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Research article

BIODEGRADATION OF TOLUENE HYDROCARBON BY A *PSEUDOMONAS* SP. ISOLATED FROM GASOLINE CONTAMINATED SOIL

Pratheesh P.T.* and Jayachandran K.

School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala, India - 686 560.
* Corresponding author, e mail: pratheeshpt@yahoo.com

ABSTRACT: In the present study efforts were made to isolate and characterize bacteria capable of aerobic biodegradation of toluene hydrocarbon from gasoline polluted soil. After initial screening, 28 different strains were isolated from soil samples and the most promising strain was selected for toluene degradation study. Biochemical and morphological characterization classified the bacterial strains selected as *Pseudomonas* sp. and was designated *Pseudomonas* sp. SBCT-17. The spectrophotometric determination of toluene monoxygenase activity, using horseradish preoxydase (HRP) assay showed 0.248 U/ml enzyme activity at 420 nm. The results obtained from enzyme assay and FT-IR degradation analysis in this study confirms that the *Pseudomonas* sp. SBCT-17 is an efficient strain capable of biodegrading toluene hydrocarbon.
Keywords: Biodegradation, toluene, gasoline pollution, horseradish peroxidase (HRP)

INTRODUCTION

Industrial revaluation has created environmental pollution and hazardous waste water, which is of a major concern for mankind. Gasoline induced pollution is one of the major problems that we are facing at present in environmental pollution. Even small releases of petroleum hydrocarbons into aquifers can lead to concentrations of dissolved hydrocarbons far in excess of regulatory limits [1]. Gasoline leaking from underground storage tanks, distribution facilities and various industrial operations represents the prime source of air, water and soil contamination. While Accidental and deliberate crude oil spills have been, and still continue to be a significant source of environmental pollution, and poses a serious environmental problem [2]. Fortunately, such incidents occur rarely, although they can result in significant contamination of ocean and shoreline environments. The most common oil based pollutants are benzene, toluene, ethylbenzene and mixture of xylene, BTEX compounds (Fig.1). BTEX compounds are monoaromatic hydrocarbons [3], they are classified as major pollutants with high frequencies of occurrence on the EPA list of priority pollutants [4].

Toluene is a methyl substitution on the aromatic benzene ring, is distributed in water, soils and industrial effluents [5]. Ground water contamination pollution is the major source of toluene [6]. Toluene is used as a solvent for paints, coatings, gums, oils, and resins. Maximum contaminant level (MCL) of toluene as per EPA is 1 mg/L. Exposure to humans can occur by either ingestion (drinking water from contaminated wells), or by inhalation (exposure to toluene contaminated water via showering or laundering). Acute exposure to gasoline and its components benzene, toluene, and xylenes has been associated with eye, nose, and throat irritation; headaches, loss of coordination, nausea; damage to liver, kidney, and central nervous system, and effects on the respiratory system [7]. Prolonged exposure to these compounds also affects these organs as well as the kidney, liver and blood systems. According to the EPA, there is sufficient evidence from both human epidemiological and animal studies that benzene is a human carcinogen. Workers exposed to high levels of toluene in occupational settings were found to have an increase in leukemia.

The process of using microbes to degrade environmental contaminants to non-harmful or less harmful products is termed as microbial biodegradation. It can occur aerobically or anaerobically. Aerobic or oxidative degradation occurs when the pollutant is oxidized using oxygen, nitrogen, iron, sulphate and manganese by the microbes and the pollutant acts as electron donor [8]. Anaerobic or reductive degradation occurs when pollutant is reduced by the microbes. The biodegradation of toluene has been well-studied at the molecular level and it thus, serves as one of the principal models for understanding the mechanisms of bacterial benzene ring metabolism [9].

