CHAPTER 6

CHARACTERIZATION AND PARTIAL PURIFICATION OF LACCASE PRODUCED DURING SSF OF 'HAMPAS'

6.0 INTRODUCTION

Recently, the laccase of the edible mushroom, *Pleurotus* sp. has attracted interest because it exhibited nearly 50% degradation of Klason lignin during solid-state fermentation (Martínez *et al.*, 1994). In nature, lignin is degraded by various microorganisms, particularly, white rot fungi. Among the fungi, *Pleurotus* sp. are well known as lignin degraders and have been used for the investigation of lignin degradation. These fungi excrete extracellular lignin-degrading enzymes such as laccase, Mn-dependent peroxidase and H_2O_2-producing oxidases (Guillén *et al.*, 1992; Gutiérrez *et al.*, 1994). Laccases in their pure form are normally blue, contain copper and are capable of catalyzing the removal of an electron and a proton from a hydroxyl group to form a free radical of *o*- and *p*-diphenols (e.g. ferulic acid, hydroquinone) and aromatic amines (Leonowicz *et al.*, 1978; Thurston, 1994). This blue copper oxidase reacts with not only diphenols but also with polyphenols. To date, laccases of several white rot fungi have been characterized and purified (Sannia *et al.*, 1986; Dubernet *et*

al., 1977; Wood, 1980b; Leonowicz and Trojanowski, 1975a; Haars and Hüttermann, 1980; Matcham and Wood, 1992).

In view of the importance of laccase in the degradation of renewable bioresources, there is thus a keen interest in the study of the properties of this enzyme by way of characterization and purification. This study was undertaken not only to enhance the immediate understanding of laccase, but also to provide a foundation for future investigations of the mechanism of action of the enzyme. In this work, some basic properties of laccase produced by *Pleurotus sajor-caju* during SSF of 'hampas' were studied. Some general kinetic properties of the crude and partially purified laccase were undertaken.

The objective of this study was to investigate:

a. simple enzyme-catalyzed reaction of the crude laccase
b. pH and temperature stability of laccase
c. partial purification of the laccase produced
d. kinetic properties of crude and partially purified laccase
e. potential inhibitors to laccase activity

6.1 MATERIALS AND METHODS

The induced crude culture extract samples used in this study were obtained from six to eight day old SSF cultures grown using 4-week old inoculum with 10% inoculum density, which gave the highest laccase activity.

6.1.1 Relationship between absorbance and time of reaction

The relationship between increase in absorbance and reaction time in the reaction mixture was studied. The reaction mixture consisted of 0.1 ml of enzyme, 0.1 ml of 0.1 mM syringaldazine in 50% ethanol and 3.0 ml of 50 mM sodium citrate buffer (pH 4.8). The formation of the purple product (tetramethoxy-azo-*bis*-methylenequinone) was monitored continuously at 25 ± 2°C by measuring the increase in absorbance at λ = 525 nm. This relationship was studied for both the crude and partially purified laccase samples. The linear portion of the initial rate of color change was determined for use as the initial rate of reaction.

6.1.2 Effect of substrate concentration

The effect of the concentration of syringaldazine (0.0125 to 0.3 mM) on the reaction rate of the crude laccase was determined at 25 ± 2°C at pH 4.8. The assay was carried out as described in Appendix A17, keeping the total volume of reaction mixture

constant while varying the syringaldazine concentration. Supernatant samples with the highest laccase activity were selected to obtain the K_m (Michaelis constant) and V_{max} (maximum forward velocity of the reaction) values from the Lineweaver-Burk (1934) plot using linear regression analysis with the Statgraphics™ software (IBM version). As K_m is independent of the substrate or enzyme concentration, it was determined with crude preparations. In contrast to K_m, V_{max} values are dependent on the enzyme concentration and enzyme purity (Werner, 1992). The V_{max} value obtained in this study relate to crude enzyme.

6.1.3 Effect of enzyme concentration

The supernatant samples with the highest laccase activity were used in this experiment. The effect of enzyme concentrations on enzyme activity was studied by adding 0.025 to 0.5 ml of crude filtrate to 0.1 ml portion of 0.1 mM syringaldazine in 50% ethanol and varying the amount of 50 mM sodium citrate buffer (pH 4.8) to keep the total volume of the reaction mixture constant. The assay was carried out at 25 ± 2°C.

6.1.4 Partial purification of laccase

The enzyme purification steps used were according to those employed by Wood (1980b). About 1000 ml of bulk crude culture filtrate obtained after six to eight days SSF of 'hampas' using a 4-week old inoculum at 10% density was used. The samples

used were those induced by vanillin which had an average laccase activity of 32.3 U/g 'hampas'.

