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Biodegradation kinetics and mechanism of 2,4,6-tribromophenol by *Bacillus* sp. GZT: A phenomenon of xenobiotic methylation during debromination

Lei Zu [a,c], Guiying Li [a], Taicheng An [a,*], Po-Keung Wong [b]

[a] State Key Laboratory of Organic Geochemistry and Guangdong Key Laboratory of Environmental Resources Utilization and Protection, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, China
[b] School of Life Sciences, The Chinese University of Hong Kong, Shatin, NT, Hong Kong Special Administrative Region, China
[c] Graduate School of Chinese Academy of Sciences, Beijing 100049, China

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ABSTRACT

A strain *Bacillus* sp. GZT capable of debrominating and mineralizing 2,4,6-tribromophenol (TBP) was isolated and characterized by morphological observation, biochemical and physiological identification as well as 16S rRNA sequence analysis. Biodegradation kinetics experiments demonstrated that initial TBP concentration had a predominant effect on degradation efficiency. Within 120 h, the highest TBP degradation and debromination efficiencies were up to 93.2% and 89.3%, respectively, under the optimum condition. Ten metabolic intermediates including five brominated compounds, three oxidative products and two cellular metabolites were all identified by gas chromatography–mass spectrometer, and six key intermediates were doubly validated by authentic standards. The proposed biodegradation mechanism inferred that reductive debromination as a major degradation pathway could simultaneously take place at *ortho*- and *para*-positions on TBP, while methylated debromination was also found as a minor degradation pathway during this process. Within 148 h degradation, nearly one-third of 3 mg/L TBP could be completely mineralized.

1. Introduction

Brominated phenols are a group of flame retardants widely used in chemical industry. These compounds can be accumulated in the food chains and concentrated into the bodies (Knob et al., 2010). As a most widely manufactured bromophenol, 2,4,6-tribromophenol (TBP) was used as one of the popular brominated flame retardants (BFRs) and wood preservative with a global consumption over 9500 tones annually in 2001 (IUCLID, 2003; WHO, 2005). Unfortunately, TBP could be ubiquitously found in aquatic and soil environments (Ronen et al., 2005), biota (Covaci et al., 2011), and even detected in human blood with a relatively high concentration (Thomsen et al., 2001). The toxicity study showed that the acute oral and dermal LD_{50} in rats for TBP were considered to be 1486 and >2000 mg/kg body weight, respectively (WHO, 2005). Thus, TBP was selected into the list of hazardous wastes by the United States Environmental Protection Agency as well as due to its acute toxicity (EPA, 1998). In addition, previous researches also revealed that, like most of BFRs, TBP could cause developmental neurotoxicity, embryotoxicity, and fetotoxicity (Lyubimov et al., 1998; Rios et al., 2003) as well as possessing estrogen-like property (Meerts et al., 2001; Legler and Brouwer, 2003).

As far as is known, a great majority researches on BFRs biodegradation were mainly focused on the isolation of various bacteria to reductively debrominate BFRs (de Wit, 2002; An et al., 2011). For instance, some microorganisms in sediment (Arbeli et al., 2006) and anaerobic soil (Nyholm et al., 2010), could debrominate tetrabromobisphenol-A into bisphenol-A. A few strains were also isolated to reductively debrominate mono-, di-, and tribromophenols, and showed the effect of position-selective on the debromination. For example, *Desulfovibrio* sp. could debrominate 2-, 4-, 2,4-, 2,6-, and 2,4,6-bromophenol to phenol, respectively, but the selectivity on 2,4,6-TBP was still not so clear (Boyle et al., 1999). However, *Ochrobactrum* sp. TB01 was also identified to successively debrominate 2,4,6-TBP to phenol but only 2,4-dibromophenol and 2-bromophenol intermediates were detected (Yamada et al., 2008). While, 4-bromophenol could be debrominated as a separate substrate by strain *Aplysina aerophoba*, but it was able to persist over the entire debromination period as the intermediate of 2,4,6-TBP debromination (Ahn et al., 2003). Hence, the regularity of position-selective on reductive debromination by different isolated strains during TBP biodegradation was a really controversial topic. Furthermore, to date, most of TBP biodegradation works were focused on the reductive debromination possibility rather than probing new debromination pathway and mineralization mechanism. According to previous

* Corresponding author. Tel.: +86 20 85291501; fax: +86 20 85290706.
E-mail address: antc99@gig.ac.cn (T. An).