The oxidative microbes degrade toluene via hydroxylation of the aromatic ring to a mixture of catechols and cresols [10]. Toluene monooxygenase, benzyl alcohol dehydrogenase, benzaldehyde dehydrogenase and catechol- 2,3-dioxygenase are enzymes involved in the degradation of toluene and are organized in two different pathways. The upper pathway codes enzymes for the conversion of aromatic alcohol to acid, while the lower pathway enzymes involved in the aromatic acid metabolism via an ortho and meta pathway [11]. The second pathway involves ring hydroxylation, yielding methyl catechols as the metabolic intermediate. The key enzyme involved in this pathway is toluene dioxygenase [12]. The aim of this study was to isolate and characterize a bacterial strain capable in degrading toluene, from gasoline contaminated soil. A simple method was used in assaying toluene dioxygenase activity, which use horseradish peroxidase (HRP) as the key compound that monitor enzyme activity by color intensity.

MATERIALS AND METHODS

Sample collection

The microorganisms used for this study were isolated from the soil sample collected from gasoline spill sites of diesel locco shed, Railway station at Thiruvananthapuram and from waste disposal ditches at Kochi Refineries Ltd. (KRL), Cochin. Kerala.

Screening of toluene tolerating strains

10 g soil collected from the gasoline contaminated area was mixed with 90 ml of sterilized distilled water containing 100 µl toluene was incubated in a rotary shaker at 150 rpm at room temperature (30 ± 2°C) for 24 hours. The soil samples were subjected to serial dilution using sterile distilled water and were pour plated with nutrient agar medium. The plates were incubated at room temperature for 48 hours and the colony count was taken. After two days of regular intervals 1ml of solution was taken out and serially diluted to isolate the organism. The concentration of toluene in the medium was increased by 100 µl each, and the process of serial dilution and increasing the concentration of toluene was continued till the concentration in stock reached 1ml.

Screening of the toluene degrading strains

The colonies isolated by soil enrichment technique were individually inoculated into 10ml of the Mineral salt toluene medium (MSTM) with 1mM toluene concentration. The composition of the basal medium used was 1g KH_2PO_4, 1g $(NH_4)_2SO_4$, 0.5 g Mg SO_4. $7H_2O$ and 0.001g $CaCl_2$ in 1 litre of the distilled water at pH 7**.** The tubes were incubated on a rotary shaker at 150 rpm at room temperature (30 ± 2°C) for 2 days .The isolates, which showed growth in the broth and also on plating with toluene containing nutrient agar medium were used to inoculate individually into MSTM with 2 µL toluene concentration. The same procedure was repeated and the isolates, which showed growth, were inoculated into MSTM with 5mM toluene concentration. The same procedure was repeated with 10 µl toluene containing MSTM. The colonies, which showed growth in MSTM enriched with 10 µl toluene concentration and the best was selected as the toluene degrading strain for the present study.

Morphological and biochemical characterization

The isolated bacterial strain was characterized based on morphological features and biochemical properties. The results were compared with Bergey's manual of determinative bacteriology [13]. The bacterial strain isolated from gasoline contaminated soil sample was tentatively identified and designated.

Optimization of growth in MSTM

The growth conditions for isolated strain was standardized using different pH (4,5,6,7,8 and 9) and temperature (25,30,35,40 and 45) conditions. The optimum growth condition for isolated strain in MSTM were standardized, and was used for the toluene degradation study.

Toluene oxygenase assay by HRP method

Toluene dioxygenase-peroxidase coupling reaction was used for degradation analysis of toluene. The enzyme assay contains 500 µl cells suspension, 450 µl sodium phosphate buffer (50 mM) and 50 µl toluene. The negative control contains 500 µl cells suspension, 450 µl sodium phosphate buffer and 50 µl distilled water. The mixture was incubated in a screw-capped bottle at 30 °C with shaking for 3 hrs at 150 rpm.

