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Bioremediation of Bisphenol A by Glycosylation with Immobilized Marine Microalga *Amphidinium crassum*

—Bioremediation of Bisphenol A by Immobilized Cells

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Abstract

Glycosylation of bisphenol A, which is an endocrine disrupting chemical, was investigated using immobilized marine microalga and plant cells from the viewpoint of bioremediation of bisphenol A. Immobilized marine microalga of *Amphidinium crassum* glucosylated bisphenol A to the corresponding glucoside. On the other hand, bisphenol A was glycosylated to its glucoside, diglycoside, gentiobioside, and gentiobiosylglucoside, which was a new compound, by immobilized plant cells of *Catharanthus roseus*.

Keywords: Glycosylation, Biosphenol A, *Amphidinium crassum*, *Catharanthus roseus*, Immobilized Cells

1. Introduction

Bisphenol A is widely used as the starting material for the production of polyacrylates, ether resins, phenol resins, photostabilizers, insecticides, fragrance ingredients, agricultural chemicals, pharmaceuticals, and coatings, and are released as pollutants and toxic compounds into rivers and seas [1]. Recently, bisphenol A has attracted considerable attention as it exhibited estrogenic activity in bioassays [2] and has been listed among "chemicals suspected of having endocrine disrupting effects" by the World Wildlife Fund, the National Institute of Environmental Health Sciences in the USA and the Japanese Environment Agency. From the viewpoint of pollution control, many studies on the biological metabolites of aromatic compounds have been reported, e.g., the benzene rings of aromatic compounds are degraded through the gentisic acid intermediate by some soil bacteria [3-8]. However, little attention has been paid to the biological degradation of endocrine disrupting chemicals. On the other hand, the metabolic pathway of aromatic compounds in plant cells is quite different from that in microorganisms; plant cells glycosylate phenols and accumulate them as glycosides in the cells [9-15].

Recently, the biotransformation of exogenous substrates by cultured marine microalga and plant cells has been reported [16,17]. These cells have the abilities of hydroxylation, glycosylation, oxido-reduction, hydrogenation, and hydrolysis for various organic compounds. Particularly, glycosylation seems to be an efficient procedure for the bioremediation of environmental pollution, because the estrogenicity of endocrine disrupting compound, *i.e.*, bisphenol A, was eliminated by formation of its glycosides [18]. This paper describes the glycosylation of bisphenol A by the immobilized marine microalga of *Amphidinium crassum* and immobilized plant cells of *Catharanthus roseus*.

2. Experimental

2.1. General

Bisphenol A was purchased from Aldrich Chemical Co. The ^{1}H and ^{13}C NMR, H-H COSY, C-H COSY, and HMBC spectra were recorded in CD_3OD using a Varian XL-400 spectrometer (Varian Inc.). The chemical shifts were expressed in δ (ppm) referring to tetramethylsilane. The FABMS spectra were measured using a JEOL MStation JMS-700 spectrometer (JEOL Ltd.). HPLC was carried out on a YMC-Pack R&D ODS column (150 × 30 mm) at 25˚C [solvent: methanol-water (9:11, v/v); detection: UV (280 nm); flow rate: 1.0 ml/min].

2.2. Cell Line and Culture Conditions

A. crassum, a gift from Ehime Prefectural Fisheries Ex-

perimental Station, Japan, cells were cultivated in a synthetic seawater (500 ml) for 2 weeks at 20˚C with constant aeration by air (1 l/min) in 1 l flasks under illumination (1000 lx). The synthetic seawater contained 20.747 g NaCl, 0.8 μg $MnCl_2 \cdot 4H_2O$, 9.474 g $MgCl_2 \cdot 6H_2O$, 1.326 g $CaCl_2 \cdot 6H_2O$, 3.505 g Na_2SO_4, 597 mg KCl, 171 mg $NaHCO_3$, 85mg KBr, 34mg $Na_2B_4O_7 \cdot 10H_2O$, 12 mg $SrCl_2$, 3 mg NaF, 1 mg LiCl, 0.07 mg KI, 0.2μg $CoCl_2 \cdot 6H_2O$, 8 μg $AlCl_3 \cdot 6H_2O$, 5 μg $FeCl_3 \cdot 6H_2O$, 0.2 μg $Na_2WO_4 \cdot 2H_2O$, 0.02 mg $(NH_4)_6Mo_7O_{24}$, 0.0045% Na_2SiO_3 and 1.07 ml of NM solution per 1 l of distilled water. The NM solution (1 l) is a kind of vitamin solutions and composed of $NaNO_3$ (150 g), Na_2HO_4 (10 g), EDTA-2Na (0.9 g), Vitamin B_{12} (1.5 mg), thiamine·HCl (75 mg), biotin (1 mg), EDTA-Fe (2.5 g), and $H_2NC(CH_3OH)_3$ (5 g) in distilled water.

