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Application of a novel gene encoding bromophenol dehalogenase from *Ochrobactrum* sp. T in TBBPA degradation

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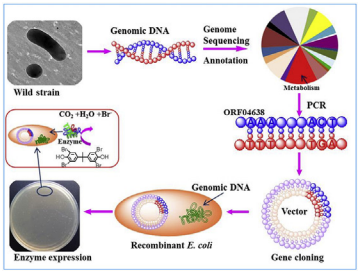
HIGHLIGHTS

- Gene *tbbpaA* encoding TBBPA degrading enzyme was cloned and overexpressed.
- Higher bromophenol dehalogenase activity was achieved in the constructed strain.
- The expression level of *tbbpaA* gene was upregulated upon the exposure to TBBPA.

GRAPHICAL ABSTRACT



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ABSTRACT

Tetrabromobisphenol-A (TBBPA), a typical brominated flame retardant, leaked from commercial products into the environments has attracted people's attention around the world. *Ochrobactrum* sp. T capable of degradation and mineralization of TBBPA was isolated in our early work. In this study, the identification of TBBPA-degrading gene from the strain was further carried out by combining whole-genome sequencing with gene cloning and expression procedures. In total, 3877 open reading frames were found within 3.9 Mb genome and seven of them were identified as dehalogenating-relating genes. One gene with a significant ability to degrade TBBPA was designated as *tbbpaA*. Sequence alignments analysis showed that it shared 100% identity with haloacid dehalogenases. Furthermore, *tbbpaA* gene was cloned and expressed into *E. coli* to achieve a constructed strain. Like the original strain, the constructed strain could degrade TBBPA (6 mg L^{-1}) with 78% of debromination efficiency and 37.8% mineralization efficiency within 96 h. Gene expression study revealed that *tbbpaA* was up-regulated in the presence of TBBPA. Overall, we report the identification of a functional TBBPA-degrading gene in an aerobe, which can deepen the knowledge of enhancing TBBPA removal by Strain T at the genetic level and facilitate in situ TBBPA bioremediation.

1. Introduction

Brominated flame retardants (BFRs) are widely used as additives in commercial products such as furniture, building materials, electronic equipment, carpets and other thermal insulation

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materials to prevent ignition (Yang et al., 2015). Given this, the leaching of BFRs into the environments during the manufacturing, transferring, recycling, and disposal of these products is inevitable. The seriousness of this problem is compounded by constantly increasing annual outputs of BFRs. It is reported that the annual global consumption of BFRs was increased from 0.13 million tons in 2002 to over 0.2 million tons in 2013 (Xiong et al., 2015). As one of the most frequently used BFRs, tetrabromobisphenol-A (TBBPA) has been detected in various environmental matrices including water, soils, sediments, aquatic animals, and even human tissues, maternal and cord serum and breast milk (Sun et al., 2014; Li et al., 2015b; Wang et al., 2015b). Due to its high toxicity, bioaccumulation and lipophilicity, significant health risk on humans and ecosystems including neurotoxicity, immunotoxicity, endocrine disruption, and hepatocytes destruction will be posed by TBBPA (Peng et al., 2017). As such, it is necessary to remove them completely and cost-effectively.

Biodegradation with reductive dehalogenation bacteria has proved as a promising way to clean up the sites contaminated with halogenated pollutants. This is because of the fact that the bromine/chlorine of these compounds can be replaced with hydrogen stemmed from molecular hydrogen or other oxidizable compounds such as succinate, propionate, pyruvate, lactate, acetate and formate by bacteria to supply energy for their growth or serve as election acceptors (Ding and He, 2012; Richardson, 2016). As a result, with the breaking of C-halide bond through reduction and the production of corresponding halide anion and the nonhalogenated product, the toxicity of these compounds is significantly reduced or completely removed (Hug et al., 2013). Recently, a growing number of bacteria were successfully isolated with the ability to degrade different contaminates and these bacterial strains include facultative anaerobe strains and obligate aerobes (*Comamonas* sp. (Peng et al., 2013), *Desulfitobacterium* (Atashgahi et al., 2016), *Ochromobacterium* sp. (Zu et al., 2014), *Bacillus* sp. (Zu et al., 2012)) and obligate anaerobe bacteria (*Dehalococcoides*, *Dehalogenimonas*, *Dehalobium*, *Dehalobacter* sp.) (Ding and He, 2012; Richardson, 2013). Among them, *Dehalococcoides* is the most well studied and documented strain because it exhibits versatile dechlorination activity to hazardous chlorine-containing chemicals such as polychlorinated biphenyls and trichloroethene (Tas et al., 2010).