After incubation, the sample was centrifuged at 14,000 rpm for 5 min to separate the cells from the supernatant. The supernatant was transferred into sterile eppendorf tubes, and after the addition of 5 µl of 0.1 ml H_2O_2 and 10 units of horseradish peroxidase (HRP), a reddish brown color was observed. The color production was allowed to proceed at room temperature for 30 minutes and intensity of the mixture was measured at 420 nm using a spectrophotometer [14]. The intensity of colour production is directly proportional to the concentration of catechol production.

FT-IR analysis

The degradation studies were carried out at 1000 ml of the MSTM under optimized conditions. After optimum incubation time the cells were removed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was extracted repeatedly with ether and the pooled ether extract was concentrated by evaporation. The concentrate ether extract was given for FT/IR analysis (Thermo Nicolet, Avatar 370).

RESULTS AND DISCUSSION

The present study was focused on isolation and characterization of an efficient bacterial strain capable of toluene biodegradation. 28 morphologically different strains were isolated from the gasoline contaminated soil sample by soil enrichment technique, capable of tolerating 1 ml of toluene concentration. These 28 cells were screened for its growth capacity in MSTM at increasing concentration and the most efficient strain was selected and used for further studies. Based on the biochemical and morphological studies the selected strain was identified as *Pseudomonas* sp. on comparison of the characters observed with Bergey's manuel of determinative bacteriology the bacterial strains, and was designated as *Pseudomonas* sp. SBCT-17 (Table –1).

Table 1 Biochemical and morphological characteristics of toluene degrading bacterial strain isolated from soil.

Biochemical characteristics		Morphological characteristics	
Growth on MacConkey Agar	+	Configuration	Round
Indole test	-		
Methyl red test	-	Margin	Entire
Voges proskauer test	+		
Citrate utilization	+	Elevation	Convex
Casein hydrolysis	-		
Starch hydrolysis	-	Surface	Smooth
Urea hydrolysis	-		
H_2S production	-	Pigments	-
Catalase test	+		
Gelatin hydrolysis	-	Gram-reaction	-
Oxidation/Fermentation	-		
Arginine dihydrolase	+	Shape	Rod
Lysine decarboxylase	-		
Nitrate reduction	+	Motility	+
Nitrite reduction	-		

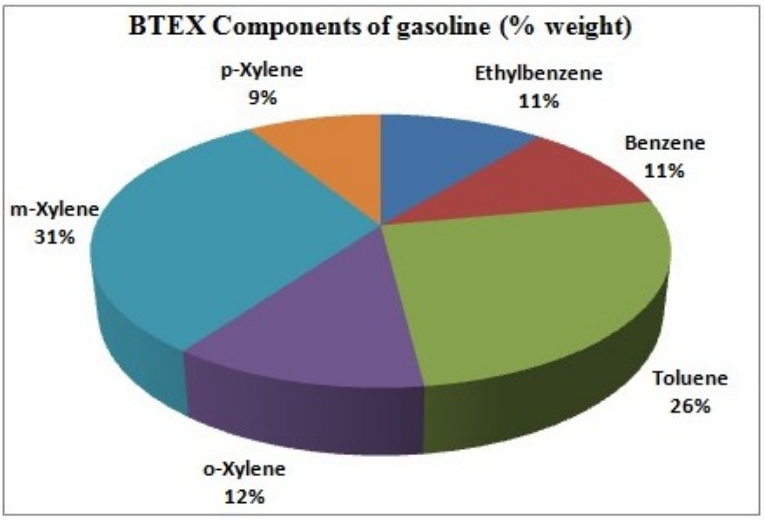

Fig. 1 Components of BTEX compounds in gasoline pollution.

The effect of substrate concentration on the toluene degradation (Figure 2) showed that up to 25 µl /100 ml concentration, degradation was 100% and any further increase in the toluene concentration from 20 µl/100 ml, resulted in decrease in the percentage of toluene degradation. 20 µl/100 ml concentration was thus selected as the optimum toluene concentration for the biodegradation studies with the isolated *Pseudomonas* strain.

