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Biodegradation of Some Aromatic Hydrocarbons (BTEXs) by a Bacterial Consortium Isolated from Polluted Site in Saudi Arabia

Mohamed A. Arafa
Biotechnology Laboratory, Petroleum and Petrochemicals Research Institute,
(KACST), Riyadh, Saudi Arabia

Abstract: A mixed bacterial culture was isolated from heavily polluted site in Eastern Region, Saudi Arabia to evaluate the potential for the biodegradation of benzene, toluene, ethylbenzene and xylenes (BTEXs). The isolated bacterial strains in this study could grow in mineral salt medium containing a mixture of BTEXs as the sole source of carbon and energy. The effect of environmental factors such as agitation, incubation temperature and medium pH on the degradation rate of BTEXs was investigated. Substantially, a greater extent of biodegradation of all the BTEXs compounds was observed in stirring liquid culture than in static. As well as, the level of degradation was relatively higher at 37°C than 45°C or 30°C and at medium pH value 7 than 8 or 6 after 21 days incubation. Toluene, ethylbenzene and xylenes were completely degraded by the mixed bacterial culture. However, a bacterial limitation concerning benzene was observed. Results obtained indicate that the mixture of microorganisms present in this study could contribute significantly to bioremediation of aromatic hydrocarbons (BTEXs) pollution.

Key words: Biodegradation, bioremediation, aromatic hydrocarbons pupation, bacterial consortium

Introduction

Petroleum hydrocarbons are common ground water pollutants as a result of leaking under-ground storage tanks and spills, release of petroleum hydrocarbons in the environment from leaks of storage tanks pollutes the soil, many petroleum hydrocarbons are soluble in water and bind strongly to soil.

A major risk of petroleum hydrocarbons release is contamination of drinking water sources by the mobile petroleum hydrocarbons, which can migrate through the soil matrix (Leahy and Colwell, 1990; Alvarez and Vogel, 1991; Dowd, 1994 and McLinn and Rehm, 1995). Biological treatments for the removal of these organic compounds from contaminated water, soil and reactors are based on the action of degrading microbial communities, so detailed knowledge of the microbiology of petroleum hydrocarbons must be clear and understood to evaluate both the biodegradability of the most petroleum hydrocarbon compounds and the specific degradative activities of the different microflorae (Solano–Serena *et al.,* 1999; Cavalca *et al.,* 2000). Benzene, Toluene, Ethylbenzene and Xylenes compounds usually are termed BTEXs; these compounds are volatile monoaromatic hydrocarbons, which are commonly found in crude petroleum and petroleum products. They are also produced on the scale of megatons per year as bulk chemicals for industrial use as solvents and starting materials for the manufacture of pesticides, plastics and synthetic fibers (Harwood *et al.,* 1997). They are considered one of the major cause of environmental pollution because of widespread occurrences of leakage from underground petroleum storage tanks and spills at petroleum production wells, refineries, pipelines and distribution terminals (Fries *et al.,* 1994). Some estimate that 35% of the 1.4 million gasoline storage tanks in the US are leaking (Harwood *et al.*, 1997). Contamination of groundwater with BTEXs compounds is difficult to remedy because these compounds are relatively soluble in water and can diffuse rapidly once introduced into an aquifer. Techniques for *in situ* bioremediation of the BTEX compounds are used to eliminate or reduce contamination levels in an aquifer. The biodegradability of the BTEX has been established using some pure bacterial strains or complex microflorae (Mallakin and Ward, 1996; Matteau and Ramsay, 1997); but little is known about the other microbial strains capacity, especially those which are wide–spreading in environment of Saudi Arabia. This work describes the capability of a degrading microbial consortium isolated from a polluted site in Saudi Arabia.

Materials and Methods

Chemicals: Benzene (purity, 99%), toluene (purity, 99.5%), ethylbenzene (Purity, 99%), o-xylene (purity 98%), m-xylene (purity 99%) and p-xylene (purity 99%) were purchased from sigma – Aldrich Chemie GmbH (D-82039

Deisenhofen, Germany). All other chemicals were of analytical grade and the highest available purity.

