

# Biodegradation of tributyl phosphate by novel bacteria isolated from enrichment cultures

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**Abstract** Tributyl phosphate (TBP) is an organophosphorous compound, used extensively (3000–5000 tonnes/annum) as a solvent for nuclear fuel processing and as a base stock in the formulation of fire-resistant aircraft hydraulic fluids and other applications. Because of its wide applications and relative stability in the natural environment TBP poses the problem of pollution and health hazards. In the present study, fifteen potent bacterial strains capable of using tributyl phosphate (TBP) as sole carbon and phosphorus source were isolated from enrichment cultures. These isolates were identified on the basis of biochemical and morphological characteristics and 16S rRNA gene sequence analysis. Phylogenetic analysis of 16S rRNA gene sequences revealed that two isolates belonged to class Bacilli and thirteen to  $\beta$  and  $\gamma$ -Proteobacteria. All these isolates were found to be members of genera *Alcaligenes*, *Providencia*, *Delftia*, *Ralstonia*, and *Bacillus*. These isolates were able to tolerate and degrade up to 5 mM TBP, the highest concentration reported to date. The GC–MS method was developed to monitor TBP degradation. Two strains, *Providencia* sp. BGW4 and *Delftia* sp. BGW1 showed respectively,

$61.0 \pm 2.8\%$  and  $57.0 \pm 2.0\%$  TBP degradation within 4 days. The degradation rate constants, calculated by first order kinetic model were between 0.0024 and  $0.0099 \text{ h}^{-1}$ . These bacterial strains are novel for TBP degradation and could be used as an important bioresource for efficient decontamination of TBP polluted waste streams.

**Keywords** Tributyl phosphate · Enrichment · GC–MS · Biodegradation · Phylogenetic analysis

## Introduction

Organophosphorus compounds are toxic compounds; they inhibit the enzyme acetylcholine esterase (AChE), an extremely important enzyme in neurotransmission in animals. Tributyl phosphate (TBP) is one such organophosphorus compound. The inhibition of AChE by TBP leads to neurotoxicity and cholinergic toxicity (Berne et al. 2007; Raushel 2002). In addition, hyperplasia of kidney and urinary bladder epithelium, hepatomegaly, papillomas and transitional cell carcinomas are also caused by TBP (Arnold et al. 1997; Auletta et al. 1998). TBP causes irritation of skin, eyes and respiratory tract (Nakamura 1991). Also acute toxicity to freshwater organisms such as fish and algae is reported to occur at concentrations as low as 2 to 10 ppm (Hernandez 2002; Michel et al. 2004).

TBP is widely mainly used as a solvent for the conventional nuclear fuel processing, solvent extractant

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of rare earth metals from ore, as a solvent for lacquers, natural gums and cellulose esters. It is also extensively used as an antifoaming agent, as a base stock in the formulation of fire-resistant aircraft hydraulic fluids, as a plasticizer in the manufacture of plastics and vinyl resins, in the manufacturing of herbicides (Nakamura 1991) and in the formulation of fluorescent dye carriers (Schulz and Navratil 1987). These wide applications of TBP have led to its worldwide production of 3000–5000 tonnes/annum (Hernandez 2002).

TBP poses the problem of environmental pollution, since it is relatively resistant to degradation in the natural environments such as soil and water leading to its long persistence therein (US EPA 1992). TBP pollution has been reported in indoor air from domestic and occupational environments (Marklund et al. 2005a), aquatic environments (Fries and Puttmann 2003) and in the influent and effluent of wastewater treatment plants (Meyer and Beser 2004; Marklund et al. 2005b).

Despite its persistence in the environment and toxicity, biodegradation of TBP has been scarcely investigated especially in comparison to other organophosphorus compounds. A few studies on TBP degradation by *Acinetobacter* sp. (Stoner and Tien 1995), *Citrobacter* sp. (Owen et al. 1992), *Serratia odorifera* and *Rhodopseudomonas palustris* (Berne et al. 2004, 2005) have been reported. Likewise, Thomas et al. (1997b) demonstrated TBP degradation using mixed cultures of Pseudomonads. In all these studies, TBP degradation up to 2 mM was reported. However, to our knowledge there are no reports on TBP degradation at higher concentrations (>2 mM). On this background of wide usage of TBP, its toxicity and persistence in environment, studies on biodegradation of TBP are becoming increasingly important. We report here the isolation, molecular characterization and phylogenetic analysis of bacteria degrading TBP at higher concentration (5 mM). In addition, the novel GC–MS method to estimate TBP in the investigation of kinetics of TBP degradation is also reported.

## Materials and methods

### Chemicals

Tributyl phosphate (99%) was obtained from Aldrich and dibutyl phosphate (97%) from Fluka, USA. The

organic solvents obtained from Merck were of highest purity and were filtered through 0.45 µm Nylon membrane filter prior to use. Primary Secondary Amine (Bondesil-PSA, 40 µm) was obtained from Varian, USA. The components used for bacteriological media, minimal media and inorganic phosphate assay were purchased from Hi-Media, SRL, India and Sigma, USA.

### Sampling

Four locations in the Pune, India (18°32'N, 73°55'E) were selected for sampling on the basis of the history of their previous exposures to various xenobiotics. Two of the sampling sites, Band Garden Bridge and Sangam Bridge were from heavily polluted Mutha river. One sampling site was agriculture college field with the history of pesticide applications. The samples were also collected from primary and secondary waste treatment plants of Padamji paper and pulp industry. All these samples were used immediately after the collection.

