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Microbial degradation of 4-monobrominated diphenyl ether with anaerobic sludge

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ABSTRACT

Polybrominated diphenyl ethers (PBDEs) are widely used flame retardant additives for many plastic and electronic products. Owing to their ubiquitous distribution in the environment, multiple toxicity to humans, and increasing accumulation in the environment, the fate of PBDEs is of serious concern for public safety. In this study, the degradation of 4-monobrominated diphenyl ether (BDE-3) in anaerobic sludge and the effect of carbon source addition were investigated. BDE-3 can be degraded by two different anaerobic sludge samples. The by-products, diphenyl ether (DE) and bromide ions, were monitored, indicating the reaction of debromination within these anaerobic samples. Co-metabolism with glucose facilitated BDE-3 biodegradation in terms of kinetics and efficiency in the Jhongsing sludge. Through the pattern of amplified 16S rRNA gene fragments in denatured gradient gel electrophoresis (DGGE), the composition of the microbial community was analyzed. Most of the predominant microbes were novel species. The fragments enriched in BDE-3-degrading anaerobic sludge samples are presumably *Clostridium* sp. This enrichment coincides with the H_2 gas generation and the facilitation of debromination during the degradation process. Findings of this study provide better understanding of the biodegradation of brominated DEs and can facilitate the prediction of the fate of PBDEs in the environment.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) have been applied in high concentrations in many industrial and daily products as flame retardants over the past three decades. In order to achieve the fire prevention capacity needed, up to 32% (by weight) of plastic products may be PBDEs [1] and the average concentrations for decabromodiphenyl ethers (deca-BDE) and octa-BDE are about 500 mg/kg in the electronic and electrical equipment waste [2]. PBDEs are not chemically bound to products in which they are used, making them more likely to leak out to the air, water and soil during their manufacture, usage or discard of consumer products. The appearance of PBDEs is ubiquitous and can be detected in dusts, water bodies, sediments and biotas [3]. For example, the total PBDEs concentration in sediment collected from downstream of a wastewater treatment plant of a plastics manufacturer ranged from 300 to over 3000 μg/g [4]. The concentration of deca-BDE in suspended particulate matter near the textile industries in the Eastern Netherland was up to 4600 μg/kg (dry weight basis) [5]. The concentrations of two kind of penta-BDE were about 14 and 76 μg/kg (dry weight basis) in the soil near a polyurethane foam production facility [6]. Since PBDEs are lipophilic with low solubility in water and accumulate easily in the food chain, the concentrations of these compounds in animals are relatively high [7–10]. PBDEs disrupt the balance of thyroid hormone, lead to developmental neurotoxicity, hepatic toxicity, immunotoxicity and reproductive toxicity in humans and mammals [11–14]. The United States Environmental Protection Agency indicates 0.007 mg/kg-day oral reference dose for deca-BDE and the United States Agency for Toxic Substances and Disease Registry also shows a minimal risk level of 10 mg/kg-day for intermediate (14–364 days) oral exposure to deca-BDE [15,16]. Although the usage of most congeners was regulated, the concentration of PBDEs in environmental samples still increased exponentially due to the long-time utilization. Therefore, PBDEs constitute a major concern for human health and the main targets in environmental remediation [17–22].

PBDEs can be degraded by zero-valent metals [23,24] or light [25–31]. Microscale or nanoscale zero-valent metals can chemically reduce PBDEs to less brominated compounds and adsorb PBDEs [23,32]. Although successful photodegradation of PBDEs has been proposed [25,28–30], the feasibility of solar irradiation can be a limitation. It has been reported that sewage sludge, the cosolvent-enhanced biomimetic system, and sediment microcosms all exhibit PBDE degradation abilities [4,33–39]. Biodegradation

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is cheaper than other physical or chemical treatments. Besides, knowing the biodegradation process more can help us to predict the fate of PBDEs in the environment. Several mixed microbes or pure bacteria strains like *Sulfurospirillum multivorans*, *Dehalococcoides* sp. have been reported bearing PBDEs degradation abilities [40–46]. Higher brominated DEs are uneasy to be degraded through anaerobic reductive dehalogenation and a long reaction time is needed [47–49]. After debromination by photolysis, anaerobic microbes and zerovalent metals, less brominated DE residuals form. Although lower brominated BDEs could be decomposed via oxidation reaction [42,44,45], the number of degraders is limited and the degradation efficiencies are various.

