Mokchae Konghak 29(3) : 112~122, 2001

Immobilization of Fungal Laccase on Keratin-Coated Soil and Glass Matrices*[1]

G. Ginalska*[2], J. Lobarzewski*[2], Nam-Seok, Cho*[3], T. H. Choi*[3], S. Ohga*[4], M. Jaszek*[2], and A. Leonowicz*[2]

ABSTRACT

Laccase enzymes from *Cerrena unicolor* and *Trametes versicolor* were immobilized on the activated glass beads (CPG), silica gel (SG) and soil (SL). The heterogeneous matrices were activated by γ-aminopropyltriethoxysilane (APTES) and glutaraldehyde (GA), and their surfaces were coated by keratin (KER) on activated or non-activated CPG, SG and SL. The laccase activities were tested in the aqueous solution for the native and immobilized preparations using different pH and temperature conditions.

By keratin coating on supports, in the cases of CPG-KER and SL-KER, the immobilization yield was increased from about 80% to 90%. Moreover, much less protein was immobilized in keratin coated matrices than in inorganic ones alone (*e.g.* on CPG-KER 57.6%, whereas on CPG alone 80.6%). Laccase immobilization on keratin coated inorganic matrices was generally more effective than that of non-coated matrices. Concerned to pH dependency, the optima pH for immobilized laccases generally shifted towards to higher values, 5.5-5.8 and even 5.9 in the case of keratin for *C. unicolor* and from 5.3 to 5.7 for *T. versicolor*, respectively, and decreased less gradually both in acidic and alkaline regions. The immobilized laccase was more stable against thermal denaturation. This seems particularly true at 75℃ in the case of *C. unicolor*, where the activity of immobilized enzyme is > 50% higher than that of the free enzyme. For *T. versicolor* the respective values were 65℃, and 50%.

Keywords : Keratin, Soil, Immobilization, Glass beads, Laccase, *Cerrena unicolor*. *Trametes versicolor*

1. INTRODUCTION

Fungal laccases (*p*-benzenediol:oxygen oxido-reductases, EC 1.10.3.2) are present in several fungal strains belonging to ascomycete, deutero-mycete, and mainly basidiomycete classes (Bollag and Leonowicz, 1984; Agematu *et al.*, 1993). The basidiomycete includes soil inhabiting fungi which decompose various wood and litter. Laccase excreted to wood or soil environment has a broad substrate specificity and is able to oxidize several phenolic and non-phenolic compounds. The oxidative capacity of laccase to produce insoluble polymers could be used in

*1 Received on August 4, 2001, accepted on September 3, 2001
*2 Department of Biochemistry, Maria Curie-Sklodowska University, Lublin, Poland
*3 School of Forest Resources, Chungbuk National University, Cheongju, Korea
*4 Department of Forest Resources Science, Kyushu University, Fukuoka, Japan

the detoxification of terrestrial and aqueous sites that contaminated by various pollutants. In soil the enzyme catalyses numerous important reactions responsible for maintaining the biological activity of soil.

The main function of laccase in soil lies in oxidative coupling of phenolic compounds which are derivatives of lignin or humic substances together with other chemical compounds. Thus the enzyme is probably involved in the formation of humus and the incorporation of organic xenobiotics into soil organic matter (Gianfreda and Bollag, 1994; Sarkar *et al.*, 1989). Besides, the broad activity of laccase on hydrogen donors provides opportunities for detoxification of some terrestrial pollutants (Bollag *et al.*, 1986) as well as for removing certain phenolic and other aromatic compounds from natural and industrial waste waters (Nannipieri and Bollag, 1991). The oxidation of phenolics generates phenoxy radicals and quinoid intermediates which are subsequently transformed to dimers or insoluble polymers with aromatic amines (*e.g.* chloroamines) (Simmons *et al.*, 1989) or aminoacids (Liu *et al.*, 1985). After sedimentation such polymers may be removed from the water environment. Terrestrial pollutants have a chance to be oxidized by laccase immobilized in the soil fractions to less toxic polymers (Leonowicz and Bollag, 1987; Sarkar *et al.*, 1989). These, after various transformation, also with other factors than laccase enzymes participating, may enrich the soil humus fraction (Gianfreda and Bollag, 1994).

