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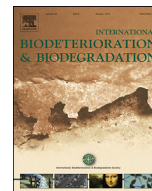

Biotransformation of tributyltin chloride to less toxic dibutyltin dichloride and monobutyltin trichloride by *Klebsiella pneumoniae* strain SD9

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ABSTRACT

A tributyltin chloride (TBTCl) resistant bacterial strain was isolated from the Zuari estuary, in Goa, India, and identified as *Klebsiella pneumoniae* based on biochemical characteristics and 16S rRNA sequence analysis, and designated as strain SD9. It could utilize TBTCl as a sole carbon source in mineral salt medium and tolerated up to 2.5 mM TBTCl with maximum growth at 2 mM. Nuclear magnetic resonance spectroscopic analysis of column purified TBTCl degradation products clearly demonstrated the presence of dibutyltin dichloride ($DBTCl_2$) and monobutyltin trichloride ($MBTCl_3$). Mass spectrometry further confirmed degradation of toxic TBTCl into its less toxic derivatives, viz., $DBTCl_2$ and $MBTCl_3$. This strain also showed enhanced siderophore production in the presence of TBTCl, which was demonstrated by chrome azurol S agar assay as an increase in diameter of the orange halo around the bacterial colony in the presence of 2 mM TBTCl; this seems to be a mechanism to counteract TBTCl toxicity. Furthermore, scanning electron microscopy revealed significant morphological alterations as shrinkage in cell size along with roughness of cell surface when bacterial cells were exposed to 2 mM TBTCl. These interesting characteristics of this estuarine bacterium make it a potential tool for bioremediation of TBTCl-contaminated sites since it possesses biotransformation capability to convert TBTCl into $DBTCl_2$ and $MBTCl_3$.

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1. Introduction

Organotins are compounds consisting of one to four organic components attached to a tin atom via carbon–tin covalent bonds (US-EPA, 2003; Lilley, 2012). Tri-organotins have a broad range of applications with an annual world production of approximating 50,000 tons yr^{-1}; they are commonly used in marine antifouling paints, in PVC stabilizers, as biocides in agriculture, and as preservatives for wood, leather, textiles, and paper; and they have been found to have a detrimental impact upon estuarine and marine biota (Bennett, 1996; Gadd, 2000; Blunden et al., 2004; Sousa et al., 2010; Cruz et al., 2010b; Lemos et al., 2011). One of the tri-organotins, tributyltin chloride (TBTCl), is an active ingredient of many bactericidal, fungicidal, insecticidal, and wood preservation products (Gadd, 2000; Lemos et al., 2011). In 1971, it replaced copper oxide as an antifoulant paint additive, and since then it has been used extensively on ship and boat hulls, cooling water pipes, docks, aquaculture cages, and buoys to prevent attachment and growth of barnacles, algae, mussels, tube worms, and other marine organisms until a partial restriction was enacted by some countries in the 1980s and early 1990s (Dubey and Roy, 2003; Bangkedphol et al., 2009; Lemos et al., 2011). Tri-organotins have also been categorized as a persistent organic pollutant (POP) and an enormous amount of this toxic substance has already been introduced into the terrestrial and aquatic environment through extensive anthropogenic activities, resulting in bioaccumulation in the food chain (Bryan et al., 1988; Dowson et al., 1996; Mendo et al., 2003; Bangkedphol et al., 2009; Ayanda et al., 2012).

In estuarine and marine environments TBTCl has been introduced as a result of leaching from ships and boats painted with TBTCl-based paints. High levels of TBTCl have been reported in estuarine, marine, and freshwater harbors and areas that are primarily associated with boating (Pain and Cooney, 1998; Mendo et al., 2003; Jadhav et al., 2009; Ramachandran and Dubey, 2009; Garg et al., 2010; Ayanda et al., 2012). It has also been reported that the coastal waters of most Asian countries are badly affected by