In this study, 4-monobrominated diphenyl ether (BDE-3) was chosen to investigate the degradation efficiency with anaerobic sludge and the effects of carbon source supplement. The predominant microbes were identified to reveal the microbial activities under different experimental settings. Our results contribute to illustrate possible players and influences in the BDE-3 degradation process under specific conditions, and also aid in the development of wastewater treatment.

2. Materials and methods

2.1. Chemicals

BDE-3 (molecular weight (MW) = 249.1, purity >99%) was purchased from Sigma–Aldrich (Milwaukee, WI, USA). Diphenyl ether (DE, MW = 170.2, 99% purity) was obtained from Acros Organics (Geel, Belgium). Hexachlorobenzene (MW = 284.8) purchased from Sigma–Aldrich was used as an internal standard. *n*-Hexane of ultra resi-analyzed grade was purchased from JT Baker Chemical Co. (Phillipsburg, NJ, USA). Water used in this study was double-distilled and then de-ionized with a Milli-Q water purification system (Millipore).

2.2. Culture medium and domestication

The medium for anaerobic culturing was prepared according to a previous report [50] and contained 47 mg L^{-1} K_2HPO_4, 27 mg L^{-1} $NaH_2PO_4{\cdot}H_2O$, 16 mg L^{-1} $NH_4H_2PO_4$, 1000 mg L^{-1} $NaHCO_3$, 3 mg L^{-1} $MgSO_4{\cdot}7H_2O$, 6 mg L^{-1} Na_2EDTA, 1 mg L^{-1} $CaCl_2{\cdot}2H_2O$, 0.2 mg L^{-1} Na_2MoO_4, 0.4 mg L^{-1} $CoCl_2{\cdot}6H_2O$, 0.066 mg L^{-1} Na_2SeO_3, 0.1 mg L^{-1} $NiSO_4{\cdot}6H_2O$, 1.4 mg L^{-1} $ZnSO_4$, 0.2 mg L^{-1} $CuCl_2{\cdot}2H_2O$, 0.85 mg L^{-1} $MnCl_2{\cdot}4H_2O$, 0.6 mg L^{-1} H_3BO_3 and 1 mg L^{-1} resazurin. It was neutralized to about 7.5 with NaOH and was prepared anaerobically under a N_2/CO_2 (80:20, v/v) atmosphere, sealed and then sterilized by autoclaving at 121 °C for 30 min. The residual amounts of BDE-3 at different time points were measured. Aliquots of the sludge were autoclaved twice to kill all microbes and used as blank controls. Further degradation reactions were then performed.

2.3. Biodegradation of BDE-3 by anaerobic sludge

The anaerobic sludge samples were obtained from the anaerobic tanks in Jhongsing and Li-Ming wastewater treatment facilities in Taiwan. The same N_2/CO_2 atmosphere was supplied during the debromination reaction in order to maintain the solution pH value [51]. Batch experiments were conducted to investigate the reactivity of sludge for debromination of BDE-3. The maximum solubility (5 mg L^{-1}) of BDE-3 was added into approximately 20 mL of anaerobic sludge in 120-mL serum bottles [46,52]. The bottles were cotton-plugged and wrapped in aluminum foil. Cultures were incubated for 16 days at 27 °C on a rotary shaker 120 rpm. For experiments with extra carbon source, 5 g L^{-1} glucose was added according to the relative experiments [53,54]. Aliquots of the sludge were autoclaved twice to kill all microbes and used as negative controls. All operations were performed at an anaerobic modular atmosphere-controlled system.

