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In situ microbial and phytoremediation of crude oil contaminated soil by *Cynodon* sp.

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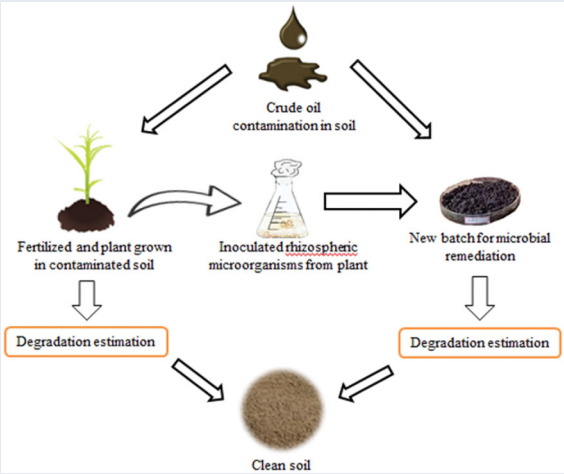
ABSTRACT

Crude oil contamination of land and water leads to their abandonment after heavy oil recovery processes. Analogous to bioremediation, phytoremediation has provided an efficient solution towards land reclamation through enhancement of flora. The present work manifests significance of phytoremediation via reclamation of crude oil contaminated soil collected from Kalol, India. The collected soil was analyzed for pH, oxidation–reduction potential, electrical conductivity (EC), bulk density, particle size, moisture. The experimental work consists three batch units; pot A, pot B and pot C with crude oil contaminated soil, fresh soil and control respectively. While observing plant growth for 120 days, Total Petroleum Hydrocarbon (TPH) was measured at determined intervals for estimation of percentage degradation. After 90 days of pot observation, contaminated soil was inoculated with rhizospheric bacterial inoculum developed from pot A which forms new batch for microbial-remediation as an additional scope to this work. Gas chromatography mass spectroscopy (GC-MS-MS) was carried out for determination of naphthalene contamination. Crude oil degradation in pot A was estimated as 82.16% followed with the affirmation given by degradation kinetics whereas, 60.68% and 36.75% degradation was observed in pot C-control and new batch respectively. *Cynodon* sp. grown in pot A was confirmed by identification as reported.

KEYWORDS

Crude oil contaminated soil; *Cynodon* sp.; first-order kinetics; *in situ* phytoremediation; naphthalene degradation; rhizospheric microorganisms

GRAPHICAL ABSTRACT



Statement of novelty

This work signifies the potential of phytoremediation by *Cynodon* sp. grown in crude oil contaminated soil fertilized with cow dung and corn silk. Although studies in this context are present in literature, this study adds an affirmation to it. Also it differs in terms of contamination percentage removal which is better compared to previous studies but the only demerit is that specific specie could not be identified due to time limitation. Also, the use of corn silk as fertilizer is new to literature. Indigenous microorganisms vary based on substrates and conditions and this experiment was performed on sweet crude oil extracted from Gujarat, India which adds new information to the literature.

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Supplemental data for this article can be accessed at publisher's website.

Introduction

Crude oil contamination gives rise to various hazards which results in compromise of comfortability, health and environment. The crude oil contamination of agricultural land caused from oil recovery operations lead to their abandonment and menace of health (Hutchinson *et al.* 2001; Fatima *et al.* 2018). The poly aromatic hydrocarbon (PAHs) present in crude oil comprise naphthalene, fluorene, pyrene, benzo[a]pyrene, anthracene, acenaphthalene, fluranthene, chrysene, phenanthrene, etc. are heavy molecular chain compounds that are complex and non-polar in nature (Wilson and Jones 1993). Among different bioremediation techniques, phytoremediation and rhizoremediation have better advantages over PAHs contamination (Abiodun 2010). The present work is focused on these two remediation types. Phytoremediation is an enhanced methodology for attenuation of heavy contamination by complex hydrocarbons, achieved from plantations of specific flora to decompose toxics into non-toxics and other harmless organic substitutes in soil (Kathi and Khan 2011; Chigbo and Nnadi 2014; Pokethitiyook 2017; Ite and Ibok 2019; Kumaria *et al.* 2021). Phytoremediation incorporates degradation process such as phytoextraction, phytostabilization, phytofilteration, phytostimulation, phytovolatilization, phytotransformation (Das and Chandran 2011; Kathi and Khan 2011; Qixing *et al.* 2011; Pokethitiyook 2017; Fatima *et al.* 2018; Ite and Ibok 2019; Kumaria *et al.* 2021). Factors such as hydrophobicity, toxic volatile components, salinity and toxic metals from crude oil inhibit germination (Zhu *et al.* 2018) despite the fact that microflora have the genetic capacity to degrade the contaminants, yet they are recalcitrant and elude microbial degradation (Correa-García *et al.* 2018). But certain plants contain the potential to grow in the presence of complex hydrocarbon contamination as it evolves from certain endophytic and rhizospheric microbial synergism. Thus, indigenous plants grown on such soil can selectively enhance favorable endophytes that produce enzymes responsible for hydrocarbon degradation via reduction in evapotranspiration and toxicity of volatile hydrocarbons (Pokethitiyook 2017). Endophytes are present inside the plant which renders survival, growth and development of plants, thus interact more closely to plants compared to rhizobacteria (Fatima *et al.* 2018). Whether the endophytic bacteria colonize intercellular or intracellular healthy plant tissue can produce extracellular enzymes including pectinase, phenoloxidase, lignin catabolic enzymes that are necessary to penetrate and colonize the host plants (Pokethitiyook 2017). Pokethitiyook (2017) stated that plants treat xenobiotics into three phases - transformation by enzymes activities; conjugation; storage of final products in vacuoles. Rhizoremediation refers to that part of phytoremediation where degradation of complex organic contaminants takes place in and on the roots of flora, with their prolific and diverse nature has some advantages towards synergic processes in biodegradation (Frick *et al.* 1999; Chaudhry *et al.* 2005; Singh *et al.* 2015; Correa-García *et al.* 2018; Fatima *et al.* 2018). Contaminants in soil undergo decomposition, transformation or stabilization by rhizobacteria present in roots (Pokethitiyook 2017; Correa-García *et al.* 2018; Ite and Ibok 2019). Plant roots penetrate in soil forming vents for air circulation and provide surface area for biochemical reactions (Kumaria *et al.* 2021). The exudates from plant roots produce enzymes that catalyzes organic carbon and oxygen to substrate as well as provides essential nutrients for endophyte synergism and enhances microbial activities in roots (Pokethitiyook 2017; Correa-García *et al.* 2018; Fatima *et al.* 2018; Ite and Ibok 2019; Kumaria *et al.* 2021; Wang *et al.* 2021). Roots improve physical structure and enrichment of soil, also with additional source of N and P showed enhanced oxygen transport to greater depths in the soil, stimulate petroleum degrading microorganisms and provide microbial access to soil micropores which results in linear relationship between plant growth and remediation (Hutchinson *et al.* 2001; Correa-García *et al.* 2018). Recent research showed that inoculation of Plant Growth Promoting Rhizobacteria (PGPR) isolates or consortia improved durability and enhanced remediation of soil due to the availability of additional nutrient source and substrate to scaled-up microorganisms (Pokethitiyook 2017; Correa-García *et al.* 2018; Ite and Ibok 2019). Baoune *et al.* (2019) investigated the synergism of PGPR and endophytes by inoculation of *Streptomyces* sp. H1W to *Zea mays* to remediate petroleum hydrocarbon contamination which resulted in 70% hydrocarbon removal and C8–C30 were efficiently degraded in plant *Streptomyces* H1h1 system. This shows degradation is better with inoculated compared to non-inoculated plants (Pokethitiyook 2017; Baoune *et al.* 2019; Ite and Ibok 2019; Kumaria *et al.* 2021).

