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Isolation, genetic identification and optimization of hydrocarbon degrading bacteria from petroleum contaminated soil in Raigad region

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ABSTRACT

In recent years, accumulation of petroleum hydrocarbons in soil and water is a global concern due to its genotoxicity and carcinogenic nature. Bioremediation of petroleum contaminates sites with natural isolates having ability to degrade hydrocarbons, are safe alternative for effective biodegradation. Present study is focused on isolation, characterization and optimization of novel isolates from petroleum contaminated soil with hydrocarbon degrading potential. Thirteen different petroleum contaminated soil sites were sampled from Raigad region to isolate indigenous hydrocarbon degrading bacteria. DCPIP screening assay was performed to select best isolates which utilize hydrocarbon as carbon source. The two best isolates S9D2 and S13D1 were identified as *Rhodococcus ruber* and *Azospirillum zeae* respectively by 16S rRNA gene-based sequencing and Phylogenetic analysis. One factor at a time (OFAT) with a spectrophotometer approach was used to optimize growth parameter. Optimum growth of *Rhodococcus ruber* was observed with 2 % diesel concentration at 37°C temperature and pH 7.0, while *Azospirillum zeae* showed better growth at 1% substrate concentration, pH 6.0. and temperature 30°C. The current study revealed that both the isolates have ability to degrade hydrocarbons present in diesel, and can be used for effective bioremediation tools.

Key words : *Characterization, Petroleum hydrocarbon, Diesel degradation, Bioremediation*

Introduction

In recent years, anthropogenic forces such as incomplete combustion, accidental spills of oil, petroleum derivatives like gasoline, diesel have created major impact on the natural ecosystems. Products obtained from petroleum, plays an important role in transportation and in other industries, among which diesel with a boiling point range from 170 °C -340 °C is massively used as fuel (Fan *et al.,* 2012). Diesel obtained during fractional distillation composed of complex hydrocarbons from C10 to C28. Release of such hydrocarbon components accidently can have carcinogenic and immune-toxicant effect on human, animals and environment (Bekele *et al.,* 2022). Various methods are available for the treatment and clean-up of petroleum contaminated sites like physical, chemical, and biological methods. Physical and chemical techniques do not assure complete restoration of impacted environment. One of the feasible, cost-effective approach for removal of hydrocarbon pollutants is bioremediation. The process of bioremediation can occur in both ways either naturally or by use of two different approaches such as biostimulation or bioaugmentation which generally applies native microbial community (Peixoto *et al.,*

2011). Microbial biodegradation of petroleum hydrocarbons is studied over several years. Various microbial community are ubiquitously distributed in hydrocarbon contaminated soils. These microbes have adapted natural mechanisms for utilizing such organic pollutant as sole source of carbon and converting them into non-toxic products through transformation and biomineralization (Ite *et al.,* 2019). Thus, isolation of such predominant indigenous microorganisms from specific contaminated sites with high degrading ability can act as promising candidate for remediation of contaminated sites (Patowary *et al.,* 2016). Bacterial hydrocarbon degraders like *Arthrobacter, Achromobacter, Brevibacterium, Bacillus, Flavobacterium, Pseudomonas, Rhodococcus* and *Stenotrophomaonas* have been reported to degrade certain types of mono or polyaromatic, aliphatic hydrocarbons (Al-Zahrani *et al.,* 2022). Various factors like temperature, nutrients, substrate, aeration play vital roles and should be maintained in favorable conditions to carry out maximum biodegradation. The ability of degrading petroleum oil depends on the composition and concentration of hydrocarbons (Xu *et al.,* 2018). The purpose of the present study is to isolate and characterize hydrocarbon degrading bacteria obtained from petroleum contaminated soil samples from Raigad district. OFAT method was adopted to optimize the growth factors of bacteria. The present study is a prerequisite for the development of a microbial consortium for effective bioremediation of petroleum contaminated soil.

Materials and Methods

Sample collection

Thirteen different soil samples were collected from a petroleum contaminated area like garages and petrol pumps nearby Raigad district, Maharashtra. The soil samples were stored in a Ziplock bag at room temperature until the further use.

Chemicals and Reagents

The chemicals used in research were of analytical grade and purchased from SRL chemicals private Ltd. India. Bushnell Haas media and other media components were purchased from Hi media private Ltd. India. Diesel used in the research was purchased from authorized petroleum station Bharat petroleum (Kalamboli) Maharashtra, India.

