Biodegradation of Endocrine-disrupting Phenolic Compounds Using Laccase Followed by Activated Sludge Treatment

Yoshitoshi Nakamura[1]* and Godliving Mtui[2]

[1] Department of Chemistry and Chemical Engineering, Faculty of Engineering, Kanazawa University, 2-40-20 Kodatsuno, Kanazawa 920-8667, Japan
[2] Applied Microbiology Unit, University of Dar es Salaam, P. O. Box 35060 Dar es Salaam, Tanzania

Abstract Endocrine-disrupting phenolic compounds in the water were degraded by laccase from *Trametes* sp. followed by activated sludge treatment. The effect of temperature on the degradation of phenolic compounds and the production of organic compounds were investigated using endocrine-disrupting chemicals such as bisphenol A, 2,4-dichlorophenol, and diethyl phthalate. Bisphenol A and 2,4-dichlorophenol disappeared completely after the laccase treatment, but no disappearance of diethyl phthalate was observed. The Michaelis-Menten type equation was proposed to represent the degradation rate of bisphenol A by the lacasse under various temperatures. After the laccase treatment of endocrine-disrupting chemicals, the activated sludge treatment was attempted and it could convert about 85 and 75% of organic compounds produced from bisphenol A and 2,4-dichlorophenol into H_2O and CO_2, respectively.

Keywords: activated sludge, biodegradation, endocrine-disrupting chemical, laccase, phenolic compound, wastewater treatment

INTRODUCTION

Recently a lot of evidence on the hormonelike effects of many xenobiotics in fish, animals, and human beings has been reported [1-3]. The endocrine-disrupting chemicals, *i.e.* chemicals that disrupt hormonal signals in living organisms, are used on factories, farmlands, golf courses, and other areas in many countries. Since factories, farmlands, and golf courses connect closely with water systems, *i.e.* rivers, lakes, and coastal seas, the endocrine-disrupting chemicals cause water pollution and provide a bad influence on the natural water ecosystem. To prevent destroy of ecosystem the direct treatment of wastewater containing endocrine-disrupting chemicals using microorganisms seems to be one of the most effective methods. However, the endocrine-disrupting chemicals have complex structures and aromatic rings that are hardly degraded by microorganisms [4,5]. On the other hand, lignin-degrading enzymes from *Basidiomycetes* such as lignin peroxidases, manganese peroxidases, and laccases have been known to cleave the aromatic rings of phenolic compounds [6-9]. Therefore, the treatment using lignin-degrading enzyme followed by microorganisms seems to be an effective method for the complete degradation of endocrine-disrupting chemicals into H_2O and CO_2.

This work investigated the operational conditions for the efficient treatment of endocrine-disrupting phenolic compounds by laccase and the degradation of organic compounds produced using microorganisms such as activated sludge.

***Corresponding author**
Tel: +81-76-234-4819 Fax: +81-76-234-4829
e-mail: ynakamu@t.kanazawa-u.ac.jp

MATERIALS AND METHODS

Materials

Bisphenol A, 2,4-dichlorophenol, and diethyl phthalate were purchased from Wako Pure Chemical Co. Ltd., Osaka, Japan.

Laccase produced from *Trametes* sp. (Laccase Daiwa, Daiwa Kasei Co. Ltd., Osaka, Japan) was used for the biodegradation of endocrine-disrupting chemicals.

Activated sludge from Kanazawa municipal wastewater treatment plant was used for the biodegradation of organic compounds produced from endocrine-disrupting chemicals by laccase treatment.

Biodegradation of Endocrine-disrupting Chemicals

Laccase treatment were carried out using the reaction mixture containing 5-100 ppm of endocrine-disrupting chemicals and 0.8 U/mL laccase in a 300 mL Erlenmeyer flask containing 100 mL of 0.1 M phosphate buffer (pH 7) at 100 rpm under various temperatures of 5-70°C. In this experiment, the laccase treatment was carried out at pH 7, because pH of river and lake water contaminated by endocrine-disrupting chemicals seems to be about 7. After the laccase treatment for 0-48 h, 1 mL activated

