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Degradation of Bisphenol A by Using an Immobilized Laccase Column.

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Application of an immobilized laccase column for degradation of bisphenol A (BPA) was investigated. Laccase from *Trametes* sp. was covalently immobilized onto alkylaminated controlled-Pore Glass (CPG). This immobilized preparation was packed into a small polymer column and then mounted in a water-jacketed holder. A biodegradation system for BPA was assembled with a peristaltic pump, a rotary injection valve with a sample loop, the enzyme column unit, a flow-though type of oxygen electrode, and a pen recorder. Since laccase demands the dissolved oxygen for oxidation of the substrate, the activity of laccase is measured by detecting the consumption of dissolved oxygen with the oxygen electrode. Various concentrations (0.1 – 1.0 mM) of BPA solutions (100 μl) were injected into this system, and amount of dissolved oxygen was monitored. A linear relationship was obtained in a range of 0.1 – 1.0 mM and the calculated coefficient was to be 0.999. This result means that the conversion of BPA by laccase was performed quantitatively. To evaluate the removal of BPA, amount of BPA before or after passing through the column was determined by HPLC. Determination of residual BPA by HPLC showed that 97 % of BPA was degraded when 0.1 mM BPA solutions was injected into this system. These results demonstrated that the immobilized laccase should be effective element in degradation of a BPA.

Keywords Flow injection analysis, bisphenol A (BPA), removal, laccase, immobilization

1. Introduction

Bisphenol A (2,2-bis(4-hydroxyphenol)propane; BPA) is widely used in a variety of industrial and residential applications, such as synthesis of polycarbonate [1]. The BPA-contained water is discharged as a by-product [1]. This causes serious environmental concern because BPA is known about one of the endocrine disrupting chemicals due to bind to estrogen receptor and to regulate the activity of estrogen responsive genes [2, 3]. Therefore, the useful method for removal of BPA from wastewater has been demanded.

Laccase and manganperoxidase are typical oxidases able to catalyze the oxidation of a wide variety of substrates [4-7], such as phenols, aromatic amines, benzenethiols, hydroxyindoles and phenothiazinic compounds, with the simultaneous reduction of oxygen to water. The low substrate specificity exhibited by laccase and its ability to oxidize priority pollutants have attracted interest for its use in wastewater treatment and bioremediation [8]. Many industrial applications of these enzymes have been reported and recognized in biomechanical pulping [9], biobleaching [10 – 12], and degradation of dioxins [13], of chlorophenols [14], of nylon [15, 16], of polyethylene [17, 18], of dyes [19], and so on. Moreover, recently, several researches to remove BPA by laccase have been reported [20 – 23]. However, in these reports, the laccase was used as the water-soluble form and applied to disporsable use. In our laboratory, immobilized laccase has been used as a recognition element for the biosensing [24, 25]. In general, immobilized enzymes can be applied to long-term operation and the small amount of the preparations is sufficient for the reaction. Therefore, use of the immobilized enzyme has many advantages over that of enzyme solution. In this study, we developed a system for removal of BPA by using immobilized laccase, and investigated the properties of the laccase activity and the stability.

2. Experimental

2.1. Materials and reagents.

Laccase, polyphenol oxidase, (EC 1.10.3.2, from *Trametes* sp.) was generously donated by Daiwa Kasei Co., Ltd. (Osaka). Controlled-Pore glass (CPG, mean pore diameter 24.2 nm, particle size 120 – 200 mesh) was purchased from Funakoshi Co., Ltd. (Tokyo). Bisphenol A (BPA) was purchased from Kanto Chemical Co. Inc., Tokyo. All other reagents were commercially available and of analytical grade. Ultrapure water with a resistivity of 18.2 MΩ-cm was obtained with an EQG-3S system (Millipore, Tokyo).

2.2. Immobilization of laccase.

Laccase was covalently immobilized onto alkylaminated CPG as described previously [26]. The glass beads (3.3 g) was boiled in nitric acid (5 %, 150 ml) for 45 min on hot plate. After allowed to stand at a room temperature, the beads were recovered with a glass filter and washed with pure water (2 l) and then dried in an oven at 95 ℃. The washed beads were alkylaminated with 10 % γ-APTES (γ-aminopropyltriethoxysilane; pH 3.45) in a shaking water-bath at 75 ℃ for 3 hours. Then, the glass beads were collected by filtration and

washed with pure water (20 x 3 ml), and the beads were dried in an oven (115 ℃ , over night). The alkylamninated glass beads (1.0 g) were activated with glutaraldchydc (2.5 %, 25 ml) undcr rcducd prcssurc, and then, laccase solution (120 mg/ml, 9.0 ml) prepared in phosphate buffer (50 mM, pH 7.0) was coupled with the glass beads with shaking at 4 ℃ for 50 hours. Finally, the immobilized preparations were treated with 10 % sodium borohydrate solution that was prepared by mixing with 0.2 M phosphate buffer (pH 5.0, 5.0 ml), 0.1 M NaH_2PO_4 (1.0 ml) and 4.0 ml pure water. The reducing process was repeated ten times and then washed with phosphate buffer (0.1 M, pH 7.0). The yield was calculated by measuring absorbance at 280 nm in the enzyme solutions before and after the coupling process. The immobilization yield was 89 %.

