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Isolation, screening and identification of benzene degrading bacterium, *pseudomonas putida*

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Abstract

The BTEX (benzene, toluene, ethylbenzene, and xylene) compounds are hydrocarbon pollutants that are considered as significant threat to the health and environment. Among BTEX, benzene is of major concern, because it is soluble, mobile, toxic, carcinogenic and one of the most stable aromatic compounds especially in ground and surface waters. Microbial degradation of benzene in aerobic environment has been successful, however, it is poorly biodegraded in anaerobic conditions. Pseudomonas species are common in aerobic bioremediation of benzene. The objective of this study was to identify the Benzene degrading *Pseudomonas putida*. Dilution plating was used to quantify the numbers of *Pseudomonas putida*, and DNA sequence analysis was used to identify *Pseudomonas putida* species. *Pseudomonas putida* was identified basing on the microscopic observations and Biochemical characterization. It was ultimately confirmed based on DNA sequence (16S rRNA sequence) analysis and finally concluded by BLAST analysis by constructing a phylogenetic tree.

INTRODUCTION

Benzene, toluene, ethylbenzene, and xylenes (BTEX) are common groundwater contaminants largely because of leaking underground gasoline storage tanks. Among them, benzene represents a particular risk to humans due to its carcinogenicity. Vast quantities of epidemiologic, clinical, and laboratory data link benzene to aplastic anemia, acute leukemia, and bone marrow abnormalities [1-2]. Benzene targets liver, kidney, lung, heart, brain and can cause DNA strand breaks, chromosomal damage, etc. Benzene has been shown to cause cancer in both sexes of multiple species of laboratory animals exposed via various routes [3-4]. Some women having breathed high levels of benzene for many months had irregular menstrual periods and a decrease in the size of their ovaries. Benzene exposure has been linked directly to the neural birth defects spina bifida and anencephaly [5]. Men exposed to high levels of benzene are more likely to have an abnormal amount of chromosomes in their sperm, which impacts fertility and fetal development [6]. Outdoor air may contain low levels of benzene from automobile service stations, wood smoke, tobacco smoke, the transfer of gasoline, exhaust from motor vehicles, and industrial emissions [7]. To understand benzene bioremediation options at contaminated sites, many aerobic benzene degrading isolates have been obtained and examined. Commonly identified isolates appear to fall within the phylum, the Proteobacteria. Proteobacteria include, for example, microorganisms within the genera *Pseudomonas* [8-13], *Burkholderia* [14], *Ralstonia* [15], *Achromobacter* [16], *Pseudoxanthomonas* [17], *Hydrogenophaga*[18], *Alicycliphilus* [19] and *Acinetobacter* [20]. Although many of these microorganisms are present in mixed cultures and at contaminated sites undergoing benzene degradation it remains technically challenging to prove which are the active and dominant *in situ* benzene degraders within such mixed community samples. The objective was to determine if the commonly recognized aerobic benzene degraders (Proteobacteria) identified via isolations and clone libraries, were responsible for benzene degradation within these mixed community samples. Thus, the work was designed to identify the organism, *Pseudomonas putida*, capable of degrading Benzene isolated from the soil and identify the species using microbial staining, biochemical analysis, and DGGE as well as DNA sequence analysis.

MATERIALS AND METHODS

Sample Collection

Samples of sewage mixed with sediment particles near petrol bunk rich in Benzene were delivered to the laboratory within one hour after their collection.

Sample Collection

Samples of sewage mixed with sediment particles near petrol bunk were aspirated from the bottom of sewers using sterile catheters applied to 150 ml sterile syringes. Samples were delivered to the laboratory within one hour after their collection.

Isolation and Screening of *Pseudomonas* for Benzene Degradation

10 ml of the sewage water sample taken in test tube and used as a stock. This sample was then serially diluted from 10^{-1} to 10^{-6} dilution and plated on a *Pseudomonas* media (Hi-Media). After confirming the isolated microorganism as *Pseudomonas* based on morphological and biochemical analysis then tested the *Pseudomonas* for benzene degradation by spreading on nutrient agar benzene media containing half strength carbon source. Secondary screening was carried out by growing in Bushnell Haas broth that lacks carbon source but it is supplemented with benzene as sole carbon source.