This enzyme not only oxidizes a large variety of aromatic hydrogen donors, mainly phenolic and methoxyphenolic acids, but also decarboxylates them (Agematu *et al.*, 1993)) and modifies their methoxyl groups by demethylation (Leonowicz *et al.*, 1984) or demethoxylation (Potthast *et al.*, 1995). These reactions constitute an important step in the initial transformation of lignin polymers. For this reason laccase together with lignin and manganese dependent peroxidases are listed among the enzymes participating in lignin degradation *in vivo* (Dittmer *et al.*, 1997; Rodriguez *et al.*, 1997; Leonowicz *et al.*, 1999; Leonowicz *et al.*, 2001). Laccase is involved in several physiological processes, which are applicable in biotechnology. For example, it is engaged in the development of fungal fruit bodies (Leatham *et al.*, 1981), pigmentation of fungal spores (Clutterbuck, 1972), pathogeneity (Rigling *et al.*, 1991), sexual differentiation (Aisemberg *et al.*, 1989) or rhizomorph formation (Worall *et al.*, 1986).

Laccase can be used as a free enzyme and an immobilized states both in water and in some organic solvents, improving in several biotechnological processes (Milstein *et al.*, 1993; Burton and Duncan, 1995, Luterek *et al.*, 1998). Of possible applications, the enzyme is considered, for example, to be a bleaching agent in the pulp and paper technology (both, through mediating factor according to Bourbonnais *et al.*, 1995, or without any mediator as reported Tagger *et al.*, 1998), a stabilizer during the must and wine processings (Lante *et al.*, 1992) or as a dechlorinating factor (Roy-Arcand and Archbald, 1991; Roper *et al.*, 1995; Cho *et al.*, 1999). In this respect, improving laccase activity and stability is a fundamental task in view of its industrial application. The purpose of this study is to explore the improvement of the activity and stability of a laccase by entrapping it in feather keratin coated CPG, silica gel and soil, and to compare the properties of the immobilized enzyme preparations with each other and with those of free enzyme.

2. MATERIAL and METHODS

2.1. Organisms and culture conditions

The fungal strains *Cerrena unicolor* (Bull.ex Fr.) Murr. No 139 and *Trametes versicolor* (L. ex Fr.) Pil. No 20 coming from our collections were maintained at 4℃ on the agar slants. Pieces of *C. unicolor* mycelia (*ca.* 0.5 cm) were transferred from the agar slant into the sterile (0.1 MP, 45 min.) liquid medium (70 ml in 250 ml conical flasks containing glass beads), prepared according to Wojtas-Wasilewska *et al.* (1983), but Difco yeast extract was omitted. Before sterilization, the pH of medium was adjusted by 0.1 M HCl to value 5.6. The culture was grown at 26℃ under static condition. When the mycelia occupied the whole surface of the liquid the mycelial mats were broken by shaking with the beads and the homogenate was transferred (10% of total volume of the medium) into 3 l of sterilized as above medium of the same composition (Luterek *et al.,* 1998). The submerged aerated culture of *C. unicolor* (30 litres of sterile air per litre of the culture per hour) was grown for 12 days at 26℃ (*T. versicolor* 4 days) and antifoam B emulsion (Sigma) was applied, if needed, to break the foam.

2.2. Determination of laccase activity

Laccase activity of the culture fluid, and purified free and immobilized enzymes was measured at 20℃ and pH 5.6 in 0.1 M McIlvaine (1921) citrate phosphate buffer, pH 5.6 for *C. unicolor* and pH 5.3 for *T. versicolor,* according to Bollag and Leonowicz (1984).

2.3. Determination of protein

The protein content both in the culture fluids and in laccase preparations was determined according to Ehresmann *et al.* (1973). Bovine albumin was used as a standard. The quantity of protein bound to the supports was calculated by subtracting the protein in the combined original filtrate and washing liquid of the immobilized enzyme from the protein used for immobilization.

2.4. Purification of laccase

For laccase purification the method of Leonowicz *et al.* (1997) with some modifications was used as follows. The aerated cultures at the tops of laccase activity were filtered through Miracloth (Calbiochem, Lucerne, Switzerland). Each filtrate was desalted on the Sephadex G-25 column. The enzyme solutions were concentrated to *ca.* one tenth of the volume at 4℃ with an Amicon ultrafiltration system equipped with a filter type PTGC (pore size 10,000 NMWL) The concentrated preparations were applied onto DEAE-Toyopearl column and eluted by a linear gradient 0 - 0.35 M NaCl in 5 mM TRIS/HCl buffer, pH 6.0. The fractions around the top of laccase activity, eluted by NaCl were collected, dialyzed to 0.5 mM TRIS/HCl buffer, pH 6 and lyophilized.