2.3. Apparatus and flow system.

The immobilized enzyme column (0.3 ml packed volume) was used as a recognition and degradation element for BPA. A schematic diagram of the flow system was shown in Fig. 1. The system was assembled with a peristaltic pump (flow rate 1.0 ml/min, Bio-minipump; ATTO Co., Tokyo), a Teflon rotary injection valve (Sample Injection Valve, Cat. No. 5020; Rheodyne Inc., California, U.S.A.) with a 100 μl sample loop, the laccase column surrounded by the water jacket maintained at 303 K, the flow-through type of a

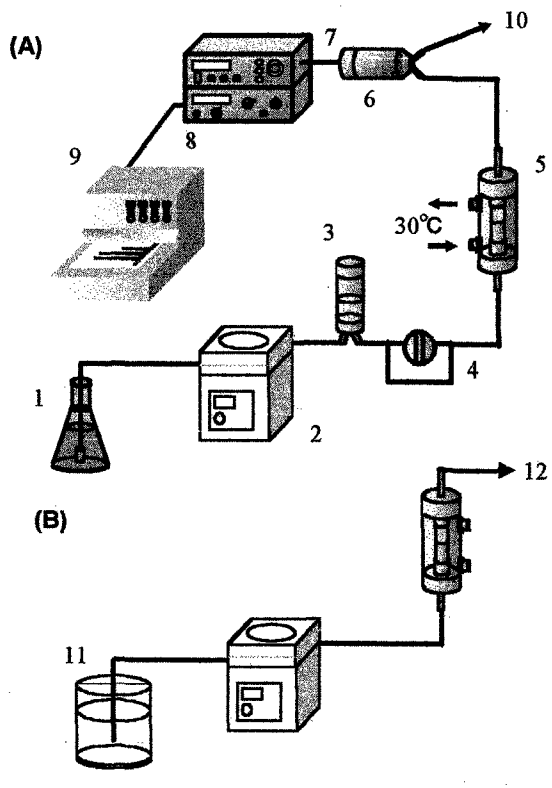

Fig. 1 System for measurement of a laccase column activity (A) and a system for removal of BPA by laccase column (B). 1) Carrier reservoir, 2) Peristaltic pump, 3) Damper, 4) Injection valve, 5) Laccase column mounted into water jacket, 6) O_2 electrode, 7) Galvanostat, 8) Electrometer, 9) Pen recorder, 10) Waste, 11) Sample reservoir, 12) collector

polarographic oxygen electrode for monitoring the dissolved oxygen enzymatically consumed, the potentio-galvanostat (Potentiostat/Galvanostat HA-301; Hokuto Denko Corporation, Tokyo, Japan), the ammeter (Zero Shunt Ammeter HM-104; Hokuto Denko Corporation) and the pen recorder (Multi-Pen Recorder; RIKADENKI Co., Ltd., Tokyo).

2.4 Evaluation of activity of the immobilized laccase column.

Citrate buffer solution (50 mM) as the carrier was continuously pumped through the system. The sample solutions were introduced into the system through the rotary injection valve. The catalytic activity of the enzyme-packed column was assessed by injecting 100 μl of BPA solutions with various concentrations. The BPA solution was dissolved with ethanol at the concentration 100 mM, and then, the solution was diluted with citrate buffer. Variation in the amount of dissolved oxygen caused by the laccase-catalyzed reaction was monitored by the polarographic oxygen electrode and then recorded.

2.5 Investigation of degradation of BPA by the immobilized laccase column.

To investigate the removal activity of the laccase column, the concentrations of BPA solutions before or after through the column was measured. Citrate buffer (50 mM, pH 5.0) was used as a carrier solution. Ten milliliter of BPA solutions at the concentration of 100 μM was introduced into the FIA system, and the solutions which passed through the column were collected. The concentration of the BPA in the collected solution was determined by reverse phase high performance liquid chromatography (RP-HPLC) analysis. The RP-HPLC was performed on a Inertsil ODS-3 column (5μ C_{18}-100 Å, 4.6 x 150 mm, GL Sciences Inc., Tokyo) in a JASCO LC-2000 Plus system (JASCO Co., Tokyo; eluent: linear gradient from 70 % to 100 % of methanol in water (for 8 min), flow rate: 1.0 ml/min). Detection was carried out with a UV detector at 276 nm (UV-970, JASCO Co., Tokyo). Gas chromatography-mass spectrometry (GC-MS) spectra used to identify the decomposed product were measured with a Hewlett Packard 5890 II gas chromatography linked to a JMS-SX102A mass spectrometer (JEOL Ltd.) (column: DB-5 column, J & W Scientific, 15 m x 0.25 mm; line: He flow at 1 ml/min; column temperature profile: 50 ℃ for 2 min, increasing from 50-120 ℃ at 20 ℃/min, and from 120-220 ℃ at 5 ℃/min, 250 ℃ for 10 min; electron potential: 70 eV).