Furthermore, with the availability of modern molecular techniques such as polymerase chain reaction (PCR)-based fingerprinting methods, high throughput techniques, microarray and next-generation sequencing, the enzymes designated as reductive dehalogenases (RDases) from these strains and the corresponding encoding genes were also studied widely, to deeply understand the molecules mechanism of these strains to degrade these compounds (Fricker et al., 2014; Liang et al., 2015). For example, previous genomic annotation has led to the discovery of 1,469,720 and 1,395,502 bp genome of *D. ethenogenes* strain 195 and strain CBDB1, which possess 17 and 32 *rdh* genes potentially encoding RDases for chlorinated compounds-degrading, respectively (Kube et al., 2005; Seshadri et al., 2005). In addition, the subsequent analysis of the draft genome (1,462,509 bp) of *Dehalococcoides mccartyi* JNA revealed the presence of 29 putative RDase genes (Wang et al., 2015a). However, as for TBBPA-degrading strain, *Ochrobactrum* sp. T (Strain T), only few recent works focused on its biodegradation kinetics and mechanisms of TBBPA (An et al., 2011; Zu et al., 2014; Xiong et al., 2015; Li et al., 2016) without further investigating its related functional dehalogenase genes.

Therefore, the primary objective of this study is to explore potential brominated compounds removal encoding genes using a series of molecular biological techniques. By genome sequencing of Strain T, the open reading frames (ORFs) related to the dehalogenase could be achieved. Then these genes were systematically cloned and expressed in *E. coli* to examine the potential of their expression enzymes for TBBPA degradation. Furthermore, the biodegradation efficiency and cell growth were extensively compared between the constructed strain with wild-type bacterium. In addition, both debromination and mineralization of TBBPA were also detected by the constructed strain cultivated with TBBPA. Lastly, the transcript expression level of the target gene during the TBBPA biodegradation process by these two strains was also analyzed using quantitative real-time PCR (qPCR). This information will help us to deeply understand the TBBPA biodegradation mechanism at the genetic level.

2. Experimental section

2.1. Chemicals, microorganisms, media and cultivation conditions

TBBPA (97%) and 2,4,6-tribromophenol (TBP, 99%) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Bisphenol A (BPA, 97%) was from Acros Organics (New Jersey, USA). All other chemicals were of analytical grade and obtained from Guangzhou Chemical Reagent Co., Inc., China.

Strain T (HM543185) was previously isolated and identified by our group (An et al., 2011) from a sludge sample collected from an electronic waste recycling site. The detail recipe of growth medium (GM) and the mineral medium (MM) used for enrichment and biodegradation of TBBPA by this strain were provided in the Supporting Information (SI). *E.coli* DH5a and *E. coli* BL21 (DE3) (Tiangen) were used as hosts for cloning and gene expression, respectively. The plasmids pMD™18-T (Takara) and pET30a (+) (Novagen) were used for cloning and expression vectors, respectively.

2.2. Genome sequencing and nucleotide sequence accession numbers

The whole genome sequencing of Strain T was conducted and compared with the sequences in the NCBI database to provide organism-specific genomic template for the molecular mechanism analysis of TBBPA degradation (details in SI). The sequence was trimmed and assembled based on a previous reference (Liang et al., 2016). This draft genome project has been deposited at DDBJ/EMBL/GenBank under the accession number of LXEK00000000.

2.3. PCR amplification, cloning and sequencing of putatively TBBPA dehalogenase gene

In total, 24 ORFs, putatively related to encode the dehalogenases capable of degrading halogenated compounds were amplified by PCR using genomic DNA as template and the specific primers. Emphasis would be given on designing specific primers targeting dehalogenases genes using Primer 3 Plus software based on selected ORFs. A total of 24 primer sets listed in Table S1 were synthesized by integrated DNA technologies and tested for the specificity using primer-BLAST on NCBI web site. *Bam*HI (in the forward primer) and *Eco*RI or *Xho*I (in the reverse primer) restriction sites were introduced to ensure the correct direction of gene insertion. Experimental details of PCR amplification were provided in SI.

The purified PCR products of individual genes amplified with specify primers were ligated into the pMD™18-T Vector (Takara, China) and transformed into *E. coli* TOP10 chemically competent cells according to the manufacturer's protocol. The blue-white screen was used for rapid detection of constructed bacteria, and white colonies on ampicillin-resistant solid plates were enriched to

extract the plasmid based on the alkaline lysis method using TaKaRa MiniBEST Plasmid Purification Kit. In all experiments, *E. coli* cells carrying empty pMD™18-T were used as a negative control. The correct insert was identified using PCR amplification and verified by DNA sequencing on an ABI 3730 xl DNA Analyzer using the M13 forward and reverse primers, which bind to sites flanking the insertion site on the plasmid.

2.4. Expression and identification of TBBPA dehalogenase

Target gene fragments were obtained from the positive clone plasmids digested with *Bam*HI and *Eco*RI or *Xho*I simultaneously. After purification, these genes were inserted into T7 expression vector pET30a (+) (Novagen), which had been predigested with the same restriction enzymes. Ligated plasmids were transformed into *E. coli* BL21 (DE3) competent cells and grew on LB plate supplemented with 30 μg mL^{-1} kanamycin to select positive constructed bacteria. Then, 100 μL of the above enriched constructed strain was inoculated into solid MM plate with 6 mg L^{-1} TBBPA for 24–48 h at 37 °C. Strain successfully survived on this plate was considered as the functional bacterium with ability to use TBBPA as carbon and energy source, which was selected as the target constructed strain. Meanwhile, one clone carrying an empty pET30a (+) was cultured under the identical conditions as the negative control. Then, PCR and sequencing of the plasmid from the constructed strain was used to verify whether the corresponding genes fragment has been successfully inserted into the expression vector. Finally, the encoding gene expression procedure was carried out according to the standard method (Michael. and Joseph., 2012). Detailed steps were provided in SI.