### Enrichment and isolation of TBP degrading bacteria

Enrichment of TBP degrading bacteria was carried out for each sample separately in the minimal medium (Thomas and Macaskie 1996) comprised ( $\text{g l}^{-1}$ ) of MOPS, 5.23;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.025;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2; NaCl, 0.1; KCl, 0.075;  $(\text{NH}_4)_2\text{SO}_4$ , 5.0;  $\text{Na}_2\text{-EDTA}$ , 0.015;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.0066;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.00171;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.0015;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.000483;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.000471;  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.000453 and yeast extract, 0.001 as a growth factor. The pH of the medium was adjusted to  $7.0 \pm 0.2$ . The enrichment was performed in two steps: In the first step, the soil or water sample (1 g soil or 1 ml water) was added in the 250 ml Erlenmeyer flasks containing 100 ml minimal medium supplemented with glucose (1%) as a source of carbon and TBP ( $0.53 \text{ g l}^{-1}$  i.e. 2 mM) as a sole source of phosphorus. The flasks were incubated for seven days on rotary shaker (150 rpm) at 30°C and the resultant culture (1 ml) was used to inoculate fresh minimal medium containing 2 mM TBP and 1% glucose. Further, this inoculum transfer from previous flask (1 ml) to the fresh minimal medium (100 ml in 250 ml flask) was carried out for subsequent eight times at regular (7 days) interval. In the second step,

the initially obtained enrichment culture was transferred to the glucose deficient minimal medium with higher TBP concentration (5 mM). Thereafter, the inoculum was transferred from previous flask (1 ml) to the fresh minimal medium containing only TBP (5 mM) for subsequent six times at regular (7 days) interval. Finally, the aliquots (100  $\mu$ l) of this secondary enrichment culture were inoculated on minimal agar medium containing 5 mM TBP as the sole source of both carbon and phosphorus and after incubation at 30°C for 24 h, morphologically distinct colonies were isolated and purified.

#### Biochemical characterization and plasmid profile of the isolates

The colony characteristics (colony size, colour, margin, opacity, consistency, elevation etc.) of selected 15 isolates were noted after the growth on nutrient agar for 24 h at 30°C. The morphological (Gram test, cell morphology and motility tests) and biochemical (catalase, oxidase, Gram and motility tests) characterization was carried out according to standard methods (Collins et al. 1995). Plasmid DNA was isolated by Qiagen plasmid purification kit according to manufacturer's protocol and subsequently electrophoresed on 0.8% agarose containing ethidium bromide (0.5  $\mu$ g ml<sup>-1</sup>). Plasmids of *Escherichia coli* V517 (Macrina et al. 1978) were used as molecular size markers.

#### PCR amplification and sequencing of 16S rRNA genes

The genomic DNA of 15 TBP degrading bacteria was extracted using Gen-Elute DNA isolation kit (Sigma, USA). The 16S rRNA genes were amplified by PCR using 16S rDNA specific universal primers 16F27N (5'-AGA GTT TGA TCC TGG CTC AG-3') (Brosius et al. 1978) and 16R1488 (5'-CGG TTA CCT TGT TAC GAC TTC ACC-3') (Lane 1991) hybridizing respectively at positions 8–27 and 1488–1511 relative to *E. coli* 16S rDNA numbering. The PCR reactions were carried out in PE 9700 thermal cycler (Perkin Elmer, USA) using following amplification conditions: an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and the final extension at 72°C for 10 min.

PCR was carried out in 25  $\mu$ l reaction mixture consisted of 10 $\times$  Taq polymerase buffer (New England Biolabs), 2 mM dNTPs, 10 pM primers, 1 unit Taq polymerase (New England Biolabs), and 10 ng DNA. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Germany) following the manufacturer's protocol and were sequenced on both strands on ABI 3730 DNA analyzer using the Big Dye terminator kit (Applied Biosystems, USA). The near full length 16S rRNA gene sequences were deposited to GenBank under the consecutive accession numbers: EU195864–EU195878.

#### Identification and phylogenetic analysis of the isolates

The 16S rRNA gene sequences of the isolates were compared with known sequences in NCBI-GenBank database (<http://www.ncbi.nlm.nih.gov>) using homology search tool BLAST. For phylogenetic analysis, the 16S rRNA gene sequences of isolates reported here and the sequences of isolates from database were aligned using the CLUSTAL-W multiple sequence alignment tool. Phylogenetic relationships between the genera were inferred using the neighbor-joining method (Saitou and Nei 1987) by MEGA v. 4.0 software (Tamura et al. 2007).

#### Biodegradation studies

All 15 isolates were grown separately in LB broth for 12–15 h under shaking conditions (150 rpm) at 30°C. Subsequently, the cell pellet of each isolate was obtained by centrifugation at 5000 $\times$ g for 5 min and was washed twice with sterile minimal medium. Then, 1 ml of cell suspension with cell density adjusted to OD<sub>600</sub>  $\approx$  1 was inoculated to 9 ml minimal medium (in 50 ml flasks) containing 5 mM TBP as a sole source of carbon and phosphorus. For each isolate, ten such sets were prepared in duplicate and incubated at 30°C for 96 h under shaking conditions (150 rpm). The uninoculated medium with TBP and medium without TBP inoculated with each isolate were used as controls. During 96 h incubation, one flask of each isolate after every 12 h interval was used for the estimation of growth (OD<sub>600</sub>), inorganic phosphate content and residual TBP. Inorganic phosphate was estimated with Katewa and Katyare (2003) method using KH<sub>2</sub>PO<sub>4</sub> as a standard. The residual TBP was

extracted and estimated by GC–MS as described in the following section. The residual TBP was calculated by following formula:

$$\text{Residual TBP (mM)} = \frac{\text{Estimated TBP (ppb)} \times 3 \times 887 \times 100}{\% \text{Recovery} \times \text{Mol. Wt. of TBP} \times 1000}$$

The growth was also monitored with 5 mM dibutyl phosphate (DBP) in similar set of experiments to trace the metabolism of TBP.

Extraction of TBP and its analysis by GC–MS method

TBP from the sample was extracted thrice using equal volume of hexane. The solvent extract was appropriately diluted with hexane to bring TBP concentration in the linear range of GC–MS analysis (50–500 ppb). Further, 2 ml of diluted extract was mixed with 25 mg Primary Secondary Amine (PSA) in order to absorb the matrix components from the extract, centrifuged at  $10,000 \times g$  for 4 min at 4°C and the supernatant was used for GC–MS analysis. Working standards were prepared by appropriate serial dilutions of stock solution of TBP with hexane.

Residual TBP analysis was carried out using the GC–MS system, consisting of Thermo Finnigan Trace GC Ultra equipped with TriPlus autosampler and PolarisQ Ion Trap MS/MS detector controlled by Xcalibur software (Thermo Electron Corporation, Italy). Using Triplus autosampler 15  $\mu\text{l}$  of sample was injected with four pre-injection and post injection washes with hexane. The autosampler and injector

parameters were as described in our earlier study (Ahire et al. 2008). The chromatographic separation of TBP was performed on a Mega-5-MS column (Mega Capillary Columns Laboratory, Italy) with  $30 \text{ m} \times 0.32 \text{ mm i.d.}$  and  $0.5 \mu\text{m}$  film thickness. Helium (99.999% purity) was used as carrier gas at flow rate of  $1 \text{ ml min}^{-1}$ . The oven temperature was programmed at 50°C initially, followed by a  $25^\circ\text{C min}^{-1}$  ramp to 285°C. Electron Impact (EI) ionization was achieved by 70 eV ionization energy with source temperature 230°C and auxiliary temperature 285°C. Positive mode full scan was performed in the mass range  $m/z$  50–450 with the solvent delay of 5 min.