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report, the physiological function of one-carbon unit, which transferred by folates (Shen and Wang, 1991), is one of the substances employed during DNA and RNA synthesis. Therefore, one-carbon unit metabolism is an essential process in most organisms and will combine to other xenobiotic to facilitate or inhibit the cell growth. For instance, some halogenated phenols can be biotransformed into corresponding methylated chemicals such as anisoles, veratroles, or guaiacols (Neilson et al., 1988; McNally and Harper, 1991). However, the xenobiotic methylation mechanism of TBP was still scarce (Donoso et al., 2008). Thus, a phenomenon of xenobiotic methylation deserved to identify during the biological debromination of TBP by the new-isolated strain *Bacillus* sp. GZT.

What's more, up till now, few works focused on the oxidative mineralization of those brominated phenols. The mineralization of TBP only occurred under the assistance with another aerobic strain (Ronen and Abeliovich, 2000) or under a chemical treatment with Hamilton system (Monrroy et al., 2007). To date, the investigation on the simultaneous debromination and mineralization of TBP *via* bromophenol as degradation intermediates were also very limited.

Thus, the main objective of this study is to isolate a bacteria strain with the ability to simultaneously decontaminate TBP, to identify of the degradation intermediates, and to probe the debromination pathway during TBP biodegradation process by this newly isolated strain. The optimal parameters affecting both debromination and biodegradation were evaluated to determine the highest degradation ability of isolated strain. To probe the degradation mechanism and specify the reductive debromination positions, the metabolic by-products of TBP by selected strain were identified by gas chromatography–mass spectrometry (GC–MS) and confirmed with the authentic standard samples. Two dibromophenols intermediates during the TBP biodegradation were also employed as the initial biodegradation substrates to indirectly clarify the pathway of methylated debromination, and the dynamically formed brominated intermediates were also identified and quantified in detail to verify the TBP degradation mechanism. Finally, a tentative biodegradation mechanism of TBP via a reductive debromination accompanying of xenobiotic methylation was proposed based on degradation intermediates and mineralization content for the first time.

2. Methods

2.1. Chemicals and growth medium

The 2,4-dibormophenol (2,4-DBP) (99%), 2-bromophenol (2-BP) (98%), 4-bromophenol (4-BP) (97%) and *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) (98%) were all purchased from Acros Organics (New Jersey, USA). The original compounds, TBP (99%), and the authentic standards of 2,6-dibromophenol (2,6-DBP) (99%) and 2,6-dibromo-4-methylphenol (98%) were purchased from Sigma–Aldrich. 1,3-Dibromo-2-methoxy-5-methylbenzene (98%) was synthesized and supplied by Suzhou Yacco Chemical Reagent Corporation. 1-(2-aminophenyl) ethanone (98%) was from J&K Chemical Ltd. All other chemicals were of analytical grade and from Guangzhou Chemical Reagent Co., Inc., China. The mineral medium (MM) was used for the isolation of TBP-degrading bacterial strains (the recipe is listed in the Supporting Information). The growth medium (GM) consisted of peptone 10.0 g, beef extract 3.0 g, NaCl 5.0 g per liter of distilled water at pH 7.0, autoclaved at 121 °C for 15 min.

2.2. Strain isolation and identification

The sludge used for bacteria isolation was sampled from a small creek near an electronic waste dismantling workshop, Guiyu Town, Guangdong, China. The strain was enriched in Luria–Bertani (LB) medium and isolated by using spread-plate method. 16S rRNA gene sequence analysis was used to identify species of the isolated strain. Genomic DNA was extracted to use the template for PCR amplification. The detailed solution component and the reaction procedure of PCR were listed in Table S1. The layout characteristics were measured by standard procedures according to Bergey's Manual of Determinative Bacteriolog (Buchanan et al., 1984), and the cell morphology was observed by transmission electron microscope (TEM, HITACHI, Japan). The detailed isolation and identification step were also specified in the Supporting Information.

2.3. TBP debromination and biodegradation

Unless otherwise specified, the debromination and biodegradation experiments were performed by inoculating of 20 mL culture, which was cultivated for 15 h in LB medium at 37 °C, pH 7.5 and 200 rpm in advance, and then added into another 100 mL of MM (within 250 mL shake flasks) containing 3 mg/L TBP at 37 °C, pH 7.0, and 200 rpm for 120 h. At biodegradation intervals of 24, 36, 48, 72, 96 and 120 h, 2 mL of the samples was collected, and filtered (0.22 μm) for concentration analysis. Another portion (100 mL) of the samples was extracted with dichlormethane/hexane (1:1, v/v) three times for mechanism studies. And all kinetics experiments were conducted in triplicate. TBP biodegradation efficiency, debromination efficiency and mineralization efficiency were calculated as follows:

