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DEGRADATION OF AROMATIC PETROLEUM HYDROCARBONS (BTEX) BY A SOLVENT TOLERANT BACTERIAL CONSORTIUM

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Abstract: Petroleum aromatic hydrocarbons like benzene, toluene, ethyl benzene and xylene, together known as BTEX, has almost the same chemical structure. These aromatic hydrocarbons are released as pollutants in the environment. This work was taken up to develop a solvent tolerant bacterial consortium that could degrade BTEX compounds as they all share a common chemical structure. We have isolated almost 60 different types of bacterial strains from different petroleum contaminated sites. Of these 60 bacterial strains almost 20 microorganisms were screened on the basis of capability to tolerate high concentration of BTEX. Ten different consortia were prepared and the compatibility of the bacterial strains within the consortia was checked by gram staining and BTEX tolerance level. Four successful microbial consortia were selected in which all the bacterial strains concomitantly grew in presence of high concentration of BTEX (10% of toluene, 10% of benzene 5% ethyl benzene and 1% xylene). Consortium #2 showed the highest growth rate in presence of BTEX. Degradation of BTEX by consortium #2 was monitored for 5 days by gradual decrease in the volume of the solvents. The maximum reduction observed was 85% in 5 days. Gas chromatography results also reveal that could completely degrade benzene and ethyl benzene within 48 hours. Almost 90% degradation of toluene and xylene in 48 hours was exhibited by consortium #2. It could also tolerate and degrade many industrial solvents such as chloroform, DMSO, acetonitrile having a wide range of log P values (0.03–3.1). Degradation of aromatic hydrocarbon like BTEX by a solvent tolerant bacterial consortium is greatly significant as it could degrade high concentration of pollutants compared to a bacterium and also reduces the time span of degradation.

Keywords: BTEX degradation; solvent tolerant; microbial consortium

INTRODUCTION

Release of petroleum hydrocarbons in the environment has of late attracted the researchers. One particular concern is the contamination of drinking water sources by the toxic, water soluble and mobile petroleum components like, benzene, toluene, ethyl benzene and xylene (BTEX). Some BTEX compounds persist in the environment at levels exceeding regulatory thresholds (Anneser *et al*., 2008).

They are widely used chemical substances in several industrial processes (Lin *et al*., 2010), besides being present in high amounts in fossil fuels (ASTDR, 2004), what determines contamination of atmosphere, soil and waters. The motility rate of such hydrocarbons in soil-water systems is related to their low octanol–water partition coefficient that leads to slow soil absorption and, consequently, a preferential water transport, thereby favoring the contamination of water.

These compounds usually occur at trace levels in superficial waters as a result of their volatility. However, they can be found in high concentrations in groundwater and are considered as the priority contaminants of such resources (Falcó & Moya, 2007). The frequency of groundwater contamination with hydrocarbons, including BTEX, has been increasing (Reusser *et al.,* 2002), demanding the development of more efficient methods to remove or minimize the damages caused by these compounds. Several factors, such as pollutant concentration, active biomass concentration, temperature, pH, availability of inorganic nutrients and electron acceptors, and microbial adaptation, influence the rate and extent of biodegradation of BTEX.

Several studies have been carried out in order to find out efficient microorganisms for BTEX degradation, so they could be used in environmental remediation for this mixture. Affinity of a bacterial strain towards a particular hydrocarbon ensures a wide choice of degradation. As most of the aromatic hydrocarbon pollutants have a similar structure it is possible that the bacterial strain showing affinity towards one hydrocarbon shows affinity towards other related aromatic hydrocarbon. Degradation of benzene in the presence of other aromatic compounds was found to be stimulated by the presence of either toluene or o-xylene (Arvin *et al.*, 1989). Several researchers have reported that toluene is degraded more readily than benzene in aquifer systems (Wilson *et al.*, 1990). However, the opposite trend was observed in other field studies (Russer *et al.*, 2002).

Recent work shows that a single bacterium cannot degrade a wide range of organic solvents. In 2000 Harwood *et al*. reported that bacteria that can tolerate and degrade a particular organic solvent shows affinity towards other organic solvents having almost similar structure. Emphasis has been given on development of microbial consortia capable of degrading a wide variety of organic solvents (Mac Carthy *et al.*, 2001; Singh *et al.,* 2003).

