Diesel degrading bacterial endophytes with plant growth promoting potential isolated from a petroleum storage facility

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Abstract

Thirteen (13) endophytic bacterial strains were isolated from *Echinochloa crus-galli* (Cockspur grass) and *Cynodon dactylon* (Bermuda grass) growing in an oil-contaminated site at a petroleum storage and transportation facility. Of the 13 strains assessed for their potential to degrade monoaromatic compounds (phenol, toluene, and xylene) and diesel and for their plant growth promoting (PGP) ability (phosphate solubilization, siderophores and 1-aminocyclopropane-1-carboxylate (ACC) deaminase production), isolate J10 (identified as *Pseudomonas* sp. by 16S rRNA gene sequencing) was found to be the best diesel biodegrader with the best PGP traits. The Monod model used for *Pseudomonas* sp. J10 growth kinetics on diesel fuel as the sole carbon source showed that the maximum specific bacterial growth rate was 0.0644 h^{-1} and the half velocity constant (K_s) was estimated as 4570 mg L^{-1} . The overall growth yield coefficient and apparent growth yield were determined to be 0.271 g h^{-1} and $0.127 \text{ g cells/g substrate}$, respectively. *Pseudomonas* sp. J10 removed 69% diesel in four days as determined by gas chromatographic (GC) analysis. These findings could assist in developing an endophyte assisted efficient diesel biodegradation system using *Pseudomonas* sp. J10 isolated from *Echinochloa crus-galli*.

Keywords Endophytic bacteria · Diesel · Phenol · Toluene · Xylene · Phytoremediation

Introduction

Petroleum industry is important economically for developing as well as developed countries. However, it has also some adverse impacts on the environment (Ledezma-Villanueva

et al. 2016; Qi et al. 2017; Iqbal et al. 2018). Petroleum extraction and refining industries produce large volumes of solid and liquid wastes, containing recalcitrant and potentially toxic organic compounds. Large refineries, capable of processing 200,000–500,000 barrels of oil per day can generate $10,000 \text{ m}^3$ of sludge per year (Van Hamme et al. 2003), whereas refining of 1-L crude oil produces approximately 1.6–2.5 L of wastewater (USEPA 1996). The sludge and wastewater from petrochemical industries contain large quantities of toxic hydrocarbons such as benzene, toluene, ethylbenzene and xylene (BTEX), phenols and polyaromatic hydrocarbons (Qi et al. 2017). Owing to their toxicity, mutagenic and carcinogenic potential, these are included in the list of priority pollutants by the United States Environmental Protection Agency (USEPA 2014).

Environmental regulations provide a framework and enforce removal of hazardous pollutants from wastewater prior to their discharge. Development of green treatment technologies has gained popularity in the recent past. Biological treatment of effluents utilizing microorganisms (i.e. bacteria and fungi) for biodegradation of recalcitrant organics has gained significant attention in recent years as

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an eco-friendly and cost-effective approach (Zhang et al. 2014; Zahid et al. 2016; Ledezma-Villanueva et al. 2016; Blain et al. 2017; Pawlik et al. 2017; Iqbal et al. 2018; Wang et al. 2018).

Bacteria dwelling within higher plants are adapted to the specific environment of the host plant (Chen et al. 2017; Sauvtre et al. 2018). Such endophytic strains have been reported to produce chemicals which can help plant growth (Iqbal et al. 2018), resistance to various diseases as well as useful in phytoremediation (Blain et al. 2017). Different processes and mechanisms taking place in root environment provide an ideal condition for hydrocarbon degradation. Extensive root growth towards deeper layers of soil benefits plants in terms of provision of water, exchange of gases and improves biological, physical and chemical properties of soil. Roots enhance the bioavailability of organic contaminants by reducing surface area and volume of soil micro-pores. This, in turn, enhances bacterial population, diversity and efficiency resulting in hydrocarbon reduction (Gaskin and Bentham 2010; Zhang et al. 2014; Chen et al. 2017). Various microorganisms naturally produce aromatic compounds (e.g., Quinones, aromatic amino acids, and phenols) that are structurally similar to aromatic contaminants from the petroleum industry. Therefore, these organisms are believed to have modified pathways to degrade the organics (Ahmad et al. 2014). Survival of these microorganisms at higher contamination levels while retaining adequate degradation potential is essential for their effectiveness of such treatment technology (Iqbal et al. 2018; Wang et al. 2018). Different studies suggest that inoculation of bacterial strains enhances the plant growth, i.e. increased biomass as well as reduced organic contaminant stress on plants (Hayat et al. 2010). In response, plants help the bacterial population to enhance their potential for the biodegradation of organic contaminants by improving soil physicochemical properties (Blain et al. 2017; Pawlik et al. 2017; Baoune et al. 2018).

