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# Microbial transformation of tributyltin chloride by *Pseudomonas aeruginosa* strain USS25 NCIM-5224

**Upal Roy* and Saroj Bhosle**

Department of Microbiology, Goa University, Taleigao plateau, Goa 403206, India

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**A bacterial isolate capable of utilizing tributyltin chloride (TBTC) as sole source of carbon was isolated from marine water samples. The isolate, producing a soluble green pigment, was identified as *Pseudomonas aeruginosa* strain USS25 NCIM-5224. The isolate showed maximum growth with 2 mM of TBTC in mineral salt medium. Time course results showed complete elimination of TBTC after 75 days of incubation. During the growth on TBTC, a product (280 mg) was found to accumulate, which was extracted with chloroform and detected on thin-layer chromatography. Based on the IR, NMR spectra and GC-MS analysis, the isolated product was identified as monobutyltin dichloro hydride. Copyright © 2005 John Wiley & Sons, Ltd.**

**KEYWORDS:** tributyltin chloride (TBTC); biodegradation; mineral salt medium (MSM); *Pseudomonas aeruginosa*

## INTRODUCTION

Organotin compounds have been employed in industry as catalysts, biocides, stabilizing agents, etc. Tributyltin compounds (TBT), such as TBTC, are commonly used as antifouling agents in marine paints[1] and have been found to have a detrimental impact upon the coastal ecosystem, mainly on living biota.[2–6,41] In the analysis of marine sediment and surface water, it has been observed that organotin is found more in sediments and the surface microlayer. The degradation rate of TBT is significantly slower within sediments than in the water column, in order of years rather than days to weeks.[7] Because of the low solubility of TBT and other properties, it binds strongly to suspended materials such as minute organic materials or inorganic sediments.[8] Reports on the persistence of TBTC showed that in laboratory conditions the half-life of TBT in freshwater sediment was 360 days (initial concentration of 450 ng/g),[9] whereas *in situ* studies on TBT degradation utilizing marine sediments revealed that TBT half-life ranges from 0.91 to 5.2 years.[9,10] Therefore the rate of TBT degradation is taken as a key element in predicting steady-state concentrations in risk assessments.

It has been recognized that even low concentration of these compounds, especially TBT, in seawater exert lethal and sub-lethal effects on wide variety of marine organisms.[11] Compounds such as TBTC and triphenyltin chloride (TPTCl) are toxic to both prokaryotes and eukaryotes.[12,13,40] The mono-, di- and tetra-organotins are almost non-toxic or barely toxic but in contrast, triorganotin compounds, whether aliphatic or aromatic, are highly toxic.[14,15]

A few reports have shown detoxification of TBT by microorganisms, but isolation of TBT-utilizing bacteria has not been successful so far,[10,13,16–20] although Barug[21] reported that several Gram-negative bacteria possess the capability to accumulate tributyltin oxide without its breakdown. As microbial degradation was observed to be the predominant process for the breakdown of TBT in near-shore waters, with dibutyltin as the major product,[22–25] it was interesting to investigate the transformation of TBTC regulated by microorganisms. Moreover TBT was found to be most abundant among the organotin compounds in Marmagao harbour sediments, Mandovi estuary and surroundings areas of the west coast of India, indicating fresh inputs and less degradation of TBT.[26] We report here studies on a natural bacterial isolate, obtained from marine surface water from the west coast of India, capable of utilizing TBTC as sole source of carbon, and characterization of its product.

## MATERIAL AND METHODS

### Culture

A bacterial culture was isolated from water samples of marine ecosystems of the west coast of India, utilizing TBTC as sole

*Correspondence to: Upal Roy, Molecular Biology Unit, National Center for cell science, Pune University Campus, Ganeshkind, Pune 411007, India.
E-mail: upal123@rediffmail.com
Contract/grant sponsor: Department of Ocean Development, New Delhi.

source of carbon.[27] This isolate was purified and maintained on mineral salts medium (MSM).[28,29] The strain was biochemically identified as *Pseudomonas aeruginosa* strain USS25,[27] and further confirmed using 16S rRNA sequencing with primer 16F27N (CCAGAGTTTGATCMTGGCTCAG), 530F (GTC CCAGCMGCCGCGG) and 16F704-A (GTAGCGGTGAAATGCGTAGA). The culture is deposited in NCIM, National Chemical Laboratory, Pune, India, accession no. NCIM 5224.

