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Original Research

Aerobic Degradation of 4-Monobrominated Diphenyl Ether: Identifying and Characterizing Three Strains Isolated from *Populus tomentosa* Phyllosphere

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Abstract

Three strains, designated A1, B2, and C3, capable of aerobic degradation of 4-monobrominated diphenyl ether (4-BDE) as sole carbon and energy source were originally isolated from *Populus tomentosa* phyllosphere. The three strains were identified as *Sphingomonas*, *Enterobacteria,* and *Curtobacterium* based on their morphology and molecular properties. The 4-BDE concentration gradient was set up and showed that the three strains could still survive at 500 mg L^{-1} 4-BDE. Their removal efficiencies were detected by utilizing GC-μECD and the analysis showed that A1, B2, and C3 were able to transform 33%, 20%, and 17% of 80 mg L^{-1} 4-BDE in 2d incubation, respectively. This is the first report of aerobic degradation of 4-BDE by three bacterial strains isolated from phyllosphere.

Keywords: 4-BDE, *Populus tomentosa*, phyllosphere, aerobic degradation

Introduction

Polybrominated diphenyl ethers (PBDEs) are a class of synthetic organic compounds consisting of 209 congeners, while the deca-, octa-, and pentabromodiphenyl ethers are most commercially used. As one kind of effective and economical flame retardant, PBDEs have been widely used in various products such as plastics, electronic appliances, furniture, and vehicles [1-2]. There is no chemical bind between PBDEs and materials, so PBDEs easily leak into the environment during the production, use, and disposal of consumer products. Now, however, PBDE congeners have been widely detected in soils, air, water, animals (even in the Arctic biosphere), and humans [3-6]. PBDEs have attracted growing concerns regarding their properties of persistence, bioaccumulation, and biotoxicity [7-9]. Even though the industrial production of some PBDEs is restricted under the Stockholm Convention, some PBDEs are still in production – like decabromodiphenyl ether (BDE209). The lower brominated diphenyl ethers (average 1-6 bromine atoms per molecule), which can be formed from photochemical and biological degradation of high brominated congeners [10-14], may carry more toxicity and bioaccumulation [10, 15, 16]. Among the lower brominated congeners, the

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monobrominated diphenyl ethers have attracted extensive attention [17-21].

Biodegradation plays a vital role in the transformation and removal of detrimental organic chemicals. For biodegradation of PBDEs, microbial anaerobic and aerobic degradation are under study. Microbial anaerobic transformation has demonstrated that, in most cases, PBDEs were debrominated to less brominated congeners, but required a relatively long time [11, 22]. Compared to anaerobic degradation, the advantages of microbial aerobic degradation on PBDEs are obvious: the degradation time cycle is shorter and the metabolites will not carry greater toxicity as the result of the characteristic degradation mechanism that PBDEs go through for hydroxylation and ring opening reaction [23-24].

The phyllosphere, aerial parts of plants dominated by the leaves, was regarded as supporting the most abundant colonizers on earth. Based on the vegetation model, the global leaf surface is as approximately vast as land surface if just one side of the leaf is in calculation [25-26]. The density of microorganisms residing on the phyllosphere (microbial epiphytes) is enormous, averaging 10^6-10^7 bacteria per square cm on leaf surface, and the species are various [27-30]. In recent years, studies about microbial epiphytes removing gaseous chemical compounds including toluene, ethylbenzene, xylene, and phenol have become available [31-34].

In this paper, 4-BDE, a model for biodegradation of lower brominated diphenyl ether, was chosen to investigate the degradation performance of the screened bacterial strains isolated from the leaves of *Populus tomentosa* [22]. Through traditional isolation and 4-BDE screening, three strains were obtained from the leaves of *Populus tomentosa* and identified. The 4-BDE concentration gradient was designed to test strains' 4-BDE tolerance ability. Based on the growth curves established on mineral salt medium containing 80mg L^{-1} 4-BDE, the three strains' 4-BDE removal efficiency was detected by a gas chromatograph (GC) coupled with a micro-electron capture detector (μECD) after 2d incubation. In this paper, we tried to prove the existence of 4-BDE-degrading strains on *Populus tomentosa* phyllosphere and investigated the corresponding removal efficiency that could expand the traditional existence state of PBDE degrading strains as well as contribute to comprehensive phytoremediation.

