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Production of Fungal Laccase and Its Immobilization and Stability

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The extracellular laccase of white–rot fungus, *Cerrena unicolor*, was purified from culture by Sephadex G–25 and ion–exchange chromatography on DEAE–Toyopearl column and immobilized on various supports. Purified laccase showed two times higher activity after desalting process and 3.7 to 13.4 times activity after DEAE chromatography. During immobilizing process, 93.8% protein and 100% of laccase activity were coupled to the supports. Following immobilization, the optimal pH (5.5) for immobilized laccase was slightly shifted wide pH values (from 5.0 to 6.0) in the case of some supports, and decreased more gradually in alkaline region. Both free and immobilized laccases showed the highest activity at 60 ˚C, however, the immobilized enzyme was more resistant to the wide range of temperature (50~80 ˚C). Even at the 90 ˚C, treated glass beads and Supp–1 maintained almost 80% activity. After 10 days of storage at 4 ˚C, the immobilized laccases retained 95~100% of its initial activity. It was also more stable during storage at 4 ˚C. While, after the same storage time only 34% the initial activity was retained by the free enzyme. Immobilized laccase on glass supports was retained 85~90% activity over 20 days storage, while free enzyme almost zero activity. The immobilized laccases which are more stable and temperature resistant than free enzyme, seem to be more useful.

INTRODUCTION

Fungal laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is present in several fungal strains belonging to various classes. The enzyme is produced by the different genera of ascomycetes (*Aspergillus nidulans* (Kurtz and Champe, 1982), *Neurospora crassa* (Froehner and Eriksson, 1974), *Podospora anserina* (Durrens, 1981; Esser *et al*., 1964), some deuteromycetes (*Botrytis cinerea*, Debernet *et al*., 1977; Gigi *et al*., 1981), and mainly basidiomycetes (Schanel, 1967). The basidiomycete class includes preferentially various wood and litter decomposing and soil inhabiting fungi (Bollag *et al*., 1979). Basidiomycete fungi are also responsible for lignin degradation. Among fungi of this class laccase is typically produced by efficient lignin degrading species. This suggests that the enzyme apparently has a significant role in lignin transformation *in vivo* (Mayer, 1987; Eriksson *et al*., 1990; Higuchi, 1993; Ishihara, 1980).

Laccase secreted into the environment catalyzes numerous important reactions that are responsible for maintaining the biological activity of a soil. In particular, it participates in the humification processes and may catalyze the coupling of some xenobiotics to the humic materials (Liu *et al*., 1985; Leonowicz and Bollag, 1987; Bollag *et al*., 1986; Bollag, 1983). Furthermore, the enzyme shows a broad substrate specificity and is able to oxidize several phenolic and non–phenolic compounds (Bollag *et al*., 1986; Shuttleworth and Bollag, 1986). The oxidation of phenols generates phenoxy radicals and quinoid intermediates which may be subsequently transformed into dimers or/and oligomers. In the oxidative process several compounds like aromatic amines or amino acids may be involved, giving rise to insoluble polymers (Liu *et al*., 1985; Simmons *et al*., 1989). Although, in vitro, laccase preferentially polymerizes lignin model compounds, it was unequivocally demonstrated that the enzyme also depolymerizes them (Leonowicz *et al*., 1985; Kawai *et al*., 1988). Consequently laccase was listed among the enzymes participating in not only lignin polymerization but also lignin degradation.

Some of laccase abilities are potentially applicable in biotechnology (Reid and Paice, 1992; Fujita *et al*., 1991). In fact, the oxidative capacity of laccase to produce insoluble polymers could be used in the detoxification of terrestrial and aqueous sites, contaminated by various pollutants (Bollag, 1983; Altlow *et al*., 1984; Nannipieri and Bollag, 1991). If the polymerization process occurs in an aqueous environment, phenolic and phenolic–like polymers can be easily removed by sedimentation. At a terrestrial site, the enzyme, present in soil as free or immobilized to soil fractions may oxidize pollutants to non–toxic polymers or entrap them in humic materials (Liu *et al*., 1985; Leonowicz and Bollag, 1987; Gianfreda and Bollag, 1994; Sarkar *et al*., 1989). The lignin transformation may be improved by performing the enzymatic process in organic solvents. Dordick *et al*. (1986) showed that lignin could be depolymerized with horseradish peroxidase in organic media. Milstein *et al*.

