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Molecular identification of the isolated diesel degrading bacteria and optimization studies

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Abstract

Diesel, a refinery product of crude oil constitutes a major source of pollution in our environment. Poor solubility in water and the higher content in sediments make diesel a potential water pollutant. Bioremediation of contaminated aquatic and soil environments has arisen as an effective technology, with a range of advantages compared to more traditional methods. A total of 9 bacteria were isolated from three petroleum contaminated soil samples and isolate 3 of sample 1 showed maximum degradation potential of diesel in both primary and secondary screening tests. Hence, it was subjected for 16srDNA study and sequence alignment by BLASTN identified the isolate as *Pseudomonas aeruginosa* strain KEB24. The reaction conditions for efficient diesel degradation by the isolate were optimized.

Key words: Bioremediation, diesel, degradation, phylogenetic tree, 16s rDNA

Introduction

Oil spilled on water spreads out in a relatively thin layer on the surface. Lighter components gradually evaporate, and some water-soluble ones dissolve. They can bioaccumulate in food chains where they disrupt biochemical or physiological activities of many organisms, thus causing carcinogenesis of some organs, mutagenesis in the genetic material, and impairment in reproductive capacity and / or causing haemorrhage in exposed population (Onwurah 2007). One of the best approaches to restoring contaminated soil is to make use of microorganisms to degrade those toxic compounds in a bioremediation process. Bioremediation is an attractive approach of cleaning up petroleum hydrocarbons because it is simple to maintain, applicable over large areas, cost effective and leads to the complete destruction of the contaminant (Kumar 2011).

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To warrant a practical application, any bioremediation process should demonstrate that removal of contaminants is the primary effect of biodegradation, and that the degradation rate is greater than the natural rate of decontamination. One of the difficulties of developing bioremediation strategies lies in achieving as good or better results in the field as in the laboratory (Nizam 2008). However, bacteria play the central role in hydrocarbon degradation. Various species of *Pseudomonas* such as *Pseudomonasstutzeri* (Vazquez 2009), *Pseudomonas fluorescens* (Olanipekun 2012), *Pseudomonas aeruginosa* (Hong 2004 and Olanipekun 2012) and *Pseudomonas* sp. strain DRYJ3 (Shukor 2008) are reported for diesel degradation. The ability of bacteria to degrade soil pollutants such as hydrocarbons is useful for bioremediation purposes and their ability to emulsify hydrocarbons has been studied to this respect, making bacteria useful in the removal of these compounds from the environment (Ganesh and Lin, 2009). The present study was designed to isolate bacteria with efficient diesel degrading ability, to identify that by phylogenetic analysis and to standardize the reaction conditions for diesel degradation.

Materials and methods

Collection of Soil Sample and Isolation of Diesel degrading bacteria

Soil samples were collected from three different garages of Bangalore, India in sterile bags and maintained in aseptic condition until use. 1g of soil from each sample was serially diluted up to 10^{-5} with saline. Microorganisms were isolated by incubation at 37°C for 24h by streak-plate method on nutrient agar with 0.5% (v/v) diesel.

Primary and Secondary Screening of the isolates

The organisms with diesel degrading ability were screened using Spirit Blue Agar media containing 0.1% (v/v) Triton-X-100 and 0.5% (v/v) of diesel. The plates were observed for colour change after incubation of 24 hrs at 37°C. Mineral Salt Media (MS media) containing 0.5% (v/v) of diesel was used for diesel degradation potential determination (Michaud 2004).

The inoculated flasks were incubated at 37°C with 150 rpm for 5days and the percent degradation was calculated.

Molecular characterization of selected bacterial isolate

The selected bacterial isolate was cultured in Luria Bertani broth and incubated at 37°C for 24 h in an orbital shaker at 150 rpm. Genomic DNA was extracted using Bacterial Genomic DNA Isolation Kit (Chromous Biotech Pvt. Ltd., Bangalore, India) according to the manufacturer instructions and visualized using 0.8% (w/v) agarose gel electrophoresis (Stover 2000).

