Monitoring the Estrogenic Activities of the Metabolites of Bisphenol A, F and S During Sphingobium Fuliginis OMI-Mediated Biodegradation

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Abstract

Bisphenol A (BPA) and its isomers: bisphenol S (BPS) and biphenol F (BPF) have been found in vast number of applications from join and coating materials to everyday items. The release of these chemicals into environments has received great concerns recently and has been reported to associate with various toxicities in animals and human. Among those toxicities, estrogenicity is in high attention since not only BPA and its isomers but also various compounds created during their metabolism can mimic the activity of estrogen in endocrine system. In this study, the estrogenic activities of the metablites created during the biodgradation of BPA, BPF and BPS were monitored. The strain Sphingobium fuliginis OMI was found efficient to remove the bisphenols from mediums. However, study also revealed that the estrogenic activity, in general, remained during the whole biodegradation experiment. It even showed a little increase in the case of BPS at 20 hours after starting the degradation.

Keywords: Bisphenol, Biodegradation, Estrogenic activity.

1. Introduction

Bisphenol A (BPA), 2,2-bis-(4-hydroxylphenyl) propane is one of chemicals with highest volume in the world. Main occurrence of BPA can be found in many resins and polycarbonate utilizing products such as epoxy surface coating, bonding and adhesive, electrical/electronic laminates and composites, food and drink storage containers, water pipes, etc. [1-3]. As being common material used in food and beverage packaging, PBA can migrate from bottles and cans to liquids and is taken into body with food. The migration process of PBA from food containers can be greatly enhanced under high temperature condition or under conditions, which the polymer making the containers is degraded by cleaning agents [4]. Some other isomers of BPA include: BPF, BPS, BPP, BPE and BPB, in which BPF is usually used in coatings, adhesives, tank linings, flooring … while BPS is used as oxidizing agent.

As being similar to estrogen in molecular structure, bisphenols have been known for their estrogenic activities. Beside of that, BPA also expresses other toxicities such as: causing abnormalities in the liver of rats and mice, adverse effects on brain, testis, kidney and pregnancy outcome, impair cell signaling, damaging DNA and genotoxicity [5]. BPA at its original form does not raise much health effects when uptake into the body due to its significantly short haft-life and usually be detoxificated by glucuronication or sulfate conjugation processes and washed out quickly from the body. An intensive study on human serum BPA concentrations has pointed out that typical serum BPA concentrations are much lower than levels measurable by modern analytical methods and below concentrations to cause estrogenicity in humans [6]. The metabolites of BPA, instead, can be consistently found in urine (>90%). However, where are the other toxicities of BPA come from? A question should point to the metabolites formed during the fast metabolism of BPA. A minor metabolic route involves nuclear oxidation of BPA to create BPA-o-quinone as a metabolite has been investigated [5]. Quinones have been well known as strong electron-transfer (ET) agents and can catalytically act in redox manner to form large qualities of reactive oxygen species (ROS) and oxidative stress (OS). The ET-ROS-OS framework, thus, provides reasonable hypothesis for BPA toxicity. According to EPA (Environmental Protection Agency), ‘‘BPA is an exogenous agent that interfaces with synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction or behavior”. Such concerns have heightened the need for novel and advanced remediation techniques for effective removal of BPA and its isomers from a variety of contaminated environments including water, wastewater, sludge, sediments and soils.

Methods for removal of BPA from the environment currently consist of photodegradation

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and photoelectrocatalytic oxidation [7-11], oxidation [3,12,13] and biodegradation using microorganism [14-16] or plant [17]. Among the methods, bacteria-mediated degradation is considered as effective method for the degradation of BPA [18]. Although there are a lot of BPA-degrading bacteria in the environment, *Sphingomonas* sp. strains are the most frequently isolated bacteria that can use BPA as a sole carbon source. The mechanism and metabolites of bacteria-mediated degradation of BPA has been well studied [18]. Microbial actions on BPA do not only result in degradation but also transformation of BPA into another forms and sometime the new forms could be more toxic than BPA itself [19]. Other reports also revealed that the metabolites formed by cellular metabolism of BPA and BPB were even more potent estrogenic than that of the parent compounds [20,21]. The BPA's metabolites in river which mainly formed by oxidation processes by bacteria and minor metabolic pathways exist in the river water has been reported for xeno-estrogenic effects [22]. Recently, a new microbial strain, *Sphingobium fuliginis* OMI, has been developed by a group at Osaka University for effective degradation of 4-tert-butylphenol (4-t-BP) via novel pathway called meta-cleavage pathway [23]. The strain also shows possible for degradation of different bisphenol isomers. Our question is that whether this bacterium during its degradation work will create metabolites with increased toxicity? Within the scope of this study, we studied the change in estrogenic activity of the metabolites during the *Sphingobium fuliginis* OMI-meditated degradation processes of BPA, BPF and BPS