The non-synergic coordination between indigenous plants and inoculated species influences the efforts of remediation (Ikhajiagbe *et al.* 2017). Despite the fact that crude oil contamination causes significant reduction in germination of grass, species such as wheat, barley, maize and sweet corn are affected less (<20%). Also variation of germination exists between species and genotypes within species (Zhu *et al.* 2018). Still grasses and legumes are widely selected for phytoremediation due to their dense, vast, fibrous roots with large porous surface area that holds abundant microbial population which may enter the soil layer 3 m deep (Razmjoo and Adavi 2012; Ikhajiagbe *et al.* 2017; Correa-García *et al.* 2018; Kumaria *et al.* 2021; Tanthry *et al.* 2020). Turf and forage specie like *Bermuda grass* is highly sod forming perennial that propagates by stolon, rhizomes and seeds in all soils from heavy clay to deep sands. It is both acid and alkaline tolerant as well as tolerant toward salt, oil, flood, cold and soil compaction (Razmjoo and Adavi 2012; Ikhajiagbe *et al.* 2017; Pokethitiyook 2017). Tolerance index for *Cyondon dactylon* under experimental condition was 94.47% as reported by Ikhajiagbe *et al.* (2017). Sonowal *et al.* (2018) reported highest superoxide dismutase activity (47 mg % pw) against oxidation stress and catalyst activity in *C. dactylon* while interaction with complex hydrocarbon contamination. To investigate the cause behind robust survival of *Bermuda grass* within such complex environment, studies were carried out by Peña-Castro *et al.* (2006) which shows DNA isolations procured from roots of *C. dactylon*,

functions in substrate metabolism, signal transduction, protein synthesis and degradation of PAHs which acts against mechanical and anoxic stress induced from PAHs. Also, *Cynodon* sp. contains antibacterial and antidiabetic property, hence, used for medicinal, forage and landscaping (Shahin and Salem 2018; Tanthry *et al.* 2020). According to Shahin and Salem (2018), mixing the hydrophobic sand with the agricultural soil improves soil–plant characteristics and conserves water use without causing any adverse effects in cultivated plants and if left unmoved, it can reach up to 2 ft height. Grasses such as *rye grass, fescue, crab grass, alfalfa, vetrivera zizaniodes, bermuda grass,* and *E. purpurea*, etc. are observed to degrade crude oil (Frick *et al.* 1999; Kathi and Khan 2011; Qixing *et al.* 2011; Pokethitiyook 2017; Shahin and Salem 2018; Ite and Ibok 2019; Kumaria *et al.* 2021). *Cynodon dactylon* also known as *bermuda grass* has efficiency to degrade complex petroleum hydrocarbon contamination in soil at moderate to high temperature zones (Frick *et al.* 1999; Basumatary and Bordoloi 2016; Ikhajiagbe *et al.* 2017; Nguemté *et al.* 2018; Pulchérie *et al.* 2018; Tanthry *et al.* 2020) using C4 metabolic pathway which consumes high water rates and provides better conversion, thus, C4 plants are better at contamination removal than C3 plants (Shahin and Salem, 2018; Sonowal *et al.* 2018). Studies reveal that *Cynodon* spp. showed highest degradation effects amongst 166 species in 125 genera and 50 families of flora (Pulchérie *et al.* 2018). The effectiveness of *Cynodon* was examined with several other species of flora. Oyedeji *et al.* (2013) found *E. indica* better than *C. dactylon* in terms of TPH, fresh and dry mass and organic carbon present in soil. While comparison of *Cynodon* spp. with *E. indica* and *A. desertorum* reveals that *E. indica* yielded better results in crude oil degradation while *Cynodon* spp. has greater impact than *A. desertorum* (Saraeian *et al.* 2018). Current work includes the use of Corn silk from maize as fertilizer. Since literature also states corn silk acts as good adsorbent of oil and also contributes toward remediation (Asadpour *et al.* 2019, 2015). Phytoremediation by maize plant is good alternative for certain concentration range (Kumaria *et al.* 2021). Ayotamuno and Kogbara (2007) have reported 60% degradation under 21% contamination. *Zea mays* also has high tolerance level toward oxidative stress induced from contamination (Ayotamuno and Kogbara 2007; Liao *et al.* 2015). According to Ayotamuno *et al.* (2006), *Zea mays* reduced contamination of crude oil from 22,000 mg/kg to 6,000 mg/kg in 6-week period. Liao *et al.* (2015) reported 73% degradation using maize and 34% in control manifests 39% increased degradation by maize.