2.5. Laccase immobilization

The porous glass beads (CPG), obtained from Cormey, Lublin, Poland as well as silica gel (SG) and soil (SL), were stirred in boiling concentrated nitric acid, washed thoroughly with distilled water up to pH 6.0 according to Sarkar *et al.* (1989). The supports were silanized by APTES (Robinson *et al.,* 1971) and activated by glutaraldehyde (Lappi *et al.,* 1976) or coated with the feathers keratin (possessing active peptide groups). Keratin obtained from hen feathers according to Lobarzewski *et al.* (1984) was joined to inorganic supports by means an

"adhesive" method as follows; keratin was dissolved in dimethylsulphoxide, precipitated with frozen acetone and kept in refrigerator at 4℃. After 24 hours the acetone was removed, keratin pellet was washed with distilled water and mixed with water in weight ratio 3 : 100. The obtained keratin - water mixture was mixed (stirring continuously) with inorganic support in the ratio 1 : 1 (volume per weight), and then evaporated to dryness under vacuum and additional dried in air.

Laccase was coupled to silanized and activated by glutaraldehyde inorganic supports according to the method of Leonowicz *et al.* (1988). The enzyme coupling to keratin coated silanized inorganic supports was performed according to Lobarzewski *et al.* (1984). The final preparations were filtered, washed with 0.1 M phosphate buffer pH 7, suspended in 0.1 M phosphate buffer, pH 6 and stored at 4℃.

2.6. Effect of temperature and pH

Activity of free and immobilized laccase was assayed at different temperatures (10 - 70℃) in 0.1 M McIlvaine (citrate phosphate) buffer, at pH optimum for particular preparations, or at different pH (3.0 - 6.6) at 20℃ in 0.1 M McIlvaine buffer.

2.7. Chemicals

All chemicals were reagent grade quality and obtained from Aldrich-Chemie (Steinheim, Germany). Fluka A.G. (Buchs, Switzerland), Merck (Darmstadt, Germany), and Sigma (St. Louis, Mo. U.S.A.). Sephadex G-25 was from Pharmacia (Uppsala, Sweden); DEAE Toyopearl 650 S was from Tosoch Corporation (Tokyo, Japan), 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde were from Sigma; silica gel, grade 22, 60-200 mesh and bovine liver catalase were from Aldrich-Chemie.

3. RESULTS and DISCUSSION

The extracellular laccases were isolated and purified from fermented cultures of both fungi (Table 1). The purification procedure was based on ion exchange chromatography on the DEAE-Toyopearl column. The method is simple and gives relative high yields in comparison with other methods (Leonowicz *et al.*, 1997, Luterek *et al.*, 1998). Applying this routine method we achieved about 18-fold purification of *C. unicolor* extracellular laccase and 23-fold in the case of *T. versicolor* (see Table 1). These data are comparable with the one obtained for *C. unicolor* laccase by Luterek *et al.* (1998).

The purified laccases were immobilized on the silanized porous glass beads (APTES-CPG), silica gel (APTES-SG) and soil (APTES-SL) after activation with glutaraldehyde. As a result of the bonding procedure in cases of CPG and SL, about 80% protein and laccase activity were coupled to the support, whereas in the case of SG only less than 0.2% activity and more than 20% of protein were immobilized as well. The final preparations contained average 2 mg protein/1 g of CPG and SL and their specific activities were around 60 units/mg protein, whereas SG showed much worse results, 0.4 mg and 0.001 units, respectively (see Table 2).

These results are in agreement with those reported by Gianfreda and Bollag (1994). It was demonstrated that about 2.3 mg of *T. versicolor* laccase was immobilized on 1 g of glass beads activated with the same procedure and retained high levels of activity. Also high laccase activity was recovered on the same support earlier, in the studies of Sarkar *et al.* (1989). Further the silanized supports were covered with keratin by using the adhesive method, and laccase preparations were immobilized to these matrices. The results are presented in Table 3.

Table 1. Laccase purification.

Purification step	Activity		Yield (%)	Purification (fold)
	Total (U x vol [ml])	Specific (U/mg protein)		
C. unicolor				
Filtrate	60,470	0.67	100.0	1.00
Sephadex G-25	38,544	1.56	63.7	2.33
DEAE-Toyopearl	10,340	12.13	17.1	18.1
T. versicolor				
Filtrate	30,840	0.49	100.0	1.00
Sephadex G-25	21,476	1.47	69.6	3.00
DEAE-Toyopearl	9,025	11.15	29.3	22.76

Table 2. Laccase immobilization on inorganic matrices.

