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## Bioremediation of Fluorophenols by Glycosylation with Immobilized Marine Microalga *Amphidinium Crassum*

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**Abstract:** Fluorophenols are used as agrochemicals and released into environment as pollutants. Cultured marine microalga *Amphidinium crassum* (*Gymnodinium*) glucosylated 2-fluorophenol (**1**), 3-fluorophenol (**2**), and 4-fluorophenol (**3**) to the corresponding  $\beta$ -D-glucosides, ie, 2-fluorophenyl  $\beta$ -D-glucoside (**4**, 60  $\mu\text{g/g}$  cells), 3-fluorophenyl  $\beta$ -D-glucoside (**5**, 20  $\mu\text{g/g}$  cells), and 4-fluorophenyl  $\beta$ -D-glucoside (**6**, 40  $\mu\text{g/g}$  cells). On the other hand, 2-, 3-, and 4-fluorophenols were efficiently converted by immobilized *A. crassum* in sodium alginate gel to give their  $\beta$ -D-glucosides in higher yields (**4**: 140  $\mu\text{g/g}$  cells; **5**: 60  $\mu\text{g/g}$  cells; **6**: 100  $\mu\text{g/g}$  cells). In repetitive batch use, the immobilized cells of *A. crassum* maintained the potential for the glucosylation of the substrate fluorophenol after 5 times of usage.

**Keywords:** glycosylation, fluorophenol;  $\beta$ -glycoside, *Amphidinium crassum*, immobilized marine microalga

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## Introduction

Fluorophenolic compounds are used to agrochemicals and pharmaceuticals, and cause serious environmental contamination.<sup>1</sup> Number of fluorine-containing agricultural chemicals has grown from 4% to approximately 9% of all agrochemicals and has increased in number faster than non-fluorinated agrochemicals over the past 15 years.<sup>1</sup> These compounds are primarily used as herbicides (48%), insecticides (23%), and fungicides (18%), and their residues are released as pollutants into rivers and seas. Many studies on biological degradation of halophenols by microorganisms have been reported.<sup>1-9</sup> However, halophenols containing stable carbon-halogen bond in their structures have been described to be much more resistant to microbial degradation than unsubstituted analogs.<sup>1</sup>

Cell cultures are ideal systems for studying biotransformations such as glycosylation reactions.<sup>10</sup> Some freshwater microalga can convert exogenous toxic compounds into glycosides.<sup>11</sup> From the viewpoint of seawater pollution control, glycosylation of halophenols by marine microalga is of importance. This study focused on the metabolism of mono-fluorophenols by cultured and immobilized marine microalga of *Amphidinium crassum*.

## Material and Methods

### General

Substrates, 2-, 3-, and 4-fluorophenols, were purchased from Aldrich Chemical Co. The <sup>1</sup>H and <sup>13</sup>C NMR, H-H COSY, C-H COSY, and HMBC spectra were recorded using a Varian XL-400 spectrometer in CD<sub>3</sub>OD solution. Chemical shifts were expressed in δ (ppm) referring to tetramethylsilane. FABMS spectra were measured using a JEOL MStation JMS-700 spectrometer. *A. crassum* cells (5 g) were cultivated in a synthetic seawater (500 ml) for 2 weeks at 20 °C with constant aeration by air (1l/min) in 1 l flasks under illumination (1000lx). Constituents of synthetic seawater are summarized in Table 1.

### Biotransformation of fluorophenols by *A. crassum*

Cultured *A. crassum* cells were harvested by centrifugation at 1000 g for 15 min and washed twice by adding 100 ml of synthetic seawater followed by centrifugation (1000 g for 15 min). To the 500 ml

