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Purification, molecular characterization and metabolic mechanism of an aerobic tetrabromobisphenol A dehalogenase, a key enzyme of halorespiration in *Ochrobactrum* sp. T

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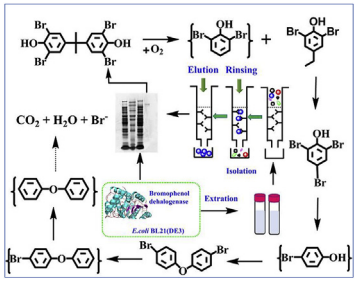
HIGHLIGHTS

- Main bromophenol dehalogenase in TBBPA degrader was first purified and characterized.
- This 117 kDa purified enzyme can remove 78% TBBPA (6 mg L^{-1}) within 4 h.
- Adding NADPH or methyl viologen significantly enhance the enzyme activity.
- The enzyme displays wide substrate specificity towards brominated compounds.
- Three metabolites of TBBPA by the dehalogenase under oxic condition were identified.

GRAPHICAL ABSTRACT



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ABSTRACT

Due to the detoxification of tetrabromobisphenol A (TBBPA) varies from different bacterial strains and depends on their specific enzymatic machinery, it is necessary to understand them for potential in situ bioremediation application. The special ability of our previously isolated *Ochrobactrum* sp. T to simultaneously debrominate and aerobic mineralize TBBPA urgent us to continuously study its degradation molecular mechanism. Herein, the purification and characterization of the dehalogenase which can debrominate TBBPA was investigated based on its corresponding encoding gene *tbbpaA*. Results showed that an enzyme with molecular mass of 117 kDa, K_m of 26.6 μM and V_{max} of 0.133 μM min^{-1} mg^{-1} was purified and designated as bromophenol dehalogenase. It was the only detected dehalogenase which exhibited TBBPA degradation ability (78%). Moreover, its activity was significantly enhanced by adding NADPH or methyl viologen to the reaction solution. The high similarity of substrate spectrum between the dehalogenase from the recombinant strain and the wild strain further indicated that it was the main dehalogenase responsible for the debromination in wild strain. Based on three identified metabolites, a metabolic pathway of TBBPA by purified enzyme under oxic condition was proposed. This study provides an excellent dehalogenase candidate for mechanistic study of aerobic dehalogenation of brominated aromatic compound.

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1. Introduction

The realization of potentially harmful impacts of brominated organics on environmental and human health in 1970s triggered extensive research on the degradation mechanisms of these organics when polybrominated biphenyls were discovered in feed for dairy cattle, livestock, and poultry in Michigan (Birnbaum and Staskal, 2004). Until now, more than 75 different brominated flame retardants (BFRs) have been commercially manufactured (Covaci et al., 2011) with global market demand of over 200,000 tons per year. Tetrabromobisphenol A (TBBPA) is the largest volume BFRs in the market with a global consumption up to 120,000 tons in 2001 (Shah, 2010). Moreover, the input of TBBPA to the environment through electronics recycling facility, recycled plastic pellets, or from disposal of components in landfills, has the potential to increase dramatically as aging computer inventories are disposed (Yong et al., 2015). So far, TBBPA has been found in various environmental matrices, including water, dust, sewage sludge, sediment, soil, air and even human and animal tissues, and raised a great threat to the world (Li et al., 2015a). Negative effects on reproduction and physiological development have been reported in aquatic species (zebra fish), frogs, terrestrial species (earthworms) and several types of plants (Malkoske et al., 2016). Therefore, it is of great significance to develop methods to efficiently remove TBBPA from the contaminated environment, considering its constantly increasing outputs and potential harmful effects, such as hepatotoxicity, cytotoxicity and immunotoxicity, on the human beings (Qu et al., 2015).

Biological strategy is an attractive method for organic pollutants degradation due to its relatively cost effective and environmentally compatible. Up to date, different species of bacteria that can catalytically remove the halogen substituents have been successfully isolated, such as *Desulfitobacterium*, *Dehalococcoides* (Fricker et al., 2014), *Dehalobacter* (Nelson et al., 2014), *Ochrobactrum* (An et al., 2011), *Bacillus* sp. (Zu et al., 2012), *Pseudomonas*, *Streptococcus* (Peng et al., 2014) and *Comamonas* sp. (Peng et al., 2013). The dehalogenation reactions catalyzed by these microorganisms are interesting from the view of genetics, biochemistry and biotechnological applications. In addition, dehalogenation reactions are also attracting from an evolutionary perspective since environmental xenobiotics were introduced in the last century (Krasotkina et al., 2001). The critical step of dehalogenation is the removal of halogens from the carbon skeleton of the molecule by oxidative/substitutive/reductive dehalogenation, dehydrohalogenation and the dehalogenation by methyl transfer (Fetzner, 1998). This step could generate intermediates with reduced recalcitrance to biodegrade and less toxicity (Vlieg et al., 2000). For example, tetrachloroethene (PCE) and trichloroethene (TCE) can be reductively dechlorinated by "*Dehalococcoides ethenogenes*" 195 to less-chlorinated intermediates, dichloroethene and vinyl chloride and then to ethene (Maymo-Gatell et al., 1999). The *Ochrobactrum* sp. T (strain T) can simultaneously debrominate and mineralize TBBPA to harmless CO_2 and H_2O in an aerobic system (An et al., 2011). The study of microbial dehalogenation of different halogenated aromatics has led to the identification and elucidation of various dehalogenases that catalytically removed the halogen atom under aerobic and anaerobic conditions. Among them, the enzyme designated as reductive dehalogenases (RDases) from the anaerobic strains and the corresponding encoding genes were the most widely studied. To be specific, the first purified RDase reported was a membrane-bound 3-chlorobenzoate dechlorinating enzyme from *Desulfomonile tiedjei* (Ni et al., 1995), and the best-characterized RDases for degradation halogenated aliphatics were those specific for chloroalkenes, PCE (*PceA*) and TCE (*TceA*) (Marzorati et al., 2007). Overall, these reported enzymes were mainly the membrane-associated enzymes with low levels of nucleotide identity and some common traits, such as two iron-sulfur clusters as prosthetic groups, a twin-arginine translocation signal peptide, and corrinoid cofactors (Maillard et al., 2005).