3. Results and Discussion

3.1. Immobilized enzyme characterization.

The optimum pH of the free laccase was already investigated previously and the value was known to be 5.0. However, in general, the optimum pH of many enzymes was altered by immobilization and then the optimum pH of the immobilized laccase column was tested. To examine the optimum pH of the immobilized laccase, 100 μl of 1.0 mM BPA solutions prepared at various pH with citrate (pH 4.0 – 6.0) and phosphate

(pH 6.0 – 7.0) buffer was injected into the FIA system. The activity of the laccase column was measured by detecting the dissolved oxygen consumed with an oxygen electrode, since laccase demands the dissolved oxygen for oxidation of the substrate. As shown in Fig.2, the laccase column was active in the observed pH range, and the peak of the laccase activity was obtained at the pH 5.0.

To evaluate the activity of the immobilized laccase column, various concentrations (0.1 – 1.0 mM) of BPA solutions (100 μl) were introduced into this FIA system. Figure 3 shows the variation in current responses of the oxygen electrode obtained in the laccase reaction. A linear relationship was observed in a range of 0.1 – 1.0 mM and the calculated coefficient was 0.999. One assay took 5 min or shorter for the responses to BPA solutions.

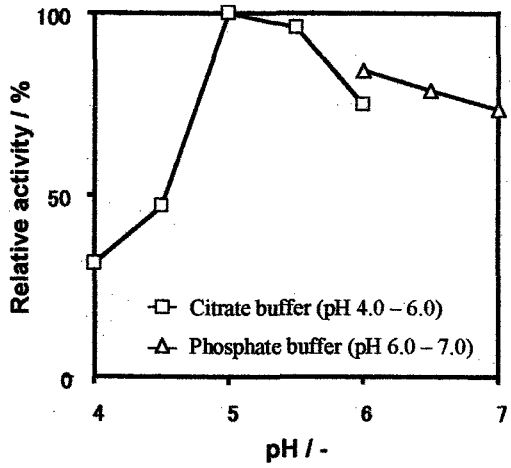

Fig. 2 Effect of the pH on the reaction rate of the immobilized laccase.

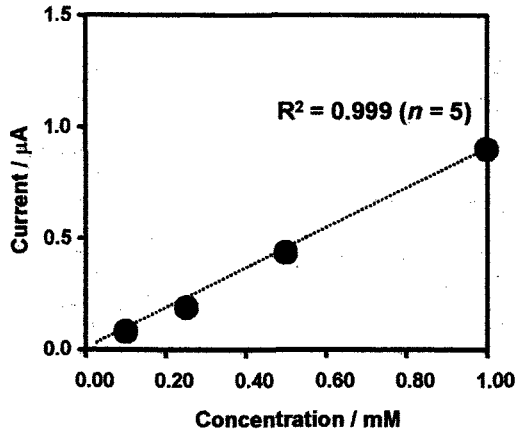

Fig. 3 Calibration graph for BPA solution by using the amperometric FIA system.

3.2. Removal of BPA by the laccase column.

The activity of removal of BPA by the immobilized laccase column was investigated by using the system shown in Fig. 1 (B). A 10 ml of 100 μM BPA solutions was introduced into the system at various flow rates, and collected to determine the amount of residual BPA. The concentration of the solution through the column was determined by injection of 100 μl of the solution into RP-HPLC system. The collected solutions were introduced into the laccase column. This procedure was repeated until the BPA couldn't be detected. As shown in Fig. 4, in each of flow rate, the amount of BPA was immediately decreased by the laccase column. When the flow rate was 0.25 ml/min, BPA was almost removed by a single introduction into the laccase column, and before the fourth time of introducing of the collected solution, BPA was removed completely. In the case of the flow rate at 2.0 ml/min, BPA was degraded more than 80 % by the first single injection of the 100 μl of 100 μM BPA solution into the column. The BPA in the solution treated with the laccase column at fifth time couldn't be detected. It means that a 134 μg of BPA was removed completely for 30 minutes. At each of flow rate, the BPA was removed completely within 90 minutes. The weight of the immobilized laccase packed in the column was calculated at 13.6 mg. Therefore, these results indicate that this laccase column reactor would be applicable to the high performance system for BPA removal.

