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Research Paper

Biotransformation of Tributyltin chloride by *Pseudomonas stutzeri* strain DN2

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Abstract

A bacterial isolate capable of utilizing tributyltin chloride (TBTCl) as sole carbon source was isolated from estuarine sediments of west coast of India and identified as *Pseudomonas stutzeri* based on biochemical tests and Fatty acid methyl ester (FAME) analysis. This isolate was designated as strain DN2. Although this bacterial isolate could resist up to 3 mM TBTCl level, it showed maximum growth at 2 mM TBTCl in mineral salt medium (MSM). *Pseudomonas stutzeri* DN2 exposed to 2 mM TBTCl revealed significant alteration in cell morphology as elongation and shrinkage in cell size along with roughness of cell surface. FTIR and NMR analysis of TBTCl degradation product extracted using chloroform and purified using column chromatography clearly revealed biotransformation of TBTCl into Dibutyltin dichloride ($DBTCl_2$) through debutylation process. Therefore, *Pseudomonas stutzeri* strain DN2 may be used as a potential bacterial strain for bioremediation of TBTCl contaminated aquatic environmental sites.

Key words: TBTCl, morphological alteration, biotransformation, $DBTCl_2$, bioremediation.

Introduction

Organotin compounds including TBTCl have been extensively employed in a variety of industrial products such as antifouling paints for boats, wood preservatives, biocides and plastic stabilizers (Gadd, 2000). A great deal of research has indicated that among the organotin compounds, TBTCl is the most toxic compound known to aquatic ecosystems (Gadd, 2000; Dubey and Roy, 2003). The biocidal properties of organotin compounds also make TBTCl an ecological threat to non target organisms exposed to it in aquatic ecosystems (Horiguchi 2006; Sousa *et al.*, 2010; Lemos *et al.*, 2011). It is found to be extremely hazardous to some aquatic organisms as it can induce imposex in female mollusks and can cause thickening of shells in oysters by being toxic even at nM concentrations in water (Bryan *et al.*, 1988; Horiguchi, 2006; Sousa *et al.*, 2010; Lemos *et al.*, 2011). TBTCl has also been identified as immune system inhibitor and endocrine disruptor in humans (Bryan *et al.*, 1988; Dubey and Roy, 2003; Horiguchi, 2006; Sousa *et al.*, 2010; Lemos *et al.*, 2011). In the aquatic environment, TBTCl is quickly removed from the water column and adheres to sediments as it has high specific gravity (*i.e.* 1.2). Its low water solubility (less than 10 mg/L at 20 °C and pH 7) is also responsible for its strong binding to suspended particulate organic and inorganic materials in the aquatic sediment. The degradation rate of TBTCl is observed to be significantly slower within sediments than in the water columns. Earlier studies have revealed that half life of TBTCl in marine sediments ranges from 0.91 to 5.2 years (Clark *et al.*, 1988; Dowson *et al.*, 1996; Ayanda *et al.*, 2012). TBTCl is known to be highly toxic to both prokaryotes and eukaryotes, while the mono-, di- and tetra-organotins are almost non toxic or barely toxic and seem to exert toxicity through their interaction with membrane lipids (Cruz *et al.*, 2010). The total butyltins in water samples vary between ~12 and 73 ng Sn L^{-1} and from 0.5 to 77 ng Sn L^{-1} ; whereas, in sediments it ranges from ~ 15 to 118 and 6 to 119 ng Sn g^{-1} of sediment for the Mandovi and the Zuari estuaries, respectively (Garg *et al.*, 2010).

In nature transformation of TBTCl into less toxic compounds ($DBTCl_2$ and $MBTCl_3$) by abiotic mechanisms such as chemical cleavage, thermal cleavage and UV irradiation has been reported (Clark *et al.*, 1988; Dowson *et al.*,