However, the obtained dehalogenated intermediates by the RDases could usually not be further degraded by anaerobes (Chen et al., 2013). That is, some other aerobes, who rarely support reductive dehalogenation but can oxidative dehalogenation, are needed to further dehalogenate and completely mineralize haloorganic compounds (Liu et al., 2013). Although, as we mentioned above, the RDases from anaerobic bacteria have been studied extensively at the molecular level including the elucidation of crystal structures, the detailed biochemical and molecular information about the enzymes from aerobic dehalogenating especially debrominating microorganisms is scarce. Up till now, only a few proteins with the ability to catalytically debrominate oligocyclic phenolic bromoaromatics were characterized (Yang et al., 2015). For example, a RDase (BhbA) from an aerobic strain of *Comamonas* sp. 7D-2 could dehalogenate bromoxynil in the presence of nicotinamide adenine dinucleotide phosphate NADPH (Chen et al., 2013) and another dehalogenase from aerobic *Bacillus* sp. GZT could dehalogenate 2,4,6-tribromophenol (TBP) (Liang et al., 2017). Recently, it was found that the purified cytochrome P450 monooxygenase (Sasaki et al., 2005) and laccase (Das et al., 2018) were involved in bisphenol A (BPA, one of TBBPA intermediates) biodegradation. Overall, very little is known regarding the dehalogenases involved in the debromination in aerobes. One of bacteria, *Ochrobactrum* sp. T, which can simultaneously degrade and mineralize TBBPA under aerobic conditions, was previously isolated by us (An et al., 2011). Its genome harbored some genes putative encoding enzymes that could degrade chlorinated compounds and phenol, of which only one gene (*tbbpaA*, GenBank accession number KY483638) was found and confirmed to be responsible for TBBPA debromination (Liang et al., 2016). However, the isolation of this TBBPA dehalogenase has never been reported and there are no other reports of purified enzyme capable of degrading TBBPA in the published literature domain.

Therefore, for the potential application of bioremediation at contaminated field sites, the major objectives of this work were to characterize a novel dehalogenase used for TBBPA debromination from the examined aerobic recombinant strain and to assess the environmental conditions under which dehalogenases are metabolically active. Furthermore, the main intermediates were firstly identified using Acquity ultra performance liquid chromatography system coupled to a Xevo TQ-S triple quadrupole mass spectrometer (UPLC/MS/MS), and then a detailed reaction pathway in an aerobic system was predicted to further confirm the function of potential genes and enzymes. This is the first enzyme identification report of BFRs-degrading strain. The information will improve our understanding of TBBPA bio-augmentation mechanism from a genetic level and eventually develop outstanding TBBPA attenuation strategies in contaminated sites.

2. Experimental section

2.1. Reagents

TBBPA (97%), TBP (99%) and 2,6-dibromophenol (2,6-DBP, 99%) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Bis(4-bromphenyl) ether (96%), 2,6-dibromo-4-(2-propenyl) phenol (98%), BPA (97%), 2,4-dibormophenol (2,4-DBP) (99%), 2-bromophenol (2-BP, 98%), 4-bromophenol (4-BP, 97%), 3-bromophenol (3-BP, 98%) and N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) (98%) were from Acros Organics (New Jersey, USA). All other chemicals were of analytical grade with more

than 99% purity and from Guangzhou Chemical Reagent Co., Inc., China.

2.2. Bacterial strains and culture conditions

Bacterial strains and culture medium: Our previously isolated bacterial strain, strain T (GenBank accession number: HM543185) (An et al., 2011), was used as a wild strain for TBBPA degradation. The recombinant strain *E. coli* BL21 (DE3) pET30a-a6 was previously constructed by us (Liang et al., 2019). The experimental growth medium (GM), mineral medium (MM) and culture conditions are described in the supporting information (SI).

The wild and recombinant strains preparation: Unless indicated otherwise, all the purification steps were carried out at 4 °C without protection against oxygen. The wild and recombinant strains were pre-cultured in GM and collected in their late logarithmic growth phase by centrifugation at 8000*g* for 5 min, then washed with MM twice. After that, the induction of the expression of TBBPA dehalogenase experiments were performed by inoculating 25 mL of harvested cultures into 100 mL MM with TBBPA (in 250 mL shake flasks) at 35 °C, pH 7.0 for 96 h under aerobic conditions. Finally, the cells were harvested by centrifugation at 10,000 g for 20 min at 4 °C and washed with buffer A (50 mM NaH_2PO_4, 300 mM NaCl and 1 mM DTT, pH 8.0). Cell pellets were stored at −80 °C until further use.