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persistent organic pollutants (POPs) due to extensive use of these chemicals in paints and agrochemicals (Jadhav et al., 2009; Garg et al., 2010). Tributyltin was found to be the most abundant among the organotin compounds in the Mandovi and Zuari estuaries (Jadhav et al., 2009; Garg et al., 2010). It has been observed that even at low concentrations TBT in seawater exerts lethal effects on a wide variety of marine organisms (Bryan et al., 1988; Ayanda et al., 2012; Lee et al., 2012). Tributyltin chloride is toxic for both eukaryotes and prokaryotes since with a partition coefficient in octanol–water mixture (log P_{ow} at 25 °C) of 3.7, allowing its dissolution into biological membranes, disturbing their integrity, and ultimately disturbing their physiological functions (Jude et al., 2004). The persistence of TBTCl in the estuarine environment is reported to have immunological, carcinogenic, and teratogenic effects on non-target organisms (Sousa et al., 2010; Lemos et al., 2011). Therefore there is pressing need to clean up TBTCl-contaminated estuarine environments using a biotechnological approach without affecting environmental sustainability. Despite TBTCl toxicity to several organisms, TBTCl resistance has been reported in some bacterial isolates (Wuertz et al., 1991; Fukagawa et al., 1994; Dubey and Roy, 2003; Mimura et al., 2008; Ramachandran and Dubey, 2009; Fukushima et al., 2012; Shamim et al., 2013). Interestingly, there are some reports on marine and estuarine bacterial strains resisting TBTCl and there is evidence suggesting that biodegradation is the major breakdown pathway in estuarine and marine sedimentary environment (Wuertz et al., 1991; Fukagawa et al., 1992; Dubey and Roy, 2003; Mendo et al., 2003; Cruz et al., 2007, 2010a,b; 2014; Lee et al., 2012; Sampath et al., 2012). Bacteria play an important role in biogeochemical cycling along with organic matter degradation and recycling of toxic compounds, including organotins, in estuarine ecosystems (Wuertz et al., 1991; Ayanda et al., 2012; Lee et al., 2012). TBTCl-resistant bacteria are able to resist very high levels of TBTCl by employing a number of mechanisms: (a) biotransformation into less toxic di- and mono-butyltins by dealkylation; (b) efflux of TBTCl outside the bacterial cell mediated by efflux pumps; (c) degradation/metabolic utilization of TBTCl as sole carbon source mediated by enzymes; (d) intracellular sequestration and bioaccumulation mediated by metallothionein-like proteins; (e) siderophore production; and (f) alteration in cell morphology (Gadd, 2000; Inoue et al., 2003; Dubey and Roy, 2003; Jude et al., 2004; Sun et al., 2006; Cruz et al., 2007; Ramachandran and Dubey, 2009; Sampath et al., 2012; Shamim et al., 2013). Bioremediation of TBTCl by employing TBTCl-resistant bacteria has already been reported to be better than using physicochemical methods (Ayanda et al., 2012; Lee et al., 2012).

Keeping in mind these multiple resistance mechanisms operational in estuarine and marine bacteria, in this study we report on the isolation, identification, and characterization of an estuarine bacterium with reference to TBTCl resistance mechanisms, viz., TBTCl biotransformation into less toxic derivatives, siderophore production, and alteration in bacterial cell morphology under TBTCl stress, and also suggest possible involvement of extracellular proteins in conferring TBTCl resistance in bacteria.

2. Materials and methods

2.1. Growth media and composition

Zobell marine broth (ZMB) and Zobell marine agar (ZMA) were purchased from Hi Media and TBTCl (98% purity) from Sigma Aldrich, USA. The composition of mineral salt medium (MSM) (1L) used for growth and biotransformation experiments was: 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).

2.2. Isolation and identification of TBTCl-resistant bacterial strain

A surface water sample from the Zuari estuary, Goa, India, was serially diluted and 100 µl of sample was plated on ZMA plates supplemented with 0.5 mM TBTCl and incubated at room temperature (28 ± 2 °C) for 24–48 h in order to screen TBTCl-resistant bacterial colonies. Colonies, which appeared on ZMA plates, were further screened for resistance at higher concentrations of TBTCl (0.5–4.5 mM). The bacterial isolate capable of tolerating the highest levels of TBTCl was selected for further characterization and designated as strain SD9. Then bacterial strain SD9 was maintained on ZMA slants containing 2 mM TBTCl at 4 °C and was then tentatively identified using Gram staining and morphological and biochemical characteristics following *Bergey's Manual of Systematic Bacteriology* (Garrity et al., 2005); identity was further confirmed by 16S rRNA sequencing and BLAST search analysis (Altschul et al., 1990).