## Results and discussion

### Enrichment and isolation of TBP degrading bacteria

Enrichment cultures were obtained from seven different soil and water samples after around 9 weeks of enrichment with TBP as an only phosphorus source. The acclimatization of these cultures to utilize TBP as the sole carbon and phosphorus source resulted in further enrichment of potent TBP degrading cultures. After rigorous enrichment, 22 morphologically different bacteria capable of growing on minimal media containing TBP were isolated. The ability of individual bacterial strain to degrade TBP was tested by the monitoring their growth spectrophotometrically for four days, which showed the substantial increase in the biomass. Amongst 22 isolates, 15 isolates showed  $\text{OD}_{600}$  more than 0.5 in minimal medium containing

**Table 1** TBP degrading bacterial isolates and their origin

Sample	Sampling site	Sample designation	Number of total isolates	Number of potential isolates <sup>a</sup>
Soil	Agriculture College	ACS	2	2
	Sangam Bridge	SBS	2	2
	Band Garden River	BGS	2	1
Water	Band Garden River	BGW	4	3
	Sangam Bridge	SBW	4	2
Primary treatment sludge	Padmaji Paper Industry	PTP	3	2
Secondary treatment sludge	Padmaji Paper Industry	STP	5	3
Total			22	15

<sup>a</sup> Screened on the basis of growth ( $\text{OD}_{600} \geq 0.5$ ) in minimal medium containing 5 mM TBP as sole carbon and phosphorous source under shaking conditions (150 rpm) at 30°C for 4 days

TBP. All the soil and water samples yielded at least one TBP degrader (Table 1).

#### Biochemical characteristics and plasmid profile of the isolates

The cultural and biochemical characteristics and plasmid profile of TBP degrading bacteria are shown in Table 2. All 15 isolates were catalase positive, 13 isolates were oxidase positive and 13 isolates were Gram negative rods. Analysis of plasmid DNA profile revealed that all 15 isolates harbored plasmids (1–3) of molecular size ranging from 4.9 to 18.4 kb (Fig. 1). Plasmids of 6.7 and 4.9 kb were most frequently detected and were found in 11 (73.33%) and 8 (53.33%) isolates respectively, whereas plasmid of 18.4 kb was detected in only one isolate. The multiple plasmids might have role in the TBP degradation. A similar phenomenon was noted previously by Thomas et al. (1997a, b), wherein the loss of degradability due to loss of one of the plasmids was observed.

#### Identification and phylogenetic analysis of the isolates

The BLAST analysis of 16S rRNA gene sequences revealed that nine out of 15 isolates belonged to subclass  $\beta$ -Proteobacteria, four to  $\gamma$ -Proteobacteria and two to Bacilli. All isolates showed 95–100% similarity to the sequences of *Alcaligenes*, *Providencia*, *Delftia*, *Ralstonia*, and *Bacillus* genera available in the GenBank database (Table 3). Amongst all, isolate PTP1 showed the least homology with *Bacillus subtilis* (95%), whereas isolate BGW1 showed the highest homology with *Delftia* sp. (100%). The phylogenetic analysis revealed that the seven isolates SBW1, SBW2, BGW3, STP11, STP12, STP13 and ACS1 clustered together and found to be closely related to genus *Alcaligenes*. The isolate BGW1 and BGS2 were closely related to *Delftia* sp. and *Ralstonia* sp. respectively (Fig. 2). Moreover, members of  $\gamma$ -Proteobacteria found to be clustered with *Providencia* sp. (Fig. 3). The isolates PTP1 and PTP2 from class

**Table 2** Morphological, biochemical and colony characteristics and plasmid profile of the potential TBP degrading bacterial isolates

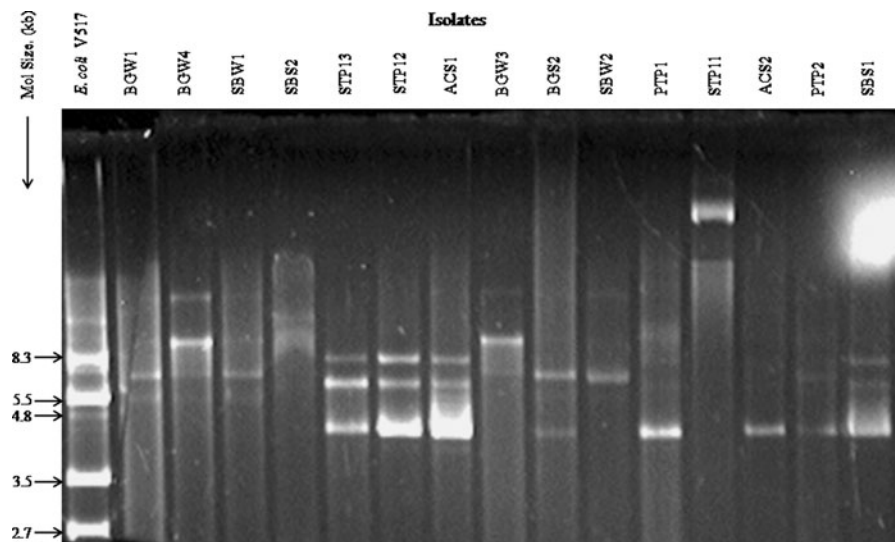
Isolate	Morphological characteristics		Biochemical characteristics		Colony characteristics			Plasmid	
	Motility <sup>a</sup>	Gram nature and cell morphology	Oxidase	Catalase	Size (mm)	Shape	Colour	Number	Size (kb)
BGW4	Motile	Gram negative rods	–	+	3	Circular	Creamy	3	11.3, 8.3, 6.7
STP13	Motile	Gram negative rods	+	+	2	Circular	Yellow	3	7.6, 6.7, 4.9
SBW2	Motile	Gram negative curved rods	+	+	1.5	Circular	Yellow	2	11.3, 6.7
PTP2	Non-motile	Gram positive rods in chain	–	–	NM	Circular	White	2	6.7, 4.9
ACS1	Motile	Gram negative rods	+	+	3	Circular	Brown	3	7.6, 6.7, 4.9
SBS2	Motile	Gram negative rods	–	+	2	Circular	Brown	1	8.3
PTP1	Motile	Gram positive rods (with spores)	–	+	3–4	Circular	White	1	4.9
STP12	Motile	Gram negative rods	++	+	2	Circular	White	3	7.6, 6.7, 4.9
STP11	Motile	Gram negative rods	+	+	2	Circular	Creamy	1	18.4
BGW3	Highly motile	Gram negative curved rods	+	+	3.5	Spindle	Creamy	3	11.3, 8.3, 6.7
BGW1	Motile	Gram negative rods	++	+	3	Irregular	Creamy	1	6.7
SBS1	Motile	Gram negative rods	–	+	3	Circular	Brown	3	7.6, 6.7, 4.9
SBW1	Motile	Gram negative rods	++	+	1	Circular	Creamy	1	6.7
BGS2	Motile	Gram negative rods	++	+	0.5	Circular	Creamy	2	6.7, 4.9
ACS2	Motile	Gram negative rods	–	+	2	Circular	Creamy	1	4.9

