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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 1and 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

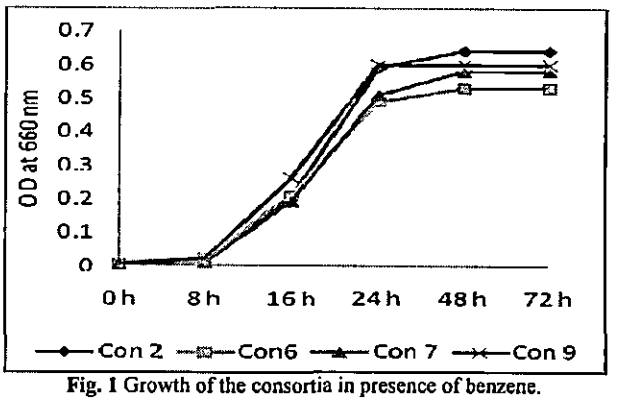

Fig. 1 Growth of the consortia in presence of benzene.

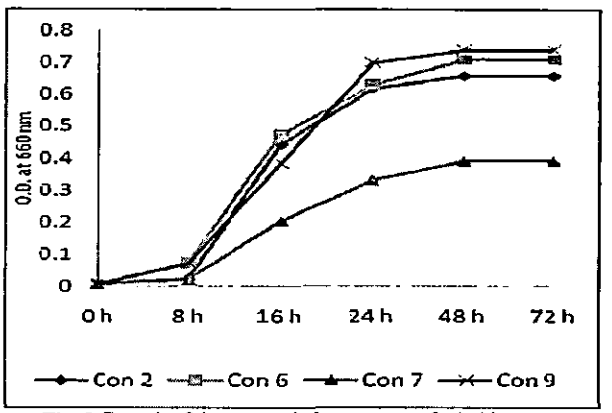

Fig. 2 Growth of the consortia in presence of ethyl benzene.

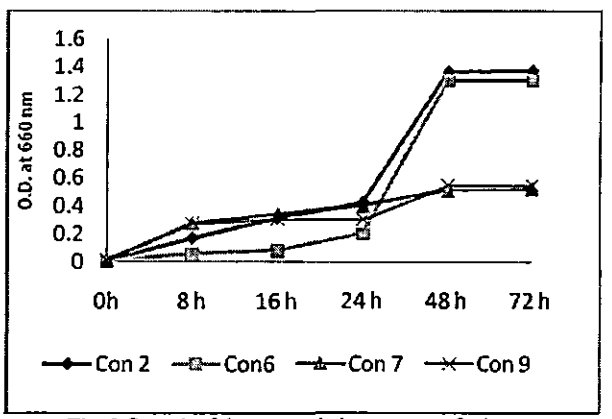

Fig. 3 Growth of the consortia in presence of toluene.

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.

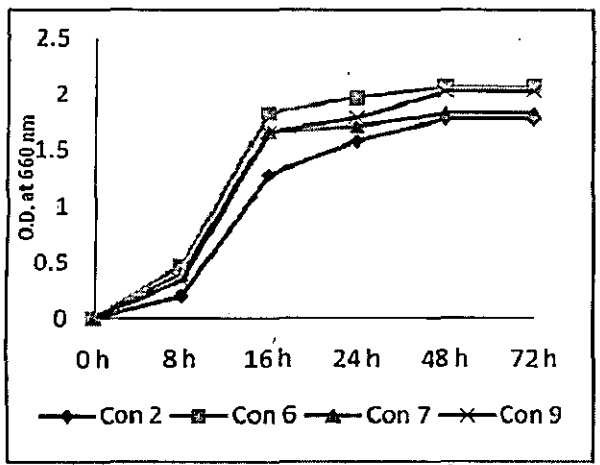

Fig. 4 Growth of the consortia in presence of o-xylene.

