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Production of Laccase and Bioremediation of Pentachlorophenol by Wood-Degrading Fungus *Trichophyton sp.* LKY-7 immobilized in Ca-Alginate Beads[*1]

Hyunchae Jung[*2], Kyuhwan Hyun[*2], and Chongyawl Park[*3]

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ABSTRACTS

The wood-degrading fungus *Trichophyton sp.* LKY-7 (*T.* LKY-7) was immobilized in ca-alginate beads for laccase production and PCP bioremediation. The immobilized *T.* LKY-7 enabled the repeated use of this fungus for laccase production and produced high amount of laccase throughout 5 cycles incubation. As a laccase inducer. oak wood meal (*Quercus variabilis*) seemed to be effective laccase inducer for *T.* LKY-7, and the optimum addition amount was 1% (W/W) in glucose-peptone medium. Bioremediation of pentachlorophenol by the immobilized *T.* LKY-7 reached an efficency of up to 90% without toxic inhibition. The immobilized *T.* LKY-7 might thus be applicable for semi-continuous laccase production and bioremediation to serve inoculum for reactor system.

1. Introduction

White-rot fungi are believed to be the only microorganisms able to selectively and efficiently degrade lignin. Its ligninolytic activities are known to be closely correlated with secretion of lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase in their ligninolytic system (1). This ability to degrade lignin has been demonstrated the potential use for bioremediation of recalcitrant aromatic compoumds such as chlorophenols known to be the most toxic and widely spread environmental pollutants (2, 3). Similarly, extracellular production of lignin-degrading enzymes is responsible for the biodegradation of chlorophenols by white-rot fungi (4, 5). Various strains of *Coriolus versicolor* are known to effectively bioremediate chlorophenol. The most active enzyme from *C. versicolor* for chlorophenol bioremediation is laccase (5, 6). The reactivity of chlorophenols with laccase depends on the number and position of substituted chlorines on aromatic rings. The lower the degree of substitution of chlorophenol, the more reactive the molecule of chlorophenol. That is, the chlorophenols substituted with *ortho-* and *para-* or *di-*, *tri-*, and *tetrachlorophenol* are bioremediated by laccase more readily than those with meta substitution or pentachlorophenol (PCP), which is known for the most recalcitrant substance (7). For the removal of chlorophenols from aqueous

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*2 College of Agriculture and Life Science, Sunchon National University
*3 Division of Forest Science, Gyeongsang National University

effluents and soils, it is most economical to utilise the whole fungus rather than purified enzymes. However, bioremediation of phenolic substances has been generally known to be inhibited by phenolic substances itself (8). By entrapping the microorganism in gel matrices in this case, the direct contact of microorganism with highly phenolated bulk liquors can be prevented, and then the microorganism can degrade phenolic substances with less substrate inhibition (3, 7).

We isolated a wood-degrading fungus *T.* LKY-7 from a hard wood chip pile. Under the culture conditions, this fungus produces large amounts of laccase without expressing detectable LiP. In previous work (9), we have demonstrated that purified laccase of *T.* LKY-7 has some properties that are different from most fungal laccases and is capable to be utilized effectively in pulp bleaching and other biocatalysts. Present work is to investigate the possibilities of mass production of laccase and to evaluate the abilities of PCP bioremediation by *T.* LKY-7, and also to study the effects of encapsulation of this fungus on laccase production and PCP bioremediation.

2. Materials & Methods

2.1. Microorganism

A new fungal strain isolated from a decayed hardwood chip pile was used in this study. This fungus was found to have a close similarity to *Trichopyton sp.* as determined by a GC-FAME (gas chromatography-fatty acid methyl ester) technique (Microbe Inotech Labs, Inc., St Louis, MO) and designated tentatively as *Trichopyton.* LKY-7. The fungal culture was maintained on potato-dextrose agar (PDA) slants grown at 29℃ and stored at 4℃. The fungus was grown on PDA plates for 5 days at 29℃. The agar plugs of 0.5cm in diameter were taken from the mycelial edge in the PDA plates and used as inoculums for liquid culture and mycelium immobilization.

2.2. Immobilization of T. LKY-7 mycelium on Ca-alginate bead

Thirty plugs (5 mm diameter) taken from the T. LKY-7 mycelial edge in PDA plates were homogenized with 40ml of glucose-peptone medium. The homogenized mycelial solution were mixed with the same volume of 2 % sodium alginate solution. The sodium alginate gel beads entrapping *T.* LKY-7 mycelium were formed by dropwise addition through a sergiological pippet into 2 % calcium chloride solution. The formed gel beads were withdrawn from the calcium chloride solution, washed with sterilised distilled water and stored at 4℃ until use.