PCR amplification

The PCR amplification reactions were performed in a total volume of 25µL containing the following solutions: 1.5 µL genomic DNA, 1 µL 10 pmol forward 16S rDNA primer (5'-AGAGTTTGATCCT GGCTCA-3'); 1µL of 10 pmol reverse 16S rDNA primer (5'-ACGGCTACCTTGTTACGACT-3'); 1 µL of 30 mMdeoxyribo-nucleoside 5'-triphosphate (N=A,T,G,C) (dNTP's); 2.5 µL of 10X PCR buffer and 1 µLTaq polymerase (1 U) (Chromous Biotech Pvt. Ltd., Bangalore, India) and water was added up to 25 µL. The thermal cycler (MJ Research PTC 200, USA) was programmed as follows: 2 min initial denaturation at 94°C, followed by 30 cycles that consisted of denaturation for 1 min at 94°C, annealing for 30 s at 57°C and extension at 74°C for 1 min and a final extension of 5 min at 74°C.The PCR amplified product was analyzed by 1.2% agarose gel electrophoresis using TAE buffer. The resulting DNA patterns were examined with UV light under transilluminator, photographed and analyzed using gel documentation system (Herolabs, Germany).

Partial 16S rDNA sequencing and analysis of sequenced data

The partial 16S rDNA sequencing of the amplified product was performed at Chromous Biotech Pvt. Ltd., Bangalore, India. The 16S rDNA sequence was aligned manually with the available nucleotide sequences retrieved from the NCBI database by using BLASTN (Altschul 1990).

Optimization of reaction conditions for efficient diesel degradation

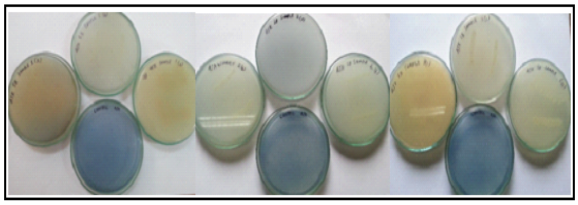
The present study assessed the impact of shaking and static condition and the size of the inoculum on the diesel degradation ability of the isolated bacteria. The MS media supplemented with 2.5% diesel was incubated for 5 days in a shaker at 150 rpm and in static condition separately at 30°C. After incubation, solvent extraction method of determining residual diesel was performed and the percentage of degradation was calculated.

Effect of inoculums size was assessed using various sizes of inoculums - 0.5%, 1%, 2%, 4%, 8% and 10% v/v. Percent degradation was calculated as above. The experiments were performed in triplicates along with control.

Results and discussion

The three garage soil samples collected had given rise to 9 colonies in total, 3 from each of the sample. The primary screening on Spirit Blue Agar media gave rise to halo around the bacterial growth. The zone of clearance confirmed the ability of the isolates to degrade diesel by causing the disappearance of the blue colour (Figure 1) while the control plate was blue in colour.

The result of the degradation potential of the isolates (Table 1) had shown isolate 3 of sample 1 with maximum degradation (95%). Hence this isolate with maximum percent degradation was subjected for biochemical tests, gram's staining method and 16srDNA study.



(a) (b) (c)

Figure 1: Isolation of bacterial isolates (with a control) on Spirit Blue Agar media containing diesel from (a) Sample 1 (b) Sample 2 (c) Sample 3

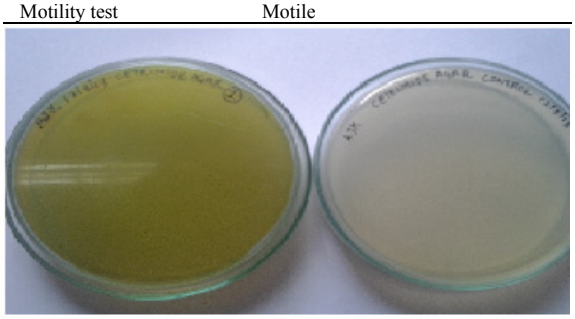
Table 2 gives the results of microscopic and biochemical tests of the isolate that showed maximum degradation ability. After identifying the isolate as *Pseudomonas,* cetrimide agar (Figure 2) test confirmed the ability of *Pseudomonas* to produce secondary metabolites (pigments) called pyocyanin.