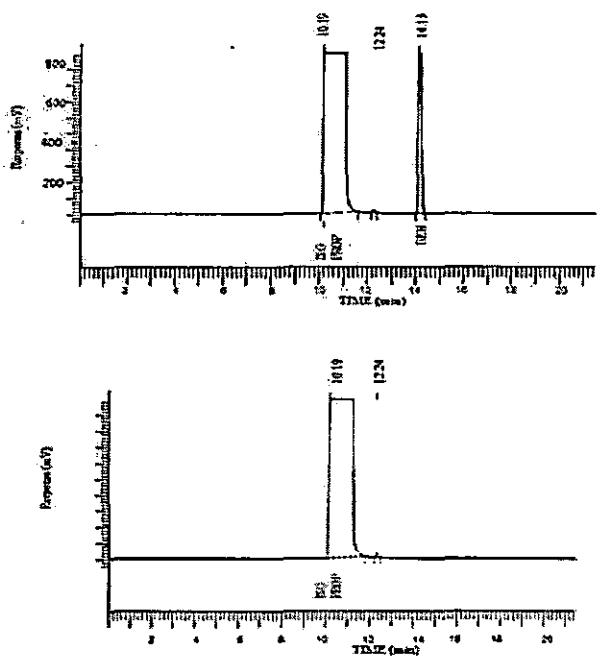

Fig. 5 GC analysis of control media having 10% (v/v) benzene, GC analysis of media having 10 (v/v) benzene inoculated with consortium #2.

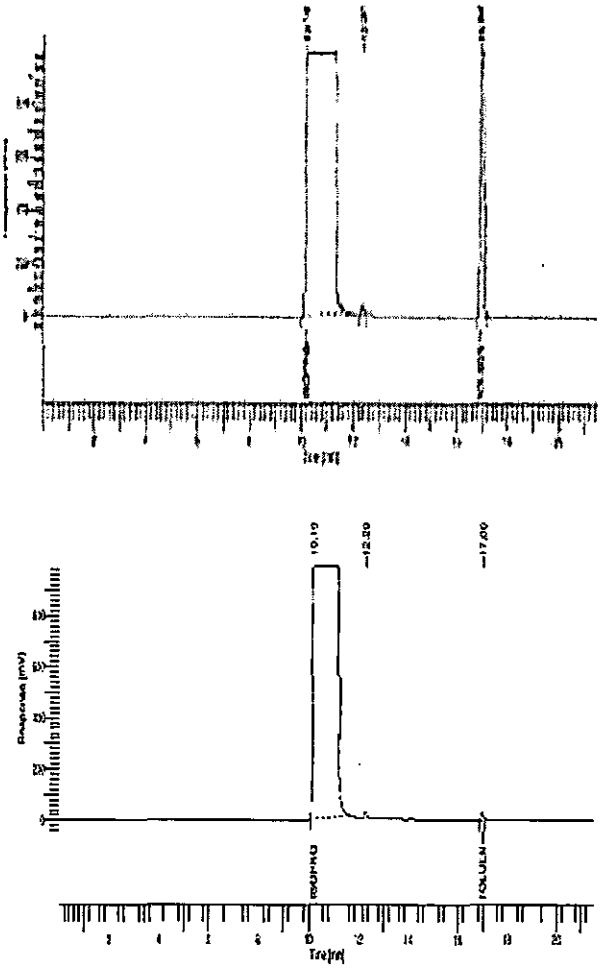

Fig. 6 GC analysis of control media having 10%(v/v) toluene, GC analysis of media having 10 (v/v) toluene inoculated with consortium #2.

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).

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RESEARCH ARTICLE

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Bioremediation potential of a newly isolate solvent tolerant strain *Bacillus thermophilus* PS11

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ABSTRACT

The increased generation of solvent waste has been stated as one of the most critical environmental problems. Though microbial bioremediation has been widely used for waste treatment but their application in solvent waste treatment is limited since the solvents have toxic effects on the microbial cells. A solvent tolerant strain of *Bacillus thermophilus* PS11 was isolated from soil by cyclohexane enrichment. Transmission electron micrograph of PS11 showed convoluted cell membrane and accumulation of solvents in the cytoplasm, indicating the adaptation of the bacterial strain to the solvent after 48h of incubation. The strain was also capable of growing in presence of wide range of other hydrophobic solvents with log P-values below 3.5. The isolate could uptake 50 ng/ml of uranium in its initial 12h of growth, exhibiting both solvent tolerance and metal resistance property. This combination of solvent tolerance and metal resistance will make the isolated *Bacillus thermophilus* PS11 a potential tool for metal bioremediation in solvent rich wastewaters.

Key words: *Bacillus thermophilus,* solvent tolerance, uranium, bioremediation

Introduction

Organic solvent wastes are an interesting topic of research due to its increasing release in the industrial effluent thus polluting the water and soil ecology (Li et al., 1998). Organic solvents are used as permeabilization agents, disinfectants, food preservatives, and industrial solvents. Though they are widely used in various industries, their uncontrolled dumping in waters, sediments and the disposal sites near rivers, oceans make them a potential environmental hazard (Bustard et al., 2002).