2.4. Chemical analysis

Every two days (except the final point, day 16), BDE-3 and DE were extracted three times with *n*-hexane (2:1, v/v). Each extraction involved a 20-min vibration by the ultrasonic disruptor (Misonix Sonicator 3000, 30 W). The concentration of BDE-3 in samples was quantified with a gas chromatograph (GC; Agilent 6890) equipped with a micro-electron capture detector (μECD), a programmable pressure on-column injection port, and a DB5-HT capillary column (length = 15 m, i.d. = 0.25 mm, film thickness = 0.1 μm). The carrier gas was nitrogen gas of chromatographic grade. The injection temperature was 250 °C, the initial oven temperature was 150 °C, maintained for 0.5 min, then programmed to 330 °C at 25 °C min^{-1}, and held for 5 min. The detector temperature was 340 °C. The molecular mass of DE produced during degradation was determined with a GC–MS (Agilent 5975 inert MSD) by using the identical column.

2.5. Bacterial community analysis

DNA was extracted from 1 g of the sludge using the Soil Genomic DNA Purification Kit (GeneMark, Tainan, Taiwan) according to the manufacturer's instructions. For 16S rRNA gene analysis, genomic DNA was amplified using primer Univ1392R (5′-ACGGGCGGTG-TGTAC-3′) and Eub968F (5′-AACGCGAAGAACCTTAC-3′) with GC-clamp (5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGC-GAAGAACCTAC-3′). PCR was performed by Minicycler PTC-150 (Bio-Rad, Hercules, CA, USA) in a 50-μL mixture, which contained 1 U Taq polymerase, 20 pmol of each primer, and 10 nmol of dNTP. The annealing temperature was 52 °C, and the reaction was carried out for 30 cycles. Triplicate PCR products were pooled, followed by DGGE analysis.

DGGE analysis was performed by the DCode Universal Mutation Detection System (Bio-Rad), with 6% acrylamide gel and 40–60% denaturant gradient. 50 ng of amplified DNA fragment was loaded and separated under 100 V for 16 h at 60 °C. DGGE bands were excised from the gel, and DNA was extracted by the Gel Elution kit (GeneMark). The extracted DNA was re-amplified by primers Univ1392R and Eub968F. Sequences of the amplified fragments were identified by Tri-I Biotech, Inc. (Taipei, Taiwan) using the Applied Biosystems Model 3730 sequencer (Foster City, USA). The obtained sequences were manually inspected to search for similar sequences in the database of the BLAST program (National Center for Biotechnology Information). A threshold of 97% similarity was taken to define the species. For sequences that could not be identified as known species (less than 97% similarity to any known taxon), their taxonomic affiliation was further estimated by the web-based Ribosome Database Project (RDP) classifier for rapid and accurate assignment of the sequences into specific bacterial taxonomic groups [55].

3. Results and discussion

3.1. Biodegradation of BDE-3

After incubation by 5 mg L^{-1} BDE-3 for 16 days, BDE-3 can be degraded by the two anaerobic sludge samples. About 77% BDE-3 was degraded by the Li-Ming sludge (Fig. 1) and 31% of BDE-3 was degraded by the Jhongsing sludge (Fig. 2). The degradation rate constants estimated were 0.26 day^{-1} for the Li-Ming sludge and 0.21 day^{-1} for the Jhongsing sludge (Table 1). Such difference

Table 1
BDE-3 degradation efficiencies under different conditions were indicated by rate constants (k), removal efficiency, Br^- generation and H_2 gas generation.

Sample	k (day^{-1})/r^2[a]	Removal efficiency (%)	Br^- (mM)	H_2 (%)
Li-Ming sludge	0.26/0.98	77	0.008	8
Li-Ming sludge with glucose	0.24/0.97	79	0.019	16
Jhongsing sludge	0.21/0.98	31	0.011	6
Jhongsing sludge with glucose	0.28/0.94	74	0.035	8

[a] r^2: the coefficient of determination of the linear regression equation by plotting residual amount of BDE-3 and degradation time for calculating the kinetics.