2.5. Growth pattern and TBBPA-degrading activity of the constructed strain

As a newly constructed strain, for further application in TBBPA degradation, its growth pattern, the debromination and mineralization activity were also determined (details in SI).

2.6. RNA extraction, genomic DNA removal and reverse transcription

Total RNA was extracted from liquid samples at each sampling time (0, 24, 48, 72 and 96 h) using Trizol reagent (Invitrogen) according to the manufacturer's instruction. Its concentration was determined at OD_{260} using a NanoDrop 2000 (Thermo), and the purity and quality were analyzed by measuring OD_{260}/OD_{280} ratios. First-strand cDNA synthesis was performed with 1 μg of total RNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, China) in a total volume of 20 μL as described by the manufacturer. Prior to qRT-PCR, purified RNA and synthesized cDNA were stored at −80 °C.

2.7. Quantitative real-time polymerase chain reaction

The qPCR analysis was performed on the CFX96™ real-time system instrument (Bio-Rad) using the SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, China), in a final volume of 25 μL, containing 2 μL of the above cDNA sample (100 ng), 12.5 μL SYBR® Premix, 8.5 μL of nuclease-free water and 1 μL of each primer (0.4 μM). The specific primers were designed using Primer 3 Plus program (Table 1). Triplicate reactions were performed for each sample using the thermal program provided in SI. Each PCR run included a no-template control (with water instead of cDNA) and a no-RT negative control (with total RNA instead of cDNA). The 16S rRNA was selected as an endogenous housekeeping gene because its average mRNA expression levels remain relatively stable (Johnson et al., 2005; Xiu et al., 2010; Li et al., 2015a; Yu et al., 2015). Therefore, to correct the variations in the amount of RNA applied to real-time PCR, the mRNA expression levels of 16S rRNA were used to normalize that of TBBPA-degrading genes in the samples. Relative mRNA expression was calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Moreover, for a valid reaction, the amplification efficiencies (E) and linear standard curves (R^2) of the target gene as well as the reference (16S rRNA) gene expression levels must be within the range of 100% ± 10% and ≥0.99, respectively (Bustin et al., 2009; Padilla-Crespo et al., 2014). Student's *t*-test was used to determine whether up-regulation or down-regulation of gene expression from different treatments is statistically significant (Li et al., 2015a). When $p < 0.05$, differences were considered statistically significant relative to baseline or control conditions.

2.8. Concurrent degradation of TBBPA

Due to most of the additional electron donor and acceptor involved in electron transfer chain are essential to organic compound metabolism, especially the redox related process (Zu et al., 2014), the effects of electron donors and acceptors on the debromination need to be systematically evaluated. The TBBPA biodegradation by the constructed strain was investigated with addition of approximately 0.1 g carbon sources and electron donors, including ethanol, glucose, NH_4NO_3, sodium acetate and sodium citrate into MM mixture (100 mL) containing 6 mg L^{-1} TBBPA. In addition, the influence of two major TBBPA biodegradation intermediates, TBP and BPA (6 mg L^{-1}), on TBBPA debromination was also investigated using a culture only added substrate TBBPA as the reference. All measurements were progressed independently three times.

2.9. TBBPA concentration, bromide concentration and TOC analysis

The TBBPA concentration, bromide concentration and TOC were also systemically analyzed during the TBBPA biodegradation process by the constructed strain. The detailed methods were provided in SI.

3. Results and discussion

3.1. Draft genome sequencing

The genome information and responding annotation are important assets to better understand the physiology and biodegrading potential of Strain T, and are valuable for future research to enhance the bioremediation of brominated contaminants. The first step to identify gene encoding for TBBPA metabolism was draft genome sequencing. Information obtained from the assembly of the Strain T genome was described in our previous paper (Liang et al., 2016). Briefly speaking, a 3.9 Mb complete genome sequence of Strain T was achieved. Among them, total 2976 proteins could be assigned to the Clusters of Orthologous Groups (COGs) families (Fig. S1). These proteins were annotated to different categories, such as metabolism, genetic information processing and environmental information processing. The proteins associated with amino acid transport and metabolism were the most abundant group of COGs (411 ORFs, accounted for 13.8% of total ORFs), followed by those associated with inorganic ion transport and metabolism (270 ORFs, 9.1%), and those proteins function unknown (323 ORFs, 10.8%). Therefore, the genes possibly responsible for the TBBPA biodegradation were analyzed in the genome of Strain T. Although no bromophenol or brominated compounds dehalogenase encoding genes was found in the genome, there do exist some

Table 1
Primers for quantitative real-time polymerase chain reaction.