2.3. Culture conditions for laccase production and PCP bioremediation

The culture mediums for laccase production and PCP bioremediation were glucose-peptone medium as previously described (9). For the laccase production, the free and the immobilized *T.* LKY-7 mycelium were used. For the free mycelium cultures, four mycelial blocks were blended with 100 ml of culture medium and then transferred to a 250-ml flask for the subsequent cultivation. And for the immobilized *T.* LKY-7, 5g of ca-alginate beads (wet state) containning *T.* LKY-7 mycelium were added to 100ml of a culture medium in a 250-ml flask. The cultures were incubated periodically at 29℃ for 9 days on shaking incubator (150 rpm). Oak woodmeal (*Quercus variabilis*) of 0.5-2% (W/W), as a putative laccase inducer, was

supplimented to culture medium to evaluate its inductive effect on laccase production.

A 100-ml of fungal cultures without oak woodmeal, inoculated with the free and the immobilized *T.* LKY-7 mycelium, were preincubated at 29℃ for 3-days, then added 50 and 100 ppm of PCP dissolved in ethanol for PCP bioremediation. The fungal cultures were incubated with shaking at 29℃ for 9-days. The inoculation was controled to be nearly same amount of mycelium in the free and the immobilized *T.* LKY-7. After incubation, the fungal cultures were filtered periodically, and the filtrates were used for laccase activity assay and analysis of residual PCP. To evaluate the binding effect of PCP on ca-alginate beads, the beads without *T.* LKY-7 mycelium were used as a control.

2.4. Enzyme assay

Laccase activity was determined spectrophotometrically by measuring the oxidation of 2,2' -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (500 μM) in a sodium tartrate buffer (50mM, pH 4.5) at 420nm (ε_{max} = 3.6 × 104M-1cm-1). One unit of laccase activity was determined as 1.0μM of product formed per minute under the assay conditions.

2.5. Analysis of PCP

Reaction mixtures, seperated from the free and the immobilized *T.* LKY-7 cultures, were acidified to pH 2.0 with 0.1 M sulfuric acid, and extracted with ethylacetate three times. The extracts were combined and evaporated on a vacumn rotary evaporator at 40℃. The residues were dissolved in a small volume of methanol and analysed by HPLC using a reversed phase column (Waters) packed with R-Sil C18 with a mobile phase of acetonitrile:water:acetic acid (75:25:0.125). Residual PCP was identified and quantified by retention time and peak area in comparison with an PCP standard. Calibration plots of peak areas of PCP standards were linear in the range of 10-100 ppm.

3. Results & Discussion

3.1. Laccase production

For application of laccase to numerous industry processes, it is important to screen the effective and active fungal strains for laccase production, and then to establish the mass production system of laccase. *T.* LKY-7 screened from hardwood chip pile did not grow well in the basal medium in which other wood-degrading fungi such as *Phanerchate chrysosporium* and *Pycnoporus cinnabarinus* grow well (10). However the fungus grew well and expressed high amounts of laccase when cultivated with the fungal mycelium in glucose-peptone medium under shaking condition (9). The fungus was immobilized in ca-alginate beads for

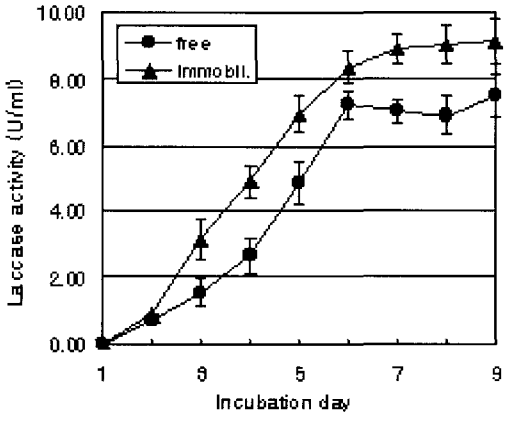

Fig. 1. Laccase production by the free and the immobilized *T.* LKY-7.

laccase production and PCP transformation. When the free and the immobilized *T.* LKY-7 were cultured in the glucose-peptone medium under a shaking condition, laccase activity was detectable after 2-days incubation, peaked with 7.5 U/ml or 9.1 U/ml on 9-days incubation, respectively (Figure 1). The *T.* LKY-7 secreted a bit higher amounts of laccase under immobilized state than under free state. The reason for this result is thought that the immobilized mycelium was preincubated for some times in refrigerater until use.