Enrichment procedure: Three of polluted soil, water and oily sludge samples were collected from Eastern Region, SA, used to isolate and select a bacterial consortium for the bioremediation of the soil contaminated by (BTEXs) benzene, toluene, ethylbenzene and xylenes in this study. All samples were mixed in one bulk, a total of 100g of whole sample was added to 900 ml of (0.85%) sterile NaCl solution and shaked for 6 hours at 30°C. the soil suspension (25 ml) was seeded in a flask containing 225 ml of the vitamin – supplemented mineral salt medium (nutrient solution) described by Izumi *et al.* (1994) with slight modification (Arafa, 2001); whereas BTEXs (600 ppm) mixture (which contained equal volumes of the individual hydrocarbons) was added to the nutrient solution as a sole source of carbon and energy instead of glucose, and incubated at 30°C for 7 days with alternative shaking (100 strokes per min). the culture was adapted to degrade the aromatic hydrocarbon mixture (BTEXs) by re-incubating 10 ml of the microbial cell suspension in 90 ml of the nutrient solution supplemented with 600 ppm of BTEXs mixture, then incubated at 30°C, this step was repeated twice.

Microorganisms identification: After performing a dilution series under aseptic conditions, 1 ml of the microbial cell suspension was plated onto nutrient agar. After 5 days incubation at 30 and 45°C, colonies that appeared on the plates were isolated. The bacterial isolates were identified using the API 20 NE system (BioMerieux, Marcyl'Etoile, France) and Biology Identification System (Biology Inc. Hayward, Calif.) according to the manufacturer's instructions. As well as, the isolates were sent to the (DSMZ) Deutsche Sammlung von Microorganismen und Zellkulturen GmbH; Mascheroder Weglb D-3300; Braunschweig; Germany, MIDI Labs; Newark; DE 19713;USA, (NCPPB); Sand Hutton; York; Y041 1LZ; United Kingdom and Bacterial Strain Identification and Mutant Analysis Service, 209 Life Sciences Bldg., Auburn University, Al 36849-5409, US. Strains were maintained in 20% glycerol stock at – 20°C after growth on aromatic compounds.

Biodegradation of BTEX by bacterial mixture: The biodegradation experiments were performed by using bioreactor (B. BRAUN'S multiple fermentor system BIOSTAT Q), which was equipped with temperature, airflow supply, pH and stirrer speed controller system. 500 ml culture vessels containing 100 ml of the vitamin – supplemented mineral salt medium were used to carry out the biodegradation experiments. 600 ppm of BTEXs were added to the culture vessels. Inocula were prepared from fresh cultures of the stock strains after growth for 48 h. on the BTEXs supplemented medium described before, cells were harvested by centrifugation for 10 min. at 15000 g and resuspended the centrifuged biomass in 10 mol l^{-1} phosphate buffer, pH 7.0 to obtain an $O.D_{600}$ of 0.7. 1 ml of the mixed bacterial cells suspension was used to incubate the medium in each culture vessel. The centrifuged biomass could be used immediately or stored at – 80°C for several months without significant loss of degradation activity. After different incubation times at different pH values and temperature degrees with and without stirring cultures, appropriate samples and control vessels were used to determine the extent of the BTEXs degradation. Samples were mixed with an equal volume of acetonitrile and the remaining hydrocarbons were extracted for 1h. under shaking. The cells were removed by centrifugation (15000 rpm for 5 min.) and 10 ml of the supernatant of each sample was injected into a HPLC [Shimadzu series 10A, SPD – 10Ai, UV-VIS detector, SIL – 10 Ai – auto injector] and detected at 246 nm. Experiments were performed in triplicate and abiotic controls were run under similar conditions.

Results and Discussion

Bacterial isolation and identification was achieved at the end of the experimentation steps of enrichment process to give an indication of the bacterial species present at steady – state conditions. 11 strains were isolated according to their different colony morphology to briefly describe the diversity of bacterial genera present in the consortium. The isolates were identified to belong to species using the API 20 NE system and Biology Identification system and then the identification was confirmed by (DSMZ) Germany, (MIDI labs) USA, (NCPPB) UK and (BSIMAS) US. The bacteria isolated were *paenibacillus pabuli, Micromonospora* spp., *Proteus mirabilis, Bacillus pumilus, Burkholderia* spp., *Xanthomonas* spp., *Bacillus coagulans, Bacillus stearothermophilus, Bacillus pallidus, Bacillus smithii* and *Klebsiella pneumoniae*. Although this information does not provide knowledge as to the relative contribution of each species to degradation of each component of BTEXs mixture. It is likely that some of the identified species were responsible for BTEXs components degradation, whereas other may metabolize the by-products of BTEXs components degradation or utilize intermediates of hydrocarbons degradation, playing a key role in production of biosurfactants, which are recognized to enhance the degradation of aliphatic and aromatic hydrocarbons in microbial populations. The

