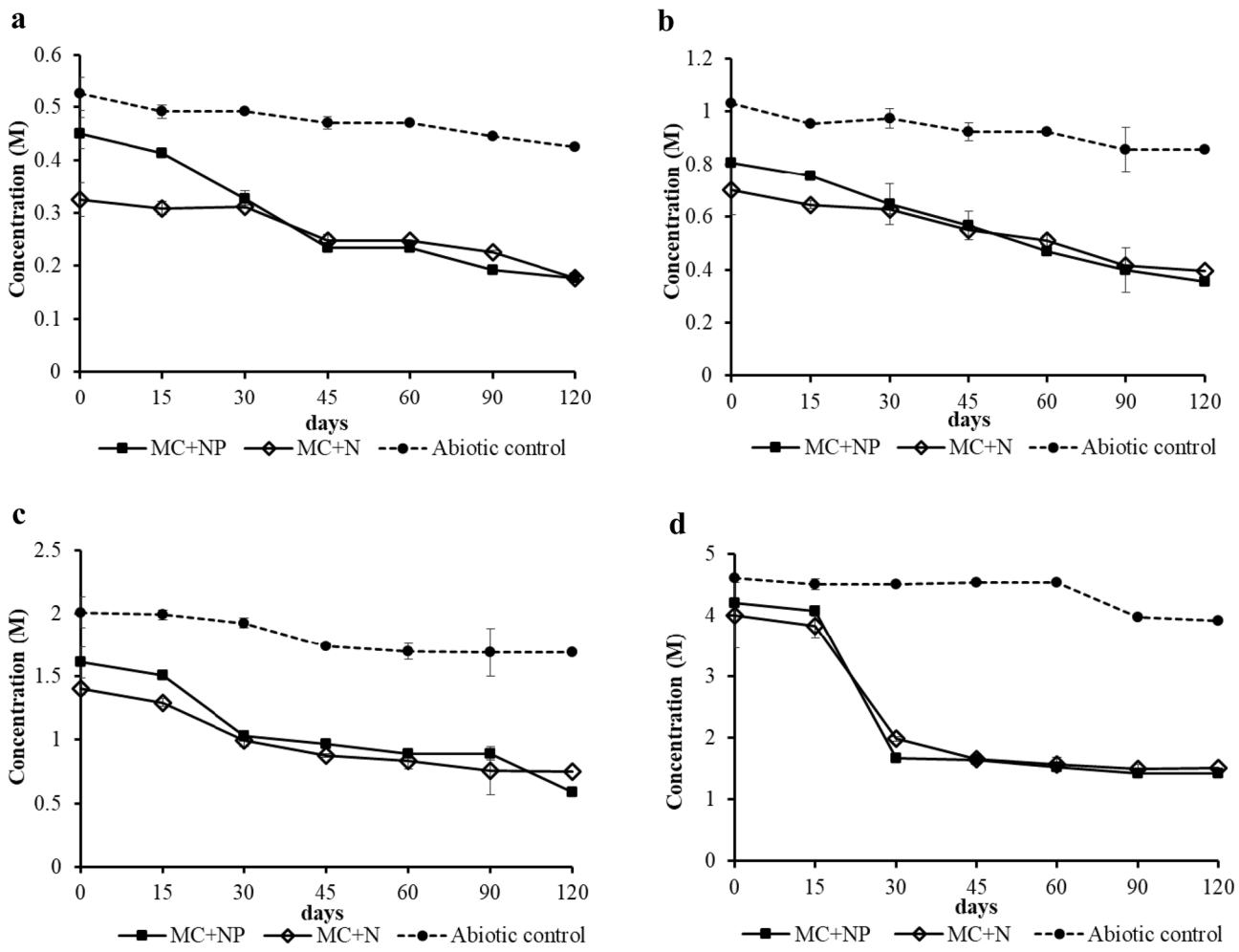

Fig. 3 Estimation of residual nitrate concentration: Residual nitrate concentration in different microcosm setups for crude oil concentration of **a** 0.25%; **b** 0.5%; **c** 1% and **d** 2%. The concentration of nitrate is plotted on y axis