2.3. Growth behavior of TBTCl-resistant isolate

Bacterial strain SD9 was grown in ZMB amended with gradually increasing concentrations of TBTCl (0–4.5 mM) at room temperature (28 ± 2 °C) and pH 7.5 with constant shaking at 150 rpm. Absorbance was recorded at 600 nm at regular 2 h intervals using a UV–Vis spectrophotometer (Shimadzu, UV-2450, Japan). Controls used were ZMB broth incorporated with respective TBTCl concentrations.

2.4. Utilisation of TBTCl as sole carbon source

Bacterial cells of the TBTCl-resistant strain were grown in MSM amended with gradually increasing levels of TBTCl (0–3 mM) and incubated for 48 h at room temperature (28 ± 2 °C) and 150 rpm. The absorbance of culture suspension was recorded at 600 nm after 48 h. The control was MSM broth incorporated with TBTCl.

2.5. Purification and analysis of TBTCl biotransformation product

The TBTCl-resistant bacterial strain SD9 was grown in a 250-ml Erlenmeyer flask containing 100 ml MSM (initial inoculum gave OD of 0.025) with 2 mM TBTCl as sole carbon source at room temperature (28 ± 2 °C) in an incubator shaker at 150 rpm for 1 wk. 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. This supernatant was extracted using a double volume of distilled chloroform. The organic layer was collected in a conical flask. The chloroform extract was reduced under vacuum and further purified through silica gel H-20 column chromatography (SIGMA, 30- x 2-cm glass column). Slurry of silica gel H-20 was prepared by mixing 9 g of silica in 20 ml of petroleum ether (40–60 °C). A glass column (15.5 cm) was packed by adding the slurry with the help of a glass rod and it was gently tapped to get rid of any void volume. The concentrated residual extract was added to the column and chloroform was passed through the column. The purity of the eluted product was checked by thin layer chromatography (TLC). The same procedure was repeated for the control flask. The eluent obtained after purification by column chromatography was further concentrated under vacuum, dried, and weighed using an electronic weighing balance. The purified,

concentrated degradation and control products were stored in a screw-capped glass vial at 4 °C until use for further analysis.

2.6. Nuclear magnetic resonance spectroscopic analysis of the biotransformation product

Nuclear magnetic resonance specta (H^1 NMR) of the column-purified control and biotransformation products 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. A sample (5 mg) was dissolved in deuterated $CDCl_3$ (0.6 ml) and placed in an NMR tube (5 mm diameter). The NMR tube was appropriately positioned in a spinner and introduced into the NMR spectrometer. All the reagents were purchased from Sigma Aldrich, USA.

2.7. Mass spectrometric analysis of the biotransformation product

Subsequent to NMR spectroscopic analysis, the column-purified biotransformation product was further analyzed by MS to confirm the presence of biotransformation products. In a typical experiment, purified biotransformation product was mixed with cyclohexane prior to injection (2 µl) into a mass selective detector (Applied Biosystem QSTAR XL, Canada) for analysis. In MS a sample is ionized by bombarding it with electrons. This may cause sample molecules to break into charged fragments. These ions are then separated according to their mass-to-charge ratio, typically by accelerating them and subjecting them to an electric or magnetic field: Ions of the same mass-to-charge ratio will undergo the same amount of deflection. The ions are detected by a mechanism capable of detecting charged particles, such as an electron multiplier. Results are displayed as spectra of the relative abundance of detected ions as a function of the mass-to-charge ratio. The atoms or molecules in the sample can be identified by correlating known masses to the identified masses or through a characteristic fragmentation pattern; this pattern is then compared with the mass library.

2.8. SDS – PAGE of TBTCl-induced extracellular proteins

The bacterial strain SD9 was grown in MSM at 28 ± 2 °C and 150 rpm, supplemented with 2 mM TBTCl, and cells were then separated from the supernatant by centrifugation at 12,000 rpm for 20 min. The cell-free supernatant was then passed through a nitrocellulose filter with pore size 0.22 µm to ensure complete elimination of cells. Filtrate (10 µl) was then analyzed using SDS PAGE (Sambrook et al., 1989). Cell-free supernatant (10 µl filtrate) of culture grown in the absence of TBTCl but in the presence of 0.1% glucose as carbon source served as a negative control. This was followed by silver staining (Laemelli, 1970). SDS PAGE gel after destaining was documented using the Alpha Innotech (USA) gel documentation system.