The current study was designed to isolate native plant endophytes from a petroleum contaminated site, with the hypothesis that these endophytes would better survive in such an environment. Bacterial growth kinetics were quantified in the context of diesel biodegradation. The widely adopted Monod model (Moliterni et al. 2012; Ahmad et al. 2014) was applied to elucidate the growth of microbial cells upon exposure to diesel as a sole source of carbon and energy. The specific objectives of this work were; (1) Isolation and characterization of endophytic bacteria from *Echinochloa crus-galli* and *Cynodon dactylon*, (2) Quantification of degradation of monoaromatic compounds (phenol, toluene and xylene) and diesel by isolated endophytic strains, and (3) Assessment of PGP traits including phosphate solubilization, siderophores production, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme production. Growth kinetics were also studied for an optimized system to

provide information for practical applications of this system in degradation activities.

Methodology

Chemicals

All the chemicals used in experimental analysis were of analytical grade and were purchased from Merck® (Darmstadt, Germany) and Sigma Aldrich Chemical® (St. Louis, MO, USA). Chemical solutions and media for bacterial growth were prepared in ultrapure water obtained from EASYpure II equipped with standard Reservoir Feed/UV Lamp. For standard growth and purification of bacterial strains, nutrient agar media (agar 15.0 g L⁻¹, beef extract 3.0 g L⁻¹, peptone 5.0 g L⁻¹, NaCl 5.0 g L⁻¹, pH 6.8 ± 0.2) were used. During isolation and degradation analysis, autoclaved M9 minimal medium (Na₂HPO₄·7H₂O 64 g L⁻¹, KH₂PO₄ 15 g L⁻¹, NaCl 2.5 g L⁻¹, NH₄Cl 5.0 g L⁻¹, 100 µL of 1M CaCl₂ and 2 mL of 1M MgSO₄) was used. Diesel was bought from Shell, College Station, Texas, USA, and analytical grade phenol, toluene and xylene were used as a sole carbon source in a separate set of experiments.

Sample collection

Plant samples (*Echinochloa crus-galli* and *Cynodon dactylon*) were collected from an oil-contaminated site at a petroleum storage and transportation facility (28°56'50.8"N 95°57'14.0"W) in Bay City near Houston, Texas, USA. All the plant samples (three plants in case of *Echinochloa crus-galli* while, around 1.5 m *Cynodon dactylon* runner) were removed aseptically, locked in zip-lock bags and were placed on ice before transfer to the laboratory for analysis. Fresh plant roots and shoots were immediately washed, and surface sterilized with 70% ethanol. These were then treated with NaClO (1%) for 5 min and washed three times with autoclaved ultrapure water for 60 s. Water from third rinse was spread on nutrient agar plates to confirm surface sterility (Iqbal et al. 2018).

For the isolation of endophytes, 4 g of surface-sterilized plant tissue (shoots or roots) were ground with a sterile pestle and mortar in 12 mL NaCl (0.9%, w/v). The mixture was submitted to constant agitation for 60 min on an orbital shaker at 30 °C, further allowed to settle and diluted from 10⁻¹ to 10⁻⁶. From each dilution, 1 mL was added to a 250 mL Erlenmeyer flask containing 60 mL of M9 minimal medium, 140 mL autoclaved ultrapure water and 1.0% filter-sterilized diesel fuel; shaken at 150 rpm on an orbital shaker at 30 °C for 4 days (Kukla et al. 2014; Ho et al. 2012). After 4 days, 100 µL were taken from the flasks, spread on nutrient agar plates and incubated for 24 h at 28 °C. All

morphologically different bacterial colonies obtained after 24 h were sub-cultured on nutrient agar media and incubated for 24 h at 28 °C for three consecutive days to confirm the purity of the strains. Biochemical assays (growth on MacConkey agar, oxidase, citrate, indole, glucose and catalase tests), Gram staining and light microscopy (to visualize bacterial cells) were performed for initial screening. The colonies thus obtained were screened for their potential to degrade single (phenol, xylene or toluene) as well as mixed (diesel) carbon source.

Screening for bacterial species

For screening experiments, solid media [(0.25 g NH_4Cl , 0.266 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3 g KH_2PO_4 , 11.32 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 15 g Agar) L^{-1}] as well as liquid media (M9 minimal medium) were used. The media were amended with one of the following contaminants: 0.4 mM phenol, 1000 mg L^{-1} xylene, 1000 mg L^{-1} toluene and 0.25% diesel. For solid media, simple agar plates without any additional nutrient amended with each of the test compounds were streaked with all morphologically different endophytic bacterial strains, to determine their survival capability and were incubated at 28 °C for 7 days (Ho et al. 2012). Streaking on simple agar plates without any contaminant served as a control for the bacterial growth.