Materials and Methods

Chemicals and Culture Mediums

4-BDE (4-bromodiphenyl ether, purity>98%) was purchased from Tokyo Kasei Kogyo Co., Ltd. (TCL) and diluted with alcohol to 80 mg mL^{-1} before use. All standards and solutions were stored hermetically in amber glass vials at 4ºC until use. All other chemicals and reagents used in this experiment were of analytical reagent grade or better. Nutrient medium was used for strain isolation and cultivation, and its composition consisted of (g L^{-1}): peptone 10, beef extract 3, agar 18, and NaCl 5.

Liquid nutrient medium (LUM) was used for strain cultivation, and its composition consisted of (g L^{-1}): peptone 10, beef extract 3, NaCl 5.

Mineral salt medium (MSM) was used as the degradation medium, and its composition consisted of (g L^{-1}): Na_2HPO_4 2.13, KH_2PO_4 1.30, NH_4Cl 0.50, and $MgSO_4 \cdot 7H_2O$ 0.20.

The PH of culture medium was all adjusted to 7.3. The culture medium and containers were previously sealed and sterilized in an autoclave at 121ºC for 20 min.

Strain Isolation

Every three healthy leaves were cut from three individuals of *Populus tomentosa*. The target trees grow in the western campus of the Agricultural University of Hebei (38°49′23.84″N, 115°26′40.52″E). The height of desired leaves was about 10 m above ground. The leaves were put in one sterile plastic bag once cut to avoid contrived contamination. In lab, the leaves were put upside down in a sterile 150 mL Erlenmeyer flask containing 80 mL MSM, and the leaf stalks were kept above the liquid level, then placed on a incubator shaker at 30ºC and 150 rpm. Eight hours later, 20 ul bacterium suspension was transferred to nutrient medium in clean bench and incubated the plate at 30ºC. Forty-eight hours later, single bacteria colonies were streaked on clean plates and repeated until they formed pure single colonies. All single colonies in 15% glycerinum were preserved at -60ºC.

Bacterial Activation

To activate bacteria, the strains were cultivated in 100 mL Erlenmeyer flasks that contained 40 mL LUM. The initial culture turbidity of bacterium suspension was modulated to OD_{600} of 0.400 and incubated on a rotary shaker at 30ºC and 150 rpm for 24h. Then the biomass was harvested and separated from the LUM by centrifugation (5,000 rpm, 10 min, 4ºC). The separated biomass was washed with MSM (5,000 rpm, 10 min, 4ºC) three times and resuspended in MSM. In this study, prior to operations including bacterial screening, 4-BDE tolerance test, growth curve establishment, and removal detection, the activated bacterium suspension was prepared and modulated to 0.100 at OD_{600} and used within 2 h. Besides that, the setting was 30ºC and 150 rpm when a rotary shaker was needed and bacterium suspension containers were 100 mL Erlenmeyer flasks, except for section 2.6, which used 60 ml tubes.

Strain Screening

We conducted strain screening to seek strains capable of metabolizing 4-BDE as the sole carbon and energy source. The screening containers contained

40 mL bacterium suspension with 20 mg L^{-1} 4-BDE and was incubated on a rotary shaker. Every 24 h, 0.700 ml bacterium suspension was taken out to measure the value of OD_{600} on a clean bench and put it back on a rotary shaker for subsequent measurement. The strain screening will be sustained for five days. The operation of OD_{600} value measurement was the same below. In this paper, all biological repeats were carried out in triplicate (including strain screening, tolerance test of strains on 4-BDE, growth curves establishment on MSM containing 80 mg L^{-1} 4-BDE and LUM, and 4-BDE removal detection).

Identification of Bacterial Strain

In addition to fundamental morphological and physiological characterizations, the 16S rDNA gene was amplified using the primer pair 27F and 1492R [35]. Sequencing was performed after recycling and purification. 16S rDNA sequences of degrading strains were compared and aligned with sequences deposited in the GenBank database using the BLAST program.