Microbial and Biochemical characterization of the isolate *Pseudomonas*

The isolated bacteria was analyzed using different staining techniques such as Gram's staining, motility test, capsule staining and different biochemical techniques such as catalase test, Urease test, Citrate utilization test, Methyl red and Voges-Proskauer test, Hydrogen sulphide production test, Oxidase test, Nitrate reduction Test, Gelatin Liquefaction test.

Molecular identification

Primer design

The primer sequence representing *Pseudomonas* species were retrieved from GenBank (National Center for Biotechnology information; http://www.ncbi.nlm.nih.gov) aligned using Claustal X [21], and the *Pseudomonas* genus-specific primers (forward: 5' -GAGTTTGATCCTGGCTCAG-3' and reverse: 5'-AGAAA GGAGGTGATCCAGCC-3') were designed based on the homologous regions specific to *Pseudomonas* genus.

DNA isolation and amplification of 16s RNA gene of *Pseudomonas putida* by Polymerase Chain Reaction (PCR)

The template genomic DNA from Pseudomonas putida was isolated following the protocol described [22-23]. In Polymerase Chain Reaction, the specific primers Forward and Reverse (Institute of Biological Sciences, Vijayawada) were used to amplify the genomic sequence of the open reading frame (ORF) of the gene. PCR conditions were 94^{0}C for 2 min, and then 94^{0}C for 1 min, 60^{0}C for 1 min, 72^{0}C for 3 min for a total of 30 cycles, with the extension at 72^{0}C for 10 min.

Agarose gel electrophoresis

Required amount of agarose (w/v) was weighed and melted in 1X TBE buffer (0.9M Tris-borate, 0.002 M EDTA, pH 8.2). Then, 1-2 µl ethidium bromide was added from the stock (10 mg/ml). After cooling, the mixture was poured into a casting tray with an appropriate comb. The comb was removed after solidification and the gel was placed in an electrophoresis chamber containing 1X TBE buffer. The products were mixed with 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) at 5:1 ratios and loaded into the well. Electrophoresis was carried out at 60V [24].

Eluting DNA from agarose gel fragments

Ethidium bromide stained agarose gel was visualized under a transilluminator. The fragment of interest was excised with a clean razor blade. After removing the excess liquid, the agarose fragment was placed in the spin column. The tube was centrifuged at 5500 rpm for **not more** than 45 seconds for the elution of DNA. The eluent was checked by running on an agarose gel and observed on a transilluminator for the presence of ethidium bromide stained DNA. The eluted DNA was used directly in manipulation reactions. This DNA fraction was subjected for sequencing (Institute of Biological Sciences, Vijayawada).

Sequencing and chimera checking

The eluted PCR product was directly sequenced using *Pseudomonas* genus-specific primers without GC-clamp at Institute of Biological Sciences, Vijayawada. Sequencing reactions were carried out with ABI PRISM Dye Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems Inc., USA). All sequences exhibiting less than 95% sequence similarity to existing sequences in GenBank were checked using CHIMERA-CHECK program at the Ribosomal Database Project (RDP) using default settings [25]. All representative sequences corresponding to bands were *Pseudomonas* species.

Phylogenetic placement

The environmental sequences were compared to the sequences in GenBank using the BLAST algorithm [26] (Altschul *et al.,* 1997) and RDP database [25] to search for close evolutionary relatives.

GenBank accession numbers

The representative sequence of the soil *Pseudomonas* species was deposited in GenBank of National Centre for Biotechnology Information (NCBI). The Gen Bank accession number is JQ350845.

RESULTS AND DISCUSSION

Isolation and Screening of *Pseudomonas* for Benzene Degradation

The organism *Pseudomonas* species was isolated by plating the serially diluted samples on *Pseudomonas* media at 10^{-6} dilution and enriching the same with Benzene as the soul carbon source.

Microbial and Biochemical characterization of the isolate *Pseudomonas*

The results of the isolated bacterium with reference to microbial and biochemical characterization was given in Table 1.