For liquid media, experimental setup was comprised of M9 minimal medium along with each of the tested endophytic bacterial strain and each contaminant individually. M9 minimal media along with each contaminant but without bacteria served as an abiotic (negative) control while M9 minimal media with each endophytic bacterial strain but no contaminant, was used as a positive control. Bacterial growth was assessed at defined time intervals using a spectrophotometer to measure turbidity/optical density ($\text{OD}_{600\text{nm}}$). To determine bacterial growth over a period of 4 days, 100 μL from each flask (experimental group, negative and positive controls) were spread on nutrient agar plates by plating technique after defined time intervals and incubated for 24 h at 28 °C. All the tests performed during the study were in triplicate.

Screening for plant growth promoting traits

Isolated bacteria were examined for phosphate solubilization (with and without bromophenol blue), siderophore production and ACC deaminase activity. All the tests performed were in triplicate. For phosphate solubilization, Pikovskaya medium having 10 g L^{-1} glucose, 5 g L^{-1} $\text{Ca}_3(\text{PO}_4)_2$, 0.5 g L^{-1} $(\text{NH}_4)_2\text{SO}_4$, 0.2 g L^{-1} NaCl , 0.1 g L^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g L^{-1} KCl , 0.002 g L^{-1} $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 20 g L^{-1} Agar, 0.5 g L^{-1} yeast extract, 0.002 g L^{-1} $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$ was used after adjusting pH to 7.0. The

bacterial isolates were screened for the formation of yellow zones/circles around the colonies after 48 h of incubation confirming the utilization of $\text{Ca}_3(\text{PO}_4)_2$ present in the media. In addition, modified Pikovskaya medium having 2.4 mg mL^{-1} bromophenol blue was also used to improve the formation of yellow zones/circles around the colonies as some bacteria can utilize various types of insoluble inorganic phosphates (Jasim et al. 2014).

For assessing siderophores production, the universal protocol, using chrome azurol S(CAS) and hexa-decyl-trimethyl-ammonium bromide (HDTMA) as indicators devised by Schwyn and Neilands (1987) and explained in detail by Loudon et al. (2011), was used.

For ACC deaminase assay, DF salts (Dworkin and Foster 1958) minimal media were used. The media composition was: glucose: 2 g L^{-1} , KH_2PO_4 : 4 g L^{-1} , Na_2HPO_4 : 6 g L^{-1} , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 0.2 g L^{-1} , $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$: 0.1 g L^{-1} , boric acid (H_3BO_3): 10 $\mu\text{g L}^{-1}$, $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$: 10 $\mu\text{g L}^{-1}$, ZnSO_4 : 70 $\mu\text{g L}^{-1}$, CuSO_4 : 50 $\mu\text{g L}^{-1}$, MoO_3 : 10 $\mu\text{g L}^{-1}$, citric acid: 2 g L^{-1} , gluconic acid: 2 g L^{-1} , agar: 12 g L^{-1} and $(\text{NH}_4)_2\text{SO}_4$: 0.2% (w/v). The bacterial endophytes which showed growth on the Petri plates after 48 h of incubation were considered as ACC deaminase positive (Jasim et al. 2014).

After analyzing PGP traits and survival on diesel, the most promising endophytic bacterial strain J10 was selected and experimented further for investigating the growth kinetics on diesel.

Diesel biodegradation and bacterial growth kinetics

All batch culture experiments were performed in 250 mL Erlenmeyer flasks, containing 60 mL of M9 minimal medium with 140 mL ultrapure autoclaved water at pH 6.5–7.5. To prepare the bacterial inoculum, fresh culture of endophytic bacterial strain J10 was removed from the Petri plates and shaken on a rotary shaker. Pellets obtained were re-suspended in autoclaved distilled water to an approximate value of 3 (OD_{600}) having 10^5 – 10^6 cells/mL. Media containing freshly grown 3% (v/v) endophytic bacterial strain J10 culture were incubated at room temperature (25 ± 1 °C) on an orbital shaker, set at 150 rpm with diesel (0.25%, 0.50%, 0.75% and 1.0%). M9 minimal media with diesel (0.25%) without bacterial inoculation served as abiotic control. M9 minimal media and endophytic bacterial strain without diesel addition served as controls for bacterial growth. After defined time intervals, the spread plate technique was used for viable cells determination (CFU/mL). Diesel degradation was estimated by gas chromatography (GC-FID). All flasks were covered externally with an aluminum foil and flask openings were closed with foam stoppers (Basak et al. 2014).