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1996; Ayanda *et al.*, 2012). Although TBTCl is highly toxic to majority of microbes some natural microbial strains employing a variety of protective biochemical and molecular genetic mechanisms can survive at very high concentrations of TBTCl without any impact on their growth and metabolism (Pain and Cooney, 1998; Dubey *et al.*, 2006; Cruz *et al.*, 2007, 2010; Krishnamurthy *et al.*, 2007; Ramachandran and Dubey, 2009; Fukushima *et al.*, 2009, 2012; Sampath *et al.*, 2012; Shamim *et al.*, 2012). Several mechanisms involved in TBTCl resistance in bacteria include (i) exclusion of the compound from the cell mediated by multidrug efflux pump (Jude *et al.*, 2004); (ii) degradation/ metabolic utilization as a carbon source (Kawai *et al.*, 1998); (iii) bioaccumulation into the cell without breakdown of the compound (Fukagawa *et al.*, 1994); (iv) unique morphological alteration in the form of long interconnected chains of bacterial cells on exposure to TBTCl (Shamim *et al.*, 2012). This unique characteristic of TBTCl resistant microbes including bacteria makes them an ideal biological tool for bioremediation of TBTCl contaminated sites and thus facilitate restoration of polluted environment.

Biotransformation of TBTCl by bacteria, algae and fungi does occur and is believed to proceed by successive debutylation reactions from TBTCl $\rightarrow$ $DBTCl_2$ $\rightarrow$ $MBTCl_3$ $\rightarrow$ inorganic tin via β-hydroxylation with appropriate dioxygenases (Clark *et al.*, 1988; Dowson *et al.*, 1996; Ayanda *et al.*, 2012). As microbial degradation is observed as a predominant biological process for breakdown of TBTCl in coastal waters (Dubey and Roy, 2003; Suehiro *et al.*, 2006; Ayanda *et al.*, 2012), it is imperative and interesting to investigate the transformation of TBTCl mediated by bacteria thriving in such econiches.

In the present communication, we have reported isolation and identification of TBTCl biotransforming marine bacterial isolate, obtained from coastal sediments of west coast of India (Goa) along with analytical characterization of degradation product of TBTCl and morphological characterization of bacteria strain exposed to TBTCl.

Materials and Methods

Isolation and Identification of TBTCl resistant bacterial strain

A bacterial strain DN2 was isolated from coastal sediments of the Zuari estuary, Goa. Serially diluted coastal sediment sample was spread plated on mineral salt medium (MSM) agar supplemented with 0.2 mM TBTCl as a sole source of carbon and plates were incubated at room temperature for a 24 h to 1 week. The isolated bacterial colonies which appear were selected as TBTCl resistant and further spot inoculated on MSM agar plates supplemented with different levels of TBTCl (0.5 mM-5 mM). The bacterial colony which grew at highest TBTCl concentration was selected for further characterization and designated as strain DN2. The composition of MSM (1 L) used for growth and biotransformation experiment consisted of ferrous sulfate (0.06 g), dipotassium hydrogen orthophosphate (12.6 g), potassium dihydrogen orthophosphate (3.64 g), ammonium nitrate (2 g), magnesium sulfate (0.2 g), manganese sulfate (0.0012 g), sodium molybdate (0.0012 g) and dehydrated calcium chloride (0.15 g) (Sigma Aldrich, USA). The isolate was maintained on MSM containing 2 mM TBTCl, pH 7.4 and stored at 4 °C. It was tentatively identified using Gram staining, morphological and biochemical characteristics following Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984), and fatty acid methyl ester (FAME) analysis (Sherlock version 6.0B).

Fatty acid methyl ester (FAME) analysis

Bacterial isolates were grown on trypticase soy agar (TSA) at their optimum growth conditions. Whole cell fatty acids were extracted from cell material and derivatised to Fatty acid methyl esters according to the MIDI protocol (Sasser, 1990). Gas chromatographic analysis of fatty acid methyl esters was performed on a GC Sherlock microbial identification system (New York, USA) fitted with cross-linked methyl silicon fused capillary column (25 m, 0.2 mm i.d.), flame ionization detector (FID) and a sampler. Helium was used as carrier gas. The sample was injected at oven temperature of 50 °C. After 1 min, the oven temperature was raised to 170 °C at the rate of 30 °C/min and then to 270 °C at the rate of 2 °C/min and finally to 300 °C at 5 °C/min. FAME profile of our sample was compared with standard FAME profile of MIDI Sherlock version 6.0B.