## Chemicals

Standard compound TBTC ($C_{12}$ $H_{27}$ ClSn) [1461-22-9], dibutyltin (DBT) and tripropyltin (TPrT) were obtained from Merck, Germany. Sodium borohydride was from SD Chemicals, India. All compounds were of 97–98% purity and were used as obtained. Sodium hydroxide and methanol were of analytical grade and were purchased from Merck. Ethanol and methanol were doubled-distilled before used. TBTC stock solution was prepared in filter-sterilized ethyl alcohol and kept in the dark at 4 °C. The experiment was performed by removing the required amount of stock solution in sterile conditions. For gas chromatographic (GC) analysis, standard stock solution was prepared separately by dissolving 10 mg in 100 ml methanol and stored at −20 °C. Suitable aliquots of the stock solution were diluted and used as standard and/or internal standard. Sodium borohydride reagent (6%) was freshly prepared everyday by dissolving 6 g in 100 ml distilled water.

## Media

MSM (single strength) for 1 l contained 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), and the final pH of the medium was 7.4. TBTC was added from the stock solution prepared in ethyl alcohol at the required concentrations of MSM.

## Transformation of TBTC

### *Extraction of butyltin for TLC analysis*

*Pseudomonas aeruginosa* strain USS25 was grown in 100 ml MSM broth with 2 mM TBTC as sole source of carbon at 28 °C in an incubator shaker at 180 rpm for 48 h. After incubation, the cell pellet was harvested by centrifugation at 8000 rpm (4 × 100, REMI, C-24, CUCT-5578) and the supernatant was separated. The cell pellet and supernatant were extracted separately with a double volume of distilled chloroform using a separating funnel. The organic layer was collected in a tube. The emulsion in chloroform was treated with Hyflo Super Cell. Chloroform extract was concentrated with nitrogen gas and the concentrated sample was analysed by thin-layer chromatography (TLC)[30] using the solvent system petroleum ether (40–60 °C) and acetic acid (9.5 : 0.5). Subsequently the TLC plate was exposed to iodine vapour to develop the spot.

### *Extraction of butyltins for GC analysis*

Culture was grown in 20 ml MSM + 2 mM TBTC in five different 50 ml flask at 28 °C in an incubator shaker at 180 rpm and culture broth was withdrawn from the flask at the respective time period, i.e. 0 h and 7, 28, 45 and 75 days.

In order to analyse total butyltin, broth samples were extracted following the method described by Matthias *et al*.[6] A suitable aliquot of culture broth (1 ml) was transferred to a separating funnel and 5 ml of dichloromethane, 1 ml of 6% (w/v) aqueous $NaBH_4$ and 500 µl containing 645 ng of the internal standard of TPrT were added. The extraction was done initially for 1 min, then vented and shaken for additional 10 min. Following a 5 min settling period, the lower organic layer was removed. The extraction was repeated with an additional 5 ml dichloromethane. The combined extract in a glass bottle was dried using anhydrous sodium sulfate and filtered through Whatman filter paper. The organic layer was reduced to 1 ml using dry nitrogen. The concentrated organic layer (1 ml) was then transferred to a polypropylene Eppendorf centrifuge tube, reduced further to 100 µl under dry nitrogen gas and analysed by gas chromatography as described below. Standard was prepared by adding 100 µl DBT (203 ng), TBT (121 ng) and TPrT (129 ng, internal standard) to 750 ml of distilled water and following the process described above.

### *Analysis of butyltins*

Separation and quantification of butyltins were performed by a capillary gas chromatograph (Agilent HP 6890 Series model) equipped with a flame photometric detector (FPD), a tin specific filter (610 nm) and a HP-5 capillary column (5 m × 530 µm × 2.65 µm). A 1 µl sample or standard mixture was injected into a programmable column injector when the initial oven temperature was 60 °C. After 2 min the oven temperature was programmed to 230 °C @ 20 °C $min^{-1}$ and held at this temperature for 8 min. Nitrogen was used as a carrier gas (1 ml $min^{-1}$). The injector was operated in track oven mode while the FPD detector was maintained at 250 °C with hydrogen and air flowing at 80 and 105 ml $min^{-1}$, respectively. Quantification of each peak in a sample was done by using the data handling system installed in the instrument.