Gene	Genbank number	Gene Primer sequence (5′→3′)		Annealing temperature (°C)	Product length (bp)	Efficiency (%)
16S rRNA	HM543185.1	Forward	GAGAGGAAGGTGGGGATGAC	60.86	131	99.6
		Reverse	CGAACTGAGATGGCTTTTGG	60.77		
tbbpaA	KY 483638	Forward	GCTTCAGAAACCGATGCTCT	59.58	118	93
		Reverse	CCTCTTCAAACGCTTTCCAC	59.85		

genes encoding enzymes that could bioremediate chlorinated compounds and phenol. As shown from Table S2, in total, seven ORFs (orf 04638, orf 05726, orf 00062, orf 04028, orf 04434, orf 01638 and orf 06062) encoding haloacid dehalogenases and two ORFs (orf 01379 and orf 00734) encoding pentachlorophenol 4-monooxygenase and monophenol monooxygenase, were found. Furthermore, four ORFs (orf 05787, orf 05349, orf 05348, orf 06285) encoding dichlorophenolindophenol oxidoreductases genes, three ORFs (orf 06401, orf 06400 and orf 05850) encoding phenolic acid decarboxylases genes and other eight ORFs encoding phenol-relevant enzymes such as hydroxylase, oxidase and hydrolase were also obtained.

In summary, these proteins were characterized as dehalogenases, monooxygenases, oxidoreductases, decarboxylases, hydroxylase, oxidase and hydrolase, and were related functionally with the degradation of environmental contaminants, including monobromoacetic acid and monochloroacetic acid (Chiba et al., 2009), phenolic and non-phenolic substrates (Coconi-Linares et al., 2015), phenol and toluene (Heinaru et al., 2016). Due to these xenobiotic biodegradation and metabolism orthology may account for more of the functionally annotated upregulated proteins than any other orthology (Zhou et al., 2015), they were most likely to be involved in TBBPA degradation, and as such were given particular focus in our work. Therefore, these ORFs were selected to identify the putative genes catalyzed the TBBPA biodegradation by the following gene cloning and expression.

3.2. Cloning, functional overexpression and confirmation of TBBPA dehalogenases-related genes

Total 24 ORFs putatively encoding TBBPA-degrading were PCR-amplified with 24 sets of specific primers. Results indicated that specific bands of correct sizes had been obtained (Fig. S2). By ligating target genes into cloning T vector and transforming into *E. coli* TOP10, many white colonies and some blue colonies were observed on the solid LB plate supplemented with Amp, X-gal and IPTG (transformation efficiency>1×10^8 colony-forming unit (cfu) μg^{-1}). The white colonies assumed as the positive constructed strains were further validated with colony PCR. The results revealed that all the positive constructed strains contained inserted fragments with correct sizes, indicating that these 24 TBBPA dehalogenase-related genes were successfully cloned. That is, they could be used to perform the following gene expression experiments.

After digesting the plasmids extracted from constructed strains and vector with *Bam*HI and *Eco*RI or *Xho*I, some bands with the size corresponding to target genes can be observed in the gel (data not shown), meaning that these plasmids have already been successfully digested. Followed by ligating with the expression vector pET30a (+), the restriction fragments were successfully expressed in *E. coli* BL21 (DE3) to generate constructed strain *E. coli* BL21 (DE3) pET30a-a1 to pET30a-a24. Due to the fact that the addition of IPTG can efficiently induce the expression of target genes (Nicolini et al., 2013), the TBBPA degradation function of constructed strain was confirmed through inoculating them into a solid MM plate with addition of IPTG and TBBPA. As Fig. S3 shows, the constructed strain *E. coli* BL21 (DE3) pET30a-a6 (hereafter called constructed strain) was able to grow on the TBBPA-containing plate, implying that this strain possessed the ability to use TBBPA as carbon and energy source. As such, ORF 04638 was regarded as the target gene and designated as *tbbpaA*. Whereas those constructed strains unable to survive on the TBBPA-containing plate were regarded as the strain not carrying the TBBPA-degrading genes. Sequencing the inserted fragment of the plasmid from constructed strain showed that a total of 1851 bp sequence was obtained, which were almost the same size as ORF 04638. Besides, no mutation had occurred during the cloning and expressing process. This confirmation has allowed us to deposit the sequence of TBBPA bromophenol dehalogenase gene at GenBank under accession number KY483638.

Moreover, SDS-PAGE analysis of the extracted total protein was carried out to identify whether the enzyme responsible for the conversion of TBBPA was expressed or not. Results showed that approximately 128.7 kDa band was present in the constructed strain, but absent from the control strain *E. coli* BL21 (DE3) pET30a(+) (hereafter called control strain) (Fig. S4), further suggesting that this protein is encoded by *tbbpaA* gene. However, this value is almost twice as the size of the TBBPA bromophenol dehalogenase (616 amino acids), which has a calculated molecular mass of 64.4 kDa, indicating that the enzyme is a dimer. Previous study also achieved similar size (117 kDa) dehalogenase (BhbA protein) used to catalyze the reductive dehalogenation reaction (Chen et al., 2013). Coincidently, this protein is also approximately twice as large as previously reported respiratory reductive dehalogenase (Bisaillon et al., 2010) and was able to catalyze the dehalogenation under the aerobic conditions (Chen et al., 2013), further implying that dehalogenation can be conducted by oxygen-tolerant reductive dehalogenase (Nijenhuis and Kuntze, 2016).