Therefore, this study was taken up to develop a bacterial consortium that can effectively degrade high concentration of BTEX compounds in short time span. Further, solvent tolerance property of the consortium was determined in presence of various industrial solvents having a wide log *P* value.

MATERIALS AND METHODS

Microbial isolates

Soil samples were collected from different hydrocarbon rich areas in Siliguri, W.B., India. The soil samples were suitably diluted using standard serial dilution procedure and inoculated into 20 ml nutrient broth over laid with 1% v/v benzene in 100 ml sealed serum bottles and incubated at 37°C with constant shaking at 120 rpm orbital shaker for 5 days. 0.1% of these 5 days old inoculum were then plated in nutrient agar plates and incubated overnight at 37°C. Growing colonies were purified by repeated streaking on agar plates. The isolated strains were maintained on nutrient agar slants at 4°C.

Culture conditions

Inoculam of the strains were prepared by inoculating them in nutrient broth overlaid (v/v) with benzene (1–10%) in 100 ml sealed serum bottles and incubated at 37°C with constant shaking at 120 rpm orbital shaker. The cultures in absence of the solvent and uninoculated media enriched with the solvent were used as control under similar condition.

Screening of BTEX tolerance strain

BTEX tolerance by bacterial strains was determined by inoculating 20 ml nutrient broth supplemented separately with benzene (1–10%), toluene (1–10%), ethylene benzene (0.1–5%) and xylene (0.5–1%) in 100 ml screw cap flask with 1% overnight grown enriched culture and incubated at 37°C at 180 rpm. All the experiments were carried in duplicate.

Preparation of Bacterial Consortia

To prepare successful microbial consortium, bacterial cultures must be compatible with each other in order to concomitantly degrade BTEX. Ten different consortia were prepared and incubated overnight at 37°C in 120 rpm. The compatibility of the bacterial strains within the consortia was checked by gram staining (Sarkar *et al.*, 2011). Microbial consortium was prepared by inoculating 5ml of overnight grown bacterial strains in 20ml of nutrient broth overlaid with 1% (v/v) benzene.

Growth of consortia in presence of BTEX

One percent of the overnight grown enriched consortia, that showed tolerance towards high concentration of BTEX, were inoculated in 20 ml nutrient broth supplemented separately with benzene (10%), toluene (10%), ethylene benzene (5%) and xylene (1%) in 100ml screw cap flask and incubated at 37°C under shaking (180 rpm) for 48 h. Growth of the bacterial consortia in presence of BTEX was determined by increase in O.D. at 660nm at a constant time interval of 8 h till 48 h. The cultures in absence of the solvent and uninoculated media enriched with the solvent were used as control under similar condition. All the experiments were carried in duplicate.

DETERMINATION OF BTEX DEGRADATION

Gas chromatography analysis of BTEX Degradation

The degradation of the BTEX was analyzed using 1% of the enriched bacterial consortium. It was inoculated in four different 100ml capacity serum bottles filled with 20 ml of nutrient broth overlaid with 10% v/v of toluene & benzene, 5% xylene and 1% ethyl benzene separately. The bottles were then closed with Teflon-coated septa and aluminum caps and were incubated for 48h at 37°C under 180 rpm. Degradation of BTEX was monitored in Perkin–Elmer 900 gas chromatograph provided with a flame ionization detector. Separation was carried out on 181m X 0.76mm stainless steel open tubular column. The temperature was programmed 20°C–130°C at 2°C /min after an initial isothermal period of 6 min. The injection temperature was 120°C and detector temperature was 140°C. A Perkin-Elmer PEP1 data processor was used for quantification and the concentration of the volatile compound was determined as parts per billion (ppb v/v). Response factor according to Dietz (1967) was used. The cultures in absence of the solvent and uninoculated media enriched with the solvent were used as control under similar condition. All the experiments were carried in duplicate.