Enrichment and Isolation of hydrocarbon degrading bacteria

Enrichment of hydrocarbon degrading bacteria was carried out in Bushnell-Haas medium (BH). One gram of soil sample was transferred into 250 ml Erlenmeyer flasks consisting 100 ml of sterile BH medium (pH 7.0), supplemented with 1% (v/v) diesel as sole source of carbon. Control flask was maintained without the addition of soil samples. Isolation was done after 7 days of incubation by spread plate technique using dilution (10^{-3} to 10^{-5}) on BH agar plates. Further plates were incubated at 30 °C for 48 hrs. Single discrete colonies obtained on BH medium were picked from the plates and purified on sterile Nutrient agar plates and stored at 4 °C until further use (Mwaura *et al.,* 2018).

Screening hydrocarbon degrading bacteria by DCPIP assay

To check the capacity of hydrocarbon degradation in isolated bacteria, qualitative DCPIP assay was performed. Isolated colonies were inoculated aseptically in 5 ml Nutrient broth media (pH 7.0) incubated at 37 °C for 24 hrs. The media was then centrifuged at 3000 rpm for 10 mins. In 2 ml of BH medium, 100 µl of pre-grown bacterial suspension (O.D. adjusted to 0.8 at 600 nm) was inoculated which was supplemented with 20 µl of diesel as carbon source, and 40 µl of DCPIP dye solution (0.01%/ 100 ml). Control tubes were also maintained without inoculum. All the tubes were incubated at 30°C for 5 days. Decolorization of the blue color of DCPIP dye was monitored on daily basis (Lima *et al.,* 2019) (Habib *et al.,* 2017). Isolates which could decolorize the DCPIP dye in a shorter period of time were selected for further studies.

Molecular identification of bacteria

Molecular identification of selected isolates was done using 16S rRNA gene sequencing at National Collection of Industrial Microorganisms (NCIM), CSIR-NCL, Pune, India. Spin column kit was used for chromosomal DNA extraction. Bacterial 16S rRNA gene (1500 bp) was amplified by PCR in a thermal cycler followed by purification using Exonuclease I -Shrimp Alkaline Phosphatase (Exo-SAP) (Clarridge, 2004) (Darby *et al.,* 2005). Sequencing of purified amplicons was done by the Sanger method using ABI 3500xL genetic analyzer (Life Technologies, USA). Sequencing files (ab1) were edited using

version 1.5 CHROMASLITE. Further analysis was done by BLAST (Basic Local Alignment Search Tool) with the closest culture sequence retrieved from the National Centre for Biotechnology Information (NCBI) database which finds regions of local similarity between sequences (Altschul *et al*., 1990). Functional and evolutionary relationships between sequences was infer using BLAST algorithms which help to identify members of gene families. Phylogenetic tree was generated using Neighbor-Joining method & the evolutionary analyses were conducted in MEGA6 (Tamura *et al.,* 2013).

Optimization of growth condition of hydrocarbon degrading bacteria

One factor at a time (OFAT) method was used to study optimization experiment. The inoculum was built for optimization by inoculating loopful of the culture in 100 ml Nutrient broth media (pH 7.0) and incubated at 37°C for 48 hrs. Further, the cells were harvested by centrifugation at 4000 rpm for 10 mins. The effect of factors like pH, temperature, substrate concentration and inoculum size were evaluated in BH medium (100 ml medium in 250 ml of conical flask incubated at 37 °C with agitation speed 100 rpm). The effect of pH on growth of bacteria was examined across range of pH 5.0 to 8.0. The effect of temperature was evaluated at 30°C, 37°C and 40°C respectively. Substrate concentration range i.e., 0.5%, 1.0 %, 2.0 % and 3.0 % were used to check the growth of isolates. To study the effect of inoculum size, BH medium was supplemented with 1% diesel and were inoculated with different inoculum size such as 0.5 %, 1.0 %, 2.0 % and 3.0 % (Habib *et al*. 2018). Bacterial growth of all the factors was monitored at 600 nm using UV-Visible spectrophotometer (JASCO V-630) for 7 days. All the experiments were carried out in triplicates (Usman *et al*. 2022).

Statistical Analysis

All the experiments were carried out in triplicates and the data was analyzed by using a two-factor analysis of variance (ANOVA) test with significance levels of $p < 0.05$ using Microsoft Excel 2019. The data obtained was in the form of mean ± standard error.