Table 1: Percentage degradation of diesel by the isolates

Isolate	Sample 1	Sample 2	Sample 3
Isolate 1	90.00	52.60	78.90
Isolate 2	37.50	70.00	85.00
Isolate 3	95.00	81.20	81.20

Table 2: Results of staining and biochemical tests of the isolate with diesel degrading ability

TESTS	RESULTS
Shape	Rod
Gram staining	Gram negative
Indole test	Negative
Voges-Proskauer test	Negative
Methyl-red test	Negative
Glucose Fermentation	Acid production was observed
Urease test	Positive
Starch hydrolysis test	Negative
Citrate test	Positive
Casein hydrolysis test	Positive
Gelatin hydrolysis test	Positive
Triple Sugar Iron test	Positive
Sucrose fermentation	Acid production
Motility test	Motile



Isolate Control

Figure 2: Cetrimide agar test of the isolated *Pseudomonas* and control

Molecular identification of the isolate with diesel degrading ability

Partial 16S rDNA sequencing and analysis of sequenced data

The amplified 500 bp long 16s rDNA was sequenced and subjected for sequence alignment. Phylogeentic tree was constructd and the isolated diesel degrading bacteria showed

99% similarity with *Pseudomonas aeruginosa* strain PAO1 following BLASTN with available sequence in NCBI database (Figure 3 to 6 and Table 3). Hence the isolated bacteria with diesel degrading ability was identified to be *Pseudomonas aeruginosa.*

AGCGGATGAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGT
GAGTAATGCCTAGGAATCTGCCCTGGTAGTGGGGGATAACGTC
CGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGT
GGGGGATCTTCGGACCTCACGCTATCAGATGAGCCCTAGGTCG
GATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCG
TAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGAC
ACGGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG
GACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGA
AGAAGGTCTTCGGATTGTTAAAGCACTTTAAGTTGGGAGGAAGG
GCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATA
AGCACCGGCTAACTTCTTGCCAGCAGCCGGCGGTAATACGAAG
GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGT
AGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTTG
GGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAG
GGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAG
GAAGGAACACCAGTTGGCGAAGGCGACCACCTGGACTGATACTG
ACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGGTCGACTAGCCGTTG
GGATCCTTGAGATCT

Figure 3: 500 base sequence of 16S rDNA from the isolated diesel degrading *Pseudomonas* sp.

Table 3: Alignment statistics for match #1

Alignment statistics for match #1				
Score	Expect	Identities	Gaps	Strand
1393 bits(754)	0.0	779/789(99%)	9/789(1%)	Plus/Plus

Petroleum hydrocarbons can be degraded by microorganisms such as bacteria which includes *Pseudomonas, Marinobacter, Alcanivorax, Microbulbifer, Sphingomonas, Micrococcus, Cellulomonas*, *Dietzia*, and *Gordonia* groups (Brito 2006), molds belonging to the genera *Aspergillus, Penicillium, Fusarium, Amorphoteca, Neosartorya, Paecilomyces, Talaromyces, Graphium*, yeasts which includes *Candida, Yarrowia* and *Pichia* and microalgae (Chaillan 2004).

However, bacteria play the central role in hydrocarbon degradation. Various species of *Pseudomonas* such as *Pseudomonas stutzeri* (Vazquez *et al* 2009), *Pseudomonas fluorescens* (Olanipekun 2012) *Pseudomonas aeruginosa* (Hong 2004 and Olanipekun *et al* 2012), *Pseudomonas* sp. strain DRYJ3 (Shukor 2008) are reported for diesel degradation. The ability of bacteria to degrade soil pollutants such as hydrocarbons is useful for bioremediation purposes and their ability to emulsify hydrocarbons has been studied to this respect, making bacteria useful in the removal of these compounds from the environment (Ganesh and Lin, 2009).

