Isolation of *Acinetobacter calcoaceticus* BP-2 Capable of Degradation of Bisphenol A

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Bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl)propane, has been widely used as a monomer for production of epoxy resins and polycarbonate plastics, and final products of BPA include adhesives, protective coatings, paints, optical lens, building materials, compact disks and other electrical parts. Since BPA is a toxic chemical to elicit acute cell cytotoxicity and chronic endocrine disrupting activity, the degradation of BPA has been focused during last decades. To overcome the problem of photo-, and chemical-degradation of BPA, in this study, a bacterium that is able to biodegrade BPA, was isolated. The bacterium, isolated from the soil of plastic factory, was identified as *Acinetobacter calcoaceticus* (strain BP-2) based on physiological and 16S rDNA sequencing analysis. *A. calcoaceticus* BP-2 was able to grow in the presence of 1140 μg ml^{-1} BPA. Biodegradation experiments showed that BP-2 mineralized BPA via 4-hydroxybenzoic acid and 4-hydroxyacetophenone, and average degradation rate was 53.3 μg ml^{-1} day^{-1} under optimal conditions (pH 7 and 30℃). In high density resting cell (3.5 g-dcw.l^{-1}) experiments, the maximal degradation rate was increased to 89.7 μg ml^{-1} h^{-1}. Our results suggest that BP-2 has high potential as a catalyst for practical BPA bioremediation.

Key words – *Acinetobacter calcoaceticus* BP-2, biodegradation, bisphenol A, detoxification

Introduction

BPA has been widely used as a monomer for the production of epoxy resins and polycarbonate plastics, and as a stabilizer for various types of plastics[1]. The annual production of BPA exceeds 640,000 metric tons in worldwide[19], and about 0.1 % of produced BPA (653 tons/year) releases environment[18]. BPA may be released from canned foods, a drinking water-tank and dental sealants, and inadvertently released as fugitive dust emissions during manufacturing, handling and processing[13]. Previous reports demonstrated that BPA causes extensive damage to various organ systems via oxidative stress[2], and acts as anti-androgenic and anti-estrogenic chemical at concentrations as low as 2~5 μg l^{-1}[3,16]. The chlorination of BPA in aqueous media resulted in the formation of more toxic and persistent chlorinated products, such as 2-chlorobisphenol A, 2,2'-dichlorobisphenol A, or 2,2',6-trichlorobisphenol A[21].

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Photodegradation of BPA into glycerol or 2-hydroxy propanoic acid was intensively studied, but the by-products, such as 4-isopropylphenol, phenol and a semiquinone derivative of bisphenol A, were still toxic[5,14,18]. Biodegradation is alternative method for BPA detoxification, because the degradation products of biodegradation, such as 4-hydroxybenzoic acid and 4-hydroxyacetophenone, had no apparent toxicity and mutagenic activity[6,12,14]. Moreover, biodegradation is more efficient and rapid compared than the photodegradation[8,12,17,18]. Different algae[4], a gram negative bacterium[12], and *Streptomyces* sp.[9] were isolated potent BPA degraders and the majority of biodegradation research used 25~50 μg ml^{-1} of BPA as test concentrations[7,8,10,19]. Considering the low volatility and high solubility of BPA (300 μg ml^{-1} at pH 7) in water and rapid migration to water from different products at room temperature[18,20], it is worth studying the biodegradation of BPA at concentration above 300 μg ml^{-1}. In fact, intensive accumulation of BPA and tetrabromobisphenol A, as high as 450 μg ml^{-1}, in soil were reported recently[14].

In this work, we isolated a bacterium to efficiently de-

grade BPA from BPA-polluted soil samples using mineral salt medium containing 500 μg ml^{-1} of BPA as sole carbon source for practical bioremediation. The results presented here suggest that the newly selected bacterium has a potential for BPA degradation.