In addition to indigenous cultivars, fertilizer addition also plays an important role in promoting root growth and reduction of recalcitrant contaminations which in turn influences the rate of degradation from double ring structures to poly ring structures of higher complexity (White *et al.* 2003, 2006; Lin *et al.* 2020). Even though phytoremediation considered better than other remediation techniques in terms of cost and space (Hutchinson *et al.* 2001; Ite and Ibok 2019) yet time resists its merits (Correa-García *et al.* 2018). The present work carries an objective to estimate the degradation potential of phytoremediation by indigenous plant fertilized with corn silk and cow dung in crude oil contaminated soil by observing reduction in TPH concentration. Also, part of the soil was observed for bioremediation resulting from consortia developed from roots of indigenously grown plant and their degradation difference was compared.

Material and methods

Raw material preparation and characterization

Around 10 kg of crude oil contaminated soil was provided by Oil and Natural Gas Corporation (ONGC) of India from well number 104 – an agricultural site in Kalol, Gujarat, India. The crude oil contamination occured to the site from oil recovery process and well reparing services. The collected raw material was present with excessive heterogeneous contamination and stickiness. Hence, the soil was initially cleaned by removing grits, immersed plastic wires and other non-degradable substances, thereafter crushed and air dried under ambient atmosphere and sunlight for 12 days prior to soil analysis. The primary estimation of physical and chemical characteristics of obtained crude oil contaminated soil was carried out. The texture of the collected soil sample was estimated according to Indian Society of Soil Science (ISSS) in International System of Classification. Sieve analysis was carried out with available mesh size of BSS (British Standard Sieves) 150, 44, 30, 16 for obtaining coarse sand, medium sand, fine layer sand and silt (DIRD, Pune Dird 2009). Whatman filter paper no. 42 with pore size 2.5 µm was used to separate very high fines/clay from bulk. Bulk density of sample was estimated by weighing bottle method which follows weight to volume ratio of sample confined using gravity bottle. Estimation of soil pH and the oxidation–reduction potential (ORP) were measured with Syntronics (µpH) system 361 (µ-controller) ORP indicator of 0.1 mV resolution. EC of soil sample was measured with Chemline CL-120, 0–200 µS/cm with cell constant 1.05 and cell range 20–200 µS/cm by immersing electrode in soil solution prepared from soil–water mixture in 1:2 ratio. The measured EC was converted to deci siemens dS/m (Carter and Gregorich 2008). The estimated data are shared from Iyer *et al.* (2020), in Table 2.

Moisture analysis

The estimation of initial moisture percentage present in soil was carried out in triplicates of 10 g each dried under oven at 63 ± 2 °C until constant mass of sample obtained. The remaining mass was deducted from initial mass and the evaporation loss was calculated as moisture present in sample soil. Water was added to the soil sample to its level of absorbing capacity and dried similarly for determination of water holding capacity of soil. Crude oil was extracted from 10 g of soil to prepare oil free soil for TDS measurement. This soil was mixed with water in 1:1 ratio and continuously stirred to form suspension while pipetting 60 ml soil into 250 ml flask containing Whatman filter paper no. 42 with

Table 1. Moisture analysis of soil sample (Iyer *et al.* 2020).

Moisture	22.5%
Water holding capacity	30.67%
Total dissolved solids	0.17%

pore size 2.5 µm. The difference observed from filtrate mass to initial sample mass is TDS. Moisture analysis data are given in Table 1 (Iyer *et al.* 2020).

TPH degradation evaluation

Degradation of crude oil was estimated at regular intervals by measuring Total Petroleum Hydrocarbon (TPH) concentration in percentage using solvent extraction followed by gravimetric analysis. Soil samples were spooned from surface, sub-surface and root-section of pot and homogenously mixed. Soil sample weighed to 10 g in triplicates were washed by 60 ml FINAR GC grade 99% sulfur/thiophene free toluene and filtered using BOROSIL 500 ml separating funnel following gravity separation method. The filtered was washed three to four times with water and allowed to settle. Due to density difference water layer settles in bottom and both oil and water was collected separately from bottom of funnel. Toluene was evaporated from recovered oil by heating at 90–100 °C using Remi heating mantle and residual oil was allowed to cool in desiccator and mass was recorded. Degradation% was calculated as given in the following equation (Prakash *et al.* 2014; John and Okpokwasili 2012):

$$\text{Degradation\%} = 100 - \left[\frac{TPH_c \text{ measured at intervals}}{\text{Initial } TPH_0} \times 100\right] \quad (1)$$

Pot preparation

Three sets of pots were prepared as A, B and C having 1 kg of soil each. Pot A and pot B were filled with crude oil contaminated soil and fresh clean soil, respectively. The fresh soil was collected from surrounding of institute premises. Pots A and B were fertilized with 10% cow dung, 5% dried corn silk and indigenous grass (present in contaminated soil) were properly tilled and watered. Pot C was prepared to contain 1 kg of crude oil contaminated soil fertilized similarly with cow dung and corn silk without sowing anything to keep it as control. The pots were placed such that air and sunlight would be sufficient and watered regularly. The pots were observed then.

