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Payel Sarkar
Shilpi Ghosh

Authors' address:
Department of Biotechnology,
University of North Bengal,
Siliguri-734013, India.

Correspondence:
Shilpi Ghosh
Department of Biotechnology,
University of North Bengal,
Siliguri-734013, India
Tel.: +91-0353 2776354
e-mail: ghoshshilpi@gmail.com

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

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.

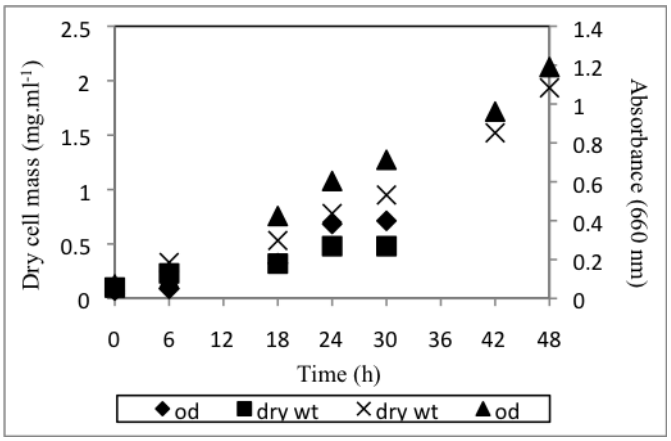

Figure 1. *Growth of B. thermophilus in the absence and presence of cyclohexane. B. thermophilus was grown in culture media in the absence and presence of cyclohexane (20% v/v). The growth (A_{660}) and dry cell mass were recorded at various time intervals. Bacterial growth in absence of cyclohexane: $OD_{660\ nm}$ (▲), 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

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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).

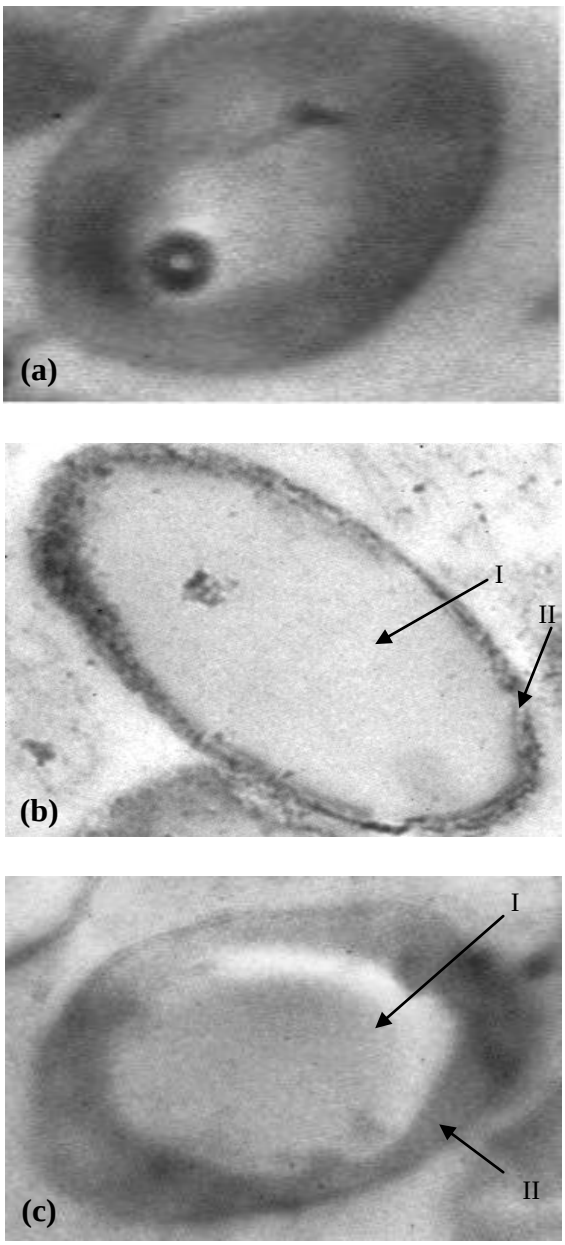

Figure 2. *Transmission electron micrograph of B. thermophilus cells:* **(a)** *in the absence of cyclohexane (exposure 21,000);* **(b)** *in the presence of 20% cyclohexane (exposure 21,000) after 48 hours incubation - (I) accumulation of solvents and (II) convoluted and disorganized cell membrane;*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

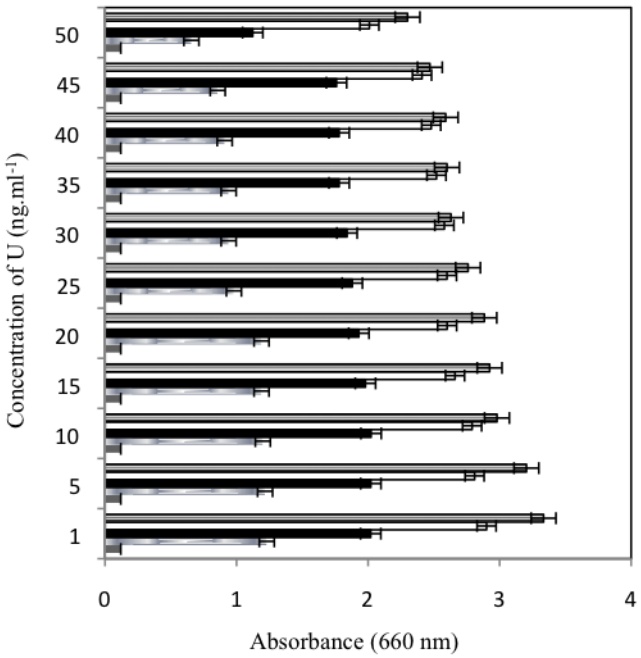

Figure 3. *Growth of B. thermophilus in varying concentration of uranium. The isolate was grown in media containing varying concentration of U (5-50 ng/ml). OD_{660nm} were recorded after 0 h (⊟), 12 h (□), 24 h (■), 48 h (◨) and 72 h (■) of growth. Each experiment was done in duplicate and the difference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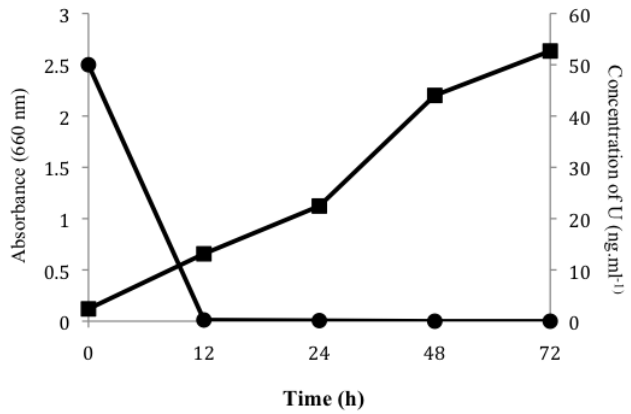

Figure 4. *Growth and uptake of uranium by B. thermophilus cells. The bacterium was grown in culture containing 50 ng/ml U. The growth (A_{660}) and concentration of the U were meadured at various time intervals. $OD_{660\ nm}$* (■), *U – ng/mL* (●). *The experiment was carried out in triplicates and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