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Flow Injection Biosensor System for 2,4-Dichlorophenoxyacetate Based on a Microbial Reactor and Tyrosinase Modified Electrode

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1. Introduction

Synthetic chlorinated organic compounds have been used extensively as herbicides and pesticides and the contamination of ecosystems with these compounds has stimulated great interest in investigation of these frequently toxic or bioaccumulatable compounds. 2,4-Dichlorophenoxyacetate (2,4-D), one of these compounds, is a synthetic phytohormone that has been widely used as a herbicide for controlling broadleaf weeds, and huge amount of 2,4-D has been released into the environment. 2,4-D is known, on the other hand, to be susceptible to rapid biological degradation in natural environments, and has been used as a model for genetic and biochemical study on the chloroaromatic degradation. A number of its degrading bacteria have been isolated worldwide from a variety of environments and extensively examined on a molecular basis (Amy et al., 1985; Sinton et al., 1986; Perkins et al., 1990; Fulthorpe et al., 1992, 1995; Ka et al., 1994; Tonso et al., 1995; Top et al., 1995; Maltseva et al., 1996; Suwa et al., 1996; Vallaeys et al., 1996; Kamagata et al., 1997; Cavalca et al., 1999; Laemmli et al., 2000; Itoh et al., 2002). One of the most extensively studied strain is *Ralstonia eutrophus* JMP134, which carries a 2,4-D-degrading gene cluster on the transmissible plasmid pJP4 (Don & Pemberton, 1981; Neilson et al., 1992; Fukumori & Hausinger, 1993a; Laemmli et al., 2000).

On the other hand, reliable determination of 2,4-D is indispensable to investigate its biological degradation. A biosensor is a device utilizing a biological sensing element, and a variety of biosensors for 2,4-D detection have been developed (Table 1). Most of sensors reported so far, however, can be classified into either immunoassay or immunoenzymatic assay. They are based on the specific interaction between 2,4-D (antigen) and its antibody. These biosensors have been demonstrated to show very high sensitivities (*e.g.* the lower detection limit of less than 1 µg/L), while these assays are generally said to be somewhat cumbersome to perform and require considerably expensive reagents. A biosensor based on the inhibition of catalytic activity of enzyme alkaline phosphatase in the presence of 2,4-D has been also proposed and a detection limit of 0.5-6 µg/L has been obtained. The enzyme inhibition effect has been also observed for another pesticide, indicating the analyte selectivity of the sensor to be not expected. On the other hand, very few attempts have been made on the biosensor employing microorganisms as the sensing component.

Bioelement	Transducer	Principle/Comment	Reference
antibody	SPR	immunoassay/ resonance angle	Starodub et al., 2005
	electrode	affinity sensor /conductance	Hianik et al., 1998,1999
	luminometer	immunoenzymatic assay/ chemiluminescence	Rubtsova et al., 1997
	QCM	immunoassay/ piezoelectricity	Horacek et al., 1997, 2000
	TIRF[a] fibre	immunoassay/optical	Mosiello et al., 1997
	electrode	immunoassay/ bi-enzyme amplifier, amperometry	Bier et al., 1997
	electrode	immunoassay/ enzymatic inhibition, amperometry	Medyantseva et al., 1997
	electrode	immunoenzymatic assay/ amperometry	Trau et al., 1997
	electrode	immunoenzymatic assay/ potentiometry	Piras et al., 1996
	electrode	immunoenzymatic assay/ amperometry	Skladal et al., 1995
	electrode	immunoenzymatic assay/ amperometry	Kalab et al., 1995
enzyme	electrode	enzymatic inhibition/ amperometry, voltammometry	Mazzei et al., 2004
micro-organism	O_2 electrode	respiration activity/ *A.eutrophus*	Beyersdorf-Radeck et al., 1993

([a]: total internal reflection fluorescence)

Table 1. Biosensors for 2,4-D analysis.

2. Principle of the present sensor system

2.1 2,4-D degrading microorganisms

As described above, a number of 2,4-D degrading bacteria have been isolated worldwide from varying environments. Bacteria capable of degrading 2,4-D are of general importance for the preservation of the environment, and microbial degradation with such bacteria has been extensively studied as a model for decomposition of hazardous chloroaromatic compounds (Häggblom,1990, 1992; van der Meer et al., 1992).

A microbial 2,4-D biosensor has been developed by employing a layer of *Alcaligenes eutrophus* (*Ralstonia eutrophus*) JMP134 (Table 1). Concerning to this sensor, the concentration of 2,4-D has been determined by monitoring the oxygen consumption with an oxygen electrode since the bacterium requires oxygen for the degradation of the xenobiotic. However, this approach can be disturbed by the presence of other oxygenases in a microbial cell.

Recent physiological and evolutional studies have demonstrated that these 2,4-D-degrading bacteria could be categorized into three groups (Ka et al., 1994; Kamagata et al., 1997; Kitagawa et al., 2002; Itoh et al., 2004). Most of the 2,4-D-degrading bacteria isolated from human-disturbed sites include copiotrophic and fast-growing genera in the β and γ subdivisions of *Proteobacteria*, which has been classified as class I 2,4-D degraders. The degradation pathway of 2,4-D has been extensively characterized with *Ralstonia eutrophus* JMP134, one of the class I degraders (Don et al., 1985; Streber et al., 1987; Perkins et al., 1990). Degradation of 2,4-D in this class of degraders is considered to be initiated by cleavage of the ether linkage to yield 2,4-dichlorophenol (2,4-DCP), which is then hydroxylated to 3,5-dichlorocatechol (3,5-DCC), followed by ring cleavage (Fig. 1).