Degradation of other organic solvents

The solvent tolerance property was determined by inoculating the bacterial consortia in nutrient broth overlaid with different organic solvents (10% v/v) with log P_{ow} values ranging 0.28–4.5, such as isooctane, dimethylsulphoxide (DMSO), xylene, acetonitrile, toluene, benzene, chloroform, 1-butanol, 2-propanol and ethanol, incubated at 37°C with shaking at 140 rpm (Sarkar & Ghosh, 2012). Evaporation of solvent was prevented by plugging the flasks with butyl-rubber stoppers. Degradation was determined by measuring the volumetric reduction of solvent in the media after the bacterial growth. The bacterial culture growing in absence of organic solvent under similar conditions served as control. Growth and dry cell mass were monitored similarly as Sarkar & Ghosh (2012).

RESULTS AND DISCUSSION

Isolation and screening of BTEX tolerant strain

About 60 different bacterial cultures were isolated from the above mentioned sites in presence of 1% (v/v) benzene. Most of the isolated strains were gram positive rods. The isolated strains were further characterized on the basis of the BTEX tolerance level. 20 bacterial strains were screened that could tolerate high concentration of BTEX (**Table 1**). Similar result was also reported by Singh et al. in 2010. An environmental contaminant acts on the microflora of the ecosystem, eliminating or selecting microorganisms in accordance to sensitivity in the presence of the toxic agent.

Among the biota present in the contaminated site, microorganisms capable of using contaminants or just resisting their toxicity can be found (Mcnaughton *et al.*, 1999). These microorganisms are able to break down compounds to be used as energy source, thereby eliminating them from contaminated environments (Pedrozo *et al*., 2002). According to Kataoka (2001), the biodegradation of organic compounds is more efficient when the microorganisms in the inoculum are pre-selected and thus become potentially more adapted to target pollutants. As BTEX is a very toxic mixture, selection of microorganisms through enrichment were carried out in this work. This initial screening step was important for successful biodegradation because the selected microorganisms were adapted to BTEX mixture. Shokrollahzadeh *et al.* (2008) also used activated sludge microflora from a petrochemical industry treatment system to biodegrade hydrocarbon contaminated wastewater.

Table 1. Morphological characteristics and BTEX tolerance level by different bacterial strains

Bacterial strains	Gram Character	Benzene (%)	Toluene (%)	Ethyl Benzene (%)	Xylene (%)
Ps1	(+) rods	7	5	0.5	0.1
Ps2	(-) rods	10	10	5	0.1
Ps3	(+) rods	1	5	1	0.2
Ps4	(+) rods	1	2	0.2	0.1
Ps5	(+) cocci	7	10	2	0.2
Ps6	(-) rods	2	2	0.2	0.1
Ps7	(+) rods	5	7	5	0.1
Ps8	(-) rods	5	7	5	0.2
Ps9	(+) cocci	7	5	2	0.1
Ps10	(-) rods	7	5	2	0.1
Ps11	(+) rods	5	2	1	0.2
Ps12	(+) cocci	2	5	1	0.2
Ps13	(+) rods	2	5	1	0.1
Ps14	(+) rods	7	7	2	0.2
Ps15	(-) rods	7	5	2	0.1
Ps16	(-) rods	5	2	1	0.1
Ps17	(+) rods	10	10	5	0.5
Ps18	(-) rods	10	7	2	0.2
Ps19	(+) cocci	5	10	2	0.5
Ps20	(+) rods	7	7	1	0.2

Preparation of bacterial consortium

The 20 BTEX tolerant bacterial strains were combined with each other by permutation combination in order to make different microbial consortia. 10 different bacterial consortia (**Table 2**) were prepared of which 4 consortia showed the best compatibility when gram staining was performed. Utilization of a consortium rather than a single microorganism has always exhibited increased rate of degradation. Many mesophilic, sulfate-reducing bacterial consortia and individual isolates have been reported to be capable of degrading BTEX-type compounds. Most of these studies were carried out with sediments containing the BTEX degraders (Lovley *et al.*, 1995).

Determination of growth

All 4 bacterial consortia exhibited almost the same growth pattern in presence of 10% (v/v) benzene and 5% (v/v) ethyl benzene (**Figs 1**and **2**). In the initial 8 hours all 4 consortia were in their lag phase as there was no gradual increase in the absorbance. All the 4 consortia attained their log phase between 8 to 24 hours of incubation and gradually entered the stationary phrase from 24 hours.

