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RESEARCH ARTICLE OPEN ACCESS

Isolation, Characterization and Optimization of Indigenous Petrol Degrading Bacteria from Oil Contaminated Soil

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Abstract

Petroleum-derived products like petrol, containing complex hydrocarbons, have catastrophic environmental effects, posing a universal challenge. Bioremediation is known as eco-friendly and safe technique for removal of hydrocarbon pollutants. In this study, indigenous bacteria were isolated from oil contaminated soils and screened for their biodegradation potential. The bacterial enrichment and isolation were done using selective media Bushnell-Haas supplemented with petrol as a sole carbon source. Out of fifteen isolated strains best two isolates S4P2 and S14P1 were selected on basis of DCPIP screening method. These isolates were identified as *Pseudomonas chengduensis* & *Pseudomonas donghuensis* by 16S rRNA gene sequencing. The growth parameters were optimized by one factor at a time. The optimum growth temperature for *P. chengduensis* & *P. donghuensis* was found to be 37°C at pH 7 with a substrate concentration of 1%.

Keywords: Enrichment, DCPIP, Optimization, Petrol Hydrocarbons

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INTRODUCTION

Petroleum is used extensively as a source of energy for transportation. The high demand of such energy leads to over-exploitation, and release of hazardous petroleum pollutant into environment.[1] Petrol, also referred to as gasoline, is an important derived liquid products fractions from petroleum used in combustion engines.[2] Anthropogenic activities like petroleum derivatives spills and incomplete combustion of fossil fuels lead to the accumulation of petroleum hydrocarbons in the environment.[3] Organic pollutant such as petroleum hydrocarbons are persistent in soil and water sources have ecotoxicological effects. Thus, there is an urgent need to eliminate this problem from the environment. Numerous methods such as chemical and physical approaches have been investigated for the removal of petroleum hydrocarbon pollutant from the soil. However, due to transportation, cost effectiveness, and excavation of contaminated materials, an alternative green technology such as bioremediation is needed.[4] Bioremediation techniques are considered sustainable, eco-friendly, and cost-effective compared to chemical-physical technologies. The method uses microorganisms that can reduced the toxicity of contaminants by converting them to lesser harmful component through biodegradation, biotransformation, and mineralization.[5] Bioremediation utilizes indigenous versatile microorganisms for the degradation of heavily contaminated sites. The emerging interest in the treatment of hydrocarbon contaminants requires biotechnological improvements such as biostimulation (i.e. addition of nutrients and oxygen) or bioaugmentation (addition of modified strains on site) for enhanced efficacy.[6] Although different hydrocarbonoclastic bacteria have been reported to detoxify organic pollutant, indigenous predominant bacteria like *Pseudomonas, Sphingomonas, Achromobacter Corynebacterium, Flavobacterium, Bacillus* and *Rhodococcus* have been reported to degrade aliphatic and aromatic (mono and poly) hydrocarbons from petroleum.[7] The purpose of the current research was to isolate, molecularly characterized, and optimized potential indigenous petrol utilizing bacteria that thrive in oil-contaminated soil from Raigad region.

MATERIALS AND METHODS

Soil sampling

Fourteen oil contaminated soil samples were obtained from areas of Raigad district, Maharashtra, India. The soil samples (2 to 5 grams) were collected with the help of spatula in Ziplock plastic bags from 5-10 cm depth below the surface (Table 1). The samples were transported back to laboratory of Changu Kana Thakur Arts, Commerce and Science college, New Panvel, and stored in cool and dried place at room temperature.

Chemicals and Media

All the media and chemicals required for study were procured from HiMedia and SRL Private Ltd. India. Petrol for the study was purchased from an authorized Bharat petroleum station in Kalamboli, Maharashtra. Bushnell-Haas (BH) medium composed of 0.02 g/l $CaCl_2$, 1 g/l K_2HPO_4, 1 g/l KH_2PO_4, 0.2 g/l $MgSO_4$, 1 g/l NH_4NO_3, 0.05 g/l $FeCl_3$ (Hi media, pH 7.0 ± 0.2), was suspended (3.27 grams) in 1000 ml distilled water and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The isolates were maintained on nutrient agar composed of peptone 10g/l, NaCl 5g/l, Beef extract 10g/l, Agar 15 g/l (Hi media, 7.3 ± 0.1). For preparation of nutrient agar 28.0 grams of media was suspended in 1000 ml of distilled water and heat until media is dissolved. The desired pH of the medium was adjusted using 0.1 N NaOH and 0.1 N HCL. Sterilization of all medium and glassware's were done in autoclave.