$$\text{TBP biodegradation efficiency}(\%) = \frac{C_0 - C}{C_0} \times 100 \quad (1)$$

$$\text{TBP debromination efficiency}(\%) = \frac{M_{Br^-}}{M_T} \times 100 \quad (2)$$

$$\text{TBP mineralization efficiency}(\%) = \frac{M - M_c}{M_0} \times 100 \quad (3)$$

where C_0 was the initial TBP concentration (mg/L), C was the TBP concentration at the time indicated (mg/L); M_{Br^-} was the produced bromide ion concentration (μM) in the degradation solution, M_T was the total bromide from the substrate (μM); M was the CO_2 concentration of sample (μM), M_c was the CO_2 concentration of the blank control (μM), M_0 was the theoretical CO_2 concentration which the substrate was totally mineralized (μM).

Bromide ion concentrations were determined using the method 4500-Br^- (APHA, 1995). TBP concentration was measured by using high performance liquid chromatography as described according to reference (An et al., 2008). Biodegradation metabolites were both identified by a GC (Agilent 7890)–MS (Agilent 5975C) with and without BSTFA derivatization by using a DB-5 column (30 m × 0.25 mm, 0.25 μm film thickness). The column temperature was programmed 60 °C for 2 min, raised at 6 °C/min to 100 °C and then 10 °C/min to 280 °C holding for 2 min. One microliter of sample was injected with the splitless model and the carrier gas was He at a flow of 1 mL/min. The injector and detector temperature were 250 and 280 °C, respectively. The concentration of the biodegradation intermediates were also quantified by GC–MS.

Concentrations of mineralized product CO_2 in the gas phase in shake flasks were determined by a GC (Shanghai Kechuang, China) coupled with a flame ionization detector (FID) equipped with a methane converter. The flow rates of the air, H_2 and N_2 were 300, 30 and 120 mL/min, respectively. 0.5 mL gas sample was injected into the TDX-01 column set at 150 °C used for separation of CO_2. The temperatures of injector and detector were 180 and 230 °C, respectively. MM containing the late log growth phase cultures without TBP was used as the blank sample to deplete other sources of CO_2. The Parafilm was used to seal the flask to ensure

a closed system. CO_2 drawn from the closed flask using an airtight syringe (Agilent 500 μL) was injected into GC for detection, and Henry's Law was used to calculate the CO_2 concentration in aqueous phase.

3. Results and discussion

3.1. Properties of TBP-degrading strain

Using TBP as the sole carbon source, bacteria with the ability to degrade TBP were successfully enriched. After 2 months cultivation, one pure colony with the highest TBP degradation efficiency was chosen and named as strain GZT. The 16S rRNA gene of strain GZT was amplified and sequenced. Phylogenetic tree was constructed by the 1641 bp nucleotide gene (GenBank accession No: HQ603747) using the Neighbor-joining method after alignment with the related sequences from the GenBank database (Fig. S1). The phylogenesis demonstrated that strain GZT belonged to the genus *Bacillus* which had a high sequence similarity to *Bacillus thuringiensis* (99%) and *B. cereus* (99%). The cell morphology was also observed by TEM (Fig. S2). The physiological and biochemical characteristics of strain GZT were further determined and summarized in Table S2. It was characterized as a rod-shaped (1.58–1.48 × 4.96–1.58 μm) Gram-positive strain without flagellum. The bacterial colonies on agar medium are typically round, non-pigmented and glossy, and about 2–3 mm in diameter. According to both gene sequence analysis and cell characteristic information above, the isolated strain GZT was classified into the genus *Bacillus* and named as *Bacillus* sp. GZT. Although genus of *Bacillus* is commonly reported for the removal of various pollutants, a strain belonging to genus *Bacillus* with the ability to degrade TBP has never been isolated to date.

As a newly isolated strain *Bacillus* sp. GZT, its growth pattern needs to be determined for further TBP degradation application. Fig. S3 shows the plot of OD_{600} against the culture time at different cultivated affecting factors, and typical lag, log, and stationary phase growth curves of the isolated strain. It can be concluded that the lag and stationary phase of *Bacillus* sp. GZT was about 3 and 15 h, respectively. The optimal cultivation condition was determined at 40 °C, pH 7.5 and 200 rpm, the strain growth could ensure enough cell amount and highest activity for the subsequent TBP degradation experiment.