2. Materials and Methods

2.1. Microbial strains and chemicals

The bisphenol A, F, and S were pure chemicals purchased from Tokyo Chemical Industry Co., Ltd (TCI). The bacterium *Sphingobium fuliginis* OMI and genetically-modified yeast *Saccharomyces cerevisiae* for degradation test and estrogenic activity test, respectively, were generously provided by the Laboratory of Bioenvironmental Engineering, Osaka University. The yeast *S. cerevisiae* was transformed with two expression plasmids, pGBT9-ERLBD and pGAAD424-TIF-2, expressing two proteins: GAL4BD-ER (GAL4DB: GAL4 DNA binding domain) and GAL4AD-Coactivator (GAL4AD: GAL4 activation domain).

2.2. Degradation test

The test was followed a standard procedure for bisphenol degradation by *Sphingobium fuliginis* OMI built and provided by the Laboratory of Bioenvironmental Engineering at Osaka University. In brief, the bacterium was firstly cultivated in minimal salts medium (MSM) (1.0 g $(NH_4)_2SO_4$, 1.0 g K_2HPO_4, 0.2 g NaH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.05 g NaCl, 0.05 g $CaCl_2$, 8.3mg $FeCl_3.6H_2O$, 1.4mg $MnCl_2.4H_2O$, 1.17mg $NaMoO_4.2H_2O$, and 1 mg $ZnCl_2$ per liter of water) containing 10 mM of glucose as a carbon source. The cell surfaces were then washed with Glucose-free MSM medium and cells were collected in 20 ml of glucose-free MSM in one tube. Cell concentration was calculated through OD_{600} using a spectrometer. The 50-ml test vials were previously prepared containing 10 ml of glucose-free MSM with nominal concentrations of the bisphenol tested. In this study, we used two nominal concentrations: 0.1 mM and 0.5 mM for each bisphenol A, F or S. The bisphenols tested were acted as sole carbon and energy sources for the bacterial use. The bacteria from the cell suspension prepared above were then added to the test vials to the OD_{600} of 1. The degradation was happened in the vials under continuous rotary shaking at 30°C, 120 rpm. At a certain time point, 700 µL of testing solution were taken out and the degradation was stopped by adding 4.9 µL of HCl 2M. Bacterial cells were removed with centrifugation at 15.000 g, 4°C for 10 minutes. Supernatants containing BPA, BPF or BPS residual and their metabolites were kept in dark vials for later analyses of estrogenic activity or measuring remained concentrations under HPLC.

2.3. Analysis of BPA, BPF and BPS with HPLC

The concentrations of the bisphenols were measured on HPLC system (Shimadzu, Japan) with an acetonitrile (HPLC grade, Cica Reagent Kanto Chemicals, Tokyo, Japan) to water ratio of 1:1 (v:v) as an elution solvent. The flow rate is 1 ml/min from a reversed-phase column (Shim-Pack guard column GVP-ODS, 10 x 4.6 mm, Shim-Pack packed column VP-ODS, 150 x 4.6 mm, both Shimadzu, Japan). The system is equipped with an SCL-10Avp controller, a DGU-14A degasser, two LC-10ADvp pumps, and a SIL-10AF auto-injector device. The column temperature is set at 40°C and peaks were detected at 280 nm by an SPD-10Avp UV-VIS detector. The retention times of the bisphenols: A, S, and F were 7.9, 4.2 and 5.7 min, respectively.

2.4. Estrogenic activity test

Estrogenic activity test was performed following procedure for yeast two-hybrid assay developed by Nishihara and co-workers [24]. In principle, the endocrine disrupting chemicals are expected to target nuclear hormone receptor, which binds specifically to steroid hormone and regulates its gene expression. Compounds that have estrogenic activity will bind to estrogenic receptor ER and, under the present of a

coactivator, will regulate the expression of a β-Galactosidase reporter gene, which is previously introduced into yeast cell. The β-Galactosidase activity is then measured and correlated to the estrogenic activity of the test compound.