Crude enzyme preparation: Whole-cell suspensions were prepared by resuspending the pellet in buffer A with a final volume approximately 1/10 of the original volume. To prepare cell-free extracts, approximately 1 g of wet cell pellet was thawed and dispersed in buffer A containing 50 mg L^{-1} lysozyme and 1 mM phenylmethylsulfonyl fluoride. Then, the cells were disrupted by sonication for 15 min at 20% amplitude on ice and centrifuged at 14,000 rpm, 20 min, 4 °C. The supernatant was regarded as crude extracts and the cell debris were further used to prepare the membrane fraction by resuspended in buffer A with 0.5% (w/v) Triton X-100. After incubating for 1 h at 4 °C, the insoluble fraction was removed from this preparation by centrifugation for 60 min at 14,000 rpm and 4 °C and the solubilized membrane enzyme was stored at 4 °C. The above fractions were subjected to test TBBPA dehalogenase activity.

2.3. Purification of TBBPA dehalogenase

The cell-free extracts which exhibited TBBPA degradation activities were used immediately for purification of the TBBPA dehalogenase after filtered through a membrane with a pore size of 0.2 μm. Due to the N-terminus of the recombinant protein was fused with poly-histidine tag, it could be purified using the His-Bind protein fusion and purification system (Marzorati et al., 2007) with Ni-NTA His-Bind resin (Novagen). To be specific, the first step was to load the crude extracts onto the His-Bind resin column, which was previously equilibrated with buffer A. After eluting the proteins with 0−500 mM imidazole in buffer A, the elution containing the target fusion protein which showed high TBBPA biodegradation activity was collected and concentrated using a 10 kDa cutoff Ultra-4 Centrifugal Filter (Merck, Germany). Protein concentrations were quantified by the Bradford method using bovine serum albumin as the standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 10% polyacrylamide separating gel and 5% stacking gel was used to check the purity of the target enzyme after stained with Coomassie brilliant blue R250. Finally, a Proteomics Analyzer Matrix-assisted laser desorption/ionization mass spectrometry with automated tandem time-off light fragmentation of selected ions (MALDI-TOF/TOF MS) was used to identify the purified recombinant protein as described in our previous work (Liang et al., 2017).

2.4. TBBPA determination by HPLC

To measure the TBBPA concentration, liquid samples in the experimental vials were collected and adjusted to pH 9.0 with 2.0 M NaOH. After filtered with 0.22 μm syringe filter, 20 μL of treated sample was injected into the high-performance liquid chromatography (HPLC, Agilent 1200 series) equipped with a diode array detector (DAD) for the analysis of TBBPA. The column used is an Agilent XDB-C18 Eclipse column (4.6 × 150 mm, 5 μm particle size). The detection conditions were similar to our previous work (Zu et al., 2014), which was set as follows: 80% methanol and 20% acetic acid (2%) for 8 min, at flow rate of 0.8 mL min^{-1} with the column temperature of 30 °C and wavelength of 230 nm.

2.5. Intermediates identification

The intermediates produced during TBBPA biodegradation process were identified and quantified by UPLC/MS/MS (Waters, Milford, MA, USA) using electrospray ionization (ESI) interface in the negative ion mode. The capillary voltage was 2 kV with a source temperature and desolvation temperature of 150 °C and 400 °C, respectively. The collision energy for the precursor ions of TBBPA, TBP, 4-BP and Bis(4-bromphenyl) ether were 22, 35,35 and 15 V, respectively (Table S1). An Acquity BEH C18 column (2.1 × 50 mm; 1.7 μm Waters) were used for UPLC separation. Gradient elution was performed using methanol and ammonia (0.05%) with initial gradient conditions set as follow: started with 40% methanol, followed by a linear increase to 60% methanol over 2.5 min, then to 100% methanol at 5 min and finally returned to the initial state to equilibrate for 2 min before the next injection. The flow rate was set at 0.4 mL min^{-1}, and the injection volume was 5 μL. The concentrations of these intermediates were determined from the peak area ratio relative to individual standard calibration curves.

Besides, at different biodegradation intervals, the intermediates were also analyzed using gas chromatography (Agilent 7890) coupled with a mass selective detector (GC/MS, Agilent 5975C) with and without BSTFA derivatization as described according to previous reference (An et al., 2011).

2.6. Enzyme activity assay

TBBPA debromination assays were carried out similar to our previous study (Liang et al., 2017) in which debromination products were assayed via HPLC after incubation. Debromination assays were performed in a 12 mL culture tube stoppered with dual cap. The standard reaction mixture (3 mL) used to detect enzyme activity contained 1 mM methyl viologen, 6 mg L^{-1} TBBPA in 50 mM Tris-HCl (pH 7.0). The reaction was initiated by adding 50 μL enzyme preparation and incubating at 30 °C, 180 rpm for 240 min. The reaction was terminated by incubation at 100 °C for 5 min. TBBPA concentration in the mixture was determined using HPLC as described above. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the degradation of 1 μM TBBPA per minute. Specific activity was expressed in units per milligram of protein. All the assays were performed triplicate, and the means and standard deviations were calculated.

2.7. Biochemical characterization of the dehalogenase

The effects of environmental factors including temperature, pH, substrate concentration, potential inhibitors and different cofactors on the TBBPA-dehalogenase activity were investigated with the above-described standard enzyme assay mixture. Initial

characterization of the enzyme was carried out with a purified enzyme obtained after chromatography on a Ni-NTA His-Bind column. Experimental details were provided in SI.