3.2. Biodegradation and debromination of TBP

The effect of the initial concentration of TBP ranging from 3 to 25 mg/L on both degradation and debromination efficiency was showed in Fig. 1a. The TBP biodegradation and debromination efficiency was calculated by Eqs. (1) and (2), respectively. With the increase of incubation time, both the degradation and debromination efficiencies increased quickly at first and then leveled off for all concentrations tested, and both the efficiencies decreased with the increase of the TBP concentration. For instance, after 120 h reaction, with the increase of TBP concentrations from 3 to 25 mg/L, the debromination efficiencies decreased from 87.7% to 62.5%, while the biodegradation efficiencies decreased from 90.6% to 67.4%. The biodegradation dynamic equation of TBP shows that the degradation kinetics of TBP followed the pseudo first-order kinetic model in the range of 3–10 mg/L (Table S3); while the linear fitting results were not so good when the TBP concentration was at 15 (R^2 = 0.8939), 20 (R^2 = 0.8840) and 25 mg/L (R^2 = 0.8815). In addition, the half-life of TBP degradation increased from 31.1 to 157.5 h when TBP concentration increased from 3 to 25 mg/L. This may explained that the inhibition effect of TBP enhanced gradually as the concentration of TBP increased. Similar phenomenon was also found during the biodegradation of bisphenol-A by *Achromobacter xylosoxidans*, and it was caused by the toxicity of substrate (Zhang et al., 2007).

For biodegradation, the inoculum volume was an important parameter to promote the degradation efficiency. Fig. 1b shows that the biodegradation efficiencies of TBP were 88.7%, 91.8%, 92.9% and 93.2% when the inoculum volume added as 10, 15, 20 and 30 mL, respectively. Following the similar results, the corresponding debromination efficiencies were obtained as 82.8%, 86.1%, 87.6% and 89.3%. From these results, it can be indicated that although the inoculum volume had a positive effect on the biodegradation, as an important parameter, its influence on biodegradation and debromination efficiencies was not as remarkable as those of substrate concentration especially when the inoculum volume was greater than 20 mL. It may be because adequate number of the bacterial cells could quickly degrade and reduce the content of TBP at a lower TBP concentration (3 mg/L), resulting in a rapid elimination of the TBP toxicity to the bacterial cells.

The effect of temperature on both biodegradation and debromination efficiency was also investigated (Fig. 1c). Results showed that the degradation and debromination efficiencies all steadily increased with the rise of temperatures from 20 to 35 °C, and the relative highest degradation efficiency of 92.5% and the highest debromination efficiency of 87.8% were both obtained at 35 °C. When the temperature was further raised to 40 °C, both efficiencies dropped slightly to 91.6% and 86.4%, respectively, but these values were still higher than those at other tested temperatures. It should be noted that although the relative higher temperature (40 °C) benefited the cell growth as described before, the degradation and debromination mainly depended on the enzyme activity of the cell which has high enzyme activity around 35 °C.

Initial pH was another significant parameter for TBP metabolism, because it might also affect both enzyme activity and solubility of TBP. The effect of pH for *Bacillus* sp. GZT to biodegrade TBP was plotted in Fig. 1d. After 120 h, the highest degradation efficiency of 92.7% and the debromination efficiency of 88.4% were both achieved at pH 7.0. At pH 8.5, both efficiencies decreased to the lowest values of 76.8% and 72.5%, indicating that the biodegradation enzymes of *Bacillus* sp. GZT did not function very well under the alkaline condition. At pH 6.5, the degradation efficiency of 92.1% and the debromination efficiency of 87.1% were slightly lower than that at pH 7.0. This may be resulted from the lower dissolved concentration of TBP in solution and then subsequently decrease the utilization of the bacterial cells because TBP was sparingly soluble below its pK_a of 6.8 (Franzen et al., 2007).

Overall, it can be concluded that the biodegradation of TBP by this new-isolated strain, *Bacillus* sp. GZT, was obviously inhibited by the high concentration of the substrate, and the promotion effect was limited by increasing the inoculum volume. Although the temperature and the initial pH could also affect the enzyme activity of the bacteria and the solubility of TBP, these influences were not as remarkable as the TBP concentration on the biodegradation kinetics. Under the optimized conditions of at 35 °C, pH 7.0, with inoculum volume of 20 and 3 mg/L TBP concentration, the degradation and debromination efficiencies of TBP by *Bacillus* sp. GZT were achieved at 93.2% and 89.3%, respectively.