Screening and isolation of petrol degrading bacteria

Enrichment and isolation of petrol degrading bacteria was done using Bushnell-Haas (BH). A 100 ml BH medium was inoculated with one gram of soil sample and supplemented with 1% (v/v) petrol as substrate. The culture medium was incubated for 7 days at ±30°C with 100 rpm agitation. Isolation of bacteria from the enriched cultured medium was carried on BH agar plates containing 1% (v/v) petrol using spread plate technique. Serial dilution using sterile saline with dilution 10^{-1} to 10^{-5} using 0.5 ml of sample of enriched flask was performed. The spreading of

sample was performed from dilution tubes 10^{-3} to 10^{-5}. Single colonies obtained were picked, marked, and purified on nutrient agar plates. Pure cultures were further maintained at 4°C.[8-10]

DCPIP (2, 6-dichlorophenolindophenol) method

The petrol utilizing capacity of the isolates was evaluated by inoculating the isolates individually in nutrient broth media and incubating them at 37°C for 24 h. Cell pellets were obtained after incubation by centrifuging the media at 3000 × g for 10 min. The bacterial pellet was resuspended in sterile saline and adjusted to optical density 0.8 at 600 nm. The assay was performed in triplicate in Eppendorf tubes containing 2 ml of BH medium, 40 µl of petrol, 100 µl of cultural suspension and 40 µl of DCPIP (0.01%) redox indicator dye along with abiotic control devoid of inoculum. The tubes were monitored for decolourization for 5 days.[11]

Bacteria Identification

Taxonomic identification of isolates by 16S rRNA gene sequencing was done at NCIM, CSIR-NCL, Pune, India. 16S rRNA gene (1500 bp) was amplified by PCR followed by purification using Exonuclease I-Shrimp Alkaline Phosphatase (Exo-SAP).[12,13] The Sanger method was employed using an ABI 3500xL genetic analyzer (Life Technologies, USA) for sequencing of the purified amplicons. Further, CHROMASLITE 1.5 version was utilized for editing resulting sequencing files (.ab1). BLAST (Basic Local Alignment Search Tool) from the National Centre for Biotechnology Information (NCBI) database was employed to find local sequence similarity. Obtained sequences were further submitted to NCBI (GenBank) for accession numbers. The Neighbor-Joining method was employed for generation of tree, which showed evolutionary analysis in MEGA6. Position including missing data and gaps were eliminated.[14]

Optimization of culture conditions

To optimize the growth of isolates, four process parameters pH, temperature, inoculum size and substrate concentration were studied using a one-factor-at-a-time (OFAT) approach. Bacterial species were cultivated in 100 ml Nutrient broth (NB) and incubated at 37°C for 48 h. The culture was then centrifuged for 10 min at 4000 × g. The pellet was collected and washed with saline to remove traces of NB broth. The inoculum size used for this study was 1% with an O.D. of 0.8 at 600 nm. All optimization studies were done in triplicates to assure reproducibility.

Effect of pH and Temperature*:* Strains were inoculated in 100 ml of BH medium supplemented with 1% petrol (v/v) as the sole source of carbon. The media was adjusted to different pH values 5.0, 6.0, 7.0, and 8.0. To adjust the desired pH level, 0.1 N of sodium hydroxide and hydrochloric acid solutions were used. All flasks were incubated at 37°C with 100 rpm agitation speed for seven days. The optimum temperature for bacterial growth was determined by incubating the cultured media at temperature 30°C, 37°C and 40°C for seven days. Optical density was measured daily at 600 nm using a UV-Visible spectrophotometer (JASCO V-630).[15]

Effect of various concentration of petrol and inoculum size*:* The influence of different concentrations of petrol (substrate) on the growth of S4P2 and S14P1 isolates was determined using 100 ml of BH medium at pH 7 supplemented with 0.5%, 1.0%, 2.0% and 3.0% of petrol. Optical density was measured at regular interval of 24 h for seven days at 600 nm.[16] To study the effect of inoculum size, BH medium was supplemented with 1% petrol was inoculated with various inoculum size: 0.5%, 1.0%, 2.0% and 3.0%. The flasks were at 37°C for seven days.