Materials and Methods

Media and chemicals

BPA, 4-hydroxybenzoic acid (4-HBA) and 4-hydroxyacetophenone (4-HAP) were purchased from Aldrich Chemical Co. (Milwaukee, USA). Ethylacetate for BPA extraction and acetonitrile for HPLC analysis were purchased from Junsei Chemical Co. (Tokyo, Japan). The liquid mineral salt medium (MSM) used contained 1g NH_4NO_3, 1g K_2HPO_4, 0.5g $MgSO_4 \cdot 7H_2O$, 0.2g $CaCO_3$ per liter of distilled water (pH 7.0). For solid medium, 1.5% (w/v) Bacto agar (Difco, Detroit, USA) was added to the liquid MSM. BPA was dissolved in DMSO at concentration of 30 g l^{-1} and added to the medium as appropriate concentration after sterilization.

Isolation of BPA degraders

About two grams of soil samples, which were collected from BPA applied field and plastic factory in Kyeongsan, Kyungpook, Korea, were put into 250 ml erlenmeyer flask containing 50 ml liquid MSM with 500 μg ml^{-1} BPA, and the cultures were incubated at 30℃ with shaking (130 rpm). After 7 days incubation, two milliliters of individual flask culture were transferred to 50ml BPA-MSM and further cultured for 7 days at 30℃. Then, culture broth was applied to solid MSM containing 500 μg ml^{-1} BPA for isolating single colonies.

Assay of BPA and its metabolites, and total organic carbon (TOC)

The isolated bacteria were incubated in BPA-MSM for 7days at 30℃ in a rotary shaker (130 rpm). And then, the total culture broth was extracted twice with ethylacetate to prevent extraction errors. After concentration with nitrogen gas, BPA and its metabolites in the extract were determined by a HPLC system; SCL-10A system controller, LC-10AD pump and SPD-10A UV detector, Shimadzu, Japan, Nova-Pak C_{18} column (Waters, USA), the eluent of 70% acetonitrile (v/v), the flow rate of 1 ml min^{-1}, the injection volume of 10 μl and the detection at 254 nm. Retention time for BPA, 4-HBA and 4-HAP were 13.04, 1.78 and 2.45 min under these analytical conditions, respectively. To confirm BPA utilization by the isolated strain, the organic carbon concentrations in the biomass and in the culture, and the CO_2 concentrations in the culture and headspace of culture, were measured using a TOC-500 analyzer as previously reported[12].

Identification of BPA degrader

To identify the isolated bacterium, physiological test based on a Bergey's manual and 16S rDNA sequence analysis were used. For sequence analysis, the same instrumentation and analytical techniques were used as previously reported[11,22]. The 16S partial sequence (493 bp) of strain BP-2 was deposited in the GenBank database under accession number AF458218.

Degradation of BPA by *A. calcoaceticus* BP-2

Isolated strains were culture in a BPA-MSM, collected by centrifugation, and washed twice in 15 mM phosphate buffer (pH 7.0). The washed cells were inoculated into the MSM containing 303 μg ml^{-1} of BPA, which adjusted to an optical density of 0.1 at 550 nm. Degradation was conducted at 30℃ in a rotary shaker (130 rpm) for 7 days, and BPA, its metabolites, culture pH, and biomass were determined at 24 h intervals. To evaluate physical loss or non-biological degradation, such as non-specific adsorption by the cell components, volatile loss and/or chemical degradation of BPA, the experiments were repeated with heat-killed cells or without inoculation. For high density resting cell biodegradation, cells were collected, washed twice in 15 mM phosphate buffer (pH 7.0) and cultured for 24 h at 30℃ in a rotary shaker using the same buffer to obtain maximum BPA degradation activity and cell growth. The starved cells were inoculated into 100 ml erlenmeyer flask containing 20 ml of phosphate buffer (15 mM, pH 7.0) with 303 μg ml^{-1} of BPA, which adjusted to optical density of 2.0 at 550 nm or dry cell weight of 3.5 $g.l^{-1}$. Degradation of BPA was conducted at 30℃ in a rotary shaker (130 rpm) for 12 h and BPA and its metabolites were measured at 3 h intervals.