The cultured plant cells of *C. roseus* have been cultivated over 20 years in our laboratory and subcultured in 300 ml conical flasks containing Schenk and Hildebrand (SH) medium (100 ml, pH 5.7) on a rotary shaker (120 rpm) at 25˚C in the dark for every 3 - 5 weeks. Part of the callus tissues (fresh weight 30 g) was transplanted to freshly prepared SH medium (100 ml in a 500 ml conical flask, pH 5.7) containing 3% sucrose and was incubated for 3 weeks prior to use for this work.

2.3. Glycosylation of Bisphenol A by *A. crassum* and *C. roseus*

Cultured *A. crassum* cells were harvested by centrifugation at 3000 rpm for 15 min and washed twice by adding 100 ml of synthetic seawater followed by centrifugation (3000 rpm for 15 min). To the 500 ml flask containing 9 g of cultured *A. crassum* cells and 300 ml of a synthetic seawater was added 0.2 mmol of bisphenol A. The cultures were incubated at 20˚C on a rotary shaker (120 rpm) for five days under illumination (1000 lx). After the incubation period, the cells and synthetic seawater were separated by centrifugation at 1000 g for 15 min. The synthetic seawater was extracted with ethylacetate and then *n*-butanol. The cells were extracted (three times) by homogenization with methanol, and the methanol fraction was concentrated and partitioned between water and ethylacetate. The ethylacetate fractions were analyzed by HPLC, combined, and concentrated. The water and *n*-butanol fractions were analyzed by HPLC, combined, evaporated, and re-dissolved in water. This water fraction was applied to a Diaion HP-20 column and the column was washed with water followed by elution with methanol. The methanol eluate was subjected to preparative HPLC [column: CAPCELLPAK R&D C18 column (250 × 30 mm); solvent: MeOH: H_2O (9:11, v/v); detection: UV (340 nm); flow rate: 1.0 ml/min] to give glycosylation products.

Bisphenol A (0.2 mmol) was administered to the 500 ml flask containing 300 ml of SH medium and 70 g of the suspension cultured cells of *C. roseus*, and the cultures were incubated at 25˚C for five days on a rotary shaker (120 rpm) under illumination (1000 lx). After the incubation, the cells and medium were separated by filtration with suction. The filtered medium was extracted with EtOAc. The medium was further extracted with *n*-BuOH. The cells were extracted (x3) by homogenization with MeOH. The MeOH fraction was concentrated and partitioned between H_2O and EtOAc. The EtOAc fractions were combined and concentrated. The H_2O fraction was applied to a Dianion HP-20 column and the column was washed with H_2O followed by elution with MeOH. The MeOH eluate was subjected to HPLC to give products.

Spectral data of a new compound, 2-(4-β-gentiobiosyloxphenyl)-2-(4-β-D-glucopyranosyloxyphenyl)propane (**5**): FAB MS: *m*/*z* 737 $[M + Na]^+$; ^{1}H NMR (400 MHz, CD_3OD, δ in ppm): δ 1.60 (s, 6H, H-1, 3), 3.25-3.90 (m, 18H, H-2', 3', 4', 5', 6', 2", 3", 4", 5", 6", 2"', 3"', 4"', 5"', 6"'), 4.50 (d, 1H, *J*=7.6 Hz, H-1"'), 4.86 (d, 1H, *J*=8.0 Hz, H-1'), 4.87 (d, 2H, *J* = 8.0 Hz, H-1"), 6.95 (d, 2H, *J* = 8.5 Hz, H-12, 14), 7.00 (d, 2H, *J* = 8.5 Hz, H-6, 8), 7.12 (d, 2H, *J* = 8.5 Hz, H-11, 15), 7.15 (d, 2H, *J* = 8.5 Hz, H-5, 9); ^{13}C NMR (CD_3OD): δ 31.5 (C-1, C-3), 42.9 (C-2), 62.3, 62.5 (C-6', C-6"'), 68.8 (C-6"), 71.2, 71.5 (C-4', C-4", C-4"'), 75.0, 75.1 (C-2', C-2", C-2"'), 77.7, 78.0, 78.1 (C-3', C-3", C-3"'), 78.2, 78.3, 78.8 (C-5', C-5", C-5"'), 102.5 (C-1', C-1"), 105.1 (C-1"'), 117.0 (C-12, C-14), 117.2 (C-6, C-8), 128.1 (C-11, C-15), 128.7 (C-5, C-9), 146.1 (C-10), 146.8 (C-4), 157.0 (C-13), 157.6 (C-7).

