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Isolation and Identification of Toluene Degrading Microbes and Detection of Catabolic Gene

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ABSTRACT

The present study reports on the isolation and characterization of new isolates from crude oil from petroleum contaminated soil. The isolates were then screened for their potential towards biodegradation of toluene by growing them in both solid as well as liquid mineral medium supplemented with toluene as a sole carbon source and further identified by GC-FAME analysis. The degradation potential of these isolates strongly suggests that the isolates have TOL like plasmid and carries xylM gene involved in the expression of the toluene monooxygenase. The primer sets were used in PCR to assess the presence of the catabolic gene in new toluene degrading isolates. The primer verification was done by using bioinformatics tools. From the results it is conclusive that isolates were able to degrade Toluene, and from PCR it is evident that the isolates contained gene coding for Toluene degradation. The test results indicate that the bacteria could contribute to bioremediation of aromatic hydrocarbon pollution.

Key words: Toluene, GC-FAME analysis, xylM gene, biodegradation, bioremediation

INTRODUCTION

Toluene is an important constituent of various commercial products including paints, dyes, cosmetics, pharmaceuticals, various chemicals and plastic articles (Bloemen and Burn, 1993; ASTDR, 1989). It is a natural component of crude petroleum and petroleum products such as gasoline, diesel fuel (Budavari, 1996) and also causes various health hazards (ASTDR, 1989; Brigmon *et al*., 2002). It contaminates surface and ground water (Lovley, 1997). Hence it was classified as priority pollutant (USEPA, 1986). To remove this volatile compound from contaminated environments, it is important to develop some good techniques. Bioremediation is one of the useful techniques that can be applied for such pollution problems (Taki *et al*., 2007). In the past few years, toluene degrading bacteria have attracted attention because of their potential to clean up spills (Bloemen and Burn, 1993; ASTDR, 1989; Brigmon *et al*., 2002). The biodegradation kinetics for toluene from gasoline-contaminated soil was recently reported (Shi *et al*., 1999). It had found that many species had capability to degrade toluene as it contain specific catabolic gene for this purpose.

In India, very little research has been done regarding the toluene degradation. The aim of this study was to isolates and characterise potent toluene degraders which would be helpful in bioremediation of environmental pollutant toluene.

MATERIALS AND METHODS

Isolation and identification of toluene degrading microorganisms: Soil sample was collected from the Automobile Garage. Soil suspension was made and toluene degraders have been isolated by spreading 10 μL of suspension on solid medium supplemented with 1 mL (wt at 20°C mL^{-1} 0.860-0.867 g) toluene and incubating plates at 28°C for 4 to 5 days (Junca and Pieper, 2003). Plates with well isolated colonies were selected for further studies. Pure cultures of these isolates were obtained and morphogical study was done. Isolates of different characteristics were selected for further cultivation and growth monitoring.

Growth estimation was done by using 50 mL MS medium broth supplimented with 1 mL of toluene. These flasks were then inoculated with 1 mL of overnight grown culture of toluene degrading isolates and were incubated at 28°C for 24 h. The growth was monitored by measuring the optical density of the culture at 600 nm (Plaza *et al*., 2007). Isolates which showed good degradation capacity in 24 h were selected (Zylstra and Gibson, 1989) and subjected for evaluation of degradation capacity for toluene.

Isolates which showed maximum degradation in 24 h selected for FAME identification. These microbes were grown on the Tryptic Soy Agar media at 28°C for 48-72 h. Cells were harvested and fatty acids were extracted in alkaline solution having methanol and distilled water by saponification. After that Methylation and extraction of fatty acids were carried out using reagents recommended by Sherlock MIDI to form Fatty Acid Methyl Esters (FAME) by lowering the pH below 1.5 with the help of Hcl and Methyl alcohol. FAME samples were redissolved in 1:1 ether-haxane as needed because of solvent evaporation. These FAME's were analyzed using Gas Chromatography with the help of MIDI Sherlock software for FAME. Aerobic library (RTBSA6.0) was referred for the analysis. The analysis was performed as per the method given in Sherlock Manual: Version 6.0 (2005) (Sasser, 2001).