Results and Discussion

Isolation and enrichment of hydrocarbon degrading bacteria

Ten bacterial isolates were isolated from the enrichment of thirteen different soil samples. Based on their origin of soil samples the isolates were designated as S1 to S13 (S1D3, S4D1, S5D1, S6D1, S6D2, S8D1, S8D2, S9D2, S10D1 and S13D1) which were selected on basis of their ability to grow on BH agar plates supplemented with 1% diesel. After isolation the bacterial strains were maintained on nutrient agar slants till further use. Periodically sub-culturing of isolates was performed as shown in Fig. 1 and Fig. 2.

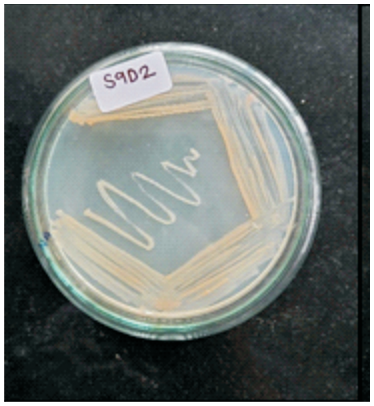

Fig. 1. Isolation of S9D2 on NA plate

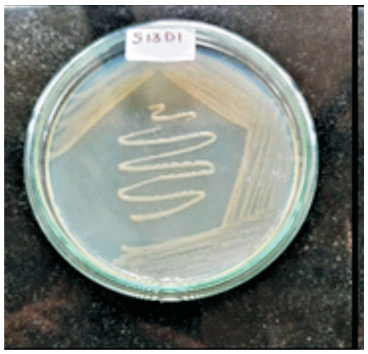

Fig. 2. Isolation of S13D1 on NA plate

Screening hydrocarbon degrading bacteria by DCPIP assay

DCPIP assay is used as pre-investigation method to check hydrocarbon degradation capacities of bacteria. Isolates (S9D2, S10D1 and S13D1) were able to decolourize the DCPIP (2,6-dichlorophenolindophenol) dye. Among three isolates S9D2 and S13D1 were selected. S9D2 was found to decolourize the blue colour within 6 hrs. of incubation period when diesel was used as hydrocarbon source as shown in Fig. 3 and 4. In, DCPIP assay, biodegradation of hydrocarbons is evaluated

by introducing DCPIP as an electron acceptor in the medium. The ability of bacterial isolates to utilize hydrocarbons is analyzed by observing the colour change of DCPIP dye from blue to colorless. Veerapagu *et al*. (2019) reported four bacteria isolates which were screened by spectrophotometric method using DCPIP as indicator. Qualitative analysis was done by Silva *et al*. (2019) for n-hexadecane degradation which evaluated six *Gordonia* isolates which were able to show color change of DCPIP dye demonstrating the abilities of degradation. According to Goveas e*t al*. (2022) findings a novel strain *Lysinibacillus sp. SS1* was able to decolourize DCPIP dye within 36 hrs of period which was used to estimate the petroleum hydrocarbon degrading capacity.

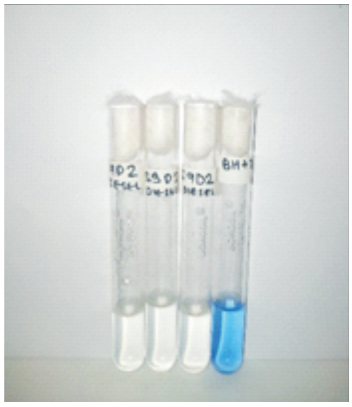

Fig. 3. DCPIP assay of S9D2

Fig. 4. DCPIP assay of S13D1

Molecular identification of bacteria

Two potent isolates with diesel utilizing capacity were selected for molecular identification. Morphological characterization of the isolates showed S9D2 as Gram positive in nature with cocci in shape while S13D1 is Gram negative in nature with short rods morphology. Both the isolates were identified by 16S rRNA gene sequencing in NCIM Pune. The results of NCBI BLAST suggested that S9D2 closely belongs to species *Rhodococcus ruber* with 100% similarity while S13D1 was identified as *Azospirillum zeae* with 98.44% similarity. Accession number was obtained from NCBI (GenBank) as OL477439 for *Rhodococcus ruber* and OL477430 for *Azospirillum zeae*.The phylogenetic tree was generated using Neighbor-Joining method. Kimura 2-parameter method was used to compute the evolutionary distances. The optimal tree with the sum of branch length 0.27064161 was used for both isolates.The Phylogeny of S9D2 shows closest homology with *Rhodococcus* sp. (Closer *ruber)* Fig .5., while for S13D1 shows closest homology with *Azospirillum sp.* (Closer to *zeae*) Fig. 6.