2.4. Preparation of Immobilized *A. crassum* and *C. roseus* in Sodium Alginate Gel

Sodium alginate (2%) was suspended in water (500 ml), which was autoclaved at 120˚C for 30 min. The cultured cells in the stationary growth phase have been used for experiments. Cultured cells of *A. crassum* (9 g) and *C. roseus* (70 g) were individually added to this solution and the mixture was stirred for 2 h until it became homogeneous. The suspension was added dropwise from a dropping funnel with a glass tube into a 5% $CaCl_2$ solution (1 l) with stirring to form pieces of spherical sodium alginate gel with 5 mm diameter immediately. Washing with water gave each immobilized cells of *A. crassum* and *C. roseus* which were used for biotransformation of bisphenol A.

2.5. Time Course Experiments

Time course experiments to examine the biotransforma-

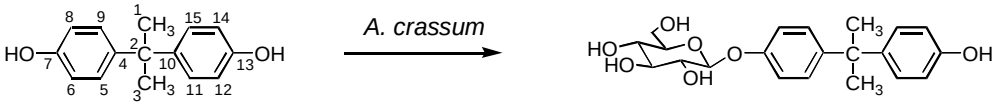
tion of bisphenol A by *A. crassum* were carried out using eight flasks containing cultured cells (9 g) or immobilized cells, which included 9 g cells. In the case of the biotransformation by the cultured and immobilized *C. roseus* cells, cultured cells (70 g) or immobilized cells, which included 70 g cells, were partitionated to each flask. Substrate (0.2 mmol) was administered to each of flasks and the mixtures were incubated on a rotary shaker at 25˚C. At a day interval, one of the flasks was taken out from the rotary shaker, and the cells (or immobilized cells) and medium were separated by filtration. The extraction and analysis procedures were same as described above. The yield of the products was determined on the basis of the peak area from HPLC and expressed as a relative percentage to the total amount of the whole reaction products extracted.

3. Results and Discussions

The biotransformation product was isolated from *A. crassum* cell cultures, which had been incubated with bisphenol A (**1**) for five days, by a combination of Diaion HP-20 column chromatography and preparative HPLC in 4% yield. The glycosylation product **2** was detected by HPLC. No additional conversion products were observed in spite of careful analyses by HPLC. Incubation of the substrate in medium without cells gave no transformation products. The structure of the product **2** was determined as 2-(4-β-D-glucopyranosyloxyphenyl)-2-hydroxyphenylpropane (bisphenol A glucoside) by FABMS, ^{1}H and ^{13}C NMR analyses (**Figure 1**). To investigate the biotransformation pathway, the time course in the conversion of **1** was followed. **Figure 2** showed that the amount of product **2** increased with time during the reaction with cultured *A. crassum* cells.

Next, *A. crassum* cells were immobilized with sodium alginate at concentrations of 2%. The immobilized *A. crassum* cells were incubated with bisphenol A (**1**) for five days. The product **2** was obtained in 6% yield. The time course of the conversion of bisphenol A (**1**) with immobilized *A. crassum* cells was investigated. As shown in **Figure 3**, the glycosylation activity for bisphenol A (**1**) was increased and the compound **2** was produced in higher yield in comparison with the case of the biotransformation using normal cells.

On the other hand, four biotransformation products **2**-**5** were isolated by a combination of Diaion HP-20 column chromatography and preparative HPLC after five days incubation of cultured plant cells of *C. roseus* with bisphenol A (**1**). The yields of **2**-**5** were 5, 12, 8, and 2%. The structures of the products **3**-**6** were identifined as 2,2-bis(4-β-D-glucopyranosyloxyphenyl)propane (diglucoside, **3**), 2-(4-β-genbiobiosyloxyphenyl)-2-(4-hydroxyphenyl) propane (gentiobioside, **4**), and 2-(4-β-gentiobiosylophenyl)-2-(4-β-D-glucopyranosyloxyphenyl) propane (gentiobiosylglucoside, **5**) (**Figure 4**). The



Bisphenol A (**1**) **2**

Figure 1. Glycosylation of bisphenol A (1) by cultured cells of *A. crassum*.