In presence of 10% (v/v) toluene consortium #7 and 9 showed the highest absorbance in initial (8h) hours of incubation but gradually in the later phase of incubation there was no constant increase in the absorbance (**Fig. 3**).

These indicated that they had a very short log phase and a prolonged stationary phase in their growth cycle. While consortium #2 and 6 had a prolonged lag phase but gradually attained their log phase from 24 hours of incubation with a sharp increase in their absorbance that continues till 48 hours.

Table 2. Different composition of the bacterial consortia

Consortia	Composition
1	Ps1, Ps6, Ps9,Ps11
2	**Ps2, Ps4, Ps7, Ps12**
3	Ps3, Ps15, Ps16, Ps9
4	Ps4, Ps17, Ps8, Ps14
5	Ps5, Ps19, Ps11, Ps1
6	**Ps6, Ps11, Ps13, Ps10**
7	**Ps7, Ps12, Ps20, Ps5**
8	Ps8, Ps18, Ps 17, Ps10
9	**Ps9, Ps3, Ps16, Ps14**
10	Ps10, Ps 3, Ps 17, Ps 1

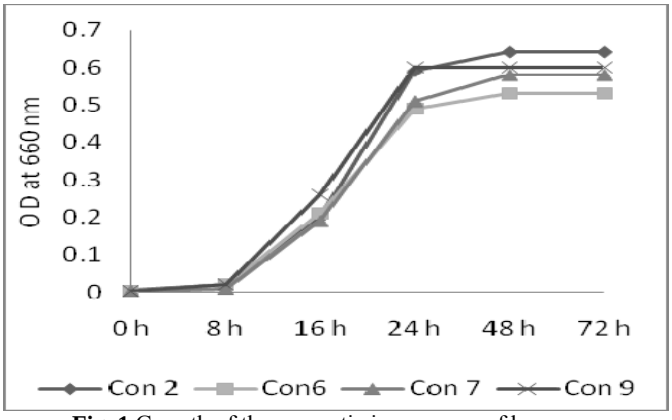

Fig. 1 Growth of the consortia in presence of benzene.

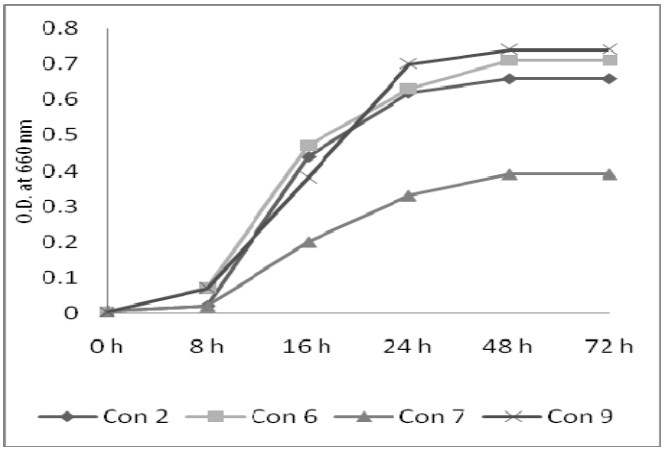

Fig. 2 Growth of the consortia in presence of ethyl benzene.

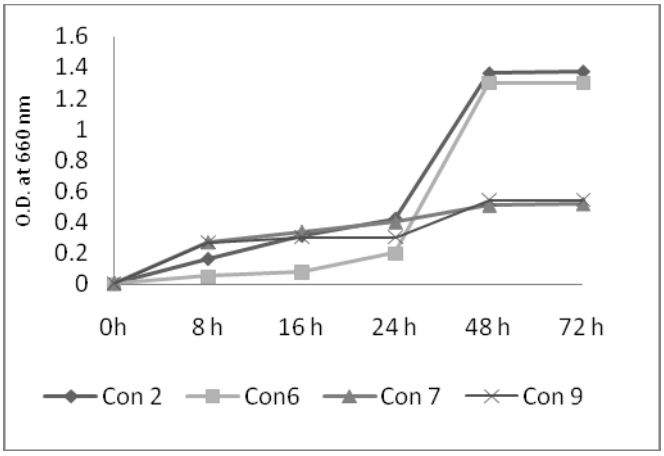

Fig. 3 Growth of the consortia in presence of toluene.