Development of rhizosphere bacterial consortium

A cut was made in pot A and shoved to the roots; little end of root was cut and spooned along with the surrounding bound soil and encapsulated in autoclaved plastic envelop as inoculum. The mineral salt medium (MSM) was prepared by diluting 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g $CaCl_2$, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 1.0 g NH_4Cl, and 0.05 g $FeCl_3$ (John and Okpokwasili 2012; Prakash *et al.* 2014; Parthipan *et al.* 2017) in 250 ml distilled water into 500 ml Erlenmeyer flask, properly mixed and autoclaved at 121 °C; 15 psi for 20 min. The autoclaved medium was inoculated with encapsulated inoculum and incubated for 6 days at room temperature maintained by continuous stirring of broth in orbital shaker at 140 ± 4 rpm.

Figure 1. New batch of crude oil contaminated soil with initial TPH of 11.7% tilled with rhizospheric bacterial consortium developed from pot A.

Preparation of new batch

New batch was prepared with 250 g of crude oil contaminated soil in 15 cm internal diameter petri dish and inoculated with 10% w/w of developed consortium from rhizosphere of pot A. The consortium–soil mixture was tilled and mixed properly. The batch was exposed to sunlight and ambient atmospheric conditions. Water spray was used once in week to regulate moisture levels (Figure 1).

Qualitative analysis

The identification and estimation of PAH present in the provided soil sample was performed using GC-MS-MS (tandem mass spectroscopy) triple quadruple acquisition (QqQ) method. Agilent 7890A GC – 7000 MS having column 1 Agilent 19091S-433UI HP-5MS of 30 m × 0.25 mm × 0.25 µm and column 2 Agilent 160-7625-5 MS inert fused silica of 5 m × 150 µm (ID) (Agilent Technologies, Palo Alto, CA). Helium was used as carrier gas as well as quenching gas with 2.25 ml/min and nitrogen as collision gas with 1.5 ml/min. The sample injection was done using split less mode with split ratio of 100 ml/min at 1 min and injection volume of 1 µl with solvent delay mode of 5 min. Mass selective detector (MSD) with transfer line temperature of 134 °C was used with electron ionization (EI) (electron energy mode; ion source temperature 300 °C) with multiple reaction monitoring (MRM) scan. Initial oven temperature for columns 1 and 2 was 70 °C for 2 min hold and ramp was provided to raise temperature by 25–150 °C, 4 °C/min to 220 °C then 10 °C/min up to 325 °C operating temperature. Column 1 is set to pressure up to 7.652 psi and flow rate 1 ml/min at average velocity of 36.445 cm/s and column 2 is set to pressure up to 10 psi and flow rate 0.39 ml/min at an average velocity of 55.51 cm/s. The commercially available certified reference material from Sigma Aldrich was used for preparation of standards. The peaks were identified based on internal library source.

Results and discussion

The crude oil contaminated soil which was collected from ONGC well decommissioned site was characterized as per ISSS classification of soil and the determined texture is given in Table 2. These data prove that the soil was suitable despite of heterogeneous distribution of contamination over its surface, it can provide channel to nutrients and water. Also the water holding capacity of the contaminated soil was about 31% as given in Table 1 is considered sufficient for plant growth (Singh and Singh 1936; Scherer *et al.* 1996). The measured ORP was positive which indicates that the soil has tendency of undergoing oxidation which is necessary for decomposition of toxicants (Table 2). The initial pH was measured as 7.5 which is favorable for growth of microorganism (Hutchinson *et al.* 2001; Vincent *et al.* 2011; Zhu *et al.* 2018). The electrical conductivity elucidates the transfer of ions and salts across the soil matrix, which also gives information about soil salinity (Carter and Gregorich 2008). The EC between 1 and 2 dS/m (Table 2) indicates that the soil might face difficulty with seed emergence (DIRD, Pune Dird 2009). The pH and EC were measured in prior and later basis.

Table 2. Initial characteristics of raw material (Iyer *et al.* 2020).