NM Not measurable

‘+’, positive reaction; ‘++’, strong positive reaction; ‘–’, negative reaction

<sup>a</sup> Motility test was performed at room temperature (25 ± 2°C)

**Fig. 1** Plasmid profile of TBP degrading bacterial isolates. The isolate names are indicated above the lane. Extreme left lane shows the molecular size markers



**Table 3** Summary of 16S rRNA gene sequence analysis of TBP degrading bacterial isolates

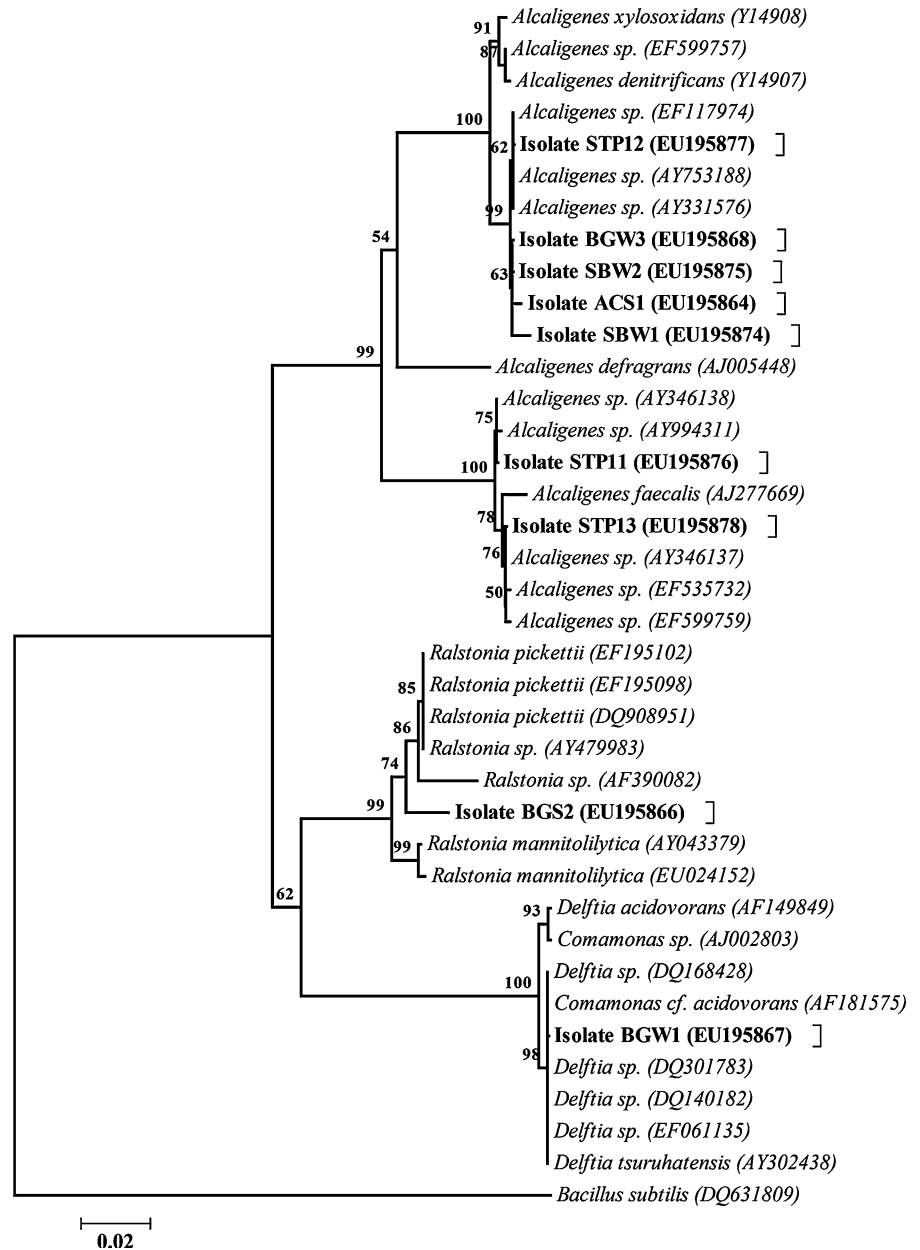
Isolate	Phylogenetic class	Homology with	Sequence similarity (%)	GenBank accession number
ACS1	$\beta$ -Proteobacteria	<i>Alcaligenes</i> sp.	98	EU195864
ACS2	$\gamma$ -Proteobacteria	<i>Providencia</i> sp.	99	EU195865
BGS2	$\beta$ -Proteobacteria	<i>Ralstonia pickettii</i>	98	EU195866
BGW1	$\beta$ -Proteobacteria	<i>Delftia</i> sp.	100	EU195867
BGW3	$\beta$ -Proteobacteria	<i>Alcaligenes</i> sp.	99	EU195868
BGW4	$\gamma$ -Proteobacteria	<i>Providencia</i> sp.	98	EU195869
PTP1	Bacilli	<i>Bacillus subtilis</i>	95	EU195870
PTP2	Bacilli	<i>Bacillus cereus</i>	97	EU195871
SBS1	$\gamma$ -Proteobacteria	<i>Providencia</i> sp.	99	EU195872
SBS2	$\gamma$ -Proteobacteria	<i>Providencia</i> sp.	99	EU195873
SBW1	$\beta$ -Proteobacteria	<i>Alcaligenes</i> sp.	99	EU195874
SBW2	$\beta$ -Proteobacteria	<i>Alcaligenes</i> sp.	99	EU195875
STP11	$\beta$ -Proteobacteria	<i>Alcaligenes</i> sp.	99	EU195876
STP12	$\beta$ -Proteobacteria	<i>Alcaligenes</i> sp.	99	EU195877
STP13	$\beta$ -Proteobacteria	<i>Alcaligenes</i> sp.	99	EU195878