Microbial bioremediation, which has been used for waste treatment in many industrial processes, is less feasible for solvent wastes because of toxic effect of solvents on the microbial cells (Isken & deBont, 1998). The extreme toxicity of organic solvents toward living microorganisms is because of their accumulation in the hydrophobic biological membranes. The toxicity of a solvent to bacteria depends upon its concentration in the membrane, which relates to its water solubility and its ability to partition from the water phase to the membrane (Zahir et al., 2006). The intrinsic toxicity of a specific organic solvent can be expressed as logarithm of its partition coefficient in n-octanol and water and termed as log P_{ow}. Solvents with log P_{ow}[s] below two are generally too hydrophilic to partition into membranes well, and solvents with log P_{ow}[s] above four are too hydrophobic to have high water solubility (Kieboom et al., 1998). It has been established that solvents with log P_{ow} values between two and four are highly toxic for microorganisms (Zahir et al., 2006)

The solvent tolerant microbes with unique ability to sustain under non-aqueous system have drawn considerable attention. Such organisms are attractive for applications in solvent bioremediation and biotransformation in non-aqueous media (Isken & deBont, 1998; Pieper & Reineke, 2000; Sardessai & Bhosle, 2004) Solvent tolerant bacteria have been isolated from ecological niche such as soil or deep sea and identified to belong to genera *Pseudomonas* (Ramos et al., 1995; Ikura et al., 1997; Tao et al., 2011), *Bacillus* (Bustard et al., 2002), *Flavobacterium* (Moriya & Horikoshi, 1993) and *Rhodococcus* (Paje et al., 1997). Recently, *Pseudomonas putida* Idaho (Tao et al., 2011), an organic-solvent-tolerant bacterium was isolated that can grow in the

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presence of more than 50% toluene, *m*-xylene, *p*-xylene 1, 2, 4-trimethylbenzene, and 3-ethyltoluene. Many of these bacteria have developed mechanisms to resist the lethal effect of organic solvents either by alterations in the composition of cytoplasmic and outer membrane in presence of organic solvents or by metabolic transformation of toxic compound into non-toxic products. Furthermore, an efflux system actively decreasing the amount of solvent in the cell has been described.

Solvent tolerant microbes often possess metal resistance. A wide range of microbes are well known for metal accumulation and detoxification but without solvent tolerance trait. The combination of the two properties has better potential in microbial metal bioremediation in solvent rich wastewaters (especially in chemical and hospital wastes), which is otherwise not possible due to microbicidal nature of solvents. The biosorption of uranium and the growth of several bacterial communities, in environments that are generally poor in nutrients, with various chemical and physical properties, is not easy to stimulate. In addition, once the uranium is immobilized, it is important to impeach its reoxidation or desorption. For these reasons, a good bioremediation strategy will always depend on a good knowledge of the microbiological, geochemical and geological properties of the site to decontaminate. Bacteria, including *Citrobacter freudii* and cell components from members of the *Firmicutes* have also been described as U(VI) biosorbents (N'Guessan et al., 2008). The biosorption efficiency seems to be positively related to temperature and can occur sometimes within hours, which is considerably faster than direct bioreduction (that can take months or years).

The present work describes isolation of solvent tolerant strain of *Bacillus thermophilus* PS11 from soil by cyclohexane enrichment. The adaptation to solvent by the bacterial strain was studied at the membrane level by transmission electron microscopy. This is the first report of solvent tolerance in *B. thermophilus* to the best of our knowledge. Growth in presence of uranium indicated uranium resistance property of the bacterial strain.