**Table 1.** The constituents of synthetic seawater.

Constituents <sup>a</sup>	Conc. (g/l)	Constituents <sup>a</sup>	Conc. (g/l)
NaCl	20.7	AlCl <sub>3</sub> ·6H <sub>2</sub> O	8 × 10 <sup>-6</sup>
MnCl <sub>2</sub> ·4H <sub>2</sub> O	1 × 10 <sup>-6</sup>	FeCl <sub>3</sub> ·6H <sub>2</sub> O	5 × 10 <sup>-6</sup>
MgCl <sub>2</sub> ·6H <sub>2</sub> O	9.5	Na <sub>2</sub> WO <sub>4</sub> ·2H <sub>2</sub> O	2 × 10 <sup>-7</sup>
CaCl <sub>2</sub> ·6H <sub>2</sub> O	1.3	(NH <sub>4</sub> ) <sub>6</sub> MO <sub>7</sub> O <sub>24</sub>	2 × 10 <sup>-5</sup>
Na <sub>2</sub> SO <sub>4</sub>	3.5	Na <sub>2</sub> SiO <sub>3</sub>	5 × 10 <sup>-2</sup>
KCl	0.6	NaNO <sub>3</sub>	0.2
NaHCO <sub>3</sub>	0.2	Na <sub>2</sub> HO <sub>4</sub>	1 × 10 <sup>-2</sup>
KBr	9 × 10 <sup>-2</sup>	EDTA-2 Na	9 × 10 <sup>-4</sup>
Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> ·10H <sub>2</sub> O	3 × 10 <sup>-2</sup>	Vitamin B <sub>12</sub>	2 × 10 <sup>-6</sup>
SrCl <sub>2</sub>	1 × 10 <sup>-2</sup>	Thiamine·HCl	8 × 10 <sup>-5</sup>
NaF	3 × 10 <sup>-3</sup>	Biotin	1 × 10 <sup>-6</sup>
LiCl	1 × 10 <sup>-3</sup>	EDTA-Fe	3 × 10 <sup>-3</sup>
KI	7 × 10 <sup>-5</sup>	H <sub>2</sub> NC(CH <sub>3</sub> OH) <sub>3</sub>	5 × 10 <sup>-3</sup>
CoCl <sub>2</sub> ·6H <sub>2</sub> O	2 × 10 <sup>-7</sup>		

**Note:** <sup>a</sup>Constituents are dissolved in distilled water.

flask containing 9 g of cultured *A. crassum* cells and 300 ml of a synthetic seawater was added 0.2 mmol of substrate. Cultures were incubated at 20 °C with aerobic shaking for five days under illumination (1000lx). After the incubation period, the cells and synthetic seawater were separated by centrifugation at 1000 g for 15 min. Synthetic seawater was extracted with ethylacetate and then *n*-butanol. Cells were extracted (three times) by homogenization with methanol, and the methanol fraction was concentrated and partitioned between water and ethylacetate. Ethylacetate fractions were analyzed by HPLC, combined, and concentrated. This water fraction and *n*-butanol fraction extracted from medium 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. Methanol eluate was subjected to preparative HPLC to give glycosylation products. Yield of the products was determined on the basis of the peak area from HPLC. Product yields in µg/g cells (dry weight) was calculated based on the starting cell concentration. Control free suspended cells, which had not been treated with substrates, were subjected to the same extraction procedures and HPLC analyses as planktonic biotransformation experiments, and no products or substrates were detected in the cells and in the medium despite careful HPLC analyses.

Spectral data of products are as follows.

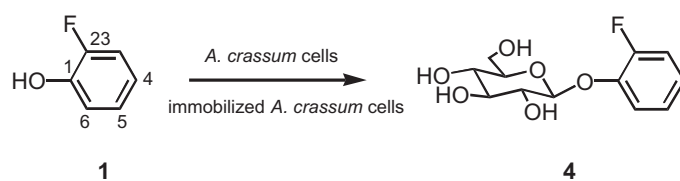
2-Fluorophenyl  $\beta$ -D-glucoside: FAB-MS  $m/z$ : 297  $[M+Na]^+$ ;  $^1H$  NMR (400 MHz,  $CD_3OD$ ,  $\delta$  in ppm): 3.41–3.89 (6H, m, H-2'-H-6'), 4.97 (1H, d,  $J = 8.0$  Hz, H-1'), 6.96–7.01 (1H, m, H-4), 7.04–7.09 (2H, m, H-3, H-6), 7.25–7.30 (1H, dd,  $J = 1.6$  Hz,  $J = 8.4$  Hz, H-5);  $^{13}C$  NMR (100 MHz,  $CD_3OD$ ,  $\delta$  in ppm): 62.3 (C-6'), 71.1 (C-4'), 74.6 (C-2'), 77.8 (C-3'), 78.0 (C-5'), 102.5 (C-1'), 117.0 (1C, d,  $J_{F-3C} = 18.9$  Hz, C-3), 119.1 (C-6), 123.8 (1C, d,  $J_{F-4C} = 6.6$  Hz, C-4), 125.4 (1C, d,  $J_{F-5C} = 4.2$  Hz, C-5), 146.3 (1C, d,  $J_{F-1C} = 10.8$  Hz, C-1), 153.9 (1C, d,  $J_{F-2C} = 244.2$  Hz, C-2).