## Purification of transformed product

### *Preparative TLC method to extract organotin compounds*[31]

The TLC plate (0.5 mm) was prepared and kept for activation in the oven. The maximum amount of sample was then loaded onto the plate and developed in the solvent system of petroleum ether (40–60 °C) and acetic acid (9.5 : 0.5). After development, the plate was removed and three-quarters of the plate covered with another glass plate to expose only part of the plate to iodine vapour for visualization of the spot. The location of the spot was marked and silica gel was then scraped off from plates leaving the exposed part.

Then product was extracted by repeated washing of silica gel with chloroform. The concentrated and residual extract was further purified by column chromatography.

### *Purification by column chromatography*

A 30 × 2 mm glass column was washed, cleaned and dried. Slurry of silica gel H-20 (SIGMA) was prepared by mixing 8 g of the gel in 20 ml of ether (40–60 °C). The column (16.5 cm) was packed by adding the slurry slowly with the help of a glass rod. The chloroform was passed through the column and the elution of product was detected by TLC. The elutent was concentrated with nitrogen gas and the purified product was analysed using IR, NMR and GC-MS.

### *Spectral analysis of the product*

IR spectra were recorded on IR Shimadzu (model 8201PC) FTIR. Nuclear magnetic resonance spectrum analysis ($H^1$