Optimization of reaction conditions

The result of shaking on diesel degradation was found to be better than the percent degradation in static condition (Figure 5). The reason could have been the maintenance of uniform temperature, nutrient, bacterial distribution and even diffusion of oxygen in shaking condition. Under static condition, diffusion of oxygen would not have been uniform and there might have been the development of oxygen tension which hindered bacterial growth there by reducing the degradation of diesel. Contrary results were obtained in another study where it was seen that static condition was better due to greater soil capacity in static culture which can provide more microbes abundant nutrients and a suitable pH for degradation of oil by microbes (Zhaung and Peng, 2008).

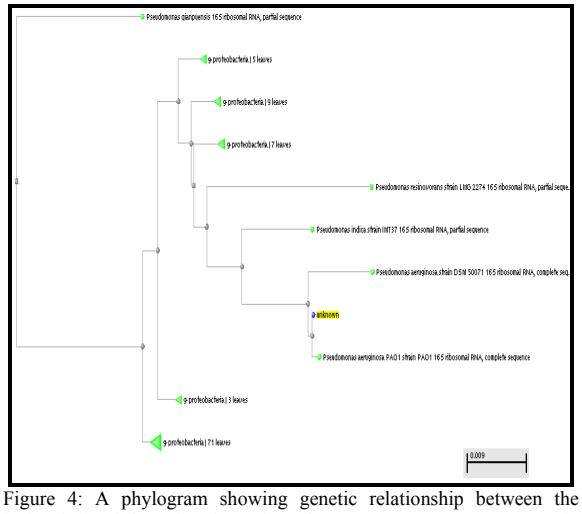


Figure 4: A phylogram showing genetic relationship between the isolated diesel degrading bacteria and *Pseudomonas aeruginosa* strain PAO1

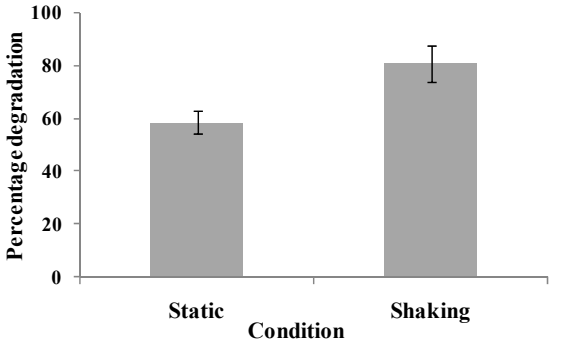


Figure 5: Percentage of diesel degradation in shaking and static conditions

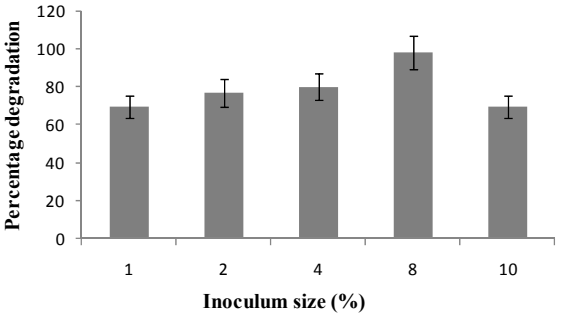


Figure 6: Percentage of diesel degradation with various inoculum size

Effect of percentage inoculums on diesel degradation gave rise to 98.06% as the maximum degradation with 8% (v/v). The least degradation 69.44% was shown by both the inoculums sizes of 1 and 10% (Figure 6). The inoculating bacteria concentration also affects the biodegradation rate of diesel oil. In a study it was found that initial bacteria concentrations of 4×10^7cells/ml was the best for diesel bioremediation (Luo, 2012).

Conclusion

The attempt in the present study in isolating diesel degrading bacteria had shown encouraging result. The bacteria was isolated successfully and identified by molecular characterization as *Pseudomonas aeruginosa*. The diesel degrading ability was optimized in shaking condition and the inoculums size being 8%.

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