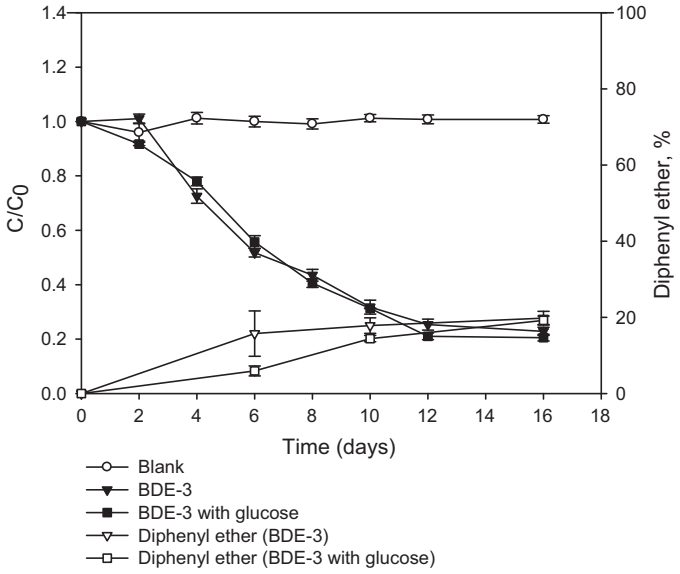

Fig. 1. Biodegradation of BDE-3 by Li-Ming sludge with/without glucose and its generated product with time.

might be due to the distinct characteristics of each sludge sample including the composition of microorganisms and the environment created by these microorganisms.

In order to evaluate the effect of carbon source addition on BDE-3 biodegradation, glucose was chosen for further investigation. After the same incubation period (16 days), the amount of BDE-3 degraded by the Li-Ming sludge did not increase with the supplement of carbon source (79%, Fig. 1). However, the BDE-3 degradation efficiency in the Jhongsing sludge increased to about 74% with additional glucose supplied. The degradation rate constant was 0.24 day^{-1} in the Li-Ming sludge and 0.28 day^{-1} in the Jhongsing sludge. The above results indicated that the Li-Ming sludge already bore high BDE-3 degradation ability, while the biodegradation ability of the Jhongsing sludge could be augmented by additional cometabolic reaction.

Previous studies have reported that the supplement of yeast extract supported and accelerated the biodegradation rate of dibromoneopentyl glycol [56]. The addition of specific carbon source also increased the degradation rate of PCBs: the addition of saccharose or agar improved PCB degradation, whereas the addition of glycerol or pyruvate reduced substantially the degradation efficiency [57].

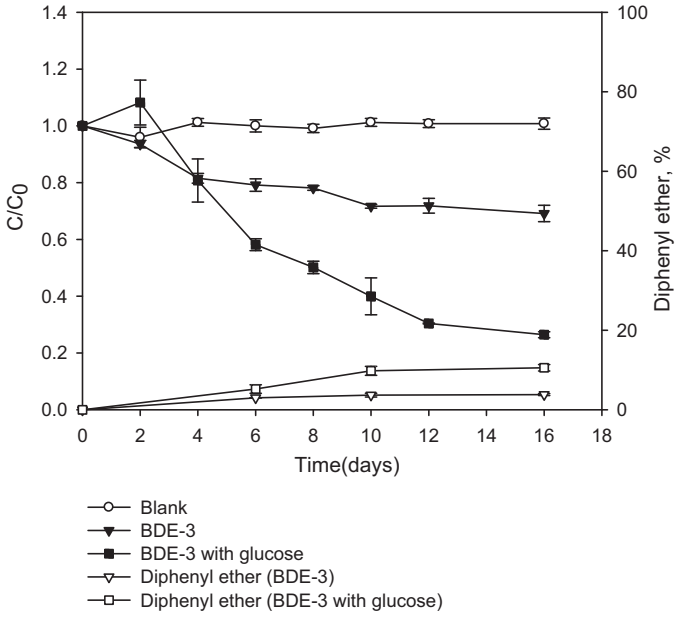

Fig. 2. Biodegradation of BDE-3 by Jhongsing sludge with/without glucose and its generated product with time.

3.2. Anaerobic degradation of BDE-3

After the debromination of BDE-3, bromide ions and DE would be generated. The existence of DE was analyzed according to the M/Z profile obtained by GC/MS. The amounts of bromide ions and DE were measured after degradation reactions. When BDE-3 was degraded by the Li-Ming sludge, there was about 19% DE generated. When glucose was added, the amount of DE increased slightly (20%). The enhancement effect was more obviously seen in the amount of bromide ions generated: the concentration of bromide ions under the supplement of glucose was higher (0.019 mM) than that in the original sludge (0.008 mM) (Table 1). After the same degradation reaction time, there was about 4% DE generated by the Jhongsing sludge. The amount of DE was increased to 11% when glucose was added. The increased concentration of bromide ions, from 0.011 mM in the original sludge to 0.035 mM with glucose added, also supported the augment effect of extra carbon source.