Materials and Methods

Isolation of solvent tolerant bacterial strains

Soil samples were collected from the proximity of a solvent extraction unit in Siliguri, India. A known amount of soil was suspended in sterilized distilled water and 250 µl of suspension was transferred to a test tube having 5.0 ml of solvent tolerance medium (STM) containing (g l^{-1}): yeast extract, 4.0; peptone, 2.5; glucose, 1; starch, 1; olive oil, 10; KH_2PO_4, 0.5; $MgSO_47H_20$, 0.5; NaCl, 0.25; cyclohexane (10%, v/v) and pH adjusted to 7.5 (Gupta et al., 2006). The test tube was incubated for 5days at 37°C with constant shaking at 220 rpm in an orbital shaker. Resultant culture fluid was spread on STM agar plate overlaid with cyclohexane. Growing colonies were further purified by repeated streaking. Finally, a solvent-tolerant strain B5 was chosen for further studies because of its highest growth rate in presence of solvent. Morphological and biochemical characteristics of the isolate were determined. The 16S rDNA of B5 strain was PCR amplified from genomic DNA using universal primers 27F (5'-AGAGTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') and the 1.4 kb PCR product was sequenced. The identity of isolate was confirmed by phylogenetic analysis of 16S rDNA sequence using the software package SeaView. It was maintained on nutrient agar at 4°C.

Growth of the isolated strain

For bacterial growth, the inoculum was prepared by inoculating a loopful of isolated cells from slant into STM followed by incubation at 37°C and 140 rpm. One millilitre of overnight grown culture having 10^6 cfu.ml^{-1} was used to inoculate 100 ml of STM overlaid with 20% v/v cyclohexane. The incubation was carried out at 37°C with constant shaking at 140 rpm in an orbital shaker. To prevent the evaporation of solvent, flasks were sealed with butyl rubber stoppers. The bacterial culture growing in absence of organic solvent under similar conditions served as control. Growth was followed by recording absorbance at 660 nm. For dry cell mass measurement, 1.0 ml culture broth was centrifuged at 10000*g* at 4°C for 10 min to pellet down the cell mass. The pellet was washed twice with distilled water and dried at 105°C till constant mass was achieved.

Organic solvent tolerance of the isolated strain

The solvent tolerance of the microorganism was checked in Erlenmeyer flasks containing STM overlaid with organic solvents (20% 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. Evaporation of solvent was prevented by plugging the flasks with butyl-rubber stoppers. The bacterial culture growing in absence of organic solvent under similar

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conditions served as control. Growth and dry cell mass were monitored similarly as described in the previous section.

Sample preparation for transmission electron microscopy (TEM)

To prepare specimens for transmission electron microscopy, cells were grown for 24h in culture medium in absence or presence of cyclohexane (20% v/v) and harvested by centrifugation at 5000 rpm for 10 min. Then cells were fixed overnight in a solution containing 2.5% (w/v) glutaraldehyde in 0.1M Na_2HPO_4/KH_2PO_4 (pH7.2) buffer at 4°C and post fixed with 1% osmium tetroxide (OsO_4). The cells were then dehydrated with ethanol and embedded in Spurr. The sections were stained with 1% (w/v) uranyl acetate and 1% (w/v) citrate and examined with a Philips model CM10 electron microscope at an accelerating voltage of 80 kV.

Uranium bioremediation by the isolated strain

Culture media containing varying amount of uranium (5-50 $ng.ml^{-1}$) was inoculated with 1% (v/v) freshly prepared inoculum of isolated strain. The inoculum preparation and culture conditions were kept same as described above for the growth of the isolate. Samples for estimating residual uranium in the media were periodically withdrawn. Total uranium contents were measured by cold vapor atomic absorption spectrometry (Hatch & Ott, 1986).

Results and Discussion

Isolation and screening of bacterial strains with solvent tolerance property

Tolerance to grow in the presence of solvents is often observed among the microbes inhabitating the soil exposed to solvents. In the present work, soil samples from the sites near to the solvent extraction unit were screened for solvent tolerant microbes. A solvent tolerant isolate B5 was chosen for further study because of its highest growth rate in presence of solvent. Similar results, mostly in case of *Pseudomonas* sp., *Flavobacterium* and *Rhodococcus,* have been also reported (Inoue & Horikoshi, 1989; Paje et al., 1997; Bustard et al., 2002).

The bacterial isolate was characterized morphologically and biochemically as aerobic, gram-positive, motile rod with very simple nutritional requirements that grow best at neutral pH and temperatures in the mesophilic range. Molecular characterization by 16S rDNA based phylogenetic analysis (accession numbers awaited) identified the bacterial isolate as *Bacillus thermophilus* and hence, tentatively named as *Bacillus thermophilus* PS11.

Growth characteristics of B. thermophilus in presence of cyclohexane

The growth curve of *Bacillus thermophilus* in the absence and presence of cyclohexane (20%, v/v) is shown in Figure 1.