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reported that immobilized laccase from Trametes versicolor could express its activity in the reaction media, where most of water has been replaced by organic solvents (Milstein *et al*., 1989). Thus it may be assumed that repolymerization processes of lignin derived phenoxy radicals, released by peroxidase or laccase, might be diminished in some organic solvents in comparison to the aqueous solutions. The aim of this study was the immobilization of *Cerrena unicolor* laccase and its thermal stability of free and immobilized enzymes in the aqueous buffer (pH 5.6).

MATERIALS AND METHODS

Cultures

The basidiomycete *Cerrena unicolor* (Bull. ex Fr.) Murr. CFC–120 was from the culture collection of the School of Forest Resources, Chungbuk National University, Cheongju, Korea. The fungus was maintained on 2% (wt/vol) malt agar slants. Pieces of mycelium (*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). Before sterilization, the pH of medium was adjusted by 1 M hydrochloric acid to value 5.6. The culture was grown at 26 ˚C under unstirred conditions. When the mycelium 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. The submerged aerated culture (30 l of steriled air per liter of the culture per hour) was grown for 12 days at 26 ˚C.

Determination of laccase activity

Laccase activity of the culture fluid and of the purified free and immobilized enzymes was measured at 20 ˚C and pH 5.6 in 0.1 M McIlvaine (citrate–phosphate) buffer with syringaldazine as a substrate (Leonowicz and Grzywnowicz, 1981; Bollag and Leonowicz, 1984; McIlvaine, 1921). The activity was measured and calculated by the following formula and expressed as nkat, *i.e*. nmoles of substrate oxidized during one second. The laccase activities (free and immobilized) in the presence of water–miscible organic solvents were measured using the reaction mixtures containing a proper concentration (v/v) of organic solvent (from 5% to 60%) and 0.1 M citrate– phosphate buffer, pH 5.6.

$$\text{Activity} = \frac{dA_{525} \times \text{total vol (ml)} \times 10^9}{E \times \text{sample volume (ml)}} \text{ (nkat/l)}$$

where : kat = mol/sec
E = 65,000(syringaldazine absorption)

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 on to the glass beads was calculated by subtracting the protein in the combined original filtrate and wash liquid of the immobilized enzyme from the protein used for immobilization.

Purification of laccase

The aerated 12 day old culture (the top of laccase activity) was filtered through Miracloth (Calbiochem, Lucerne, Switzerland). The filtrate was desalted on the Sephadex G–25 column. The enzyme solutions was concentrated to ca. one tenth of the volume at 4 ˚C with an Amicon ultrafiltration system equipped with a filter type PTGC (pore size 10,000 NMWL) The concentrated preparation was 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.

Laccase immobilization

Four immobilizing beads, the controlled porous glass (CPG) beads which are obtained from Cormey–Lublin, Poland, and Supp1, Supp 2 and Supp 3 which are prepared from soils, were stirred in boiling 5% nitric acid, washed thoroughly with distilled water and dried at 180 ˚C. The support was activated using 3–aminopropyltriethoxysilane (APTES) as described by Robinson *et al*. (1971). Laccase was coupled to APTES–CPG using glutaraldehyde according to Lappi *et al*. (1976). The final preparation was filtered, washed with 0.1 M phosphate buffer pH 7, suspended in 0.1 M phosphate buffer, pH 6 and stored at 4 ˚C.

Effect of temperature and pH

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

RESULTS AND DISCUSSION

Isolation and purification of laccase enzyme

Cerrena unicolor extracellular laccase was isolated and purified from fermented, 12 day old culture. The purification procedure was based on ion–exchange chromatography on the DEAE–Toyopearl column. Using this routine method we got the enzyme preparation purified about 13.4 fold (Table 1). The protein with high laccase activity was eluted by 0.2 M NaCl concentration. Table 1 showed increases of specific laccase activities at various purification steps, e.g., 0.79×10^5nkat/mg of culture filterates, almost double activity (1.53×10^5nkat/mg) by desalting step, 10.61×10^5nkat/mg by DEAE Cellulose chromatography. Despite using only two steps of purification, this laccase was sufficiently purified to carry out the investigations whose are presented in this paper. It should be noted that Bekker *et al*. (1990) obtained from the same organism the homogeneous laccase with the 1.5–fold purification, using the procedure consisting of 3 steps.