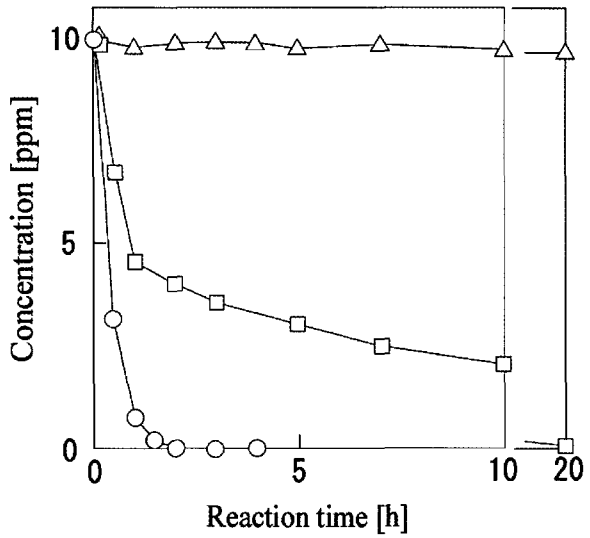

Fig. 1. Degradation of bisphenol A, 2,4-dichrolophenol, and diethyl phthalate by laccase at 30°C. Symbols: ○, bisphenol A; □, 2,4-dichrolophenol; △, diethyl phthalate.

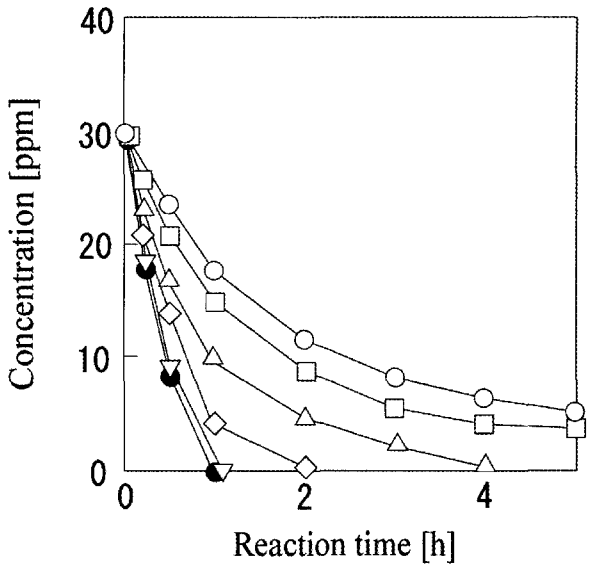

Fig. 2. Degradation of bisphenol A by laccase under various temperatures. Symbols: ○, 5°C; □, 10°C; △, 20°C; ◇, 30°C; ▽, 40°C; ●, 50°C.

sludge was added into the reaction mixture and then it was incubated at 37°C for 0-72 h.

Analysis

The concentrations of endocrine-disrupting chemicals were measured by high performance liquid chromatography (HPLC) with Wakosil Agri-9 column (Wako Pure Chemical Co. Ltd., Osaka, Japan). The sample was eluted with 45% acetonitrile containing 50 mM KH_2PO_4, H_3PO_4 buffer (pH 3.7) at a rate of 1.0 mL/min (LC-10AD, Shimadzu Co. Ltd., Kyoto, Japan) and analyzed at 275 nm by a UV detector (SPD-10AV). Chemical oxygen demand (COD) of sample was measured following Japanese Industrial Standards method (JIS K0102).

RESULTS AND DISCUSSION

Treatment of Endocrine-disrupting Chemicals by Laccase

Fig. 1 shows the degradation of endocrine-disrupting chemicals, *i.e.* bisphenol A, 2,4-dichlorophenol, and diethyl phthalate, by laccase at pH 7 and 30°C. The substrate concentration and the laccase concentration were 10 ppm and 0.8 U/mL, respectively. The concentrations of bisphenol A and 2,4-dichlorophenol decreased with the increase of reaction time reaching almost 0 at a reaction time of 2 and 24 h, respectively. The concentration of diethyl phthalate, however, remained 10 ppm without degradation even for 24 h of reaction time. The reason why no disappearance of diethyl phthalate was observed by laccase treatment seems to be that the laccase can oxidize phenolic compounds while the diethyl phthalate does not have a phenolic structure [10]. Furthermore, the slow disappearance rate of 2,4-dichlorophenol depended on the fact that it has a chlorine group tolerable to degradation by lignin-degrading enzyme [11,12].

Fig. 2 shows the time courses of bisphenol A concentration in the laccase treatment under various temperatures from 5 to 50°C. The bisphenol A concentration decreased gradually from its initial value of 30 ppm with the increase of reaction time and then reached almost 0 after a few hours. The disappearance rate of bisphenol A became greater with the increase of temperature and reached a maximum value beyond 40°C. At high temperatures of 40 and 50°C, the bisphenol A concentration became almost 0 after only an hour of reaction time. On the other hand, since the complete disappearance of bisphenol A was also observed at 5 or 10°C, it seems that the laccase treatment can be applicable to the degradation of bisphenol A even under a comparatively low temperature environment.