All operations were carried out at 5°C. Samples of this bulk culture filtrate were dispensed into Spectra/Por 6 Molecular porous dialysis membrane (MWCO = 50 000) measuring 15 cm in length. The tubes were filled to about half full, suspended in five liters of 0.01M phosphate buffer (pH 6.0) and dialyzed for 48 h against two changes of similar buffer (Wood, 1980b). Then the tubes were removed and carefully buried in a jar containing sucrose for about 4 h to concentrate the filtrate to approximately one-seventh volume. This was carried out after unsuccessful attempts at concentrating the enzyme by ultrafiltration in Vivaspin (15 ml) ultrafiltration cell using a Polyethersulfone membrane (MWCO, 10 000). Laccase was precipitated using solid ammonium sulfate up to 80% saturation which was added to the filtrate. The precipitate was collected by centrifugation (15 000 *g* for 30 min.). All of the enzyme activity was retained in the precipitate, as the yellow colored supernatant had no enzyme activity. For each fraction, 1.0 ml portion was saved for protein and laccase assays which were carried out as described in Appendices A12 and A17.

6.1.5 Determination of pH stability

The stability of crude and partially purified laccase at different pH-values was determined by preincubating the enzymes using buffers systems of pH range from 3

to 10. The buffers used were citrate buffer (pH 3 to 7) and glycine-NaOH buffer (pH 9 to 10) (Appendix B). To 1.0 ml portion of the enzyme, 1.0 ml of suitable buffer was added. Each mixture was incubated for 2 h in a 30°C waterbath with moderate shaking, whereafter the measurement was carried out under normal assay conditions. The results of pH stability are expressed as residual activities that is as a percentage of untreated control.

6.1.6 Determination of temperature stability

The temperature stability of the crude and partially purified laccase was studied by assaying the enzyme at temperatures from 30 to 70°C, with 5°C increments. Thermostability was measured by incubating 1.0 ml enzyme with 1.0 ml of 50 mM sodium citrate buffer (pH 4.8) for 2 h at set temperatures in a waterbath with moderate shaking. After incubation, the supernatant containing the enzyme solution was quickly cooled under running tap water. The residual activity was immediately determined and expressed as percentage of the original activities assayed at room temperature of 25 ± 2°C.

6.1.7 Effect of potential inhibitors on laccase activity

Several reported inhibitors, metal-binding agents and detergents were tested for their action on the partially purified laccase. The inhibitors selected in this study were sodium azide at 0.1 mM and 1 mM; ethylenediaminetetra-acetic acid disodium salt

(EDTA) at 0.1 mM and 1 mM; cetyltrimethylammonium bromide (CTAB) at 0.1 mM and 1 mM; methanol at 50% and 100% and acetone at 50% and 100%.

A fraction of the precipitate was dissolved in an equal volume of 0.01M phosphate buffer (pH 6.0) and used in this study. To 1.0 ml portion of the partially purified laccase, 1.0 ml of the potential inhibitor was added. Each test was carried out in triplicates and incubated for 2 h in a 30°C waterbath with moderate shaking. The percentage inhibition of laccase activity was measured under normal assay conditions.

6.2 RESULTS AND DISCUSSION

6.2.1 Relationship between absorbance and reaction time

In this experiment, an arbitrary eight minutes incubation time was used beyond which no increase in color change was noted with the absorbance readings tapering off. The time course of crude and purified laccase reaction is shown in Fig. 23. The rate of color change was observed in the absorbance due to the formation of pink to purple colored tetramethoxy-azo-*bis*-methylenequinone from the reaction of laccase with syringaldazine (Harkin and Obst, 1973). A time of one minute was fixed to represent the linear portion of the reaction for both the crude and partially purified enzyme.