Monod kinetic model (Eq. 1) was applied to study the bacterial growth kinetics using diesel as a sole source of carbon and energy (Monod 1949).

$$\mu = \mu_{\max} \frac{S}{K_s + S}, \quad (1)$$

where μ = Specific growth rate (h^{-1}) of the endophytic bacterial isolate (strain J10), $\mu_{\max}$ = maximum specific growth rate (h^{-1}) of the endophytic bacteria (strain J10), S = diesel concentration for bacterial growth (mg L^{-1}), K_s = Half velocity constant or saturation constant (mg L^{-1}).

Biomass was calculated using CFU/mL vs. biomass correlation. While ignoring the lag phase where no net increase in the biomass was observed, log “X” vs. “t” curve of the log phase would give the specific growth rate (slope of the curve) of the endophytic bacteria used in the system. A linear regression was used for “1/ μ ” vs. “1/S”. Maximum specific growth rate ($\mu_{\max}$) was calculated from the intercept (1/ $\mu_{\max}$) on the abscissa (Ahmad et al. 2014). Half velocity constant (K_s) was calculated using Eq. 2.

$$K_s = \frac{\mu_{\max} S}{\mu} - S. \quad (2)$$

Overall growth yield coefficient and apparent growth yield were calculated using Eqs 3 and 4, where the subscript f denotes the final values and 0 stands for initial values (Crueger and Crueger 1990)

$$\text{Overall growth yield coefficient} = \frac{X_f - X_0}{t_f - t_0}, \quad (3)$$

$$\text{Apparent growth yield} = \frac{\Delta X}{\Delta S}. \quad (4)$$

The specific diesel degradation rate (V_{diesel}) was calculated from time-series data of cell concentration (X) and residual diesel concentration (C_{diesel}) using Eqs. 5 and 6 (Moliterni et al. 2012; Ahmad et al. 2014):

$$\mu = \frac{1}{X} \frac{dX}{dt}, \quad (5)$$

$$V_{\text{diesel}} = -\frac{1}{X} \frac{dC_{\text{diesel}}}{dt} \bigg|_{t \rightarrow 0}. \quad (6)$$

Extraction and analysis of residual diesel concentration

Extraction of residual diesel oil was carried out using solid phase extraction (SPE) cartridges with a typical solid phase extraction vacuum manifold (Diaz-Ramos et al. 2012). Acetone was used as the extraction solvent. Visiprep solid phase extraction (SPE) vacuum manifold equipped with

HLB cartridges (Waters Oasis, 12 cc) was used for extraction (using manufacturer’s standard protocol). Analytical standards of diesel were prepared. Area response attributed to diesel was determined and integrated for resolved peaks with respect to the retention times for less than ± 3 deviation (for individual peaks). Residual diesel concentration was analyzed with a gas chromatograph equipped with a flame ionization detector (GC-FID, Agilent 6890), using a 30 m capillary column (HP5) with a film thickness of 0.25 μm and nominal diameter of 250 μm . Carrier gas utilized was Helium (He), while the injection volume was 2 μL , and a split injection mode was utilized with split ratio 5:1. For better peak resolution, initial oven temperature was set at 50 $^{\circ}\text{C}$, with hold time of 2 min; ramping up to 320 $^{\circ}\text{C}$ with a total run time of 17.5 min. The injector as well as the detector temperatures were set at 300 $^{\circ}\text{C}$.

Molecular characterization and phylogenetic analysis

The most efficient diesel degrading endophytic bacterial strain (J10) was identified by 16S rRNA sequencing using universal primers by Macrogen®, USA. Genomic DNA isolation followed by PCR amplification was performed using universal primers 27F (5’AGAGTTTGATCMTGGCTCAG3’) (Barns et al. 1999) and 1492R (5’TACGGYTACCTTGTTACGACTT3’) (Lane 1991). PCR products were then purified using X kit (Macrogen®, USA) according to manufacturer’s protocol. Amplicons were then sequenced by Macrogen®, USA using 518F (5’CCAGCAGCCGCGGTAATACG3’) and 800R (5’TACCAGGGTATCTAATCC3’) primers. Sequencing reactions were performed using BigDye® v3.1 (Life Technologies, Applied Biosystems) as per the manufacturer’s protocol. Sequence detection was performed by capillary electrophoresis on a 3730xl Genetic Analyzer (Life Technologies, Applied Biosystems) using a 50 cm array, the Long DNA sequencing module (LongSeq50_POP7) and the KB analysis protocol (KB basecaller) with the default instrument settings. Post-detection, raw signal data was initially processed on the 3730xl Genetic Analyzer computer using Sequencing Analysis v5.3.1 (Life Technologies, Applied Biosystems) from Macrogen®, USA.