Sequence alignments in Fig. 1 showed that this dehalogenase shared 100% identity with the haloacid dehalogenases from *Ochrobactrum* sp. (WP 021588323) and *Ochrobactrum* intermedium (WP 025091885). Thus, the above results indicated that the enzyme encoding by gene *tbbpaA* is an oxygen-tolerant dehalogenase responsible for the conversion of TBBPA. This is contradicted with other studies, which reported a two-step process including anaerobic debromination and aerobic mineralization for biodegradation of TBBPA by sequential anaerobic-aerobic strains (Ronen and Abeliovich, 2000; Liu et al., 2013). Whereas in accordance with our previous finding, which revealed that the novel wild strain T has the ability to debrominate and mineralize TBBPA in a one-step process under aerobic conditions (An et al., 2011). This oxygen-tolerant dehalogenase were identified further verified this one step TBBPA decontamination mechanism, suggesting that it may be functioned as both dehydrogenase and debrominase, thus enabling it to simultaneously reductively debrominate and oxidatively mineralize bromide phenols. For future application, the dehalogenase activities of the enzyme from constructed strain need to be further analyzed.

3.3. The growth of the constructed strain, biodegradation and debromination of TBBPA

The growth conditions of the constructed strain are important

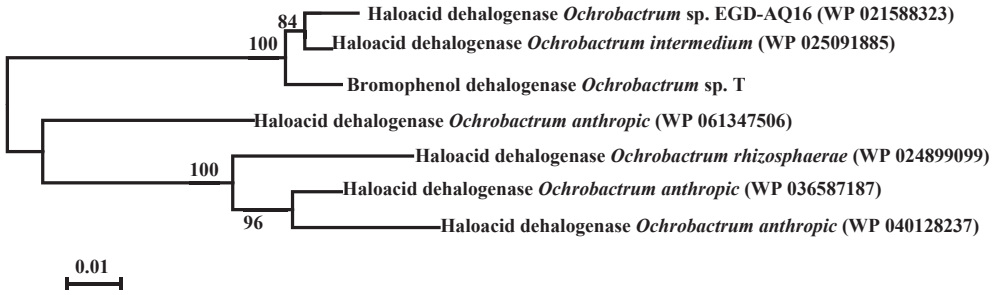

Fig. 1. Phylogenetic tree of bromophenol dehalogenase (ORF 04638) from Strain T and other related dehalogenases based on amino acid sequences by the neighbor-joining method on the program MEGA5.2. The numbers shown next to the nodes indicated the bootstrap values of 1000 in percentage.

parameters to obtain high biodegradation efficiency of TBBPA. Therefore, the dependence of debromination activity of constructed strain and Strain T on the cell growth was further compared. As Fig. S5 shows, all the strains have almost the same growth trend with lag and stationary phases of 4 and 16 h, respectively, and the highest OD_{600} was obtained approximately 1.2 under the optimal conditions of pH 7.0 and 30 °C. Considering the appropriate strain growth could ensure enough cell amount and highest activity for the subsequent substrate degradation experiment (Stasinakis et al., 2010), 14 h of the culture time, which was close to the end stage of the log phase of the strain, was chosen as the enrichment time. Besides, the results of the genetic stability of constructed strain showed that after 10, 20 and 30 passages, no significant difference was found in the biodegradation efficiency for these constructed strains compared with the initial passage (Fig. S6). Further, PCR amplification and sequencing results showed no mutation of the *tbbpaA* gene sequence, indicating that constructed plasmid pET30a-a6 had high heredity stability in *E. coli* BL21 (DE3). All the above results proved that the obtained constructed strain was a potential stable bacterium with TBBPA removal activity.

TBBPA biodegradation profiles by these three bacteria were also compared. As shown in Fig. 2a, different trends of the biodegradation activity existed among them, although these strains behaved similarly during the enrichment process. To be specific, the constructed strain exhibited much higher TBBPA biodegradation activity than the original Strain T. Complete removal of TBBPA was seen within 96 h by constructed strain, while relatively slow removal of TBBPA (89.7%) and no removal of TBBPA were observed by the Strain T and control strain, respectively, under the same conditions.