Kumari *et al.* (2016) isolated and identified *Rhodococcus pyridinivorans* and *Psuedomonas stutzeri* and reported higher degradation of TPH in crude oil. Bekele *et al.* (2022) also reported bacteria belonging to genera *Pseudomonas, Bacillus, Providencia, Roseomonas, Achromobacter, and Stenotrophomonas*, among which *Bacillus subtilis* and *Pseudomonas aeruginosa* showed diesel degrading efficiency of 80% and above. *Azospirillum* strains are recently used in degradation of petroleum hydrocarbons. The two most species described and studied in xenobiotics compounds are *Azospirillum lipoferum* and *Azospirillum brasilense* (Cruz-Hernández *et al*., 2022).

Optimization studies

To determine the influence of pH on the growth of *R. ruber* and *A. zeae* isolates, the growth was analyzed on different pH ranges of 5.0, 6.0, 7.0 and 8.0. The optimum pH of for S9D2 was found to be pH 7 (Fig.7). While the optimum pH for growth of S13D1 was at pH 6 respectively (Fig.8). Considering the identified bacteria, the temperature range for optimization was selected as 30°C, 37°C and 40°C respectively. The optimum temperature for S9D2 was recorded as 37°C (Fig. 9.) while S13D1 showed significant better growth at 30 °C. (Fig.10). AlThukair *et al*. (2020) similarly reported best biodegradation of naphthalene by *Rhodococcus qinshengi* at optimum temperature of 37°C.

Mehnaz *et al*. (2007) performed studies on isolation *Azospirillum zeae* species from rhizosphere soil