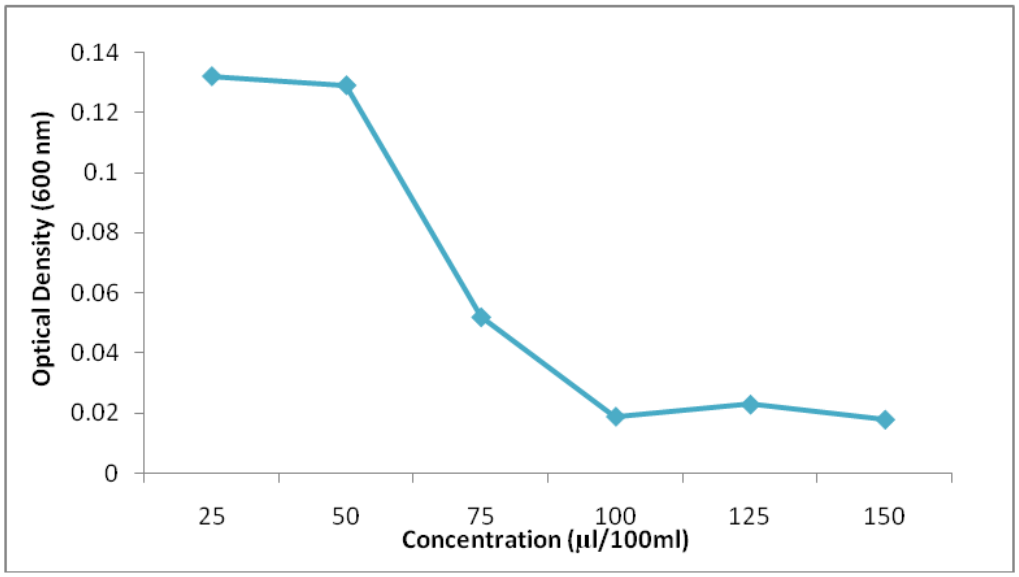

Fig. 2 Effect of substrate concentration on the degradation of toluene by the *Pseudomonas* sp. SBCT-17 in mineral salt media after 32 hrs of incubation.

Growth condition for *Pseudomonas* sp SBCT-17 in MSTM wwere evaluated at different pH ant temperatures, pH 7 (Figure 3) and 35 ºC (Figure 4) were found to be ideal for the study. The *Pseudomonas* sp SBCT-17 showed a steady increase in the degradation of toluene along with the increase in the incubation time and indicated 100% degradation after 32 hrs (Figure 5), which was considered as the optimum condition for the biodegradation of toluene. Growth studies of *Pseudomonas* sp SBCT-17 in MSTM revealed that a maximum cell concentration could be achieved in 32 hrs, which was also the optimum time for the maximum biodegradation of toluene.

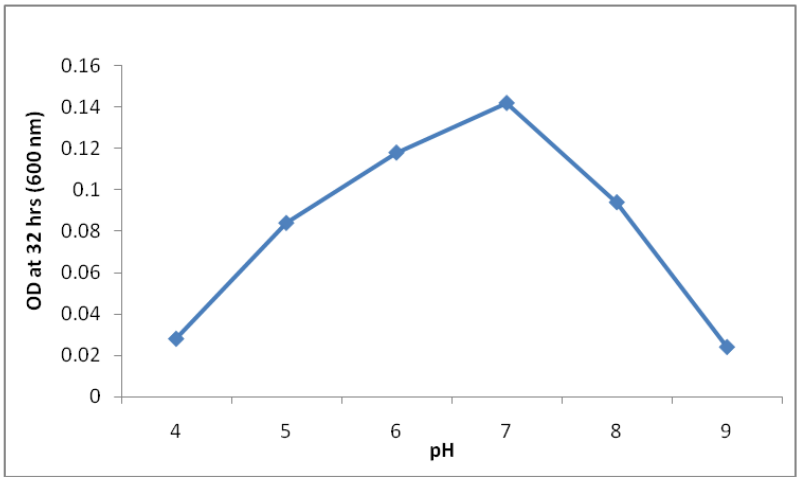

Fig. 3 Effect of pH on the growth and toluene degradation by the selected *Pseudomonas* sp. SBCT-17 in MSTM.