Results and Discussion

Screening of BPA degrader and identification of strain BP-2

From 30 BPA-polluted soil samples, about 80 bacteria were isolated using solid MSM containing 500 μg ml^{-1} of BPA. Biodegradabilities of isolated bacteria were evaluated using liquid MSM containing 500 μg ml^{-1} of BPA for 7 days (data not shown), and 4 bacteria among them, strain BP-2, BP-5, BP-13 and BP-21, showed high degradation activities (the degradation ratios of strain BP-2, BP-5, BP-13 and BP-21 were 92%, 68%, 79% and 69%, respectively), and strain BP-2 exhibiting the highest activity was finally selected for the further study. No growth was observed when BPA was omitted from the MSM media and pH stayed constant at 7.2, indicating that strain BP-2 could not grow with DMSO. Without inoculation of strain BP-2, no decrease of BPA was observed, suggesting that losses due to volatility or non-biological degradation were not important under the culture conditions employed. The finally selected BP-2 was able to grow in the presence of 1140 μg ml^{-1} BPA (result not shown).

Strain BP-2 was gram-negative, oxidase test-negative, and urea test-negative bacteria. Analysis of a Vitek Gram-Negative Identification (+) Kit showed similarity of 95% to *Acinetobacter sp.*. Further, a 493-base segment of the 16S rDNA [AF458218 in GenBank(http://www.ncbi.nlm.nih.gov/entrez/)] showed similarity of 99% to that of *Acinetobacter calcoaceticus*. From these results, strain BP-2 was identified as *A. calcoaceticus*. Although many bacteria capable of degrading BPA were reported[4,6,7-9,14], *A. calcoaceticus* as a BPA degrader has not been reported until now.

BPA degradation by *A. calcoaceticus* BP-2

Total carbon analysis during BPA degradation at pH 7.0 and 30℃ demonstrated that 72.2% of BPA added (218.7 μg $ml^{-1}$of 303 μg ml^{-1}) was converted to biomass (140.98 μg ml^{-1}) and CO_2 (77.75 μg ml^{-1}). The remained soluble organic carbon (BPA) in the culture was 17.8% (53.9 μg ml^{-1}). These results further suggest that the decrease of BPA in the culture is due to the mineralization and assimilation of BPA by the *A. calcoaceticus* BP-2.

To characterize the BPA degradation by *A. calcoaceticus* BP-2, the degradation activity was measured at different initial pHs and culture temperatures using MSM containing 303 μg ml^{-1} of BPA. As shown in Fig. 1, maximum degradation activity was observed at pH 7.0 and 30℃, respectively (the degradation ratios were above 93.5%). The cell growth showed a good correlation with BPA degradation activity. Although maximum degradation activity was shown at 30℃, the strain BP-2 had a potent BPA degradation activity at temperature ranges of 20~40℃. The growths and degradation activities were severely decreased at pH 5 and pH 3, suggested that the BP-2 needs adjustment of pH to neutral before BPA degradation.

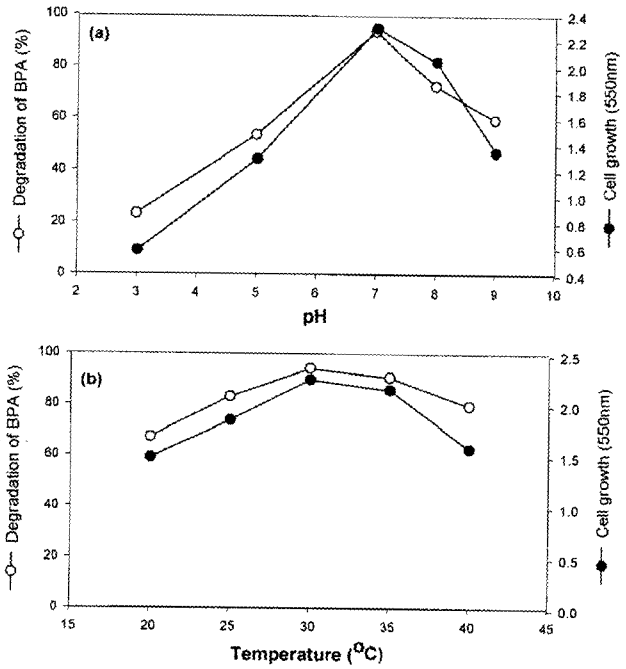

Fig. 1. Effect of culture pH (a) and temperature (b) on BPA degradation and cell growth of *A. calcoaceticus* BP-2. The initial concentration of BPA in the mineral salt medium was adjusted to 303 μg ml^{-1}. After 7 days cultivation, the cell growth and remained BPA were determined.