Furthermore, a positive correlation of TBBPA removal and bromide ion production were also evident. As shown in Fig. 2b, the bromide ion concentration increased in a time dependent manner for different strains. For example, under the aerobic conditions, as reaction proceeded from 24 to 96 h by the strain T, the Br^- concentrations increased from 10.3 to 33.2 μM corresponding to the debromination efficiencies from 23.3% to 75.2%, with the biodegradation efficiencies of TBBPA increased from 57.0% to 89.8%. While for the constructed strain, after 96 h biodegradation of 6 mg L^{-1} TBBPA, 34.4 μM Br^- was produced, which is correspondent to the debromination efficiency of 78.0%. These results suggested that most bromine atoms on the TBBPA molecule could be converted into Br^- by the constructed strain. Comparatively, the constructed strain had an equal but a slightly faster degradation and debromination of TBBPA function than Strain T. Similar results of increase enzyme activity also found in the constructed *Trichoderma reesei* strains (Xiong et al., 2016). One possible explanation is that the specific enzyme (dehalogenase) related to TBBPA bioremediation from the constructed bacteria were extensively upregulated in

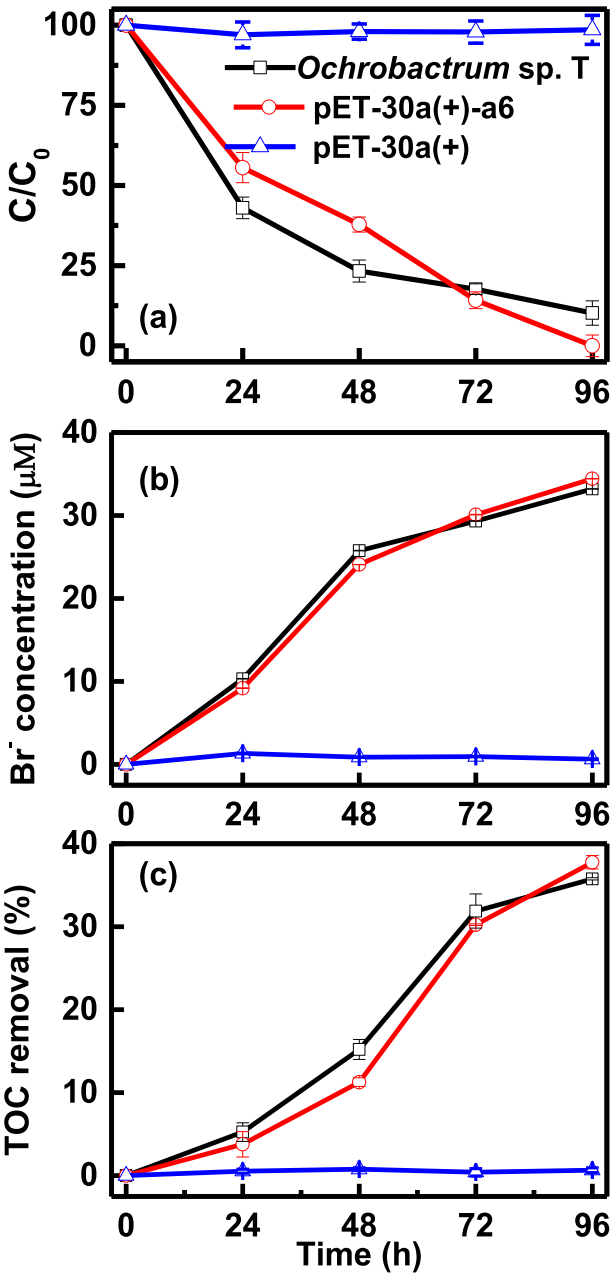

Fig. 2. Comparison of (a) temporal decrease of TBBPA, (b) formation of bromide and (c) loss of total organic carbon (TOC) during the course of biodegradation of TBBPA by constructed strain with other bacteria. Error bars represent standard deviations of triplicate cultures.

response to TBBPA exposure as compared with the Strain T (Zhou et al., 2015). However, considering the previous papers demonstrating that the microbial debromination was functioned by the dehalogenase originated from anaerobic bacteria (Voordeckers et al., 2002; Arbeli et al., 2006), the results that a constructed strain containing a dehalogenase encoding gene can degrade TBBPA under the oxic condition was contradicted with them. Thus, from other aspect, further verified that the dehalogenation can occur under both aerobic and anaerobic conditions (Arora and Bae, 2014; Nijenhuis and Kuntze, 2016).

As revealed by our previous study that the Strain T could simultaneously debrominate and mineralize TBBPA (An et al., 2011), the mineralization of TBBPA was also compared between the constructed strain and Strain T through analyzing TOC removal. As shown in Fig. 2c, TBBPA mineralization efficiencies increased slightly during the first 24 h, followed by increased rapidly during the next 48 h, and then leveled off with further prolonging of the reaction time. In total, up to 37.8% and 35.7% of carbon contents of TBBPA (6 mg L^{-1}) were mineralized by the constructed strain and Strain T, respectively. This means that, similar with the Strain T (Zu et al., 2012), the constructed strain could also simultaneously debrominate and mineralize TBBPA. The reason why the degradation efficiency (100%) well exceeded the mineralization efficiency may be attributed to the formation of some recalcitrant intermediates during the biodegradation processes (Horikoshi et al., 2008). In addition, partial cleavage rather than complete degradation of the TBBPA molecule during TBBPA biodegradation could also result in lower mineralization and higher removal of TBBPA (Guo et al., 2014).