Soil properties	Values
Coarse sand in wt%	24.59
Medium sand in wt%	45.39
Fine sand in wt%	16.01
Silt in wt%	12.74
Clay in wt%	1.27
Bulk density in kg/l	1.433
pH	7.5
ORP in mV	+135
Electrical conductivity (EC) in dS/m	1.7
TPH %	11.7

The fresh soil in pot B with pH 7.9 had similar texture of finely grained homogenous mixture of sand, silt and high clay as determined from sieve analysis analogous to raw material. Fertilizer boosts biochemical activities with additional source of nutrients and substrate to improve hydrocarbon degradation (Lin *et al.* 2020; Kumaria *et al.* 2021). Fertilization was done with corn silk as it contains phosphorus in abundance and other nutrients vitamins, minerals, proteins (Kumar and Jhariya 2013; Rahman and Wan Rosli 2014) and mixed with cow dung to provide nutrient matrix to the soil. Cow dung was used here as fertilizer due to its effective source of enhancement and development of microbial growth for hydrocarbon degradation since it contains carbon–nitrogen compounds that provides sufficient nitrogen to carbon source in soil matrix (Obire *et al.* 2008; Nduka *et al.* 2012; Adeleke *et al.* 2016). Corn silk is an excellent source of steroids, alkaloids, volatile oils, natural antioxidants, flavonoids, etc. Its flat structure with huge hollowness provides good buoyancy, low density, easily available and biodegradable, hence, useful as natural sorbent for removal of oil contamination. A recent study showed that acetylation of corn silk improved the adsorption efficiency by more than 50% (Asadpour *et al.* 2015, 2019). Pot B showed first baby shoot after 5 days whereas pot A had no growth till the first week which gives an idea that the germination in pot A was slow initially. This might be due to hydrophobicity, toxic volatile components, salinity from crude oil provide resistance to germination (Zhu *et al.* 2018). After 8 weeks of observation pot B had shown 38–40 cm height plant growth and pot A was measured with

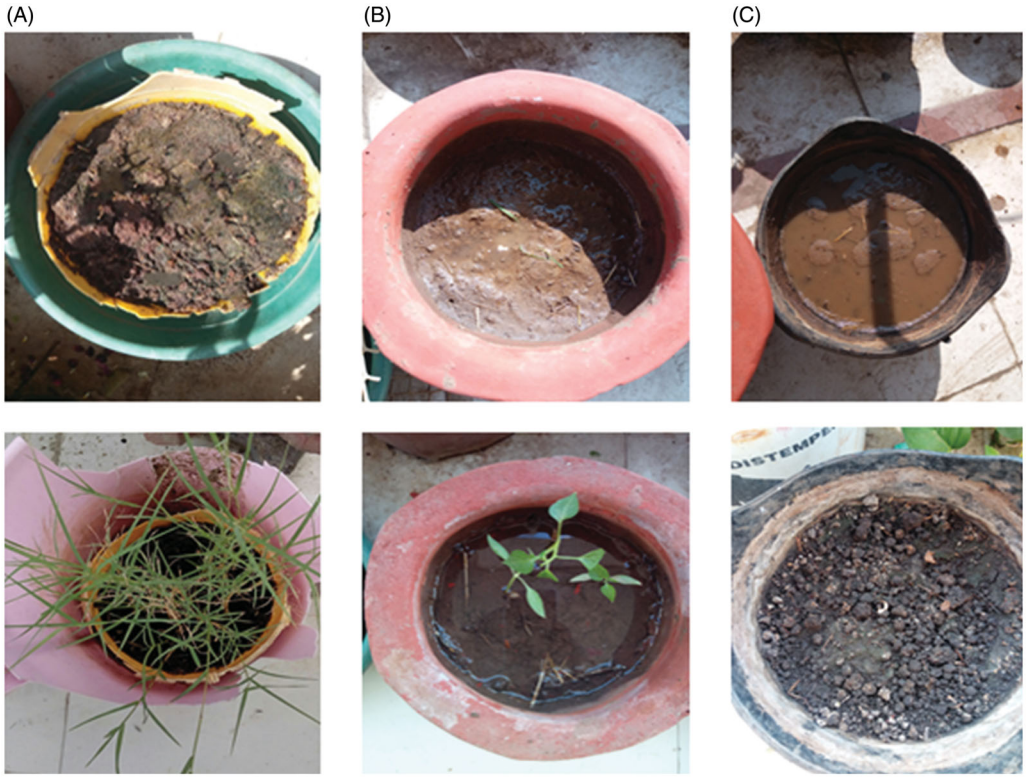

Figure 2. Plant growth in pots A, B and C. Pots appear from initial stage before growth of plant (above); pots show growth of plant after 60 days (below).

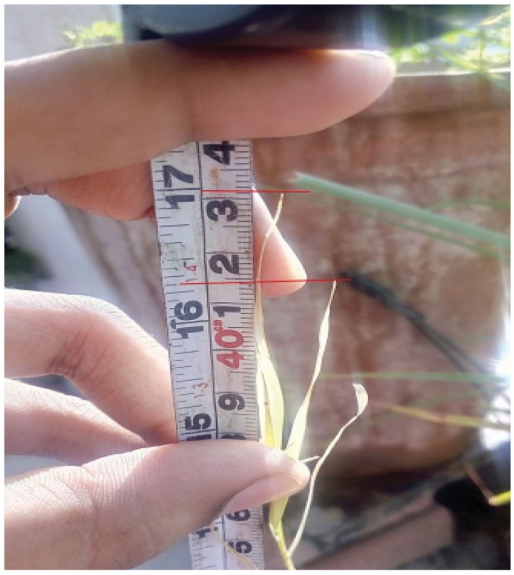

Figure 3. Height of plant grown in pot A after 8 weeks.

40–42.5 cm height of plant growth from top surface layer of soil (Figure 2). There were differences in plant morphology grown in both the pots A and B even though they were fertilized similarly under same environmental conditions as shown in Figure 3 and the growth was natural, no artificial lights were provided at additional hours. Due to oil present in pot A, no other plant except *Cynodon* sp. grew whereas growth of some other plant was observed in pot B containing normal soil. No growth was observed in pot C (control) where nothing was sowed except fertilized and watered daily under the same environmental conditions. After 8–10 weeks small clods as salts appeared on top soil surface in pot A which proves the stabilization of nutrients into salts and formation of other metabolites that must have faced difficulty in translocation as well as penetration into the layer underneath soil due to oil bonded to soil heterogeneously (Basumatary and Bordoloi 2016), such oxidizing reactions in phytochemical processes were elucidated by Sonowal *et al.* (2018) and Correa-García *et al.* (2018). The leaves began to dry early with simultaneous growth of new leaves observed in pot A which shows the essence of phytovolatilization (Ayotamuno and Kogbara 2007).