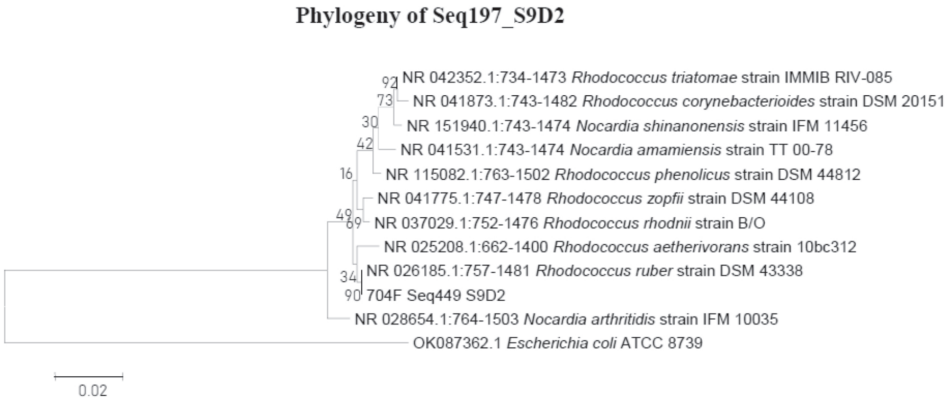

Fig. 5. Phylogenetic analysis of S9D2

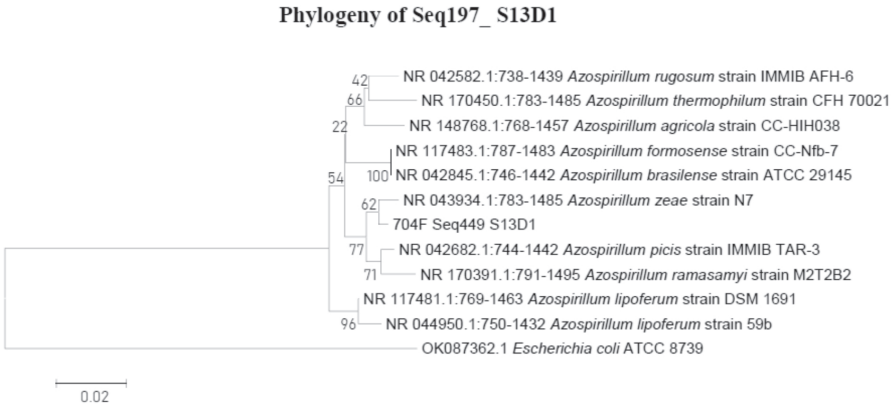

Fig. 6. Phylogenetic analysis of S13D1

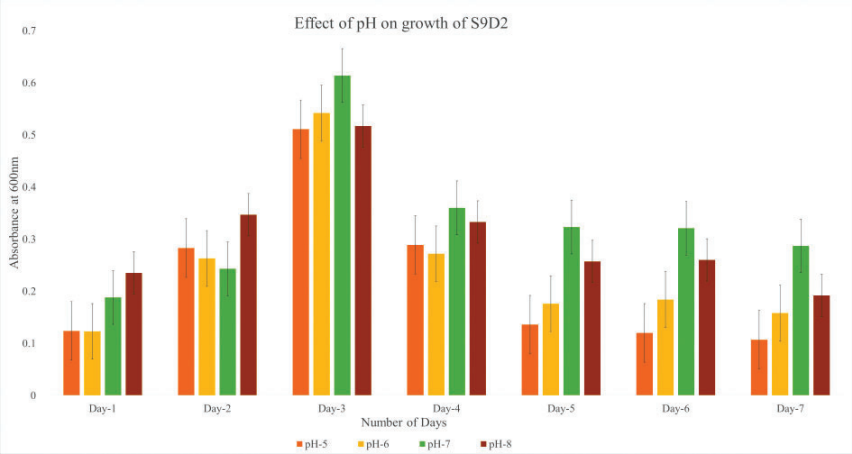

Fig. 7. Effect of pH on growth of S9D2

region of corn and reported favorable growth conditions like temperature of 30 °C with pH range 5.0-7.0. *Azospirillum zeae* (S13D1) isolated in these studies have shown similar growth conditions such as temperature 30 °C and pH of 6.0. in presence of diesel. Significant difference was observed in the microbial growth at various diesel concentrations. S9D2 was seen growing best at 2.0 % substrate concentration. While S13D1 showed the highest growth at 1.0% diesel concentration. Inoculum size was also optimized and found to be 1 % for *R. ruber* and 2 % for *A. zeae*.