2.9. Production of siderophore under TBTCl stress

The chrome azurol S (CAS) agar assay (Schwyn and Neilands, 1987) was used to detect siderophores produced by the TBTCl-resistant strain SD9. Bacterial culture was spot-inoculated on the CAS agar (blue agar) plate and the plate was incubated at room temperature (28 ± 2 °C) for 24–48 h. The results were interpreted based on the color change of CAS agar from intense blue to an orange zone surrounding the bacterial colony. A similar experiment was repeated by amending 2 mM TBTCl in CAS agar to check siderophore production under TBTCl stress and the diameter of the orange halo around the bacterial colony was recorded. A CAS agar plate amended with 0 mM TBTCl was used as the control.

2.10. Morphological characterization of TBTCl-resistant strain

In order to detect any morphological alterations in the presence of TBTCl, scanning electron microscopic analysis was performed using the bacterial strain SD9 grown in MSM supplemented with 2 mM TBTCl, and bacterial strain grown in MSM with 0.1% glucose was used as a control. A culture smear was prepared on a glass slide, air-dried, and fixed in 3% glutaraldehyde overnight with 50 mM potassium phosphate buffer at 4 °C. The glass slide was then washed three times 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 a vacuum chamber prior to scanning electron microscopy (Naik and Dubey, 2011).

3. Results

3.1. Isolation and identification of TBTCl-resistant bacterial strain

The TBTCl-resistant bacterial strain SD9, isolated from the Zuari estuary, was Gram-negative, non-motile, short rod, and fermentative. Indole and methyl red tests were negative but the bacterial isolate metabolized citrate and the Voges Proskaeur's test was found positive. The bacterial isolate was urease-positive and showed presence of a capsule. Based on biochemical characteristics and using *Bergey's Manual of Systematic Bacteriology*, as well as 16S rRNA sequencing and BLAST search analysis, strain SD9 was identified as *Klebsiella pneumoniae* (Altschul et al., 1990; Garrity et al., 2005). The 16S rRNA sequence of the strain received GenBank Accession No. JQ993102. Phylogenetic analysis revealed its evolutionary relatedness to *K. pneumoniae* and showed 100% homology with *K. pneumoniae* strain ATCC13884T (Fig. 1).

3.2. Growth behavior of TBTCl-resistant bacterial isolate

The optical density (OD) at time zero was 0.025; this gradually increased with time. *K. pneumoniae* strain SD9 showed the best growth in ZMB with 2 mM TBTCl and, interestingly, showed a very short lag phase. Although at TBTCl concentrations higher than 2 mM a significant lag of 4–8 h was observed, the strain could tolerate TBTCl even up to 4 mM. This clearly indicates that a TBTCl concentration above 2 mM was toxic to the bacteria in ZMB. It was also noted that at higher concentrations of TBTCl, a shorter exponential phase and a faster death phase occurred, further revealing the toxicity of TBTCl to bacterial cells (Fig. 2).

3.3. Utilization of TBTCl as a sole carbon source

K. pneumoniae strain SD9 showed the best growth in MSM with 2 mM TBTCl as a sole carbon source but could tolerate TBTCl up to 2.5 mM (Fig. 3). Utilization of TBTCl as a sole source of carbon clearly demonstrated that the test organism was a TBTCl degrader.

3.4. Nuclear magnetic resonance spectroscopic analysis of the biotransformation product

The quantity of purified biotransformed product of TBTCl obtained after subsequent purification steps was 252 ± 0.85 mg l^{-1}.

Nuclear magnetic resonance spectrum analysis (H^1 – NMR) of the column-purified biotransformation products clearly revealed a mixture of TBTCl biotransformation products, viz., $DBTCl_2$ and $MBTCl_3$. The NMR spectrum of the control sample showed