Study of growth behaviour and TBTCl resistance limit

Growth behaviour and TBTCl (Sigma Aldrich, USA) resistance limit of the selected bacterial isolate was studied in Mineral salt medium supplemented with varying concentrations of TBTCl (0.5, 1, 2, 3, and 4 mM) as a sole carbon source to find out the optimum concentration of TBTCl for cell growth at 28 °C, pH 7.4 with constant shaking at 150 rpm in an Erlenmeyer flask. As a negative control MSM was inoculated with the bacterial strain without any carbon source. Absorbance of the culture suspension was recorded at definite time intervals of 6 hrs at OD 600 nm using UV-Vis spectrophotometer (Shimadzu, UV-2450, Japan) and graph was plotted between absorbance and time interval. Growth of the isolated was also recorded as cell dry weight in terms of g/L.

Scanning electron microscopy

In order to reveal morphological alterations in presence of TBTCl, scanning electron microscopic (SEM) analysis (JEOL JSM-5800LV, Japan) was performed using the bacterial strain grown in MSM supplemented with 2 mM TBTCl. Bacterial strain grown in MSM with 0.1% glucose as sole carbon source was taken as control. Culture smear was prepared on a glass slide, air dried and then fixed in 3% glutaraldehyde overnight with 50 mM potassium phosphate

buffer. The glass slide was then washed thrice with phosphate buffer and dehydrated in gradually increasing concentrations of ethanol, *i.e.*, 10, 20, 50, 70, 80, 90, 95, and 100% for 15 min each. The glass slide was subsequently air dried and stored in vacuum chamber prior to SEM analysis (Naik and Dubey, 2011).

Biotransformation studies

Extraction and thin layer chromatographic (TLC) analysis of biotransformation product

Bacterial strain DN2 was grown in 250 mL Erlenmeyer flask containing 100 mL MSM (starting inoculum size; OD of 0.025 which is equivalent to 0.007 gm/L) with 2 mM TBTCl as a sole source of carbon at 28 °C in an incubator shaker at 150 rpm for 1 week. Similarly, an uninoculated flask containing MSM (100 mL) with 2 mM TBTCl was used as a control. After incubation the cell pellet was harvested by centrifugation at 8000 rpm and the cell free supernatant was separated. The cell free supernatant was extracted using double volume of distilled chloroform. The organic layer was collected in a conical flask. The chloroform extract was reduced under vacuum and the concentrated sample was then loaded on a pre activated TLC plate and developed using the solvent system, petroleum ether: acetic acid (9.5:0.5). The TLC plate was subsequently exposed to iodine vapours to develop the spot. The location of the spot was marked and scraped off from the TLC plates. The product was extracted by repeated washing of silica gel with chloroform. The concentrated and residual extract was further purified by silica gel H-20 column chromatography (SIGMA, 30 x 2 cm glass column). Slurry of silica gel H-20 was prepared by mixing 9 gm of silica in 20 mL of ether (40-60 °C). A glass column (15.5 cm) was packed by adding the slurry with the help of a glass rod and gently tapped to avoid any void volume. The concentrated residual extract was added to the column and chloroform was passed through the column. Purity of eluted product was checked by TLC. The same procedure was repeated for the control flask.

FTIR and UV-Vis Spectrophotometric analysis of purified biotransformation product

The eluent obtained after purification by column chromatography was further concentrated under vacuum, dried and weighed using electronic weighing machine. The purified product was stored in a glass screw capped vial at 4 °C until use. The purified biotransformation product was analyzed in the region 400-4,000 cm^{-1} using (SHIMADZU-FTIR 8201 PC instrument, Japan) in order to find out different transformation products of TBTCl. The purified product was also analyzed spectrophotometrically using UV-Vis Spectrophotometer (Shimadzu UV-2450, Japan) at 236 nm.

NMR analysis of purified biotransformation product

Nuclear magnetic resonance spectrum analysis (H^1 NMR) of the control product and purified biotransformation product were recorded with the help of an NMR spectrometer (BRUKER WT, 300 MHz) in deuterated chloroform ($CDCl_3$) with tetramethyl silane (TMS) as an internal standard. Sample (5 mg) to be analysed was dissolved in deuterated $CDCl_3$ (0.6 mL) and placed in an NMR tube (5 mm diameter). The NMR tube was then appropriately positioned in a spinner and introduced into the NMR spectrometer. All the reagents were purchased from Sigma Aldrich, USA. (Devi *et al.*, 2010).