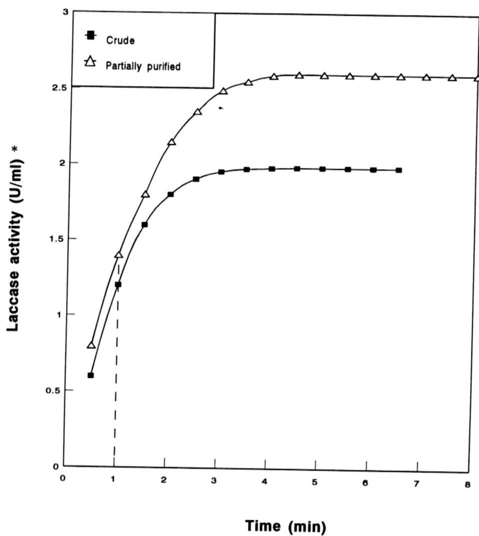


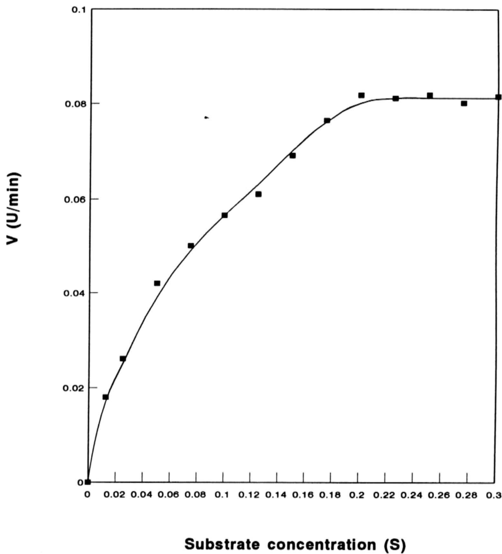
Figure 23. Time course of laccase reaction

*Refer to Appendix A17

6.2.2 Effect of substrate concentration

The relationship between substrate concentration and rate of crude laccase activity is shown in Fig. 24. The syringaldazine concentration was not a limiting factor until the value reached 0.2 mM and the simple enzyme-catalyzed reaction of the crude laccase obeyed Michaelis-Menten kinetics. This is typical as an enzyme has a fixed number of active sites to which substrates can bind (Aiba *et al.*, 1973). The V_{max} value cannot be determined accurately from plot of V against S (substrate concentration). The double-reciprocal (Lineweaver-Burk) plot which gives better estimates on V_{max} was plotted (Fig. 25). The apparent K_m and V_{max} calculated from linear regression analysis of the Lineweaver-Burk plot were 0.073 mM and 0.962 U/min. for syringaldazine, respectively.

Several authors have reported K_m values of pure or partially purified laccase from various microorganisms. The K_m values of laccases reported in literature with different substrates varied from 0.011 to 0.77 mM (Table 15). According to Dubernet *et al.* (1977) the K_m value of the enzyme does not change during purification as it is independent of the substrate or enzyme concentration. Hence, the K_m and V_{max} values of partially purified laccase were not determined.



Substrate concentration (S)

Figure 24. Effect of substrate concentration on crude laccase activity

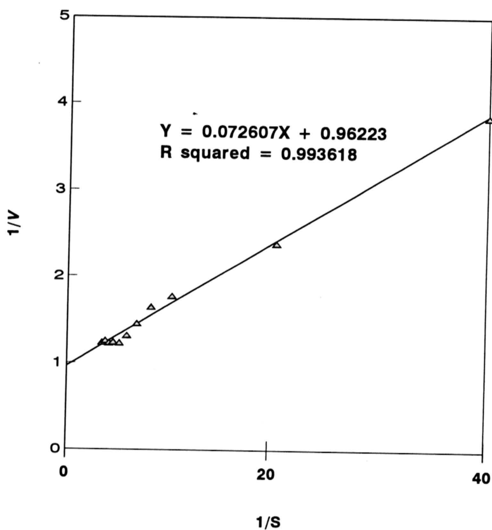


Figure 25. Double-reciprocal (Lineweaver-Burk) plot

Table 15. Apparent K_m values of laccases of various fungi towards different substrates

Substrate	*K_m (mM)*	*Fungi*	*Reference*
p-phenylenediamine	0.1	*Agaricus bisporus*	Wood (1980b)
quinol	0.19	*Botrytis cinerea*	Dubernet *et al.* (1977)
4-methylcatechol	0.045		
p-cresol	0.67		
ascorbic acid	0.77		
syringaldazine	0.0172	*Pleurotus ostreatus*	Leonowicz & Grzywnowicz (1981)
p-phenylenediamine	0.0588		
3,3'-dimethylbenzidine	0.011	*Lentinus edodes*	Leatham & Stahmann (1981)
ABTS	0.280	*Pleurotus ostreatus*	Palmieri *et al.* (1993)
syringaldazine	0.015		
guaiacol	0.650		
Ferulic acid	0.048	*Pleurotus ostreatus*	Youn *et al.* (1995)
Syringic acid	0.089		

ABTS = 2,2'-azino-*bis*-3-ethylbenzathiazoline-6-sulfonic acid

6.2.3 Effect of enzyme concentration

The relationship between volume of crude enzyme concentrate versus laccase activity is shown in Fig. 26. The graph produced is a rectangular hyperbolic curve similar to the substrate concentration graph. It was noted that for 0.1 mM of syringaldazine as substrate, an amount 0.5 ml of crude enzyme gave maximum laccase activity.