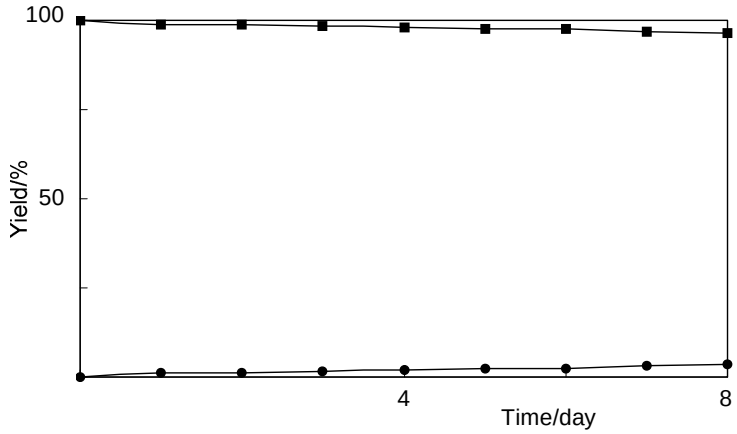


Figure 2. Time course of the glycosylation of bisphenol A (1) by the cultured cells of *A. crassum*. Yields of 1 (■) and 2 (●) are plotted.

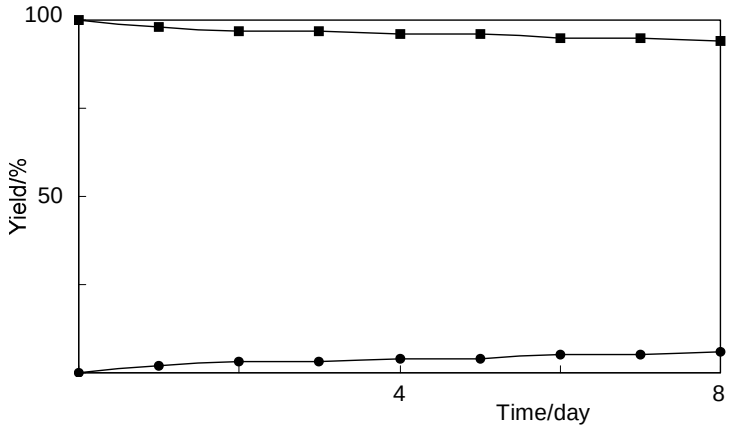


Figure 3. Time course of the glycosylation of bisphenol A (1) by the immobilized cells of *A. crassum*. Yields of 1 (■) and 2 (●) are plotted.

product **5** was a new compound. To investigate the biotransformation pathway, the time course in the conversion of **1** by cultured *C. roseus* cells was examined. Products **2**, **3**, and **4** were produced at an early stage of incubation. On the other hand, **5** was accumulated after 3 days of incubation (**Figure 5**). These findings indicated that **1** was first converted to **2**-**4** and further glycosylation gave **5** as shown in **Figure 4**.

Immobilized *C. roseusm* cells were tested for their ability to convert bisphenol A (**1**). The substrate, bisphenol A (**1**), was converted into products **2**-**5** in 7, 17, 11, and 4% yields by five days incubation. The time course of the conversion of bisphenol A (**1**) with immobilized *A. crassum* cells was investigated. As shown in **Figure 6**, the products **2**-**5** were obtained in higher yields in comparison with the case of the biotransformation using nor-

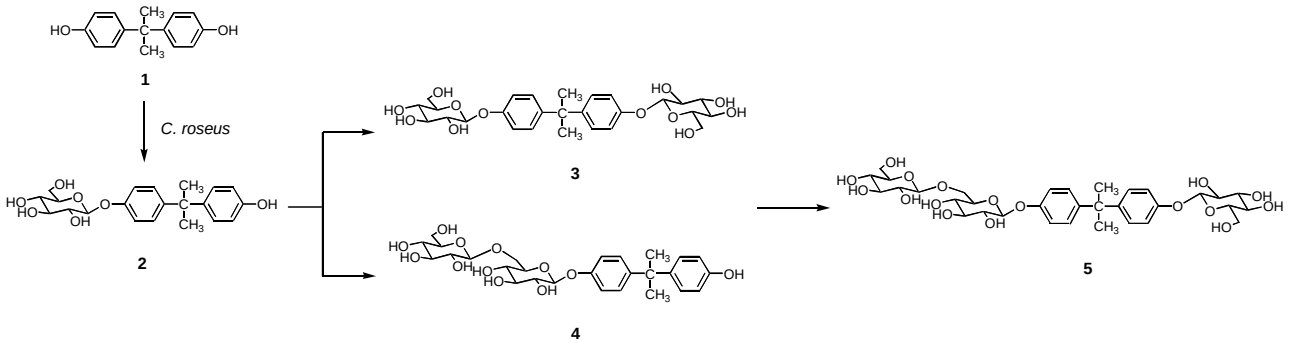


Figure 4. Glycosylation of bisphenol A (1) by cultured cells of *C. roseus*.