Results and Discussion

Identification of TBTCl resistant bacterial strain

The TBTCl resistant bacterial strain DN2 was isolated from coastal sediments of the Zuari estuary, Goa, India since it is reported to be highly contaminated with TBTCl (> 1.3 ng Sn/g) (Jadhav *et al.*, 2009). These TBTCl contaminated estuarine sediments are responsible for natural enrichment of TBTCl resistant microbes including bacteria. The bacterial strain DN2 was Gram negative, motile, rod shaped, and oxidative. It also showed presence of enzymes viz. oxidase, catalase and gelatinase. Indole, methyl red and Voges Proskaeur's tests were found negative but it utilized citrate. Bacterial strain DN2 didn't produce any pigment. Based on biochemical and morphological characteristics strain DN2 was identified as *Pseudomonas stutzeri* and further confirmed by FAME analysis.

Growth behaviour and TBTCl resistance limit

Pseudomonas stutzeri strain DN2 evidently showed an initial lag of 6 h, followed by a long exponential phase in presence of 0.5, 1 and 2 mM TBTCl respectively, whereas 3 mM TBTCl induced an extended lag of 24 hr. It showed best growth in presence of 2 mM TBTCl among the tested concentrations whereas it could resist TBTCl up to 3 mM while 4 mM level of this biocide proved lethal due to cytotoxic effects. (Figure 1). *Pseudomonas stutzeri* strain DN2 didn't show any growth in MSM without TBTCl (Figure1). There are very few reports on TBTCl resistant estuarine bacteria which include TBTCl resistant estuarine *Aeromonas caviae* strain KS-1 from Mandovi estuary, Goa, India which tolerates TBTCl up to 1.0 mM (Shamim *et al.*, 2012). *Aeromonas veronii*, a tributyltin (TBT) degrading bacterium isolated from an estuarine environment of Ria de Aveiro in Portugal can tolerate TBT up to 3 mM in nutrient rich Trypticase Soy Broth medium (Cruz *et al.*, 2007). Tributyltin chloride degrading *Pseudomonas* spp. capable of growth in MSM containing 2 mM TBTCl as a sole source of carbon, have also been isolated earlier from Indian coastal waters (Sampath *et al.*, 2012). Suehiro *et al.*, (2006) reported degradation of tributyltin in microcosm using Mekong river sediments. Tributyltin resistant estuarine

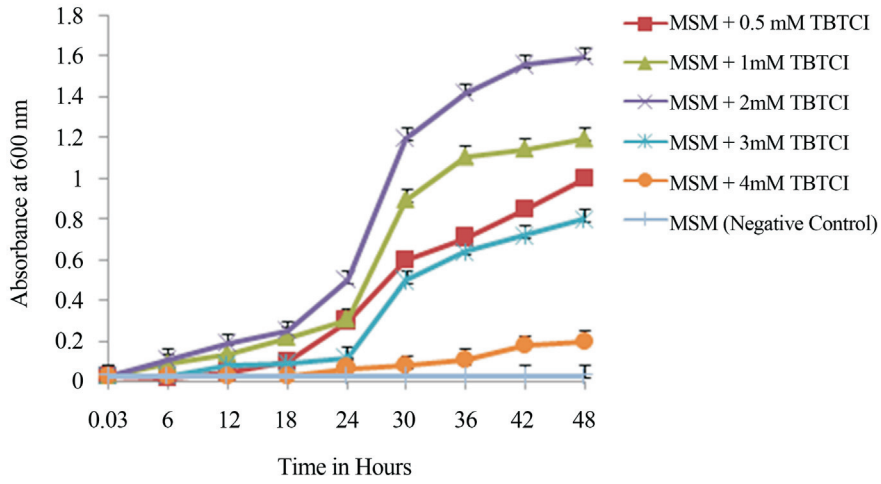

Figure 1 - Growth behaviour of *Pseudomonas stutzeri* strain DN2 in MSM amended with different concentrations of tributyltin chloride as a sole carbon source. Bacteria inoculated in plain MSM without TBTCl is considered as negative control.

bacterial isolates capable of growing on 8.4 µM TBT were isolated from Boston Harbor which belonged to *Pseudomonas* spp. and *Enterobacteriaceae* family (Wuertz *et al.*, 1991). *Shewanella putrefaciens* also showed growth in sea water medium supplemented with low levels of tributyltin (Lee *et al.*, 2012). But till date TBTCl biotransformation product generated by resistant estuarine bacteria has not been analysed yet.