was interesting to observe that the consumption of nitrate in MC + NP and MC + N sets were similar for all the crude oil concentrations used. Nitrate serves as an important nitrogenous nutrient as well as a terminal electron acceptor for many microorganisms. Thus, in nitrogen-limited condition, input of nitrate could influence nitrogen cycling and subsequent biogeochemical processes which are mostly driven by microorganisms (Galloway et al. 2008). Due to its thermodynamic favorability as an electron acceptor, large number of microorganisms during anaerobic respiration reduce nitrate in the process of oxidizing organic matter or other reduced substrates. Nitrate amendments in contaminated sediments for in situ bioremediation have been demonstrated in quite a number of studies which suggested its role in promoting degradation of organic carbon (Cunningham et al. 2001; Kutvonen et al. 2015; Xu et al. 2015; Romantschuk et al. 2023).

Concentration of residual phosphate in microcosms showed a gradual decrease over the entire bioremediation treatment process when compared to the abiotic control. Reports suggest that microbial metabolic traits shift with phosphate availability and the amount of phosphate present acts as a critical controller of hydrocarbon degradation (Oliverio et al. 2020). Siciliano et al. (2016) established the essential role of phosphate in enhancing the catabolic potential of microorganisms. Phosphate was consumed more for the MC + P set than MC + NP for all the crude oil concentrations used. Changes in the concentration of phosphate (plotted on y-axis) in microcosms have been presented in Fig. 4.

This finding could suggest that in the presence of both N and P as nutrient, microorganisms would prefer using N over P. This could probably happen due to the complex formation between phosphorus and different metals present in crude oil making the phosphate less available for utilization (Mattingly 1975; USEPA 1985, 2013; Fragkou et al. 2021).

