Biodegradation of bisphenol A by algal-bacterial systems

藻類・バクテリア共存系を用いたビスフェノール A の生分解

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SYNOPSIS

ビスフェノール A（BPA）は内分泌かく乱物質の一種であり、主にポリカーボネートの原料として世界中で用いられている。現在、ポリカーボネート工場から排出される BPA 含有排水は、薬剤を用いた処理や曝気を伴う活性汚泥法等により、高いエネルギーとコストをかけて処理されている。近年、低エネルギー・低コスト型の排水処理法として、曝気の代わりに藻類の光合成によって生成された酸素を利用して汚水処理を行う微細藻類・バクテリア共存系システムが注目されている。本研究では、活性汚泥中のバクテリアによる BPA 分解実験（回分）、微細藻類 *Chlorella sorokiniana* および *C. vulgaris* の BPA への耐性実験（回分）、藻類・バクテリア共存系システムによる BPA 分解実験（回分及び半連続）を実施した。基質中の BPA 濃度は回分実験では 0, 10, 20, 50 mg L^{-1} の 4 条件、半連続実験では 50 mg L^{-1} とした。バクテリア単独での BPA 分解実験では全条件において、実験開始から 96～120 時間目に BPA 濃度は検出限界以下まで減少した。藻類単独での BPA 耐性実験では、藻類種に関わらず、全条件において投入 BPA の 50%以上が分解されずに残存したため、藻類単独での効率的な BPA の除去は困難であることが示された。藻類・バクテリア共存系システムによる分解実験（回分）では、両種・全条件において BPA 濃度が検出限界以下まで減少した。また、バクテリア単独より藻類・バクテリア共存系システムのほうが高い BPA 除去速度を示した。藻類・バクテリア共存系システムによる半連続 BPA 分解実験では、両種とも 15 日間の実験期間を通してほぼ 100%の高い BPA 除去率が維持された。以上のことから、藻類・バクテリア共存系システムによる、曝気を伴わない BPA 含有排水の効率的な処理が可能であることが明らかとなった。

Keywords: Algal-bacterial system, bacterial consortium, biodegradation, bisphenol A, biodegradation intermediates, photo-oxygenation

INTRODUCTION

Emerging pollutants in water, such as endocrine-disrupting compounds have generated significant interest because of their adverse effect on wildlife. Bisphenol A (BPA) is toxic to aquatic organism (Alexander at al., 1988), and exhibits oestrogenic activity (Krishnan et al., 1993) that is widely used in the raw materials for polycarbonate production and epoxy resins. Due to its high demand and high supply, the BPA global market is estimated to exceed 9,619-kilo tons by 2020. As such, the amount of wastewater carrying BPA from plastic manufacturing plants will also increase. In many developing countries, domestic and industrial wastewater, as well as landfill leachate that is contaminated with BPA, are being discharged directly into water environments without treatment because they cannot afford the high treatment cost. Nevertheless, the BPA concentration of landfill leachate has been reported to be 5-17 mg L^{-1} (Yamamoto et al., 2001). These concentrations are acutely toxic to the aquatic organism and algae at EC 50 of 1 mg L^{-1} (Staples et al., 1998). Therefore, low-energy biological treatment is required to treat BPA-containing wastewater before discharging it into the environment.

Until now, BPA biodegradation studies mainly focused on a single-strain of bacteria, but rarely on bacterial consortium (Roh et al., 2009). However, employing a single-strain of bacteria in the wastewater treatment process is not practical because of the difficulty in maintaining a pure culture and amplifying the pure strain to augment a practical wastewater process. The bacterial consortia in wastewater treatment plants are comprised of a broad spectrum of bacterial species treating various types of wastewater. Therefore, they may have a good resistance to substrate inhibition (Marrot et al., 2006). Moreover, they are more efficient in the utilization of organic compounds and potentially toxic biodegradation intermediates (Hanson et al., 1999). A variety of bacteria with different degradation pathways are expected to degrade BPA to its intermediates that are then utilized by other bacteria. However, bacterial BPA degradation produced some oestrogenic biodegradation intermediates that may cause secondary pollution. For these reasons, BPA biodegradation intermediates produced by bacterial consortium should be evaluated.