Bacilli showed clustering with *Bacillus subtilis* and *Bacillus cereus* respectively (Fig. 4). Although the isolates were obtained from different environmental samples (Table 1), their phylogenetic analysis demonstrated that some isolates such as ACS1, BGW3 and STP12 were very closely related irrespective of their sampling site (Table 3). In previous studies, the involvement of *Alcaligenes* sp. in the degradation of organophosphorus pesticides such as glyphosate, chlorpyrifos and other xenobiotic compounds (Tolbot et al. 1984; Singh and Walker 2006) has been reported.

Likewise, in our studies seven out of 15 isolates were *Alcaligenes* sp. enriched on TBP further confirmed their ability of organophosphorous degradation. Although *Providencia* sp. have been reported for the reduction of explosives like RDX and HMX (Kitts et al. 1994), there is no report on the involvement of this genus in the degradation of organophosphorus compounds. However, some of our isolates which were identified as *Providencia* sp. were observed to be potent degraders of TBP. Although, *Ralstonia* sp. has been reported for carbendazim, chlorobenzene, 2,6-



**Fig. 2** Phylogenetic relationships between nine TBP degrading isolates (in *bold*) and other members belonging to  $\beta$ -Proteobacteria, inferred from 16S rRNA gene sequences using the Neighbor-Joining method. *Bacillus subtilis* was used as an outgroup. The number at each node indicates the percentage of 500 bootstrap replicates and *bar* represents the number of base substitutions per site

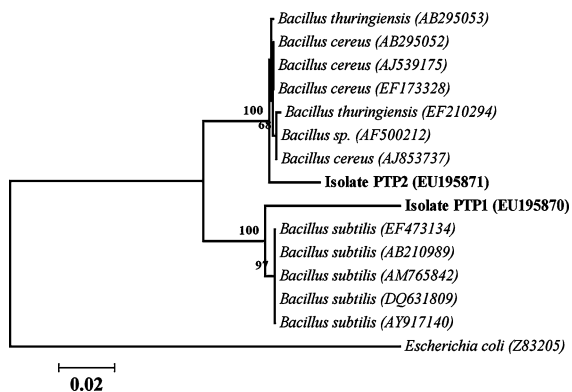
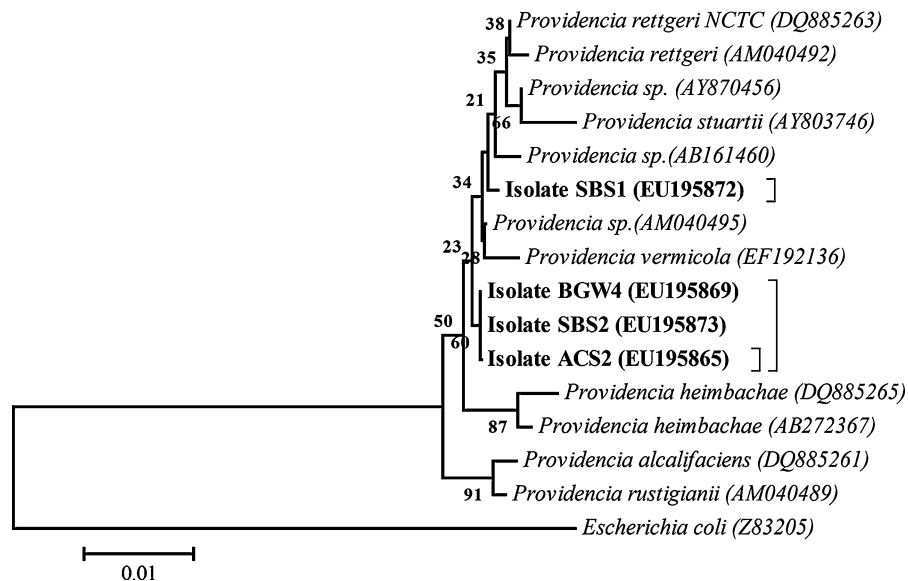


dichlorophenol and 3,5,6-trichloro-2-pyridinol degradation (Zhang et al. 2005; Li et al. 2010), to the best of our knowledge this is the first report on its involvement in TBP degradation. *Bacillus* is well known for the degradation of organophosphorus pesticides such as parathion, glyphosate, monocrotophos etc. (Singh and Walker 2006), however, two *Bacillus* sp. (PTP1 and PTP2) in our study showed comparatively poor TBP degradation than other isolates.

#### Novel GC–MS method for TBP analysis

Among six organic solvents (hexane, acetone, ethyl acetate, dichloromethane, iso-octane, diethyl ether, and toluene) used for TBP extraction, the extraction efficiency with hexane was maximum ( $96.92 \pm 2.69\%$  RSD) (data not shown) and hence it was used as a solvent for further experiments. The % recovery was calculated by following formula:

**Fig. 3** Phylogenetic relationships between four TBP degrading isolates and other members belonging to  $\gamma$ -Proteobacteria. *Escherichia coli* was used as an outgroup



**Fig. 4** Phylogenetic relationships between two TBP degrading isolates and other members belonging to class Bacilli. *Escherichia coli* was used as an outgroup

$$\% \text{Recovery} = \frac{\text{Measured concentration}}{\text{Theoretical concentration}} \times 100$$

The previously reported GC–MS method for analysis of TBP was for portable field GC–MS system (Makas and Troshkov 2004). The parameters and working principles (such as multiple module GC system, compact magnetic mass-spectrometer and multicollector ion detector) of portable field GC–MS system are far different than those used in regular research and analytical laboratories. Moreover, this system is rarely used in regular research laboratories. Therefore, the efforts were made to develop new GC–MS method for the analysis of TBP. The calibration curve of TBP

verses peak area obtained with developed GC–MS method showed excellent linearity in the concentration range of 50–500 ppb ( $r^2 = 0.995$ ). Although, other than dilution no offline sample preparation was carried out, the matrix effects caused by salts and other biomolecules were least. In addition, the developed method has the strength of rapid, accurate and reproducible analysis of TBP at trace levels with higher sensitivity (LOD = 1 ppb, S/N more than 3) than the previously reported methods (Lamouroux et al. 2000 and references therein; Dodi and Verda 2001; Makas and Troshkov 2004).