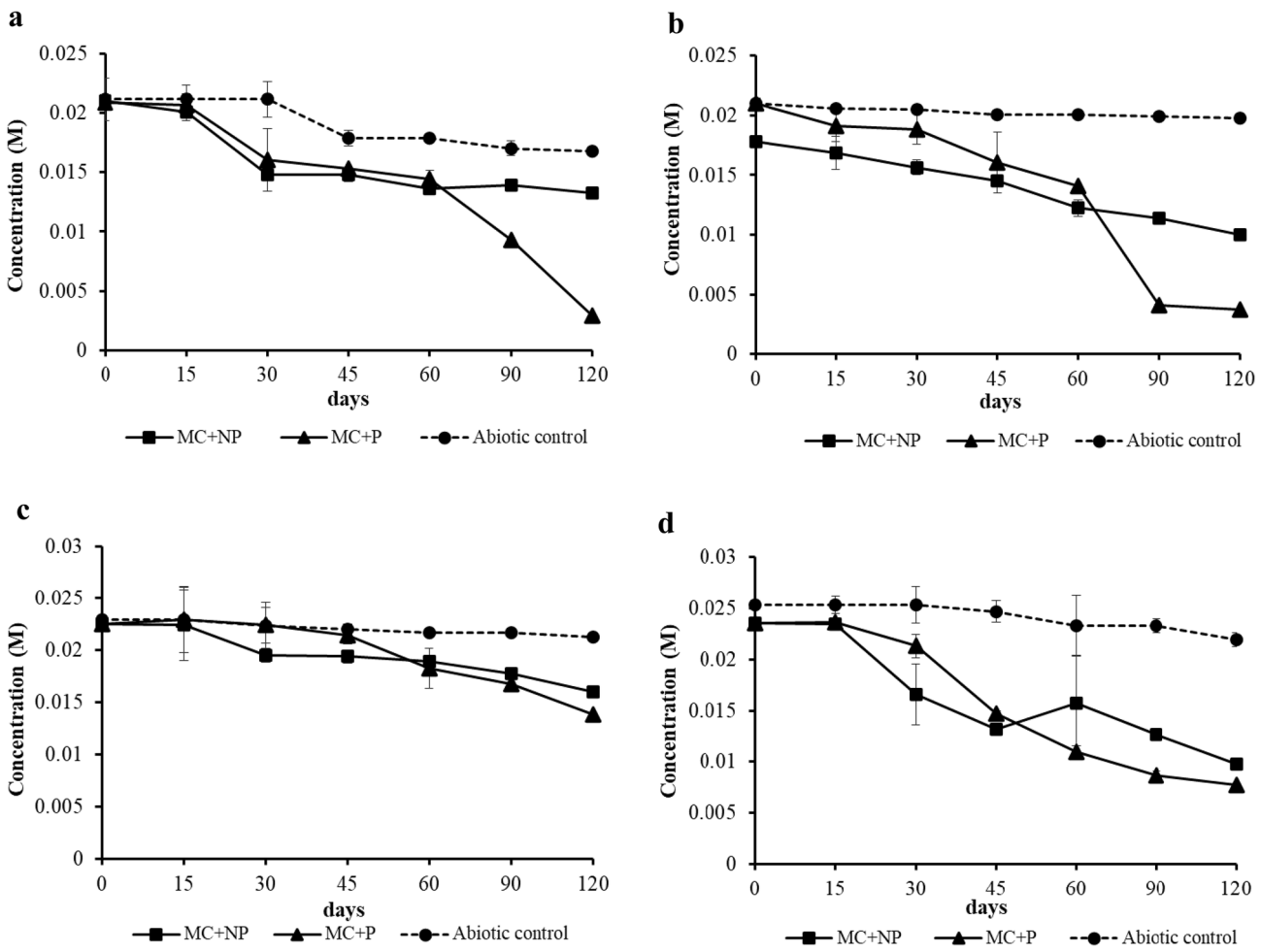

Fig. 4 Estimation of residual phosphate concentration: Residual phosphate concentration in different microcosm setups for crude oil concentration of **a** 0.25%; **b** 0.5%; **c** 1% and **d** 2%. The concentration of phosphate is plotted on y axis

Assessment of crude oil mineralization via CO_2 evolution

All the microcosm setups containing different concentrations of crude oil showed higher mineralization activities, compared to abiotic controls during the 120 days of the experiment indicating the utilization of crude oil TPH as the sole carbon source by the amended microbial community (Fig. 5). The highest mineralization occurred in the treatments of 0.25% crude oil by MC + P setup (28.6 ± 0 mg/mL of evolved CO_2) followed up by MC + N (24.2 ± 0 mg/mL), which also showed the maximum cell yield and TPH