Table 1. Isolation and purification of extracellular laccase from *Cerrena unicolor*

Purification step	Laccase activity		Purification factor	Yield (%)
	total (nkat×10^5)	specific (nkat/mg protein×10^5)		
Filtrate	530.8	0.79	1.0	100
Sephadex G–25	288.7	1.53	1.9	54.4
DEAE–Toyopearl				
Fraction No 52	14.6	2.92	3.7	2.75
Fraction No 58	83.7	10.61	13.4	15.8

Immobilization of laccase

The purified laccase was immobilized on the silanized porous glass beads(200–400 mesh), Supp1, Supp2 and Supp3, after activation with glutaldehyde. The specific surface area, mean pore diameter, and porosity (pore volume) of glass used as a support were 97.5 m^2/g, 488 nm, and 1.21 cm^3/g, respectively. Such pores are available even for Lambda virus, *i.e.*, for proteins of MW about 10^7 daltons. Therefore the support was useful for the enzyme (MW: 2×10^5 D) and could not act as a macromolecular sieve. The porosity of the glass was higher than that of commercially available glass. Such high porosity allows full utilization of the volume of glass particles for immobilization. As a result of the bonding procedure 93.8% of protein and 100% of laccase activity were coupled to the supports. The final preparation contained *ca.* 2.4 mg protein per 1 g of glass beads. Its specific activity was 0.6×10 units/mg protein. These results are in agreement with those reported by Gianfreda and Bollag (1994). It was demonstrated that about 2.3 mg of *Cerrena unicolor* laccase was immobilized on 1 g of glass beads activated with the same procedure and retained high levels of activity. Almost all laccase activity was recovered on the same support in the studies of Sarkar *et al.* (1989), Rogalski *et al.* (1985) and Leonowicz *et al.* (1988). This results are comparable to that obtained earlier for other fungal enzymes, *e.g.*, cellulase complex (71.6~100% activity retained after immobilization on the porous glass) (Rogalski *et al.*, 1985), laccase (95~100%) (Ishihara, 1980), peroxidase (45%) (Leonowicz *et al.*, 1988), or glucoamylase (11.3~18.17%) (Rogalski *et al.*, 1995).

Effect of pH on laccase activity

The effect of pH on the free and immobilized laccase activity is shown in Fig. 1. Free laccase showed optimum pH with syringaldazine at 5.5. Following immobilization, the optimal pH for immobilized laccase was slightly shifted wide pH values (from 5.0 to 6.0) in the case of some supports, and decreased more gradually in alkaline region. The activity of the immobilized enzyme remained almost constant in the pH range 5.0~6.0 of glass beads and Supp3, and was about 87~95% at pH 6.0 (at the same pH free enzyme showed 55% activity). Such results have previously been detected for various immobilized enzymes (Rogalski *et al.*, 1985; Lobarzewski, 1981; Karube e*t al.*, 1977). This phenomenon can be explained by the partitioning of protons at the active sites of an enzyme when affected by ionized active groups of the support(Leonowicz *et al.*, 1985). It is also interesting to point out that the activity of immobilized laccase was higher at more than 4.5 pH zones. For example, free laccase displayed 46% of activity at a pH of 4.5, whereas 55~90% high activities were shown by immobilized enzyme. Similar pH shift were previously observed for various immobilized enzymes (Woitas–Wasilewska *et al.*, 1988; Lobarzewski 1981; Rogalski *et al.*, 1985), also for *Trametes versicolor* laccase (Leonowicz *et al.*, 1988). A similar change in the pH activity profile for free and immobilized D–xylanase and alkaline phosphatase on porous glass have been reported (Rogalski *et al.*, 1985; Weetall, 1969).

Effect of temperature on laccase activity

The activity–temperature profile is reported in Fig. 2. Both free and immobilized laccase showed the highest activity at 60 ˚C (Fig. 2). However, at any temperature, the activity of immobilized enzyme was higher than that of free enzyme at the same temperature. All immobilized enzymes showed relatively stable at the wide

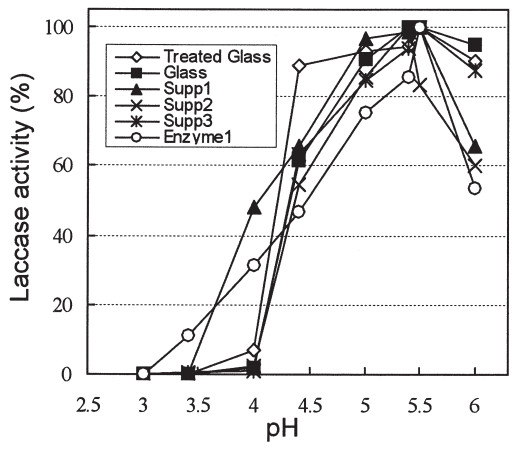

Fig. 1. The effect of pH on activities of free and immobilized laccases.

