Degradation of Toluene by Highly Efficient Indigenous Isolate

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ABSTRACT

Screening soil samples collected from Isfahan petroleum refineries led us to isolate eight toluene-utilizing bacteria. They were isolated by 2 methods: exposure of the samples to vapor pressure of toluene (1-100%); and addition of toluene to Minimal Salt Medium (10-200 mg l-1) as the sole source of carbon and energy sources. The highly efficient strain was identified as Bacillus pumilus by biochemical characteristics and 16S rRNA sequencing. Ortho or Meta pathways were detected for degradation of toluene by the isolated strains. The results showed that Catechol 2, 3-dioxygenase was responsible for toluene degradation through meta-cleavage pathway. Results showed that the isolate had good potential for application in bioremediation of toluene contaminated environments.

INTRODUCTION

Benzene, toluene, ethylbenzene and xylene isomers (BTEX) are the major components of crude petroleum and petroleum products. BTEXs are common environmental pollutants resulting from spillages of fuels, solvents or chemicals from storage tanks, pipelines and refineries. These components represent a significant environmental hazard for humans, have potential toxicity, mutagenicity and carcinogenicity, and affect the reproduction and mortality rates of aquatic animals. Because of their high water solubility and their acute toxicity and genotoxicity, BTEX components are classified as priority pollutants by the U.S. Environmental Protection Agency [1,2].

Several ways have been so far recognized for monoaromatic compounds removal but microbial degradation represents the major route responsible for removing these pollutants. Include fungi, aerobic and anaerobic bacteria and mainly belong to the genera pseudomonas, Sphingomonas, Bacillus, Mycobacterium, Alcaligenes [3,4,5].

In aerobic metabolic pathways to degrade BTEX, two enzymic systems (dioxygenases and monooxygenases), have been identified. The monooxygenase attacks methyl or ethyl substituents of the aromatic ring but dioxygenase attacks aromatic ring with the formation of 2-hydroxy-substituted compounds. Intermediates of the “upper” pathway are then mineralized by either Ortho- or Meta-ring cleavage (“lower” pathway). Catechol-1, 2-dioxygenase is responsible for degrading through Ortho-cleavage pathway. In contrast, some strains show activity with the enzyme Catechol-2, 3-dioxygenase, catalyzing the second step degradation through meta-cleavage pathway [6,7].

Methyl-substituted aromatic substrates such as toluene are generally degraded via meta-ring fission. Catechol is oxidized to 2-hydroxymuconic semialdehyde. The final products of both the pathways are molecules that can enter the Tricarboxylic acid cycle [8,9].

The present work has focused on isolation and characterization of bacterial strains that can degrade toluene and detection of enzyme pathway to find appropriate strains for effectively treating environment contamination.

MATERIALS AND METHODS

Growth media and culture conditions:


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Minimal salt (MS) medium for hydrocarbon degradation assessment was composed of 4 g NaNO3, 1.5 g KH2PO4, 0.5 g Na2HPO4, 0.0011 g FeSO4·7H2O, 0.2 g MgSO4·7H2O and 0.01 g CaCl2 per liter at pH 7.0 (MS medium). MS medium was supplemented with 15 g of agar per liter to provide solid medium [10].

The soil samples was collected from and Isfahan (Isfahan, Iran) oil refinery areas. Serial dilutions of the soil samples were prepared in ddH2O and plated on minimal salt medium agar. Plates were incubated at room temperature (~26°C) in 5-liter glass desiccators with a crystallizing dish containing various mixtures of toluene and vacuum pump oil (vpo): 0% (150 g of vpo); 0.01% (0.05 g of toluene; 500 g of vpo); 0.1% (0.5 g of toluene; 499.5 g of vpo); 1% (2.5 g of toluene; 247.5 g of vpo); 10% (10 g of toluene; 90 g of vpo); and 100% (15 g of toluene).

In the other method, 50 ml MS media (pH=7) containing different concentrations of toluene (10-200 mg l−1) in a 250CC screw bottle was inoculated with 1 ml of overnight culture (OD600=0.5) of individual isolates from the soil samples on the Nutrient agar. Microorganisms were incubated in shaker incubator at 30 °C and 150 rpm for 72 hours. Growth of the cultures was monitored spectrophotometrically at 600nm [11,12,13].

Identification of toluene utilizing isolates:

The selected toluene-utilizing isolates were identified by biochemical tests including gram-staining, Nitrate reduction, Citrate and different sugars (D-glucose, D-xylose, D-maunitol, D-fructose, L- arabinoce), utilization, Casein, Gelatin and Starch hydrolysis, Catalase and Oxidase activity, Methyl red test, Voges-Proskauer reaction, H2S and Indole production and sequencing techniques. DNA extraction, 16S rDNA amplification and sequencing were carried out as described previously [14].

The fD1 (5'AGAGTTTGGATCCTGGTACG3') and rd2 (5' TAAGAGGGTGATCCAGGC3') primers (provided by Sinagen, Iran) were used for PCR amplification of the 16S ribosomal DNA. DNA amplifications were carried out in 50 µL reaction mixture containing 5 µL of 10xPCR buffer, 2 µL dNTP mixture (10mM), 1.5 µL MgCl2 (50mM), 2 µL of each primer (10 Pmol µL−1), 1µL of DNA template (5-10ng) and 0.6 µL Taq DNA polymerase (5U µL−1). The thermo cycling conditions consisted of a denaturing step at 94 °C for 5 min, 30 amplification cycles of 94 °C for 1 min, 48 °C for 40 s, 72 °C for 90 s and a final polymerization at 72 °C for 10 min. The PCR products were purified from the Agarose gel using DNA extraction kit (Roche, Germany) and cloned into plasmid pBluescript II vector (Fermentase, Germany) prior to sequencing. Several clones containing a 1.5-kb insert were isolated and sequenced by using dideoxy nucleotid method [15]. Sequence similarities were determined by using Gene bank database and BLAST software at http://www.ncbi.nih.gov/blast.