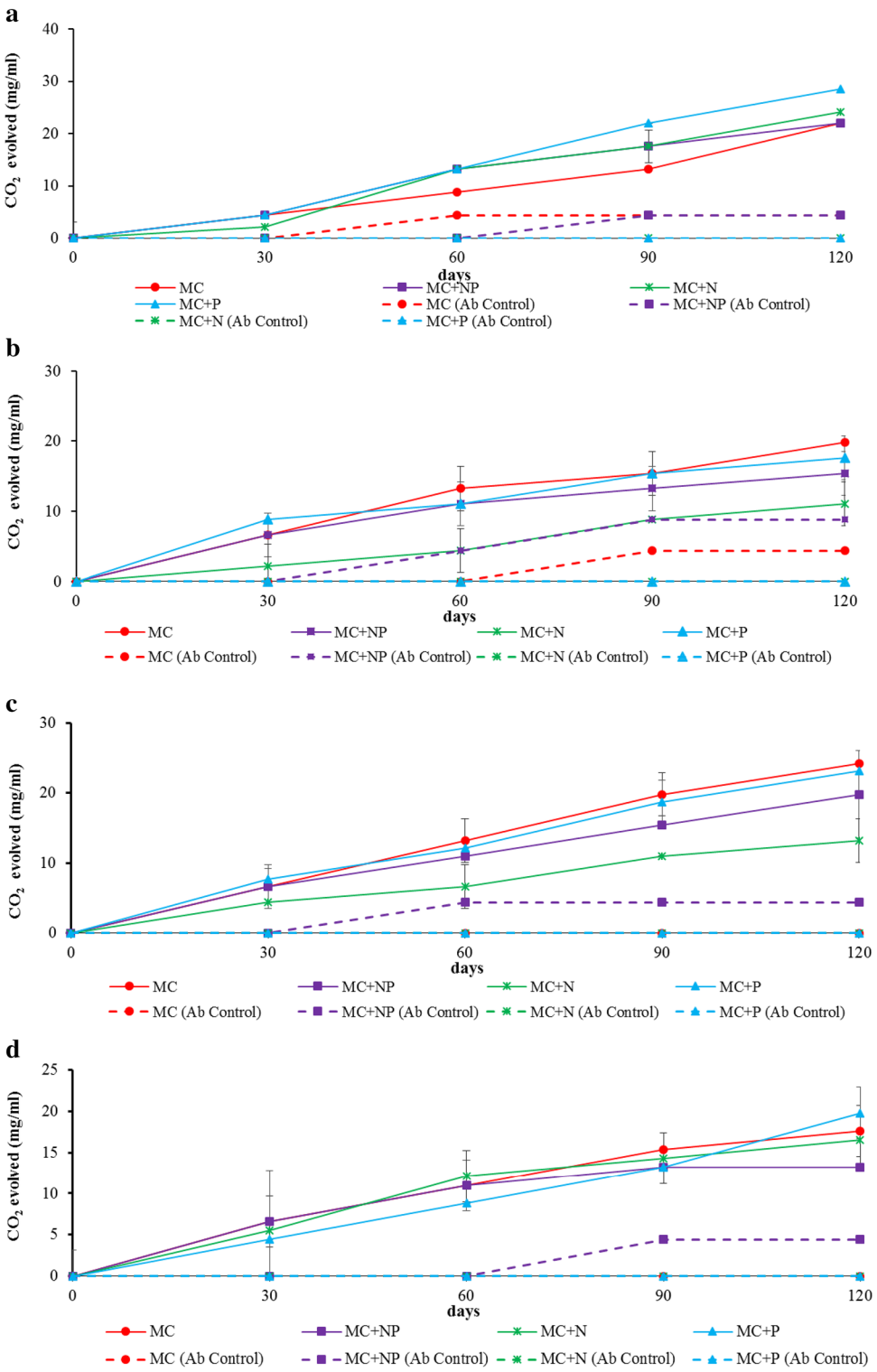

Fig. 5 CO_2 estimation: Estimation of evolved CO_2 by different microcosm setups for crude oil concentration of **a** 0.25%; **b** 0.5%; **c** 1% and **d** 2%

degradation. For 0.5% and 1% crude oil, MC setup showed the highest mineralization of 24.2 ± 0 mg/mL of CO_2 evolution, whereas the MC + P set of 2% crude oil yielded 19.8 ± 0 mg/mL of evolved CO_2 as the highest during the 120 days. Quantification of microbial CO_2 evolution and the results obtained from crude oil TPH degradation through gas chromatography highlighted the important role of autochthonous microorganisms in TPH removal.

Previous investigators have also demonstrated that addition of hydrocarbon-degrading bacteria to the petroleum-contaminated soil could result in a substantial increase in TPH mineralization and removal, which strongly and positively correlated with the overall outcome of the hydrocarbon removal process (Tahhan et al. 2011; Abena et al. 2019). The degrading bacteria can follow various pathways of assimilation, metabolism, and cellular decomposition to degrade individual crude oil components following the formation for CO_2. Alkanes are either converted to alcohol which gets oxidized to an alkanal and dehydrogenated by aldehyde dehydrogenase to the corresponding fatty acid, or, the aldehyde is further oxidized to generate alcohol and acetic acid that undergoes terminal oxidation to generate fatty acid. The fatty acid formed undergoes beta-oxidation with the generation of fatty acyl-CoA and acetyl-CoA. The fatty acyl-CoA continues to be oxidized to generate carbon dioxide and water (Varjani and Upasani 2017). Cycloalkanes are successively oxidized to cycloalkanol, cycloalkanone and then finally to adipic acid. Adipic acid goes directly for β-oxidation, where it is oxidatively decomposed into water and carbon dioxide (Abbasian et al. 2015; Hazaimeh and Ahmed 2021; Xue et al. 2015). Monocyclic aromatic compounds are catalyzed by oxygenases and dehydrogenase to produce catechol which undergoes further cleavage. Intermediate products undergo oxidation and the TCA cycle, which eventually mineralize compounds into carbon dioxide and water (Chunyan et al. 2023).