3.3. Mechanisms of TBP metabolism

To better understand the TBP environmental fate and the interaction with the cell metabolism, the TBP biodegradation intermediates produced by isolated *Bacillus* sp. GZT were identified using GC–MS (Fig. S4). Results showed that five intermediates had the bromine atom isotopic characteristic peak. Two biodegradation intermediates (A) (retention time (RT) = 14.891 min) and (B) (RT = 15.339 min) with almost the same mass spectra (m/z = 250/

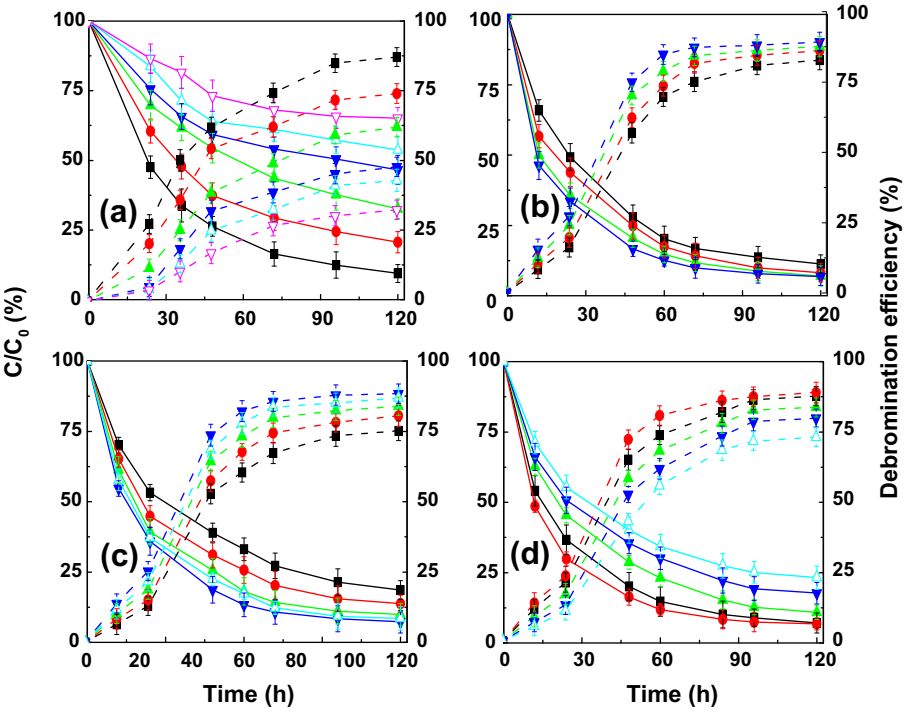


Fig. 1. The effect of (a) TBP concentrations (mg/L): (●) 3, (▲) 5, (▼) 10, (△) 15, (▽) 20, (▽) 25; (b) inoculum volume (ml): (●) 10, (▲) 15, (▼) 20, (△) 30; (c) temperature: (●) 20 °C, (▲) 25 °C, (▼) 30 °C, (△) 35 °C, (▽) 40 °C; (d) pH value: (●) 6.5, (▲) 7.0, (▼) 7.5, (△) 8.0, (▽) 8.5 on TBP biodegradation efficiency (solid lines) and debromination efficiency (dashed lines). The error bar value presents the standard deviation of triplicates.

252/254 [M^+]) could be detected and determined as dibromophenols according to the isotopic characteristic peak m/z (M−2):M:(M + 2) = 1:2:1. Another intermediate molecular ion cluster at m/z = 172/174 [M^+] was tentatively determined as mono-BP (C). To properly distinguish the two dibromophenol isomers of (A) and (B), two authentic standards of 2,4-DBP and 2,6-DBP was employed to identify each other in GC–MS analysis (Fig. S5). Validated with the authentic samples, intermediates (A) and (B) were identified as 2,4-DBP and 2,6-DBP, respectively. For mono-BP analysis, the authentic standards mono-BP of 2-BP and 4-BP also were injected separately (Fig. S6), and 2-BP was finally determined to be the TBP debrominated intermediate based on the comparison of the retention time.