A very short lag phase was noted in all 4 consortia in presence of 1% xylene. There was a sharp increase in growth from 8 hours and it lasted till 48 hours. All the consortia entered their stationary phase after 48 hours (**Fig. 4**).

In all the cases the consortia could utilize benzene, toluene, ethyl benzene and xylene as sole source of carbon and energy. The results are in agreement with Chen and Taylor (1997). They developed two thermophilic bacterial consortia that could utilize BTEX as sole carbon and energy source.

Gas chromatography analysis of BTEX degradation

As consortium #2 was the best degrader of BTEX among the four consortia so it was selected for further analysis of BTEX degradation by Gas Chromatography. GC analysis revealed consortium #2 degrades different BTEX compounds .at different rate in the same time span. The selected consortium could degrade 100% benzene (10%) and ethyl benzene (5%) present in the growth medium. 100% degradation was confirmed by the absence of the benzene (**Fig. 5**) and ethyl benzene peak in the GC chromatogram. while the toluene (10%) and xylene (1%) was degraded almost up to 90% (**Fig. 6**) as the peak was significantly shorter compared to both the controls without the inoculam.

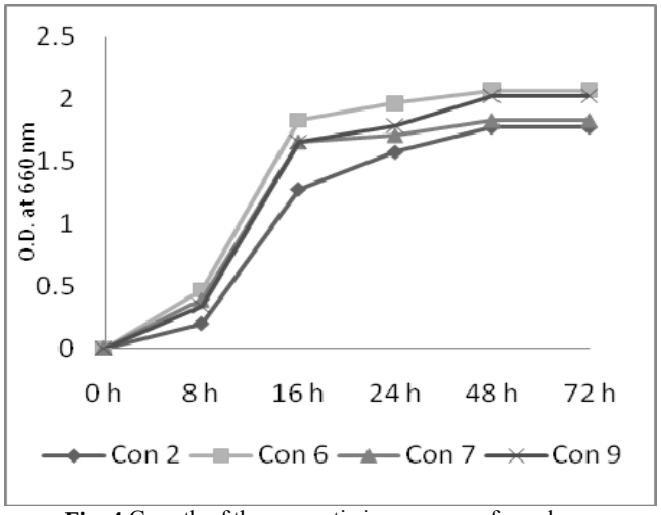

Fig. 4 Growth of the consortia in presence of o-xylene.

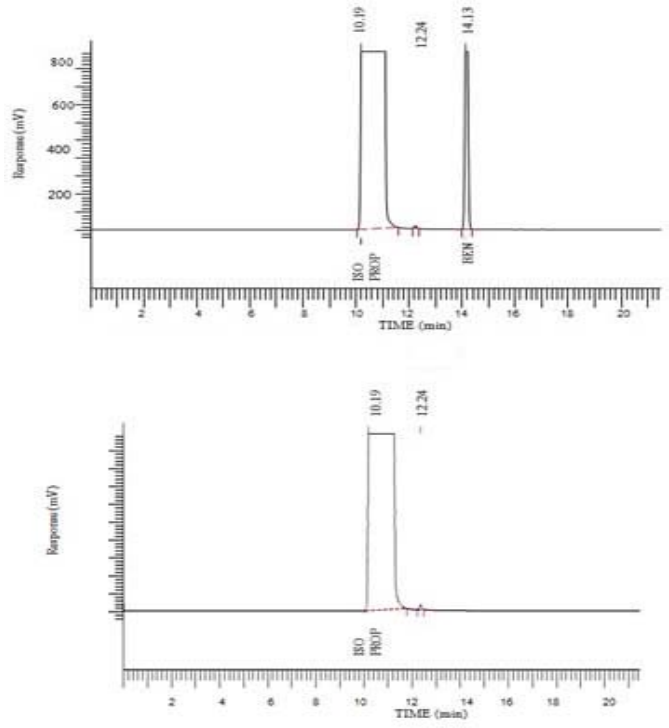

Fig. 5 GC analysis of control media having 10% (v/v) benzene, GC analysis of media having 10 (v/v) benzene inoculated with consortium #2.