Wastewater contaminated with BPA can be primarily treated by bacteria under aerobic conditions, but not under anaerobic conditions (Mohapatra et al., 2011). However, the aerobic process is costly due to the energy required for the aeration and stirring. In reality, the aeration system consumes approximately 50 to 65% of the net power demand of a typical wastewater treatment plant. Therefore, an alternative low-cost aerobic system is required. An algal-bacterial system represents one type of low-cost biological treatment system. In this system, algae supply the oxygen necessary for the heterotrophic bacteria to degrade organic pollutants, and then produce the carbon dioxide needed by the algae for photosynthesis. In this context, the cost of external aeration required for the conventional aerobic system could greatly be reduced. Recently, the application of algal-bacterial systems have been expanded to treat industrial wastewater and the removal of nutrients from livestock effluent (Muñoz et al., 2004; Muñoz and Guieysse 2006). Despite the significant development of research into nutrient removal, there is a lack of knowledge regarding the application of the algal-bacterial system for a more toxic pollutant biodegradation. In fact, algae are usually limited by their low tolerance of toxic pollutants (Muñoz et al. 2003).

Chlorella is known to be one of the fast-growing microalgae genera. Of the *Chlorella* species, *Chlorella sorokiniana* is a fast growing strain with a good photosynthetic productivity (Li et al. 2013). Moreover, *C. vulgaris* has often been used to remove

nitrogen, phosphorus, and heavy metals from tertiary wastewater (Gonzalez et al., 1997). However, these algae together with bacteria have not been used to degrade BPA. Hence, the feasibility and implications of low-energy photosynthetic algal-bacterial systems are unclear. This study provides an insight into the relationship between algae and bacteria in an enclosed system during BPA biodegradation.

The present study examined the feasibility of algal-bacterial systems to degrade toxic and oestrogenic compound of BPA. The specific objectives of the study were (1) to investigate the BPA degradation capability of a bacterial consortium in the light and dark conditions, and (2) to investigate the BPA degradation capability of an algal-bacterial system, and to evaluate the feasibility of an algal-bacterial system for biodegrading BPA in a semi-continuous BPA loading.

MATERIALS AND METHODS

In Study 1, bacterial BPA degradation assay was conducted under the light and dark conditions. In Study 2, algal BPA inhibition of *C. sorokiniana* and *C. vulgaris* was tested. The algal-bacterial BPA degradation assay for *C. sorokiniana* + Bacteria and *C. vulgaris* + Bacteria were also evaluated. In order to determine the feasibility of using the algal-bacterial system, semi-continuous BPA loading experiments were conducted.

All of the experiments were performed in triplicate and incubated at 25 ±1 ℃ in a 12-h light/12-h dark cycle with a light intensity of 300 μmol photons $m^{-2}s^{-1}$ for 168 hours (unless otherwise specified).

Microbial community analysis using 16S rDNA gene sequencing was performed to identify the bacteria of the inoculum in the batch and semi-continuous experiments.

Study 1. Bacterial BPA degradation in the light and dark conditions

The bacterial consortium capable of degrading BPA used in this study was enriched from the activated sludge of Kitano Wastewater Treatment Plan Hachioji, Tokyo. The activated sludge bacteria were acclimatised with 50 mg L^{-1} BPA in mineral salt medium (MSM). The culture was incubated at 25 ± 1 ℃ in the dark under continuous aeration. The bacteria were fed with BPA 50 mg L^{-1} once a week for three months. The bacterial BPA degradation test was performed in serum bottles containing 100 mL of MSM. BPA at concentrations of 0, 10, 20, and 50 mg L^{-1} were added to the acclimatized bacteria, and the initial bacterial cell dry weight was adjusted to approximately 0.006 μg mL^{-1}. The bottles were then closed with rubber septa, and mechanical aeration was supplied through glass tubes with the flow rate of 0.2 L min^{-1}. Milli-Q water was added daily to compensate for the evaporation. Samples (1.5 mL) were withdrawn daily to measure the cell density and BPA concentration. The same experimental design was conducted in the dark as a control.