Biodegradation of PBDEs could happen either in aerobic or anaerobic environments. Deca-BDE could be degraded into octa- and nona-BDE congeners under anaerobic conditions [35]. Di-BDE underwent sequential reductive debromination to BDE-3 and DE by anaerobic microcosm in the fixed-film plug-flow biological reactor [38]. Higher brominated DEs are debrominated through reductive dehalogenation and the degradation rate was higher in lower brominated ones [41,47–49]. Our results coincided with the possible degradation mechanism under anaerobic condition. The mass balance was also conserved.

We also measured the amount of H_2 gas generated under the debromination process. The relative amount of H_2 gas was about 8% after BDE-3 degraded by the Li-Ming sludge. When glucose was added, H_2 gas was increased to 16%. In the Jhongsing sludge, the amount of H_2 gas was at first about 6% and became 8% when glucose was added. The increase in H_2 gas with addition of glucose coincided with the previous observation that the hydrogen-producing efficiency of *Clostridium* sp. increased according to the concentration of glucose [58]. Since hydrogen-producing populations would support the reductive dehalogenators [49], the increase in H_2 gas indicated the enrichment of supporters and could explain the augment effect from glucose addition.

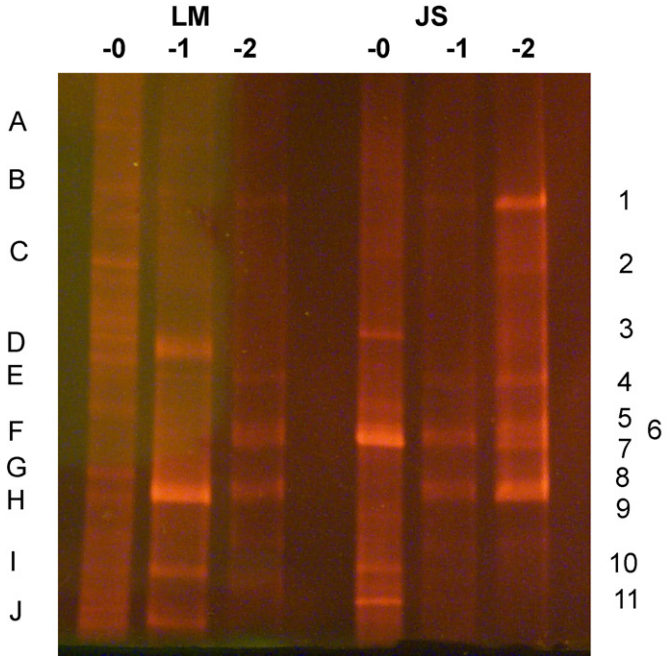

Fig. 3. DGGE profile of 16S rDNA genes from two sludges before and after the BDE-3 degradation. LM, Li Ming community; JS, Jhongsing village; −0, original anaerobic sludge; −1, after BDE-3 degradation reaction; −2, after BDE-3 degradation reaction with the supplement of glucose.

With the transfer of excess electrons, PBDEs could be reduced by zero-valent iron (ZVI). 90% deca-BDE could be removed by nanoscale ZVI within 40 min, while it takes 40 days with microscale ZVI to remove similar amount of deca-BDE [23,32]. Similar removal efficiency could happen when deca-BDE is degraded by solar light within 60 min [30]. The abiotic degradation is generally faster than biodegradation. The degradation abilities of the polychlorinated biphenyl degrading bacteria *Rhodococcus jostii* RHA1 and *Burkholderia xenovorans* LB400 toward mono-BDE are almost 100% within 3 days [42], which is faster and larger than ours. However, Kim et al. has reported that 23% of mono-BDE or 14% di-BDE could be degraded within 8 days by *Sphingomonas* sp. PH-07 [44]. *Rhodococcus* sp. RR1 and the ether degrading bacteria *Pseudonocardia dioxanivorans* LB400 only can transform 20% mono- and di-BDE within 3 days [42]. Their efficiency of degradation is less than ours.