Pseudomonas stutzeri strain DN2 was identified as a potent TBTCl degrader as it could grow best in MSM with 2 mM TBTCl among the tested concentrations as a sole carbon source. It has been reported that oraganotin compounds are toxic to both gram positive as well as gram negative bacteria isolated from sediments; nevertheless, the former showed increased sensitivity to organotins while gram negative bacteria were found to be more resistant to this biocide (Mendo *et al.*, 2003).

Scanning electron microscopy

Scanning Electron Microscopy of *Pseudomonas stutzeri* strain DN2 cells exposed to 2 mM TBTCl clearly demonstrated significant morphological alterations as cell elongation, wrinkling and shrinkage (supplementary Figure 1) . While cells grown in absence of TBTCl showed a normal morphology and cells inoculated in plane MSM didn't show growth. Alterations in cell morphology as cell elongation, wrinkling and shrinkage leads to possible decrease in the surface area of the cells thereby resulting in reduced adsorption capacity of the cells to TBTCl. Similar findings have suggested that bacterial cells grown in presence of TBTCl were smaller than normal size and appeared aggregated (Cruz *et al.*, 2007). Our earlier studies have also demonstrated that *Aeromonas caviae* strain KS-1 under the stress of TBTCl protects itself by forming long chains of cells which reduces the surface to volume ratio and results in reducing the exposed cell surface for TBTCl (Shamim *et al.*, 2012). It has also been demonstrated that certain components on the cell surface of TBTCl resistant *Pseudoalteromonas* sp. possess capability to adsorb toxic TBTCl in marine environments (Mimura *et al.*, 2008). Therefore alteration in cell morphology due to TBTCl exposure may be a protective mechanism adapted by the TBTCl resistant *Pseudomonas stutzeri* strain DN2.

Biotransformation of TBTCl

The quantity of purified biotransformed product of TBTCl obtained after subsequent purification steps was 237 ± 0.85 mg/L.

FTIR and UV-Vis Spectrophotometric analysis

The FTIR spectrum of the purified biotransformation product of TBTCl clearly revealed presence of butyl group giving characteristic bands at 2958.80, 2926.01, 2872.01 and 2856.58 cm^{-1} (Supplementary figure 2). Prominent peak around 1450 cm^{-1} is due to C-H bending vibrations of butyl group. A doublet at 705.95 and 669.30 cm^{-1} is also characteristic of $DBTCl_2$ moiety. The comparison of the FTIR spectrum of the biotransformation product with standard TBTCl and $DBTCl_2$ (SIGMA-ALDRICH, USA) with Pubchem substance ID 24900253 and 24852336 respectively clearly demonstrated that the biotransformation product is different from pure TBTCl and matched with standard $DBTCl_2$. Biotransformation product was also further confirmed by spectrophotometric analysis which showed a characteristic peak at 236 nm corresponding to $DBTCl_2$.

NMR analysis

^1H NMR spectra of the control product showed characteristic peaks at 1.582, 1.313, 1.265 and 0.910 cm^{-1}, whereas ^1H NMR spectra of purified biotransformation product gave peaks at 0.960, 1.392, 1.463 and 1.800 cm^{-1}