3-Fluorophenyl  $\beta$ -D-glucoside; FAB-MS  $m/z$ : 297  $[M+Na]^+$ ;  $^1H$  NMR (400 MHz,  $CD_3OD$ ,  $\delta$  in ppm): 3.39–3.90 (6H, m, H-2'-H-6'), 4.92 (1H, d,  $J = 7.2$  Hz, H-1'), 6.74 (1H, m, H-4), 6.85 (1H, m, H-2), 6.90 (1H, dd,  $J = 2.2$  Hz,  $J = 8.0$  Hz, H-6), 7.27 (1H, m, H-5);  $^{13}C$  NMR (100 MHz,  $CD_3OD$ ,  $\delta$  in ppm): 62.3 (C-6'), 71.2 (C-4'), 74.7 (C-2'), 77.7 (C-3'), 78.0 (C-5'), 102.1 (C-1'), 105.2 (1C, d,  $J_{F-4C} = 25.5$  Hz, C-4), 109.7 (1C, d,  $J_{F-2C} = 20.6$  Hz, C-2), 113.4 (1C, d,  $J_{F-6C} = 2.4$  Hz, C-6), 131.3 (1C, d,  $J_{F-5C} = 9.9$  Hz, C-5), 160.1 (1C, d,  $J_{F-1C} = 10.5$  Hz, C-1), 164.5 (1C, d,  $J_{F-3C} = 241.7$  Hz, C-3).

4-Fluorophenyl  $\beta$ -D-glucoside; FAB-MS  $m/z$ : 297  $[M + Na]^+$ ;  $^1H$  NMR (400 MHz,  $CD_3OD$ ,  $\delta$  in ppm): 3.35–3.90 (6H, m, H-2'-H-6'), 4.85 (1H, d,  $J = 7.2$  Hz, H-1'), 6.95–7.01 (2H, m, H-3, H-5), 7.08–7.12 (2H, m, H-2, H-6);  $^{13}C$  NMR (100 MHz,  $CD_3OD$ ,  $\delta$  in ppm): 62.4 (C-6'), 71.2 (C-4'), 74.7 (C-2'), 77.8 (C-3'), 78.0 (C-5'), 102.9 (C-1'), 116.4 (2C, d,  $J_{F-3,5C} = 23.1$  Hz, C-3, C-5), 119.3 (2C, d,  $J_{F-2,6C} = 8.3$  Hz, C-2, C-6), 155.2 (1C, d,  $J_{F-1C} = 2.5$  Hz, C-1), 159.4 (1C, d,  $J_{F-4C} = 237.6$  Hz, C-4).

### Biotransformation of fluorophenols by immobilized *A. crassum* cells in sodium alginate gel

Sodium alginate (2%) was suspended in water (50 ml), which was autoclaved at 120 °C for 30 min. Cultured *A. crassum* cells (9 g) were added to this solution and the mixture was stirred for 2h until it became homogeneous. Suspension was added drop-wise from a dropping funnel with a glass tube into a 5%  $CaCl_2$  solution (11) with stirring to form pieces



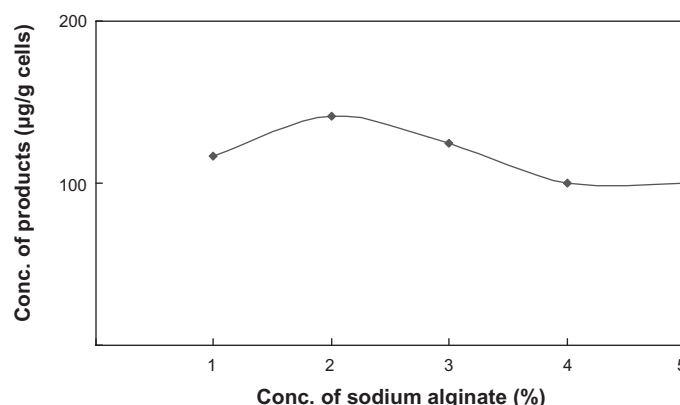
**Figure 1.** Biotransformation of 2-fluorophenol (1) to 2-fluorophenyl  $\beta$ -D-glucoside (4) by cultured and immobilized cells of *A. crassum*.

of spherical sodium alginate gel with 5 mm diameter immediately. After washing with a synthetic seawater, immobilized *A. crassum* cells were used for biotransformation experiments.