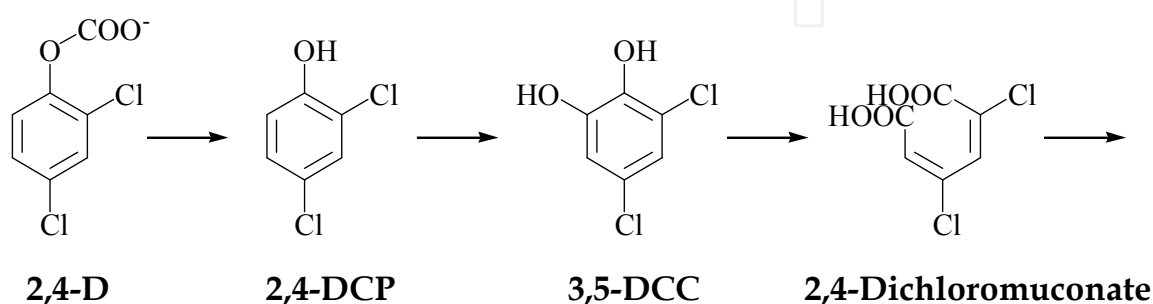**2,4-D**   **2,4-DCP**   **3,5-DCC**   **2,4-Dichloromuconate**

Fig. 1. Aerobic degradation pathway of 2,4-D for class I degraders.

2.2 Biosensors detecting metabolic intermediates

It is noteworthy that the phenolic and the catecholic compounds are produced as the first and the second degradation products, respectively, in the pathway described above, while these compounds are metabolized finally to carbon dioxide. This implies that the concentration of 2,4-D might be estimated by determining these intermediates with a sensing device. Microbial biosensors based on the determination of the metabolic intermediate have not been reported for 2,4-D, while such a type of whole cell amperometric biosensors have been reported for *p*-nitrophenol (Lei et al., 2004; Mulchandani et al., 2005). A tyrosinase-modified electrode is one of effective transducers for phenols or catechols detection, for which active studies have been made on the development of highly efficient tyrosinase electrodes (for example, Notsu & Tatsuma, 2004; Mailley et al., 2004; Stanca & Popescu, 2004; Gutés et al., 2005; Carralero Sanz et al., 2005; Liu et al., 2005). Developments of flow injection analysis with tyrosinase electrode have been also attempted (Dall' Orto et al., 1999; Li & Tan, 2000; Notsu et al., 2002; Serra et al., 2003).

Then, in the present work a novel biosensor system was constructed with a fixed-bed reactor packed with immobilized microbes and a tyrosinase-modified graphite electrode. This sensor system monitors phenolic and catecholic compounds produced in the first two steps by the microbial degradation of 2,4-D.

3. Construction of sensor system

3.1 Isolation of 2,4-D-degrading bacteria and confirmation of degradation

In order to isolate 2,4-D-degrading bacteria, soil samples were collected from various environments such as mountains, farmlands, riversides, and industrial areas in Northern Kyushu. They were mixed with a mineral salt (MS) medium (0.5 g K_2HPO_4, 0.5 g KH_2PO_4,

0.25 g NH_4NO_3, 0.002 g $NaMoO_4 \cdot 2H_2O$, 0.001 g $FeSO_4 \cdot 7H_2O$, and 0.001 g $MnSO_4 \cdot 7H_2O$ per liter) containing 2,4-D (0.005 g) as the sole carbon source, and enrichment culture was performed at 30 °C for 7 d. The culture broth was centrifuged, and the concentration of 2,4-D in the supernatant was measured using a spectrophotometer at 284 nm and a high-performance liquid chromatography (HPLC) (LaChrom series, Hitachi, Tokyo, Japan). The culture broths with decreased 2,4-D concentration were then transferred to basal agar plates containing 2,4-D. Finally, we could obtain 11 isolates. 2,4-D degradation ability of the strains was confirmed by the decrease in the amount of remaining substrate, which was measured with HPLC. Identification of the strains was performed by 16S rDNA sequence analysis. Among them the bacterium identified as *Ralstonia* sp., which showed relatively high degradation capability and rapid growth in a medium containing 2,4-D as a sole carbon source, was employed for the sensor system. The strain was found to have 97% sequence similarity to *Ralstonia* sp. JMP134.
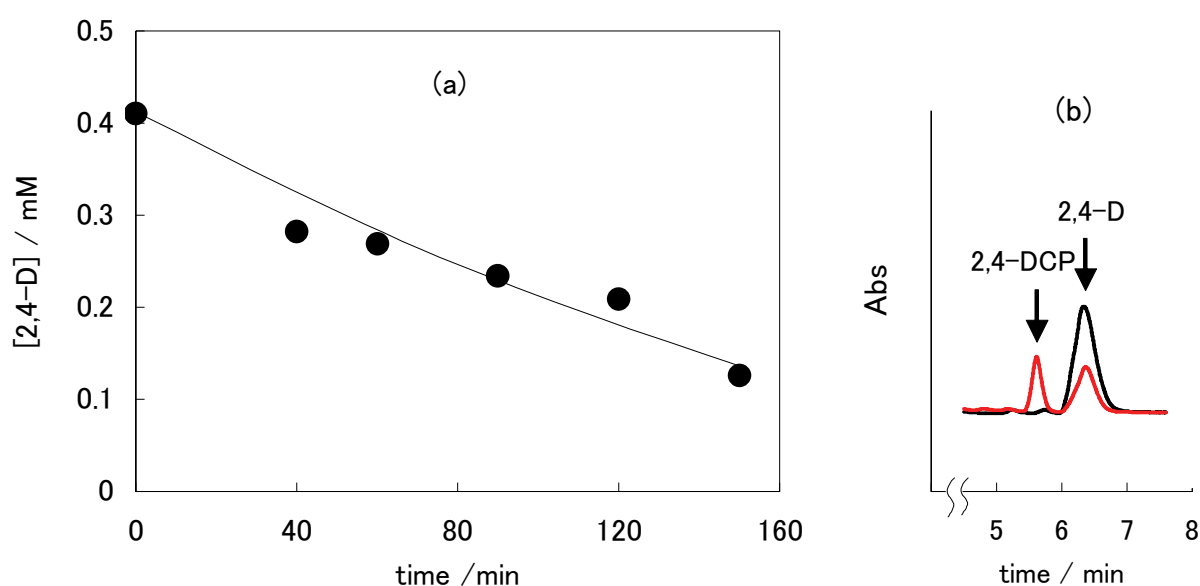