Tolerance Test of Strains on 4-BDE

The pollutant concentration gradient was set as 40, 80, 120, 160, 200, 300, 400, and 500 mg L^{-1}. The 60 ml tube containing 10 ml bacterium suspension served as an incubation container. The parallel controls were set without the addition of 4-BDE. These incubation systems were cultivated on a rotary shaker. The value of OD_{600} was measured at intervals of 24 h.

Growth Curve Establishment on MSM Containing 80 mg L^{-1} 4-BDE and LUM

To investigate the growth dynamic of strains on different nutrition conditions, a growth curve establishment was conducted. In MSM, 4-BDE was added to the 40 ml bacterium suspension with final concentration of 80 mg L^{-1}. To further verify the 4-BDE function as the sole carbon and energy source, the same amount of 4-BDE was added to the incubation systems at 90h. In LUM, the initial OD_{600} value of the bacterial suspension was also adjusted to 0.100. Place all incubation systems on a rotary shaker. OD_{600} value measurement was conducted at intervals of 6h within the desired time.

4-BDE Removal Detection

Every incubation container containing 40 mL bacterium suspension with 80 mg L^{-1} 4-BDE. Place the incubation systems on a rotary shaker. The parallel controls were set without the addition of bacterial biomass. After 48h incubation, 10 mL n-hexane was added to each of the incubation systems and all systems were set on rotary shaker at 20ºC and 150 rpm for 30 min. After shaking, 1 mL supernate was extracted from the suspensions. The removal efficiency was analyzed by gas chromatograph (Agilent Technologies 6890N) coupled with a micro-electron capture detector (Agilent Technologies, USA; GC-μECD). A high-performance capillary column (Agilent J&W DE-5MS UI: 30 m·0.25 mm, 0.25 μm film thickness) was used. For temperature setting, the oven temperature was initiated at 80ºC, ramped up 10ºC/min to 180ºC, 4ºC/min to 250ºC, and then up to 300ºC at 5ºC/min, and held for 3 min. Automatic injection of 1 μL sample was conducted in the splitless mode. Chromatographic-grade helium was used as the carrier gas at a flow rate of 1.1 mL min^{-1}. The injector temperature was fixed at 220ºC and the ECD was maintained at 280ºC.

Statistical Methods

In statistical analysis process, Origin 8.5 was performed to calculate the mean value and standard deviation (SD) for three replicates and form figures. Specifically, the 4-BDE removal efficiency was first calculated the removal efficiency for each replicate and then we calculated the mean value and SD for three replicates by Origin 8.5.

Results and Discussion

The Screening and Identification of the 4-BDE Degrading Strains

After three times sampling we obtained three aerobic strains capable of utilizing 4-BDE as sole carbon and energy source. The strains were designated A1, B2, and C3 in time sequence. Colonies of strain A1 on the nutrient medium showed a white pigmentation without mucus, B2 showed white pigmentation with mucus, and C3 showed pink pigmentation with mucus. For gram staining, strains A1, B2, and C3 were Gram negative, negative, and positive, respectively. The 16S rDNA analysis revealed that strains A1, B2, and C3 exhibit the highest sequence similarity with *Sphingomonas echinoides* strain NRRL B-3127 (KP208156.1, 99% homology), *Enterobacter* sp. SJZ-5 (LC014954.1, 94% homology), and *Curtobacterium* sp. MR MD2014 (KU740254.1, 99% homology) according to blast search using the NCBI Genbank. Through elementary identification, strains A1, B2, and C3 were identified as genus *Sphingomonas* sp., *Enterobacter* sp., and *Curtobacterium* sp. The phylogentic trees of A1, B2, and C3 were generated by MEGA5 and were described in Figs 1(a-c). The phylogentic trees were constructed by the amplified 16S rRNA sequence fragments of A1, B2, and C3, and their neighbors in GenBank via BLAST (the statistical method: neighbor-joining; bootstrap method: 1,000 re-samplings; scale bar: percent similarity; number in parentheses: accession number).