Study 2. BPA degradation by an algal-bacterial system

The BPA inhibition study was performed in serum bottles containing 100 mL of MSM. The algae were cultivated with 10, 20, and 50 mg L^{-1} BPA. The control was conducted under the same culture conditions without BPA. The algae were inoculated into the bottle with an initial Chl *a* concentration of 0.5 mg L^{-1} for both *C. sorokiniana* and *C. vulgaris*. To create an enclosed system, the headspaces of the bottles were filled with N_2/CO_2 (70/30 v/v) and sealed with rubber septa and aluminum caps. Samples (1.5 mL) were withdrawn daily using a sterilized syringe to monitor the algal growth and BPA removal. The abiotic removal of 10, 20, and 50 mg L^{-1} BPA in MSM without microbial addition was determined during the experiment period.

The algal-bacterial BPA degradation assay was performed in serum bottles containing 100 mL of MSM. The bottles were filled with MSM and BPA at a final concentration of 0, 10, 20, or 50 mg L^{-1}. The algal and bacterial cells were then inoculated into the bottle at the same initial microbial cell density as in the experiment for the algal BPA inhibition assay and the bacterial BPA degradation assay. In order to investigate the symbiotic relationship between the algae and the bacteria, the headspace was filled with air, and the bottles were then sealed with rubber septa to preclude external gas exchange. Samples (2.5 mL) were withdrawn daily to measure the cell density and BPA concentration. For the semi-continuous algal-bacterial experiment, both *C. sorokiniana*-bacteria and *C. vulgaris*-bacteria were used to clarify the feasibility of applying algal-bacterial system for BPA biodegradation. A semi-continuous algal-bacterial system was performed in a 1L round flask with BPA concentration of 50 mg L^{-1}. BPA was later injected to the same initial concentration when it was depleted.

RESULTS AND DISCUSSION

Study 1. Bacterial BPA degradation in the light and dark conditions

The bacteria dry weight increased to a stationary phase in the dark at 0.03, 0.05, 0.06, and 0.08 μg mL^{-1} with the initial BPA concentrations of 0, 10, 20, and 50 mg L^{-1}, respectively (Fig. 1a). Similarly, the maximum growth of the bacteria cultured in the lig ht at the dry weight of 0.07, 0.09, 0.10, and 0.12 μg mL^{-1} with the initial BPA concentrations of 0, 10, 20, and 50 mg L^{-1}, respectively (Fig. 1a). Furthermore, BPA was removed to below the detection limit both in the light and dark regardless of the initial BPA concentrations (Fig. 1b). BPA degradation followed first-order kinetics both in the light and dark. Based on the degradation kinetics, the degradation rate constants of BPA ranged from 0.0317 to 0.0549 h^{-1} in the light, and from 0.0529 to 0.0680 h^{-1} in the dark for the initial BPA concentrations of 10 to 50 mg L^{-1}. However, Zhang et al. (2007) reported that a BPA

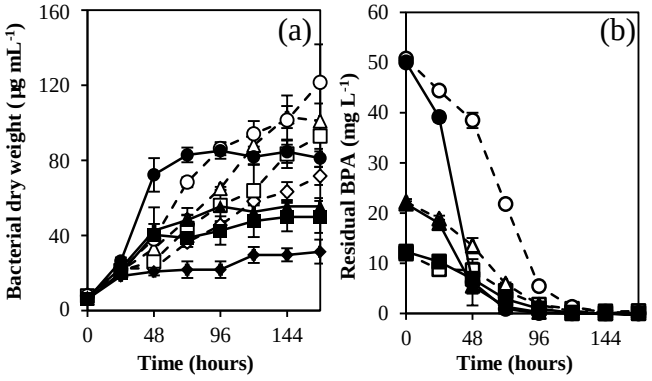

Fig. 1. Bacterial dry weight (a) and residual BPA (b) for initial BPA concentrations of (mg L^{-1} **):** (◇) 0; (□) 10; (△) 20; (○) 50 in the light (a) and (◆) 0; (■) 10; (▲) 20; (●) 50 in the dark (b).

acclimatized single-strain, *Achromobacter xylosoxidans* strain B-16, degraded BPA with the degradation rate constants of 0.0004-0.0021 h^{-1}. In comparison to the bacterial consortium in the present study, a significantly higher BPA degradation rate constant was observed in the present study. This finding indicates that bacterial consortium is more efficient than a single-strain bacteria for the degradation of BPA.