Under optimum culture conditions, the growth of *A. calcoaceticus* BP-2 reached a maximum at 5 days and afterwards decreased in some degree (Fig. 2a). The culture pH decreased from 7.0 to 5.6 as cell growth. After 5 days, the pH was slightly increased inversely proportional to cell growth. The BPA concentration in culture decreased continuously with concomitant to cell growth; the final concentration of BPA was less than 15 μg ml^{-1} and the average degradation rate during 5 day cultivation was 53.3 μg ml^{-1} day^{-1} (Fig. 2b). 4-HBA, as a reported non-toxic metabolite[12,14], was identified during BPA degradation. The maximum concentration, 15.99 μg ml^{-1}, was found at 6 day and the average accumulation rate was 2.66 μg ml^{-1} day^{-1}. 4-HAP, another reported non-toxic metabolite, was detected after 2 day, and the maximum concentration was 0.34 μg ml^{-1}. These results are agreement with previous re-

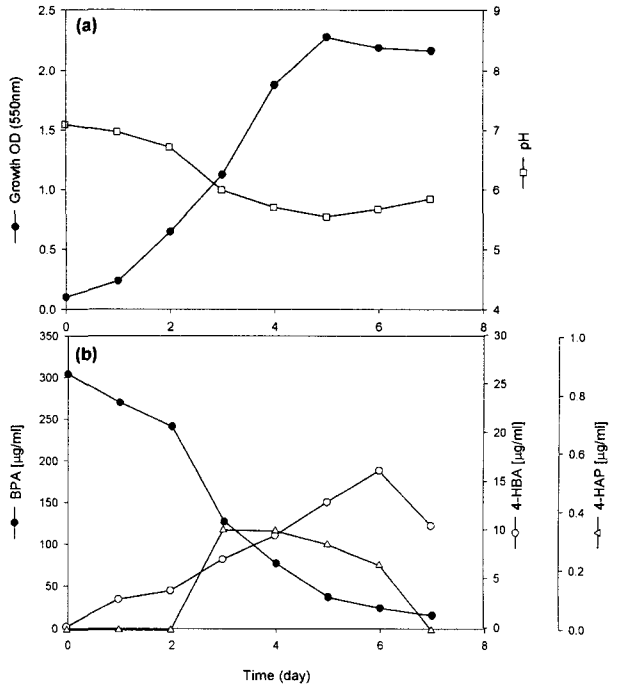

Fig. 2. Biodegradation of BPA by *A. calcoaceticus* BP-2. (a) Growth of *A. calcoaceticus* BP-2 (●) and culture pH (□) in MSM containing 303 μg-BPA ml^{-1} during 7 days cultivation at initial pH 7 and 30℃ with shaking (130 rpm). (b) Degradation of BPA (●) and production of 4-HBA (○) and 4-HAP (△) in BPA-MSM.

ports that gram-negative bacteria MV1 and WH1 mineralize BPA via 4-HBA or 4-HAP[14,20]. After 6 day, when the concentration of BPA decreased below 24 μg ml^{-1}, the concentration of 4-HBA and 4-HAP were rapidly decreased, suggesting that 4-HBA and 4-HAP also degraded and mineralized. These results suggest that *A. calcoaceticus* BP-2 mineralize and assimilate BPA via mainly 4-HBA and 4-HAP as non-toxic metabolites and *A. calcoaceticus* BP-2 has high potential for practical application in BPA bioremediation.