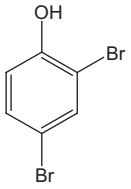
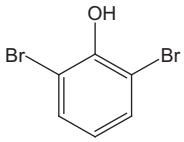
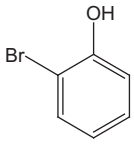
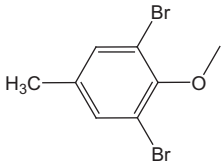
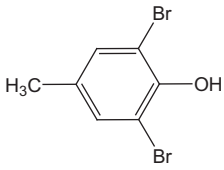
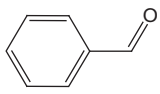
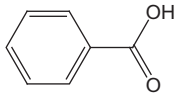
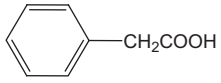
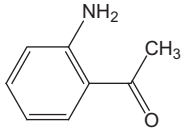
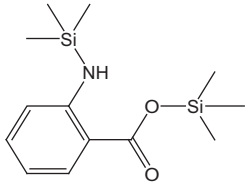
Another notable phenomenon is that two dibrominated intermediates containing methyl group were also detected during the biodegradation of TBP. Based on their mass spectra (m/z = 278/280/282, m/z = 264/266/268 [M^+]) and validated with the authentic standards of them, they were identified as 1,3-dibromo-2-methoxy-5-methylbenzene (D) and 2,6-dibromo-4-methylphenol (E), respectively (Fig. S7). Also, the authentic standard of 1-(2-aminophenyl) ethanone (I) confirmed the generation of the amino acid byproduct during this biodegradation (Fig. S5). In summary, ten intermediates associated with the chemical structures, the retention times, and the mass spectra are all demonstrated in Table 1. They were finally identified as 2,4-DBP (A), 2,6-DBP (B), 2-BP (C), 1,3-dibromo-2-methoxy-5-methylbenzene (D), 2,6-dibromo-4-methylphenol (E), benzaldehyde (F), benzoic acid (G), phenylacetic acid (H), 1-(2-aminophenyl) ethanone (I) and 2-aminobenzoic acid (J) (only detected after BSTFA derivatization), respectively.

Meanwhile, the evolution curves of the five brominated intermediates were plotted during the TBP biodegradation (Fig. 2a) and the concentrations quantified based on their standard curves (Fig. S8). Results showed that intermediates (A), (B), (D) and (E) were the four intermediates during the first 12 h of TBP biodegradation. The concentrations of two methylated intermediates (D) and (E) were very low (about 6.5%) compared with other two first-step debromination products (A) and (B) (about 93.5%). Intermediate (C) was produced later with the decrease of (A) and (B), indicating that (C) may be the daughter product of intermediates (A) and (B).

Although the evolution curves were studied during the TBP biodegradation, the detailed pathway where two methylated intermediates (D) and (E) originated from was still unclear. That is, they may produce directly from TBP biodegradation or via the addition of methyl group onto two dibromophenols intermediates. Therefore, in order to verify the source of this two methylated intermediates and to distinguish from these two different pathways, the primary metabolism intermediates from TBP, 2,4-DBP and 2,6-DBP, were also used as the start biodegradation substrate by *Bacillus* sp. GZT, respectively, and the results showed that only one brominated product 2-BP was detected during the biodegradation of two dibromophenols intermediates (Fig. 2b). Hence, it can be concluded that the methylation intermediates should be produced directly from the biodegradation of TBP rather than from either of two dibromophenols intermediates.

Thus, the novel debromination methylation pathway was worth discussing. The xenobiotic methylation has never been reported during TBP biodegradation process. As known, one-carbon unit metabolism is the major way to produce methyl group for DNA or RNA synthesis, and the source of one-carbon unit can be from the metabolism of amino acids such as Gly, His, Ser and Trp, etc. Two metabolites, 1-(2-aminophenyl) ethanone (I) and 2-aminobenzoic acid (J) which had been identified in the TBP biodegradation sample were supposed to be the by-products of Trp catabolism: intermediate (I) should be from kynurenine which is the important metabolite of Trp, and further oxidized to intermediate (J). Meanwhile, formic acid is formed accompanying with the production of kynurenine and become the one-carbon unit source to participate in the metabolism. According to the hypothesis, in this biodegradation process, one of the methylation sources may come from the Trp catabolism

Table 1
Retention time, mass spectra and structure of the identified metabolites by GC–MSD analysis.

	Intermediates	Retention time (min)	Chemical structure	*m/z*
A	2,4-Dibromophenol	14.891		252* [M$^+$], 173 [M$^+$-Br], 144 [173-COH]
B	2,6-Dibromophenol	15.339		252* [M$^+$], 173 [M$^+$-Br], 144 [173-COH]
C	2-Bromophenol	9.283		174* [M$^+$], 94 [M$^+$-Br], 77 [M$^+$-OH]
D	1,3-Dibromo-2-methoxy-5-methylbenzene	17.429		280* [M$^+$], 265 [M$^+$-CH_3], 199 [M$^+$-Br]
E	2,6-Dibromo-4-methylphenol	16.250		266* [M$^+$], 185 [M$^+$-Br], 168 [185-OH]
F	Benzaldehyde	6.607		106* [M$^+$],77 [105-CO], 51 [77-C_2H_2]
G	Benzoic acid	11.777		122* [M$^+$], 105 [M$^+$-OH], 77 [105-CO]
H	Phenylacetic acid	13.291		136* [M$^+$], 91 [M$^+$-COOH], 77 [M$^+$-CH_2COOH]
I	1-(2-Aminophenyl) ethanone	14.211		135* [M$^+$], 120 [M$^+$-CH_3], 92 [120-CO]
J	2-Aminobenzoic acid (BSTFA derivatization)	18.551		281* [M$^+$], 266 [M$^+$-CH_3]