6.2.4 Determination of pH stability

The pH stability for *P. sajor-caju* laccase activity in the crude culture extract and in the partially purified form is shown in Fig. 27. The pH dependence of both the forms of laccase, crude as well as partially purified, was not distinctly different. It is interesting to note that the partially purified laccase showed a broader pH stability as compared to the crude form. Inactivation occurred both in crude and partially purified extracts. As reported by Dubernet *et al.* (1977), it is therefore unlikely that inactivation could be ascribed to proteolytic breakdown of the enzyme.

The activity of laccase reached its maximum at pH 6 with syringaldazine as substrate. The crude laccase was 100% stable for at least 2 h when incubated at pH of 4.5 and 9.5 at 30°C while the purified enzyme was totally stable between pH 4 and 10. The residual activities of more than 100% demonstrated that laccase activity in the test

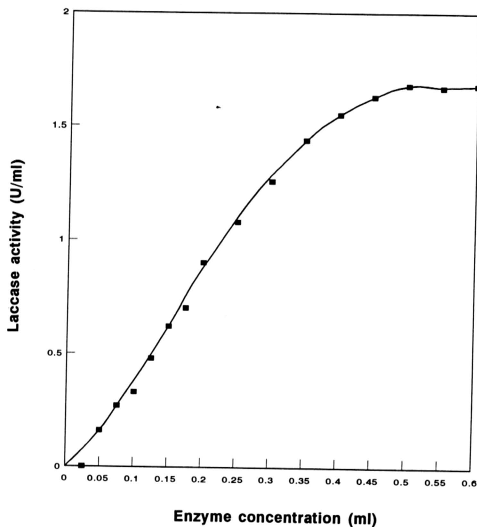


Figure 26. Effect of crude culture filtrate on laccase activity

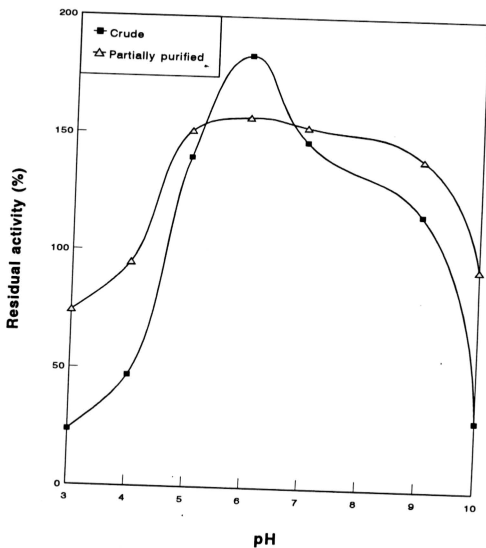


Figure 27. Effect of pH on the residual activity of crude and partially purified laccase

solution was higher than in the control which was maintained at pH 4.8 with 50 mM sodium citrate buffer.

Bonnen *et al.* (1994) studying lignin-degrading enzymes of the commercial button mushroom, *Agaricus bisporus* also reported a pH optimum of 6.0 for laccase with syringaldazine as the substrate. However, pH optimum for laccase from different basidiomycete fungi varied with the substrate used and generally was in the acidic range, viz. 4.7 (Dubernet *et al.*, 1977), 5.0 (Nishizawa *et al.*, 1995), 5.3 (Leonowicz and Grzywnowicz, 1981), 5.6 (Wood, 1980b), 6.0 (Ling, 1994). This lower pH stability of laccase of most white-rot fungi is physiologically significant as most lignocellulosic wastes contain organic acids and the enzyme would not be inactivated easily. The differences in pH optimum may be due to the fact that the enzymes were isolated from different substrates (solid versus liquid media) and were in different states of purification (crude versus purified).

6.2.5 Determination of temperature stability

The effect of temperature on the stability of laccase activities is shown in Fig. 28. Similar to the trend observed in pH stability, the partially purified laccase exhibited a broader temperature stability compared to the crude laccase. The activity of the crude laccase increased steadily with increase in temperature up to 50°C, and then it dropped sharply. The partially purified enzyme retained full activity between 30 and 55°C after