Data analysis

The data obtained in triplicates were analysed using a Two way-ANOVA test with a significance level of $p < 0.05$ using Microsoft Excel 2019. The data obtained was presented in as mean ± standard error (n=3).

RESULTS AND DISCUSSION

Screening and isolation of petrol degrading bacteria

Fifteen strains were isolated from oil contaminated soil samples. The isolates were named from S1 to S14 based on number of soil sample collected from different locations, where P represents for petrol and numbers 1,2 are sample's serial number of each isolate (S1P1, S3P1, S4P1, S4P2, S6P1, S6P2, S8P1, S9P1, S10P1,

S10P2, S11P1, S11P2, S12P1, S12P2 and S14P1). The preliminary screening of isolates was done on basis of their ability to grow on selective BH agar plate supplemented with 1% petrol as sole carbon source. The strains were maintained on Nutrient agar plates periodically and stored at 4°C (Figure 1).

DCPIP assay

Petrol degradation was evaluated using DCPIP assay. S4P2, S10P1 and S14P1 isolates were capable to decolorize DCPIP (2, 6-dichlorophenolindophenol). The decolourization of blue colour was observed for 5 days, with isolates achieving DCPIP dye decolorization within a 72 h incubation period. The time required by above *Pseudomonas* sp. to decolourize DCPIP dye was shorter. In the DCPIP assay, microbial oxidation of petroleum resulted in electron transfer to DCPIP dye, changing the color from blue (oxidized) to colorless (reduced). The decolorization of the redox indicator DCPIP is an interpretation of bacterial capabilities of degrading petroleum hydrocarbon.[17] Similar studies have been conducted for *Brevibacillus agri, Brevibacillus formosus,* and *Burkholderia pyrrocinia* achieving up to 69.5% petrol removal efficiency.[9]

Bacterial Identification

In current studies, potential isolates were sent for molecular identification by 16S rRNA gene sequencing at NCIM, CSIR-NCL, Pune, India. According to NCBI BLAST results, S4P2 belongs to *Pseudomonas chengduensis* with 99.75%

Table 1. Sites of soil sample collection

No.	Oil contaminated samples	Source (Location)
1.	S1, S2 and S7	Kamothe repairing garages site
2.	S3	ONGC (Uran)
3.	S4	Petroleum pump (Alibaug)
4.	S5, S6, and S11	Kalamboli, Khanda colony (Panvel)
5.	S8 and S9	Khandeshwar (garages)
6.	S10	Taloja repairing (garage)
7.	S12	Khanda colony (garage)
8.	S13	Nhava Seva
9.	S14	Steel market, Kalamboli

Table 2. Molecular Characterization of bacteria

Isolates	Accession number received from NCBI (GenBank)	Closet phylogenetic relative	Similarity
S4P2	OL477418	*Pseudomonas chengduensis*	790/792 (99.75%)
S14P1	OL477420	*Pseudomonas donghuensis*	696/697 (99.86%)