To the immobilized *A. crassum* cells which included 9 g of *A. crassum* cells with 250 ml of the synthetic seawater in a 500 ml flask was added 0.2 mmol of substrate. Effect of sodium alginate concentration on the glycosylation of 2-fluorophenol (1) was examined by using the cells immobilized with sodium alginate at final concentrations of 1, 2, 3, 4, and 5%. Flask was incubated at 20 °C with aerobic shaking for five days under illumination (1000lx). Isolation of products was carried out by the similar procedures to planktonic biotransformation experiment described above.

### Results and Discussion

Biotransformation product was isolated from the cultured cells of *A. crassum*, which had been treated with 2-fluorophenol (1) for 48 h. Potentially glucosylated product (4) was obtained, and no additional conversion



**Figure 2.** Effects of sodium alginate concentration on the glucosylation activity of the immobilized cells of *A. crassum* against 2-fluorophenol (1). Concentration ( $\mu\text{g/g cells}$ ) of 2-fluorophenyl  $\beta$ -D-glucoside (4) ( $\blacklozenge$ ) is plotted.

**Table 2.** The production of **4** by immobilized *A. crassum*.

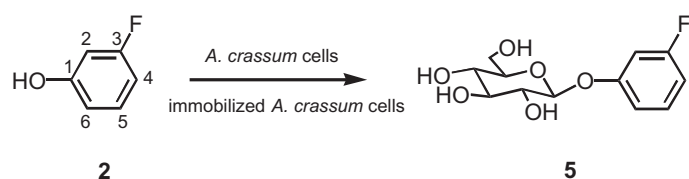
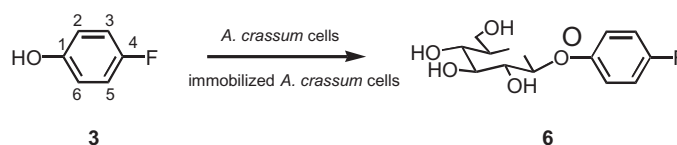
Batch	Conc. of products ( $\mu\text{g/g cells}$ ) <sup>a</sup>
1	147
2	143
3	123
4	117
5	90

**Note:** <sup>a</sup>Results are expressed as average of three times experiments.

products were observed in spite of the careful HPLC analyses. Chemical structure of the product was determined on the basis of their FABMS, <sup>1</sup>H and <sup>13</sup>C NMR, H-H COSY, C-H COSY, and HMBC spectra as 2-fluorophenyl  $\beta$ -D-glucoside (60  $\mu\text{g/g cells}$ ) (Fig. 1). Other microalga, ie, *Chattonella* sp. and *Heterosigma* sp., showed no glycosylation action against **1**.

In addition, 2-fluorophenol (**1**) was subjected to the biotransformation by immobilized *A. crassum* cells with sodium alginate. Fig. 2 shows that the glycosylation activity of immobilized cells was enhanced at 2% sodium alginate concentration. Immobilized *A. crassum* cells, which had been prepared at 2% sodium alginate concentration, were used for biotransformation experiments. After 48 h incubation of 2-fluorophenol (**1**), 2-fluorophenyl  $\beta$ -D-glucoside (140  $\mu\text{g/g cells}$ ) was obtained in higher yield in comparison with the case of the biotransformation using planktonic cells. Repetitive use of the immobilized cells was carried out. Yields of the product **4** obtained after each batch reaction were shown in Table 2. Experiments were repeated three times, and average is shown in Table 2. In repetitive batch use, the immobilized cells of *A. crassum* maintained the potential for the glycosylation of **1** (100  $\mu\text{g/g cells}$ ) after 5 times of usage.