The 4-BDE Tolerance Test

The growth dynamic curves of three strains on 4-BDE gradient concentration were shown in Figs 2(a-c), which show that the biomass of the parallel controls showed

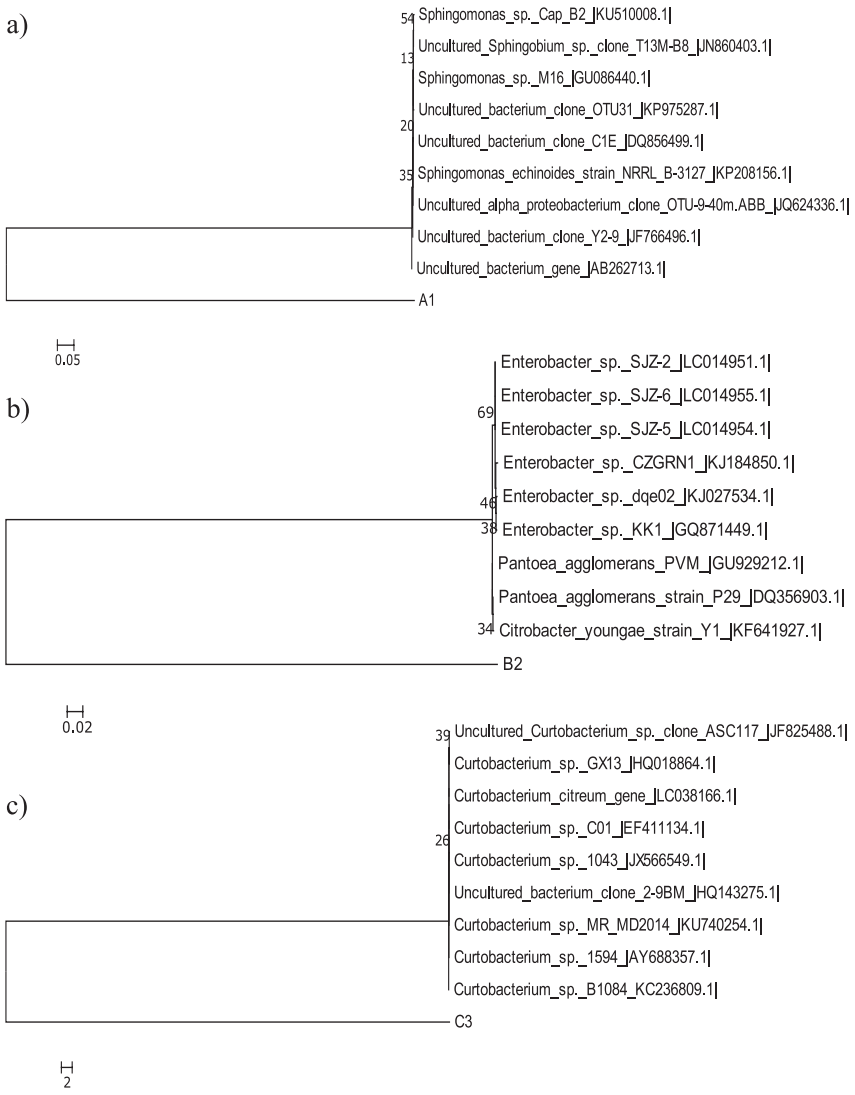

Fig. 1. The phylogentic trees of strains: a) A1, b) B2, c) C3

significant decline within 6d. In Fig. 2a), A1's biomass in high 4-BDE concentration (300, 400, and 500 mg L^{-1}) is lower than low 4-BDE concentration within the first 2d. After 3d, the incubation systems under 200 mg L^{-1} 4-BDE stepped into stationary phase but concentration over 200 mg L^{-1} emerged with a strong upward trend to a different extent. Different from A1, B2 showed no temporal biomass overlap as depicted in Fig. 2b). Earlier than A1, all incubation systems of B2 stepped into stationary or decline phase after 2d. In Fig. 2c), in spite of the 4-BDE concentration difference, all culture turbidity of C3 was in the range of 0.15~0.20 at OD_{600} within the 1d, except for the 40 mg L^{-1} group.