BioEdit software was used to assemble the contig sequences of 16S rRNA gene of strain J10. The consensus sequence of 1372 nucleotides obtained was submitted to DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp/>) under the GenBank/EMBL/DBJ accession number of LC440022. The strain J10 was identified using this sequence of 16S rRNA gene on Ez-Taxon Server (<http://eztaxon-e.ezbiocloud.net>). The sequence data compared with other representative 16S rRNA gene sequences of more closely related organisms. The sequence data of closely related validly named type strains were retrieved from the database of the

Table 1 Growth response of endophytic bacterial isolates for single and mixed carbon source using solid media

Plant species:	<i>Cynodon dactylon</i>								<i>Echinochloa crus-galli</i>				
Endophytic bacterial strains:	A1	B2	C3	D4	E5	F6	G7	H8	I9	J10	K11	L12	M13
Agar (control)	–	–	–	–	–	–	–	–	–	–	–	–	–
Diesel (0.25%)	+	–	+	–	+	+	+	–	–	+	+	–	+
Phenol (0.4 mM)	–	–	–	–	–	–	–	–	–	–	–	–	–
Toluene (1000 mg L ⁻¹)	–	–	–	–	–	–	–	–	–	–	–	–	–
Xylene (1000 mg L ⁻¹)	–	–	–	–	–	–	–	–	–	–	–	–	–

All the tests performed were in triplicate

Growth (+), no growth (–)

Table 2 Growth response of endophytic bacterial isolates for single and mixed carbon source using liquid media

Plant species:	<i>Cynodon dactylon</i>								<i>Echinochloa crus-galli</i>				
Endophytic bacterial strains:	A1	B2	C3	D4	E5	F6	G7	H8	I9	J10	K11	L12	M13
Abiotic control	–	–	–	–	–	–	–	–	–	–	–	–	–
Positive control	–	–	–	–	–	–	–	–	–	–	–	–	–
Diesel (0.25%)	+	–	–	–	–	–	–	–	–	+	–	–	+
Phenol (0.4 mM)	–	–	–	–	–	–	–	–	–	–	–	–	–
Toluene (1000 mg L ⁻¹)	–	–	–	–	–	–	–	–	–	–	–	–	–
Xylene (1000 mg L ⁻¹)	–	–	–	–	–	–	–	–	–	–	–	–	–

All the tests performed were in triplicate

Growth (+), no growth (–)

EzTaxon Server and alignment was performed using Clustal W. Gaps and ambiguous data were deleted in the alignment as described previously (Ahmed et al. 2014) and neighbor joining (NJ) phylogenetic tree was constructed using the Kimura 2-parameter model contained in MEGA 7.0 software package (Kumar et al. 2016). Bootstrap analysis was done to access the stability of the relationship by performing 1000 re-sampling for the tree topology.

Results

Screening for bacterial species

After 48 h of incubation on solid media, eight of the 13-isolated endophytic bacterial strains showed significant growth on diesel, but none of the strains grew on agar plates amended with phenol, xylene or toluene (Table 1). There was no growth on agar plates without inoculation (control group). For the liquid media, bacterial growth was observed for 5 consecutive days; after every 2 h using a UV–Visible spectrophotometer to measure optical density at 600 nm. However, no significant growth was observed for phenol, xylene or toluene in any experimental group. On the other hand, three out of 13 bacterial strains (A1, J10 and M13) showed considerable growth on diesel (Table 2). From the findings of the screening experiment, it was deduced that our isolated endophytes were unable to thrive on these

Fig. 1 Screening of three bacterial strains (J10, M13, A1) for growth on diesel using M9 minimal media (error bars represent standard error of the means among three replicates)

compounds as a single carbon source or may be intolerant to these organic compounds. Out of all the tested isolates, eight grew on diesel-amended solid media while only three isolates grew in diesel-amended liquid media (Fig. 1).

Plant growth promoting (PGP) traits

Fresh (unaged) diesel fuel has been observed to be toxic to plants (Cruz et al. 2014). However, it was hypothesized

that native plants can survive such lethal concentrations of diesel in soil in the presence of endophytic bacteria that can potentially reduce the stress by biodegradation into less toxic molecules as well as facilitating plant growth (Wang et al. 2018). Eleven of the 13 isolated bacteria were able to efficiently solubilize phosphate (on Pikovskaya medium and modified Pikovskaya medium), nine had the potential to produce ACC deaminase enzyme while seven could produce siderophores (Table 3). These findings further strengthened our resolve to test our hypothesis that bacterial isolates have potential for diesel biodegradation.

Molecular characterization and phylogenetic analysis

From the 13 bacterial isolates, strain J10 was selected based on the screening process (as the best survivor on diesel and its potential to show PGP traits) for further kinetic studies. When grown on nutrient agar plates for 3 days, colonies were in clear circles with clear edges, about 0.3–0.5 cm (diameter), even and greenish-yellow in appearance.