3.3. Fluctuation in microbial community

The composition of microbial community in sludge samples under different degradation conditions were analyzed by DGGE (Fig. 3). In the original Li-Ming sludge, there were multiple predominant microbes (marked as bands A, B, C, G and H in lane LM-0 in Fig. 3). After 16 days of BDE-3 degradation, the predominant bacteria type marked as band B remained the same while band H became more enriched. Other previously predominant bacteria types disappeared but three new bacteria types evolved (marked as bands D, I and J in lane LM-1 in Fig. 3). With the addition of 5 g L^{-1} of glucose, the microbial community was further changed and shifted to different types. The predominant bacteria types marked as band B and H were still the same as in the original sludge. Other predominant bacteria types presented in the BDE-3 environment disappeared. In addition, there were two new bacteria types showed up (marked as bands E and F in lane LM-2 in Fig. 3). The microbial community compositions in the Jhongsing sludge during BDE-3 degradation processes also fluctuated. There were fewer major types of bacteria in the original Jhongsing sludge than in the original Li-Ming sludge (marked as bands 3, 6, 10 and 11 in lane JS-0 in Fig. 3). After 16 days of BDE-3 degradation, most of the predominant microbial types disappeared; only band 6 still existed. Some new types evolved (marked as bands 1, 4, 8 and 9 in lane JS-2 in Fig. 3). Similarly, when the sludge was co-incubated with glucose to degrade BDE-3 for the same period, the composition of the microbial community was partially altered. Some microbial types remained the same as in the non-glucose condition (marked as bands 1, 4, 6, 8 and 9 in lane JS-2 in Fig. 3) and some were newly evolved (marked as bands 2, 5 and 7 in lane JS-3 in Fig. 3).

Table 2
Identification of bacteria strains in Li-Ming anaerobic sludges.

Samples	Strains	Similarity	Accession
Band A	*Salmonella chingola*	97%	U92192.1
Band B	*Sulfurovum lithotrophicum* strain 42BKT	94%	NR_024802.1
Band C	*Methylobacter marinus* strain A45	97%	NR_025132.1
Band D	*Clostridium acetobutylicum* NCIMB 8653	77%	U17099.1
Band E	*Clostridium diolis*	98%	AB610548.1
Band F	*Escherichia coli* DH1	87%	AP012030.1
Band G	*Sulfurovum lithotrophicum* strain 42BKT	95%	NR_024802.1
Band H	*Geobacillus caldoxylosilyticus* strain S35	76%	FJ823099.2
Band I	*Parabacteroides gordonii*	76%	AB470345.1
Band J	*Bacteroides thetaiotaomicron* strain UTM FZZ25	94%	HQ235061.1

According to the sequence of each amplified PCR fragment, the exact species of each predominant microbial type in the sludge has been identified (Tables 2 and 3). In the original Li-Ming sludge, band A was 97% identical to *Salmonella chingola* (accession number U92192.1). Band C was 97% identical to *Methylobacter marinus* (strain A45, accession number NR_025132.1). Band G was 95% identical to *Sulfurovum lithotrophicum* (strain 42BKT, accession number NR_024802.1). They no longer existed when BDE-3 were co-incubated; toxicity might be the reason. Band B was 94% identical to *S. lithotrophicum* strain (strain 42BKT, accession number NR_024802.1). It could be present in all the experimental conditions with similar enrichment level; therefore, it should not be the same type as band G. Band H was enriched in the original sludge but became more enriched after BDE-3 degradation and showed relative low identity with *Geobacillus caldoxylosilyticus* (76%, strain S35s, accession number FJ823099.2). Band D was enriched after BDE-3 degradation and showed 77% identity with *Clostridium acetobutylicum* (strain NCIMB 8653, accession number U17099.1), a known hydrogen generation bacteria [58]. According to the 16S rRNA gene sequences analysis, the genus *Clostridium* is extremely heterogenous, with 80–99% interrelationship within the same cluster and less than 74% between different clusters [59]. Band I also showed low identity with *Parabacteroides gordonii* (76%, accession number AB470345.1) while Band J showed 94% identity with *Bacteroides thetaiotaomicron* (strain UTM FZZ25, accession number HQ235061.1). Bands E and F were enriched after glucose addition

Table 3
Identification of bacteria strains in Jhongsing anaerobic sludges.