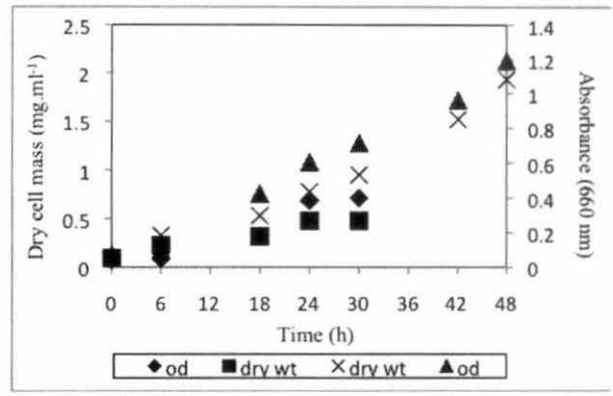

Figure 1. *Growth of B. thermophilus in the absence and presence of cyclohexane. B. thermophilus was grown in culture media in the absence and presence of cyclohexane (20% v/v). The growth (A_{660}) and dry cell mass were recorded at various time intervals. Bacterial growth in absence of cyclohexane: $OD_{660\ nm}$ (▲), dry cell mass (✕) and growth in presence of cyclohexane: $OD_{660\ nm}$ (♦), dry cell mass (■). The experiment was carried out in triplicates and the difference in the individual results was less than 3%.*

Incorporation of cyclohexane into the media served as a factor for screening of solvent tolerant strain. The isolated strain PS11 showed delayed growth pattern in presence of cyclohexane and log phase started after 18h, as compared to 6h in its absence. Since butyl rubber covered flasks were used in both cases, availability of oxygen may not be responsible for lesser growth in the presence of solvent. The reason may be the direct effect of cyclohexane on cells. The dry cell mass of the culture in the presence of cyclohexane was about double of that in its absence. Similar growth behavior was reported in the case of *P. aeruginosa* PST-01 while 23% cyclohexane was incorporated in the media (Ogino et al. 1995, 1999).

Growth characteristics of B. thermophilus in presence of other organic solvents

The response of *Bacillus thermophilus* towards other

solvents was studied by monitoring its growth in medium broth overlaid with solvents of varying log P_{ow} values. The log *P*-value is the index for measuring the toxicity of solvents. Solvents with log P_{ow} values between two and four, are highly toxic for microorganisms (Torres et al., 2009). However, the results summarized in Table 1 show that the isolated strain PS11 could grow in solvents having higher log *P*-value, but surprisingly the alcohols having very low log *P*-value inhibited the growth. Similar finding of growth pattern in presence of various solvents was also reported in case of *B. cereus* R1 strain (Matsumoto et al., 2002). Bacterium grew well on the medium plates overlaid with cyclohexane, DMSO, toluene, chloroform, benzene, hexane, xylene but did not grow in the presence of isopropanol, 1-butanol and ethanol thus indicating its tolerance for hydrophobic solvents rather than hydrophilic. The cell mass in presence of DMSO was comparable to that in cyclohexane overlaid medium, whereas cell growth was least in presence of acetonitrile.

Table 1. *Growth of B. thermophilus in presence of organic solvents.*

Solvent	Log *P*	OD_{660} [a]	Dry cell mass (mg.ml^{-1})[a]
Control [b]		2.69	1.47
Isooctane	4.50	*	*
DMSO	-1.35	1.78	1.19
Xylene	3.10	0.41	0.54
Acetonitrile	0.03	0.30	0.13
Cyclohexane	3.20	1.67	1.05
Toluene	2.50	0.89	0.76
Benzene	2.00	0.74	0.68
Chloroform	2.00	0.76	0.68
1-Butanol	0.80	*	*
2-Propanol	0.28	-	-
Ethanol	-0.24	-	-

Legend: * OD_{660} value < 0.1 and dry cell mass (mg.ml^{-1}) < 0.05 after 24h of growth; [a] After 24h of growth; [b] without solvent.

Transmission electron micrograph of B. thermophilus

Transmission electron micrographs of the *Bacillus* cells growing in the absence and presence of cyclohexane are shown in Figure 2. Transmission electron micrograph of *B. thermophilus* cells in the presence of 20% cyclohexane showed accumulation of solvents and convoluted, disorganized cell membrane. Similar cellular changes have been reported for *Pseudomonas* sp. cells grown in *p*-xylene and *Enterobacter sp.* grown in the presence of cyclohexane (Gupta et al., 2005, 2006).