The mineralization rate was more in the initial 60 days of experimental setup probably because the more labile fraction in the crude oil could have been readily used as a source of carbon and energy for the microorganisms, which also corroborated with the maximum microbial population that was observed in this particular period. Marin et al. (2005) showed that the CO_2 emissions gradually decreased while the most labile hydrocarbon fractions disappeared, leaving behind the unutilized recalcitrant fraction. Furthermore, it was observed that the setups with lower concentrations of crude oil showed higher mineralization rate than the treatments with higher nutrient amounts. Similar behavior was observed for the rate of mineralization in the work realized by Tahhan and Abu-Ateih (2009), wherein the inhibitory effect of the added nutrients (NH_4NO_3 and KH_2PO_4) was observed during the mineralization of TPH from the oily sludge. Since nutrients were added as a ratio of the added carbon, inhibition was the greatest with the highest TPH treatment. This could be attributed to the possibility of biodegradation inhibition by high concentrations of nutrients. Earlier reports also suggest similar attributes where there was a permanent inhibition of hydrocarbons assimilation with a high input of nutrients thereby revealing that different nutrient supplies should be added in an optimum for the unhindered continuation of the degradation process (Chaineau et al. 2005).

Ecotoxicity bioassay by seed germination and plant growth

Data for the seed germination bioassay of treated and untreated crude oil has been summarized in Table 4. Germination studies have been considered as a primary assay for determining the reduction in toxicity of pollutants (Roy et al. 2014). The seed germination results corresponded well with the findings on TPH degradation. No seed germination took place for untreated crude oil setups (abiotic controls) whereas all seeds germinated in uncontaminated garden soil. Maximum seed germination occurred for the treated 0.25% and 0.5% crude oil sets suggesting that these crude oil concentrations were the most suitable for bacterial degradation and utilization for 120-day period in static conditions. This ecotoxicity study provided two highlights (i) the reduction in toxicity of the treated sludge following the effective TPH

Table 4 Percentage of *Sinapis alba* seeds germinated with different crude oil concentrations undergoing different treatments

Treatments	Crude oil concentration							
	0.25%		0.5%		1%		2%	
	Sample: Soil ratio							
	1:1	1:2	1:1	1:2	1:1	1:2	1:1	1:2
Abiotic control	0	0	0	0	0	0	0	0
MC	66	66	100	100	66	66	0	0
MC + NP	100	100	100	33	0	0	0	0
MC + N	66	100	100	100	0	66	0	0
MC + P	100	100	66	100	0	66	0	0

removal and (ii) renewed activities of introduced bioaugmented bacteria (Roy et al. 2014). Along with the decrease in TPH content, the reduction of heavy metal toxicity due to the introduction of microorganisms could also be a reason for the germination of seeds. Improved root and shoot lengths were observed at a lower concentration ratio (1:2) than in the case of 1:1 ratio for the lowest (0.25%) concentrations of crude oil (Fig. 6).

Root shoot length for MC setup degrading 0.5% crude oil (in both 1:1 and 1:2 ratio) was similar to that of seeds growing in uncontaminated garden soil. Growth of seedlings was also significant for MC setup while degrading 1% crude oil. Amendments with N or P also showed significant germination and growth of seedlings. It has been reported that petroleum hydrocarbon content in the soil could negatively affect plant germination and growth (Tang et al. 2011). In the present experiment, the higher concentrations of crude oil inhibited the seed germination as their degradation was also lesser compared to lower crude oil concentrations. These findings have been consistent with previous reports, which documented an improved germination and plant growth on applying bioremediation (Wang and Bartha 1990; Salanitro et al. 1997; Saterbak et al. 2000; Roy et al. 2014).

Conclusion

The present study deals with the exploitation of native resident bacteria from crude oil-contaminated site for bioremediation procedures. Microbial consortium formulated from fifteen isolated bacterial strains (out of forty-four, selected based on their metabolic potential) showed their inherent potential of crude oil biodegradation and mineralization in microcosms with or without nutrient amendment. Throughout the 120-day degradation study, 60–65% of TPH was removed for 0.25% and 0.5% crude oil. However, for 1% and 2% crude oil, 30–40% of TPH could be reduced by the microbial consortium. Germination of *Sinapis alba* seeds suggested the reduction in toxicity of crude oil components. Overall, the study concluded that the consortium developed from diverse indigenous microorganisms of crude oil-impacted regions could be a potential tool for in-situ bioremediation of crude oil-contaminated sites. With the emergence of high-throughput omics techniques (e.g., genomics and metagenomics), the individual bio-degraders, hydrocarbon-degrading microbial communities, metabolic pathways and interactions can