Enzyme assay:

Preparation of cell extracts:

Bacteria cells from 50 mL MS medium culture containing 50 ppm toluene were removed by centrifugation at 4,000 g for 20 min at 4°C and suspended in Tris-HCl buffer (50 mM; pH 7.5) containing 1 mM Ascorbate (buffer A). After centrifugation at 4,000 g for 20 min, the cells were suspended in 9 ml buffer A. The cells were broken by sonication, and the debris was removed by centrifugation at 10000g for 60 min at 4 °C [8]

Assay:

Catechol 2, 3-dioxygenase was measured by a modified method of Nozaki (16). The reaction mixture contained 50 µmol of phosphate buffer (pH 7.4) and 0.1 µmol of catechol in a total volume of 1 ml. After addition of enzyme, the increase in optical density at 375 nm (corresponding to the formation of 2-hydroxymuconic semialdehyde) was measured in a silica cuvette with a 1.0-cm light path. One unit of activity was defined as the amount of enzyme required to form 1 µmol of product per min under the assay conditions Activity condition (pH, temperature) and substrate specificity of the enzyme were also determined [9,12, 7].

Results:

Isolation and identification of toluene-utilizing bacteria:

In screening soil samples from Isfahan oil refinery, 8 promising toluene-utilizing isolates were selected that grew in the presence of toluene vapor in the toluene containing MS medium. The most tolerant strain from soil sample was selected for further studies.

The highly tolerant isolate grew in the presence of 10% toluene vapor and 200 mg l−1 toluene in the MS medium. The toluene-utilizing isolate with the highest toluene utilization, chosen for further characterization.

Biochemical and biological properties as well as 16S rDNA sequences of the selected isolates were considered to identify the taxonomy of the strains. The compiled data indicated that the M and I isolates should be the strains of Bacillus cereus ANY and B.pumilus species, respectively. 16S rDNA sequences of the strains (Gene bank accession No GQ375259, GU 191909) were in agreement with the biochemical tests. 16S rDNA sequencing showed this strain has 99% B. pumilus species.
The optimum conditions also was done for growth this bacteria. The suitable temperature for growth B.pumilus is 25°C conditions respectively, PH=7, 150 RPM and low concentration of toluene are the best condition for maximum growing of this bacteria.

**Enzyme assay:**

Assay of catechol-2, 3-dioxygenase activity was done. Reaction mixture was incubated for 72 hours at 30°C. Optical densities were measured every 12 hours. Enzyme showed maximum activity in 24 hours after incubation and constant up to 36 h, and then it decreased.

The effect of pH on the catechol-2, 3-dioxygenase activity was investigated by varying the pH of buffer in the range of 5-8, keeping all other parameters constant (temperature 30°C, toluene concentration and incubation 2 h). The results showed that the enzyme activity increased up to 7 and then decreased when pH increased. The enzyme had maximum activity in pH=7.6. Also the effect of temperature on activity of catechol-2,3-dioxygenase was measured by varying the temperatures of incubation in range of 25°C to 80°C in optimal conditions. Results show that in temperature 50°C B.pumilus has the maximum the rate of activity.

**Discussion:**

In this study, 8 strains was isolated from oil contaminated soils, used toluene as the only source of carbon and energy. The highly tolerate isolates; B.pumilus could grow in presence of highest concentration of toluene (200 mg l⁻¹)

Ability of degradation toluene was investigated through assay of enzyme catechol 2, 3 dioxygenase converted Catechol to 2-hydroxymuconic semialdehyde and give yellow color. The intensities of color generation indicate the total activity of enzyme that was monitored by spectrophotometer at wavelength 375 nm (17, 18).

Assay of enzyme for 8 isolates indicated Bacillus pumilus has the Maximum activity among 8 isolates (about 0.12U/mL), enzyme activity for other isolates were beween 0.01_0.08 U/mL.

In the same study ability of isolates; B.cereus, B.pumilus and Bacillus megaterium to degrade the broad range of aromatic substrates was investigated through the assay of specific activities of phenol hydroxylase, catechol 1, 2-dioxygenase and catechol 2, 3-dioxygenase. Results determined the Meta pathway used for metabolized phenol in B.cereus, B.pumilus [19,20].

In Zaki's study, Catechol2, 3-dioxygenas activity was detected in the cells of isolates; Bacillus, Stenotrophomonas, Klebsiella and Ralstonia [9].

In Mathur et al. research, assay of oxygenase in thermophilic isolates and optimal conditions for enzyme activity was investigated. Results show that in three of bacteria, oxygenase has maximum activity in pH=7.6 and 50 °C and one of them in pH =6.8 and 60 °C [12]. In this study optimal condition are pH=7 and temperature 50 °C for B.pumilus.

Results from the present study show that this isolate has a good potential to utilize the toluene in high concentration and if the optimal conditions were prepared, this strain can be used for bioremediation goals efficiently.

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**REFERENCES**