Fig. 2. Time course of 2,4-D concentration (a) and an example of chromatogram (b) obtained for degradation experiment with cell suspension; reaction time —: 0 min, —: 150 min.

The 2,4-D degradation activity of the strain was examined by monitoring the reduction of the amount of remaining substrate in a culture. The microorganism was grown aerobically in Luria-Bertani (LB) medium (NaCl, 10; Tryptone, 10; Yeast Extract, 5 g/L) containing 0.10 g/L (0.4 mM) 2,4-D at 30°C for 20 h to a late-exponential phase. The cells were harvested by centrifugation and washed thoroughly with sterile saline, which was then resuspended in the same solution. Degradation experiment initiated by transferring a fixed amount of the cell suspension into MS medium supplemented with 2,4-D (0.10 g/L), was performed at 30°C on a reciprocating shaker. The 2,4-D content was measured periodically with HPLC.

The time course of decrease in 2,4-D concentration and an example of HPLC chromatogram are shown in Fig. 2(a) and (b), respectively. It is seen that the 2,4-D content decreases smoothly with increasing the incubation time. Further, a decrease in peak-height for 2,4-D and an appearance of 2,4-DCP peak were confirmed by comparing the chromatograms obtained at the incubation time of 0 and 150 min, while variation of the amount of 2,4-DCP could not be followed accurately since most of their peaks were too small.

3.2 immobilization of microbes

Silica gel particles (Wako Gel type G, 300-600 μm, Wako Pure Chemical Industries Ltd., Tokyo, Japan) were used as the carrier for immobilization of bacteria. LB medium containing 0.05 g/L 2,4-D was used for cultivation and immobilization. A continuous flow reactor was constructed with a glass column (13 mmϕ × 80 mm) in which silica gel particles were packed. A sterile medium (100 mL) prepared in a reservoir (250 mL- glass bottle) was circulated with a peristaltic pump at a flow rate of 1 mL/min, followed by inoculation with the pre-incubated culture (1 mL). After the incubation at 30°C for 72 h the cells were harvested and washed thoroughly with 0.1 M sodium phosphate buffer (PBS; pH 7.0). Then, the immobilized microbes particles were suspended in sterile 0.9 % NaCl solution and transferred into a centrifuge tube. The immobilized microbes particles thus obtained were stored in a refrigerator for at least 24 h before use.

3.3 Instrumentation

A schematic diagram of the reactor type biosensor system is illustrated in Fig. 3. The system consists of a peristaltic pump (model 7553-80, Masterflex, Cole-Parmer Instrument Co., Vernon Hills, IL, USA), a sample injector with a 50 μL loop, a 6-way valve, a microbial reactor, and a flow cell. Two PTFE rotary valves (Rheodyne, Rhonert Park, CA, USA) were used as the injector and the valve. The 6-way valve was switched to the reactor port when the response to 2,4-D was measured. On the other hand, the valve was switched to the flow cell port when the sensitivity of enzyme electrode was checked. The microbial reactor, flow cell, and carrier reservoir were placed in an incubator maintained at 30°C.

Enlarged schematics of the microbial reactor and the flow cell are illustrated in Figs. 4(a) and 4(b), respectively. The parts of the reactor body were made of a PTFE rod (60 mmϕ). Silica gel particles with immobilized microbes were packed in the cell holder (20 mmϕ) and supported with two pieces of SUS mesh screen. In the present work the cell holders of 3 and 12 mm in height (*H*) were used. The flow cell was made of a PTFE sheet and a 3 mm- thick silicone gasket. The cell volume was estimated to be about 0.7 mL. A platinum plate (20×10×0.5 mm) and an Ag/AgCl electrode (1 mmϕ) were used as the auxiliary and the reference electrode, respectively. The microbial reactor and the flow cell were kept at 30°C in an incubator.
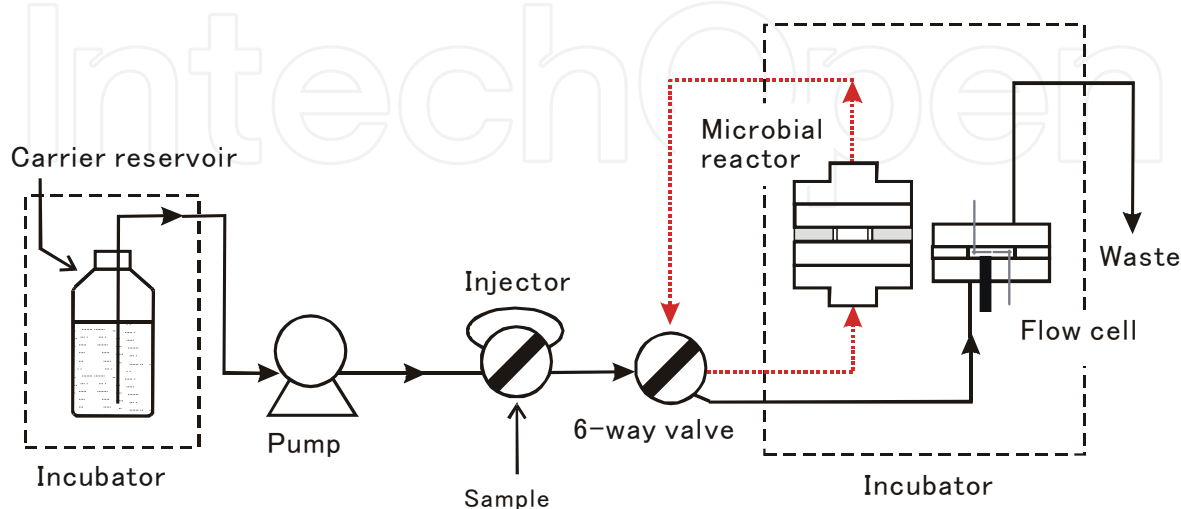