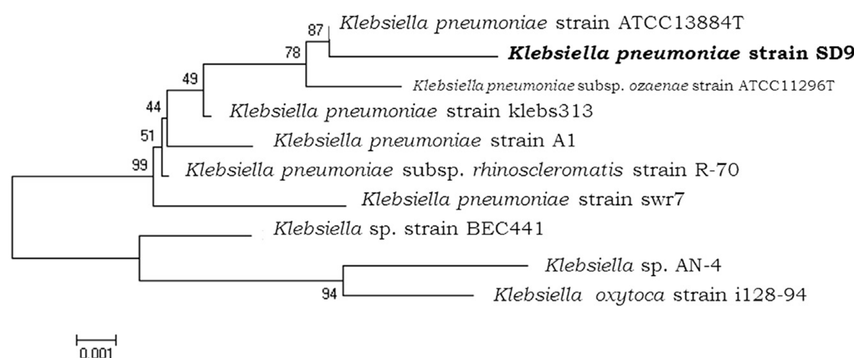


Fig. 1. Phylogenetic tree showing evolutionary relationship between *Klebsiella pneumoniae* strain SD9 and other *Klebsiella* spp. using the neighbor-joining method. The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test is shown next to the branches.

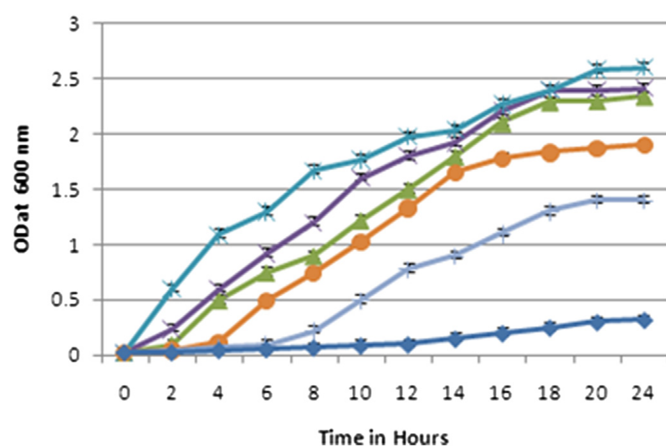
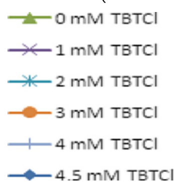


Fig. 2. Growth profile of *Klebsiella pneumoniae* strain SD9 grown in Zobell marine broth with varying concentrations of TBTCl (0–4.5 mM).



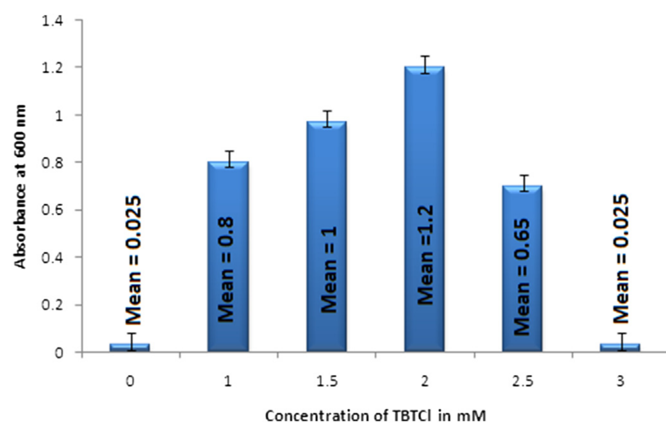


Fig. 3. Growth of *Klebsiella pneumoniae* strain SD9 in terms of absorbance (600 nm) at varying TBTCl concentrations in MSM.

characteristic peaks at 1.562 cm^{-1}, 1.313 cm^{-1}, 1.265 cm^{-1}, and 0.910 cm^{-1} (Fig. 4a). This was compared with the peaks of biotransformed products obtained after 1-wk incubation period (Fig. 4b). The biotransformation product showed peaks at 3.143 cm^{-1}, 2.407 cm^{-1}, 1.834 cm^{-1}, and 1.573 cm^{-1} (Fig. 4b). The ^{1}H NMR data of TBTCl used in our experiment and the purified transformation product (Fig. 4a,b) were also compared against the standard ^{1}H NMR of TBTCl, $DBTCl_2$, and $MBTCl_3$ in the spectral database for organic compounds SDBS (http://sdbs.riodb.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi). The ^{1}H NMR of TBTCl (control) used in our experiment matched with the standard TBTCl SDBS-^{1}H NMR No. 6438HSP-01-479. The ^{1}H NMR spectrum of biotransformation product showed a significant difference in chemical shift from the standard TBTCl. The peak obtained by the biotransformation product at 1.573 corresponds to TBTCl while the peak at 1.834 specifically corresponds to $DBTCl_2$ (SDBS-^{1}H NMR No. 3555HSP-00-035), whereas the singlet at 3.143 and the peak at 2.407 are attributed to $MBTCl_3$ (Fig. 4b). The singlet peak at 3.143 revealed the presence of an organic compound bonded to a Sn (tin) moiety, whereas the peak at 2.407 revealed Sn further attached to three chloride atoms, thereby confirming that the final degradation product was a monobutyltin trichloride entity. The control sample didn't show any peaks beyond 1.562 cm^{-1}, indicating the absence of degradation of TBTCl in control conditions (Fig. 4a).