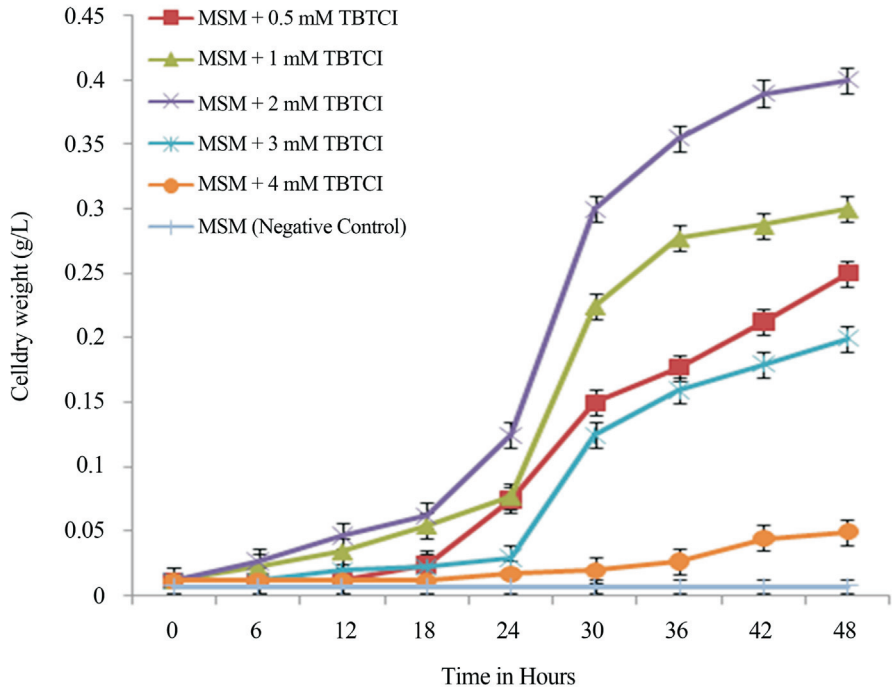

Figure 2 - Growth behaviour of *Pseudomonas stutzeri* strain DN2 in MSM amended with different concentrations of tributyltin chloride as a sole carbon source in terms of Cell dry weight (g/L) Vs Time in hours. Bacteria inoculated in plain MSM without TBTCl is considered as negative control.

(Figure 3. A, B). ^{1}H NMR spectral data of control and purified transformation products (Figure 3. A, B) were compared against standard ^{1}H NMR of TBTCl and $DBTCl_2$ respectively, in the NMR Spectral database for organic compounds (SDBS). Purified biotransformation product significantly matched with standard $DBTCl_2$ (SDBS-^{1}H NMR No. 3555HSP-00-035) which clearly confirmed it to be $DBTCl_2$. The ^{1}H NMR of control used in our experiment also matched with standard TBTCl (SDBS-^{1}H NMR No. 6438HSP-01-479). Small singlet peak at 7.26 cm^{-1} is due to solvent $CDCl_3$ (Figure 3. B). Peak at 1.800 cm^{-1} specifically corresponds to the biotransformation product, $DBTCl_2$ whereas peak at 1.582 corresponds to TBTCl. ^{1}H NMR spectrum of purified product showed slight differences in chemical shift (ppm) of protons from standard TBTCl which further confirmed TBTCl transformation product as