In an effort to enhance the laccase production by *T.* LKY-7, various phenolic compounds were added to the fungal cultures in previous study (9). However, the effect of laccase induction by phenolic compounds was not significant. Especially, the inductive effect of 2,5-xylidine known for effective inducer of laccase in lignin-degrading fungi was not as significant as on other fungi (11). In this study, oak wood meal was used as a putative laccase inducer. The addition of oak wood meal resulted in a two or threefolds increase in extracellular laccase activity and its effect was slightly higher in the free *T.* LKY-7 than that of the immobilized *T.* LKY-7, as shown in Figure 2. That is, after 7-days cultivation of the free or the immobilized *T.* LKY-7, the laccase activities in the medium supplimented with 0.5% oak wood meal were 18.9 and 16.5 U/ml respectively, which was twofolds higher than that without oak wood meal. With 1-2% oak wood meal, laccase activities increased to 23.4-24.5 U/ml in the free *T.* LKY-7 and 19.5-20.6 U/ml in the immobilized *T.* LKY-7, which was threefolds increase compared with control (without oak wood meal). Various compounds have been reported as an effective laccase inducer, including copper, veratryl alcohol, phenolic compounds, lignin preparations, and various agricultural residues. However, oak wood meal seemed to be effective laccase inducer for *T.* LKY-7 through the present and the previous studies. And the optimum addition amount was 1% (W/W) in glucose-peptone medium.

The immobilized *T.* LKY-7 enable repeated use of this fungus for the production of laccase. The semicontinuous laccase production by the immobilized *T.* LKY-7 was carried out by replacing the culture medium containing 1% oak wood meal (W/W) with fresh one in every 7-days incubation. Under

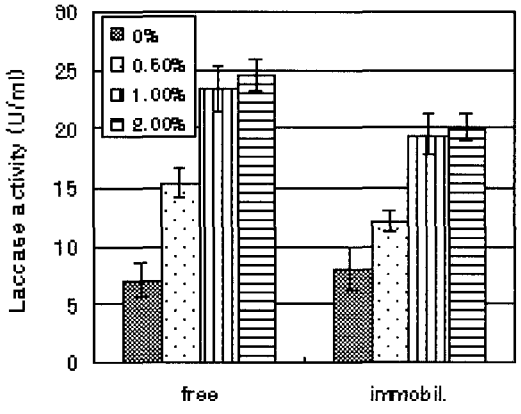

Fig. 2. The effect of oak wood meal on laccase production of the free and the immobilized *T.* LKY-7.

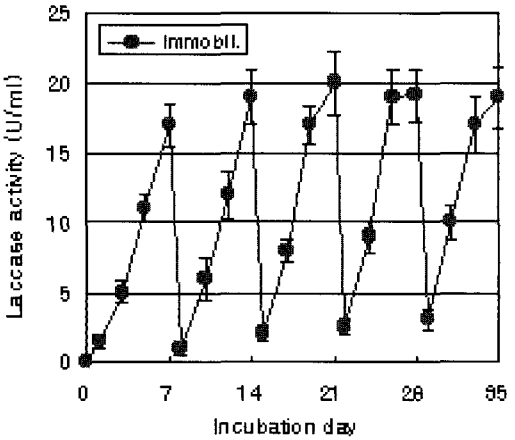

Fig. 3. Semicontinuous production of laccase by the immobilized *T.* LKY-7.

these conditions, the immobilized *T.* LKY-7 produced high amount of laccase throughout 5 cycles incubation, as shown in Figure 3. After first 7-days incubation, the culture medium was harvested, of which laccase activity was about 17 U/ml. After the culture medium was replaced by fresh one, laccase level was reached again within the next 7-days incubation. The next third cycle gave similar results with laccase activity of about 20 U/ml. The continuous laccase production with the immobilized T. LKY-7 was stopped in fifth cycle incubation because gel matrix was broken by over growth of *T.* LKY-7 mycelium during sixth incubation.