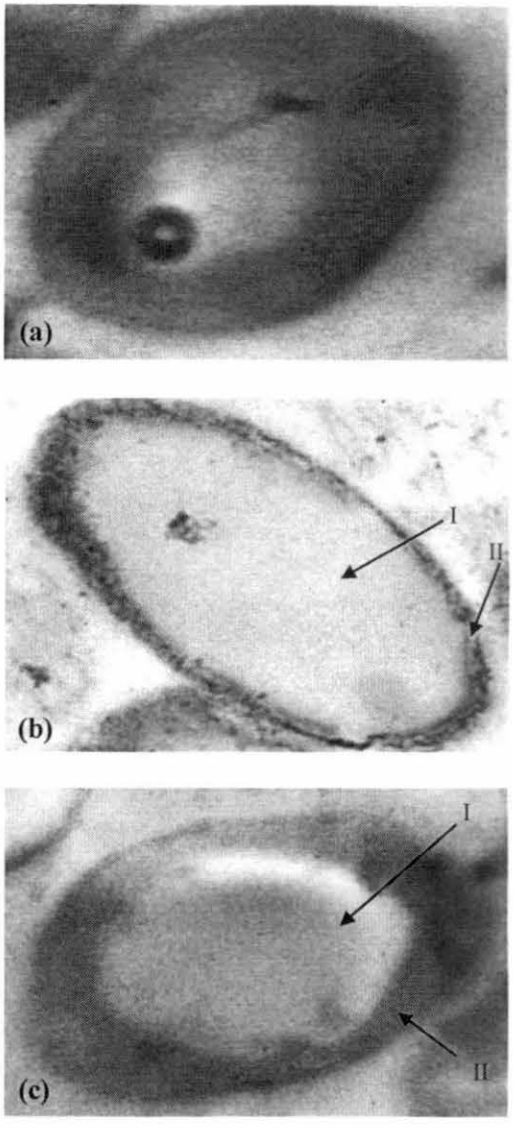

Figure 2. *Transmission electron micrograph of B. thermophilus cells:* **(a)** *in the absence of cyclohexane (exposure 21,000);* **(b)** *in the presence of 20% cyclohexane (exposure 21,000) after 48 hours incubation - (I) accumulation of solvents and (II) convoluted and disorganized cell membrane;* **(c)** *in the presence of 20% cyclohexane (exposure 21,000) after 96 hours incubation. (I) and (II) regeneration of cytoplasm and cell wall.*

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Solvents are reported to damage the integrity of cell membrane structure resulting in loss of permeability regulations. In extreme cases leakage of cell RNA, phospholipid and protein also take place (Sikkema et al., 1995). Moreover, it is clearly visible that the solvent accumulation inside the cell was initially increased occupying the entire cytosolic region at 48h of incubation (Figure 2b). The decline in solvent accumulation and reorganization of cell membrane were observed on further incubation till 96 hours (Figure 2c).

Structural changes seen in TEM of *Bacillus* cells suggest that organic solvent affected the membrane system. The solvent tolerant nature of the bacterial strain is evident from the reorganization of cell membrane and decline in cytosolic accumulation of solvent during prolonged exposure to solvent. Hence, solvent adaptation property of *Bacillus thermophilus* PS11 seems to be related to both restoration of membrane fluidity and metabolic transformation of hydrophobic solvent to less toxic products. Cell morphology alterations and filamentous growth were observed in solvent resistant bacteria in response to environmental stress, including organic solvents (Toress et al, 2009). Efflux pumps are reported to be one of the main mechanisms of solvent tolerance and this mechanism may have the effect of diminishing the solvent concentration in the cytoplasm. Efflux systems in case of gram positive bacteria are either secondary transporters or ATP binding cassette (ABC) type transporters (Bolhuis et al., 1997). Solvent tolerant cells adapt by making changes in fatty acid composition and protein/lipid ratio in cell membrane to restore the fluidity. They also have capability of metabolic transformation of toxic compound into non-toxic products. However, the variety of mechanisms that could confer adaptation to organic solvents implies that bacterial solvent tolerance cannot only possibly be provided by a single one type mechanism (Heipieper et al., 2007). It is very likely that the combination of different metabolic strategies leads to cellular solvent tolerance.