byproduct formic acid. This result is a little different from the reports the chloromethane was the methyl donor in an *o*-methylation occurred on phenol catalyzed by the fungus *Phellinus pomaceus* (Harper et al., 1989; McNally and Harper, 1991). Compared with fungus, the difference may be due to that bacterium *Bacillus* sp. GZT may possess various enzyme systems and have different metabolism mechanisms. In addition, most research works have preferred S-adenosyl-L-methionine (SAM), which was produced from methionine (Met), to be the intermediary to induce the reaction of xenobiotic methylation through the Met cycle (Finkelstein, 1990; Reed et al., 2004). Recent studies showed that some halogenated phenols also could induce the SAM-dependent methyltransferase to catalyze *o*-methylation (Neilson et al., 1988; Alvarez-Rodriguez et al., 2002; Coque et al., 2003). Accordingly, in this work, it can be proposed that during the TBP methylation, the methyl originating from the formic acid was transferred to the xenobiotic substrate TBP via the Met cycle, and the reactions including the *o*-methylation and the debromination co-occurred during the xenobiotic metabolism (Neilson et al., 1988; Harper et al., 1989; Alvarez-Rodriguez et al., 2002; Coque et al., 2003).

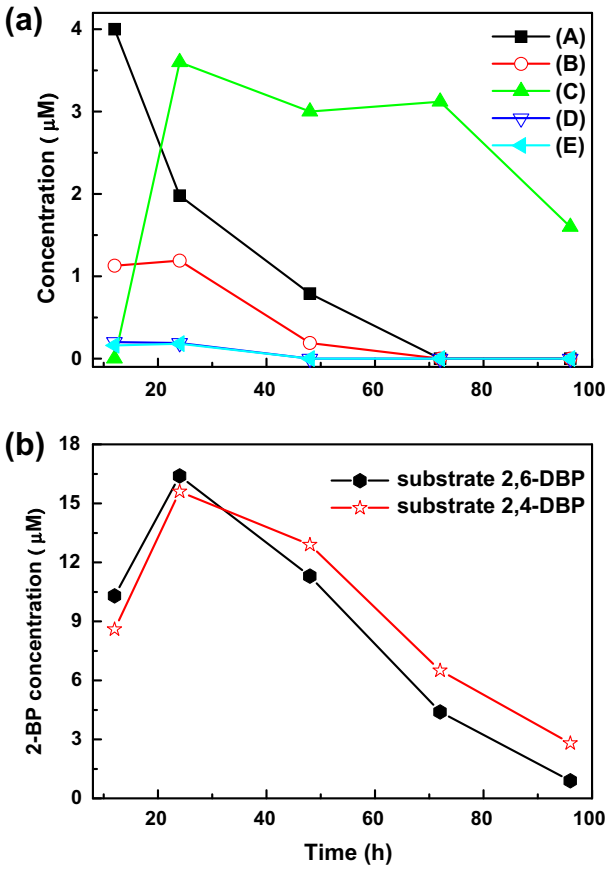


Fig. 2. (a) Evolution curves of TBP biodegradation brominated intermediates observed from GC–MSD analysis. (A) 2,4-DBP; (B) 2,6-DBP; (C) 2-BP; (D) 1,3-dibromo-2-methoxy-5-methylbenzene; (E) 2,6-dibromo-4-methylphenol. (b) Evolution curves of 2,4-DBP and 2,6-DBP biodegradation brominated intermediates 2-BP observed from GC–MSD analysis.