result of isolated species provides useful information for comparison with other studies, some of the isolated bacteria in this study have been reported to degrade a wide range of hydrocarbons, whereas others have not been reported (Hook *et al.,* 1992; Golovleva *et al.,* 1992; Desai and Banat, 1997; Cavalca *et al.,* 2000).

The Kinetics of BTEXs degradation were first investigated by determining the residual components of BTEXs through experimental period, which reached to 21 day. Liquid culture studies in bioreactor were conducted to determine BTEXs degradation under different environmental conditions. In order to study the effect of different temperature degrees on BTEXs degradation, 21 day experiments in a fermentor at 30, 37 and 45°C with culture media pH 7 were carried out as described in materials and methods. The obtained results are presented in Figs. 1, 2 and 3, the kinetics of BTEXs degradation were observed. At end of the 3rd day, the degradation rate percentage of benzene, toluene, ethylbenzene and xylene isomers (o-x, m-x and p-x) reached to 4, 52, 41, 32, 35 and 42% at 30°C; 8, 70, 61, 43, 45 and 52% at 37°C and 6, 62, 50, 35, 42 and 51 at 45°C respectively. It is clear from the results in Table 1 that the degradation of the hydrocarbon compounds except benzene was fast through the first 3 days, followed by a slow degradation rate till end of the experiment of 21 days.

Figs. 1, 2, 3 and Table 2 show the residual amounts of the BTEXs components in culture media during the experimental period of 21 day incubation with a bacterial consortium in the bioreactor for abiotic and for test vessels at 30, 37 and 45°C. also the mentioned results indicate that the degradation activity of the bacterial consortium was better at 37°C than at 30 or 45°C. Fig. 4 shows the effect of culture media pH on the biodegradation activity of the bacterial consortium for BTEXs components. It is clear from the data in Fig. 4, that there are significant differences of degradation activity values between the three treatments 6, 7 and pH 8 when the culture media pH values in the fermentor vessels were adjusted at 6, 7 and 8 during the experimental period of 21 days. Also the results in Fig. 4 indicate that the biodegradation activity of the bacterial consortium was superior at pH 7 than at pH 6 or pH 8.

In an experiment to study the effect of agitation in the culture media on the biodegradation activity of bacterial consortium under investigation, fermentor vessels were supplied with stirrers and air flow rate at 70 standard liter per minute (slpm) and other vessels were not supplied, the result of Fig. 5 indicates that, a greater extent of biodegradation of all the BTEXs components was observed in the stirring liquid cultures than in the static liquid cultures, as well as supplying with air flow was

Table 1: Degradation of BTEXs after 3 and 21 day of incubation at 37°C and pH 7

		Degradation rate (%)♣	
BTEXs components	Initial amount (ppm)	* After 3d	* After 21d
B	100	8	25
T	100	70	99
E	100	60	99
oX	100	40	90
mX	100	45	99
pX	100	50	99

♣ The degradation rate was determined as the amounts of BTEXs degraded to the initial amounts. * Mean value of three replicates

Table 2: Residual BTEXs in cultures after 21 day of incubation for abiotic and for test vessels at different temperature degrees and pH 7

	Amount in abiotic vessels (ppm)*			Amount in test vessels (ppm)*		
Components	30°C	37°C	45°C	30°C	37°C	45°C
B	98	97	96	84	75	78
T	99	99	99	0	0	0
E	99	98	98	7	0	0
oX	99	99	99	30	10	20
mX	99	99	99	15	0	6
pX	99	99	99	10	0	5