Next, *A. crassum* cells were incubated with 3-fluorophenol (**2**) for 48h in the same biotransformation conditions as 2-fluorophenol (**1**). Glycosylation

**Figure 3.** Biotransformation of 3-fluorophenol (**2**) to 3-fluorophenyl  $\beta$ -D-glucoside (**5**) by cultured and immobilized cells of *A. crassum*.**Figure 4.** Biotransformation of 4-fluorophenol (**3**) to 4-fluorophenyl  $\beta$ -D-glucoside (**6**) by cultured and immobilized cells of *A. crassum*.

product was isolated from the cell cultures of *A. crassum* by preparative HPLC and was identified as 3-fluorophenyl  $\beta$ -D-glucoside (**5**, 20  $\mu\text{g/g cells}$ ) by spectroscopic methods (Fig. 3). Use of the immobilized *A. crassum* cells, which had been prepared at 2% sodium alginate concentration, improved the conversion yield of the product to 60  $\mu\text{g/g cells}$ . When the substrate 4-fluorophenol (**3**) was used for the biotransformation experiment, 4-fluorophenyl  $\beta$ -D-glucoside (**6**) was obtained in 40  $\mu\text{g/g cells}$  (Fig. 4). Incubation of immobilized *A. crassum* cells prepared at 2% sodium alginate concentration with 4-fluorophenol (**3**) afforded 4-fluorophenyl  $\beta$ -D-glucoside (**6**) in 100  $\mu\text{g/g cells}$ .

This study demonstrated that the cultured cells of *A. crassum* can convert 2-, 3-, and 4-fluorophenols into the corresponding  $\beta$ -D-glucosides. Highest yield was found in the case of the biotransformation of 2-fluorophenol with *ortho*-phenolic hydroxyl group. Results of this experiment revealed that cultured cells of *A. crassum* can incorporate the environmentally toxic compounds such as 2-, 3-, and 4-fluorophenol and biotransform into  $\beta$ -D-glucosides. Yield of  $\beta$ -D-glucosides produced from fluorophenols by immobilized *A. crassum* cells in sodium alginate gel was improved in comparison with the case of biotransformation by planktonic *A. crassum* cells. Immobilized cells of *A. crassum* would be useful to detox fluorophenols to stable glycosides. This procedure is a simple operation and is environmentally friendly. Further studies on the enzymes which catalyze the glycosylation of fluorophenols are now in progress.

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## Disclosure

This manuscript has been read and approved by all authors. This paper is unique and is not under





consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

## References

1. Key BD, Howell RD, Criddle CS. Fluorinated organics in the biosphere. *Environ Sci Technol.* 1997;31:2445–54.
2. Haggblom MM. Microbial breakdown of halogenated aromatic pesticides and related compounds. *FEMS Microbiol Rev.* 1992;9:29–71.
3. Kiyohara H, Hatta T, Ogawa Y, Kakuda T, Yokoyama H, Takizawa N. Isolation of *Pseudomonas pickettii* strains that degrade 2,4,6-trichlorophenol and their dechlorination of chlorophenols. *Appl Environ Microbiol.* 1992;58:1276–83.
4. Hofrichter M, Bublitz F, Fritsche W. Unspecific degradation of halogenated phenols by the soil fungus *Penicillium frequentans* Bi 7/2. *J Basic Microbiol.* 1994;34:163–72.
5. Bae HS, Lee JM, Lee ST. Biodegradation of 4-chlorophenol via a hydroquinone pathway by *Arthrobacter ureafaciens* CPR706. *FEMS Microbiol Lett.* 1996;145:125–9.
6. Hollender J, Hopp J, Dott W. Degradation of 4-chlorophenol via the meta cleavage pathway by *Comamonas testosteroni* JH5. *Appl Environ Microbiol.* 1997;63:4567–72.
7. Boersma MG, Dinarieva TY, Middelhoven WJ, et al. <sup>19</sup>F nuclear magnetic resonance as a tool to investigate microbial degradation of fluorophenols to fluorocatechols and fluoromuconates. *Appl Environ Microbiol.* 1998;64:1256–63.
8. Bondar VS, Boersma MG, van Berkel WJ, et al. Preferential oxidative dehalogenation upon conversion of 2-halophenols by *Rhodococcus opacus* 1G. *FEMS Microbiol Lett.* 1999;181:73–82.
9. Boersma MG, Solyanikova IP, van Berkel WJ, Vervoort J, Golovleva LA, Rietjens IM. <sup>19</sup>F NMR metabolomics for the elucidation of microbial degradation pathways of fluorophenols. *J Ind Microbiol Biotechnol.* 2001;26:22–34.
10. Ishihara K, Hamada H, Hirata T, Nakajima N. Biotransformation using plant cultured cells. *J Mol Catal B Enz.* 2003;23:145–70.
11. Nakajima N, Teramoto T, Kasai F, et al. Glycosylation of bisphenol A by freshwater microalgae. *Chemosphere.* 2007;69:934–41.

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