CHAPTER

EFFECT OF PHENOLIC MONOMERS ON GROWTH OF *Pleurotus sajor-caju* AND ON LACCASE PRODUCTION

5.0 INTRODUCTION

Laccase is produced by several basidiomycetes and ascomycetes. It was reported by Leonowicz and Trojanowski (1975b) that new forms of laccase were induced by ferulic acid in *Coriolus versicolor*, *Pleurotus ostreatus* and *Pholiota mutabilis*. Leonowicz *et al.* (1978) later demonstrated that the induced forms of laccase had several-fold higher activity than the constitutive laccase towards degradation of toxic phenolic compounds. *De novo* biosynthesis of laccase was indicated by an increase in its activity by the effect of ferulic acid on *Pleurotus ostreatus* and was preceded by an increase of mRNA biosynthesis (Leonowicz and Trojanowski, 1975a).

In the natural habitat, *P. sajor-caju* grows on wood or plant litter and comes in contact with various phenols of plant origin. Phenolic monomers are commonly found in lignocellulosic wastes. Extracellular fungal laccases, by oxidizing these compounds, play a role as detoxifying agents. The amounts of laccase produced vary tremendously as enzyme synthesis is regulated in both positive and negative directions by control mechanisms such as induction, end product repression and catabolite repression.

Inducible extracellular enzymes are normally synthesized at a lower rate when the substrate is absent, but the synthesis may increase several thousand-fold upon induction (Ward, 1989). Control mechanisms in 'overproduction' of enzymes can be influenced by environmental conditions such as addition of inducers and decreasing repressor concentrations and genetic manipulations such as constitutive mutants and increasing copies of enzyme producing genes (Demain, 1971; Dhaliwal *et al.*, 1992). In order to assess the importance of phenolic monomers in the development of a suitable 'hampas'-substrate for the growth of *P. sajor-caju* and for laccase production, some lignin derived phenols were used in these studies.

The objectives of this study include:

a. assessing the effect of selected lignin-derived phenolic monomers on the growth of *P. sajor-caju*
b. studying laccase production by *P. sajor-caju* during SSF of 'hampas' in the presence of phenolic monomers
c. analyzing the fibrous composition of spent 'hampas'

5.1 MATERIALS AND METHODS

5.1.1 Effects of phenolic monomers on radial growth of *P. sajor-caju*

The effects of selected phenolic monomers on the growth of *P. sajor-caju* was studied. The aromatic compounds tested were ferulic acid (4-Hydroxy-3-methoxycinnamic acid); vanillin (4-Hydroxy-3-methoxybenzaldehyde); and 2,5-xylidine (2,5-Dimethylaniline). These three lignin-derived monomers were selected, among others, as they have been reported in literature to induce laccases of basidiomycetes (Table 12).

Ferulic acid is soluble in hot water, alcohol and ethyl acetate and is used as food preservative (Merck, 1989). Stock solution of 20 mM ferulic acid was prepared in 50% ethanol and filter-sterilized.

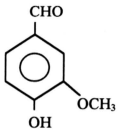
Vanillin is freely soluble in alcohol, chloroform, ether, carbon disulfide, glacial acetic acid and pyridine. About one gram of vanillin dissolves in 100 ml water (Merck, 1989). A stock solution of 20 mM Vanillin was prepared in distilled water and filter sterilized.

Table 12. Some phenolic compounds reported to induce laccase in Basidiomycetes

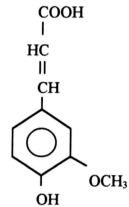
Fungi	*Inducer*	*Reference*
Polyporus versicolor	2,5-xylidine	Fåhraeus *et al.* (1958)
Pleurotus ostreatus	ferulic acid	Leonowicz & Trojanowski (1975a)
Coriolus versicolor	ferulic acid	Leonowicz & Trojanowski (1975b)
Pleurotus ostreatus		
Fomes annosus	2,5-xylidine	Bollag & Leonowicz (1984)
Pholiota mutabilis		
Pleurotus ostreatus		
Pleurotus sajor-caju	ferulic, vanillic, caffeic, *p*-coumaric acid & vanillin	Cai *et al.* (1993)

2,5-Xylidine is a dark colored liquid above 20°C, immiscible with water, but soluble in alcohol and forms more or less soluble salts with strong mineral acids (Merck, 1989). A stock solution of 50 mM of xylidine was prepared in 50% ethanol and filter-sterilized.