Immobilization efficiency	Enzyme		Yield (%)	Protein /g support (mg)	Specific activity (units/mg protein)
	Free	Immobilized			
C. unicolor					
CPG					
activity	6.33[1]	5.11[1]	80.7		61.23
protein	11.32[2]	9.12[2]	80.6	2.21	
SG					
activity	5.97[1]	0.01[1]	0.17		0.001
protein	11.23[2]	2.32[2]	20.7	0.44	
SL					
activity	6.21[1]	4.84[1]	77.9		59.12
protein	10.98[2]	8.58[2]	78.1	2.12	
T. versicolor					
CPG					
activity	5.32[1]	4.25[1]	79.9		63.24
protein	13.21[2]	11.13[2]	84.3	1.89	
SG					
activity	5.75[1]	0.01[1]	0.17		0.001
protein	12.93[2]	2.14[2]	16.6	0.33	
SL					
activity	5.11[1]	4.10[1]	80.2		57.13
protein	12.67[2]	10.15[2]	80.1	1.80	

[1] total activity (U x 10^6)
[2] total protein (mg)

Laccase immobilization on keratin coated inorganic matrices was generally more effective than that on inorganic matrices. Exception was silica gel, where laccase could not be immobilized at all. In the cases of CPG and SL, the immobilization yield was increased from about 80% to 90%. Moreover, much less protein was immobilized in keratin coated matrices than in the inorganic ones alone (*e.g.* on CPG-keratin 57.6%, whereas on CPG alone 80.6%). The

Table 3. Laccase immobilization on keratin (KER) coated inorganic matrices

Immobilization efficiency	Enzyme		Yield (%)	Protein /g support (mg)	Specific activity (units/mg protein)
	Free	Immobilized			
C. unicolor					
CPG-KER					
activity	7.26[1]	6.84[1]	94.2		71.45
protein	16.26[2]	9.39[2]	57.6	1.58	
SG-KER					
activity	8.24[1]	0.01[1]	0.12		0.001
protein	15.27[2]	3.01[2]	19.7	0.42	
SL-KER					
activity	6.97[1]	6.29[1]	90.2		68.46
protein	13.79[2]	8.97[2]	57.8	1.65	
T. versicolor					
CPG-KER					
activity	5.97[1]	5.42[1]	90.8		71.87
protein	14.17[2]	7.27[2]	51.3	1.20	
SG-KER					
activity	6.24[1]	0.01[1]	0.16		0.001
protein	13.97[2]	2.11[2]	15.1	0.17	
SL-KER					
activity	5.21[1]	4.79[1]	91.9		65.47
protein	13.47[2]	6.59[2]	48.9	1.10	

[1] total activity (U x 10^6)
[2] total protein (mg)

immobilization yield on keratin-soil was comparable to one on keratin-glass beads. It means that soil, which is much cheaper than CPG, is also a good support for immobilization, and can be used as matrix for coating by keratin. These results confirm and coincide the results reported by Sarkar *et al.* (1989) and Gianfreda and Bollag (1994).

In addition, the some properties of obtained immobilized laccase preparations on CPG and SL (also keratin coated) were discussed in comparison with free enzyme. The effect of pH on the free and immobilized laccase activity is shown in Fig. 1 and Fig. 2.

Free laccases were shown optima pH in the range of 5.4 - 5.6 for *C. unicolor* and 5.2 - 5.4 for *T. versicolor*. Following immobilization, the optima pH for the immobilized laccases generally shifted towards higher values (5.5 - 5.8 and even 5.9 in the case of keratin for *C. unicolor* and from 5.3 to 5.6 (5.7) for *T. versicolor*, respectively), and decreased less gradually both in acidic and alkaline regions. The activity of the immobilized *C. unicolor* laccase remained almost constant in the pH range 5.4 - 6.0, and was about 90% at pH 6.2

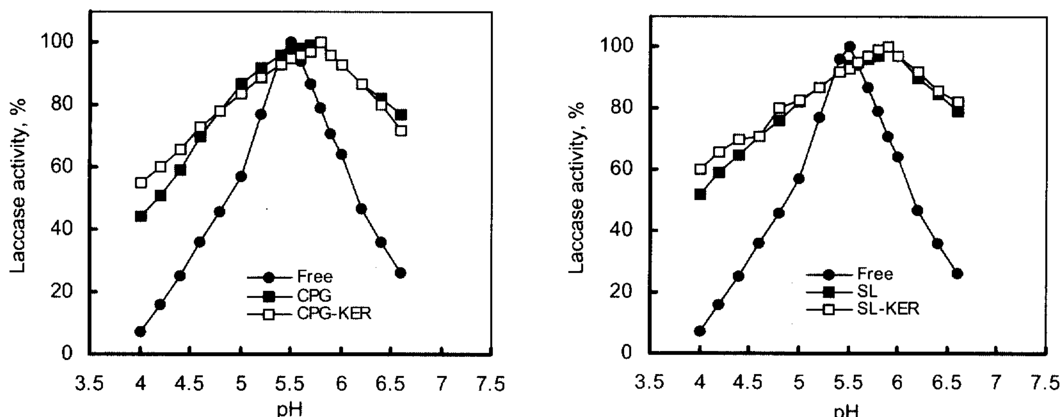

Fig. 1. The effect of pH on the activity of *C. unicolor* laccase free and immobilized on inorganic matrices and keratin coated inorganic matrices.