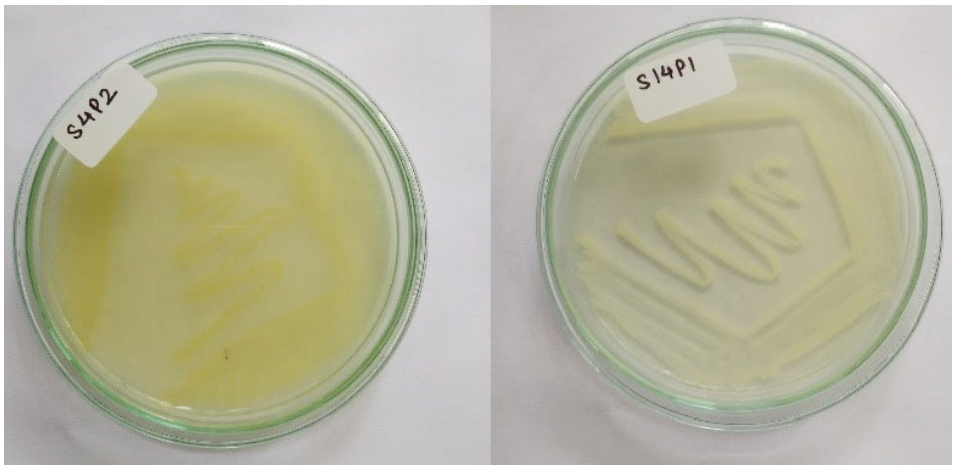

Figure 1. Isolates S4P2 and S14P1 on Nutrient agar plates

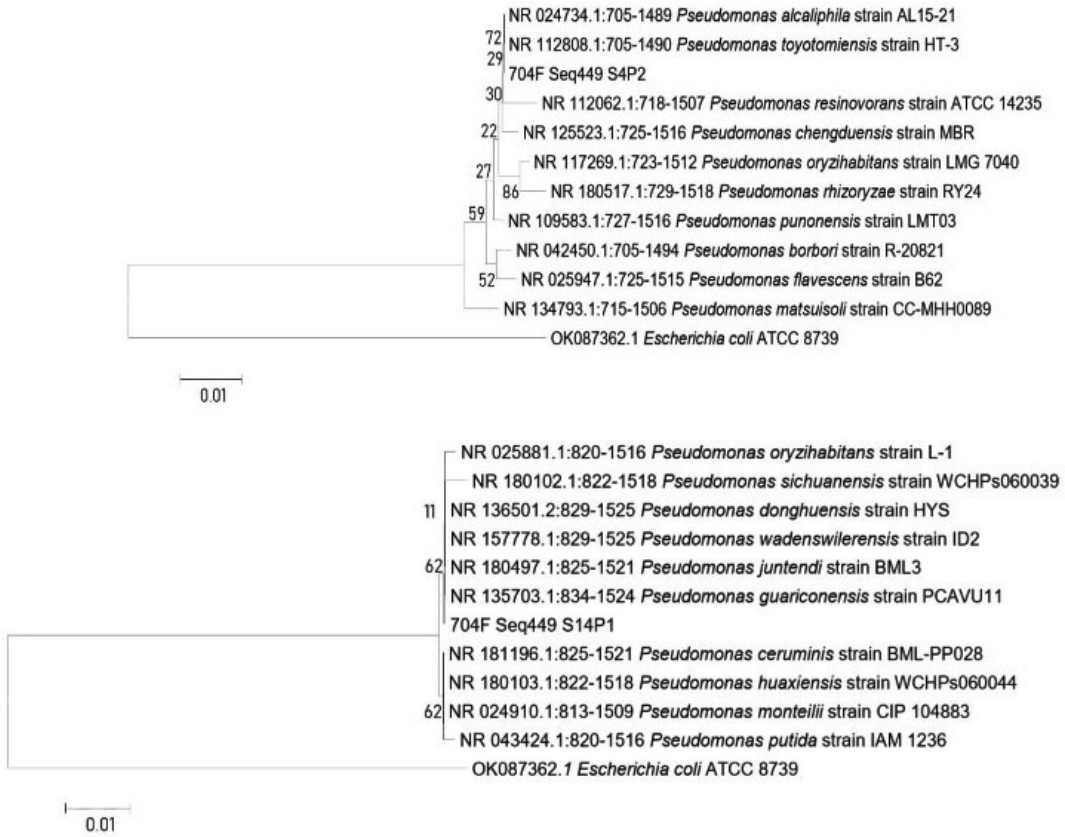

Figure 2. Phylogentic analysis of isolates S4P2 and S14P1

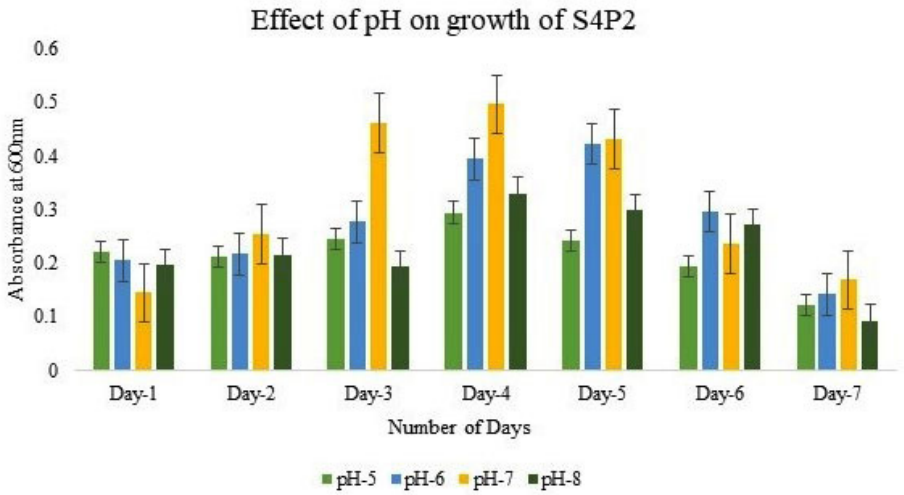

Figure 3. Effect of pH on growth of S4P2 isolate

similarity, while S10P1 & S14P1 were identified as *Pseudomonas aeruginosa,* and *Pseudomonas donghuensis* with 99.72% and 99.86% similarity respectively (Table 2). Based on previous work reported on *Pseudomonas aeruginosa*, only two isolates S4P2 and S14P1 were selected for further analysis. Cunha *et al.,*[8] reported three-gram-negative species, *Burkholderia cepacia, Klebsiella pneumoniae* and *Pseudomonas alcaligenes* isolated from soil samples contaminated with gasoline. Similar findings were reported by Sajib *et al*.[18] The authors isolated ten different types of bacterial species on BH agar plates supplemented with petrol, diesel, and octane as sole source of carbon, with *Pseudomonas* sp. being most prominent. According to the phylogenetic identification of strains reported by Perdigao *et al*.[19] all isolated strains in their study belonging to *Pseudomonas* and *Rhodococcus* genera were involved in developing hydrocarbon degrading consortia. In the present investigation a novel strain, *Pseudomonas donghuensis* has been found as efficient as *Pseudomonas aeruginosa* which is widely applied in bioremediation of hydrocarbons.

A phylogenetic tree based on 16S rRNA gene results was constructed (Figure 2). The

