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

정 현 채[1] · 현규환[1] · 박종열[2]
[1]순천대학교 농업생명과학대학 · [2]경상대학교 산림과학부

ABSTRACTS

The lignin-degrading fungus *Trichophyton* sp. LKY-7 was immobilized in ca-alginate beads for laccase production and PCP remediation. The immobilized *Trichophyton* sp. 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 *Trichophyton* sp. LKY-7, and the optimum addition amount was 1% (W/W) in glucose-peptone medium. Biotransformation of pentachlorophenol by immobilized *Trichophyton* sp. LKY-7 reached an efficency of up to 90% without toxic inhibition. Immobilized *Trichophyton* sp. LKY-7 might thus be applicable for semicontinuous laccase production and bioremediation to serve inoculum for reactor system.

1. Introduction

The ability of white-rot fungi to degrade lignin have 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 (1, 4, 9). Similarly, extracellular production of lignin-degrading enzymes is responsible for the biodegradation of chlorophenols by white-rot fungi (5, 7). Various strains of *Coriolus versicolor* are known to effectively bioremediate

chlorophenol. And laccase is known to be the most active enzyme of *C. versicolor* for chlorophenol bioremediation (3, 6, 7, 11). However, bioremediation of phenolic substances have been generally known to be inhibited by phenolic substances itself (8, 10). Therefore, 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.

We isolated a wood-degrading fungus *Trichopyton* sp. 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 (2), we have demonstrated that purified laccase of *Trichopyton* sp. 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 *Trichopyton* sp. LKY-7, and also to study the effects of encapsulation of this fungus on PCP bioremediation.

2. Materials and Methods

2.1. Microorganism

A new fungal strain isolated from a decayed hardwood chip 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 was designated tentatively as *Trichopyton* sp. LKY-7 (*T.* LKY-7).

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

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, washed with sterilised distilled water and stored at 4℃ until use.

2.3. Culture conditions for laccase production and PCP bioremediation

T. LKY-7 grew well and expressed high amounts of laccase when cultivated with the fungal mycelium in glucose-peptone medium under shaking condition (2). For the laccase production, free and immobilized *T.* LKY-7 mycelium were inoculated to glucose-peptone medium. The fungal cultures were incubated periodically at 29℃ for 9 days on shaking incubator (150 rpm). Oak woodmeal (*Quercus variabilis*) of 0.5-2% (W/W), a putative laccase inducer, was supplimented to culture medium to study its inductive effect on laccase production. 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 7days incubation.

For PCP bioremediation, fungal cultures inoculated with free and immobilized *T.* LKY-7 mycelium were preincubated at 29℃ for 3-days, then added 50 and 100 ppm of PCP dissolved in ethanol. The fungal cultures were incubated with shaking at 29℃ for 9-days. 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, same amount of beads without *T.* LKY-7 mycelium were used as a control.

Laccase activity was determined spectrophotometrically by measuring the oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (500μM) in a sodium tartrate buffer (50mM, pH 4.5) at 420nm (ε_{max} = $3.6\times10^{4}M^{-1}cm^{-1}$).

The residual PCP was 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).

3. Results and Discussion

3.1. Laccase production

The fungus was immobilized on ca-alginate beads for laccase production and PCP remediation. When the free and 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. (Fig. 1). The *T.* LKY-7 secreted a bit higher amounts of laccase under immobilized state than under free state.

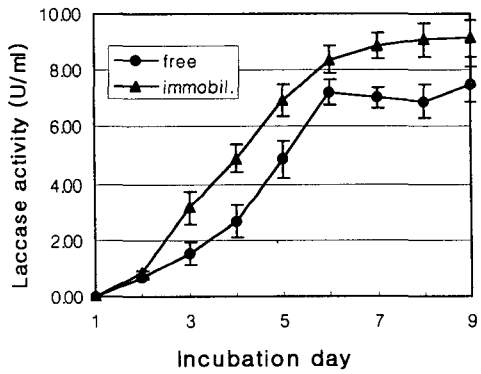

Fig. 1. Laccase production by free and immobilized T. LKY-7.