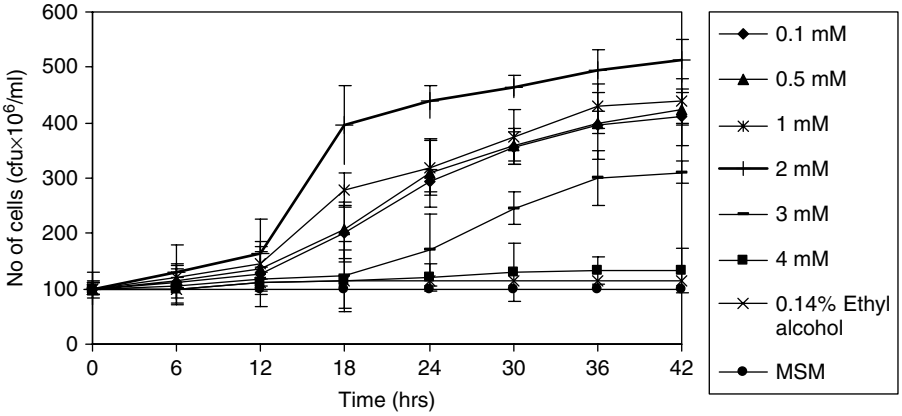


**Figure 1.** Growth behaviour of *Pseudomonas aeruginosa* strain USS25 in different concentrations of TBTC in MSM.

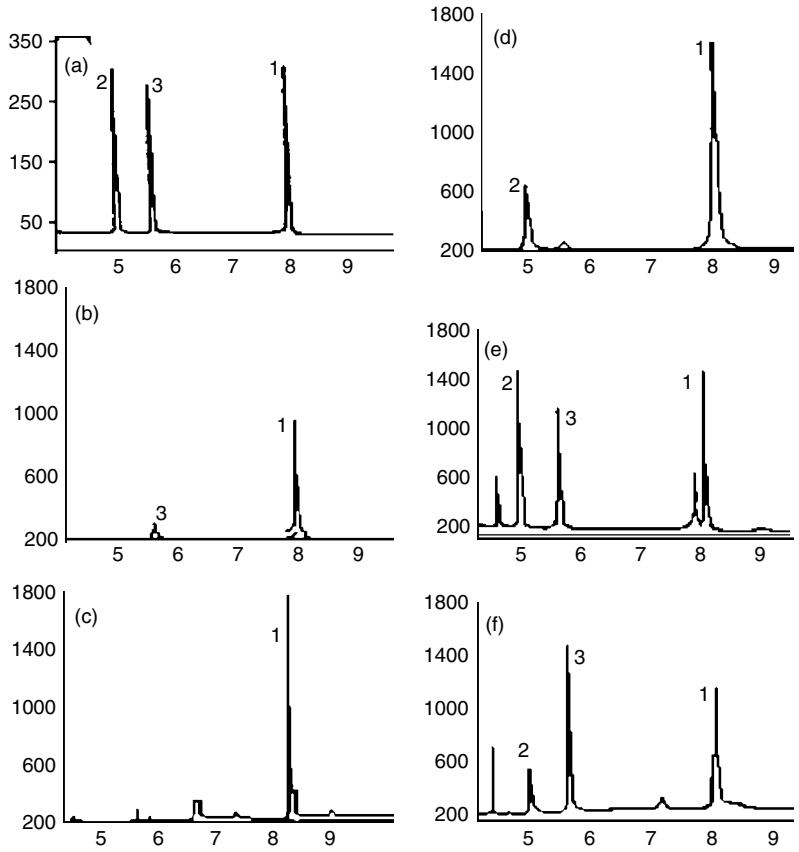


**Figure 2.** (a) Gas chromatogram (HP6890 series model) of standard TBTC (1), DBT (2), TPrT (3). (b) Bacterial culture broth (0 h); (c) 7 days; (d) 28 days; (e) 45 days; (f) 75 days; (g) by use of HP-5 capillary column.

NMR) was recorded on FT NMR Bruker WT (300 MHz) in $CDCl_3$ with tetramethyl silane (TMS) as an internal standard.

Mass spectroscopy (Thermofinigan) was performed in trace GC with trace MS plus. The column used was a cap column HP-5 (30 m × 0.2 μm) at temperature 180–225 °C. The injection temperature, interphase temperature and source temperature were 220, 275 and 180 °C, respectively.

## RESULTS AND DISCUSSION

Our earlier studies resulted in the isolation of bacterial cultures utilizing TBTC as sole source of carbon in mineral salt medium and among the those isolates the most potent was identified as *Pseudomonas aeruginosa* strain USS25.[27] The present work on transformation of TBTC was carried out using this culture, which showed better utilization of TBTC in MSM broth with TBTC as carbon source. Interestingly, the control flask with MSM + ethyl alcohol (0.14%, v/v) did not support visible growth of the present isolate (Fig. 1). It has been reported that TBTC tolerant bacteria are present in seawater[34] and *Pseudomonas aeruginosa* strains are reported to be capable of degrading tributyltin oxide when the compound is present at concentration of 2.5 ppm,[18] but the efficiency of degradation and its characterization have still not been reported.

The growth study of the organism shows an initial lag period of 6–8 h, following which a long exponential phase was observed (Fig. 1). In order to determine the optimum concentration level of TBTC for growth of the bacterial