In brief, the cells were preincubated overnight at 30°C in SD medium free from tryptophan and leucine. After the pre-incubation, 250 µl of the cell culture in a small test tube was then mixed with 2.5 µl of test chemical pre-dissolved in DMSO solution and incubated for 4 h at 30°C. After the incubation, 150 µl of solution in the test tube was moved to a 96-well microplate for measuring ABS620 on a microplate reader (BioTek PowerWave HT, BioTek Instrument Inc., USA). The 100 µl solution left in tube was centrifuged to collect cells. The cells were then digested enzymatically by incubation with 1 mg/ml Zymolyase 20T (Nacalai Tesque Inc., Tokyo, Japan) (200 µl) at 30°C for 15 min. The lysate was mixed with 4 mg/ml ONPG (40 µl) and reacted until development of a yellow color (20 min) before the reaction was stopped by the addition of Na_2CO_3 1M (100 µl). An aliquot (150 µl) was taken into each of 96 wells of a microplate. Absorbances at 420 and 550 nm were read on a microplate reader to estimate estrogenic activity. The β-Galactosidase was calculated following the equation: β-Galactosidase activity (Miller units) = $1000x(ABS_{414}-1.75xABS_{540})/txVxABS_{620}$.

In which:

- ABS414 is an absorbance of *o*-nitrophenol at 414 nm.
- ABS540 is an absorbance of cell debris at 540 nm.
- 1.75 is a factor to correlate the absorbance of cell debris from 540 nm to 414 nm.
- ABS 620 is an absorbance of cells at 620 nm.
- *t*: reaction time (min) = 20 min.
- *V*: start volume of cell culture for cell lysis (ml) = 0.1/2 = 0.05 (ml)

2.5. Data analysis

Data were calculated and plotted using Microsoft Excel. An analysis of variance (ANOVA) was used to determine significant differences between a group of data of one nominal concentration. The ANOVA analysis was conducted in StatPlus:mac LE ver. 5.9.50.

3. Results and discussion

3.1. Sphingobium fuliginis OMI-mediated biodegradation of bisphenol A, F and S

In this experiment, we tested the biodegradation capacities of the strain *Sphingobium fuliginis* OMI on BPA and its two derivatives: BPS and BPF at two starting concentrations: 0.1 and 0.5 mM. As mentioned in the introduction, the bacterial strain was isolated for degradation of 4-tert-butylphenol (4-t-BP), the experiment showed that the strain can also be used for efficient removals of PBA, PBS and PBF (Fig. 1). In the figure, the drastic decreases in concentrations of the three bisphenols within the first 10 minutes after the bacterial was added to bisphenol-containing mediums could be explained as an absorption activity across cell membrane of the bisphenols (Fig. 1a). While inside, the degradation of the bisphenols will be facilitated by endogenous enzymes to form metabolites. Figure 1b showed the appearances of peaks representing the formation of metabolites during the biodegradations.

The figure showed that the biodegradation efficiency of the *Sphingobium fuliginis* OMI strain could be highest with BPA and lowest with BPS, which indicated in 0.5 mM cases where most of BPA was removed after 6 hours. At 20 hours after starting the degradation tests, around 60% of BPS and more than 20% of BPF still remained. The less effective removal of BPS in this experiment could be explained by the nature of the compounds. While BPA and BPF are widely used in coating, adhesive materials, BPS is mainly use in oxidizing agent. Thus in toxicological aspect, BPS could directly harm the cells. Nevertheless, the data suggested that this *Sphingobium* strain is efficient microbial tool for removal of BPA, BPF and BPS from the environment.

3.2. Estrogenic activity changes during Sphingobium fuliginis OMI-mediated degradation of bisphenol A, F and S

This experiment assessed changes in estrogenic activities during the biodegradation of BPA, BPF and BPS mediated by the bacterium *Sphingobium fuliginis* OMI. The β-galactosidase activities representing the expression level of β -galactosidase reporter genes were monitored during the degradation processes of the bisphenols. Data shown in the figure 2 indicated, in general for the all three bisphenols, β-galactosidase did not change two much. It means that the total estrogenic activities of the bisphenols and their metabolites did not show significant change during the whole degradation processes. An analysis of variances (ANOVA) was employed to find significant differences. In the case of BPF, the *p*