3.5. Mass spectrometric analysis of the biotransformation product

The biotransformation product was further confirmed by mass spectrometric detection of column-purified degradation product. A peak of *m*/*z* 178.9055, corresponds to monobutyltin and peaks having *m*/*z* values 234.9403 and 290.9760 revealed the presence of dibutyltin and tributyltin, respectively (Fig. 5). A peak of *m*/*z* 122.8734 corresponds to the Sn^+ isotope (Fig. 5). These results go hand in hand with earlier findings of Banaub et al. (2004) and clearly revealed that *K. pneumoniae* strain SD9 biotransforms TBTCl into $DBTCl_2$ and finally into $MBTCl_3$ by utilizing it as a sole carbon source in MSM.

3.6. Characterization of TBTCl-induced extracellular proteins

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of extracellular proteins of TBTCl degrading *K. pneumoniae* strain SD9 clearly revealed up-regulation of 54 kDa and 66 kDa polypeptides and induction of 15 kDa extracellular polypeptide when bacterial cells were grown in the presence of 2 mM TBTCl, confirming their possible role in TBTCl resistance.

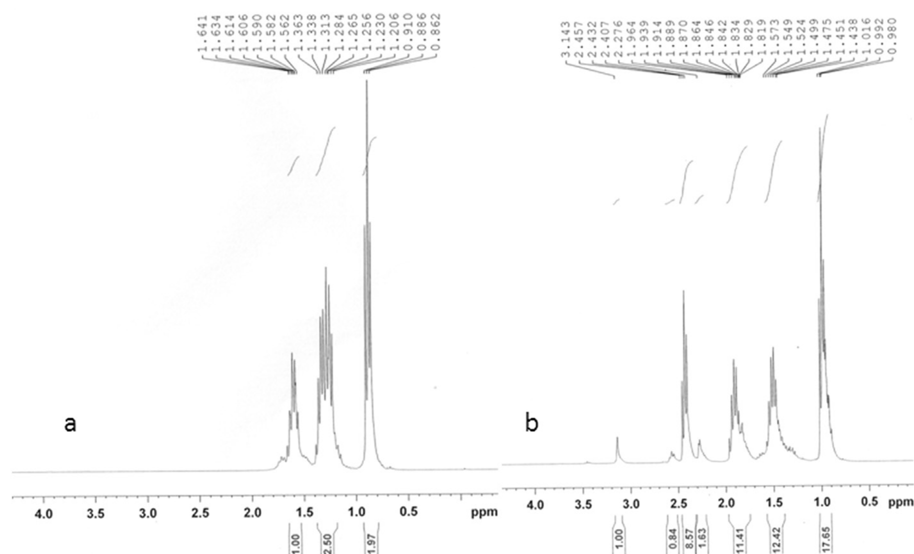


Fig. 4. ^{1}HNMR spectrum of (a) control (purified product from the sample devoid of bacterial cells); and (b) purified transformation product obtained after growing *Klebsiella pneumoniae* strain SD9 in MSM supplemented with 2 mM TBTCl.