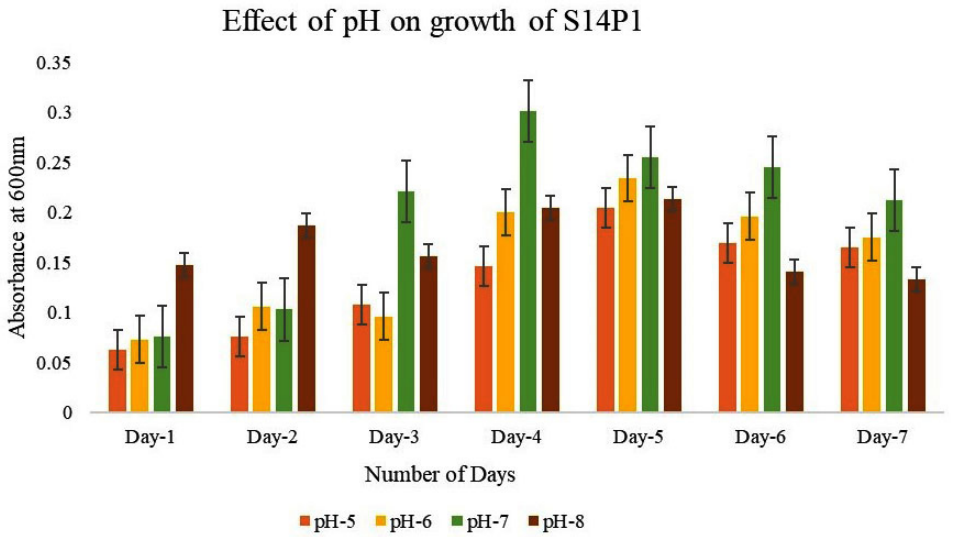

Figure 4. Effect of pH on growth of S14P1 isolate

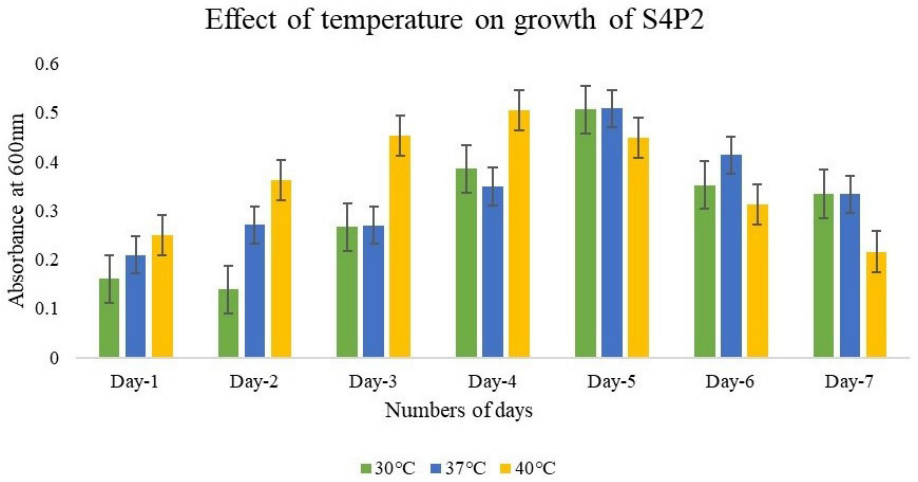

Figure 5. Effect of temperature on growth of S4P2 isolate

phylogenetic analysis revealed that S4P2 and S14P1 are closely related to *Pseudomonas* sp. Ebrahimi et al.[20] reported closely related type strain *P. chengduensis* isolated from Khazar lake for PAH degradation. In contrast, current study isolated *Pseudomonas chengduensis* from petroleum pump in Alibaug Raigad region, hence signifying the presence of this strain in petroleum contaminated sites. Safari *et al.*[21] also isolated indigenous strain of *Pseudomonas* from southwest of Iran, characterized as *Pseudomonas* YKJ which was reported to degrade certain aromatic compounds. Hence, all above reported studies shows the dominance and potential of *Pseudomonas* sp. in different geographical area. Combination of co-culture *Bacillus subtilis* and *Pseudomonas aeruginosa* was isolated from Daqing Oilfield in China which showed 63.05% hydrocarbons degradation.[22] The two main strains, *Bacillus licheniformis* and *Pseudomonas aeruginosa*, are the most reported species in petroleum bioremediation. In this study, an unreported strain of *Pseudomonas donghuensis* was identified from contaminated soil samples in the Raigad region.