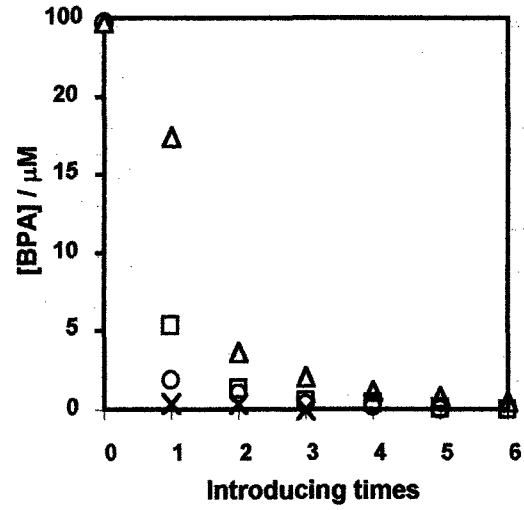

Fig. 4 Evaluation of removal of BPA by the immobilized laccase column and effect of flow rates on the activity. The flow rate as follows: 0.25 ml/min (×), 0.5 ml/min (○), 1.0 ml/min (□), 2.0 ml/min (△).

3.3. Stability of the laccase column in comparison with free laccase.

The stability of the immobilized laccase was evaluated and compared with that of free laccase. The ability of removal of BPA by the laccase column when 100 μl of 100 mM BPA solutions introduced into the column (flow rate: 1.0 ml/min) was shown in Figure 5 (A). No noticeable differences in the activity of the laccase column before and after 50 times injections of BPA solutions were exhibited.

Figure. 5 (B) shows changes in the activity of the immobilized and free laccase at a room temperature during 10 days. The activity of the free laccase decreased immediately and the activity was reduced to below half within only first 2 days. On the other hand,

the immobilized laccase kept the initial activity during 10 days. Further investigations showed that the laccase column didn't vary in its activity more than 2 months.

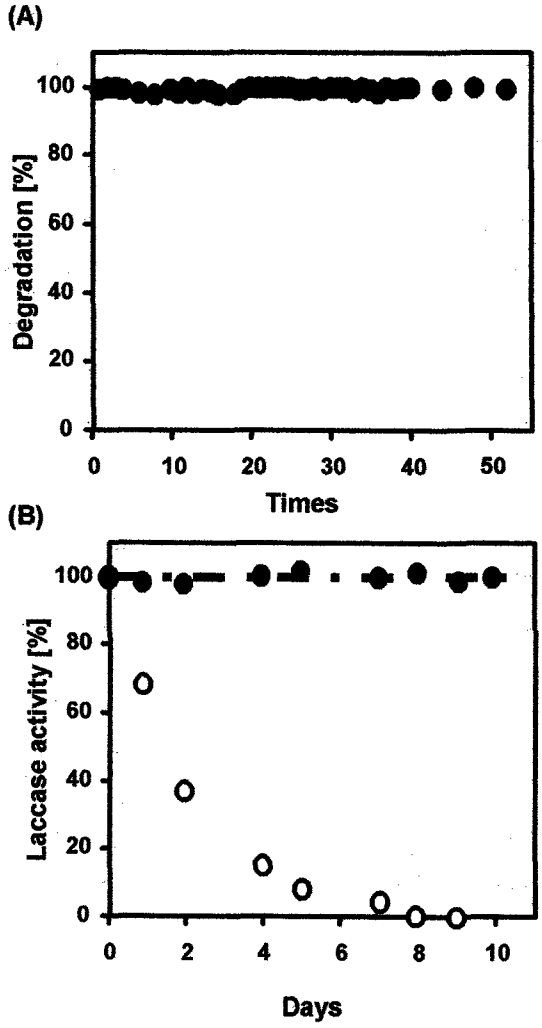

Fig. 5 Stability of the laccase activity for removal of BPA. (A) Repeatability of the activity by injection of BPA in many times into the immobilized laccase column. (B) Comparison of the stability with the immobilized laccase and the free laccase. ●: immobilized laccase, ○: free laccase

4. Conclusion

In this study, we developed the effective system for removal of BPA by using the immobilized laccase and investigated the characteristic performance of the system. The optimum pH for the laccase column was to be 5.0. A ten milliliter injection of 100 μM BPA solution caused complete removal of BPA attributable to catalytic activity of the small laccase column (packed volume was 0.5 ml, the immobilized amount was 13.6 mg) within 30 minutes. The activity of the column was kept constant more than 2 months. These results demonstrate that the proposed laccase column should be a powerful tool for removal of BPA.

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