values for groups of data 0.1 and 0.5 mM are both higher than the critical value indicating no significant difference within these groups was found. In BPA case, there is no significant difference found for 0.1 mM group (p = 0.226 > 0.05) while there is a difference found in 0.5 mM group at 0 hour of the time point. The activity reduced drastically during the first 10 minutes of degradation process and then got balance. ANOVA analysis for this group without 0-hour time point indicated no significant difference (p = 0.056 > 0.05). This could be explained by that the estrogenic activity of BPA is normally high and during the first 10 minutes of degradation process, BPA was absorbed into the bacterial cells very fast causing drastic reduction of BPA concentration in incubating medium. After 10 minutes, the metabolites were constantly formed, thus balanced with BPA left over in the medium. In the case of BPS, there is no significant difference found for both groups until 20 hours. The 20-hour time point experienced an increase in the β-galactosidase activity of 0.5 mM group while no significance found for 0.1 mM group during the whole process. This could be an interesting data because this increase in the β-galactosidase activity should indicate an increase in estrogenic activity in the medium (containing BPS residual and its metabolites).

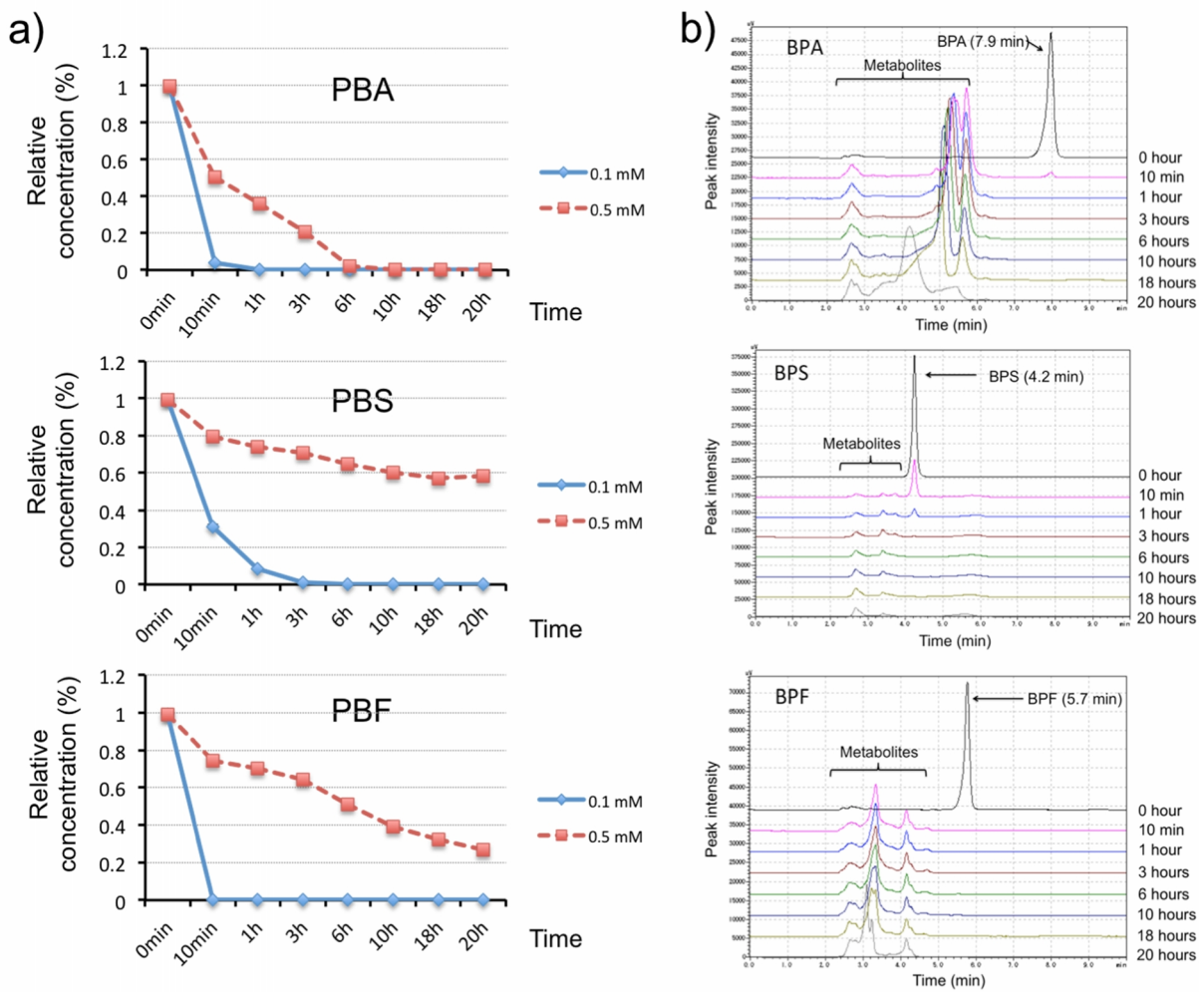

Fig. 1. Biodegradation of BPA, BPS and BPF mediated by the strain *Sphingobium fuliginis* OMI. a) relative reduction of BPA, BPS and BPF during the biodegradation. b) HPLC chromatograms of the degrading solutions during the biodegradation of BPA, BPS and BPF from the starting concentrations of 0.1mM.