Gram's staining determined the cells as Gram negative and rod shaped. Cells were oxidase and catalase positive (Table 4). Sequencing of 16S rRNA gene identified the bacterial strain J10 as a *Pseudomonas* sp. (Fig. 2) showing 99.48% sequence similarity with *Pseudomonas aeruginosa*

JCM 5962^T (BAMA01000316). Previous studies by Zhang et al. (2012) also found that three strains of *Pseudomonas aeruginosa* can biodegrade petrochemicals, but their biodegradation capacities were different.

Diesel biodegradation and bacterial growth kinetics

The Monod equation is primarily considered to describe the microbial growth for a specific substrate under optimized conditions (Moliterni et al. 2012). The rate and degree of diesel biodegradation were estimated using peak area from gas chromatograms for residual diesel concentrations. Linear correlation was observed between bacterial growth and diesel degradation, suggesting that bacteria effectively utilized diesel as their carbon source to increase biomass. K_s was calculated as 4570 mg L⁻¹, overall growth yield coefficient and apparent growth yield were estimated to be 0.271 g h⁻¹ and 0.127 g cells/g substrate, respectively.

Bacterial specific growth (μ) for initial diesel concentrations of 0.25%, 0.50%, 0.75% and 1.0% (v/v) were found as 0.0376, 0.0543, 0.0657 and 0.0665 h⁻¹, respectively. The Monod model efficiently represented the bacterial efficiency for diesel biodegradation. Bacterial specific growth rate was comparatively less for 0.25% as compared to other concentrations used (0.50%, 0.75% and 1.0% diesel). However, it reached to nearly constant values for 0.75% and

Table 3 Plant growth promoting (PGP) traits of isolated endophytic bacteria

Plant species:	<i>Cynodon dactylon</i>								<i>Echinochloa crus-galli</i>				
Endophytic bacterial strains:	A1	B2	C3	D4	E5	F6	G7	H8	I9	J10	K11	L12	M13
ACC* deaminase assay	+	–	+	+	+	–	–	+	+	+	–	+	+
Phosphate solubilization with BP**	+	+	+	+	–	+	–	+	+	+	+	+	+
Phosphate solubilization without BP	+	+	+	+	–	+	–	+	+	+	+	+	+
Siderophore production	+	–	–	+	+	–	–	+	+	+	–	–	+

All the tests performed were in triplicate

Growth (+), no growth (–)

*1-Aminocyclopropane-1-carboxylate (ACC) deaminase enzyme

**Bromophenol blue

Table 4 Biochemical characteristics of all isolated endophytic bacteria

Plant species:	<i>Cynodon dactylon</i>								<i>Echinochloa crus-galli</i>				
Endophytic bacterial strains:	A1	B2	C3	D4	E5	F6	G7	H8	I9	J10	K11	L12	M13
Gram stain	+	+	+	–	–	–	–	–	–	–	–	–	–
McConkey agar growth	+	+	–	–	+	–	–	–	+	+	–	+	+
Oxidase test	–	–	–	–	–	+	+	–	+	+	+	+	–
Citrate test	+	+	–	+	+	+	+	–	+	+	+	–	+
Indole test	–	–	+	+	–	–	–	+	+	–	–	–	+
Glucose test	+	–	+	–	+	+	–	+	–	–	+	–	+
Catalase test	+	+	+	–	–	–	–	–	–	+	–	+	+

All the tests performed were in triplicate

Positive (+), negative (–)

Fig. 2 Neighbour-joining phylogenetic tree showing inter-relationship of the strain *Pseudomonas* sp. J10 with the most closely related type species inferred from sequences of 16S rRNA gene. Data with gaps were removed during alignment for the construction of tree, which is generated using the MEGA (version 7.0) software package Kumar et al. (2016) based on a comparison of approximately 1308 nts. Bootstrap values (only > 50% are shown), expressed as a percentage of 1000 replications, are given at the branching points. The sequence of Bar, 0.5% sequence divergence. The accession number of each type strain is shown in parentheses

Fig. 3 Strain J10 growth kinetics at various initial concentrations of diesel under optimized conditions. (Error bars represent standard errors of the means among three replicates. Maximum specific growth rate ($\mu_{\max}$) was calculated to be 0.0644 h⁻¹)

1.0% diesel concentrations (Fig. 3). Analogous behavior was observed in other biodegradation experiments reported in literature (Paslawski et al. 2009; Moliterni et al. 2012) suggesting improved tolerance of strain J10 (*Pseudomonas* sp.) over a wide range of initial diesel concentration. The stabilization of bacterial growth rate at

higher concentration, i.e. 1.0% (v/v) could be attributed to interaction of cell membrane proteins with the contaminant that may result in protein malfunction as suggested by Moliterni et al. (2012).