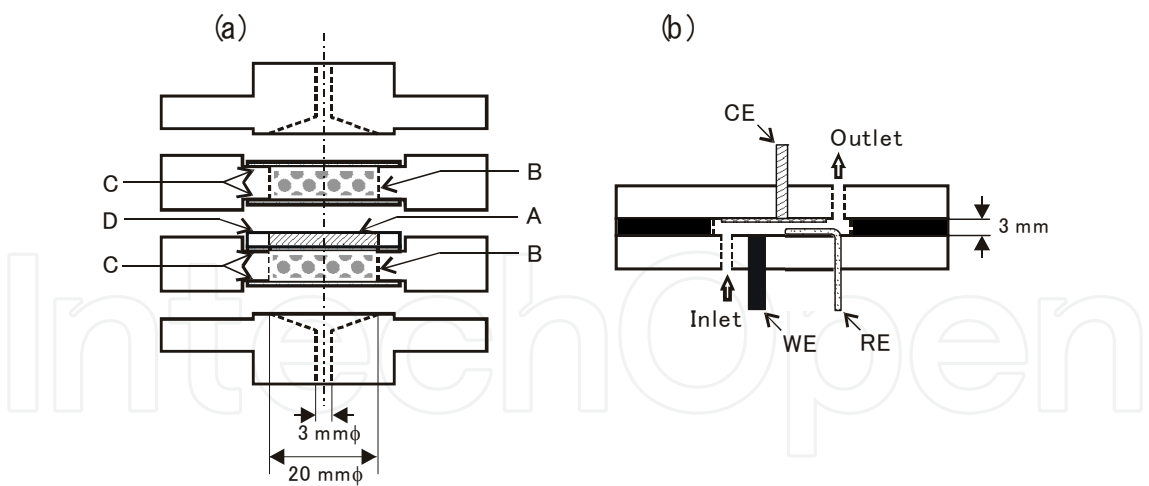Fig. 3. Schematic diagram of the 2,4-D sensor system.

Fig. 4. Schematics of the microbial reactor (a) and the flow cell (b). A: immobilized microbe, B: glass beads, C: SUS mesh screen, D: immobilized microbes holder.

Graphite electrodes were polished with 0.1 μm alumina powder, and rinsed thoroughly with deionized water. Then, these electrodes were sonicated in acetone and deionized water successively, and allowed to dry at room temperature. Tyrosinase (from mushroom, E.C. 1.14.18.1) was purchased from Sigma (St. Louis, MO, USA) and used without further purification. The enzyme activity was determined by using tyrosine as a substrate. An enzyme solution was prepared by dissolving 3.0 mg tyrosinase in 1.0 mL PBS (0.1 M; pH 7.0). The 20 μL of the tyrosinase solution (about 36 units) were deposited with a microsyringe on a graphite electrode surface, and allowed to dry at 4°C. Finally, the 20 μL of Nafion solution (Sigma-Aldrich, 0.5%) were deposited on the tyrosinase-coated electrode surface, and allowed to dry at 4°C for an overnight. The enzyme electrodes were stored in PBS (0.1 M, pH 7.0) at 4°C when not in use.
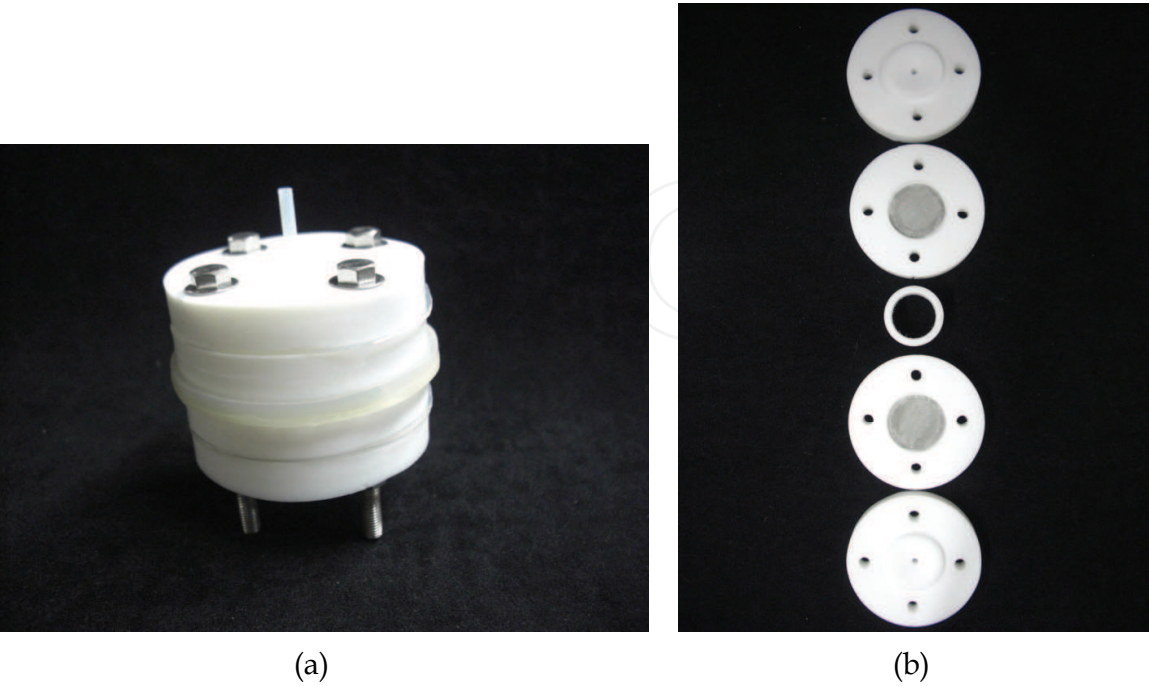