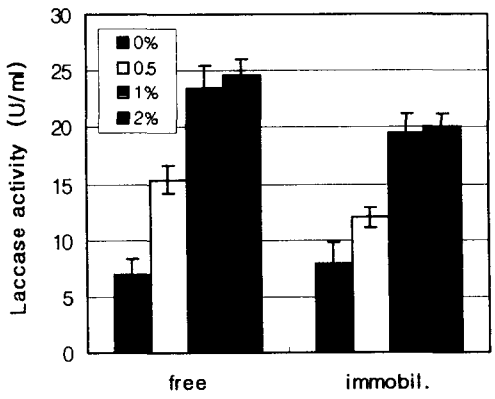

Fig. 2. The effect of oak wood meal on laccase production of free and immobilized *T.* LKY-7

Oak wood meal was used as a putative laccase inducer. The addition of oak woodmeal resulted in a two or threefold increase in extra-cellular laccase activity and its effect was slightly higher in free *T.* LKY-7 than that of immobilized *T.* LKY-7 (Figure 2). That is, after 7-days cultivation of free or immobilized *T.* LKY-7, laccase activities of the cultures supplimented with 0.5% oak wood meal were 18.9 and 16.5 U/ml respectively, which was about twofolds higher than that of culture without oak wood meal. With 1-2% oak wood meal, laccase activities increased to 23.4-24.5 U/ml in free *T.* LKY-7 and 19.5-20.6 U/ml in 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. However, 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 of this fungus for laccase production and produced high amount of laccase throughout 5 cycles incubation as shown in Fig. 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 medium, laccase level was reached again within the next 7days incubation. The next three cycles gave similar results with laccase activities of about 20 U/ml. The incubation time which is reached to the optimum laccase activity was found to be decreased according to repeated use of immobilized fungus.

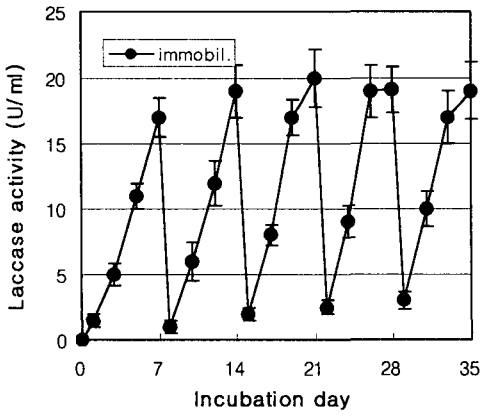

Fig. 3. Semicontinuous production of laccase by immobilized *T.* LKY-7.

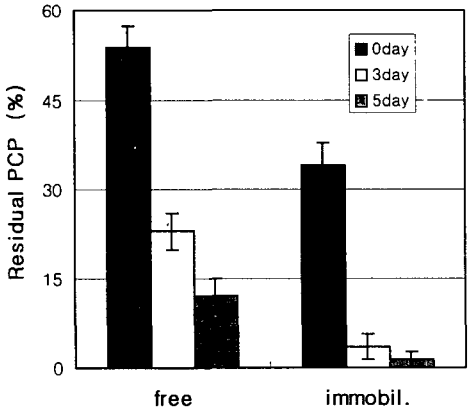

Fig. 4. The effect of preincubation day of T. LKY-7 on transformation of PCP(50ppm).

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

To investigate the effect of pre-incubation time of *T.* LKY-7 on PCP bio-remediation, 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 free and 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, free and immobilized *T.* LKY-7 were inocubated to 100 ml glucose-peptone medium and preincubated for 3 days and then added 100 ppm PCP. The fungal cultures were incubated periodically at 29℃ for 7 days.

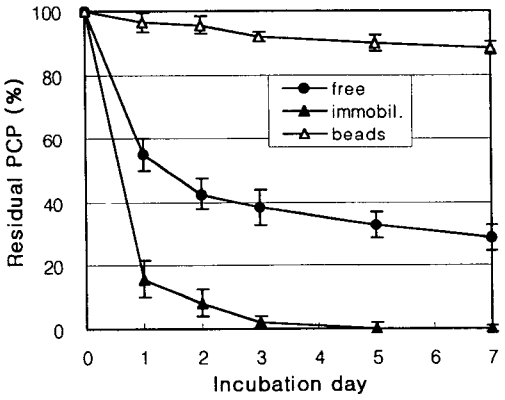

Fig. 5. Transformation of PCP (100 ppm) with free and immobilized *T.* LKY-7 preincubated for 3 days.