During the lag phase (from 0 to 20 h) for 1.0% diesel, no significant increase in biomass was observed as the lag phase could be attributed to the period of acclimatization of the endophytic bacterial strain (*Pseudomonas* sp.) and induction of enzymes for substrate (diesel) consumption. The bacterial growth rate accelerated and reached up to a maximum value of 0.0665 h⁻¹ during 22–36 h with substrate (diesel) utilization rate of 0.0289 h⁻¹ (Fig. 4). During this time period, bacterial biomass increased exponentially, being independent of nutrients and substrate (diesel) concentration (Yates and Smotzer 2007) with cell doubling time of 10.42 h. The deceleration phase lasted from 36 to 40 h where microbial biomass started decreasing, that could be attributed to low nutrient levels, addition of toxic growth by-products, or unstable growth pattern and metabolism shift for survival (Yates and Smotzer 2007; Ledezma-Villanueva et al. 2016).

Deceleration phase was followed by stationary phase, which lasted from 40 to 46 h where no net increase in cell biomass was observed. Death phase started after 46 h and followed first order kinetics for rate of cell decline (Yates and Smotzer 2007; Moliterni et al. 2012). These results were in good agreement with the estimated understanding of the Monod equation that biomass production is directly

Fig. 4 Growth of strain J10 in CFU mL⁻¹ at 1.0% diesel concentration (circles). Diamonds represent the control group inoculated with strain J10 but without any carbon source. Vertical lines from 22 to

36 h time period present the log phase (error bars represent standard errors of the means among three replicates)

proportional to substrate (diesel) utilization as depicted by gas chromatograms (Fig. 5).

Biodegradation of diesel was investigated over a period of 4 days. Samples were drawn after regular intervals for extraction followed by gas chromatographic analysis of residual diesel concentration during the period of incubation with *Pseudomonas* sp. strain J10. It was found that our endophytic bacterial strain was an efficient diesel degrader with about 69% degradation during the experimental period (Fig. 5).

During the log phase, diesel degradation rate was optimum. Gas chromatograms of the samples exhibited reduction in the area of major peaks of main hydrocarbons with time. GC analysis revealed the potential of our endophytic bacterial strain J10 to efficiently biodegrade main constituents of diesel, which was not significant in the abiotic control group. The diminutive reduction in the peak area of the abiotic control group can be attributed to little volatilization of total petroleum hydrocarbon (TPH) (less than 4.3%). Figure 6 shows the distribution characteristics of *n*-alkanes in the experimental group. Overall, there was a reduction in the relative content of the *n*-alkanes. Reduction in major hydrocarbon peaks for diesel degradation was observed by Ahmad et al. (2014) upon exposure to *Burkholderia* sp. DRY 27. Growth kinetics of *Burkholderia* sp. DRY 27 on diesel was best explained by Haldane model in comparison to the Monod or Luong models with R^2 value of 0.99 (Ahmad et al. 2014).

Discussion

None of the endophytic bacterial strains was capable of consuming every hydrocarbon as a sole source of carbon and energy as depicted by their selective growth behaviors. Hydrocarbons have various extents for biodegradation pertaining to the microbial enzymatic machinery involved (Lumactud et al. 2016; Sharma et al. 2018). Microbes are specific in utilization of hydrocarbons owing to their tendency to produce various enzymes to degrade these into even smaller molecules (Peng et al. 2015).

Baek et al. (2004) investigated the phytotoxic effects of crude oil (10,000 mg L⁻¹) on *Zea mays* and *Phaseolus nipoensis* (OWH1) and, reported reduced growth rate in the exposed plants. Similarly, Chaîneau et al. (1997) studied the phytotoxic effects of fuel oil (3000–12,000 mg L⁻¹) on *Helianthus annuus*, *Zea mays*, *Lactuca sativa*, *Phaseolus vulgaris*, *Triticum* sp. and *Trifolium* sp. They found stunted plant growth and inhibitory effects on seed germination. Conversely, common weeds and herbaceous plants are often found growing abundantly near oil wells and spill sites (Lumactud et al. 2016; Pawlik et al. 2017) with TPH concentration up to 30,000 mg L⁻¹ (Liu et al. 2012).