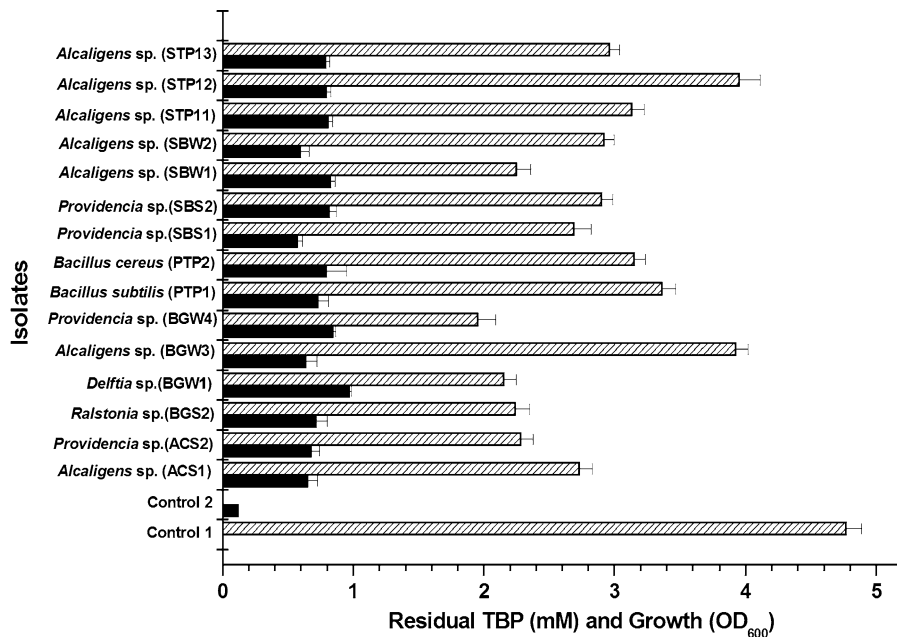
Furthermore, attempts were made to investigate the degradation products of TBP from the medium by GC–MS method. However, the degradation products of TBP could not be recovered, most probably due to their concentrations below the detection limit in the extract. This might be because of their utilization by bacteria for the growth, as TBP was the only carbon and phosphorus source in the medium. Therefore, further investigations to explore the intracellular enzyme systems of these bacteria are proposed.

#### Biodegradation of TBP

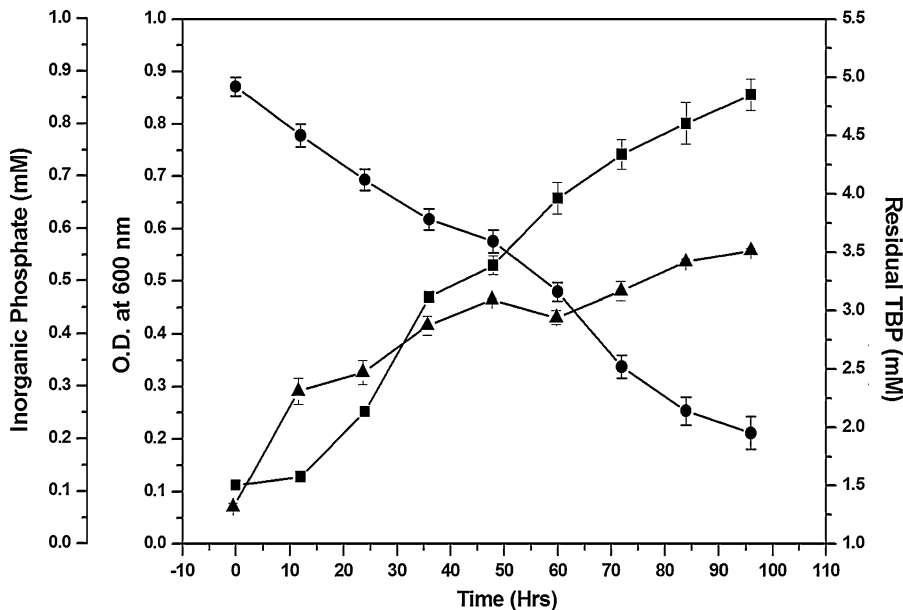
All 15 isolates showed 21–61% degradation of TBP with the initial concentration of 5 mM within 4-days of incubation (Fig. 5). Kinetics of TBP degradation by *Providencia* sp. BGW4 showed the growth with concurrent depletion of TBP from the minimal



**Fig. 5** Growth and kinetics of TBP degradation by bacterial isolates. Growth (filled square) was estimated spectrophotometrically and residual TBP (square with upper right to lower left fill) was estimated by GC–MS after 4 days incubation at 30°C under shaking conditions (150 rpm). Control 1 was sterile minimal medium containing TBP and Control 2 was medium without TBP inoculated with isolate



**Fig. 6** Kinetics of TBP degradation by *Providencia* sp. strain BGW4 at 30°C in the minimal medium containing 5 mM TBP. The growth (filled square), residual TBP (filled circle) and the inorganic phosphate (filled triangle) in the medium are shown. The data points are mean ± SD (indicated by vertical bars) of duplicate or triplicate sets



medium (Fig. 6). In addition, the gradual increase in inorganic phosphate clearly indicated the degradation of TBP. Furthermore, all isolates showed OD<sub>600</sub> more than 0.5 (Fig. 5), which was significantly more than that in control flasks lacking any substrate (OD<sub>600</sub> ≈ 0.1). Two isolates, *Providencia* sp. BGW4 and *Delftia* sp. BGW1 showed very low residual TBP as 1.95 mM (61.0 ± 2.8% degradation) and 2.15 mM (57.0 ± 2.0% degradation) respectively (Fig. 5). The TBP

degradation rate constants (k) for all isolates, calculated using first-order kinetic equation of the form  $C_t = C_0 e^{-kt}$  (Xu et al. 2005 and references therein) (where C<sub>0</sub> is concentration of TBP at time 0, C<sub>t</sub> is concentration of TBP at time t) ranged from 0.0024 to 0.0099 h<sup>-1</sup> (Table 4). The degradation rates of TBP by all isolates were four to tenfold more than that reported for *Acinetobacter* sp. (0.8 μmol ml<sup>-1</sup> h<sup>-1</sup>) by Stoner and Tien (1995). Berne et al. (2004) reported