Taking the three strains' growth conditions into account, we found that more biomass yield was produced along with the 4-BDE concentration increase. Among the three strains, A1 showed the most powerful degradation ability. Another difference is that at high 4-BDE concentration, the culture turbidity of B2 and C3 declined after 3d, but A1 still showed a strong upward trend. For the highest culture turbidity at 500 mg L^{-1} 4-BDE, A1, B2, and C3 reached about 0.48 at 6d, 0.414 at 2d, and 0.363 at 3d, respectively (at OD_{600}). Actually, the aqueous solubility of monobrominated diphenyl ethers ranges from 1 to 10 mg L^{-1}, and the positive correlation of biomass yield to 4-BDE concentration may reveal that the 4-BDE unsolvable in the bacterium suspension can also be utilized.

Growth Curve Establishment on LUM and MSM Containing 4-BDE

In Fig. 3a), strain A1 showed the most powerful multiplication capacity in LUM, followed by B2 and C3, which reached the highest culture turbidity at 36 h of 1.048, 24 h of 0.757, and 54 h of 0.629, respectively (at OD_{600}). At 36 h, strains A1 and C3 stepped on stationary phases and strain B2 did at 12 h.

Fig. 3b) exhibited the growth curves of three strains in MSM containing 4-BDE for 120 h. At first, 4-BDE was added to incubation systems at the concentration of 80 mg/L and at 90h. To further verify 4-BDE's role as

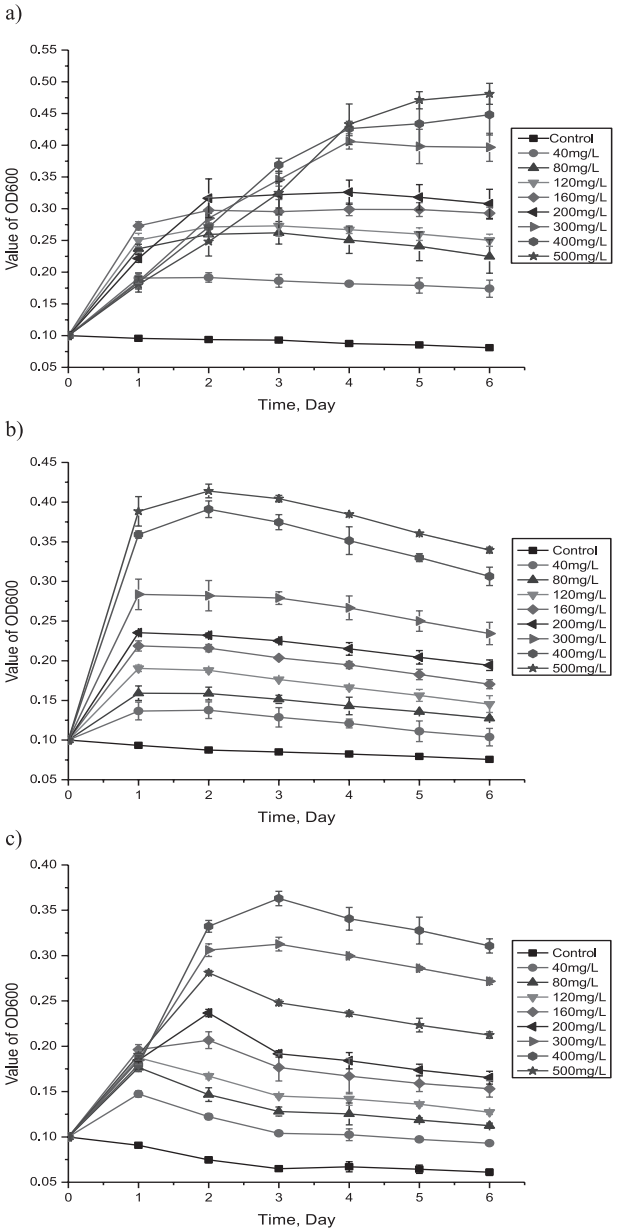

Fig. 2. Growth dynamic curves on 4-BDE gradient concentration strain: a) A1, b) B2, c) C3.