Crude oil degradation

After 60 days of plant growth in pot A, soil samples were spooned and mixed homogenously from top soil, underneath top soil and near shoot section for TPH estimation. No estimation was done for pot B as it contained fresh soil without contamination. The initial TPH in pot A was estimated to be 11.7% which was reduced to 6% after 60 days, 4.2% after 90 days and 2.087% in 120 days. The reduction in TPH concentration elucidates increase in percentage of degradation estimated as 48.72% in 60 days, 64.10% in 90 days and 82.16% after 120 days. Similarly, Hutchinson *et al.* (2001) showed that *Cynodon dactylon* enhanced TPH degradation by 68% in 1 year. Onwuka *et al.* (2012) has reported higher results showing 56–86% TPH degradation using *Cynodon dactylon* in four batch studies. Razmjoo and Adavi (2012) experimented with ten cultivars of bermuda grass which showed 38–45% remediation in six months. Basumatary and Bordoloi (2016) reported crude oil degradation up to 38.2–46.7% by *C. dactylon* in 180 days upon soil from Assam, India, which differs by 40% compared to current work based on soil from Gujarat, India. Observations from Nguemté et al. (2018) manifest phytoremediation by *E. indica, C. dactylon* and *A. sessilis* in which *C. dactylon* gives 80% contamination removal by involving only with rhizodegradation. Lin *et al.* (2020) studied the inoculation of *Cynodon D.* species with *Sporobascillus* sp., *prevotella* sp. and *Clostridium* sp. have increased the removal rate of diesel by 16%. The pot C – control was analyzed for initial and final measurement of TPH after 120 days. Sampling was done from pot C in triplicates. After 120 days of observation pot C showed no plant growth with reduction in TPH concentration from 11.70% to 4.60% which gives 60.68% degradation. The provided nutrients in control did not render plant growth but was responsible for developing indigenous microorganisms which lead to natural attenuation of crude oil (Ikhajiagbe *et al.* 2017). The resulting degradation about 60% in pot C was due to microbial remediation by microflora which might have scaled up by available nutrients from fertilizer (Obire *et al.* 2008; Nduka *et al.* 2012). No degradation was observed in bottom most layers in control. This could be due to less availability of oxygen at deeper part may prompt less growth of organisms compare to upper layer. The toxicity raised from slow degradation inhibits new microorganisms to develop (Saraeian *et al.* 2018). The difference of percentage oil degradation between pot A and pot C was about 22% which could be the contribution of *Cynodon* sp. plant as in these two pots all other conditions were similar except *Cynodon* sp. growth in pot A and not in pot C.

Kinetics of crude oil degradation

Mineral composition in nutrients harness plant growth supporting better degradation rates (Shahin and Salem 2018). Based on the estimated data of TPH% reduction in soil at every 30 days, degradation rate was calculated based on integral method; $TPH_C = TPH_0 e^{kt}$ and polynomial method (by polynomial curve fitting) using Excel software (Abbassi and Shquirat 2008; Yudono *et al.* 2010). Logarithm of TPH_C/TPH_0 was plotted against intervals in days to calculate rate constant $k = 0.0138\ day^{-1}$ assuming order $n = 1$ according to integral method, resulting in linear plot with $R^2 = 0.9654$. This shows that the rate of phytodegradation obeys first-order kinetics with an affirmation to Vincent *et al.* (2011). Now with the same data, the reduction in TPH concentration over time curve was fitted using the second-order polynomial function and differentiated with respect to time (t) which gives new function for rate of degradation (dC_{TPH}/dt). Using this new function, linear representation with $R^2 = 0.99$ was deduced from the logarithmic plot of $\ln(dC_{TPH}/dt)$ versus ln(TPH) concentration estimating rate constant $k = 0.0022\ g^{(1-n)}\ day^{-1}$ and order $n = 0.38$. The