An increased of bacterial density with decreased of BPA concentrations indicates that bacteria used BPA as a carbon source for their growth. The carbon conversion efficiency of the bacteria consortium showed a strong correlation between the bacterial dry weight and BPA initial BPA concentration, both in the light and dark (Fig. 2). The slopes of the equations represent the cell yields gained from BPA. The average yield was 0.951 g-cell g-BPA^{-1} in the light and 0.973 g-cell g-BPA^{-1} in the dark. The parallel cell yields of both bacterial cultures in the light and dark suggest that light did not affect the carbon conversion efficiency of the bacteria feeding on BPA. However, the bacterial dry weight in the light was 47.6 mg mL^{-1} higher than in the dark for all the BPA concentration. This results suggested that photolysis could be occurred. The photolysed compound indirectly support the growth of bacterial in the light. On the other hand, based on the bacterial cell yield, 65.5% of the carbon in BPA carbon was found in the cell biomass, and 34.5% was found in the dissolved organic carbon and carbon dioxide (CO_2). When compared with the single-strain bacteria, *Sphingomonas* sp. MV1, mineralized 60% of the carbon in BPA to CO_2 and approximately 20% to dissolved organic carbon. The remaining 20% were used to maintain the bacterial biomass (Lobos et al., 1992). The higher fraction cell yield of BPA in this study suggests that the bacterial consortium efficiently degraded BPA and converted the carbon of BPA to maintain the cell yield.

Previous studies reported that *Sphingomonas* sp. MV1 and *Sphingomonas* sp. strain AO1, rapidly degraded BPA to *p*-hydroxyacetophenone (*p*-HAP). However, because of the absence of a catechol pathway in the bacterial strains, *p*-HAP was accumulated to an inhibitory level (Lobos et al., 1992). Therefore, the efficiency of the bacterial consortium in this study was likely because of the existence of various bacteria with different enzymes that may allow the utilization of BPA and its biodegradation intermediates.

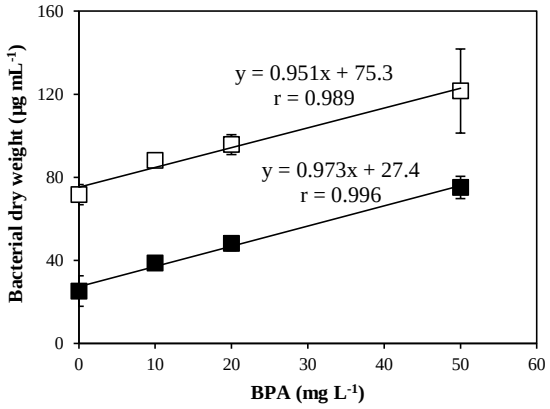

Fig. 2. Growth of the bacteria on BPA in the light condition (open symbols) and in the dark (closed symbols).

Study 2. BPA degradation by an algal-bacterial system

The BPA algal inhibition study showed that the Chl *a* concentration of *C. sorokiniana* was significantly lower at BPA concentrations of 20 and 50 mg L^{-1} ($p < 0.05$) than the control. Similarly, the Chl *a* concentration of *C. vulgaris* was significantly lower than the control at BPA 50 mg L^{-1} ($p < 0.05$). These results suggest that BPA are toxic to both *C. sorokiniana* and *C. vulgaris* at higher BPA concentrations.

The Chl *a* concentration of the algal-bacterial system significantly increased ($p < 0.05$) at the end of the experiment for both *C. sorokiniana* + Bacterial and *C. vulgaris* + Bacteria. Most notably, the algae grew significantly ($p < 0.05$) at an initial BPA concentration of 20 and 50 mg L^{-1} when growth of *C. sorokiniana* in monoculture was otherwise limited. These findings indicate that the bacteria in the algal-bacterial system reduce the inhibitory effect of BPA on the growth of the algae. This was probably due to the rapid reduction of BPA by the bacteria.