3. Results and discussion

3.1. Localization of the TBBPA dehalogenase

Previously, we analyzed the genomic DNA of our previously isolated strain T, an outstanding TBBPA debromination and aerobic mineralization bacterium, and found that some open reading frames (ORFs) were similar to the genes encoding dehalogenases (Liang et al., 2016). These encoding genes were also further PCR amplified, cloned and overexpressed in *E. coli* BL21 (DE3) and it was found that the recombinant strain could degrade TBBPA (Liang et al., 2019). In this study, the TBBPA dehalogenase activities from different fractions of the recombinant strain and wild strain T were compared to further explore the functional protein. As Fig. 1a shows, the dehalogenation of TBBPA was observed in the whole cell suspension of recombinant strain under aerobic condition, as evidenced by approximately 85% loss of the parent substrate. Comparatively, only 4.4% of TBBPA was degraded after 240 min incubation by the membrane fraction of the recombinant strain, whereas 78% of TBBPA removal was associated with the cell-free fraction, which was nearly equal to that of the whole cell suspension. Furthermore, the addition of membrane fraction in the cell-free fraction did not stimulate TBBPA dehalogenation. All these results suggested that the cell-free extracts were capable of dehalogenation of TBBPA, whereas essentially no dehalogenation was detected in the membrane fraction. These results of the recombinant strain were consistent with findings for the wild strain T. As Fig. 1b shows, the highest debromination efficiency (75%) were achieved with the cell-free fraction of strain T, indicating that recombinant strain expressed the same active TBBPA dehalogenase with the wild strain T. However, these results contradicted with some previous reports that dehalogenases were usually membrane associated and oxygen sensitive proteins from anaerobic bacteria (Chen et al., 2013; Payne et al., 2015). Considering the enzyme found by us was from an aerobic bacterial strain, we concluded that it might be a novel soluble, oxygen-tolerant TBBPA dehalogenase. Therefore, the non-membrane associated extracts were subjected to perform the subsequent purification and dehalogenation assays to verify this hypothesis.

3.2. Bromophenol dehalogenase purification and characterization

As TBBPA dehalogenase activity was readily found in the cell extracts of isopropyl-β-D-thiogalactopyranoside -induced recombinant strain, a Ni-NTA resin was used to purify the target dehalogenase from the crude extracts based on its N-terminus polyhistidine tag which was introduced during the construction of the recombinant strain. After specifically bound to Ni-NTA resin in the buffer A containing 10 mM imidazole, the His-tagged targeted dehalogenase was eluted with 500 mM imidazole. The purification scheme for the His-tagged dehalogenase is summarized in Table 1. Throughout the purification procedure, a total of 10.2-fold enrichment with a yield of 58.5% protein was achieved and the specific activity increased from 0.3 (before purification) to 3.06 (after purification) units mg^{-1} protein. As Fig. 2 shows, the crude extract of the recombinant contained an protein with a size of 117 kDa band, which was absent from that of the control strain *E. coli* BL21 (DE3) pET30a(+), further suggesting that this protein is encoded by *tbbpaA* gene. After purification, SDS-PAGE revealed only one band with a molecular mass estimated to be 117 kDa, indicating that the enzyme was successfully purified. This size is corresponding to the apparent molecular mass detected by MALDI-TOF analysis. In addition, as Table S2 shows, computer database matching resulted in a score of 714 for the purified dehalogenase with a sequence coverage of 15% of the total amino acids. The ion score of 64 in the Mascot search revealed it to be ($p < 0.05$) a haloacid dehalogenase. Based on these results and our previous founding (Liang et al., 2019) which showed that this protein shared 100% identity with the haloacid dehalogenase, this newly-isolated enzyme was designated as bromophenol dehalogenase (accession number: ARV76518).

Furthermore, the multiple sequence alignment of dehalogenase with other haloacid dehalogenases was carried out. This alignment has a minimum sequence length of 611 and maximum sequence length of 638. As Fig. S1 shows, the pairwise results of haloacid dehalogenase from *Ochrobactrum* sp. T and other organisms gave 59% sequence identity. Amino acids with high consensus value are D (Asp), E (Glu), R (Arg), K(Lys), T (Thr), N(Asn), S (Ser) and Q (Gln). According to previous study, amino acids D (Asp), R (Arg) and S (Ser) was played important role in the haloacid dehalogenase mechanism (Adamu et al., 2016, Azza Hanif Harisna et al. 2017). This suggested that this purified dehalogenase may have the same functional residues responsible for the dehalogenation process as the above dehalogenase.

The above study showed that no TBBPA dehalogenation activity was found in the membrane fraction, but it was detected in the cell-free extract, indicating that this bromophenol enzyme is a cytoplasmic enzyme. Therefore, to achieve best TBBPA biodegradation efficiency by the purified enzyme, it is necessary to characterize the target protein and perform the enzyme assay under the optimal