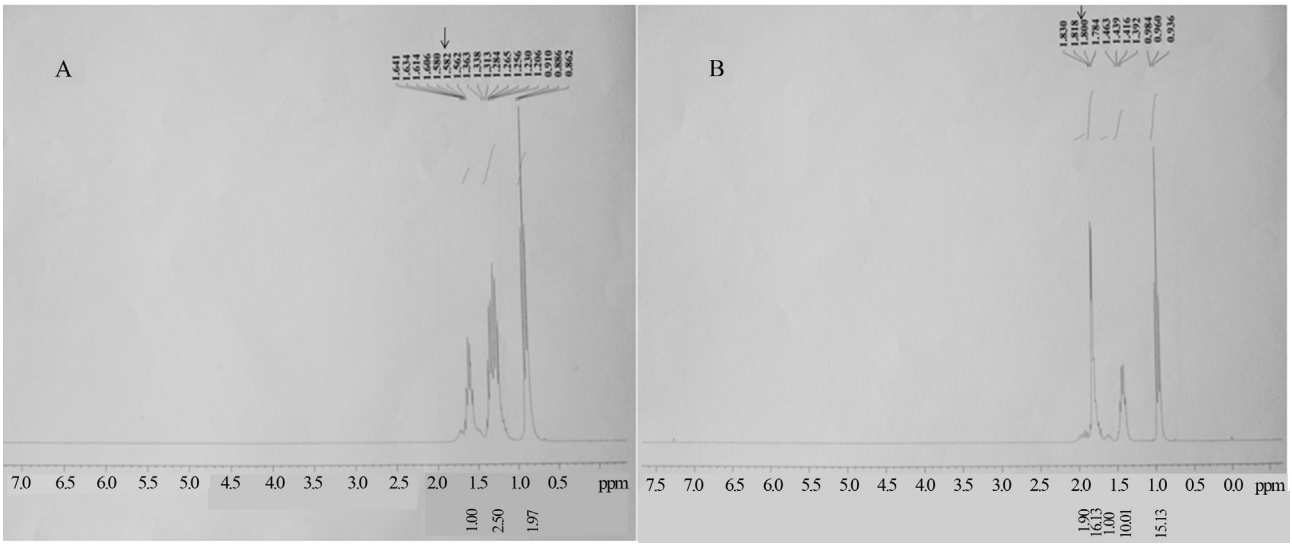

Figure 3 - ^{1}HNMR spectrum of (A) Control (Purified product from the sample devoid of bacterial cells) (B) Purified transformation product obtained after growing *Pseudomonas stutzeri* strain DN2 in MSM supplemented with 2 mM TBTCl. Arrows (↓) indicate TBTCl and $DBTCl_2$ specific peaks at 1.582 cm^{-1} and 1.800 cm^{-1} respectively.

$DBTCl_2$ (Figure 3. A, B). These studies have clearly demonstrated the biotransformation of TBTCl into less toxic product $DBTCl_2$ by *Pseudomonas stutzeri* strain DN2 isolated from coastal sediments of Goa, India.

Biotransformation of TBTCl by bacteria has been reported to be through successive debutylation reactions from $TBTCl \rightarrow DBTCl_2 \rightarrow MBTCl_3 \rightarrow$ inorganic tin via β-hydroxylation involving dioxygenases (Dowson *et al.*, 1996). There are very few reports on TBTCl degradation by estuarine bacteria (Kawai *et al.*, 1998; Mendo *et al.*, 2003; Suehiro *et al.*, 2006; Shamim *et al.*, 2012), but detailed studies on biotransformation of TBTCl to less toxic compounds by bacteria isolated from TBTCl contaminated estuarine sediments have not been conducted so far. Several reports are available on TBTCl resistant bacteria but very little is known about resistance mechanisms adapted by these bacteria to overcome TBTCl stress and toxicity along with biotransformation mechanisms (Barug, 1981; Fukagawa *et al.*, 1994; Jude *et al.*, 2004; Ramachandran and Dubey, 2009; Fukushima *et al.*, 2012; Sampath *et al.*, 2012; Shamim *et al.*, 2012). In the present communication we have clearly demonstrated TBTCl biotransformation potential of *Pseudomonas stutzeri* strain DN2 into its less toxic derivative $DBTCl_2$ through successive debutylation which was further confirmed by NMR, FTIR and UV-Vis spectroscopic analysis of biotransformation product. It is interesting to note that *Pseudomonas stutzeri* strain DN2 can transform TBTCl into its less toxic derivative, $DBTCl_2$ whereas it has been reported earlier to resist TBTCl through efflux mechanism (Jude *et al.*, 2004). It is evident from our studies that *Pseudomonas stutzeri* strain DN2 tolerates high concentrations of TBTCl since it degrades it into less toxic $DBTCl_2$ and also confirms that TBTCl degradation may serve as an additional resistance mechanism besides efflux based resistance mechanism in *Pseudomonas stutzeri*.

This study has evidently demonstrated that *Pseudomonas stutzeri* strain DN2 possesses other TBTCl resistance mechanisms along with biotransformation of TBTCl into $DBTCl_2$. These mechanisms include metabolic utilization of TBTCl as a sole carbon source and significant morphological alterations as cell elongation and shrinkage. These studies have clearly suggested that this sediment bacterial strain may be employed as a potential microorganism to clean up TBTCl contaminated environmental sites.

Pseudomonas stutzeri strain DN2 possessing significant potential to biotransform TBTCl to less toxic $DBTCl_2$ and utilizing it as sole carbon source proves to be an important microorganism which could be used to further characterise TBTCl resistance mechanisms at molecular level. We are trying to explore molecular mechanisms of TBTCl biotransformation operational in this bacterial strain. Our studies on *Pseudomonas stutzeri* strain DN2 have clearly demonstrated that this strain can be employed for environmental cleanup of TBTCl contaminated estuarine sites.

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