**Table 4** Kinetic equations of tributyl phosphate biodegradation by various isolates

Isolates	Kinetic equations <sup>a</sup>	Rate constants ( $k$ ) ( $\text{h}^{-1}$ )	$t_{1/2}$ (h)	$r^2$
<i>Alcaligenes</i> sp. (ACS1)	$\ln C_t = -0.0070t + 1.6603$	0.0070	99.02	0.9868
<i>Providencia</i> sp. (ACS2)	$\ln C_t = -0.0081t + 1.6784$	0.0081	85.57	0.9731
<i>Ralstonia pickettii</i> (BGS2)	$\ln C_t = -0.0086t + 1.6678$	0.0086	80.60	0.9664
<i>Delftia</i> sp. (BGW1)	$\ln C_t = -0.0092t + 1.6602$	0.0092	75.34	0.9803
<i>Alcaligenes</i> sp. (BGW3)	$\ln C_t = -0.0025t + 1.5814$	0.0025	277.26	0.9639
<i>Providencia</i> sp. (BGW4)	$\ln C_t = -0.0099t + 1.6534$	0.0099	70.01	0.9665
<i>Bacillus subtilis</i> (PTP1)	$\ln C_t = -0.0031t + 1.5947$	0.0031	223.60	0.9789
<i>Bacillus cereus</i> (PTP2)	$\ln C_t = -0.0044t + 1.618$	0.0044	157.53	0.9629
<i>Providencia</i> sp. (SBS1)	$\ln C_t = -0.0067t + 1.6344$	0.0067	103.45	0.9903
<i>Providencia</i> sp. (SBS2)	$\ln C_t = -0.0075t + 1.6542$	0.0075	130.78	0.9852
<i>Alcaligenes</i> sp. (SBW1)	$\ln C_t = -0.0053t + 1.6116$	0.0053	92.42	0.9306
<i>Alcaligenes</i> sp. (SBW2)	$\ln C_t = -0.0056t + 1.6166$	0.0056	123.78	0.9918
<i>Alcaligenes</i> sp. (STP11)	$\ln C_t = -0.0047t + 1.5962$	0.0047	147.48	0.9854
<i>Alcaligenes</i> sp. (STP12)	$\ln C_t = -0.0024t + 1.5769$	0.0024	288.81	0.9445
<i>Alcaligenes</i> sp. (STP13)	$\ln C_t = -0.0050t + 1.6267$	0.0050	138.63	0.9709

<sup>a</sup> Degradation studies were performed for 96 h at 30°C with the initial TBP concentration of 5 mM

inability of *S. odorifera* to degrade TBP unless the media was supplemented with 1 mM glucose and 50 mM phosphate. In comparison to this, the isolates reported here were able to use TBP as sole source of carbon and phosphorus. Thomas and Macaskie (1998) reported degradation of TBP by *Pseudomonas* sp. ( $15 \mu\text{mol ml}^{-1} \text{h}^{-1}$ ), which was slightly higher than the degradation rates of isolates in this study. However, the spontaneous loss of TBP degrading ability after eight sub culturing was evident in their studies. The authors further mentioned that it was not possible to stabilize the cultures and DNA profile with respect to TBP utilization. In contrast, the isolates reported in our study were stable for their TBP degradation ability even after several subculturing (over 2 years) in liquid and solid media (data not shown). The estimated half-life of TBP degradation was 70–288 h for all isolates (Table 4). The isolates obtained in this study exhibited higher TBP tolerance (5 mM) than earlier reported 2 mM concentration (Thomas et al. 1997a, b; Berne et al. 2004, 2005). Additional studies with DBP (one of the proposed intermediates of TBP degradation) revealed that only five isolates viz., ACS1, BGW4, SBS2, SBW1, STP13 could grow well on DBP. However, other isolates showed moderate or no growth on DBP (data not shown). These observations suggest the possible involvement of alternative pathways in TBP degradation and/or differential TBP/DBP

transport mechanism across the cell membrane. To elucidate this further studies on transport of TBP/DBP across the cell membrane are desirable.

## Conclusions

In summary, the bacteria capable of degrading tributyl phosphate at higher concentrations (5 mM) were isolated by extensive enrichment culture technique. The prominent TBP degraders were identified as members of *Delftia*, *Ralstonia*, *Providencia*, *Alcaligenes* and *Bacillus* genera using biochemical, molecular and phylogenetic analysis. Further, biodegradation studies highlighted the novelty and stability of these bacterial isolates with respect to TBP degradation since, the isolates showed consistent ability to degrade TBP even after several subculturing. The developed GC–MS method demonstrated its feasibility and utility for quantitative estimation of TBP.