3.2. PCP bioremediation by the free and the immobilized T. LKY-7

To investigate the effect of preincubation time of *T.* LKY-7 on PCP bioremediation, 50 ppm PCP was added to culture medium of *T.* LKY-7 preincubated for 0, 3, 5 days and then the reaction products were filtered after 3-days incubation. Figure 4 shows the residual PCP from 100ml volumes of 50 ppm PCP. Without preincubation of culture medium, 45% and 66% of 50 ppm PCP were removed by the free and the immobilized *T.* LKY-7. On the contrary, with 3 or 5-day-old culture, over 80% of PCP was removed by the free *T.* LKY-7 and almost complete removal of PCP was observed in the immobilized *T.* LKY-7.

For bioremediation of PCP by *T.* LKY-7, the free and the immobilized T. LKY-7 were inoculated to 100 ml glucose-peptone medium without laccase inducer. After 3-days preincubation, 100 ppm PCP was added to culture medium. And the culture medium was incubated periodically at 29℃ for 7 days. As shown in figure 5, PCP was almost removed by immobilized *T.* LKY-7 in 3-days incubation, while about 70% of PCP was removed by the free *T.* LKY-7 in this time and 80% of PCP was removed in 7-days incubation. Bioremediation of phenolic substances has been generally known to be inhibited by phenolic substances itself (3). Concerning the removal of PCP, it was shown that *T.* LKY-7 was able to remove PCP effectively but the addition of PCP led to inhibit mycelial growth (data not shown). To decrease the inhibition effect of toxic PCP, it is demanded that the direct contact of fungal mycelium is prevented. It can be prevented the direct contact with PCP by

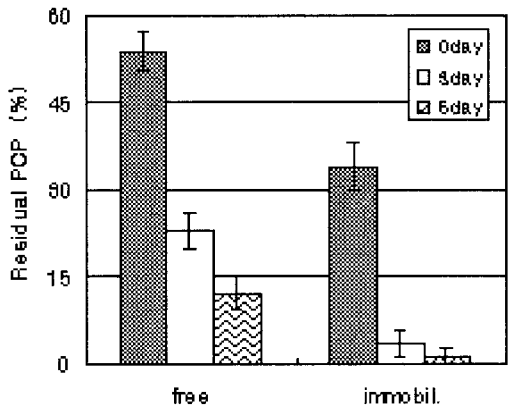

Fig. 4. The effect of production day of *T.* LKY-7 on bioremediation of PCP(50ppm).

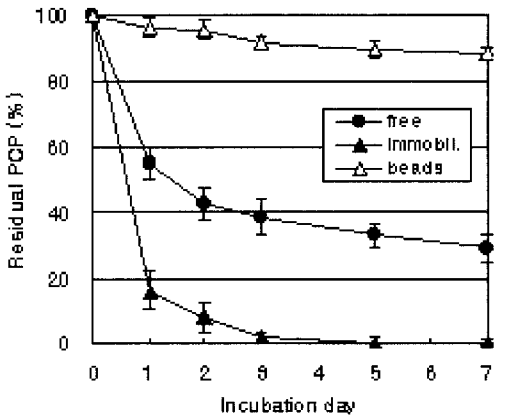

Fig. 5. Bioremediation of PCP(100ppm) with the free and the immobilized *T.* LKY-7.

entrapping the *T.* LKY-7 mycelium in ca-alginate beads. Thus, the immobilized *T.* LKY-7 removed PCP effectively with less inhibition, compared the free *T.* LKY-7. Binding of chlorophenols to organic matrices has been observed (3, 12). To evaluate the binding effect of PCP to ca-alginate beads, the beads without *T.* LKY-7 mycelium were used. When PCP solution was incubated with the free mycelium beads, the binding of PCP appeared to be low level with below 10% throughout the entire incubation days.

Of the ligninolytic enzymes, laccase is a predominant enzyme for the removal of PCP in many white-rot species such as *Coriolus versicolor* and Pleurotus ostreatus (13, 14). Likewise, laccase was the major enzyme in the bioremediation of PCP by the free and the immobilized *T.* LKY-7, whereas LiP and MnP activity was not detectable. Figure 6 shows the extracellular laccase activity according to the incubation times. Generally, in the degradation of phenolic compounds by lignin-degrading fungi, laccase activity has been known to increase throughout reaction time. However, the laccase activity measured in PCP culture solution decreased continuously throughout