Samples	Strains	Similarity	Accession
Band 4	*Clostridium tyrobutyricum* 5S	88%	L08062.1
Band 5	*Bacteroides ovatus* strain JCM 5824	94%	NR_040865.1
Band 6	*Bacteroides ovatus* strain JCM 5824	93%	NR_040865.1
Band 7	*Bacteroides ovatus* strain JCM 5824	94%	NR_040865.1
Band 8	*Bacteroides ovatus* strain JCM 5824	94%	NR_040865.1
Band 9	*Bacteroides ovatus* strain JCM 5824	94%	NR_040865.1
Band 10	*Bacteroides ovatus* strain JCM 5824	93%	NR_040865.1
Band 11	*Bacteroides eggerthii strain JCM12986*	96%	EU136695.1

and showed identity with *Clostridium diolis* (98%, accession number AB610548.1) and *Escherichia coli* (87%, strain DH1, accession number AP012030.1). *C. diolis* was also a known hydrogen generation bacterium [60].

In the original Jhongsing sludge, bands 1 and 3 had low similarity with known bacterial 16S rRNA genes, indicating that these sequences may represent as-yet-identified novel bacteria. Bands 10 and 11 were identical to *Bacteroides ovatus* (93%, strain JCM 5824, accession NR_040865.1) and *B. eggerthii* (96%, strain JCM12986, accession EU136695.1), respectively. Band 6 was 93% identical to *B. ovatus* (strain JCM 5824, accession number NR_040865.1) and could be present in all the experimental conditions with little change in relative amount. Several bands were immerged after BDE-3 degradation. Band 4 was 88% identical to *Clostridium tyrobutyricum* (strain 5S, accession number L08062.1), which was a known hydrogen generation bacterium [61]. Bands 8 and 9 show 94% identity with *B. ovatus* (strain JCM 5824, accession number NR_040865.1). When glucose was added, these bands all existed in BDE-3 degradation. Bands 5 and 7 were also identical to *B. ovatus* (strain JCM 5824, accession number NR_040865.1) with 94% identity. Band 2 showed low similarity with any known bacteria sequence, indicating a novel bacterial lineage. Under all the experimental conditions, about 64% (7/11) predominant bands showed more than 93% identity with the genus *Bacteroides*. These types were presented in the original sludge and could survive under the BDE-3 degradation condition.

None of these above microorganisms has been reported as PBDE degraders. The emergence of bacteria type capable of hydrogen generation after BDE-3 degradation in the two sludge samples supported our observation of hydrogen generation (Table 1). The *Clostridium* sp. type was altered when glucose was added to the Li-Ming sludge. Such change might lead to great enhancement in H_2 gas generation efficiency. However, both BDE-3 degradation and bromide ion generation were kept at the maximum rates. In contrast, the *Clostridium* sp. type remained when glucose was added in the Jhongsing sludge. However, the increase in H_2 gas indicated more numbered hydrogen-generating microbes or higher H_2 gas generation efficiency. This might contribute to enhance BDE-3 degradation in such environment because H_2 molecules can facilitate the dehalogenation reaction [49].

4. Conclusion

We have demonstrated that effective BDE-3 biodegradation can be achieved in anaerobic sludge through co-metabolism with glucose. According to the measured BDE-3 removal as well as generation of DE, bromide ions and hydrogen gas, the degradation efficiencies were evaluated. The reductive degradation of BDE-3 reached a high rate in the Li-Ming anaerobic sludge but the enhancement due to glucose addition was limited. In the Jhongsing sludge, the degradation ability was only half of that in the Li-Ming sludge. However, with the supplement of glucose, the degradation ability elevated to the level similar to that in the Li-Ming sludge. One responsible bacterial species is *Clostridium* sp., which can survive under the BDE-3 degradation condition and supported the dehalogenation through the generation of H_2 gas. *Clostridium* sp. even became more enriched after glucose addition and augmented the debromination reaction. From this study, the supplement of glucose in anaerobic sludge enhances the BDE-3 biodegradation capability and offers a potential way for future environmental pollution processing.

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