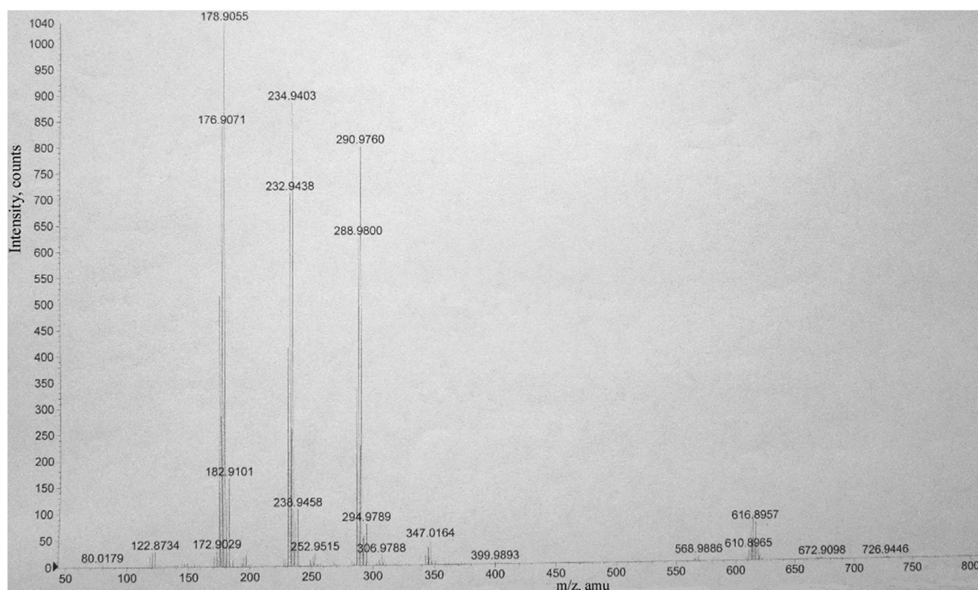


Fig. 5. Mass spectroscopic profile of purified transformation product obtained after growing *Klebsiella pneumoniae* strain SD9 in MSM supplemented with 2 mM TBTCl.

3.7. Production of siderophores under TBTCl stress

A CAS agar assay clearly revealed orange zones around the bacterial colony, confirming siderophore production in the plates amended with TBTCl, and those without TBTCl. The diameter of the orange zone was observed to be much larger (4.2 cm $\pm$ 0.3) in the case of plates supplemented with 2 mM TBTCl as compared to control plates devoid of TBTCl (2.5 cm $\pm$ 0.4).

3.8. Morphological characterization of TBTCl-resistant strain

Scanning electron microscopic analysis of *K. pneumoniae* strain SD9 exposed to 2 mM TBTCl showed significant morphological alterations. Changes in cell morphology were evident as shrinkage and significant reduction in cell size when cells were exposed to 2 mM TBTCl.

4. Discussion

Several bacterial strains can tolerate high levels of TBTCl, but very few can utilize it as a sole carbon source following its degradation (Barug, 1981; Dubey and Roy, 2003; Cruz et al., 2007; Krishnamurthy et al., 2007; Sampath et al., 2012). It is interesting to note that *Klebsiella pneumonia*e strain SD9 can successfully utilize 2.0 mM TBTCl as a sole carbon source. *Klebsiella pneumonia*e strain SD9 also showed tolerance of up to 4 mM TBTCl in ZMB as compared to 2.5 mM in MSM, which indicates that toxicity of TBTCl in nutrient-rich medium was lower than that in minimal medium (Figs. 2 and 3). The nutrient-rich ZMB provides protection to bacterial cells from TBTCl stress, as they can utilize other carbon sources (Ramamoorthy and Kushner, 1975; Cruz et al., 2010b, 2012). Growth of strain SD9 in ZMB with 2 mM TBTCl is better than that of the cells grown in 1 mM TBTCl and those of the control (ZMB

without TBTCl), suggesting utilization of TBTCl as carbon source along with other carbon sources present in nutrient-rich ZMB through co-metabolism (Kawai et al., 1998; Cruz et al., 2012) (Fig. 2).

Some microorganisms are known to utilize TBTCl as a sole carbon source and also transform it into its less toxic derivatives, viz., $DBTCl_2$ and $MBTCl_3$ (Barug, 1981; Fukagawa et al., 1994; Jude et al., 2004; Ramachandran and Dubey, 2009; Sampath et al., 2012), but a detailed study of transformation products and mechanisms behind this have still not been explored. Here, we have reported on a TBTCl-resistant *K. pneumoniae* strain, SD9, which is able to utilize TBTCl as a sole carbon source in MSM and also biotransform it into less toxic $DBTCl_2$ and $MBTCl_3$ as revealed by $H^1 - $NMR analysis and MS analysis of the purified biotransformation product. The presence of $DBTCl_2$ and $MBTCl_3$ specific peaks in the purified cell-free supernatant confirms successful biotransformation of TBTCl into these less toxic derivatives by *K. pneumoniae* strain SD9.