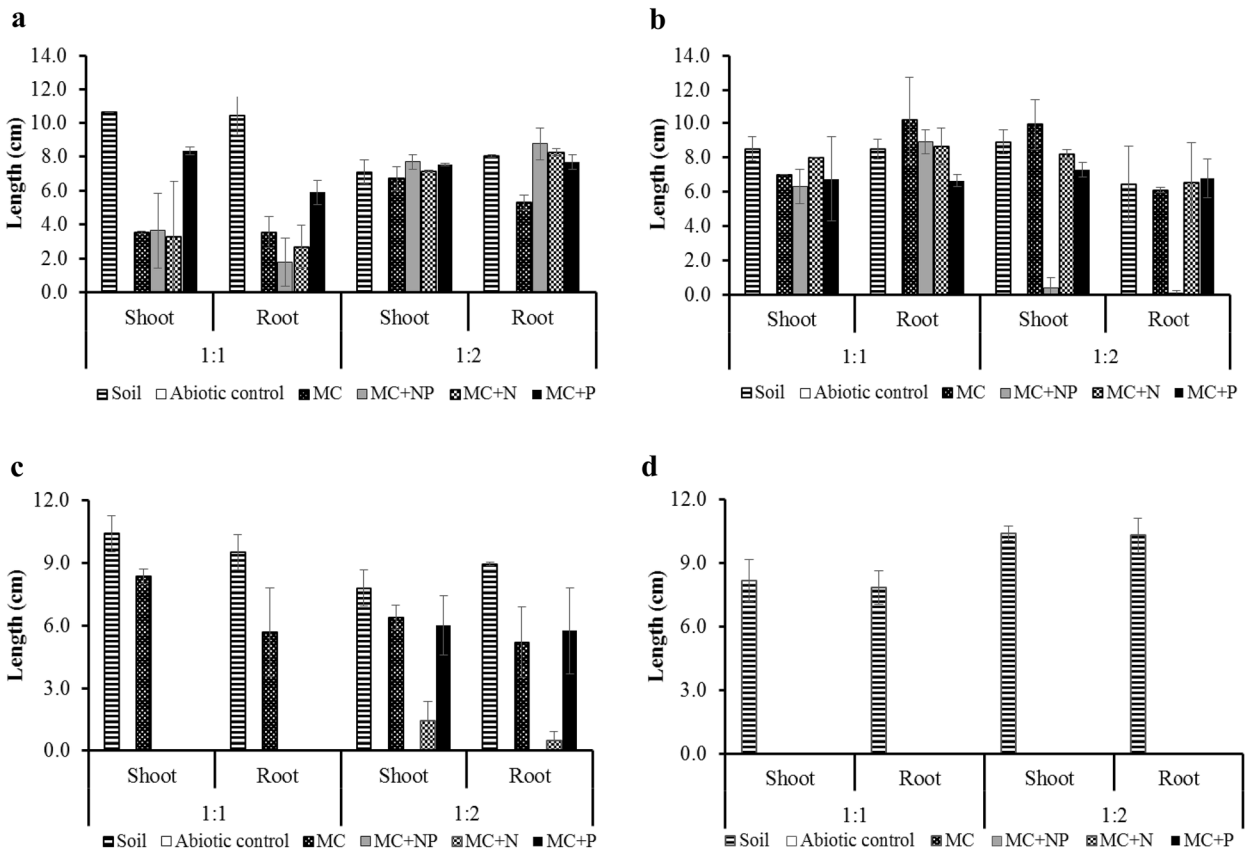

Fig. 6 Plant germination assay: Shoot and root length of germinated seeds from treated microcosms with crude oil concentration of **a** 0.25%; **b** 0.5%; **c** 1% and **d** 2%

be described at a contaminated site. Single microorganisms or microbial communities can be examined at the system level and the metabolic networks, interspecies interactions during hydrocarbon mineralization can be elucidated. Genomic studies of the microbial isolates to identify the active genes and proteins responsible for the inherent capability of hydrocarbon degradation could help us in amplifying the biodegradation potential of the formulated consortium. Moreover, in-depth analysis of the crude oil sample may help unravel novel unculturable hydrocarbon-degrading strains and enzymes which could be fitted into the metabolic networks of the community, which in turn would support the scaling up of these microcosm studies for ex-situ applications and field trials.

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Author contributions Not Applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest in the publication.

Accession numbers Not applicable.

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