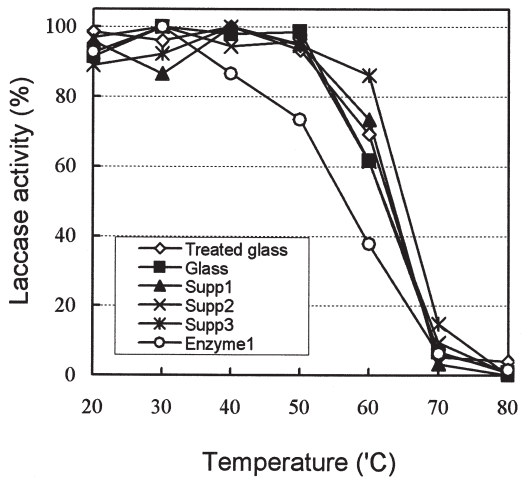

Fig. 2. The effect of temperature on activities of free and immobilized laccases.

range of temperature(50~80 ˚C) compared to the free enzyme. Even at the 90 ˚C, treated glass and Supp1 maintained almost 80% activity. These result could suggest that immobilized laccase was more stable against thermal denaturation. This seems particularly true at 80 ˚C, where the activity of immobilized enzyme is 30~60% higher than the free enzyme. Broader temperature optima were also reported for pronase (Cresswell and Sanderson, 1970) and pepsin (Nishida *et al*., 1988), which were covalently attached to the cellulose and to glass supports, respectively.

Figure 3 referred laccase activity at various different temperatures for 1 hour. At 20 ˚C for 1hr, both immobilized and free enzymes retained more than 90%, these phenomena maintained upto 50 ˚C, and 60 ~87% activity at 60 ˚C. The free enzyme started the activity loss from 30 ˚C, showed 65% activity at 50 ˚C, and only 12% activity retained at 60 ˚C. But the immobilized enzyme was shown higher thermal stability than the free enzyme.

Stability of laccase during storage

The effect of storage of free and immobilized laccases at 4 ˚C is presented in Fig. 4. It can be seen that the immobilized laccase was more stable to the storage than the free enzyme. After 10 days of storage the immobilized laccase retained 95~100% of its initial activity. On the contrary, after the same storage time only 60% the initial activity was retained by the free enzyme. The activities suddenly were decreased after 10days, and reached to 40~60% in the case of immobilized enzymes after 20days, while only 20% activity retained in the free enzyme. At 25days, all activities completely disappeared. 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 *Trametes versicolor*, respectively. An increase in storage stability had also been observed with lactate dehydrogenase immobilized on porous glass (Dixon *et al*., 1973), and with trypsin, papain, and ficin, immobilized by various methods using different carriers (Weetall, 1970). The enzyme immobilized on glass was shown higher thermal stability than those on organic carriers. The reason for inactivation of the enzyme after freeze–drying was not clearly yet determined. The immobilized laccase completely lost its activity after freeze–drying. However, no loss in activity occurred when it was stored at 4 ˚C for 90 days. Immobilized pepsin on porous glass also was shown complete inactivation after freeze–drying, but there was no changes in its activity when stored at 6 ˚C (Line *et al*., 1971).

Figure 5 was shown the effect of storage of free and immobilized laccase at 24 ˚C. The immobilized laccase was more stable to the storage than the free enzyme. After 5 days of storage the immobilized laccases retained 90~100% of its initial activity. On the contrary, after the same storage time only 34% the initial activity was retained by the free enzyme. Immobilized laccase on Glass supports was retained 85~90% activity over 20 days storage, while free enzyme almost zero activity. The activities were abruptly decreased after 10 days storage, and reached to 40~60% in the case of immobilized enzymes after 20 days, while only 20% activity

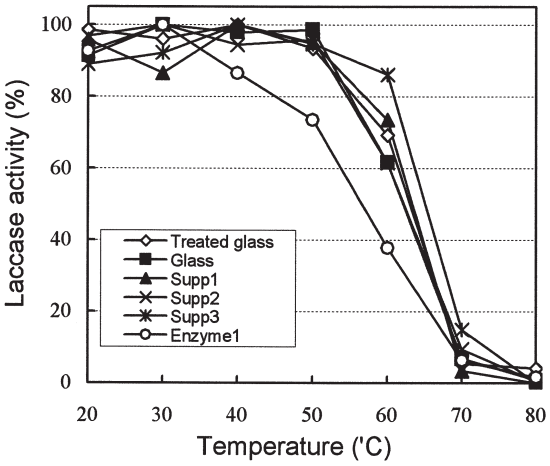

Fig. 3. The effect of temperature (1 hr exposure) on activities of free and immobilized laccases.