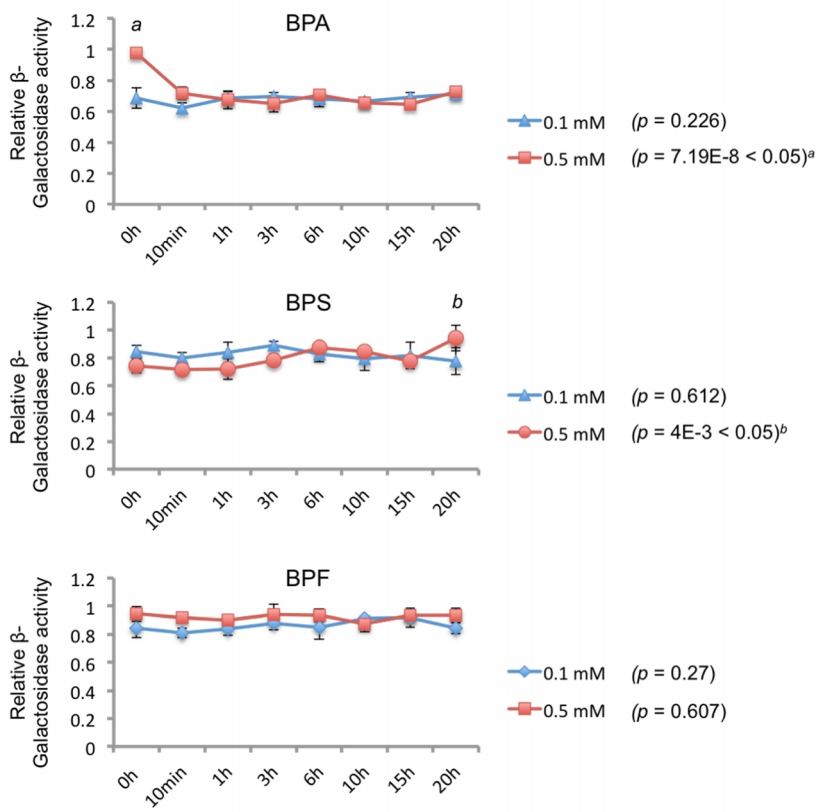

Fig. 2. Relative β-galactosidase activity at different time points during *Sphingobium fuliginis* OMI-mediated biodegradations of BPA, BPF and BPS. ANOVA critical value was set at $\alpha = 0.05$. *a* and *b* indicate points of significant differences.

According to the methods for bisphenol degradation and measuring estrogenic activity, the β-galatosidase activity, which could be correlated to the estrogenic activity, here, represents the total estrogenic activity. It means that the activity here should be additive activity of bisphenol residual and its metabolites. When the total estrogenic activity remains unchanged during the degradation process, it suggests that the metabolites formed by degradation of the bisphenols also express estrogenic activity. A reduction in the activity could be expected for complete degradation where the metabolites are CO2 and water. However, many reports previously have suggested that microbial degradations of bisphenols and other organic contaminants are always happened in several pathways simultaneously, in which the parent compounds could be degraded or transformed into another forms. Some other reports also indicated an increased toxicity of the metabolites during microbial degradation of bisphenols. The *Sphingobium fuliginis* OMI-mediated metabolic pathway is a novel degradation pathway for alkylphenols and bisphenols that have never been monitored for toxicity. If the metabolites formed by this pathway express even higher toxicity than parent compounds, then degradation method should be brought into reconsideration.

4. Conclusions

In conclusion, the biodegradation of bisphenol A, F and S mediated by the bacterium *Sphingobium fuliginis* OMI has been tested and showed efficient at removing the bisphenols from the environment. However, the observation of estrogenic activity changes has indicated no significant reduction of the activity, in general, but even a little increase in the case of BPS at 20 hours of the degradation process. Nevertheless, this study should be continued to monitor the toxicity of the metabolites at longer time scales as well as determine bisphenol residuals and metabolic components for identifying the main causes producing estrogenic activity.

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