Evaluation of degradation capacity: Toluene degradation capacity was checked with different concentrations of toluene. For that 50 mL Minimal Salt medium (Bushnell Haas Broth) was inoculated with 5 mL of culture. Different concentrations (2.0, 3.0, 4.0 g mL^{-1}) of toluene were added in each set and all flasks were incubated at 28°C. Absorbance of culture from each flask was taken at 24 h interval for 5 to 6 days at 600 nm (Junca and Pieper, 2003).

Plasmid DNA isolation and amplification of catabolic gene: For plasmid Isolation enrichment is required. Bacterial cells were first grown in nutrient broth for 48 h and then cells were harvested. These cells were inoculated in 50 mL selective medium that contains Mineral medium with 1 mL toluene for enrichment. Medium was incubated at 28°C for 3 to 4 days. Plasmid DNA was then isolated from this culture using Mini-prep method and DNA was confirmed on 0.8% agarose Gel. PCR amplification was carried out in Applied Biosystem machine, using primer set TOL-F (5'TGAGGCTGAAACTTTACGTAGA3')/TOLR (3'CTCACCTGGAGTTGCGTAC5') as described by Baldwin *et al*. (2003). To check whether the PCR generated the anticipated DNA fragments (amplicon), the product was run on 1.5% Agarose Gel.

RESULTS AND DISCUSSION

Isolation and identification: To screen for different microbial diversity for toluene degradation, microbial isolation was carried out from petroleum contaminated soil sample. Total seven bacterial isolates were obtained by growing them on solid medium enriched with 1 mL toluene for 4-5 day.

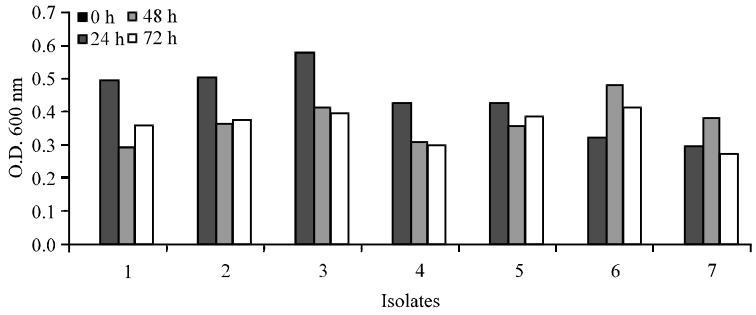

Fig. 1: Growth (O.D. 600 nm) in 1 mL of toluene isolates at 24, 48, 72 h interval

Table 1: Nme of identified organism with their similarity index

Sample name	Identified organism	Similarity index
Isolate 1	*Bacillus badius*	0.101
Isolate 2	*Brevibacterium liquefaciens*	0.619
Isolate 3	*Pseudomonas mendocina*	0.403
Isolate 4	*Pseudoxanthomonas mexicana*	0.491
Isolate 5	No match in RSTAB6 library	0.000

It was reported that *pseudomonas* species has capability for degrading hydrocarbon (Olsen *et al.*, 1994; Whited and Gibson, 1991; Zylstra and Gibson, 1989). From the Gram staining and Motility test it was found that out of seven isolates, four were Gram-negative isolates and three Gram positive isolates. Secondary and tertiary screening then used to characterize most potent organisms.

These seven isolates were then screened for maximum toluene degradation with in 24 h by growing them in liquid mineral medium with desired toluene concentration. Their Optical Density (OD) was taken at 24 h interval for three days. During this study it was observed that only five isolates showed maximum degradation with in first 24 h and then their growth declined.

As showed in Fig. 1, first five isolates achieved maximum degradation with in first 24 h. Their ODs were 0.494, 0.505, 0.58, 0.424 and 0.427, respectively in presence of supplimented hydrocarbon.