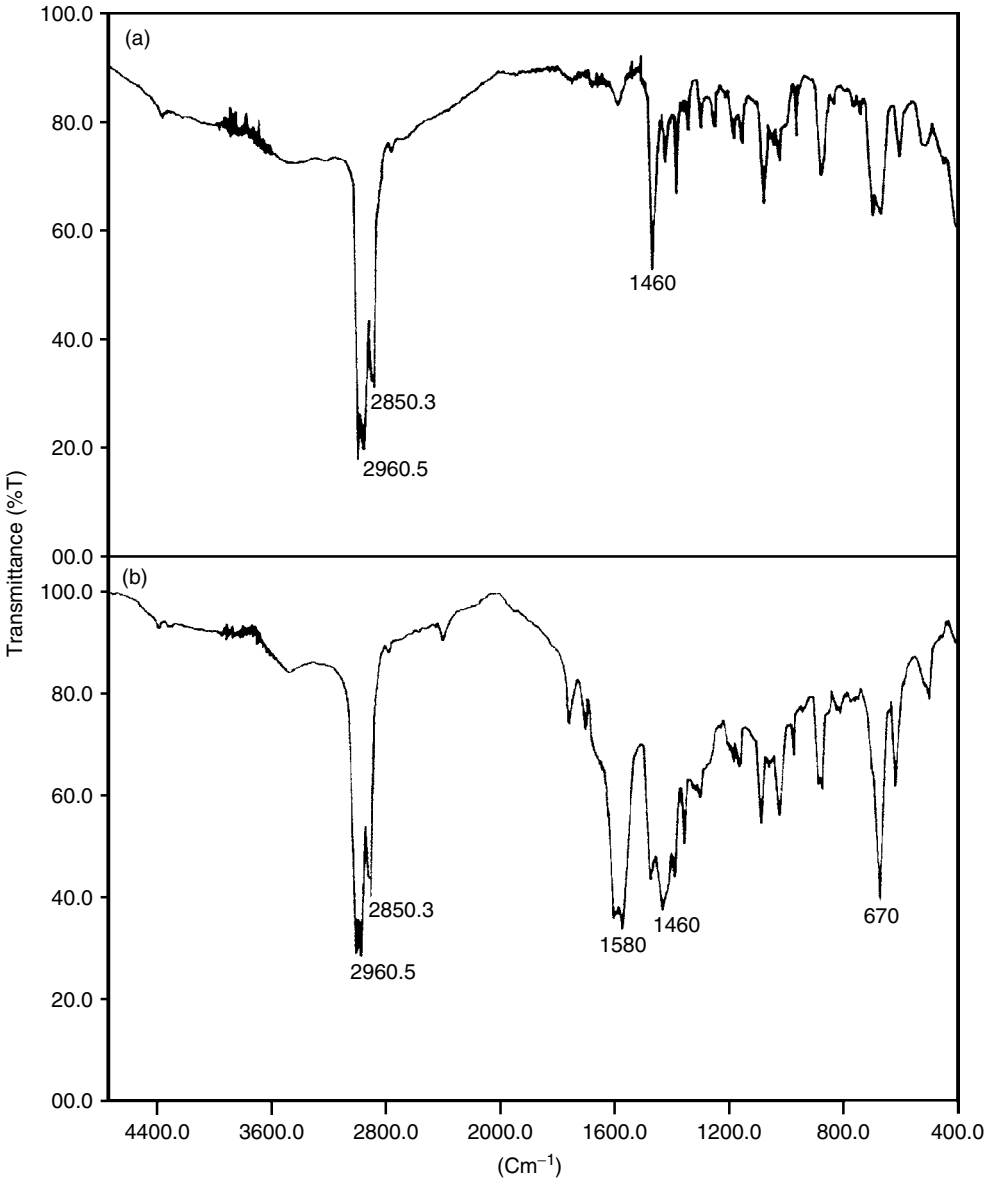


**Figure 3.** Infrared spectra of (a) standard TBTC and (b) degradation product.

isolate, it was grown separately in MSM broth with different concentration of TBTC ranging from 0.1 to 4 mM. It was interesting to note that an initial lag phase was similar in 0.1–2 mM up to 6 h and growth yield was maximum with 2 mM of TBTC in MSM broth. The higher concentration of TBTC, i.e. 3 and 4 mM, showed growth with an extended lag phase of more than 12 h (Fig. 1). The growth behavior of the culture reflects on the mechanisms of reducing the toxicity up to certain concentrations. Several mechanisms have been proposed for survival of bacteria in the presence of TBTC such as an efflux pump system,[32] biosorption,[19,33] bioaccumulation and detoxification.[15] Higher-level exposure of TBTC seems to be inhibitory to cells due to cytotoxic effects on cell metabolism. Further, at lower concentration the organism is capable of utilizing TBTC as sole source of carbon, either by an inducible/constitutive enzymes system or by reducing the concentration to below a level which results in lag phase during growth. Hence, the optimum level for growth of the isolate was considered to be 2 mM TBTC in MSM broth, although the culture can survive up to 4 mM of TBTC, i.e. 40 times higher TBTC tolerance as compared with other TBTC-tolerant Gram-positive or Gram-negative bacterial strains, viz. *Bacillus*, *Alcaligenes*, *Alteromonas*, *Vibrio* and *Pseudomonas*, as it is known that these could tolerate only up to 100 μM TBTC.[13,18,34,35]

GC analysis of culture broth, shown in Fig. 2, represents data of TBTC concentration in culture broth extracted after different periods of time. The standards were run in GC to check the ideal retention time of standard compound. The GC standards of TBT, TPrT (internal standard) and DBT had retention times near to each other [Fig. 2 (a)]. The prevailing GC peak at $8 \pm 0.4$ min showed the presence of TBTC in high concentrations (650.9 μg/μl) initially (0 h) in culture broth, which was diluted up to 100 times to get the peak in range [Fig. 2(b)]. The data with respect to main peak area and amount of TBTC obtained in GC of the culture broth after 7, 28 and 45 days [Fig. 2(c–e)] showed that there was an 85% transformation of TBTC to DBT and some other metabolites in broth within 75 days of incubation. The appearance of an additional peak in 45- and 75-day extracts [Fig. 2(e, f)] indicated the subsequent transformation of DBT to some other organotin derivative, as an additional peak increased with the decrease in DBT peak, indicating degradation of TBTC by *Pseudomonas aeruginosa* USS25. A control experiment with MSM and TBTC alone did not show a difference in concentration of TBTC; care was taken to protect the flask from any photochemical effects. Several studies have been attempted to identify the mechanisms of degradation of TBTC to determine if it is successively de-alkylated from the tri- to di- to monobutyltin (MBT) and finally a form of tin or whether TBT is converted directly to MBT. Dibutyltin was the initial product in Toronto harbour sediments,[36] whilst MBT was the principal initial product in San Diego Bay,[21,37] but microbial degradation of TBTC by bacteria has not been investigated in detail until recently.[23]