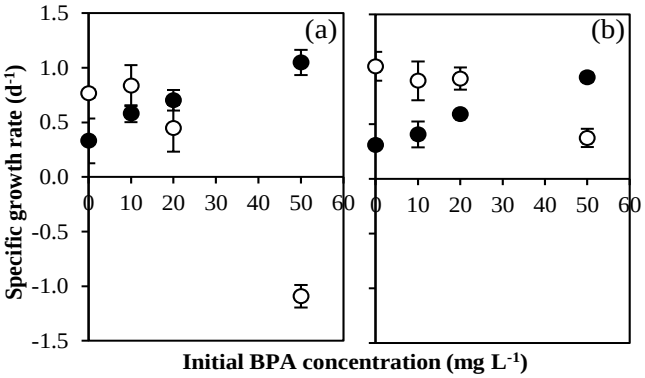

Fig. 3. Effect of BPA concentrations on algal specific growth rate (a) *C. sorokiniana* and (b) *C. vulgaris*. The open symbols represent the algal system, and the closed symbols represent the algal-bacterial system.

To further determine the growth of algae in both an algal system and an algal-bacterial system, specific growth rate (SGR) was calculated (Fig. 3). The specific growth rate of the two algal species decline when increased of BPA concentrations in the algal system. This finding indicates the toxicity of BPA on algal growth. On the other hand, the SGR of the algae in the algal-bacterial system, increased with increasing BPA concentrations. The correlation between BPA concentrations and the specific growth rate of algae in algal-bacterial system was probably stimulated by the bacteria. Since it is an enclosed system, the CO_2 needed for algal growth was provided by the bacteria. Therefore the higher oxygen (O_2) production rate may induce higher SGR of the algae.

A hyphothetical mass balance of O_2 and CO_2 was calculated, with an assumption that BPA has degraded either into bacterial biomass or carbon dioxide. The stoichiometry shows that the amount of O_2 demanded by the bacteria is insufficient for BPA degradation. These results agreed with a previous study that an external O_2 or CO_2 source is needed for complete pollutant degradation (Bordel et al., 2009). However, in this study, instead of supplying additional O_2 or CO_2, adding some other organic pollutant as an additional carbon source for bacteria would compensate for the CO_2 shortage.

BPA was removed to below the detection limit in the both algal-bacterial systems for all BPA concentrations. These results agree with the findings reported by previous studies that

photosynthetic oxygenation efficiently supports the degradation of organic pollutants by the bacteria in the absence of mechanical aeration (Borde et al., 2003).

According to previous studies, there are biodegradation intermediates that exhibit oestrogenic activity and non-oestrogenic activity (Suzuki et al. 2004; Yoshihara et al. 2001). BPA biodegradation intermediates that exhibit oestrogenic activity are *p*-HAP, 2,2-bis(4-hydroxyphenyl) propanoic acid, hydroquinone, and hydroxyl-BPA. The oestrogenic BPA biodegradation intermediates increased initially during the BPA biodegradation but then decreased to a lower level at the end of the experiment. These biodegradation intermediates have been shown to have lower oestrogenic than the mother compound, BPA (Suzuki et al., 2004). The results therefore suggest that the algal-bacterial system detoxified BPA to its biodegradation intermediates that is lower oestrogenic than BPA or non-estrogenic and –toxic.

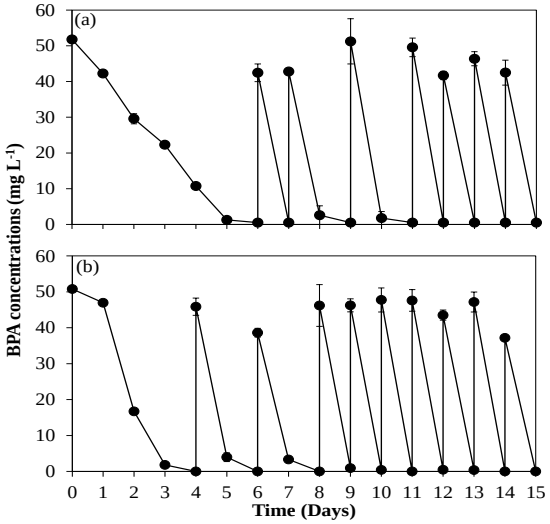

Fig. 4. The changes of dissolved oxygen in (a) *C. sorokiniana*-bacteria and (b) *C. vulgaris*-bacteria during BPA degradation.