There are very few reports on expression of TBTCl-induced proteins that may have been involved in resistance or degradation. The up-regulation of two proteins has been reported in TBTCl-resistant *Vibrio* sp. 30 kDa and 12 kDa, which were observed when cells were grown in the presence of 125 μM TBTCl (Fukagawa et al., 1992). The expression of TBTCl induced three periplasmic proteins of 43 kDa, 63 kDa and 68 kDa; this has also been reported in TBTCl-resistant marine sediment isolate *Alcaligenes* sp. which may be responsible for TBTCl resistance (Ramachandran and Dubey, 2009). Our studies reveal up-regulation of 54 kDa and 66 kDa extracellular proteins with the induction of a 15 kDa protein in the presence of 2 mM TBTCl. Thus a significant involvement of these TBTCl-regulated extracellular proteins in governing resistance to TBTCl in *K. pneumoniae* strain SD9 may be hypothesized. However, it is imperative to carry out further characterization of these extracellular TBTCl-induced and up-regulated proteins to confirm their role in TBTCl degradation. Therefore more research is being carried out of this TBTCl degrader at the molecular level to explore functions of extracellular proteins in TBTCl resistance.

Microbial siderophores are known to play an important role in iron acquisition (Neilands, 1995). But involvement of pyoverdine siderophore in degradation of toxic triphenyltin (TPT) in *Pseudomonas chlororaphis* CNR15 has also been reported (Inoue et al., 2003). The role of pyochelin in conferring resistance to TPT has also been demonstrated in *Pseudomonas aeruginosa* (Sun et al., 2006). Both pyochelin and pyoverdine siderophores have also been implicated in lead resistance in *P. aeruginosa* strain 4EA (Naik and Dubey, 2011). Similarly, TBTCl-degrading marine *Pseudomonas* spp. also produced siderophores in the supernatants (Sampath et al., 2012). However, research on siderophores involved in TBTCl resistance mechanisms is lacking. In the present study, an increase in siderophore production in the presence of 2 mM TBTCl clearly revealed the involvement of TBTCl in up-regulation of siderophore synthesis in *K. pneumoniae* strain SD9 and its role in TBTCl resistance. It is noteworthy that *K. pneumoniae* strain SD9 showed TBTCl-induced up-regulation of siderophore synthesis along with up-regulation of two extracellular polypeptides (54 kDa and 66 kDa) and induction of one polypeptide (15 kDa), suggesting their possible role in TBTCl resistance, degradation (biotransformation), and utilization.

Scanning electron micrographs reveal that cells in the presence of TBTCl had a rougher cell surface along with a smaller cell size than controls. Such drastic alterations in cell size are a protective mechanism in several bacteria subjected to heavy-metal and organotin stress (Mimura et al., 2008; Naik and Dubey, 2011; Shamim et al., 2013). A similar response was observed in *P. aeruginosa* strain 4EA under the stress of lead (Naik and Dubey, 2011). Therefore, morphological alterations observed in the present study, such as reduction in bacterial cell size, wrinkling, and roughness on cell surfaces, may be adaptive resistance mechanisms in *K. pneumoniae* strain SD9 to counteract TBTCl stress.

5. Conclusion

Removal of organotins, specifically TBTCl, from estuarine environments is a challenging environmental problem. There is a pressing need to clean up TBTCl-contaminated estuarine areas using a biotechnological approach without affecting environmental sustainability. Therefore it is imperative to employ TBTCl-resistant bacteria in bioremediation of contaminated sites since the approach is economically viable and ecofriendly.

The present investigation demonstrates a TBTCl-degrading estuarine bacterial isolate, *K. pneumoniae* strain SD9, that biotransforms this pollutant into its less toxic derivatives, viz., $DBTCl_2$ and $MBTCl_3$. The bacterial strain demonstrated induction and up-regulation of extracellular proteins as well as alteration in cell morphology and enhanced siderophore synthesis when subjected to TBTCl stress. Therefore *K. pneumoniae* strain SD9 proves its potential as a tool to bioremediate TBTCl-contaminated estuarine environmental sites.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibiod.2015.04.030.

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