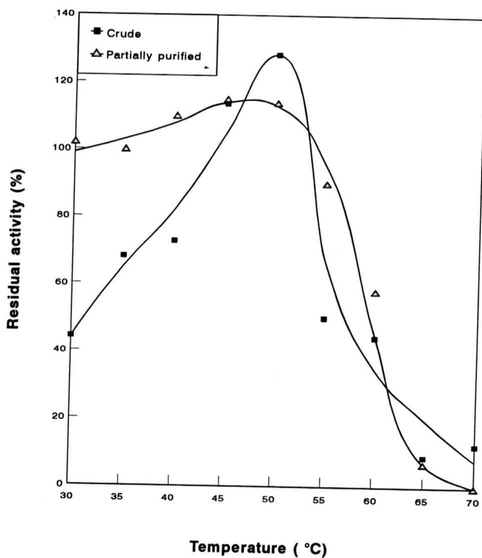


Figure 28. Temperature stability of crude and partially purified laccase

2 h incubation. Somewhat similar results were reported by Ling (1994) while studying the growth of *P. sajor-caju* on oil palm frond parenchyma tissue. Ling also observed that the laccase was 100% thermostable at temperatures of 30 to 50°C for 2 h.

6.2.6 Partial Purification of Laccase and Effects of some Inhibitors

The results of the partial purification for the extracellular laccase from *P. sajor-caju* are shown in Table 16. At the end of the simple three step purification, the laccase was purified about 3-fold from the culture filtrate with 81% activity yield. The most marked increase in laccase activity occurred with ammonium sulfate precipitation. The precipitate had 12-fold higher laccase activity than the crude filtrate.

Table 16. Partial purification for extracellular laccase from *Pleurotus sajor-caju*

Fraction	*Laccase activity (U/g)*	*Protein (mg/g)*	*Specific activity (U/mg)*	*Yield (%)*	*Purification factor*
Culture filtrate	32.3	13.24	2.44	100	1.0
Dialysis	96.9	18.31	5.29	85	2.2
$(NH_4)_2SO_4$ precipitation	387.6	49.61	7.81	81	3.2

The final enzyme precipitate was brown in contrast to other fungal laccases ranging from pale yellow (Wood, 1980b), blue-green (Matcham and Wood, 1992) and blue (Malström *et al.*, 1975; Dubernet *et al.*, 1977). The purification can be improved by raising the initial extraction efficiency with suitable means as proposed by Matcham and Wood (1992). Further studies on purification of crude laccase of *P. sajor-caju* are in progress in this laboratory.

The inactivation of laccase by various potential inhibitors with two concentrations each is shown in Table 17. The enzyme was completely inhibited by sodium azide (1mM), which is a known inhibitor of copper containing oxidases (Bollag and Leonowicz, 1984). Methonal or acetone at 100 % also completely inhibited the laccase from *Pleurotus sajor-caju*, whereas EDTA affected laccase activity to a lesser extent. The least effective inhibition was with the detergent, CTAB. Comparable and somewhat similar findings were reported by Bollag and Leonowicz (1984), Wood (1980b), Haars and Hüttermann (1980) and Dubernet *et al.* (1977).

The information obtained in this study on the properties of laccase produced by *Pleurotus sajor-caju* could lead to the understanding of the functional role, not yet fully understood, for the oxidative processes performed by this fungus. The other hydrolytic and oxidative enzymes produced by this fungus could also be used for large

Table 17. Percentage inhibition of *Pleurotus sajor-caju* laccase by potential inhibitors

Potential inhibitor	*Present study*	*Wood (1980b)*[#]	*Bollag & Leonowicz (1984)*[†]	*Palmieri et al. (1993)* *
Sodium azide (1 mM)	100	100	100	na
Sodium azide (0.1 mM)	86	90	100	na
Methanol (100%)	100	na	na	92
Methanol (50%)	56	na	na	na
Acetone (100%)	96	na	na	70
Acetone (50%)	25	na	na	na
EDTA (1 mM)	26	26	25	na
EDTA (0.1 mM)	17	5	7	na
CTAB (1 mM)	0	80	na	na
CTAB (0.1 mM)	9	5	na	na

Values represent the relative activity with respect to that of the enzyme not incubated with the potential inhibitor.
na = not available
[#] laccase from *Agaricus bisporus*
[†] Incubation for 15 min. at 24°C, laccase from *Pleurotus ostreatus*
* Incubation for 60 min. in 50% water/organic solvent at 25°C, laccase from *P. ostreatus*

scale bioprocessing as suggested by Matcham and Wood (1992). Leonowicz *et al.* (1972) reported that laccase and peroxidase were especially active in the demethylation and degradation processes of lignin. The purified laccase after immobilization can be used in bioreactors for treatment of wastewaters containing phenolics and lignin.

Further purification of laccase, isolation of its respective genes, and identification of other enzymes involved in 'hampas' breakdown would be important for further characterization of the lignin-degrading system of *P. sajor-caju*.

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