In the semi-continuous algal-bacterial BPA degradation experiment, *C. sorokiniana* + Bacteria decreased 50 mg L^{-1} of BPA to below the detection limit after 6 days of inoculation (Fig. 4a). The subsequent BPA removal was rapidly achieved in one day of the new injection, but the degradation rate could not be maintained. The following BPA concentration was removed within two days of the new injections and continued for two injections. After day 11, BPA was added to maintain the BPA concentration at 50 mg L^{-1} daily four days. In the semi-continuous algal-bacterial BPA degradation experiment, *C. vulgaris* + Bacteria decreased BPA to below the detection limit after 4 days of inoculation (Fig. 4b). The subsequent BPA was injected on days 4 and 6 of two days intervals. On the second day of both injections, BPA concentrations decreased to below the detection limit. On day 8, BPA was injected daily to maintain the BPA concentration at 50 mg L^{-1}. Semi-continuous biodegradation of BPA proved the feasibility of the mutualistic relationship between the algae and the BPA-degrading bacteria consortium for the degradation of BPA.

The partial 16S rDNA sequencing of randomly selected colonies showed that 17 and 15 phylotypes were found in batch and semi-continuous experiments, respectively. The microbial community from the batch experiments showed that the bacterial clones were belong to four phyla. Proteobacteria was the dominant phylum that accounted for 90% of the clones. Amongst the bacteria, *Sphingobium* sp. the dominant speacies represent 42% of the clones. This bacteria is known as a 3-phenoxybenzoic acid-degrading bacteria, which degraded the compound that is similar in chemical structure to BPA. Therefore, it is speculated that *Sphingobium* sp. degraded BPA. On the other hand, the bacteria that were isolated from the semi-continuous experiments belong to two Phyla, which were 6% and 94% of the Bacteroidetes and Proteo bacteria isolates, respectively. Amongst the Proteobacteria, Betaproteobacteria was the dominant class, represented by 48% of the isolates and Oxalobacteraceae was the dominant genus. The results suggest that almost 90% of the bacterial isolates belonged to the phylum Proteobacteria in both the batch and the semi-continuous experiment. These results agreed with previous studies that used single-strain bacteria for the degradation of BPA (Lobos et al., 1992; Zhang et al., 2013). A total of 7 phylotypes was detected in both the batch and the semi-continuous experiment. Hence, these phylotypes may be functionally important bacteria for the degradation of BPA and its biodegradation intermediates.

SUMMARY

The present study clarified the BPA removal efficiency of bacterial consortium. To evaluate BPA biodegradation capability of an algal-bacterial system, the BPA degrading bacterial consortium were mixed with *Chlorellla* spp.. In order the investigate the feasibility of an algal-bacterial system, a semi-continuous experiment was conducted. The bacterial consortium biodegraded BPA to below the detection limit in all BPA concentrations under mechanical aeration. Similarly, the algal-bacterial system also degraded BPA to below the detection limit in all BPA concentrations under photosynthetic oxygenation. This results suggest that photosynthetic oxygen provided by the algae support the degradation of BPA. The semi-continuous experiment of an algal-bacterial system proved the feasibility of the mutualistic relationship between the algae and the BPA-degrading bacteria consortium for the degradation of BPA. The partial 16S rDNA sequencing results show that the BPA-degrading bacteria consortium that was used in the batch experiment and the semi-continuous experiment were dominated by the phylum Proteobacteria. The results demonstrate that with the BPA-degrading bacteria consortium, an algal-bacterial system could be an efficient method to treat BPA contaminated wastewater.

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