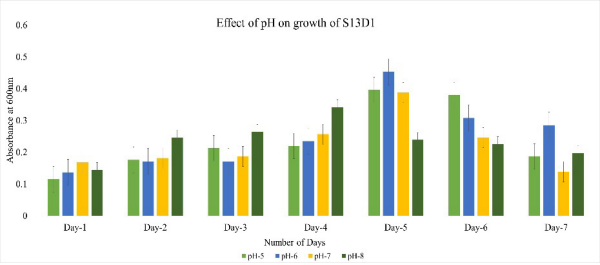

Fig. 8. Effect of pH on growth of S13D1

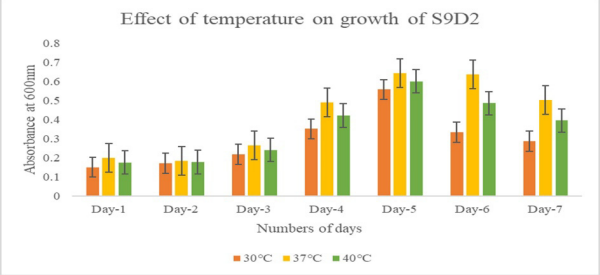

Fig. 9. Effect of temperature on growth of S9D2

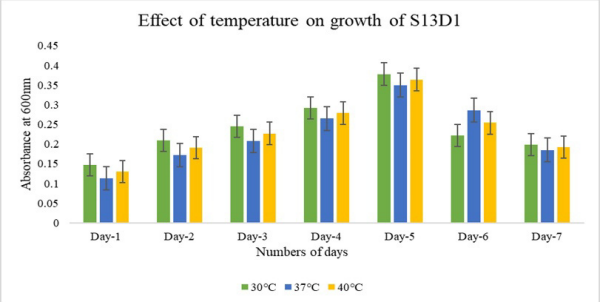

Fig. 10. Effect of temperature on growth of S13D1

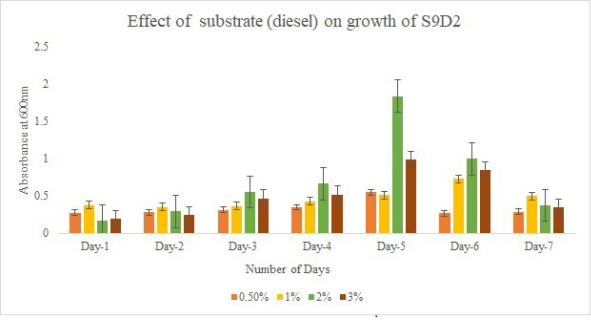

Fig. 11. Effect of substrate concentration (diesel) on growth of S9D2

Our results are in agreement with Makut *et al.* (2022). They carried out similar microbial growth assessment by measuring the optical density for 7 days at 580 nm by using UV-Visible Spectrophotometer. It is observed that in most of the petroleum

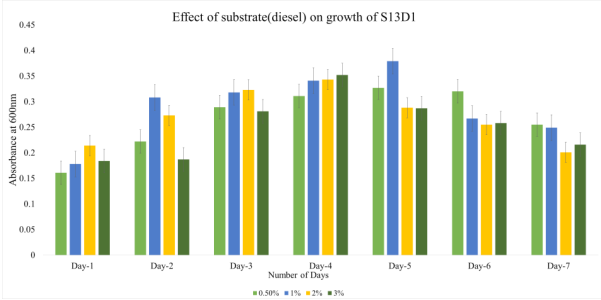

Fig. 12. Effect of substrate concentration (diesel) on growth of S13D1

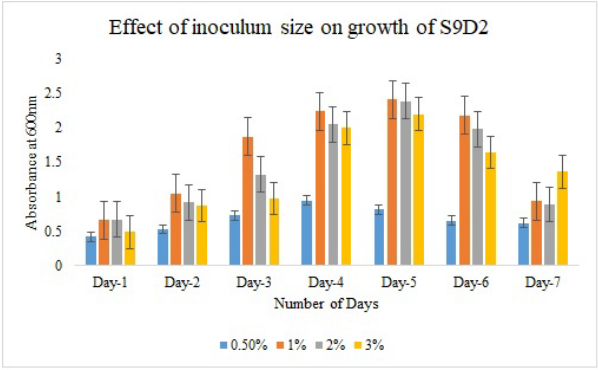

Fig. 13. Effect of inoculum size on growth of S9D2

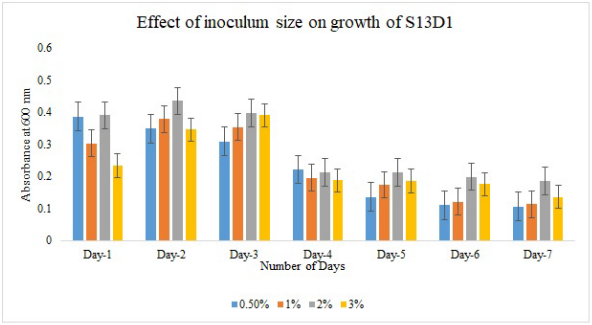

Fig. 14. Effect of inoculum size on growth of S13D1

hydrocarbon degradation research work done the most predominant bacteria isolated and reported belongs to genus *Pseudomonas* and *Bacillus*. This is first report to explore the potential of species *Azospirillum zeae* in diesel hydrocarbons degradation.

Statistical Analysis

Two factor analysis of variance (ANOVA) test was carried out on Microsoft Excel 2019 with significance levels of $p < 0.05$. There was significant differ-

ence in the temperature, pH, substrate and inoculum requirements by both the isolates (supplementary data in Table 1-8).

Conclusion

In this present investigation, two promising efficient hydrocarbons degrading bacterial strains *Rhodococcus ruber and Azospirillum zeae* were isolated from petroleum oil contaminated sites of the Raigad region, Maharashtra, India. The optimization studies were carried using OFAT approach which revealed that *Rhodococcus ruber* can take up maximum diesel concentration up to 2%, in contrast *Azospirillum zeae* showed best growth at 1 %. Most of the work have been reported on other *Azospirillum sp.* for xenobiotics degradation, more work needs to be explored for *Azospirillum zeae*. Furthermore, the DCPIP assay confirmed the ability of both isolates to utilize and degrade the hydrocarbons. In future studies, the consortium of both can be developed for enhanced biodegradation of petroleum hydrocarbons.

Acknowledgements

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Conflict of interest

The authors have no conflict of interest.

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