Fig. 3 shows the time courses of bisphenol A concentration in the laccase treatment under various initial concentrations from 5-100 ppm at 30°C. The bisphenol A concentration decreased with the reaction time and then reached almost 0 after a reaction time of 0.5-2.5 h. The complete disappearance of 100 ppm bisphenol A for 2.5 h of reaction time suggests that the laccase treatment is effective for bioremediation of water or soil contaminated with a comparatively high concentration of bisphenol A.

In order to express the disappearance rate equation of bisphenol A by laccase, Michaelis-Menten equation [13], was used:

$$V = \frac{V_m S}{V_L + S} \quad (1)$$

where V, V_m, K_m, and S are reaction rate, maximum re-

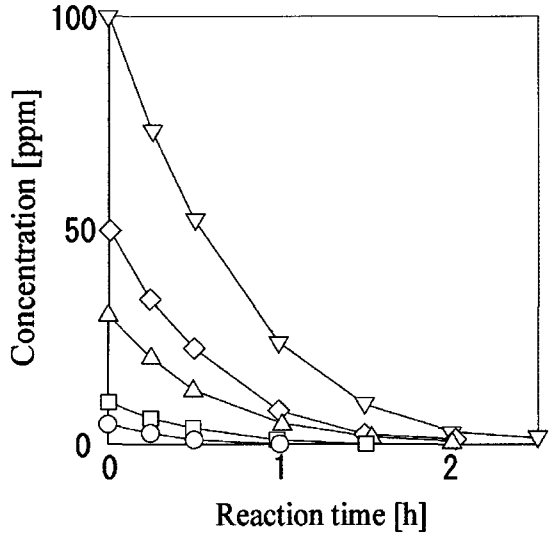

Fig. 3. Degradation of bisphenol A by laccase under various initial concentrations of bisphenol A at 30°C. Symbols: ○, 5 ppm; □, 10 ppm; △, 30 ppm; ◇, 50 ppm; ▽, 100 ppm.

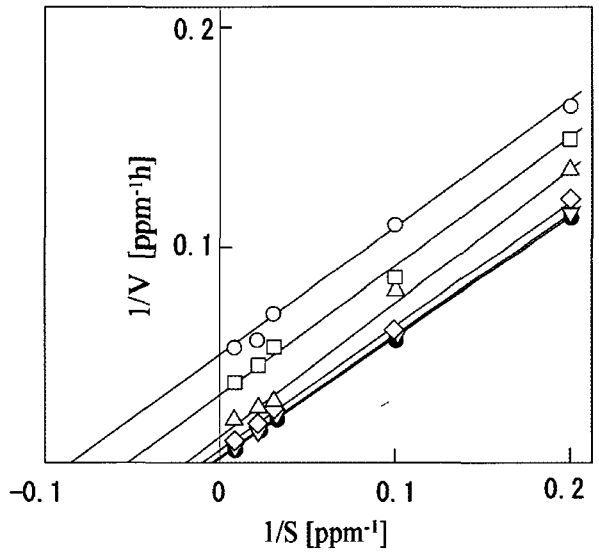

Fig. 4. Linweaver-Burk plots between reaction rate, V, and bisphenol A concentration, S, under various temperatures. Symbols: ○, 5°C; □, 10°C; △, 20°C; ◇, 30°C; ▽, 40°C; ●, 50°C.

action rate, Michaelis constant, and substrate concentration, respectively. Straight correlations between $1/V$ and $1/S$ were confirmed under various temperatures of 5-50°C and the parameter values of V_m and K_m were determined directly from Fig. 4.

Fig. 5 plots the maximum reaction rate and the Michaelis constant against a reciprocal of temperature. These plots from 3.6×10^{-3} K^{-1} (T=5°C) to 3.2×10^{-3} K^{-1} (T=40°C) obeyed Arrhenius reaction, *i.e.* straight relationships were obtained, and the activation energies concerning maximum reaction rate and the Michaelis constant calculated from the slopes are 12,720 and 12,410 cal/mol, respectively. Since the plots at 3.12×10^{-3} K^{-1} (T=50°C) were away from the straight lines; they were almost same as those at 3.22×10^{-3} K^{-1} (T=40°C), it was confirmed that Arrhenius reaction held from 5 to 40°C in the disappearance of bisphenol A by laccase.