Fig. 5. Photographs of the reactor (a) and its parts (b).

3.4 Response measurement

0.1 M PBS (pH 6.0, 6.5, and 7.0) containing 0.1 M NaCl was used as the mobile phase. The carrier reservoir was hold in an incubator kept at 30°C, and the carrier solution was made to flow through the system at a constant flow rate. Amperometric measurements for the sensor system were made by applying a given potential on the working electrode (enzyme electrode) with a potentiostat (model HECS 318C, Huso Electro Chemical Systems, Kawasaki, Japan) connected to a personal computer. 2,4-D, 2,4-DCP and 3,5-DCC standard solutions for flow injection measurements were prepared with the PBS used as the mobile phase. The microbial reactor was filled with the mobile phase solution employed and left at 30 °C when not used for the measurements.

4. Performance and characteristics of sensor system

4.1 Sensor response of the tyrosinase-modified electrode

The enzyme tyrosinase catalyses two reactions; the hydroxylation of phenols to give catechols and the oxidation of catechols to o-quinones in the presence of oxygen. The determination of phenolic compounds with the tyrosinase modified electrodes can be based either on the electrochemical oxidation of catechols or the electrochemical reduction of quinones.
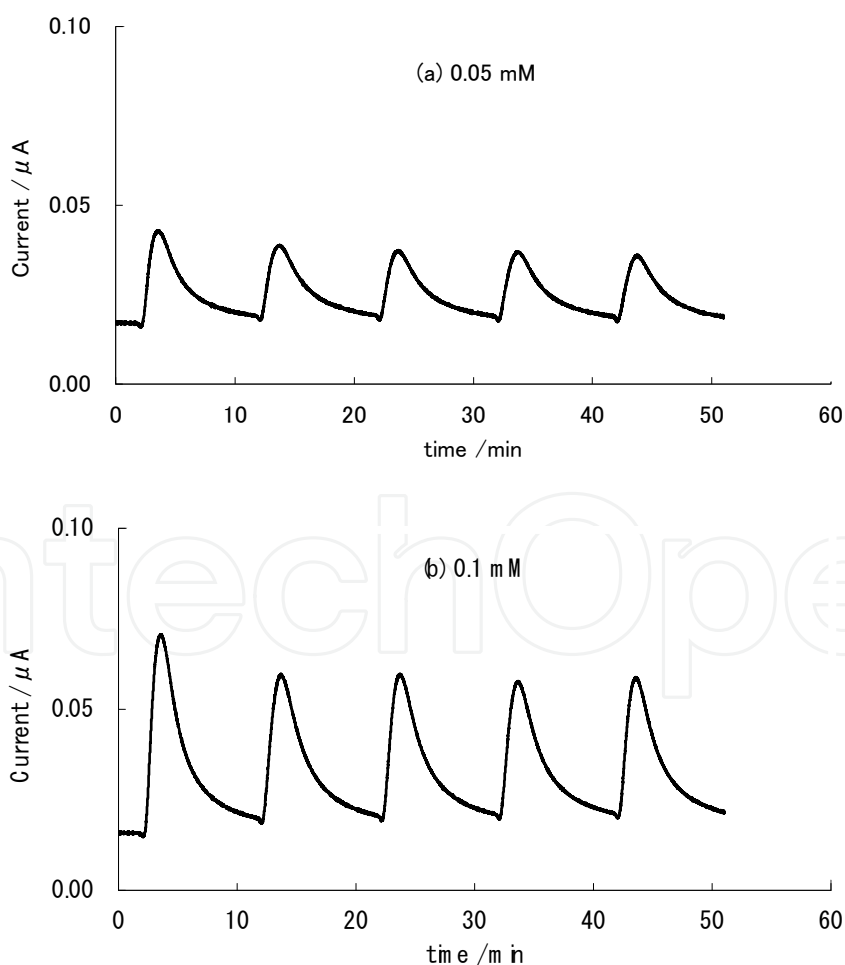

Fig. 6. Diagrams of amperometric responses obtained with the flow cell for successive injections of (a) 0.05 mM and (b) 0.1 mM 2,4-DCP (pH 6.5; H = 3 mm; flow rate 1.0 mL/min).

The oxidation on the electrode occurs at a relatively high potential, while the reduction at a low potential. Then, effects of the applied potential on the amperometric response for the tyrosinase electrode were first examined by applying a potential of ±0, +0.2, and +0.5 V (*vs.* Ag/AgCl). In this case the microbial reactor was bypassed and the substrate was sent directly to the flow cell. Consequently, the magnitudes of both the response and the residual currents were found to be affected seriously by the electrode potential, and the best sensitivity and baseline stability were obtained at +0.5 V as compared with those at ±0 and +0.2 V. Then the potential was fixed at +0.5 V and anodic output currents were monitored throughout the measurements.