3.4. Cell density and the tbbpaA gene expression after exposure to TBBPA

Analysis of the cell density revealed that the debromination of TBBPA was accompanied by the growth of constructed strain. In cultures with initial TBBPA concentration of 6 mg L^{-1}, the cell densities of constructed strain increased from 1.5×10^6 to 3.3×10^6 cfu mL^{-1} as the incubation time rise from 24 to 72 h (Fig. 3a), and these values were comparable to those of Strain T. However, the cell densities did not increase significantly during the first 48 h incubation time, suggesting that the cell growth was suppressed by the toxicity of TBBPA to some extent (Zhang et al., 2007). Subsequently, a steep increase of the cell density was found for both bacterial strains during 48–72 h after a period of steadily adapting to the TBBPA environments. It should be noted that, during the later stage, the densities of the constructed strain were higher than that of Strain T, which is matching well with the degradation curve of TBBPA (Fig. 2a). Eventually, the cell densities decreased as the TBBPA was gradually consumed. This is an additional evidence for the growth of both bacterial strains by using TBBPA as respiratory electron acceptor and carbon source (Yang et al., 2015).

The transcript levels of the TBBPA-degrading gene in both strains were assayed using qPCR. As shown in Fig. 3b, significant up-regulation of *tbbpaA* gene was observed following the exposure to TBBPA (6 mg L^{-1}) ($p < 0.05$). After 24 h, the *tbbpaA* gene expression relative to the housekeeping gene (16S rRNA) increased 2.2-fold and 1.6-fold for the Strain T and constructed strain, respectively, as compared with the time 0 h. This up-regulation was likely due to the presence of TBBPA in the mineral medium inducing the expression of *tbbpaA* gene. Coincidentally, the trend of *tbbpaA* gene expression was similar with that of the *tceA* and *vcrA* genes in *Dehalococcoides* spp, which were also up-regulated by trichloroethylene exposure for 24 h (Xiu et al., 2010). Besides, this result also fitted well with the up-regulation of the *bvcA* and *vcrA* gene expression upon exposed to vinyl chloride (Baelum et al., 2013).

Compare to the *tbbpaA* gene expression of Strain T (6.6-fold up-regulation), a relative higher degree of up-regulation was observed for constructed strain with maximum 10-fold up-regulation within 72 h. These results were consistent with our abovementioned finding that TBBPA removal efficiency was accelerated by the constructed strain (Fig. 2a). Whereas, the expression of *tbbpaA* decreased to 5.7-fold and 4.2-fold for constructed strain and Strain T, respectively, with further prolonging the biodegradation time to 96 h. One possible explanation for this phenomenon is that, during this period, both bacterial strain began to enter their anaphase of the stationary period or in prophase of the death period (Annweiler et al., 2000). This result can also be confirmed by the decreased densities of both bacterial strains at 96 h.

Overall, the above obtained results revealed that the key step of TBBPA removal by the constructed bacteria is catalyzed by specific enzyme encoded by the *tbbpaA* gene in this work. The enhanced biodegradation of TBBPA by constructed strain is due to the up-regulated expression of the *tbbpaA* gene from 24 h to 72 h.

3.5. The effects of various additives on the aerobic TBBPA biodegradation

Previous study showed that most microorganisms could obtain the redox power needed for the contaminant metabolized through the electron transfer chain (Zu et al., 2014). Therefore, it is worth exploring the effect of various carbon sources and electron donors, such as ethanol, glucose, NH_4NO_3, sodium acetate and sodium citrate on the removal activity of the constructed strain. As shown

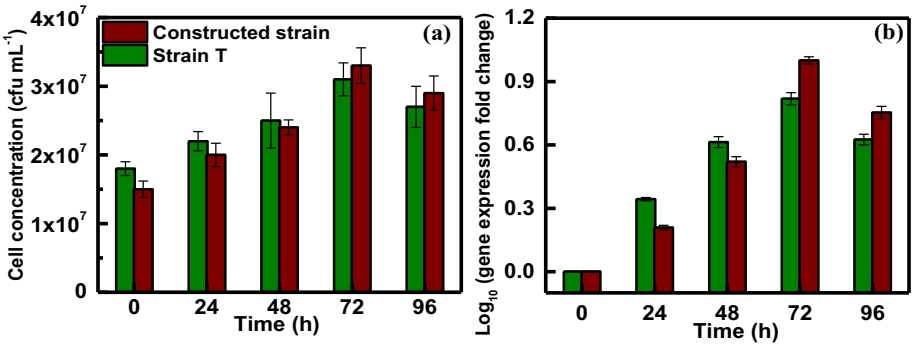

Fig. 3. The growth (a) and relative *tbbpaA* gene expression fold changes (b) of constructed strain and Strain T after the exposure to TBBPA. Fold changes of target genes for each time were normalized to initial conditions (time 0 h). The 16s rRNA was used as the reference gene. All data points represent average values from triplicate samples, and error bars represent one standard deviation.