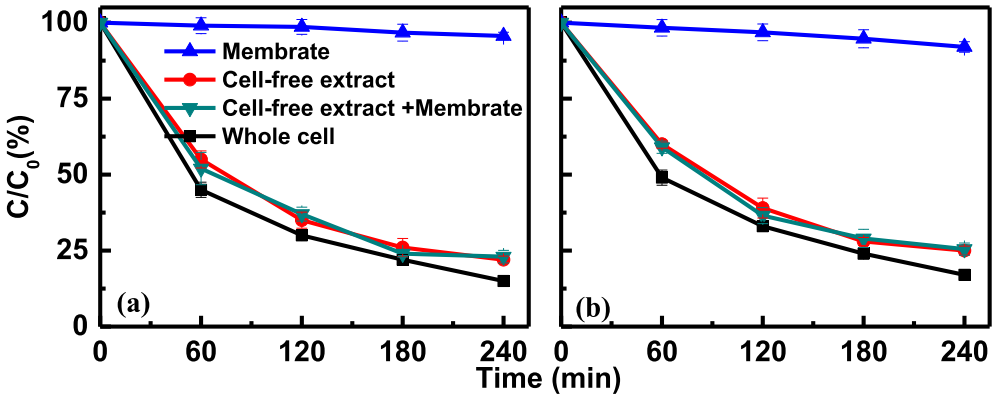


Fig. 1. Debromination of TBBPA by different subcellular fractions of (a) recombinant strain and (b) wild strain T.

Table 1
The purification scheme for the His-Tagged dehalogenase.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1})	Yield (%)	Purification factor (fold)
Crude extract	352	117	0.3	100	1
Ni-NTA	22.4	68.4	3.06	58.5	10.2

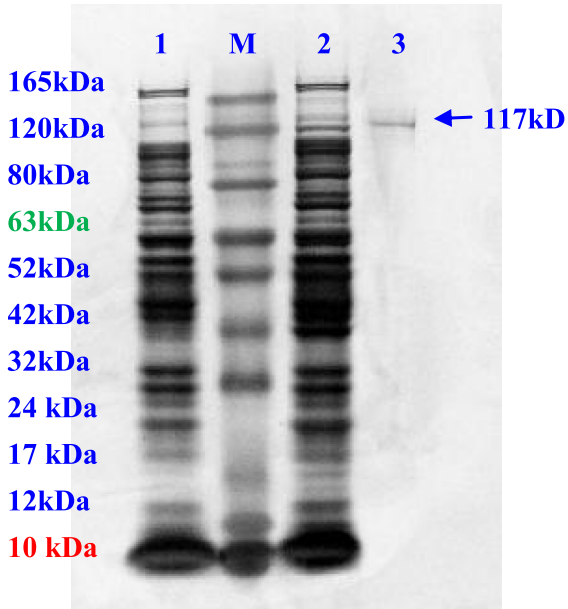


Fig. 2. SDS-PAGE analysis of the purified bromophenol dehalogenase from recombinant strain. Lane M: protein marker; Lane 1: protein extract of control strain *E. coli* pET30a (+); Lane 2: protein extract of recombinant strain; Lane 3: purified bromophenol dehalogenase.

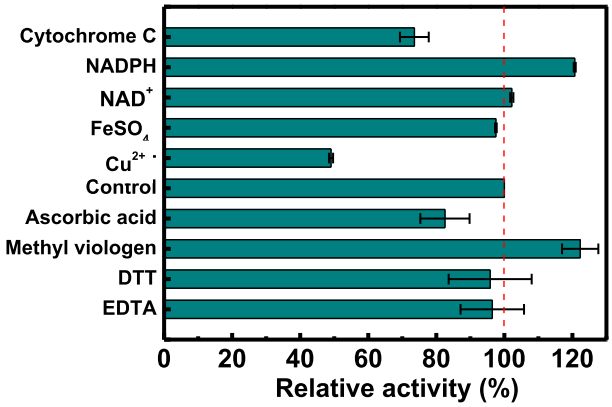


Fig. 3. The effect of different additives on the activity of bromophenol dehalogenase from recombinant strain. The data is represented as the mean ± standard deviation for triplicate incubations.

condition. As Fig. 3 shows, the activity of purified enzyme was greatly increased by the addition of cofactor NADPH, suggesting that NADPH was served as the electron donors in this dehalogenation process. Similar result was also found for RDase from *Comamonas* sp. 7D-2, an aerobic strain which was able to degrade the brominated aromatic herbicide bromoxynil completely (Chen et al., 2013). In addition, TBBPA dehalogenation in this study also increased in the presence of methyl viologen. This result was consistent with previous dehalogenation studies, which proved that methyl viologen can accelerate the dechlorination of vinyl chloride and chlorinated propenes by vinyl chloride RDase (VcrA) and PCE RDase, respectively (Parthasarathy et al., 2015; Schmitz et al., 2007). All these indicated that methyl viologen might serve as an artificial electron donor (Nijenhuis and Zinder, 2005). When methyl viologen was used as the electron donor in this study, the relative enzyme activity was slightly (1-fold) higher than that of NADPH. As such, methyl viologen was regarded as a better electron donor of bromophenol dehalogenase in vivo. Comparatively, the incubation of the dehalogenase with DTT, EDTA, NAD^+ or $FeSO_4$ had no significance effect on the enzyme activity. This may be suggesting that they were not involved in the electron transport system of dehalogenase and chelating reaction (Das et al., 2018). However, the addition of Cu^{2+} to the enzyme mixtures showed strong inhibition effects in this study, which is possibly due to the toxicity of Cu^{2+} to dehalogenase (Ma et al., 2017). The inhibition effect by Cu^{2+} and the lack of inhibition by EDTA and NAD^+ were consistent with the report of 2-haloacid dehalogenase and dichlorophenol RDase (Thibodeau et al., 2004; Zhang et al., 2013).