Examples of amperometric responses obtained with the tyrosinase-modified electrode for successive injections of 2,4-DCP standard solutions with PBS (pH 7.0) containing 0.1 M NaCl as the mobile phase at the applied potential of 0.5 V and the flow rate of 1.0 mL/min are shown in Fig. 6(a) and 6(b). With an injection of the substrate, as it can be seen, an anodic peak was obtained as the electrochemical response. Both the peak current (height) and the peak area were found to increase with the concentration of 2,4-DCP injected. The average peak area determined for quintuplicate output signals was used to depict the calibration curve (Fig. 7). It can be seen that there exists not appreciable difference between the sensitivity for 2,4-DCP and that for 3,5-DCC. The lower detection limit for both compounds was 0.01 mM.

On the other hand, appreciable response current could not be observed when 1.0 mM 2,4-D standard solutions injected did not passthrough the microbial reactor and was sent directly to the tyrosinase electrode. This implies that 2,4-D is not detected electrochemically with the enzyme-modified electrode and either 2,4-DCP or 3,5-DCP is not significantly contained in the 2,4-D standard solution as an impurity.
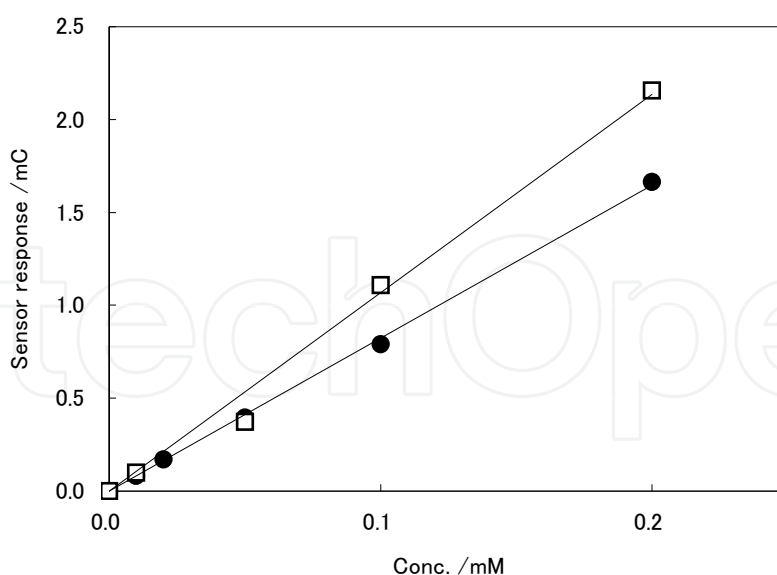

Fig. 7. Calibration plots for 2,4-DCP (•) and 3,5-DCC (□) determined with the flow cell.

4.2 Performance of 2,4-D biosensor system

The 2,4-D degradation activity of the strain was examined, as described before, using suspension of *Ralstonia* sp. in MS medium containing 2,4-D as a sole carbon source. In the

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case of immobilized cells, however, it is necessary to take into account the possibility of adsorption of the substrate by carrier particles employed for immobilization. Then, a fixed-bed reactor was constructed by replacing the glass column (8 mmϕ × 50 mm) of the system utilized for cell immobilization. After the immobilized cells or bare silica gel particles were packed into the column, a sterile MS medium (100 mL) containing 2,4-D prepared in a reservoir was circulated at a flow rate of 1.5 mL/min at 30°C for 12 h. The 2,4-D content was analyzed periodically with HPLC.

The time course of decrease in 2,4-D concentration observed for the immobilized microbial reactor is shown in Fig. 8. It is seen that 2,4-D is appreciably adsorbed by silica gel particles and its concentration is reduced to almost one-half its initial value for 12 h. The amount of adsorbed 2,4-D would decrease when microorganisms are immobilized onto the carrier particles. In the assay of the culture, on the other hand, the signal of 2,4-DCP could not be observed in the chromatogram. This may be due to the possibilities of adsorption of 2,4-DCP by the solid carrier and/or limitation of the detector of HPLC. Moreover, the initial rates of the decrease in 2,4-D content for both cases could be considered to be almost the same, suggesting that the degradation and the adsorption of 2,4-D might occur competitively when the substrate and the immobilized microbe particles come into contact in the reactor. Then, in order to eliminate the effect of adsorption on the silica gel surface, successive injections of the substrate was continued until stable response was obtained in the amperometric measurements.
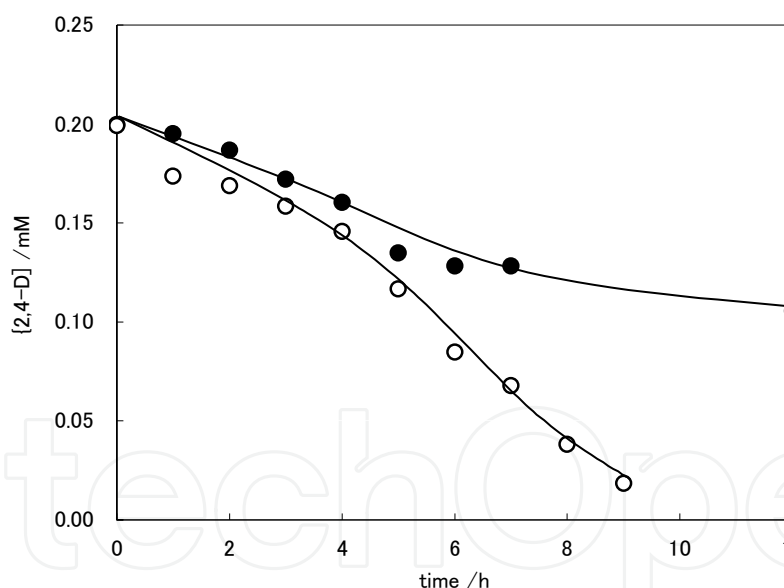

Fig. 8. Time course of 2,4-D concentration obtained for the fixed bed reactor packed with immobilized cells (○) and carrier particles alone (•).