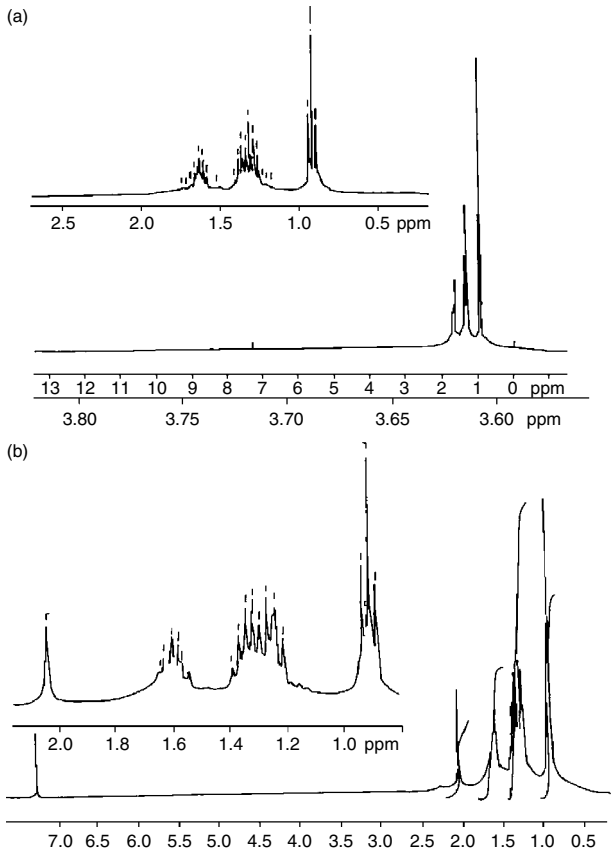


**Figure 4.** $^1$H NMR spectra of (a) standard TBTC and (b) degradation product.

The product was initially detected on TLC and purified by preparative TLC to remove the cell component, which was subsequently purified by column chromatography. The yield of the compound was found to be 280 mg $g^{-1}$ of TBTC. Further, the sodium fusion test[38] revealed the presence of chlorine atom in the molecule. The IR and NMR spectra (Figs 3 and 4) of standard TBTC and the product indicated the presence of organic moiety, namely butyl group. Both the spectra give characteristic bands at 2850.3, 2927.7 and 2960.5 $cm^{-1}$ that are assignable to C–H stretching vibration. A comparison of IR spectra (Fig. 3) of standard TBTC and degradation compounds clearly indicates that the product is quite different from that of pure TBTC. The IR spectrum of the product exhibits a doublet at around 1580 $cm^{-1}$, whereas the reference compound (TBTC) does not show any signals at that region. The band at 1460 $cm^{-1}$ in the reference compound is observed as a triplet for the product. Similarly, a strong band is seen at around 670 $cm^{-1}$ for the product, which is seen as a doublet for TBTC. These differences can be attributed to the presence of at least one butyl group in the product. The $^1$HNMR spectra are presented and the chemical shifts of various protons are summarized in Table 1 and the labelling scheme for the NMR spectral assignment is shown in Fig. 4. The spectrum of purified product showed a slight difference in chemical shifts (Fig. 4) from the standard TBTC and DBT.[39]