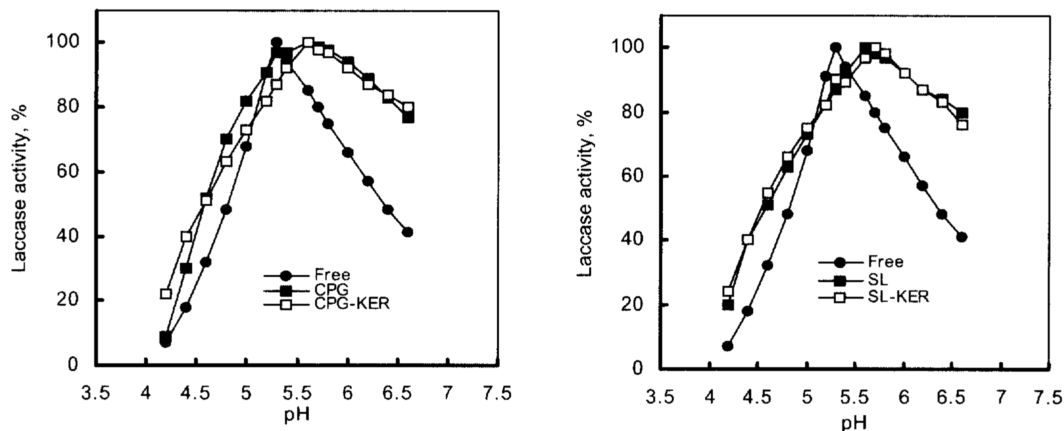

Fig. 2. The effect of pH on the activity of *T. versicolor* laccase free and immobilized on inorganic matrices and keratin coated inorganic matrices.

(the free enzyme showed 47% activity at the same pH). In the case of *T. versicolor* the respective values were 5.3 - 5.8 and 90 - 57%. Even better result was present at any pH in the acidic zone below the optimum. For example, free *C. unicolor* laccase displayed 7% of activity at a pH 4.0, whereas immobilized one at the same pH was shown 44 - 60%. In the case of *T. versicolor* the respective values at pH 4.2 were 7% and 9 - 24% depending on the supports. As shown in Fig. 1 and Fig. 2, the pH shifts were previously observed for various immobilized enzymes, also for *C. unicolor* and *T. versicolor* laccase (Luterek *et al.*, 1998 and Rogalski *et al.*, 1990).

The laccase activity-temperature profiles are shown in Fig. 3 and Fig. 4. Both free and immobilized *C. unicolor* laccases showed the highest activity at 60℃ (Figure 3), *T. versicolor* 55℃ (Figure 4). However, at any temperature, the activity of immobilized enzymes were higher than that of free enzymes at the same temperature. These results suggest that immobilized laccase was more stable against thermal denatura-

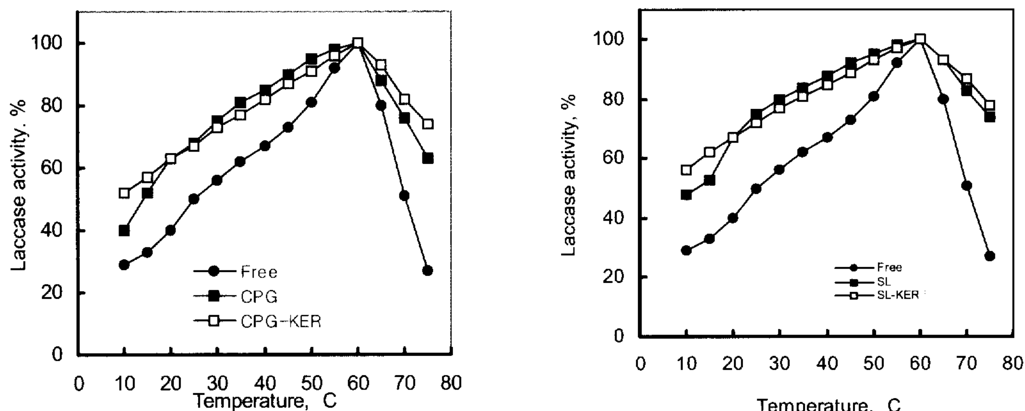

Fig. 3. The effect of temperature on the activity of *C. unicolor* laccase free and immobilized on inorganic matrices and keratin coated inorganic matrices.