Table: 1 Microbial and biochemical characterization of *pseudomonas.*

S.No	Test	Result
1	Gram staining	Positive
2	Motility test	Positive
3	Catalase test	Positive
4	H_2S production	Negative
5	Gelatin test	Positive
6	Oxidase test	Negative
7	Urease test	Negative
8	Methyl red test	Negative
9	Voges proskauer	Negative
10	Citrate utilization	Positive
11	Nitrate reductase	Negative

Molecular identification

Amplification of DNA coding for 16s r RNA

The genomic DNA of the organism screened was subjected for the isolation of the DNA coding for 16s rRNA by using Polymerase chain reaction Fig.1. The bands were cut and eluted and the DNA so obtained was subjected for sequencing.

Sequence analysis

The sequence analysis demonstrated that all the corresponding bands on agarose gel belonged to *Pseudomonas putida.* Upon sequencing of the amplified DNA, the data obtained corresponds to 1414 bases for *Pseudomonas putida.* The sequence so obtained was as follows.

ATGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCG
GTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGGGTGA
GTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCG
GAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCA

GGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGG
ATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATC
CGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAG
GGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTG
TGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGG
AAGGGCAGTTACCTAAGATTGTTTTGACGTTACCGACAGAAT
AAGCACCGGCTAACTCTGTGCCAGCAGAACCGCGGTAATACA
GAGGGTGCAAGCGTTAATCGGAATTACTCCGGGCGTAAAGCG
CGCGTAGGTGGTTAGTTAAGTTGGATGTGAAATCCCCGGGCT
CAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGG
TAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAG
ATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACT
GATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGA
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAG
CCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCATT
AAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
AATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGT
TTAATTCGAAGCAAGAAGAACCTTACCAGGCCTTGACATCCA
ATGAACTTTCTAGAGATAGATTGGTGCCTTCGGGAACATTGA
GACAGGTGCTGCATGGCGTCAGCTCGTGTCGTGAGATGTTTT
GGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTAC
CAGCACGTAATGGTGGGCACTCTAAGGAGACTGCCGGTGACA
AACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCT
TACGGCCTGGGCTACATGCTACAATGGTCGGTACAGAGGGTT
GCCAAGCCGCGAGGTGGAGCTAATCCCAGAAAACCGATCGTA
GTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAAT
CGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCG
GCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCA
CCAGAAGTAGCTAGTCTAACCTTCGGGGGG

Phylogenetic affiliation of the *Pseudomonas putida* based on the ITS sequences

Sequences of the dominant DGGE bands revealed that *Pseudomonas* species in tested soil was *Pseudomonas putida* (100% similarity to the ITS sequences of *Pseudomonas putida*) with the accession no. JQ350845. This sequence can be found in NCBI Genome Databank. The phylogenetic tree was shown in Fig 2.

Indigenous *Pseudomonas putida* species are known to have

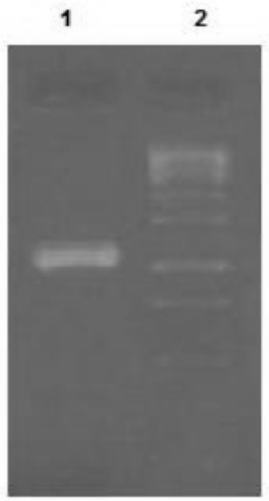

Fig.1: Agarose gel showing amplified 16s DNA. Well 1: Amplified DNA of *Pseudomonas putida;* Well 2: DNA Ladder.