sole carbon and energy source, the same amount of 4-BDE was added to incubation systems again. From Fig. 3b), It can be seen that three strains were in the lag phase in the first 6 h. The growth trend of three strains in the MSM containing 4-BDE was similar in LUM. After the second 4-BDE addition, the culture turbidity of all incubation systems went up significantly upon the addition without lag phase and from this result it can be expected that the 4-BDE dose acts as the sole carbon and energy source. Compared to the first 6h, no appearance of lag phase after the second addition may signify that the strains had been adaptable to the 4-BDE aqueous environment. Besides, two differences can be seen from Fig. 3b): less biomass yield and earlier arrival on stationary phase compared to the first 4-BDE addition.

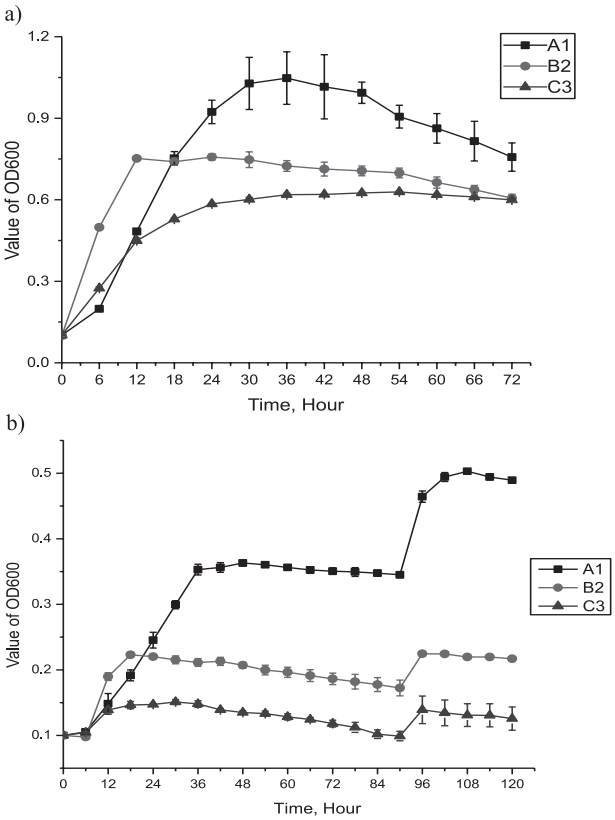

Fig. 3. Growth curves of three strains: a) on liquid nutrition medium (LUM), b) in MSM containing 80 mg L^{-1} 4-BDE and with the same amount of 4-BDE addition at 90 h.

4-BDE Removal Efficiency

After 48h incubation, the 4-BDE removal efficiency was shown in Table 1 and the result showed that 33%, 20%, and 17% of 80 mg L^{-1} 4-BDE was transformed by A1, B2, and C3. Removal efficiency was the positive relationship to the growth curves of three strains on LUM and 80 mg L^{-1} 4-BDE MSM. In parallel control, no significant 4-BDE removal occurred within the same time, implicating that possible volatilization or non-biological degradation was negligible. For 4-BDE biodegradation, the three strains isolated from the phyllosphere have at least three advantages.

First, 4-BDE can be the sole source of carbon and energy for the three strains, which means no growth substrate or co-metabolism substrate was necessary. *Lysinibacillus fusiformis* strain DB-1 can transform deca-BDE to lower brominated BDEs in aerobic conditions under the premise of using lactate, pyruvate, or acetate as carbon sources [36]. *Sphingomonas* sp. PH-07 could transform five selected PBDEs by using diphenyl ether as a carbon source [37]. For further application of microbial degradation on halogenated hydrocarbon pollutants, the carbon source addition would increase the economic burden of remediation and may worsen environment pollution if the carbon source itself is toxic.

Second, the 4-BDE removal efficiency of three strains – 33% for A1, 20% for B2, and 17% for C3 in

Table 1. Removal efficiency of strains A1, B2, and C3 on 80 mg L^{-1} 4-BDE after 48 h.