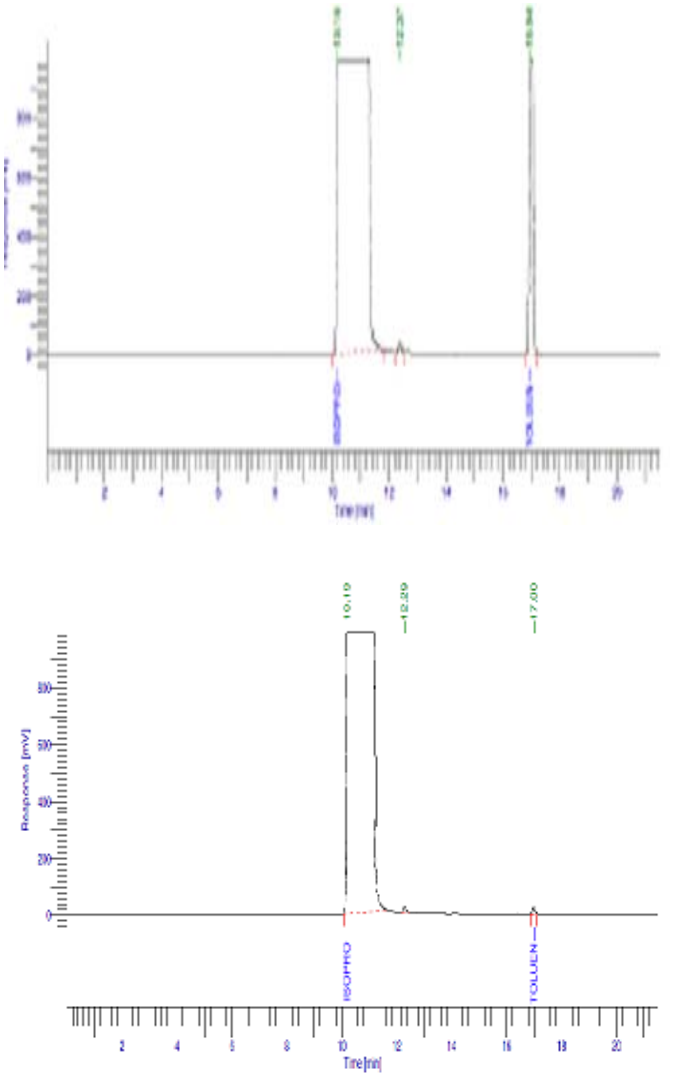

Fig. 6 GC analysis of control media having 10%(v/v) toluene, GC analysis of media having 10 (v/v) toluene inoculated with consortium #2.

Degradation of other organic solvents

The response of consortium #2 towards other solvents was studied by monitoring its growth and degradation capabilities in medium broth overlaid with solvents of varying log *Pow* values. The log *P*-value is defined as the index for measuring toxicity of solvents. Solvents with log *P*ow values between two and four, are highly toxic for microorganisms (Torres *et al.*, 2009). Degradation was determined by measuring the volumetric reduction of solvent in the media after the bacterial growth. The selected consortium could grow in solvents having higher log *P*-value, but surprisingly the alcohols having very low log *P*-value inhibited the growth (**Table 3**).

CONCLUSION

Biodegradation time tested was insufficient for the total elimination of solvents other than BTEX, implying the need for periods exceeding 5 days in

Table 3. Growth of consortium #2 in presence of organic solvents and its degradation capabilities

Solvent	log *P*	O D [a]$_{660}$	% degradation
Control[b]		1.99	–
Isooctane	4.5	*	–
DMSO	-1.35	1.8	57
Xylene	3.1	1.24	90
Acetonitrile	0.03	0.208	42
Cyclohexane	3.2	1.57	83
Toluene	2.5	1.05	90
Benzene	2	1.84	100
Chloroform	2	0.97	70
1-Butanol	0.8	*	–
2-Propanol	0.28	–	–
Ethanol	-0.24	–	–

* O D $_{660}$ value < 0.1 after 5days of growth, [b]without solvent

order to achieve this process' effectiveness. Effective bioremediation of highly recalcitrant compounds like BTEX, is most likely to rely on a consortia of microorganism rather than on the action of a single microorganism (Sarkar *et al.*, 2011). The selected consortium could grow and degrade a wide range of solvents. From the application point of view, this consortium could be a promising tool for aromatic monohydrocarbon degradation and solvent waste management.