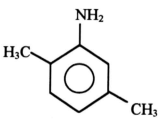
All chemicals, purchased from commercial sources and of the highest purity available, were used without further purification. The chemical structures of the phenolic compounds used in this study and some of their properties are shown in Figure 21.



Vanillin
4-Hydroxy-3-methoxybenzaldehyde;
Methylprotocatechuic aldehyde;
Vanillic aldehyde
$C_8H_8O_3$ MW = 152.15 g/mol



Ferulic acid
4-Hydroxy-3-methoxycinnamic acid;
Caffeic acid 3-methyl ether
$C_{10}H_{10}O_4$ MW = 194.18 g/mol



2,5-Xylidine
2,5,-Dimethylaniline;
Aminodimethylbenzene
$C_8H_{11}N$ MW = 121.18 g/mol

Figure 21. Chemical structure of lignin-related phenolic monomers

Pleurotus sajor-caju maintained on (PDA) medium at 4°C was used. Prior to use, the slants were brought to room temperature and then point inoculated on PDA plates to examine for purity.

An exploratory study showed that *P. sajor-caju* exhibited poor growth on potato dextrose agar (PDA) supplemented with these phenolic monomers with concentrations of 1, 2, 5 and 10 mM. Known volumes of PDA (adjusted to pH 6.0) were then autoclaved and appropriate amounts of filter-sterilized aqueous solutions of phenolic monomers stocks were added aseptically to the cooled medium to give final concentrations of 0.2, 0.4, 0.6 and 0.8 mM. For all the concentrations of phenolic monomers tested, PDA with the respective solvents were used as 'positive control'.

Each petri dish was inoculated at its center with a 6 mm. agar disc (face up) cut from the vegetative margin of a seven day old culture grown in the absence of phenolic compounds. Six replicate plates for each concentration were used to assess the effect of the added aromatic compound. The fungal cultures were incubated in a 27°C for eight days and the colony diameters, measured at two day intervals, were taken as the mean of two diameters at right angles to each other.

5.1.2 SSF of 'hampas' with inducers

About 10 g of autoclaved 'hampas' was supplemented with mineral nutrients as outlined in Table 8. Solutions containing mineral nutrients were prepared, adjusted to pH 6.0 with 1M NaOH and filter sterilized. A filter sterilized stock solution of 20 mM vanillin was incorporated into the mineral nutrients to give a final concentration of 0.2 mM vanillin in each culture flask. In the case of ferulic acid, a stock solution of 80 mM ferulic acid in 50% ethanol was added to the mineral nutrient solution to give a final concentration of 0.2 mM in all the culture flasks. The contents of the flasks were thoroughly mixed with a sterile spatula and allowed to stand for half an hour.

Each flask was then inoculated with 4-week old *P. sajor-caju* koji at 10% density prepared as outlined in Chapter 4, pp. 83. The flasks were incubated without any shaking at 25 ± 2°C for 12 days. The total number of flasks incubated were 84 [(2 inducers + control) x 7 samplings x 4 replicates]. All the flasks were arranged in a Completely Randomized Design (CRD). Four random culture flasks were sampled at regular intervals on day 0, 2, 4, 6, 8, 10 and 12. The control flasks were prepared in the same manner but in the absence of phenolic compound.

5.1.3 Laccase Assay and Chemical Analysis of Spent 'Hampas'

About 100 ml of 50 mM sodium citrate buffer (pH 6.0) was used for extracting the crude laccase from the culture flasks at each sampling (Fig. 12). Extracellular laccase activity of *P. sajor-caju* in the supernatant was determined using standard methods (Appendix A17). The data on laccase activity obtained were analyzed for significance by using one way ANOVA. Means of sample days with maximum laccase activity were tested for significance using the 'Contrast' method. Two contrasts were tested, that is (1) 'Vn. vs. Fer.', to evaluate whether there were any differences between vanillin and ferulic acid and (2) '(Vn. & Fer.) vs. Cont' to evaluate whether there were any differences in the means of laccase activity between with and without phenolics.