BPA degradation by high density resting cell

Since the degradation rate of BPA is proportionally related to cell concentration, the rapid degradation using high density resting cell was investigated. As shown in Fig. 3, the BPA in phosphate buffer was rapidly decreased from 303 μg ml^{-1} to 13.5 μg ml^{-1} by high density resting cell (3.5 g-dcw.l^{-1}) during 12 h incubation. Without cell inoculation, the BPA concentration did not show apparent changes. During first 3 h incubation, about 89% of BPA was degraded and the maximum degradation rate was 89.7 μg

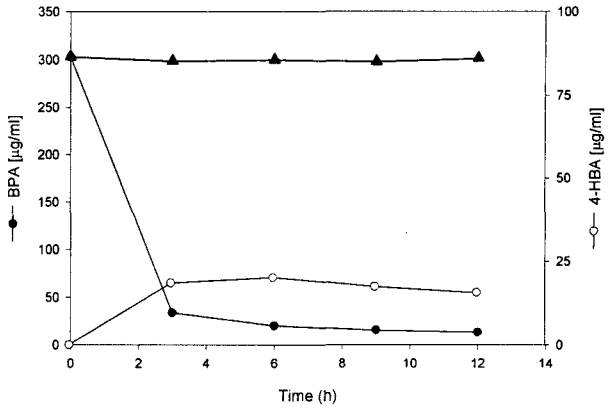

Fig. 3. Biodegradation of BPA by high density resting cells of *A. calcoaceticus* BP-2. Concentration of BPA (●) and 4-HBA (○) with inoculation of resting cells, and BPA (▲) concentration without inoculation of cells in 15 mM phosphate buffer (pH 7.0) containing 303 μg ml^{-1}BPA. Cells were cultured in a BPA-MSM, collected, washed twice in 15 mM phosphate buffer (pH 7.0) and starved for 24 h at 30℃ in a rotary shaker (130 rpm) using the same buffer. The starved cells were inoculated which adjusted to optical density of 2.0 at 550 nm (3.5 g-dcw.l^{-1}).

ml^{-1} h^{-1}. In metabolites analysis, only 4-HBA was detected. Its concentration rapidly increased to 18.5 μg ml^{-1} at 3 h and maintained without apparent changes for 12 h. These results suggest that *A. calcoaceticus* BP-2 could successfully apply to high cell density-rapid degradation of BPA under environmental conditions.

The rapid degradation of BPA is important in treatment of drinking water. Because, BPA is easily chlorinated with residual chlorine in the water, that has been added as a disinfectant, and transformed to more toxic and recalcitrant chlorinated BPAs, such as 2,2',6,6'-tetrachlorobisphenol A, 2,6-dichlorobisphenol A, and 2,2',6-trichlorobisphenol A [15,21]. Therefore, rapid BPA degradation before chlorination in water may have advantages in public health. The maintaining of high cell density is not easy for practical application. The high cell density immobilization of strain BP-2 and application of plug flow type reactor against chlorinated- or non- chlorinated water are under way in our laboratory. For practical application of BP-2, in wastewater or soils, further studies on interactions between indigenous microflora and *A. calcoaceticus* BP-2, and on maintaining of degradation activity at broad pH are necessary.

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초록 : Bisphenol A 분해균주 *Acinetobacter calcoaceticus* BP-2의 분리 및 bisphenol A 분해 특성

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BPA는 에폭시 수지 및 플라스틱 생산의 단량체로서 사용되어 왔으며, 접착제, 페인트, 광학렌즈, 건축자재, 전자제품 소재 등 다양한 제품을 생산하는 데 사용되고 있다. 그러나 BPA의 급성세포독성 및 내분비교란활성이 보고되면서 BPA의 분해에 대한 연구가 집중되고 있다. 본 연구에서는 BPA의 광분해 및 화학적 분해의 문제점을 극복하고, 실제적 BPA의 생물학적 분해를 목표로 BPA 분해균을 플라스틱 공장의 토양으로부터 분리하였다. 분리균주 중 가장 활성이 우수한 BP-2는 5 mM의 BPA처리에서 성장할 수 있었으며, pH 7, 30℃의 최적 배양조건에서 53.3 μg ml^{-1} day^{-1} 의 분해속도를 나타내었다. 균주 동정결과 BP-2는 *Acinetobacter calcoaceticus*로 확인되었으며, 3.5 g-건조중량l^{-1}의 고농도 휴식 세포 반응 결과 89.7 μg ml^{-1} h^{-1}의 BPA 분해속도를 나타내었다. 이러한 결과는 고농도 세포농도를 유지하는 경우, BP-2 균주가 실제적 BPA분해를 위한 생물촉매로 사용될 수 있음을 제시하고 있다.