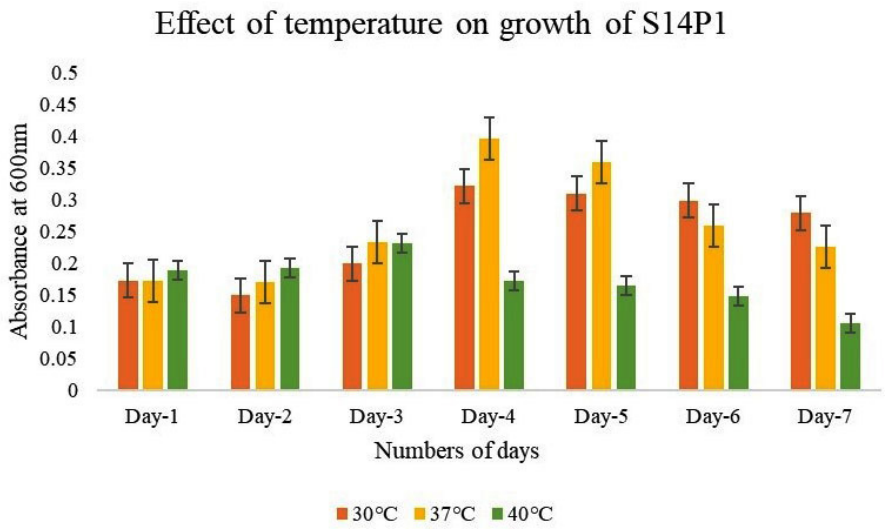

Figure 6. Effect of temperature on growth of S14P1 isolate

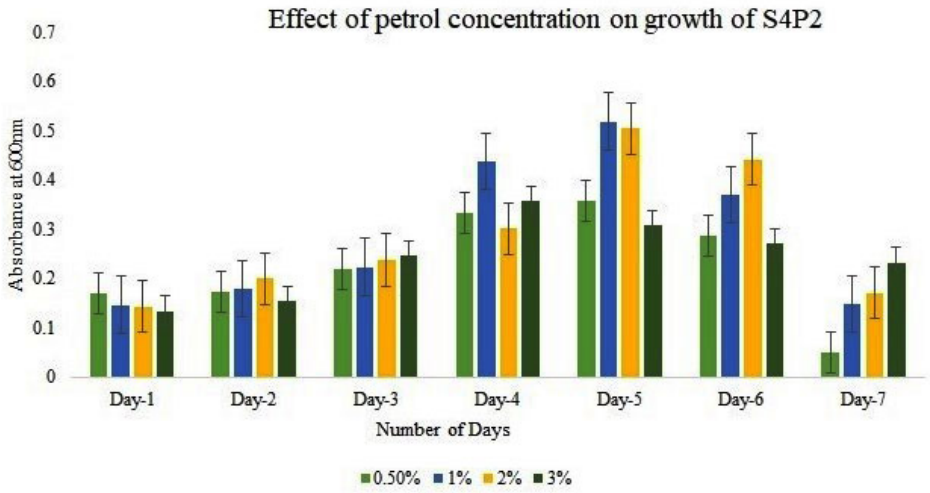

Figure 7. Effect of petrol concentration on growth of S4P2 isolate

Optimization

The effect of pH and temperature for growth of *P. chengduensis* (S4P2) & *P. donghuensis* (S14P1) was evaluated. Figure 3 & 4 shows the effect of different pH levels on growth of S4P2 and S14P1 in BH medium supplemented with 1% petrol. According to the results, maximum growth of *P. chengduensis* and *P. donghuensis* was observed at pH 7.0, suggesting that these bacteria performed optimally at neutral pH. According to reported studies, *Pseudomonas* sp. often grows best at 37°C. Our data revealed that optimum temperature of 37°C was documented for both *Pseudomonas* strains (Figure 5 and 6).

In accordance with Hossain *et al*.[23] who reported similar findings with isolates belonging to *Pseudomonas* sp., *Acinetobacter* sp., and *Enterobacter* sp., all three isolates showed the best growth at 37°C and pH 7.0 by taking absorbance at 600 nm. Significant difference in microbial growth were observed at various petrol concentrations. Maximum growth for both the isolates was observed at 1.0% concentration (Figure 7 and 8). The effect of inoculum size on *P. chengduensis*