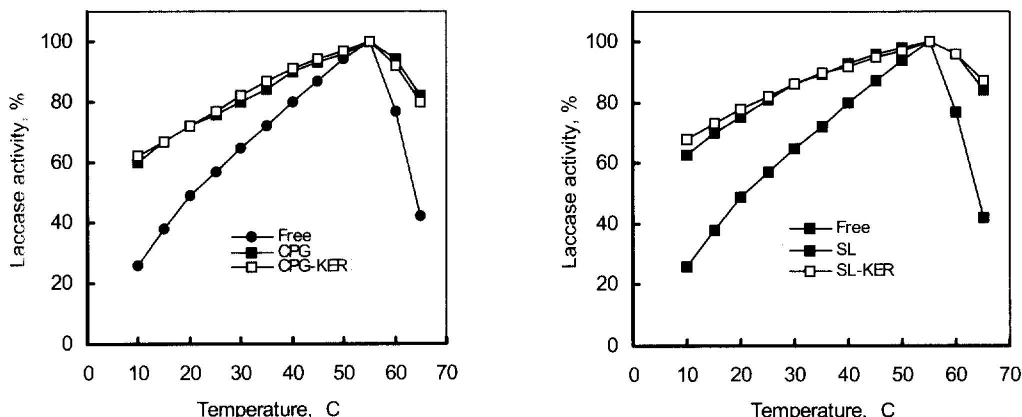

Fig. 4. The effect of temperature on the activity of *T. versicolor* laccase free and immobilized on inorganic matrices and keratin coated inorganic matrices.

tion. This seems particularly true at 75℃ in the case of *C. unicolor*, where the activity of immobilized enzyme is > 50% higher than that of the free enzyme. For *T. versicolor* the respective values were 65℃, and 50%. These results are very similar to those obtained by Rogalski *et al.* (1995) and Gianfreda and Bollag (1994) in studies on free and glass beads immobilized laccase from *Phlebia radiata*, and *T. versicolor*, respectively. High activity and considerable resistance for temperature and pH stresses may have a potential usage of the enzyme for detoxification of the environment polluted by phenolic derivatives of natural and industrial origins as well as the transformation of xenobioties to humic materials.

4. CONCLUSIONS

This study has demonstrated a convenient method for immobilization of extracellular laccase on home made and very cheep glass beads of controlled porosity (CPG), cheep keratin (KER) made from hen feathers and also very cheap soil support (SL) prepared. In cases of CPG and SL, about 80% protein and laccase

activity were coupled to the support, whereas in the case of SG only less than 0.2% activity and more than 20% of protein were immobilized as well. The final preparations contained average 2 mg protein/g of CPG and SL and their specific activities were around 60 units/mg protein, whereas SG showed much worse results, 0.4 mg and 0.001 units, respectively.

The immobilization yield was increased from about 80% to 90% in the cases of CPG-KER and SL-KER by keratin coating on supports. Moreover, much less protein was immobilized in keratin coated matrices than in the non-coated ones (*e.g.* on CPG-KER 57.6%, whereas on CPG alone 80.6%). Laccase immobilization on keratin coated inorganic matrices was generally more effective than that on inorganic matrices. The optima pH for the immobilized laccases generally shifted towards higher values, 5.5 - 5.8 and even 5.9 in the case of keratin for *C. unicolor* and from 5.3 to 5.7 for *T. versicolor*, respectively, and decreased less gradually both in acidic and alkaline regions. The immobilized laccase was more stable against thermal denaturation. This is particularly true at 75℃ in the case of *C. unicolor*, where the activity of immobilized enzyme is > 50% higher than that of the free enzyme. For *T. versicolor* the respective values were 65℃, and 50%.

ACKNOWLEDGEMENTS

This work was supported by Korean Research Foundation (KRF Grant 1998-010-92) and European Community (Contract ICA2-CT-2000-10050) with the Polish Committee of Scientific Investigations (Contracts 139/E-339/SPUB-M-5PR-UE/DZ 280/200, BS/BiNoZ/4, BW/BiNoZ/ 4).