This indicated that the selected consortium could grow and degrade hydrophobic solvents rather than hydrophilic. Similar finding of growth pattern in presence of various solvents was also reported in case of B. thermophilus PS11 strain (Sarkar & Ghosh, 2012).

REFERENCES

Anneser, B., Einsiedl, F., Meckenstock, R.U., Richters, L.,Wisotzky, F., Griebler, C. (2008) High-resolution monitoring of biogeochemical gradients in a tar oil-contaminated aquifer. *Appl. Geochem*; **23**, 1715–1730.

ATSDR. Agency for Toxic Substances and Disease Registry. Interaction profile for: benzene, toluene, ethylbenzene, and xylenes (BTEX). Atlanta: U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry; 2004.

Arvin, E., Jensen, B. K. & Gunderssen, A. T. (1989) Substrate interactions during aerobic biodegradation of benzene. *Appl. Environ. Microbiol.,* **55**, 3221-3225.

Falcó, I.P., Moya, M.N. (2007) Analysis of volatile organic compounds in water. In: Nollet LML, editor. Handbook of water analysis. New York: CRC Press. 599–666.

Kataoka, A.P.A.G. (2001) Biodegradação de resíduo oleoso de refinaria de petróleo por microrganismos isolados de "landfarming". Rio Claro, Brasil (PhD Thesis — Instituto de Biociências, Unesp — Rio Claro).

Lin, C.W., Chen, L.H., YetPole, I., Lai, C.Y. (2010) Microbial communities and biodegradation in lab-scale BTEX-contaminatedSubstances and Disease Registry. Interaction profile for: benzene, toluene, ethylbenzene, and xylenes (BTEX). (2004) Atlanta: U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Register.

Mcnaughton, S.J., Stephen, J.R., Venosa, A.D., Davis, G.A., Chang, Y.J., White, D.C. (1999) Microbial population changes during bioremediation of an experimental oil spill. *Appl. Environ. Microbiol*; **65**, 3566–3574.

Pedrozo, M.F.M., Barbosa, E.M., Corseuil, H.X., Schneider, M.R., Linhares, M.M. (2002) Ecotoxicologia e avaliação de risco do petróleo. Salvador: NEAMA.

Reusser D.E., Istok, J.D., Beller, H.R., Field, J.A. (2002) In situ transformation of deuterated toluene and xylene to benzylsuccinic acid analogues in BTEX-contaminated aquifers. *Environ. Sci. Technol*; **36**, 4127–4134.

Sarkar. P., Meghvansi, M.K. and Singh, R. (2011) Microbial Consortium: Synergistic Approach in Biodegradation of Organic Kitchen Wastes. *Int. J. Env. Sc. Dev*; **2** (3)

Sarkar, P., & Ghosh, S. (2012**)** Bioremediation potential of a newly isolate solvent tolerant strain *Bacillus thermophilus* PS11 *J. BioSci. Biotech;* **1**(2), 141-147.

Shokrollahzadeh, S., Azizmohseni, F., Golmohammad, F., Shokouhi, H., Khademhaghighat, F. (2008) Biodegradation potential and bacterial diversity of a petrochemical wastewater treatment plant in Iran. *Bioresour Technol*; **99**, 6127–33.

Torres, S., Martínez, M.A., Pandey, A., Castro, G.R. (2009). An organic–solvent-tolerant esterase from thermophilic *Bacillus licheniformis* S-86. Bioresour. Technol., 100(2): 896-902.

Wilson, J., Leach, L., Michalowski, J., Vandergrift S., Callaway. R., (1990). In situ reclamation of spills from underground storage tanks: new approaches for site characterization, project design, and evaluation of performance, p. 30-1-30-16. In I. P. Muraka and S. Cordle (ed.), Proceedings: Environmental Research Conference on Groundwater Quality and Waste Disposal.Washington, D.C.