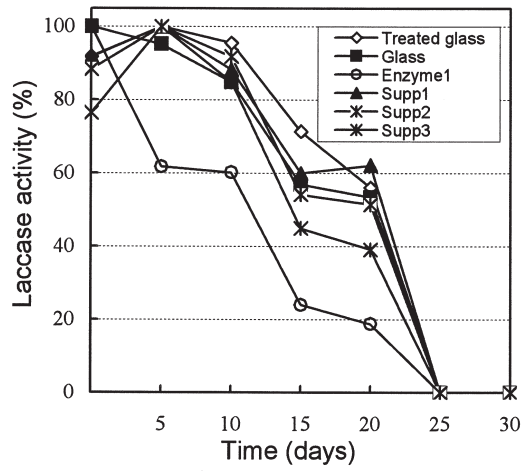

Fig. 4. The effect of storage temperature (4 ˚C) on activities of free and immobilized laccases.

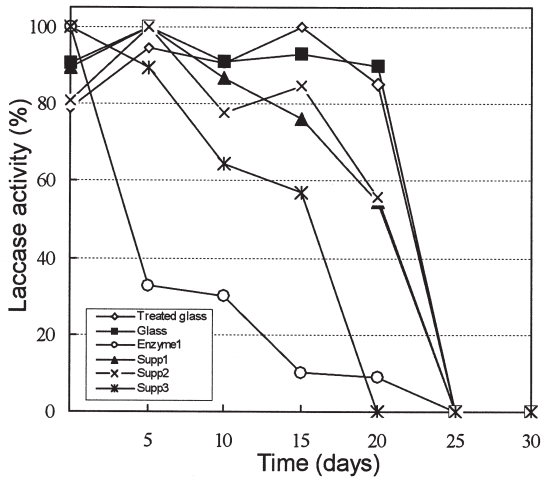

Fig. 5. The effect of storage temperature (24 ˚C) on activities of free and immobilized laccases.

retained in the free enzyme. At 25 days, all activities completely disappeared. 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 *Trametes versicolor,* respectively.

CONCLUSIONS

Cerrena unicolor extracellular laccase was isolated and purified. The purification procedure was based on ion–exchange chromatography on the DEAE–Toyopearl column. Using this routine method we got the enzyme purified about 13.4 fold. The specific laccase activities were increased at various purification steps, *e.g*., 0.79×10^5 nkat/mg of culture filterates, almost double activity (1.53×10^5 nkat/mg) by desalting step, 10.61×10^5 nkat/mg by DEAE–cellulose chromatography. As a result of the immobilization procedure, 93.8% of protein and 100% of laccase activity were coupled to the supports. The final enzyme contained *ca*. 2.4 mg protein per 1 g of glass beads. Its specific activity was 0.6×10 U/mg protein. Free laccase showed optimum pH with syringaldazine at 5.5. Following immobilization, the optimal pH for immobilized laccase was slightly shifted to wide pH values (from 5.0 to 6.0) in the case of some supports, and moved gradually in alkaline region.

Both free and immobilized laccases showed the highest activity at 60 ˚C. However, the activity of immobilized enzyme was higher than that of the free enzyme at the same temperature. All immobilized enzymes showed relatively stable at the wide range of temperature (50~80 ˚C) compared to the free enzyme. Even at the 90 ˚C, the enzymes immobilized on treated glass beads and Supp–1 maintained almost 80% activity. After 10 days of storage at 4 ˚C, the immobilized laccases retained 95~100% of its initial activity. In the case of free enzyme, after the same storage time at 4 ˚C, only 60% of the initial activity was retained. The activities suddenly were decreased after 10days, and reached to 40~60% in the case of immobilized enzymes after 20 days, while only 20% activity retained in the free enzyme. The immobilized laccase was more stable to the storage than the free enzyme at 24 ˚C. After 5 days of storage at 24 ˚C, the immobilized laccases retained 90~100% of its initial activity. While, after the same storage time only 34% the initial activity was retained by the free enzyme. Immobilized laccase on Glass supports was retained 85~90% activity over 20 days storage, while free enzyme almost zero activity. At 25 days, all activities completely disappeared. The oxidizing capacity of free and immobilized laccase was slightly higher for the free enzyme.

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