Furthermore, the effects of temperature, rotation speed and pH on the dehalogenase activity were assayed with the purified enzyme. As Fig. S2 shows, more than 80% relative activity was observed at temperature ranging from 25 to 35 °C (maximum activity at 30 °C) (Fig. S2a), and the optimal rotation speed for the enzyme activity was 180 rpm (Fig. S2b). In addition, bromophenol dehalogenase showed high activity at pH 6.5–7.0, with an optimum pH of 6.5, and its activity was inhibited under weak acidic (pH = 6.0) or alkaline (pH = 8) condition (Fig. S2c). This result was in good agreement with a previous report that the optimal temperature and pH for removal of butachlor by the recombinant debutoxylase was 30 °C and pH 6.5, respectively (Gao et al., 2015), whereas different from that of the wild strain with the optimal condition of pH 7.0 and 35 °C, respectively (Gao et al., 2015). The wide difference of the optimal pH and temperature for the same enzyme from inside and outside of the cell was not expected. One possible explanation is that the oxidative state of the enzyme might be different between the outside and inside of the cell, which affected its thermal and pH stability, and, therefore resulted in varied responses to the pH and temperature (Habash et al., 2002). Overall, these characteristics are important for the practical application of this enzyme, taking the complexity of the natural environment into consideration.

3.3. Bromophenol dehalogenase substrate specificity and kinetics analysis

The substrate specificity of the purified bromophenol dehalogenase was further investigated in order to identify its catalytic activities, which may be related to its biological function, such as biodegradation and biostimulation (Sfetsas et al., 2009). To this end, a broad range of substrates was examined under the condition that the relative activity of the dehalogenase for the substrate TBBPA was labeled as 100%, whereas other substrates activities were expressed as the percentages of the activity found with TBBPA. As Table S3 shows, bromophenol dehalogenase exhibited significant differences of relative activities towards these substrates. To be specific, the highest enzyme activity level was observed with 2,6-DBP (138 ± 8.4%), and the remarkable debromination at the para-

position was also detected with 4-BP (133 ± 7.7%). Other individual substrates including TBP, 2,6-dibromo-4-(prop-1-en2-yl) phenol and 4-bromodiphenyl ether could also be degraded by the purified dehalogenase with a lower degradation activity (approximately 80% of TBBPA). Further, even lower specific activity of the purified enzyme was found towards bis(4-bromophenyl) ether (65.72 ± 3.4%) and diphenyl ether (56.5 ± 3.7%). No biodegradation was detected for 2,4-DBP, 3-BP, 2-BP and BPA with the purified enzyme, suggesting that they are not the substrates of the dehalogenase. Comparatively, the overall substrate specificity profile of the dehalogenase from the recombinant strain resembled that of its wild strain. For example, both of them could degrade TBP, 2,6-DBP and 4-BP. This is not surprising since the encoding gene of recombinant enzyme are from the wild strain T, therefore some features of the recombinant enzyme are functionally similar to those of the enzymes of wild strain. However, still some substrates such as BPA could only be degraded by wild strain rather than by the purified enzyme from the recombinant strain. One possible explanation is that, in the wild strain, the debromination of these organohalides is a collective contribution of the whole bacterial enzyme system with different enzymes, which are responsible for the conversion of different substrates (Sfetsas et al., 2009). The purified enzyme is only partial of the whole enzymes. But overall, bromophenol dehalogenase is in agreement with other characterized dehalogenases like PceA from *Desulfitobacterium* sp. Y51 and TceA from *Dehalococcoides ethenogenes* (Jugder et al., 2016), which exhibited wide substrate specificity and could catalyze a broad range of reactions.

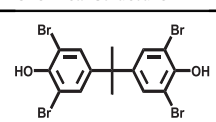
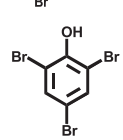
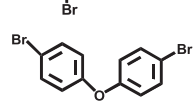
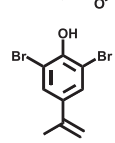
In addition, to assess how efficiently the dehalogenase degraded TBBPA, the specificity constant (K_m and V_{max}) was measured using Lineweaver-Burk equations (Roberts et al., 2004). The results showed that the purified enzyme had a *Km* value of 26.6 μM. Considering previous studies have reported that the K_m value of the purified enzyme was higher than that of the crude cell extract (Fetzner, 1998; Wong et al., 2016), in this study, we also speculated that the purified enzyme had a higher K_m value than the crude extract. This is because the surrounding environment (e.g., buffers, detergents, presence of other proteins and membrane components) of the enzyme can adversely affect the conformation of the protein (Jugder et al., 2017). We also found that its K_m (26.6 μM) in this study is significantly lower than that of reported values of the 1,2-dibromoethane (100 μM) (Grostern et al., 2009), pentachlorophenol (46.7 μM) (Bisaillon et al., 2010), PCE (105.7 μM) and TCE (535.3 μM) (Suyama et al., 2002) when dehalogenation by the anaerobic RDases. It has to be mentioned that the V_{max} (0.133 μM min^{-1} mg^{-1}) of the bromophenol dehalogenase in this study is comparable to that reported for RDase of *Desulfitobacterium frappieri* for 3,5-dicholorophenol (Thibodeau et al., 2004). These subtle differences in kinetics of dehalogenases may give insight into how aerobic and anaerobic dehalogenases differ to each other.