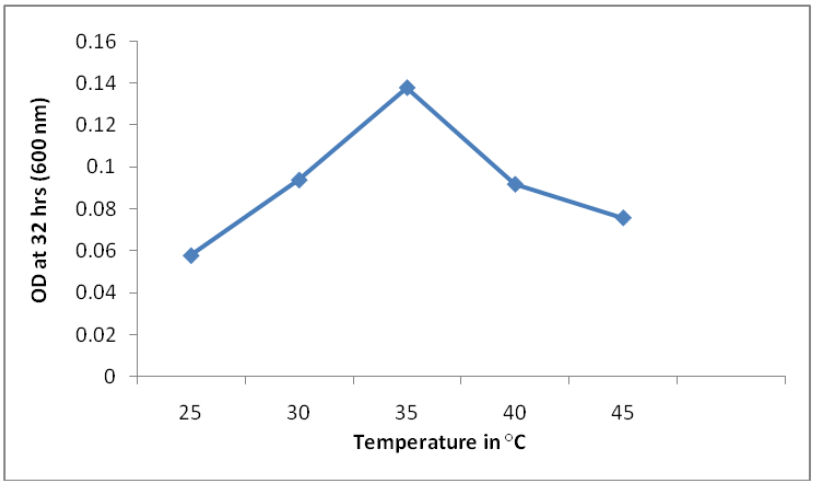

Fig. 4 Effect of temperature on the growth and toluene degradation by the selected *Pseudomonas* sp. SBCT-17 in MSTM.

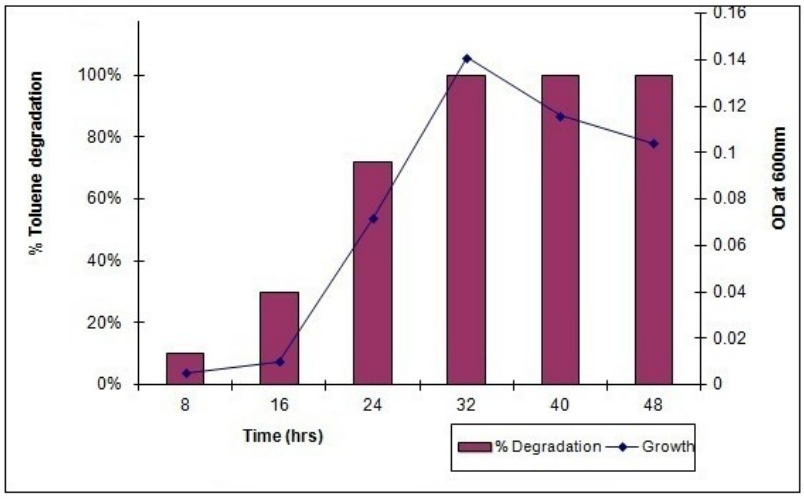

Fig. 5 Biodegradation of toluene with respect to growth of *Pseudomonas* sp. SBCT-17

The toluene degradation ability of isolated *Pseudomonas* sp. SBCT-17 was monitored using horsersdish peroxidase (HRP) method. HRP was coupled with hydroxylation of aromatic substrates which give colored compounds. In the present study toluene was converted into catechol when it was added to cell suspension. Catechol reacted to H_2O_2 and also HRP, which produced the colored product that can be monitored by spectrophotometer at wavelength 420 nm. The intensity of the color generated indicates the total activity of oxygenase [14, 15]. The *Pseudomonas* strain isolated in this study showed an enzyme activity 0.248 U/ml on HPR assay.