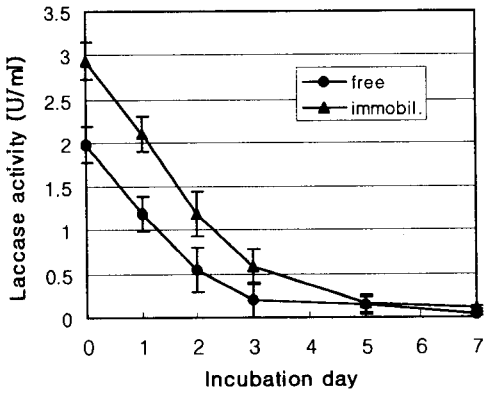

Fig. 6. Laccase activity in PCP transformaion by free and immobilized *T.* LKY-7 preincubated for 3 days.

As shown in figure 5, PCP was almost removed by immobilized *T.* LKY-7 in 3-days incubation, while about 60% of PCP was removed by free *T.* LKY-7 in this time and 70% of PCP was removed in 7-days incubation. When PCP solution was incubated with the free mycelium beads, the binding of PCP on ca-alginate beads appeared to be low level with below 10 % throughout the entire incubation days.

In the bioremediation of PCP by free and immobilized *T.* LKY-7, laccase was the major enzyme, whereas LiP and MnP activity was not detectable (data not shown). 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 (3, 10). However, the laccase activity measured in PCP culture solution decreased continuously throughout reaction time. It was not known precisely from this results which facters were responsible for the decrease of extracellular laccase in the PCP remediation by *T.* LKY-7.

CONCLUSIONS

The wood-degrading fungus *T.* LKY-7 was immobilized in ca-alginate beads for laccase production and PCP remediation. When the free and 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. With 1-2%(W/W) oak wood meal suppliment, laccase activiy was found to be threefolds increase compared with control (without oak wood meal). The immobilized *T.* LKY-7 enabled the repeated use for laccase production and produced high amount of laccase throughout 5 cycles incubation.

With 3 or 5-day-old fungal cultures by preincubation, over 80% of 50 ppm PCP was removed by the free *T.* LKY-7 and almost complete removal of PCP was observed in the immobilized *T.* LKY-7 in 3 or 5 days incubation. After 3-days preincubation, when 100 ppm PCP was added to fungal cultures, PCP was almost removed by immobilized *T.* LKY-7 in 3-days incubation, while about 60% of PCP was removed by 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.

REFERENCES

1. Alleman, B. C., B. E. Logan and R. I. Gilbertson. 1995. Degradation of pentachlorophenol by fixed films of white rot fungi in rotating tube bioreactors. Water Res. 29:61-67.
2. Hyunchae, J.,
3. Leidig, E., U. Prusse, K. D. Vorlop and J. Winter. 1999. Biotransformation of poly R-478 by continuous cultures of PVAL-encapsulated *Trametes versicolor* under non-sterile conditions. Bioprocess engineering 21:5-12.
4. Lamar, R. T. and D. M. Dietrich. 1990. In situ depletion of pentachlorophenol from contaminated soil by Phanerochate spp. Appl. Environ. Microbiol. 56:3093-3100.
5. Mileski, G. J., J. A. Bumpus, M. A. Jurek and S. D. Aust. 1988. Biodegradation of pentachlorophenol by the whiterot fungus *Phanerochate chrysosphorium.* Biotech. Bioeng. 35:1125-1134.
6. Ricotta, A., R. F. Unz and J-M. Borag. 1996. Role of laccase in the degradation of pentachlorophenol. Bull. Environ. Contam. Toxicol. 59:230-237.
7. Roy-Arcand, L. and F. S. Archibald. 1991. Direct dechlorination of chlorophenolic compounds by laccase from *Trametes versicolor.* Enzyme Microb. Technol. 13:194-203.
8. Ruttimann, J. C. and R. T. Lamar. 1996. Polymerization of pentachlorophenol and ferulic acid by fungal extracellular lignin-degrading enzymes. Appl. Environ. Microbiol. 62:3890-3893.
9. Shuttleworth, K. L. and J. M. Bollag. 1986. Soluble and immobilized laccase as catalysts for the transformation of substituted phenols. Enzyme Microb. Technol. 8:171-177.
10. Ullah, M. A., H. Kadhim, R. A. Rastall and C. S. Evans. 2000. Evaluation of solid substrate for enzyme production by *Coriolus versicolor*, for use in bioremediation of chlorophenols in aqueous effluents. Appl. Microbiol. Biotechnol. 54:832-837.
11. Ullah, M. A., C. T. Bedford and C. S. Evans. 2000. Reactions of pentachlorophenol with laccase from *Coriolus versicolor.* Appl. Microbiol. Biotechnol. 53:230-234.