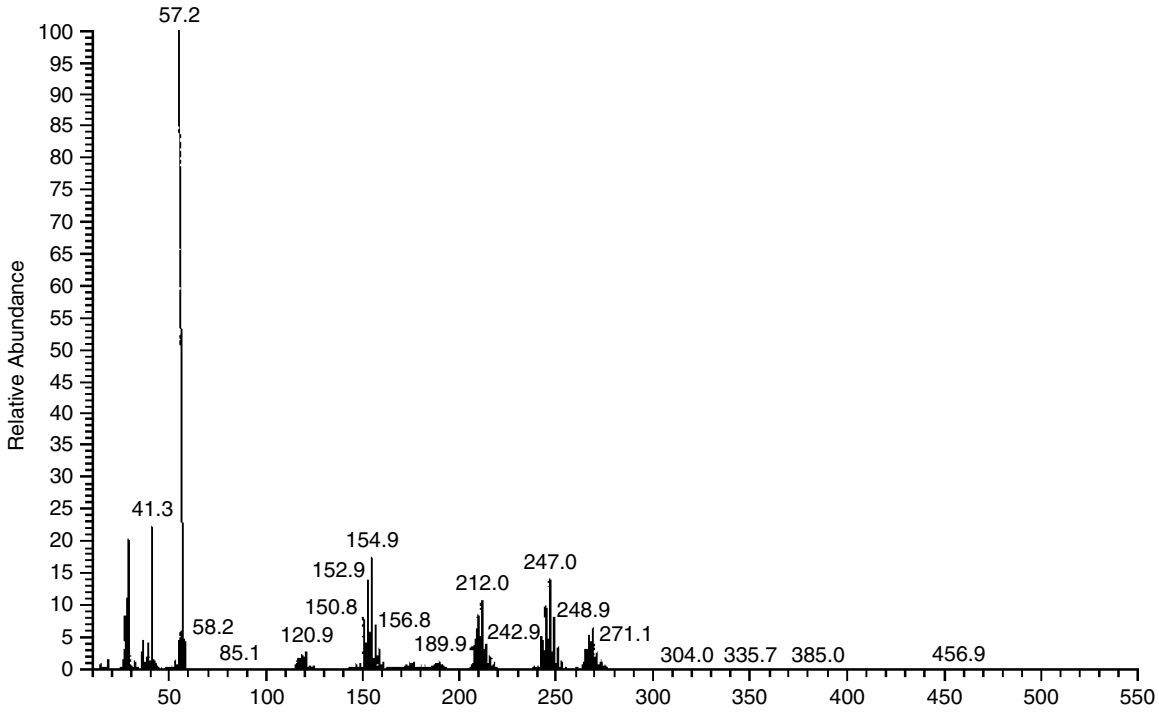


**Figure 5.** MS spectrum of degradation product.

**Table 1.** NMR ($H^1$) analysis: chemical shift of TBTC, DBT and degradation product in $CDCl_3$ ($\delta$ 7.26)

| | TBTC $Bu_3SnCl$ | DBT $Bu_2SnCl_2$ | Degradation compound |
|---|---|---|---|
| H | – | – | 2.040(S,1H) |
| $\alpha$ | 1.282(t,6H) | 1.216 | 1.211(t,2H) |
| $\beta$ | 1.632(quintet,2H) | 1.781(quintet,4H) | 1.588(quintet,2H) |
| $\gamma$ | 1.309(sextet,6H) | 1.427(sextet,4H) | 1.315(sextet,2H) |
| $\delta$ | 0.908(t,9H) | 0.959(t,6H) | 0.916(t,3H) |

This can be attributed to solvent effect, as earlier reported spectra were recorded in DMSO-$d_6$,[39] whereas in the present study the spectra were recorded in $CDCl_3$.

The $^1H$ NMR spectrum of the product showed singles at $\delta$1.597 (m, 2H), $\delta$1.339 (m, 2H), 1.290 (m, 2H) and 0.904 (t, 3H), which is similar to TBTC signals, but in addition to this, the spectrum also showed a singlet at $\delta$2.288 ($\delta$, 1H). This downfield signal showed that the proton is directly attached to tin. On the basis of integration it was observed that the molecule consist of one butyl and one hydrogen group. In addition, a positive chlorine test was observed for the product. Thus, the molecular structure of the compound was derived as $BuSnHCl_2$ (monobutyltin dichloro hydride). The structure of the degradation compound was further confirmed by GCMS. The compound molecular ion peak values $(M + H)^+$ at 248.9 (calculated value 248.7) confirmed the molecular weight of the compound to be 247.72. The corresponding fragment ion peaks were 212 $(M - Cl)^+$, 247.0 $(M - H)^+$, 154.8 $(M - Bu - Cl)^+$ and 189.9 $(M - Bu - H)^+$ (Fig. 5).

The isolate *Pseudomonas aeruginosa* strain USS25 was found to be a novel culture transforming TBTC and utilizing it as a source of the carbon and further transforming it to monobutyltin cholorohydride under laboratory conditions.

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