Fig. 9 shows an example of amperometric response obtained with the sensor system employing *Ralstonia* sp. for which the immobilized cell holder of 3 mm in height was used. Using 0.1 M PBS (pH 6.5) containing 0.1 M NaCl as the mobile phase, 1.0 mM 2,4-D was injected successively at the flow rate of 1.0 mL/min. An anodic peak was observed each time with an injection of the substrate. It can be also seen not to take more than 10 min for each measurement. Response current could not be observed, as described before, when the substrate did not pass through the microbial reactor. This indicates the response of the

sensor system to be induced by the electrode reaction of electrochemically active substances such as phenolic and/or catecholic compounds, which could be produced by the microbial degradation of 2,4-D in the reactor. Then, the average peak area determined for triplicate output signals was used as the response of the sensor system.
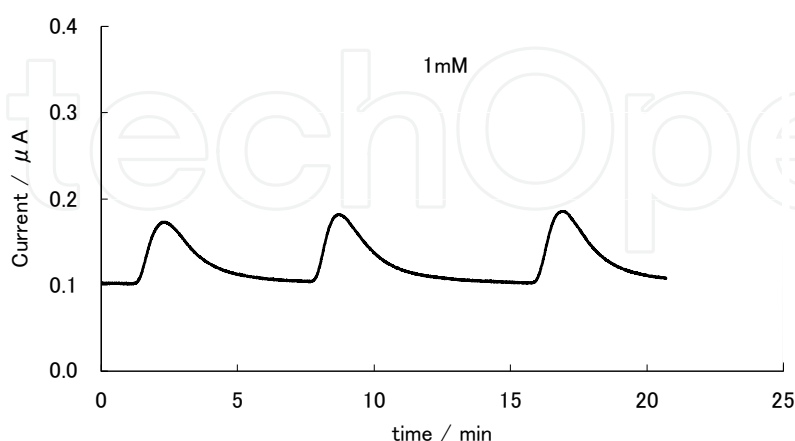

Fig. 9. Diagram of amperometric response obtained with the sensor system for successive injections of 2,4-D standard solution (pH 6.5; H = 3mm; flow rate 1.0 mL/min).
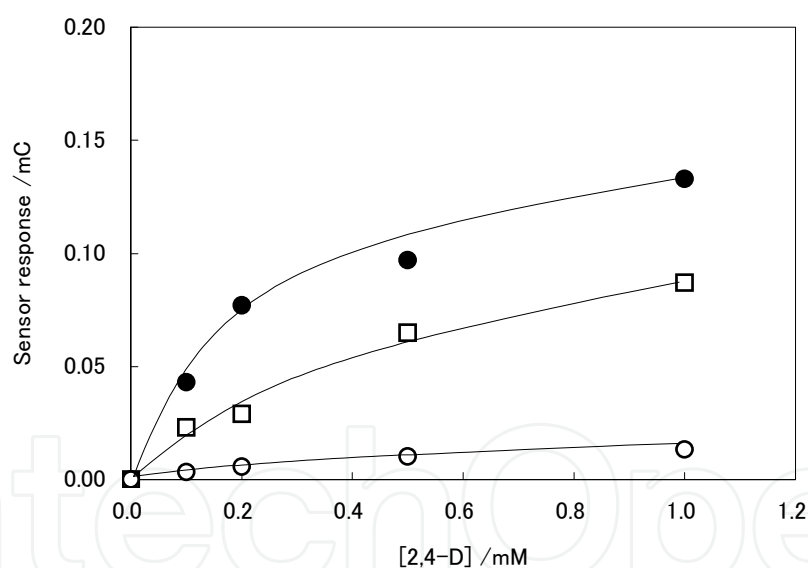

Fig. 10. Effect of the pH of carrier solution on the response of sensor system for 2,4-D; (●) pH 7.0, (□) pH 6.5, (○) pH 6.0 (H = 3 mm; flow rate 1.0 mL/min).

The effect of the pH of mobile phase solution on the response of sensor system was investigated using carrier solutions of pH 6.0, 6.5 and 7.0. The results are depicted in Fig. 10. The sensor response can be seen to increase with increasing pH of carrier solution in the range applied here. It has been reported that 2,4-dichlorophenoxyacetate/α-ketoglutarate dioxygenase, the enzyme responsible for the first step of degradation pathway for 2,4-D exhibits maximum activity at pH 6.5-7 (Fukumori & Hausinger, 1993b). On the other hand, the sensitivity of the tyrosinase-modified electrode for 2,4-DCP has been also observed to exibit almost the same pH dependence (the data are not shown). The pH dependence of the

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responsibility of present sensor system would result from the synergetic effect of the pH dependencies of enzyme activities for 2,4-D degradation and product detection.

Effect of the height of immobilized microbes bed on the responsibility of sensor system is shown in Fig. 11. It is seen that the response obtained by using the bed of 12 mm in height is considerably enhanced when compared with that observed with the bed of 3 mm in height. As the immobilized microbes bed becomes higher both the residence time and the amount of biocatalyst in the reactor increase. It is reasonable that the amount of degradation product increase with increasing the height of biocatalyst bed. Thus, employment of higher bed in the reactor is considerably effective to enhance the sensitivity of the sensor system, while it has been found to require longer time for a measurement (about 20 min at flow rate of 1.0 mL/min).