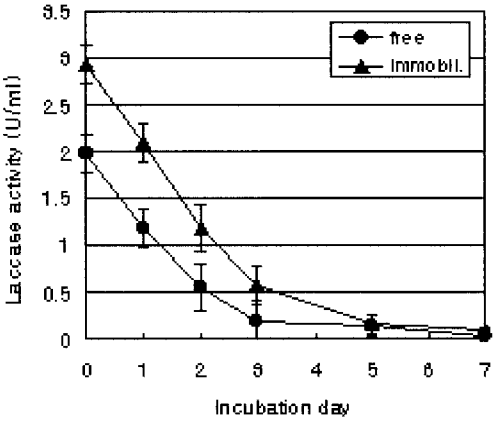

Fig. 6. Laccase activity in PCP remediation by the free and the immobilized *T.* LKY-7.

incubation time. It is not clear the reason why the extracellular laccase activity decreased. Production of large amounts of polymeric materials in reactions of laccase with phenolic substrates is well known phenomenon. Dec and Bollag (15) reported that laccase reduces acute toxicity by polymerizing and rendering less soluble many of the chlorinated compounds and also showed that dechlorination of chlorophenols occurs via an enzymatic reaction accompanied by two oxidative coupling processes. It is therefore obvious that the primary product of PCP bioremediation by *T.* LKY-7 would be a high molecular weight polymer, resulting the oxidative coupling processes by laccase. So, the laccase activity in PCP culture solution might be inhibited by these polymeric materials.

4. Conclusions

The wood-degrading fungus *T.* LKY-7 was immobilized in ca-alginate beads for laccase production and PCP remediation. When the free and the immobilized *T.* LKY-7 were cultured in the glucose-peptone medium under a shaking condition, laccase activity was detectable after 2 days, peaked with 7.5 U/ml or 9.1 U/ml on day 9, respectively. As a laccase inducer. oak wood meal seemed to be effective laccase inducer for *T.* LKY-7, and the optimum addition amount was 1% (W/W) in glucose-peptone medium. The immobilized *T.* LKY-7 enabled the repeated use for laccase production and produced high amount of laccase throughout 5 cycles incubation.

After 3-days preincubation, when 100 ppm PCP was added to fungal cultures, PCP was almost removed by the immobilized *T.* LKY-7 in 3-days incubation, while about 60% of PCP was removed by the free *T.* LKY-7 in this time and 70% of PCP in 7-days

incubation. And the binding of PCP on ca-alginate beads appeared to be low level with below 10 % throughout the entire incubation days. In the remediation of PCP by free and immobilized *T.* LKY-7, laccase was the major enzyme, whereas LiP and MnP activity was not detectable. The laccase activity measured in PCP culture solution decreased continuously throughout the entirely reaction time.

Literature Cited

1. Eriksson, K-EL., lanchette, R. A. and Ander, P., New York : Springer-Verlag(1990).
2. Lamar, R. T. and Dietrich, D. M., Appl. Environ. Microbiol. 56:3093-3100 (1990).
3. Alleman, B. C., Logan, B. E. and Gilbertson, R. I., Water Res. 29:61-67 (1995).
4. Mileski, G. J., Bumpus, J. A., Jurek, M. A. and Aust S. D., Biotech. Bioeng. 35:1125-1134 (1998).
5. Roy-Arcand, L. and Archibald F. S., Enzyme Microb. Technol. 13:194-203(1991).
6. Ullah, M. A., Bedford, C. T. and Evans, C. S., Appl. Microbiol. Biotechnol. 53:230-234 (2000).
7. Shuttleworth, K. L. and Bollag J. M., Enzyme Microb. Technol. 8:171-177 (1986).
8. Ullah, M. A., Kadhim, H. R., Rastall, A. and Evans, C. S., Appl. Microbiol. Biotechnol. 54:832-837 (2000).
9. Jung, H. C., Xu, F. and Li, K., 2002. Enzyme Microb. Technol. 30:161-168 (2002).
10. Kirk, T. K., Schultz, E., Connors, W. J., Lorenz, L. F. Zeikus, J. G., Arch. Microbiol. 85:117-277 (1978).
11. Eggert, C., Temp, U., Eriksson, K-EL., Appl. Environ. Microbiol. 62:1151-1158 (1996).
12. Leidig, E., Prusse, U., Vorlop, K. D. and Winter, J., Bioprocess engineering 21:5-12 (1999).
13. Ricotta, A., Unz, R. F., and Borag, J. M., 59:230-237 (1996).
14. Ruttimann, J. C. and Lamar, R. T., Appl. Environ. Microbiol. 62:3890-3893 (1996).
15. Dec, J. and Bollag, J. M. Arch. Environ. Contam. Toxicol. 19:543-550 (1990).