The analysis of the degraded products of toluene by *Pseudomonas* sp. SBCT-17 was done by FT-IR. It was done to confirm the changes of the degraded product with respect to chemical structure and the functional groups of the parent compound due to biodegradation. In the FT-IR analysis an uninoculated medium extracted with ether was used as the control, the mega absorption band at 3324.77 cm^{-1} in control is due to aromatic ring of toluene (Fig. 6a). The biodergraded sample sample was also ether extracted and tested. In the FT-IR result the change in chemical structures of the aromatic compound was evident (Fig. 6b). The FT-IR results confirm the degradation on toluene by *Pseudomonas* sp. SBCT-17. The current work is only a stepping stone in understanding the degradative properties of the bacterial strains isolated and more studies need to be conducted to exploit the properties of the organism.

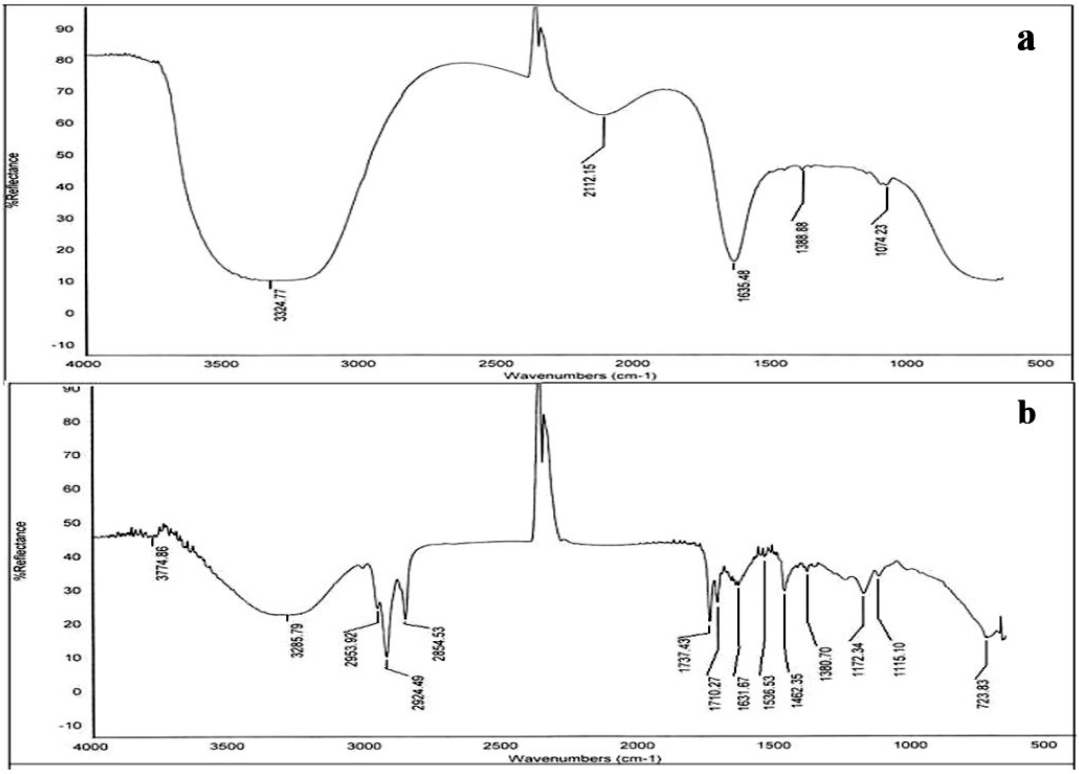

Fig. 6 FT-IR analysis of toluene biodegradation by *Pseudomonas* sp. SBCT-17

CONCLUSION

The biodegradation of monoaromatic compounds has been studied in detail in the last several years. The *Pseudomonas* sp. SBCT-17 isolated from gasoline polluted soil in this study is a highly potent strain. The results of the work suggest that the organism is capable of effectively degrading toluene hydrocarbon, this property can very well be utilized for the wastewater treatment and biodegradation processes in large scale to protect environment from toluene pollution.

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