* Mean value of three replicates

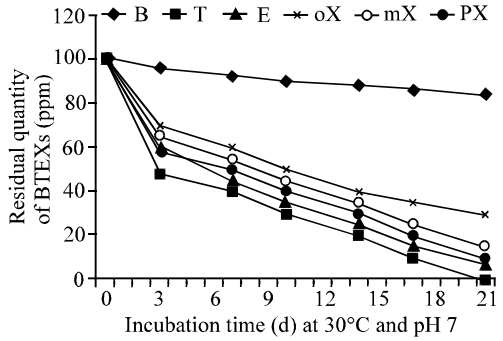

Fig. 1: Kinetics of BTEXs degradation during the course of the experiment

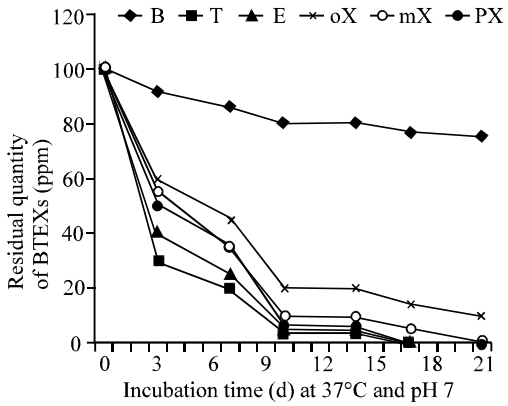

Fig. 2: Kinetics of BTEXs degradation during the course of the experiment

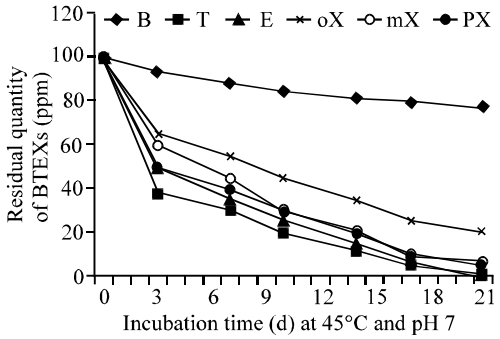

Fig. 3: Kinetics of BTEXs degradation during the course of the experiment

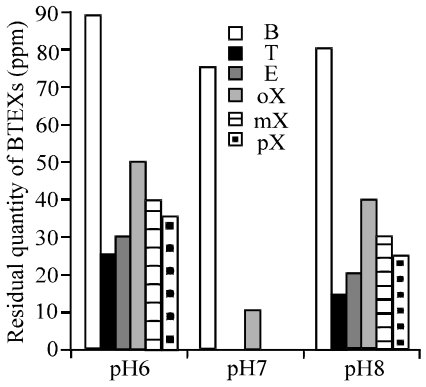

Fig. 4: Effect of culture media pH on the BTEXs degradation
The final amounts of BTEXs were determined after 21 days of incubation at 37°C

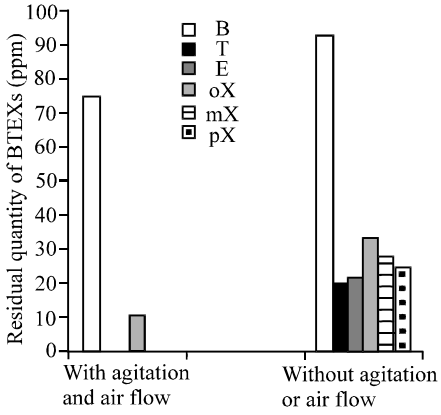

Fig. 5: Effect of agitation and airflow on the degradation of BTEXs
The final amounts of BTEXs were determined after 21 days of incubation at 37°C and pH 7

enhanced the biodegradation of the bacterial consortium, Fig. 5 shows the residual amounts of BTEXs components after 21 day incubation with agitation and air flow supplement, as well as without agitation or air flow supplement.

Finally, the BTEXs test appears quite useful to assess the potential for natural attenuation of polluted sites. Concerning the limitation in degradative capacities observed for benzene, the test allowed a fine discrimination of the specific capacities for the degradation of toluene, ethylbenzene and xylene isomers. From an applied point of view, the results indicate that, for remediation purposes, the microbiological treatment has to take into account the slow degradation of benzene and o-xylene (Zhou and Crawford, 1995; Nielsen *et al.*, 1995; Yerushalmi and Guiot, 1998; Solano–Serena, 1999). Results obtained indicate that the mixture of microorganisms present in this study could contribute significantly to bioremediation of aromatic hydrocarbons pollution.

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