Identification of selected toluene degraders by GC-FAME analysis: Five bacterial isolates that having good toluene degradation capacity were identified by GC-FAME analyser. The MIDI Sherlock microbial identification system used RTSBA6 method and identified the organisms by their Similarity Index (SI) with stored library. By using this method four organisms out of five have been identified as shown in Table 1. However isolate-5 did not find any matching chromatogram pattern in FAME RTBSA6 6.0 library. The Sherlock Microbial Identification System is an accurate and automated gas chromatographic system, which identifies over 1,500 bacterial species based on their unique fatty acid profiles. Identification of bacteria by conventional methods usually requires 48 h after a colony has been isolated. The identification of slow-growing or biochemically inert microbes to the species level is difficult and time-consuming by conventional methods so we used GC-FAME for microbial identification.

Evaluation of degradation capacity: These five bacteria's were then selected for the Degradation Assay and allowed to grow in different hydrocarbon concentrations (2, 3, 4 g mL^{-1}).

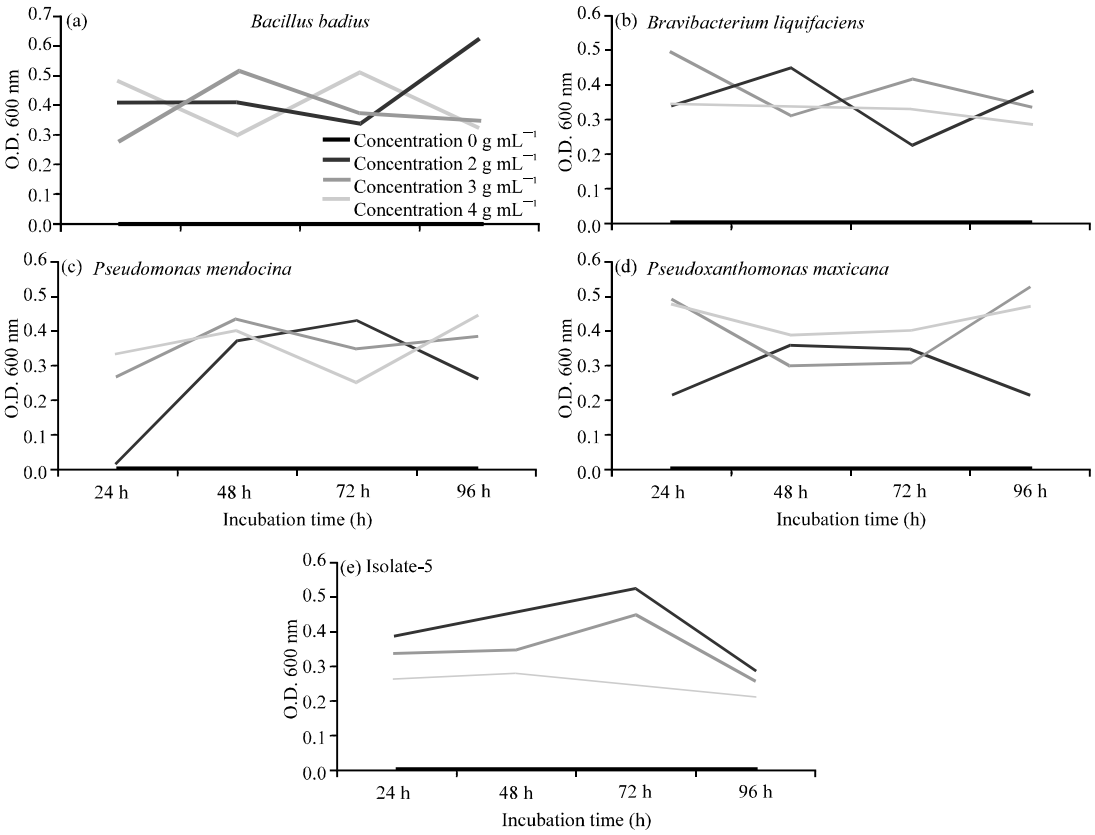

Fig. 2(a-e): Growth profile of different isolates in different hydrocarbon concentrations (2,3,4 g mL^{-1}), (a) *Bacillus* shows maximum degradation upto 48 h in 3 g mL^{-1} concentration, (b) *Bravibacterium* shows maximum growth up to 48 h in 2 g mL^{-1}, (c) *Pseudomonas* shows exponential growth upto 72 h, (d) *Pseudoxanthomonas* shows exponential growth in 2 mg mL^{-1} concentration of toluene upto 48 h. After that the growth shows stationary phase upto 72 h and then declined and (e) In above graph isolate-5 shows initial exponential growth in 2 mg mL^{-1} concentration of toluene upto 72 h, after then growth declined

Degradation capacity was checked by measuring the O.D 600 nm at 24 h interval for 4 days. The maximum O.D. indicates the maximum growth of those bacteria in respective toluene concentration.