At the end of the 12 day SSF, the spent 'hampas' samples were oven dried at 60°C for 48 h and then ground in a Waring blender. The Acid Detergent Fiber (ADF), lignin, cellulose and insoluble ash contents of the spent 'hampas' were determined using the methods outlined by Van Soest and Wine (1968) (Appendix A6-A8). All analyses were carried out in triplicates with each of the four culture flasks.

5.2 RESULTS AND DISCUSSION

5.2.1 Radial growth

The effects of various concentrations of phenolic monomers on the growth of *P. sajor-caju* are shown in Table 13. *Pleurotus sajor-caju* exhibited different sensitivity profiles to vanillin, xylidine and ferulic acid and seemed to be tolerant to the concentrations of phenolics tested as reported earlier by Cai *et al.* (1993). However, the variations are not adequate enough to conclude that the occurrence of these compounds in lignocellulosic wastes alone is responsible for the conspicuous differences in the ability of *P. sajor-caju* to grow on a particular lignocellulosic substrate. The ability of the mushroom fungi to produce ligninolytic and other related enzymes which degrade and/or detoxify inhibitory substrates may also account for the difference.

Of the three compounds tested, only xylidine at 0.8 mM suppressed mycelial growth by 16%. Supplementation of the culture medium with 0.2 to 0.8 mM ferulic acid stimulated growth by 27 to 45%. However, the reverse was observed with vanillin, growth being inversely proportional to increase in vanillin concentration. *Pleurotus sajor-caju* is known to produce phenoloxidases and other extracellular enzymes that have been found to degrade not only ferulic but also vanillic and protocatechuic acids (Hussein *et al.*, 1988) apart from lignin degradation (Royse, 1992; Ortega *et al.*, 1992).

Table 13. Effect of lignin-related phenolic monomers on the growth of *Pleurotus sajor-caju*

Compound	*Concentration (mM)*	*Growth(% of control value)*
Vanillin	0.2	129
	0.4	123
	0.6	114
	0.8	107
Xylidine	0.2	115
	0.4	118
	0.6	102
	0.8	84
Ferulic acid	0.2	127
	0.4	136
	0.6	145
	0.8	141

Values represent the mean of six replicates. The growth was recorded after 6 days where growth rates were linear with time.

In the present study, *P. sajor-caju* produced color reactions with xylidine, especially with concentrations higher than 0.4 mM (Plate 7). These dark diffusion zones, indicated release of phenoloxidases and were found around and beneath the mycelium on the PDA plates. These reactions are similar to those found with *Pleurotus ostreatus* (Kirk and Kelman, 1965) and *Pleurotus flabellatus* (Rajarathnam *et al.*, 1987). Higher concentrations of xylidine were found to be toxic and inhibited the growth of *P. sajor-caju* (Table 12). Laccase induced in the presence of phenols has been reported to cause the detoxification of phenols(Johansson and Hägerby, 1974)

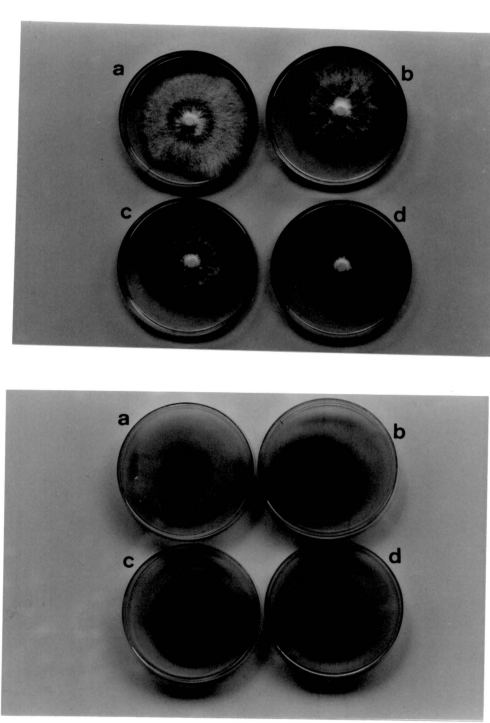


Plate 7. Effect of various concentrations of xylidine on growth of *P. sajor-caju*
a = 0.2 mM, b = 0.4 mM, c = 0.6 mM, d = 0.8 mM
A = top view B = reverse view of PDA plates