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## References

- Ahire KC, Arora MS, Mukherjee SN (2008) Residue analysis of Lufenuron in milled wheat by gas chromatography–mass spectrometry (GC–MS) and monitoring its bio-efficacy against *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). *J Chromatogr B* 861:16–21
- Arnold LL, Christenson MC, John MK, Wahale BS, Cohen SM (1997) Tributyl phosphate effects on urine and bladder epithelium in male Sprague-Dawley rats. *Fundam Appl Toxicol* 40:247–255
- Auletta CS, Weiner ML, Richter WR (1998) A dietary toxicity/ oncogenicity study of tributyl phosphate in the rat. *Toxicology* 128:125–134
- Berne C, Montjarret B, Guounti Y, Garcia D (2004) Tributyl phosphate degradation by *Serratia odorifera*. *Biotechnol Lett* 26:681–686
- Berne C, Allainmat B, Garcia D (2005) Tributyl phosphate degradation by *Rhodospseudomonas palustris* and other photosynthetic bacteria. *Biotechnol Lett* 27:561–566
- Berne C, Pignol D, Lavergne J, Garcia D (2007) CYP201A2, a cytochrome P450 from *Rhodospseudomonas palustris*, plays a key role in the biodegradation of tributyl phosphate. *Appl Microbiol Biotechnol* 77:135–144
- Brosius J, Palmer ML, Kennedy PJ, Noller HF (1978) Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci USA* 75:4801–4805
- Collins CH, Lyne PM, Grange JM (1995) Collins and Lyne's microbiological methods, 7th edn. Butterworth-Heinemann, London, U.K
- Dodi A, Verda G (2001) Improved determination of tributyl phosphate degradation products (mono- and dibutyl phosphates) by ion chromatography. *J Chromatogr A* 920: 275–281
- Fries E, Puttmann W (2003) Monitoring of the three organophosphate esters TBP, TCEP and TBEP in river water and ground water (Oder, Germany). *J Environ Monit* 5:346–352
- Hernandez O (2002) Tributyl phosphate—SIDS initial assessment report for 12th SIAM. UNEP Publications. <http://www.chem.unep.ch/irptc/sids/OECD/SIDS/126-73-8.pdf>. Accessed 13 March 2008
- Katewa SD, Katyare SS (2003) A simplified method for inorganic phosphate determination and its application for phosphate analysis in enzyme assays. *Anal Biochem* 323:180–187
- Kitts CL, Cunningham DP, Unkefer PJ (1994) Isolation of three hexahydro-1,3,5-trinitro-1,3,5-triazine-degrading species of the family Enterobacteriaceae from nitramine explosive-contaminated soil. *Appl Environ Microbiol* 60: 4608–4611
- Lamouroux C, Virelizier H, Moulin C, Tabet JC, Jankowski CK (2000) Direct determination of dibutyl and monobutyl phosphate in a tributyl phosphate/nitric aqueous-phase system by electrospray mass spectroscopy. *Anal Chem* 72:1186–1191
- Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) *Nucleic acid techniques in bacterial systematics*. John Wiley & Sons Inc., Chichester, pp 115–175
- Li J, Liu J, Shen W, Zhao X, Hou Y, Cao H, Cui Z (2010) Isolation and characterization of 3,5,6-trichloro-2-pyridinol-degrading *Ralstonia* sp. strain T6. *Bioresour Technol* 101:7479–7483
- Macrina FL, Kopecko DJ, Jones KR, Ayers DJ, McCowen SM (1978) A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. *Plasmid* 1:417–420
- Makas AL, Troshkov ML (2004) Field gas chromatography–mass spectrometry for fast analysis. *J Chromatogr B* 800:55–61
- Marklund A, Andersson B, Haglund P (2005a) Organophosphorus flame retardants and plasticizers in air from various indoor environments. *J Environ Monit* 7:814–819
- Marklund A, Andersson B, Haglund P (2005b) Organophosphorus flame retardants and plasticizers in Swedish sewage treatment plants. *Environ Sci Technol* 39:7423–7429
- Meyer J, Beser K (2004) Organophosphate flame retardants and plasticizers in wastewater treatment plants. *J Environ Monit* 6:599–605
- Michel K, Brinkmann C, Hahn S, Dott W, Eisentraeger A (2004) Acute toxicity investigations of ester based lubricants by using biotests with algae and bacteria. *Environ Toxicol* 19:445–448
- Nakamura A (1991) International programme on chemical safety—environmental health criteria 112-tri-*n*-butyl phosphate. World Health Organisation Publication, Geneva. [http://whqlibdoc.who.int/ehc/WHO\\_EHC\\_112\\_eng.pdf](http://whqlibdoc.who.int/ehc/WHO_EHC_112_eng.pdf)
- Owen S, Jeong BC, Poole PS, Macaskie LE (1992) Tributyl phosphate degradation by immobilized cells of *Citrobacter* sp. *Appl Biochem Biotechnol* 34:693–707
- Rauschel FM (2002) Bacterial detoxification of organophosphate nerve agents. *Curr Opin Microbiol* 5:288–295
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
- Schulz WW, Navratil JD (eds) (1987) Science and technology of tributyl phosphate, vol 2, part B. CRC Press Inc., Boca Raton, FL
- Singh BK, Walker A (2006) Microbial degradation of organophosphorus compounds. *FEMS Microbiol Rev* 30: 428–471
- Stoner D, Tien A (1995) Method and compositions for the degradation of tributyl phosphate in chemical waste mixtures. US Patent 5,453,375
- Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 24:1596–1599
- Thomas RA, Macaskie LE (1996) Biodegradation of tributyl phosphate by naturally occurring microbial isolates and coupling to the removal of uranium from aqueous solution. *Environ Sci Technol* 30:2371–2375
- Thomas RA, Macaskie LE (1998) The effect of growth conditions on the biodegradation of tributyl phosphate and potential for the remediation of acid mine drainage waters by a naturally-occurring mixed microbial culture. *Appl Microbiol Biotechnol* 49:202–209
- Thomas RAP, Greated A, Lawlor K, Bailey M, Macaskie LE (1997a) Stabilisation of tributyl phosphate-biodegradative

- ability of naturally-occurring pseudomonads using ampicillin. *Biotechnol Tech* 11:781–785
- Thomas RAP, Morby AP, Macaskie LE (1997b) The biodegradation of tributyl phosphate by naturally occurring microbial isolates. *FEMS Microbiol Lett* 155:155–159
- Tolbot HW, Johnson LM, Munneck DM (1984) Glyphosate utilization by *Pseudomonas* sp. and *Alcaligenes* sp. isolated from environmental sources. *Curr Microbiol* 10:255–260
- US EPA (1992) Chemical testing & data collection—tributyl phosphate test results. US Environmental Protection Agency Publication, Washington. <http://www.epa.gov/opptintr/chemtest/pubs/tributph.html>. Accessed 16 February 2009
- Xu XR, Li HB, Gu JD (2005) Biodegradation of an endocrine-disrupting chemical di-*n*-butyl phthalate ester by *Pseudomonas fluorescens* B-1. *Int Biodeter Biodegrad* 55:9–15
- Zhang GS, Jia XM, Cheng TF, Ma XH, Zhao YH (2005) Isolation and characterization of a new carbendazim-degrading *Ralstonia* sp. strain. *World J Microbiol Biotechnol* 21:265–269