Zhang et al. (2014) isolated bacterial endophytes (*Pseudomonas* sp. J4AJ and *Bacillus subtilis* U-3) from *Scirpus triquetra* from a wetland, estuary of Huangpu-Yangtze River, China. Utilization of both the strains for diesel

Fig. 5 GC-FID profiles of abiotic control as well as initial and residual diesel concentration from the experimental group at specific time intervals corresponding to significant bacterial growth pattern with 69% diesel degradation in 96 h

Fig. 6 Distribution characteristics of *n*-alkanes at 1.0% diesel (v/v) concentration at specific time intervals

degradation showed promising results even under extreme pH, temperature and salinity conditions, suggesting these endophytes suitable for large scale in situ applications. Gas chromatographic analysis showed visible decrease in *n*-alkanes (decrease in major peaks) as discussed by Zhang et al. (2016). They used three different *Bacillus* strains for enhanced oil recovery. Relative variations in the total number and peak area of gasifiable *n*-alkanes showed a strong ability of these strains to convert diesel into lighter fractions (Zhang et al. 2016).

In the current study, it was found that the specific growth rate of strain J10 (*Pseudomonas* sp.) corresponded well with the substrate (diesel) degradation rate. The Monod equation effectively correlated mechanism for microbial growth under diesel stress as well as biodegradation linked microbial growth. Bacterial growth along lag, log and stationary phases reflected the complicated nature of the process where various enzymes would have been involved. Furthermore, the gas chromatographic results supported that the study was a significant contribution as compared to previous lab-scale investigation for TPH removal.

The isolated strain *Pseudomonas* sp. J10 degraded the carbon compounds in diesel, and after 96 h, a considerable decrease in the major peaks was observed. Under optimized temperature, pH and salinity conditions, Ahmad et al. (2014) observed reduction in major diesel hydrocarbon peaks upon exposure to *Burkholderia* sp. DRY 27 (about 96.5% degradation) for 7 days. Mohanty and Mukherji (2007) observed reduction in major diesel hydrocarbon peaks by *B. cepacia* by 51.37% after 15 days of exposure. Increase or decrease in the peak area over a period of time suggested the utilization of diesel as a carbon and energy source by our isolated strain (*Pseudomonas* sp.). The peak at C₂₁ was present at the start of the experiment and this again reappeared in the sample taken at 30 h and after that, the peak for C₂₁ was not observed in any of the samples (Fig. 6).

Zhang et al. (2012) studied the ability of three bacterial isolates (*Pseudomonas aeruginosa*) to biodegrade alkanes in crude oil up to *n*-C₄₀. After 7 days treatment, the degradation efficiency ranged between 36% and 46%. The endophytic bacterial isolate strain J10 (*Pseudomonas* sp.) degraded about 69% diesel in 4 days that could be attributed to improved resistance and acclimatization of isolated bacterial associate while within the plant. *P. aeruginosa* RRI was able to grow on alkanes due to the presence of *alkBI* and *alkB2* (alkanes hydroxylase gene), *alkG1* and *alkG2* (rubredoxins gene), and *alkT* (rubredoxin reductase gene) but was unable to consume benzene (C₆H₆), toluene (C₆H₅CH₃), naphthalene (C₁₀H₈), phenanthrene (C₁₄H₁₀) and pyrene (C₁₆H₁₀) (Marin et al. 2003). In another investigation, *P. aeruginosa* CM323 was able to utilize toluene and ethylbenzene but not the benzene and xylene (Cavalva et al. 2000).

The analysis revealed that bacterial growth rate was in good agreement with diesel biodegradation. When our isolated endophytic bacterial strain J10 was further investigated for survival on diesel in plant–microbe system in soil (data not shown here), it effectively enhanced the plant growth under stress conditions and facilitated diesel biodegradation, expressing *alkb* (alkane monooxygenase) gene responsible for hydrocarbon degradation. It is highly desirable to investigate new native bacterial isolates that could efficiently metabolize highly persistent constituents of diesel fuel and to test them for their potential to be utilized for in situ phytoremediation.

Conclusions

The current investigation resulted in the isolation of 13 different endophytic bacterial strains from *Echinochloa crus-galli* and *Cynodon dactylon* plants growing on an oil contaminated site/petroleum storage facility. Out of these 13 bacterial isolates, *Pseudomonas* sp. J10 had the ability to degrade diesel and was capable of phosphate solubilization, siderophores production and ACC deaminase activity. Having high rate of diesel degradation and promising performance, endophytic strain J10 could be effectively utilized in association with different plant species for developing an efficient phytoremediation system.

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Author contributions AI: performed lab and field work, analyzed the data and wrote the manuscript. MA: supervised, advised lab work and data analyses. RK: advised biodegradation experiments, GC analysis and data analyses. TJG: advised lab work, experimental design and data analysis, JR: helped in GC and kinetic analysis, IA: helped in phylogenetic analysis of the strain, APS: supervised field work and data analyses.

Compliance with ethical standards

Conflict of interest Authors declare that there are no competing financial as well as individual interests.

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