Duration(h)	Percent removal[a] (%)		
	A1	B2	C3
48	33.65±8.39	20.44±3.02	17.14±0.24

[a]Values shown are mean ± SD for three replicates

2d incubation – was comparable or superior to most 4-BDE anaerobic and aerobic microbial or community removal efficiencies if taking into consideration the initial bacterium suspension optical density of 0.100 and the initial 4-BDE concentration of 80 mg/L. For example, *Rhodococcus* sp. RR1 could aerobically transform 20% of 17 ug L^{-1} mono- and di-BDE within three days [23]. *Sphingomonas* sp. PH-07 could aerobically degrade 23% of mono-BDE or 14% di-BDE within eight days with biphenyl ether addition, and the pollutant concentration was fairly high at 1 g/L [37]. Recently, *Pseudomonas putida*, an aromatics-degrading strain, was used for the aerobic biodegradation of 4-BDE, and the degradation efficiency of 2 mg/L 4-BDE was 42.5% after 10d treatment [38]. Two anaerobic sludge samples, which were obtained from the anaerobic tanks in two wastewater treatment facilities, were employed for 4-BDE degradation and after incubation by 5 mgL^{-1} 4-BDE for 16 days, about 77% of 4-BDE was degraded by Li-Ming sludge and 31% of 4-BDE was degraded by Jhongsing sludge [22].

Third, different from the degrading strains isolated from the pollutant-contaminated soil, sewage treatment plant or places with contamination history [36, 37, 39-40], the three strains were from the green and healthy *Populus tomentosa* phyllosphere, which means strains capable of degrading PBDEs are not exclusive to bacteria surviving in a heavily contaminated environment. For future phytoremediation which contains (but is not limited to) plant bodies and related microbial epiphytes, the original degrading strains from microbial epiphytes have one huge advantage: they have been a part of the bacterial community on the phyllosphere and they have adapted to the leaf surface environment. But for the addition of degrading strains (even possessing better degradation efficiency) which are isolated from other biotic districts to the phyllosphere, the introduced degrading strains may not survive or degrade pollutants on the leaf surface as there maybe not niches for them. And it has been proven that *Sphingomonas* sp., a versatile degrading genus and A1's genus, is widely distributed in some plants' phyllospheres [41-43].

As a class of semi-volatile organic compounds (SVOCs), the fastest way to spread regionally and globally for PBDEs is through the atmosphere [44]. The existing state of bromominated biphenyl ethers in air can be gaseous or adherency to fine particulate matters, and both can migrate for long distances [45-47]. It has been reported that gaseous pollutants are prone to accumulate on the leaf surface, several to dozens of times higher than in the air, which is favorable to be accessible to and degraded by microbial epiphytes [33]. But the degradation experiment conducted in this paper was in the aqueous phase, so the degrading strain was symmetrical, which is different from the bacterial aggregates in phyllosphere aboriginality [48]. Additionally, the 4-BDE concentration performed in this study was definitely higher compared to environmental air [49-50]. So for further research it is necessary to investigate the removal effect of gaseous PBDEs as well as PBDEs absorbed on particulate matter by microbial epiphytes at a larger scale and *in situ*.

Conclusions

Form *Populus tomentosa* phyllosphere, we obtained three bacterial strains (A1, B2, and C3) that are able to utilize 4-bromodiphenyl ether (4-BDE) as the sole carbon and energy source. Based on the elementary morphology and molecular properties, the three strains are identified as *Sphingomonas*, *Enterobacteria*, and *Curtobacterium*, respectively. To test 4-BDE tolerance property, we set up a concentration gradient and the result showed that 500 mg L^{-1} 4-BDE in MSM is not the tolerance limit point for three strains. By utilizing GC-μECD, removal efficiency was analyzed. A1, B2, and C3 were able to transform 33%, 20%, and 17% of 80 mgL^{-1} 4-BDE in 2d incubation. This is the first report of PBDE biodegradation by microbial epiphytes, which will expand the existing field of PBDE degrading strains. We expect that the result of the experiment will promote the PBDE microbial epiphytes degradation in situ and benefit integrated phytoremediation.

Acknowledgements

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