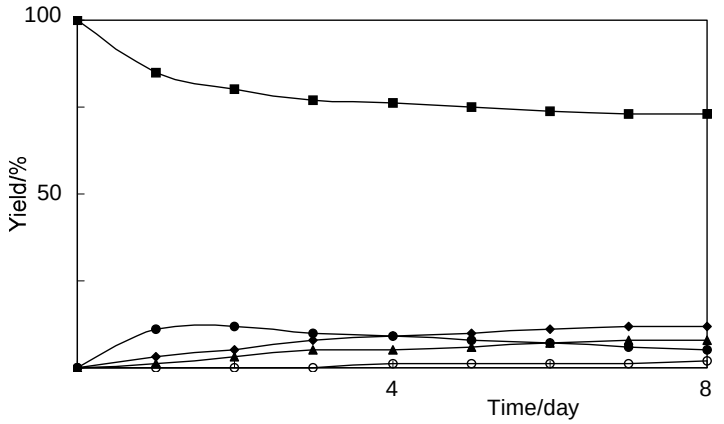


Figure 5. Time course of the glycosylation of bisphenol A (1) by the cultured cells of *C. roseus*. Yields of 1 (■), 2 (●), 3 (♦), 4 (▲), and 5 (○) are plotted.

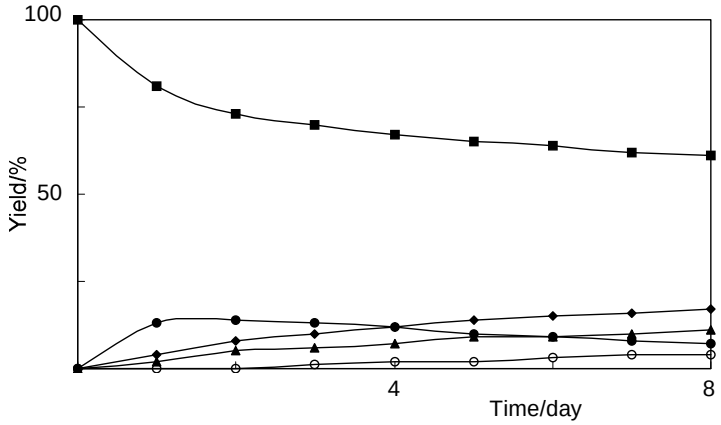


Figure 6. Time course of the glycosylation of bisphenol A (1) by the immobilized cells of *C. roseus*. Yields of 1 (■), 2 (●), 3 (♦), 4 (▲), and 5 (○) are plotted.

mal cells.

The results of this experiment demonstrate that cultured marine microalga of *A. crassum* converted bisphenol A into its glucoside and that cultured plant cells of *C. roseus* glycosylate bisphenol A to its glucoside, diglucoside, gentiobioside, gentiobiosylglucoside.The use of immobilized cells of both *A. crassum* and *C. roseus* in sodium alginate gel much improved the yield of the products.

Recently, it has been reported that freshwater microalga of *Pseudokirchneriella subcapitata*, *Scenedesmusacutus*, and *Coelastrum reticulatum* converted bisphenol A into its glucoside [19]. Also, bisphenol A was shown to be transformed to its glucoside, diglucoside, gentiobioside, and trisaccharide, *i.e.*, *O*-β-D-glucopysyl-(1→4)-[β-D-glucopyranosyl-(1→6)]β-D-glucopyranoside, by cultured plant cells of *Nicotiana tabacum* [20]. On the other hand, recent paper revealed that estrogenicity of bisphenol A was eliminated by formation of the diglucoside and that reduced activity remained in the glucoside [18]. These studies demonstrate that metabolism of bisphenol A by freshwater microalga and plants offers the possibility of bioremediation of contaminated water. The present study showed that immobilized marine microalga of *A. crassum* and plant cells of *C. roseus* are useful bioreactors for bioremediation of bisphenol A, which is an environmental pollutant released into seas and rivers. Studies of the physiological activities of bisphenol A glycosides, such as gentiobioside and gentiobiosylglucoside, are now in progress.

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5. References

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