REFERENCES

1. Agematu, H., N. Shibamoto, H. Nishida, R. Okamoto, T. Shin and S. Murao. 1993. Oxidative decarboxylation of 4-hydroxymandelic acid and 2-(4-hydroxyphenyl)glycine by laccase from *Coriolus versicolor* and bilirubin oxidases from *Trachyderma tsunodae* and *Myrothecium verrucaria.* Biosci. Biotech. Biochem. *57,* 1877-1881.
2. Aisemberg, G. O., E. Grorewold, G. E. Taccioli, N. D. Judewicz and A. Major. 1989. Transcript in the response of *Neurospora crassa* to protein synthesis inhibition by cycloheximide. Exp. Mycol. *13,* 121-128.
3. Bollag, J. M. and A. Leonowicz. 1984. Comparative studies of extracellular fungal laccases. Appl. Env. Microbiol. *48,* 849-854.
4. Bollag, J. M, K. L. Shuttleworth and D. H. Anderson. 1986. Laccase mediated detoxification of phenolic compounds. Appl. Environ. Microbiol. *54,* 3086-3091.
5. Bourbonnais, R., M. G. Paice, I. D. Reid, P. Lanthier and M. Yaguchi. 1995. Lignin oxidation by laccase isozymes from *Trametes versicolor* and role of the mediator 2,2'-azino-bis(3-ethyl benzthiazoline-6-sulfonate) in kraft lignin depolymerization. Appl. Env. Microbiol. *61,* 1876-1880.
6. Burton, S. G. and J. R. Duncan. 1995. Activation of mushroom polyphenol oxidase in organic medium by the detergent SDS. Biotechnol. Lett. *17,* 627-630.
7. Cho, N.-S., J. Rogalski, M. Jaszek, J. Luterek, M. Wojtas-Wasilewska, E. Malarczyk, M. Fink Boots and A. Leonowicz. 1999. Effect of coniferyl alcohol on removal of chlorophenols from water effluent by fungal laccase. Mokuzai Gakkaishi (in press).
8. Clutterbuck, A. J.1972. Absence of laccase from yellow spored mutants of *Aspergillus nidulans*, J. Gen. Microbiol., *70,* 423-435.
9. Dittmer, J. K., N. J. Patel, S. W. Dhawale and S. S. Dhawale. 1997. Production of multiple laccase isoforms by *Phanerochaete chrysosporium* grown under nutrient sufficiency. FEMS Microbiol. Lett., *195,* 65-70.
10. Ehresmann, B., P. Imbault and J. H. Weil. 1973. Spectrophotometric determination of protein concentration in cell extracts containing tRNAs and rRNAs. Anal. Biochem. *54,* 454-463.
11. Gianfreda, L. and J. M. Bollag. 1994. Effect of

soils on the behavior of immobilized enzymes. Soil Sci. Soc. Am. J. *58,* 1672-1681.