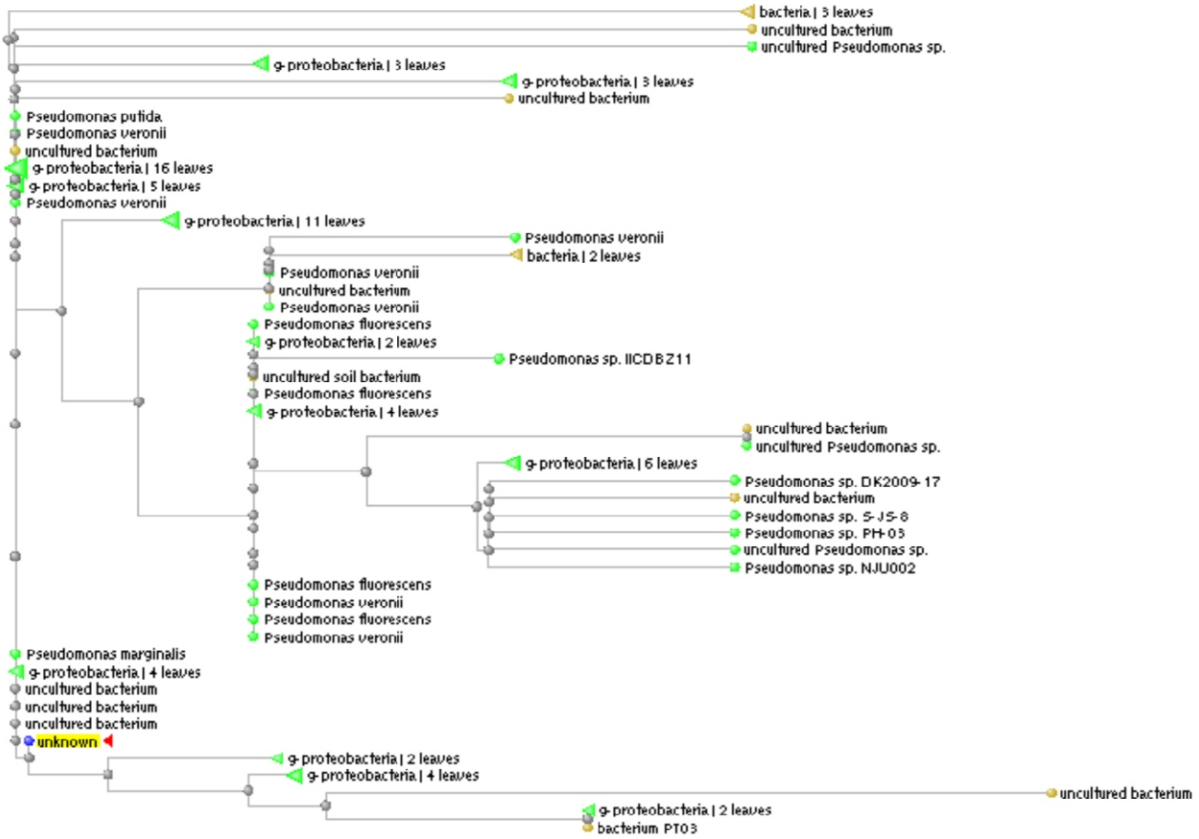

Fig.2: Phylogenetic affiliation of *Pseudomonas putida*

greater tolerance for Benzene and like compounds than many other soil microorganisms [27]. We found that the ITS sequences of the saved colonies from soil dilution plating generally matched with those of the excised bands. Our results demonstrated that the 16s rRNA primers enabled us to amplify *Pseudomonas putida* species from oil contaminated soils and confirm their identity by BLAST search. The concentration of the gel and running time for electrophoresis should always be adjusted based on the GC content of the target DNA; these procedures will eventually maximize the band separation on agarose gel, and ensure that all related soil *Pseudomonas putida* species have been included [28]. Although degradation genes can be located on either chromosome or plasmid, Phenanthrene degrading *Pseudomonas* from crude oil contaminated soil samples collected in a petroleum refining area were isolated and characterized [29]. They concluded that hydrocarbon degradation routes are frequently located on plasmid.

CONCLUSION

In general, oil contaminated soils are rich in microbial diversity. Therefore benzene, which is a product of petroleum product, has to be degraded in the soil. *Pseudomonas putida*, a biocontrol agent. In addition, our work suggest that the combination of soil dilution plating, DGGE and DNA sequence analysis are effective approaches to facilitate extensive examinations of the propagule numbers of *Pseudomonas putida*, to reliably identify *Pseudomonas putida* in soils.

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