3.4. Metabolites of TBBPA by bromophenol dehalogenase

Due to the reductive dehalogenation of TBBPA by dehalogenase is originally thought to rarely occur in aerobes, most mechanism studies were mainly focused on the dehalogenation under the anaerobic condition (Hug et al., 2013; Zhao et al., 2018). Therefore, it is of rather importance to elucidate the aerobic biodegradation pathway of TBBPA by purified enzyme. In this study, the metabolites in the enzyme extracts at different incubation time intervals were identified. Both UPLC/MS/MS and GC/MS were used to detect these metabolites based on the bromine isotope pattern and the resulting characteristic mass fragments (the mass spectra of TBBPA, TBP, bis (4-bromophenyl) ether and 4-propenyl-2,6-dibromophenol phenol are shown in Fig. S3). For the parent TBBPA, an abundant $[M-2H]^-$ ion at *m/z* 270.7 with two product ions at *m/z* 419.6 and 80.9 was observed (Table S1). The retention time (1.96 min) of TBBPA is shorter than that of TBP (2.29 min) because of its higher boiling point and lower polarity (Konoz et al., 2013). The detection of TBP was based on the signal from the *m/z* 330.4 → 80.9 transition, with $[M-H]^-$ corresponding to *m/z* 81.0 being used as the confirmation transition. A peak (0.53 min), corresponding to metabolite bis (4-bromophenyl) ether with a molecular ion peak at *m/z* 162.7 was also formed with the further cleavage of TBBPA. Other intermediates that could not be detected using UPLC/MS/MS probably due to their low concentration were further analyzed using GC/MS. One degradation product with a molecular ion $[M^+]$ at *m/z* 292.0 and the fragment ions at *m/z* 277.0 $[M^+-CH_3]$, 212.0 $[M^+-Br]$, 196.0 $[M^+-CH_3Br]$ was tentatively identified as 4-propenyl-2,6-dibromophenol (Table 2).

Meanwhile, the evolution curves of these brominated intermediates were plotted during TBBPA biodegradation process by dehalogenases (Fig. 4). Results revealed that, during the first 60 min of TBBPA degradation by the purified enzyme, the intermediate 4-propenyl-2,6-dibromophenol phenol accounted for a large

Table 2
Retention time, mass spectra and structure of TBBPA and intermediate products.

Compounds	Molecular weight	Retention time (min)	Chemical structure	Fragmentation ions (*m/z*)
TBBPA	543.8	1.96		250.5 (MS^2 fragments of *m/z* 270.7)
TBP	330.8	2.29		264.5, 134.9 (MS^2 fragments of *m/z* 330.4)
Bis(4-bromophenyl) ether	328	0.53		188.9 (MS^2 fragments of *m/z* 162.7)
2,6-Dibromo-4-(2-propenyl) phenol	292	32.32		277, 212, 196, 132

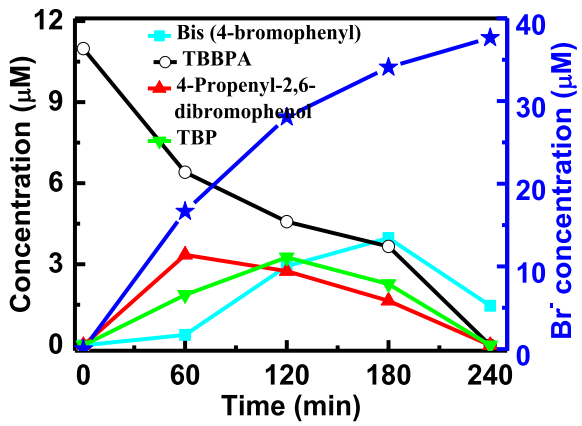
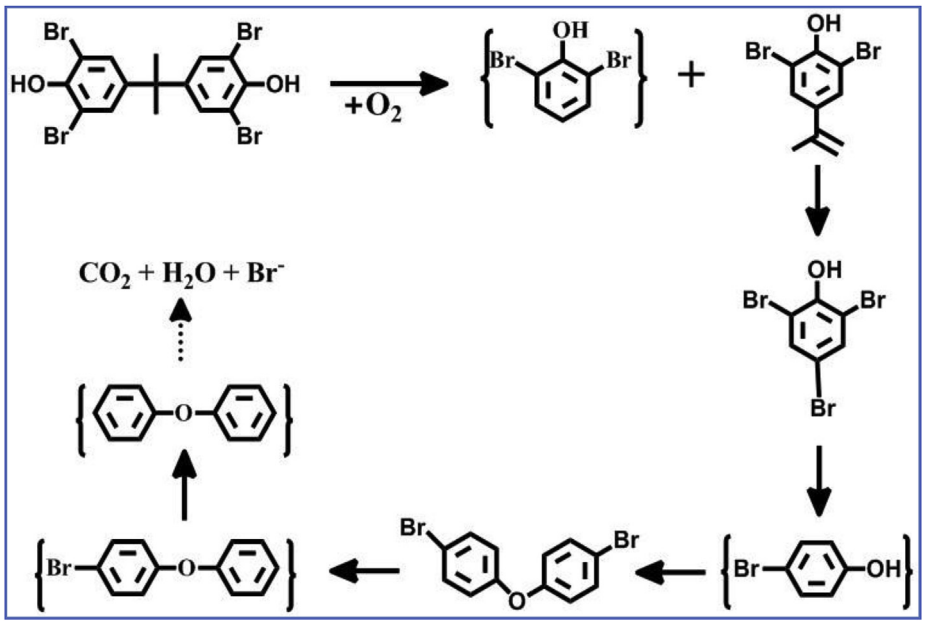