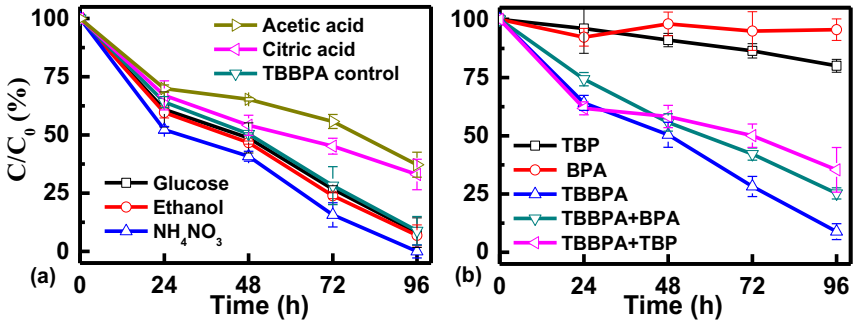

Fig. 4. The addition of different energy sources (a), TBP and BPA (b) on the biodegradation efficiency of TBBPA by constructed strain. Dates are the means of triplicates.

in Fig. 4a, compared with the inoculated control (91.1%), higher aerobic degradation efficiencies of 100%, 93.1% and 91.9% were observed in the system with addition of NH_4NO_3, ethanol and glucose, respectively. These were consistent with a previous work that the addition of ethanol, pyruvate and glucose can enhance biodegradation of TBBPA in sediment by halorespiring bacteria (Arbeli et al., 2006). Therefore, we concluded that they might also be used directly by the TBBPA debromination bacteria as the carbon and energy sources or electron donors. This result also agreed with the report that the supply of $NaNO_3$ and NH_4Cl can effectively improve the degradation efficiencies of TBBPA from 19.1% to 77.6% and 83.9%, respectively (Peng and Jia, 2013). Nevertheless, the increase of degradation efficiencies was not so obvious with the addition of glucose, indicating that no noticeable stimulation of the microbial activity existed in this system. This property of the constructed strain was also compared with the original Strain T (Zu et al., 2014), further confirming that the constructed strain retained some characteristics of the Strain T. In contrast, the use of citric acid and acetic acid will greatly decrease the biodegradation efficiency from 91.1% to 67.0 and 62.8% at 96 h, respectively, probably because these two additives do not function as electron donors by the constructed strain (Chang et al., 2012).

Based on our previous work, two main debromination intermediates, TBP and BPA were produced during degradation of TBBPA by Strain T (An et al., 2011). Therefore, the influence of these two compounds on the biodegradation efficiency was also investigated when TBBPA was degraded by the constructed strain. As shown in Fig. 4b, when BPA or TBP alone was subjected to the biodegradation systems of TBBPA by the constructed strain, BPA could not be eliminated, and only 20.0% TBP was removed after 96 h reaction, suggesting that TBP can slowly be used by the constructed strain. These results were different from those of Strain T probably due to that the degradation of these organics by these two strains was under the regulation of different enzymes (Zu et al., 2014). Furthermore, in TBBPA + BPA system, the addition of BPA could obviously inhibit the TBBPA degradation by constructed strain from degrading TBBPA (74.7% efficiency after 96 h). The inhibition effect is due to the estrogen-like effect of BPA, a well-known estrogen disrupter, on the constructed strain (Olsen et al., 2003; Uhnakova et al., 2011). In addition, a gradual decrease trend of biodegradation efficiency in the TBBPA + BPA system is different from the results obtained in original Strain T system, which decreased rapidly within first 36 h, then exhibited an increase trend and finally declined at the stage of 36–60 h (Zu et al., 2014). This may explain that no BPA could be produced during the biodegradation of TBBPA by constructed strain. Different from the BPA + TBBPA system, the evolution curve in the TBBPA + TBP system by the constructed strain can be described in two stages. First stage, within 24 h inoculation, faster degradation efficiency of TBBPA was observed in TBBPA + TBP system than that in TBBPA alone system; Second stage, after that, with the increase of the reaction time from 24 to 96 h, the degradation efficiency is reversed due to the toxicity of the increase concentration of TBP to the TBBPA biodegradation strain (Arbeli et al., 2006). As a result, we conclude from the above different effects of various additives on the aerobic TBBPA biodegradation between constructed strain and Strain T that, except for the enzyme encoding by the *tbbpaA* gene, there also existed other enzymes in the strain T associated with the fully degradation of TBBPA.

4. Conclusions

In sum, a novel TBBPA-degrading gene (*tbbpaA*) was cloned from the genomic DNA of Strain T after draft genomic sequencing. Subsequently, this gene was successfully expressed in the *E. coli* BL21 (DE3) to generate constructed strain, which exhibited faster debromination and mineralization activity as well as more robust growth than Strain T with TBBPA as the sole carbon and energy source. NH_4NO_3, ethanol and glucose addition promoted the biodegradation of TBBPA, while the addition of citric acid and acetic acid inhibited the biodegradation by this constructed strain. Much higher transcription expression level of *tbbpaA* suggested that the up-regulated expression of *tbbpaA* would enhance the TBBPA biodegradation. These results provide sound basis knowledge for future studies aimed to better understand the molecular principles of TBBPA biodegradation. Meanwhile, it also opens up possibilities for the potential application of bioremediation of TBBPA in situ environments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2018.11.004.

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