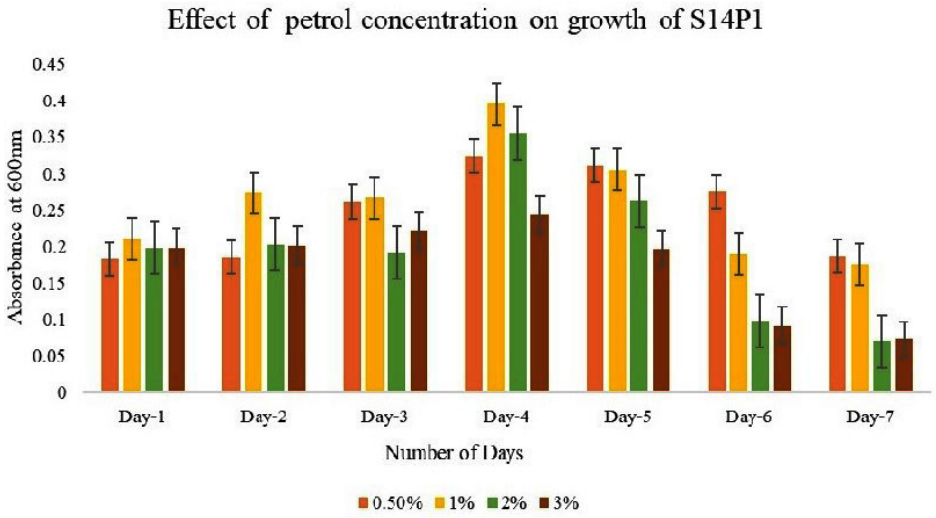

Figure 8. Effect of petrol concentration on growth of S14P1 isolate

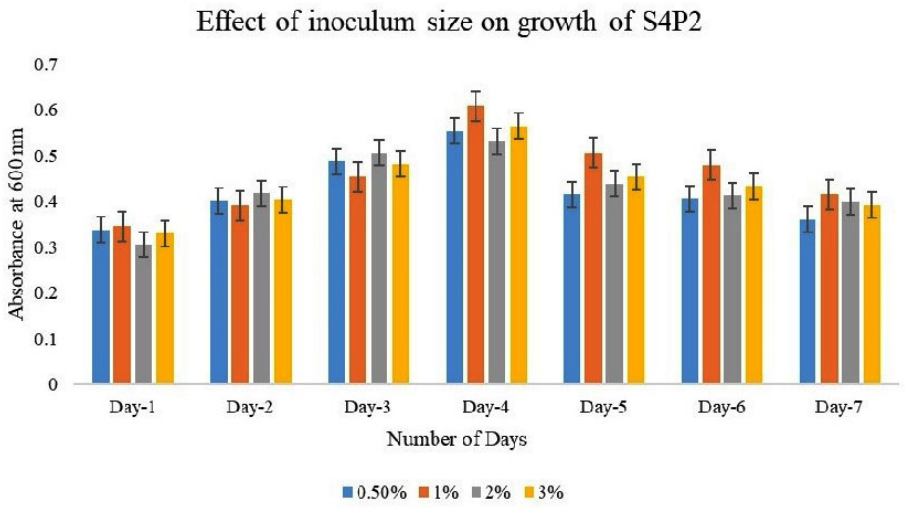

Figure 9. Effect of inoculum size on growth of S4P2 isolate

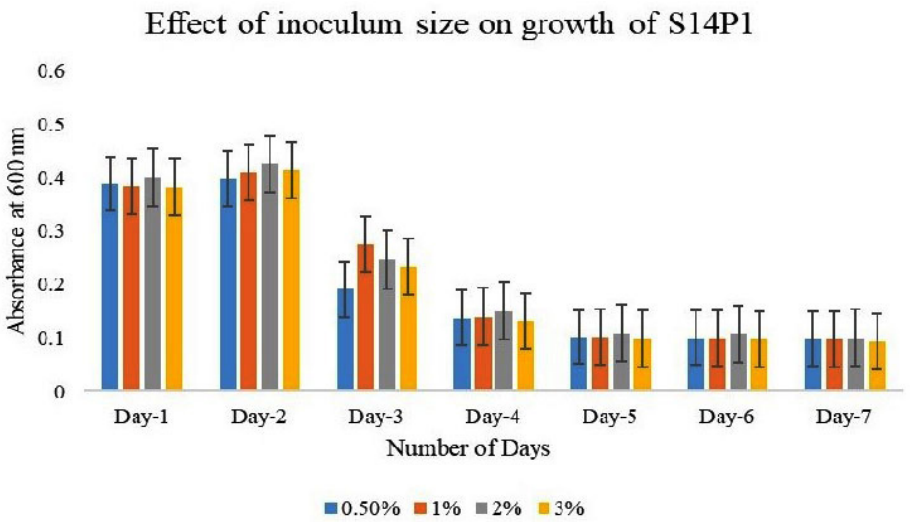

Figure 10. Effect of inoculum size on growth of S14P1 isolate

(S4P2) was studied with range of 0.5-3.0%. As per Figure 9, maximum absorbance was measured at 1% inoculum size with highest absorbance of 0.608 ± 0.01 ($P < 0.05$). The effect of inoculum size on (S14P1) *P. donghuensis* was studied with range of 0.5-3.0% (Figure 10). Maximum absorbance was estimated at 2% inoculum size with highest absorbance of 0.426 ± 0.02 ($P < 0.05$). A similar work was conducted on parametric optimization of bacterial isolates *Bacillus, Parapusillimonas, Pseudoxanthomonas* and *Halomonas* isolated from oil field of ONGC, Rajasthan, India. Growth parameters such as pH, salt concentration, carbon source and nitrogen source were studied.[24] The main aim of optimization was to identify the most favourable growth conditions for these bacteria to achieve the highest biodegradation of hydrocarbons. In this study, pH, temperature, substrate concentration and inoculum size are crucial factors for enhancing bacterial growth and improving the biodegradation of contaminated sites.

Data analysis

Two-factor analysis of variance (ANOVA) test was carried out on Microsoft Excel 2019 with significance levels of $p < 0.05$. The results obtained revealed that both S4P2 and S14P1 were found to be significantly different.

CONCLUSION

In current research, we isolated, characterized, and optimized native autochthonous petrol degrading bacteria obtained from contaminated soil in Raigad region. Two bacterial isolates S4P2 and S14P1 were identified as *Pseudomonas chengduensis* & *Pseudomonas donghuensis,* respectively. Both isolates were able to grow in presence of petrol. DCPIP assay results demonstrated the hydrocarbon-degrading capacity of both isolates. The current study characterized and optimized a novel strain *Pseudomonas donghuensis* with the potential of petrol hydrocarbon degradation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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