12. Lante, A., A. Crapisi, G. Pasini, A. Zamorani and P. Spettoli. 1992. Immobilized laccase for must and wine processing. Eds. D. S. Clark and D. A. Estel. *In:* Annals of the New York Academy of Sciences Vol. 672, Enzyme Engineering 11, The New York Academy of Sciences New York, New York. pp. 558-561.
13. Lappi, D. A., F. E. Stolzenbach, N. O. Kaplan and M. D. Kamen. 1976. Immobilization of hydrogenase on glass beads. Biochem. Biophys. Res. Commun. *69*, 878-884.
14. Leatham, G. F. and M. A. Stahmann. 1981. Studies on laccase of *Lentinus edodes*: Specificity, localization and association with the development of fruiting bodies, J. Gen. Microbiol. *125*, 147-157.
15. Leonowicz, A. and K. Grzywnowicz. 1981. Quantitative estimation of laccase forms in some white-rot fungi using syringaldazine as a substrate. Enzyme Microb. Technol. *3*, 55-58.
16. Leonowicz, A., J. M. Sarkar and J. M. Bollag. 1988. Improvement in stability of an immobilized fungal laccase. Appl. Microbiol. Biotechnol. *29*, 129-135.
17. Leonowicz, A., J. Rogalski, M. Jaszek, J. Luterek, M. Wojtas-Wasilewska, E. Malarczyk, G. Ginalska, M. Fink-Boots, and N.-S. Cho. 1999. Cooperation of fungal laccase and glucose 1-oxidase in transformation of Bjorkman lignin and some phenolic compounds. Holzforschung 53, 376-380.
18. Leonowicz, A., L. Gianfreda, J. Rogalski, M. Jaszek, J. Luterek, M. Wojtas-Wasilewska, E. Malarczyk, A. Dawidowicz, M. Fink-Boots, G. Ginalska, A. Leonowicz and N. S. Cho. 1997. Purification of extracellular *Cerrena unicolor* laccase by means of affinity chromatography. J. Korea Tappi 29, 7-17.
19. Leonowicz A, N.-S. Cho, J. Luterek, A. Wilkolazka, M. Wojtas-Wasilewska, A. Matuszewska, M. Hofrichter, D. Wesenberg and J. Rogalski J. 2001. Fungal laccase: properties and activity on lignin. J. Basic Microbiol 41, 183-225.
20. Leonowicz, A., R. U. Edgechill and J.-M. Bollag. 1984. The Effect on the transformation of syringic and vanillic acids by the laccases of Rhizoctonia praticola and Trametes versicolor. Arch. Microbiol. 137, 89-96.
21. Liu, S. Y., A. F. Freyer, R. D. Minard and J. M. Bollag. 1985. Enzyme catalyzed complex formation of amino acid esters and phenolic humus constituents. Soil Sci. Soc. Am. J. 49, 337-342.
22. Lobarzewski, J., A. Paszczynski, T. Wolski and J. Fiedurek. 1984. Keratin and polyamide-coated inorganic matrices as supports for glucoamylase immobilization. Bioch. Bioph. Res. Comm. *121*, 220-228.
23. Luterek, J., L. Gianfreda, M. Wojtas-Wasilewska, N. S. Cho, J. Rogalski, M. Jaszek, E. Malarczyk, M. Staszczak, M. Fink-Boots and A. Leonowicz. 1998. Activity of free and immobilized extracellular *Cerrena unicolor* laccase in water miscible organic solvents. Holzforschung *52,* 589-595.
24. McIlvaine, T. C. 1921. A buffer solution for colorimetric comparison. J. Biol. Chem. *49,* 183-186.
25. Milstein, O., A. Huttermann, H. D. Ludemann, A. Majcherczyk and K. Schulze. 1993. Transformation of lignin related compounds with laccase in organic solvents. J. Biotechnol. *30,* 37-47.
26. Nannipieri, P. and J. M. Bollag. 1991. Use of enzymes to detoxify pesticide contaminated soils and waters. J. Environm. Quality *20,* 510-517.
27. Potthast, A., T. Rosenau, C. L. Chen and J. S. Gratzl. 1995. Selective enzymatic oxidation of aromatic methyl groups to aldehydes. J. Org. Chem. *60,* 4320-4321.
28. Rigling, D. and N. K. Van Alfen. 1991. Regulation of laccase biosynthesis in the plant pathogenic fungus *Cryptonectria parasitica* by double stranded RNA. J. Bacteriol. *173,* 8000-8003.
29. Robinson, O. J., P. Dunnill and M. D. Lilly. 1971. Porous glass as a solid support for immobilization or affinity chromatography of enzymes. Biochim. Biophys. Acta *242*, 659-661.
30. Rodriguez, C. S., R. Santoro, C. Cameselle and A. Saroman. 1997. Laccase production in semi-solid cultures of *Phanerochaete chryso-*

sporium. Biotechnol. Lett., *19*, 995-998.

31. Rogalski, J., A. Dawidowicz and A. Leonowicz. 1990. Purification and immobilization of the inducible form of extracellular laccase of the fungus *Trametes versicolor*. Acta Biotechnol. *10*, 261-269.
32. Rogalski, J., E. Jozwik, A. Hatakka and A. Leonowicz. 1995. Immobilization of laccase from *Phlebia radiata* on controlled porosity glass. J. Mol. Cat. *95*, 99-108.
33. Roper, J. C., J. M. Sarkar, J. Dec and J. M. Bollag, 1995. Enchanced enzymatic removal of chlorophenols in the presence of co-substrares. Wat. Res. *29*, 2720-2724.
34. RoyArcand, L. and F. S. Archibald. 1991. Direct dechlorination of chlorophenolic compounds by laccases from *Trametes versicolor*. Enzyme Microb. Technol. *13*, 194-203.
35. Sarkar, J. M., A. Leonowicz and J. M. Bollag. 1989. Immobilization of enzymes on clays and soils. Soil Biol. Biochem. *21*, 223-230.
36. Simmons, K. E., R. D. Minard and J. M. Bollag. 1989. Oxidative co-oligomerization of guaiacol and 4-chloroaniline. Envron. Sci. Technol. *23*, 115-121.
37. Tagger, S., C. Perissol, G. Gil, G. Vogt and J. Le Petit. 1998. Phenoloxidases of the white-rot fungus *Marasmius quercophilus* isolated from an evergreen oak litter (*Quercus ilex* L.). Enz. Microb. Techn. *23*, 372-379.
38. Wojtas Wasilewska, M., J. Trojanowski and J. Luterek. 1983. Aromatic ring cleavage of protocatechuic acid by the white-rot fungus *Pleurotus ostreatus*. Acta Biochim. Polon. *30*, 291-301.
39. Worrall, J. J., I. Chet and A. Huttermann. 1986. Association of rhizomorph formation with laccase activity in *Armillariella* sp. J. Gen. Microbiol. *132*, 2527-2533.