Figure 2 shows maximum growth efficiency of different strains in individual manner with increased hydrocarbon source. From the above graph it is found out that, isolate-5 shows exponential growth upto 72 h with maximum OD 0.613 and 0.524 in 2 and 3 g mL^{-1}, respectively, which indicates that this isolate having maximum ability to degrade 2, 3 g mL^{-1} toluene. Whereas, *Pseudoxanthomonas* shows minimum growth at $OD_{0.358}$ in 2 g mL^{-1} toluene in compare to isolate-1,2 and 3 with 0.410, 0.447, 0.428 in same amount of hydrocarbon, respectively.

Plasmid DNA isolation and amplification of catabolic gene: Baldwin *et al.* (2003) demonstrated that PCR based detection of monooxygenases from petroleum contaminated soil can be used to study diversity of catabolic genes for toluene degradation. This can give a better

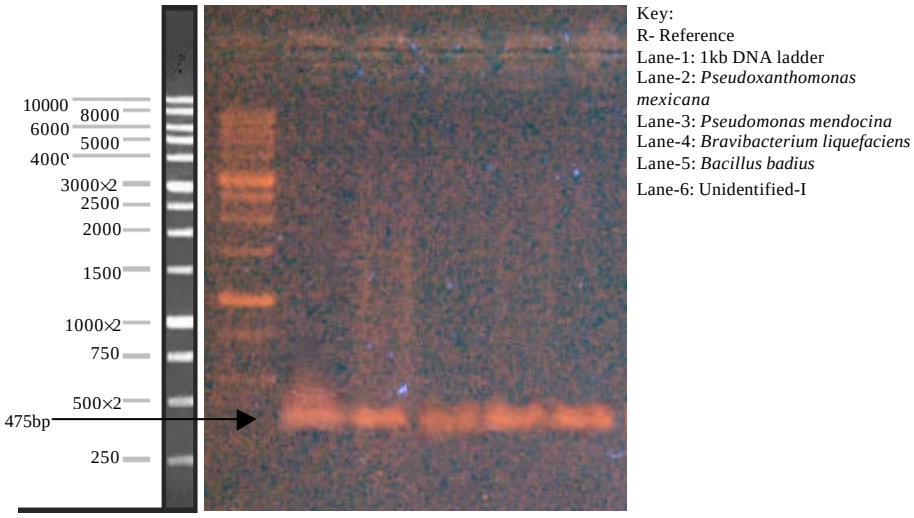

Fig. 3: PCR product on 1.5% agarose gel

perspective of microbial ecology of contaminated soil. The genes for catabolism of toluene are found to be contained in TOL plasmid. Hence, to demonstrate that isolated genotypes have catabolic genes for toluene degradation plasmid DNA isolation was carried out from all the isolated bacterium strains. DNA was isolated by Plasmid mini-prep method and samples were run on 0.5% agarose gel. Reference ladder of 1 kb had the largest linear fragment size of 10 kb. The isolated plasmid DNA bands migrated slower than 10 kb fragment so DNA band shows no match with in the ladder pattern. Moreover, super-coiled form of plasmid that is same size as linear would run faster than linear fragment, and hence plasmid much larger than 10 kb might run as fast as 10 kb fragment of linear DNA. Protein contaminants were removed by proteinase K treatment which was immediately followed by PCR. After PCR amplification band pattern separated on 1.5% agarose gel (Fig. 3), showed migration rate match with 500 bp fragment, slightly slower. From primer design it was approximated that amplified product should be of 475 bp. Gel electrophoresis suggested the presence of the product; Toluene degrading gene.

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