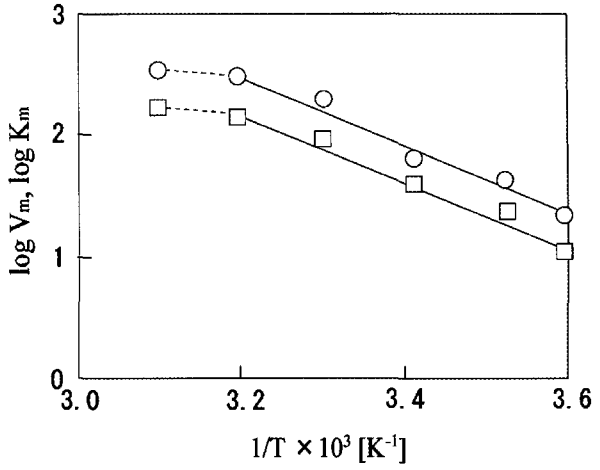

Fig. 5. Effect of temperature, T, on maximum reaction rate, V_m, and Michaelis-Menten constant, K_m, in the degradation of bisphenol A by laccase. Symbols: ○, V_m; □, K_m.

Activated Sludge Treatment of Endocrine-disrupting Chemicals Treated by Laccase

Fig. 6 shows the change of COD in the activated sludge treatment of endocrine-disrupting chemicals, *i.e.* bisphenol A and 2,4-dichlorophenol, treated by laccase. The laccase and activated sludge treatments were carried out at 30 and 37°C, respectively. The same initial values of COD of untreated and treated endocrine-disrupting chemicals suggest that the laccase treatment could extinguish endocrine-disrupting chemicals but not converted them up to H_2O and CO_2, so for the complete degradation of endocrine-disrupting chemicals a further degradation treatment, *i.e.* an activated sludge treatment, is necessary and carried out in this work. No decrease in COD of both untreated bisphenol A and untreated 2,4-dichlorophenol were observed even if the activated sludge treatment was carried out for a comparatively long time, *i.e.* 3 days. This means that the activated sludge that contained many microorganisms could not degrade bisphenol A and 2,4-dichlorophenol at all due to their aromatic ring structures reluctant to microbial degradation. The COD of bisphenol A treated by laccase for 1, 2, and 6 h decreased from its initial value of 120 ppm with the increase of incubation time and then reached their constant values, 40, 25, and 20 ppm, respectively. On the other hand, the COD of 2,4-dichlorophenol treated by laccase for 6, 24, and 48 h decreased from its initial value of 140 ppm with the increase of incubation time and then reached their constant values, 60, 35, and 35 ppm, re-

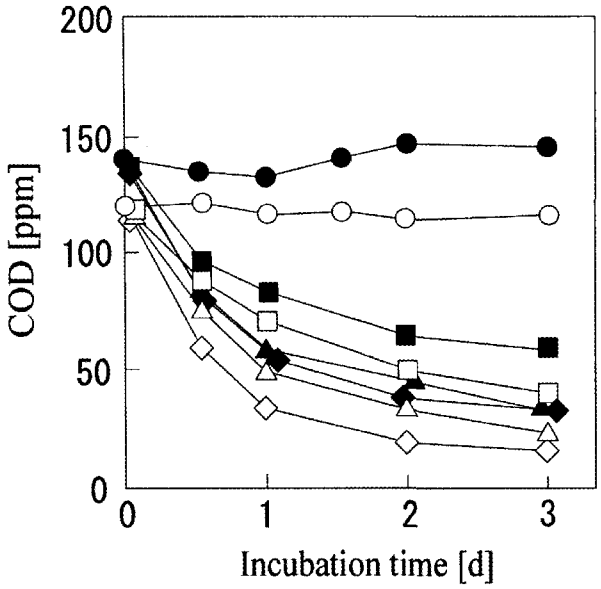

Fig. 6. Time course of COD in the activated sludge treatment of bisphenol A and 2,4-dichlorophenol treated by laccase. Symbols: ○, untreated bisphenol A; □, bisphenol A treated for 1 h; △, bisphenol A treated for 2 h; ◇, bisphenol A treated for 6 h; ●, untreated 2,4-dichlorophenol; ■, 2,4-dichlorophenol treated for 6 h: ▲, 2,4-dichlorophenol treated for 24 h; ◆, 2,4-dichlorophenol treated for 48 h.

spectively. The reason why the time courses of COD in the 2,4-dichlorophenol treated by laccase for 24 and 48 h were almost same seems to be that no further degradation of 2,4-dichlorophenol into lower-molecular organic compounds occurred beyond 24 h of laccase treatment. Since the laccase treatment and then activated sludge treatment decreased COD values of bisphenol A and 2,4-dichlorophenol up to 15 and 25% of their initial values, respectively, it seems to be that 85 and 75% of organic compounds produced from bisphenol A and 2,4-dichlorophenol by laccase treatment were converted into H_2O and CO_2. As a result, it was found that this consecutive treatment is a very effective method for the almost complete degradation of bisphenol A and 2,4-dichlorophenol. Future study will be focused on the identification of lower-molecular organic compounds produced from endocrine-disrupting chemicals by laccase treatment and the screening of microorganisms that can degrade the compounds rapidly and efficiently.