Uranium bioremediation by B. thermophilus

Solvent tolerant strains are often endowed with heavy metal resistance (Chang & Hong, 1995; Wagner-Döbler, 2003). To check the simultaneous effect of these two traits, this solvent tolerant isolate of *Bacillus thermophilus* was grown up to 50 ng/ml of U in media (Figure 3). The bacterium could uptake uranium in its initial 12h of growth. During this time the growth rate is less compared to the growth rate when uranium is up taken by the cells. The lag phase prolonged with increasing uranium concentration, which is a common pattern in microbial metal resistance (Wagner-Döbler, 2003).

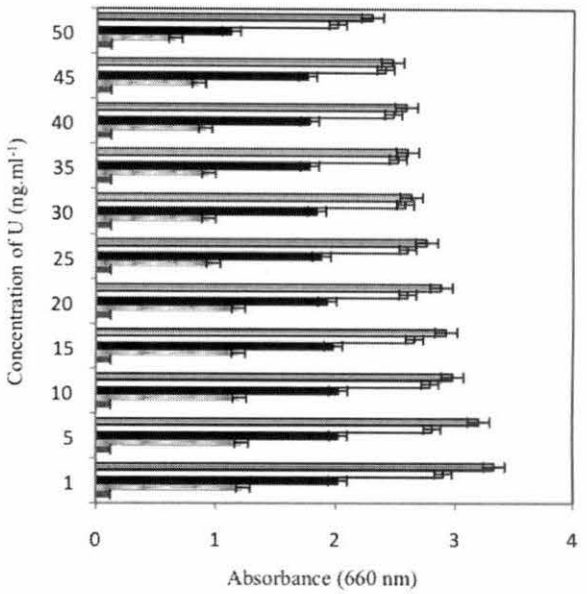

Figure 3. *Growth of B. thermophilus in varying concentration of uranium. The isolate was grown in media containing varying concentration of U (5-50 ng/ml). OD_{660nm} were recorded after 0 h (▤), 12 h (□), 24 h (■), 48 h (▥) and 72 h (▩) of growth. Each experiment was done in duplicate and the difference between two sets of experiments was less than 3%.*

The level of uranium in the medium decreased with bacterial growth, thus confirming its uptake by the microorganism (Figure 4). The observed U resistance by the test isolates may be explained by uranium-bacteria interaction mechanisms resulting in cell surface or intracellular sequestration of uranium as a means to limit U toxicity, which may be followed by bioprecipitation and biomineralization (Merroun et al., 2008). Similar uranium biomineralization by a metal-resistant *Pseudomonas aeruginosa* strain was recently reported (Choudhary et al., 2011). Mechanism of uranium detoxification is presumed to be by the metallic reductase enzyme or due to metal resistant genes encoding efflux pumps present in the microbes. Further works regarding the reasons behind heavy metal tolerance is under process.

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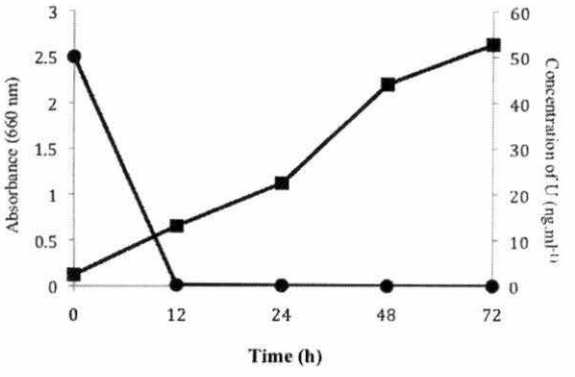

Figure 4. *Growth and uptake of uranium by B. thermophilus cells. The bacterium was grown in culture containing 50 ng/ml U. The growth (A_{660}) and concentration of the U were meadured at various time intervals. $OD_{660\ nm}$ (■), U – ng/mL (●). The experiment was carried out in triplicates and the difference in the individual results was less than 3%.*

Conclusion

The isolated gram positive solvent tolerant strain of *Bacillus thermophilus* PS11 could grow in presence of various solvents having varying log P value. Change in the membrane structure was observed as an adaptive feature when grown in presence of solvents. The strain could also uptake uranium from the media. Thus, this strain could be promising for treatment of solvent wastes and detoxification of uranium.

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