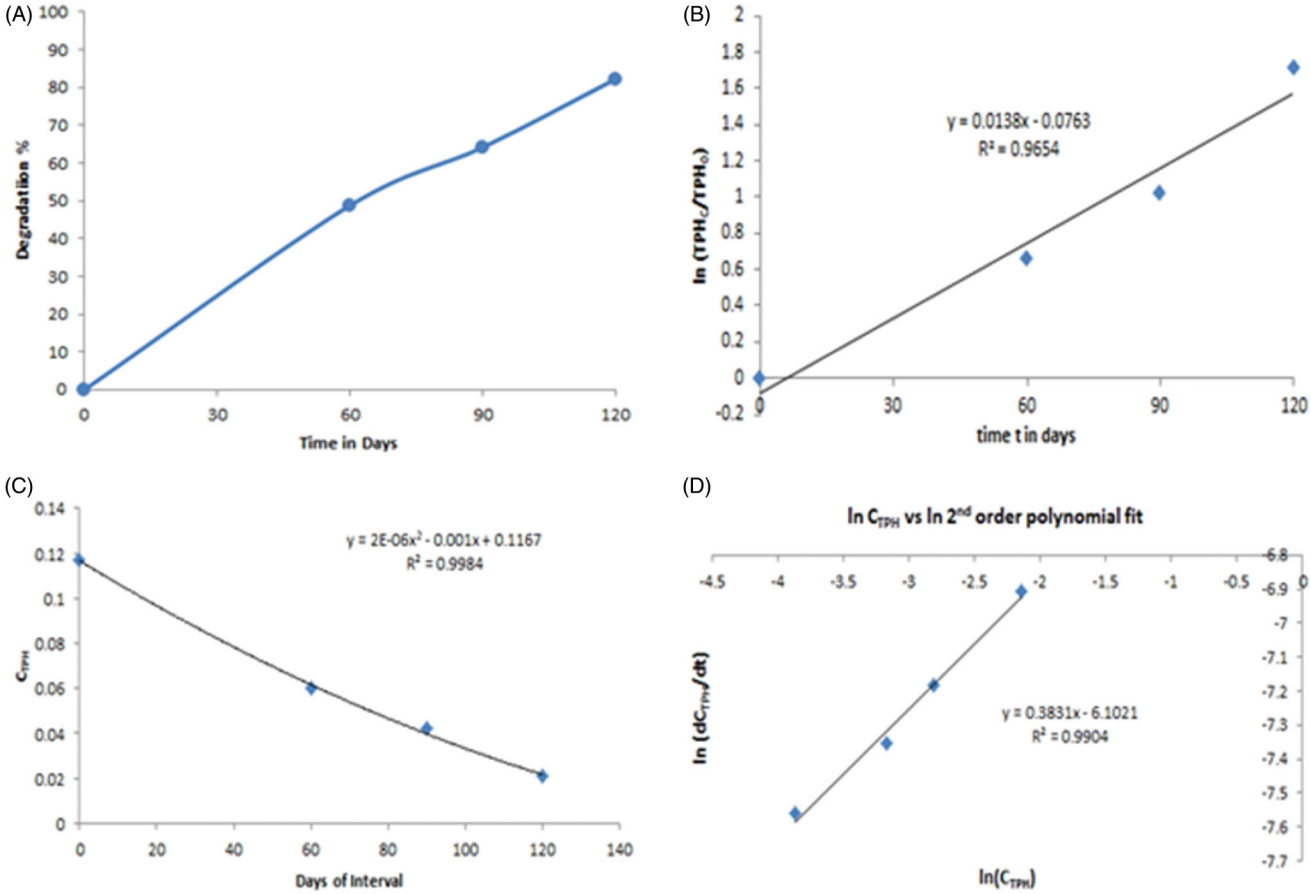

Figure 4. Graphical representation of TPH degradation in pot A; (a) degradation plotted against time *t* intervals in days; (b) Plot of degradation rate using integral method assuming first order kinetics; (c) Plot of reduction in TPH concentration over time (days) is shown with second order polynomial curve fitting; (d) Plot of degradation rate using polynomial method - logarithmic rate of degradation against logarithmic TPH concentration.

determination of order and rate constant using polynomial method differ from integral method due to the shift in existing data points causing formation of new data points from curve fitting. With very minimal difference in rate constant *k* from integral and polynomial methods followed the order of reaction from zero to one. Since the polynomial method does not state complete zero order, the generated results can be seen as pseudo-first order to first-order kinetics (Karamalidis *et al.* 2010; Vincent *et al.* 2011), refer Figure 4.

Bioremediation via rhizomicrobes

Plants employ rhizosphere microorganisms for decomposition of carbon substrates into production of organic metabolites, water and carbon dioxide (Peña-Castro *et al.* 2006). As specific plant roots exudates provide useful conditions for microbial scale-up, colonization and to utilize the contaminant as substrate makes rhizosphere (area near roots) rich in microbes that effectively decompose the substrate secreting necessary enzymes (Correa-García *et al.* 2018; Fatima *et al.* 2018; Ite and Ibok 2019; Kumaria *et al.* 2021; Wang *et al.* 2021). After 90 days of plant growth in pots, an additional new batch of contaminated soil was prepared to observe bioremediation by microorganisms from the rhizosphere of *Cynodon* sp. plant as shown in Figure 1. This batch was recorded to contain 7.5 pH. The TPH was determined using solvent extraction with only one interval having 30 days because of the stipulated time planned for this research. The estimation of reduction in TPH concentration from 11.70% to 7.40% after 30 days, gives 36.75% degradation in TPH concentration within 30 days. This degradation was due to only microbial activity inoculated from pot A. The applied inoculum was well acquainted with oil contamination as it was obtained from pot A. The fact that the microorganisms were adapted to oil contamination, also the available surface area was more and depth was less in petri plate compared to pot which could be the reason for much faster degradation of oil. These results encourage toward further studies in achieving better and faster *in situ* oil degradation.

After 120 days of observation in all pots, the pH recorded in pots A, B and C were 8.0, 8.8 and 7.0, respectively, whereas in new batch, no significant change in pH was observed after 30 days. The pH did not significantly change in control either while pH in pot A with grown plant shifted slightly toward alkalinity i.e. from 7.5 to 8.0. This observed shift might be because of alkaline intermediates that settled upon the soil surface. EC recorded as 0.26 dS/m in pot A in the end manifests the improvement in soil susceptibility to contain microbial environment with good seed emergence. Similar EC as 0.235 dS/m was reported by Zhu *et al.* (2018) but for fresh soil prior spiking with hydrocarbons.

Table 3. GCMS/MS analysis of crude oil contaminated soil sample for control (pot C) and treated (pot A) with grown plant.