CONCLUSION

The effect of laccase treatment on the degradation of endocrine-disrupting chemicals and the activated sludge treatment of organic compounds produced by laccase treatment were investigated experimentally. The following findings were obtained.

(1) The concentrations of bisphenol A and 2,4-dichlorophenol decreased and then reached almost 0 by laccase treatment, but no decrease in the concentration of diethyl phthalate was observed.

(2) The enzymatic reaction rate equation was proposed for presenting the disappearance of bisphenol A by laccase treatment and the temperature dependencies of kinetic parameters were confirmed from 5-40°C.

(3) The laccase treatment followed by activated treatment could degrade and convert bisphenol A and 2,4-dichlorophenol into H_2O and CO_2 almost completely; *i.e.* about 85 and 75% of their initial COD values were disappeared by this consecutive treatment.

REFERENCES

[1] Colborn, T., F. S. vom Saal, and A. M. Soto (1993) Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health. Perspect.* 101: 378-384.

[2] Soto, A. N., C. S. Sonnenschein, K. L. Chung, M. F. Fernandez, N. Olea, and F. O. Serrano (1995) The E-SCRREN assay as a tool to identify estrogens: An update on estrogenic environmental pollutants. *Environ. Health. Perspect.* 103 (Suppl 7): 113-122.

[3] Hammond, B., B. S. Katzenellenbogen, N. Kranthammer, and J. McConnell (1979) Estrogenic activity of the insecticide chlordecone (keptone) and interaction with uterine estrogen receptors. *Proc. Natl. Acad. Sci. USA* 76: 6642-6645.

[4] Ronen, Z. and A. Abeliovich (2000) Anaerobic-aerobic process for microbial degradation of tetrabromobisphenol A. *Appl. Environ. Microbiol.* 66: 2372-2377.

[5] Spivack, J., T. K. Leib, and J. H. Lobos (1994) Novel pathway for bacterial metabolism of bisphenol A. *J. Biol. Chem.* 269: 7323-7329.

[6] Hirano, T., Y. Honda, T. Watanabe, and M. Kuwahara (2000) Degradation of bisphenol A by the lignin-degrading enzyme, manganese peroxidase, produced by the white-rot basidiomycete, *Pleurotusostreatus*. *Biosci. Biotechnol. Biochem.* 64: 1958-1962.

[7] Tsutsumi, Y., T. Haneda, and T. Nishida (2001) Removal of estrogenic activities of bisphenol A and nonylphenol by oxidative enzymes from lignin-degrading basidiomycetes. *Chemosphere* 42: 271-276.

[8] Koroleva, O. V., E. V. Stepanova, V. P. Gavrilova, N. S. Yakovleva, E. O. Landesman, I. S. Yavmetdinov, and A. I. Yaropolov (2002) Laccase and Mn-peroxidase production by *Coriolushirsutus* strain 075 in a jar fermentor. *J. Biosci. Bioeng.* 93: 449-455.

[9] Nakamura, Y., M.G. Sungusia, T. Sawada, and M. Kuwahara (1999) Lignin-degrading enzyme production by *Bjerkandera adusta* immobilized on polyurethane foam. *J. Biosci. Bioeng.* 88: 35-41.

[10] Ghose, T. K. (1989) *Bioprocess Engineering*. pp. 39-67. Ellis Horwood Limited, Chichester, England.

[11] Leatham G. F., R. L. Crawford, and T. K. Kirk (1983) Degradation of phenolic compounds and ringcleavage of catechol by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 46: 191-197.

[12] Bumpus, J. A., M. Tien, D. Wright, and S. D. Aust (1985)

Oxidation of persistent environmental pollutants by a white rot fungus. *Science* 228: 1434-1436.
[13] Bailey, J. E. and D. F. Ollis (1986) *Biochemical Engineering Fundamentals*. pp. 100-105. McGraw-Hill, Inc., Singapore.

[Received May 26, 2003; accepted July 12, 2003]