Fig. 4. Evolution curves of TBBPA biodegradation brominated intermediates by purified enzyme.

proportion. Then the concentration of TBP and bis (4-bromophenyl) ether rose significantly with the further loss of TBBPA. The highest concentration of bis (4-bromophenyl) ether could be detected at incubation time of 180 min. With the prolongation of degradation reaction, these intermediates together with TBBPA residue can be mineralized by dehalogenase. Simultaneously, the Br^- concentration during the removal process was also conducted quantitatively. As operation time increased from 60 to 240 min, the accumulated amount of Br^- increased from 16.7 to 37.7 μM, suggesting that the biodegradation of TBBPA was accompanied with the release of bromide into the mixture solution and Br^- was the main final metabolite.

The one-ring phenolic compound 4-propenyl-2,6-dibromophenol was usually detected as an important cleavage intermediate of TBBPA in an aerobic soil and activated sludge (Li et al. 2014, 2015a), by isolated strain T (An et al., 2011) or laccase (Feng et al., 2013). Therefore, according to the plausible degradation pathways of TBBPA under the oxic condition and the identified metabolites in the enzyme reaction mixture elaborated above in this study, a tentative transformation pathway was proposed for the biodegradation of TBBPA by this isolated bromophenol dehalogenase. As Scheme 1 shows, with the help of oxygen, TBBPA was initially oxidized by dehalogenase to deprive an electron and proceeded via beta-cleavage (cleavage between one of the benzene rings and the isopropyl group of TBBPA) to form 4-propenyl-2,6-dibromophenol and 2,6-DBP (not detected). This degradation pathway is in accordance with the observation of Li et al., (2015a), who accelerated the transformation of TBBPA by nitrifiers and emphasized the importance of oxidative cleavage of the alkyl chain and ipso-hydroxylation in aerobic TBBPA degradation in nitrifying activated sludge. In addition, similar transformation pathway was also observed in TBBPA biodegradation by ipso-substitution in submerged soil (Sun et al., 2014) and oxic sandy soil (Li et al., 2015b). Further, 4-propenyl-2,6-dibromophenol was converted to TBP by interacting with a bromine radical produced during the cleavage of a carbon-bromine bond. The formation of TBP in the TBBPA degradation process has also been reported previously (Barontini et al., 2004; Qu et al., 2015). Coincidentally, these two steps were found to be similar to the TBBPA biodegradation by strain T (An et al., 2011), meaning that a common dehalogenase was involved in the initial ipso-hydroxylation of TBBPA and the production of TBP. This result further proved that this purified dehalogenase was from strain T. No BPA (a complete reductive debromination intermediate of TBBPA) was detected in this reaction indicated that other enzyme complex might exist in strain T besides the purified enzyme. Even though previous report has confirmed that the degradation intermediates of TBP by *Ochrobactrum* sp strain TB01 was 2,4-DBP and 2-BP (Yamada et al., 2008; Zu et al., 2012), the debromination of TBP in this study led to the production of 4-BP. The reason for no 4-BP detection in this study is that it might quickly be transformed to bis (4-bromophenoyl) ether through mutual chemical reaction by bromophenol dehalogenase. That is, further debromination of TBP readily occurred, suggesting that the rate-limiting step for aerobic TBBPA degradation is the conversion of TBP to bis (4-bromophenoyl) ether. Although the formation of bis (4-bromophenoyl) ether was not reported in previous paper, the 4-BP was produced by metabolism of TBP with sediment microorganisms (Ronen and Abeliovich, 2000). After that, sequentially reductive debromination of bis (4-bromophenoyl)



Scheme 1. Proposed pathway of TBBPA biodegradation by newly-purified bromophenol dehalogenase.

ether to 4-bromodiphenyl ether and diphenyl ether by replacement of a Br atom with a H atom were also deduced based on the microbial degradation pathways of 4,4-dibromodiphenyl ether (Rayne et al., 2003). The lack of 4-bromodiphenyl ether and diphenyl ether during this enzyme degradation process might be attributed to the low concentration of these compounds. Besides, the above substrate specificity analysis also provided evidence that 4-bromodiphenyl ether and diphenyl ether can be degraded by the purified enzyme.

4. Conclusion

In the present report, firstly, we described the purification of an aerobic bromophenol dehalogenase from the recombinant with a TBBPA-degrading encoding gene of wild strain T. This purified enzyme with molecular mass of 117 kDa exhibited high TBBPA degradation ability (78%). Then, the results of biochemical characterization showed that the presence of NADPH or methyl viologen as electron donors in purified enzyme could greatly improve the TBBPA degradation activity. Besides, high similarity was observed between this dehalogenase with wild strain T in substrate activities and biochemical properties, indicating that it retained the characteristic of the wild strain T. Finally, the reaction mechanism is proposed and it is somehow different from that of the wild strain T, suggesting that, in the wild strain T, more than one enzyme is involved in the TBBPA reduction. Overall, the purification and biochemical characterization of TBBPA debromination enzyme and the corresponding metabolic products and pathway by purified bromophenol dehalogenase is the first report. It suggested that the biotic degradation by dehalogenase extracts may significantly impact the fate of TBBPA in TBBPA-contaminated wastewater or polluted environmental matrices, contributing to the overall environmental attenuation of this important anthropogenic contaminant.

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Appendix A. Supplementary data

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