The flow rate of carrier solution has been also found to considerably affect the sensor response. Calibration plots obtained by the sensor system for 2,4-D with different flow rates are shown in Fig. 12. As can be seen from the figure, the sensitivity of the sensor system was appreciably enhanced by lowering the flow rate. The residence time in the reactor increases with decreasing the flow rate, which exerts almost the same effect as that of the height of biocatalyst bed. It is also confirmed that lowering the flow rate enhances the sensitivity of tyrosinase-modified electrode in the flow cell. The result obtained here is considered to be induced by combined effects of the flow rate on the reactor and the flow cell. In any event, it is evident that the sensitivity of sensor system can be improved by making the carrier solution to flow at a reduced rate, while it takes longer time for a measurement.
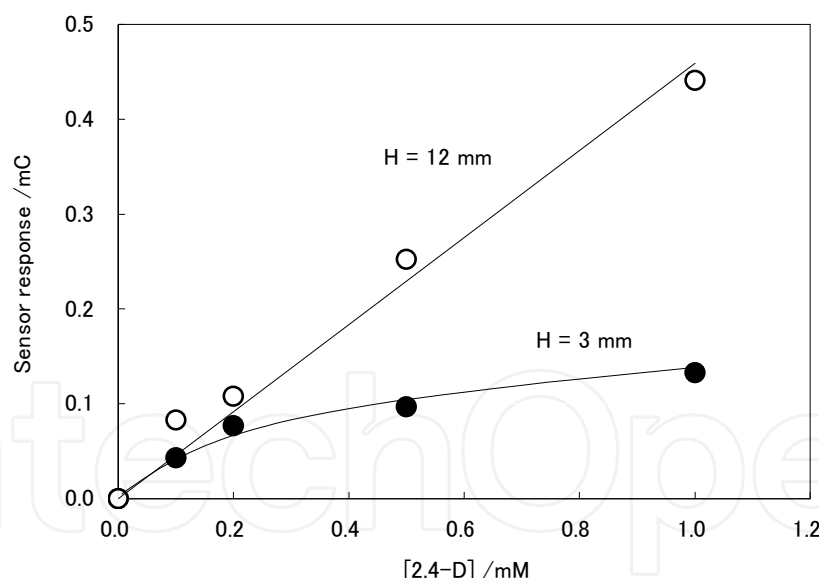

Fig. 11. Effect of the height of immobilized microorganisms bed on the response of sensor system for 2,4-D (pH 7.0; flow rate 1.0 mL/min).

The sensitivity of the sensor system was found to be almost unchanged during the measurements for one week when the reactor and the tyrosinase electrode were stored as described before. The lower detection limit and the detection range at the present state were 0.1 mM and 0.1 – 1 mM, respectively for 2,4-D. There is a possibility, however, that the sensitivity of the present sensor system can be enhanced by improving the performance of biocatalyst and/or by applying more effective detection method.
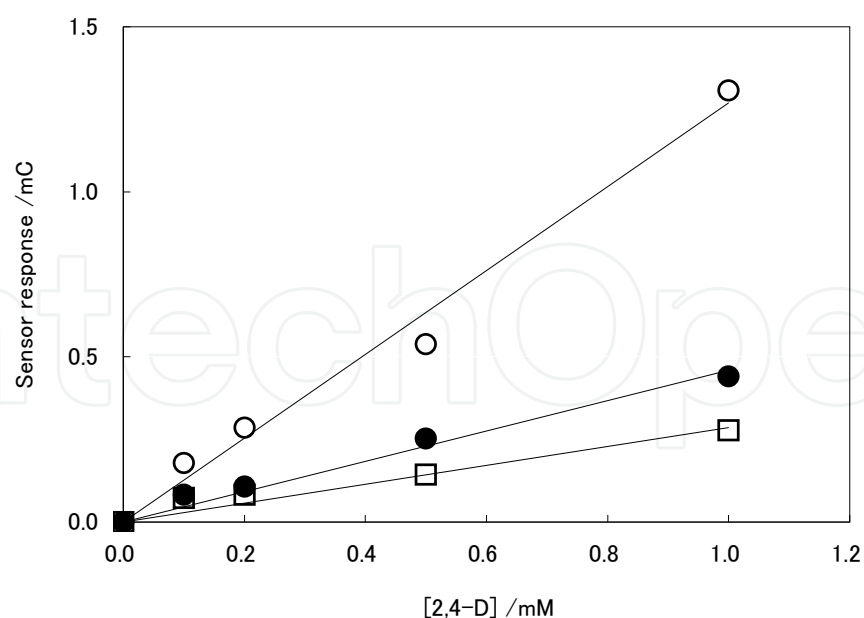
Fig. 12. Effect of the flow rate of carrier solution on sensitivities of the sensor system for 2,4-D; (○) 0.5, (●) 1.0, (□) 2.0 mL/min. (*H* = 12 mm, pH 7.0).

5. Conclusions

A novel flow-injection biosensor system for 2,4-D detection consisting of the microbial reactor for substrate degradation and the enzyme electrode for product detection was demonstrated. The most remarkable feature of the sensor system is to utilize a 2,4-D-degrading microorganism such as *Ralstonia* sp. which produces phenolic compounds at initial stages in the degradation pathway. The 2,4-D degrader was immobilized on silica gel particles and a fixed-bed bioreactor was constructed. The resulting 2,4-DCP and/or 3,5-DCC were detected amperometricaly with the tyrosinase-modified electrode. The sensitivity of the system was found to be considerably affected by pH and the flow rate of carrier solution. The height of immobilized microorganism bed also exerted reasonable effect on the responsibility. Although the sensitivity is not high enough for microanalysis at the present stage, further improvement of the responsibility is possible. The strategy proposed here can be easily extended to other biosensor development.

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