			Control			Treated		
Compound	Molecular ion, *m/z*	Fragment ion, *m/z*	Abundance	Retention time	Peak area	Abundance	Retention time	Peak area
Naphthalene	128.1	78.1; 102.1	2,566.66	6.907	7,495	2,916.09	6.905	5,738
Fluorene	166.1	164.01; 165.09	451.01	12.268	37,198	178.22	11.569	3,345
Acenaphthene	152.1	127; 126	259.29	12.274	10,716	137.26	12.269	500
Acenaphthylene	152.1	102.1; 126	1,684.25	12.278	13,720	87.77	12.27	526
Phenanthrene	178.1	152.1; 150.1	750.14	18.501	60,152	147.33	14.376	3,862
Anthracene	178.1	151.09 ; 152.1	1,605.52	18.503	83,157	344.29	14.379	3,869
Fluranthene	201.1	200.1; 152.1	408.94	25.209	12,566	130.36	24.42	195
Benz(a)anthracene	114	226.1; 101.1	29,134.44	29.002	56,383	8,618.54	28.975	12,496
Phyrene	201.1	200; 174	215	29.002	1,002	78.1	28.974	168
Chrysene	113.1	112.1; 226.1	4,305.61	29.003	14,810	1,183.95	28.975	1,611
Benzo(b)fluranthene	126	113.1; 250.1	5,369.21	31.615	23,594	1,617.38	31.765	5,973
Benzo(a)pyrene	125	124.1; 250.1	5,293.97	31.618	33,637	3,069.63	31.586	7,677
Indenopyrene (1,2,3)	137	136; 137.09	8,928.63	35.423	25,520	4,549.29	34.207	10,086
Benzo(g,h,i)pyrelene	138	136; 137	9,488.53	35.423	28,596	4,180.33	35.399	11,476
Dibenzo(a,h)anthracene	125	276.1; 124.1	4,212.69	35.422	12,878	1,664.42	35.4	5,545

Naphthalene degradation

Homogenized samples were collected from pot A (treated) and C (control) to perform GC-MS-MS (tandem mass spectroscopy) before and after treatment for the identification and quantification of PAH in received crude oil contaminated soil. Triple quadrupole (QqQ) acquisition, MS detector type with MRM mode was used for mass analysis. MRM enhances the detection limit across interferences and EI was applied at second quadrupole to ionize parent ions (precursor ions) to maximize the vision of detection (Vekey 2001). Relative response factor with five point calibration was plot for the analytes of interest with standard PAH solution to obtain necessary data about analyte concentration (McDonald *et al.* 2000). The following compounds in PAHs were identified as given in Table 3 where higher carbon chain compounds were present in lower detection limits. With MRM + EI scan cycles 95–250 were applied to focus C_2 aromatic hydrocarbon for observing degradation effect on the particular. Naphthalene (in given scan) was identified as major contamination present in the given soil sample with peak area 6871 and retention time 6.868 before treatment. The analysis after treatment shows identification of naphthalene present in soil samples with no change in TPH concentration of control and 17.19% degradation in TPH concentration of treated from obtained GCMS reports. Whereas the calculated naphthalene degradation percentage in treated soil and control soil were 16.50% and 9.08%, respectively, this was deduced using the received data from reports including peak area and relative abundance given in Table 3. The difference in results of laboratory chemical extraction technique and GCMS analysis is due to the variation in sample preparation. Determination of PAH present in harbor sediments and their potential effects and interferences explored by Dong *et al.* (2012) elucidates identification of PAH contamination and their treatment as reported in literature. Similarly, Balachandran *et al.* (2012) reported the effectiveness of *Streptomyces* sp. isolate ERI-CPDA-1 on naphthalene, anthracene degradation using GC whereas the current work focused on indigenous microorganisms. From the results obtained via tandem mass spectroscopy as mentioned shows that higher degradation of naphthalene present in crude oil contamination took place in pot A-treated soil compared to control although rate of degradation was slow.

Plant identification

The grown plant in pot A was identified based on its morphological characteristics. The identification was carried out externally at Department of Botany, Gujarat University, Ahmedabad, Gujarat, India. The identification results concluded that the given plant belongs to *Cynodon* genus in the *Poaceae* family. The identification of its belonging specie was not possible as it bore no flower within the stipulated time of this research.

Conclusion

Conducted pot studies showed that pot A with grown plant was able to degrade 82.16% crude oil from soil after 120 days of phyto-treatment. The estimated degradation in pot C – control after 120 days was 60.68% with the presence of indigenous microorganisms but without plant growth. Under same environment, pot B was observed to grow different plant compared to pot A. After 90 days of observation in pots, the inoculated new batch of contaminated soil showed 36.75% degradation in 30 days. Hence, the difference in degradation from pots A, C and new batch manifests 22–30% effective degradation by *Cynodon* sp. Determination of degradation rate using integral and polynomial method shows first-order kinetics with estimated rate constants k as 0.0138 and 0.0022 day^{-1} respectively. GS-MS-MS confirms the presence of naphthalene in higer concentration and its eventual degradation. The grown plant was confirmed as *Cynodon* sp. after identification. EC of the contaminated soil sample decreased to 0.26 dS/m from 1.7 dS/m after remediation and pH turned slightly alkaline indicating improved seed emergence. Facts from the observations conclude that phytoremediation using *Cynodon* sp. is an efficient and environment friendly option for land reclamation contaminated with crude oil.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The GCMS reports and RRF calibration for PAHs data are available at Figshare data repository under this link - https://doi.org/10.6084/m9.figshare.14919465.v1. The supporting calculations are available as Supplementary Files.

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