Based on all above results, the metabolism mechanism of TBP by *Bacillus* sp. GZT was also proposed and demonstrated in Fig. S9. TBP biodegradation process can be roughly group as two steps. That is, the debromination is the first step and further degradation of debromination intermediates is second step. However, the debromination step also included two pathways, the reductive debromination (major) and the methylated debromination (minor) based on the produced by-products concentrations (Fig. 2a). As showed in Fig. S9, the pathway of reductive debromination of one bromine atom at both *ortho*- and *para*-positions to give 2,4-DBP and 2,6-DBP, respectively, was successfully deduced in the first part of mechanism discussion. This result is different from a previous study (Yamada et al., 2008) which detected only one debromination byproduct 2,4-DBP. From Fig. 2a, it can also be found that the concentration of 2,4-DBP was much higher than that of 2,6-DBP, indicating that *ortho*-position bromine was more easily to be removed than that of *para*-position. This result also agreed very well with the work about bromophenols debromination by a marine sponge (Ahn et al., 2003). The subsequent debromination to yield 2-BP from both 2,4- and 2,6-DBP which was also confirmed by using these two isomers as the biodegradation substrate respectively. This result also coincided with a previous study that 2,4-DBP was also debrominated to 2-BP rather than 4-BP during TBP biodegradation (Yamada et al., 2008). Furthermore, from the evolution curves of 2-BP during 2,4- and 2,6-DBP biodegradation (Fig. 2b), it can be found that the debromination of 2,6-DPB was slightly faster than 2,4-DBP, further confirming the result that the debromination occurred on *ortho*-position was more easily than on *para*-position.

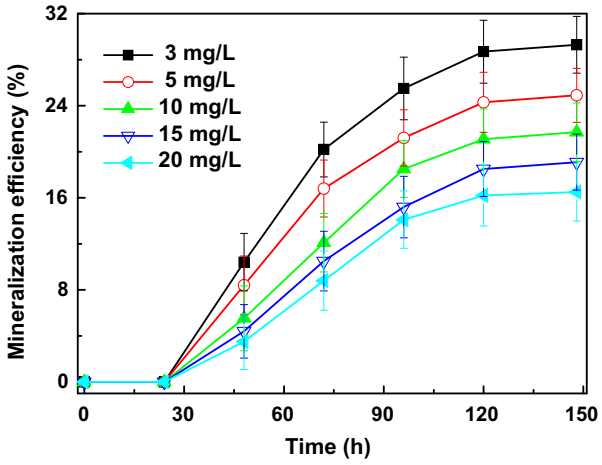


Fig. 3. TBP mineralization by *Bacillus sp.* GZT. The error bar value presents the standard deviation of triplicates.

As for the further debromination and oxidation step, the intermediates (F), (G) and (H) and phenol were also formed as the completely debromination by-products. It must note that although phenol was not detected during TBP biodegradation process due to low concentration as a grand-daughter product, it was indeed detected during the biodegradation of two debromination products 2,4-DBP and 2,6-DBP and double confirmed by its authentic standard (Fig. S10). Thus, it can be deduced that phenol also was the metabolic intermediates resulted from the biodegradation of TBP *via* dibromophenols and then to monobromophenol. The similar reductive debromination process was also reported previously (Yamada et al., 2008) except with the different debromination positions with different strain.

3.4. Mineralization of TBP

To verify the complete mineralization of TBP biodegradation intermediates, the degree of TBP metabolism with *Bacillus* sp. GZT was investigated in terms of the mineralization efficiency (Fig. 3). It can be seen that the mineralization final product CO_2 was not detected at the first 24 h as the TBP concentration ranged from 3 to 20 mg/L, which is similar to the evolution of the degradation intermediates. However, with the further increase of the degradation time from 24 to 120 h, CO_2 was produced and accumulated steadily. After 120 h, the production of CO_2 was leveled off. The highest mineralization efficiency value of 29.3% was achieved at 148 h from 3 mg/L TBP solution. With the TBP concentration increasing, the mineralization efficiency decreased dramatically, which was consisted with the effect of the TBP concentration on the biodegradation because of the substrate inhibition. Based on the evidence of CO_2 generation and Br^- evolution during the biodegradation, the terminal biodegradation products of TBP were proposed to be CO_2, Br^- and H_2O.

4. Conclusions

A pure strain was isolated from the sludge of an electronic waste recycling site, and identified as *Bacillus* sp. GZT, which has an excellent ability of simultaneous debromination and mineralization of TBP. Furthermore, the metabolism mechanism of TBP by the strain demonstrated that the reductive debromination as the major pathway of debromination could occur on both *ortho*- and *para*-positions, while the xenobiotic methylation debromination was believed as a minor and novel debromination pathway

during the biodegradation of TBP with novel bacterial strain of *Bacillus* sp. GZT. The mineralization and Br^- evolution experiment indicated that part of the brominated and debrominated by-products can be converted into CO_2, Br^- and H_2O.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2012.01.131.

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