but Haars and Hüttermann (1980) were of the opinion that not the phenol itself, but quinone, the colored-oxidation product is toxic to the fungus. In the present study, laccase activity was detected in the cultures of *P. sajor-caju* and this enzyme could have led to the color reactions with xylidine. However, these qualitative observations and reasoning need further verification.

Another interesting observation was that *P. sajor-caju* produced aerial and sparse mycelium in the culture plates with higher concentration (more than 0.4 mM) of phenolic monomers compared with unsupplemented controls. In the presence of phenolics, the mycelial growth was irregular on the periphery, making radial growth measurements less precise, at times. It was most evident from the data that *P. sajor-caju* showed higher tolerance towards vanillin and ferulic acid than towards xylidine. The lowest concentrations of 0.2 mM of both vanillin and ferulic acid markedly stimulated growth. However, highest increase in growth occurred with 0.6 mM ferulic acid but growth was inhibited slightly at 0.8 mM ferulic acid.

As pointed out by Demain (1971), some inducers may not be readily available and be costly in increasing enzyme production. It would therefore be wise to optimize other physiological parameters and incorporate minimal quantities of inducers to obtain maximum end-product. It may also be possible to substitute a compound that can be converted by the organism to the required inducer. Considering the cost factor and the relative toxicity of the phenolics at higher concentrations to the growth of *P. sajor-*

caju and enzyme production, the lowest concentration of 0.2 mM ferulic acid and vanillin were chosen for further studies in the development of suitable 'hampas'-substrate for induction of laccase.

5.2.2 Growth of *P. sajor-caju* on 'hampas' with inducers

The results of the effect of vanillin and ferulic acid on laccase activity through SSF are summarized in Fig. 22. Rapid colonization of the fungal mycelium was noted in all flasks after the second day of SSF. Visually, no difference was noted in the mycelial colonization between the induced and control flasks. The mycelium of *P. sajor-caju* colonized the 'hampas' in all the culture flasks by day seven. The rapid growth of thick white mycelium from the koji completely covered the flask bottom by day eight (Plate 8). The thick white mycelium also grew up on the sides of the culture flasks (Plate 9) but no primordiation was observed throughout the study of 12 days.

5.2.3 Laccase activity and changes in fibrous component of 'hampas'

Laccase activity in the presence of phenolic monomers was found to gradually increase with the duration of fermentation with a maximum activity of about 31.2 - 34.8 U/g 'hampas' between six and eight days of SSF (Fig. 22). As found in the earlier study (Chapter 4, pp. 104), maximum laccase production of 17.7 U/g was observed in the

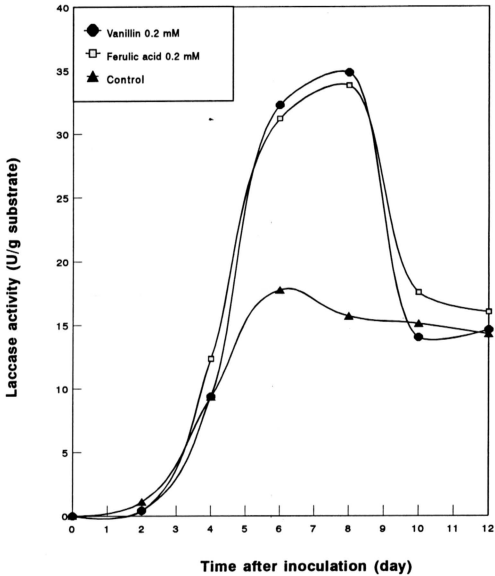


Figure 22. Laccase activity of crude culture extract with lignin-related phenolics

Plate 8. Colonization of 'hampas' by *Pleurotus sajor-caju* after 8 days of SSF
a = raw sago 'hampas' b = upgraded 'hampas'

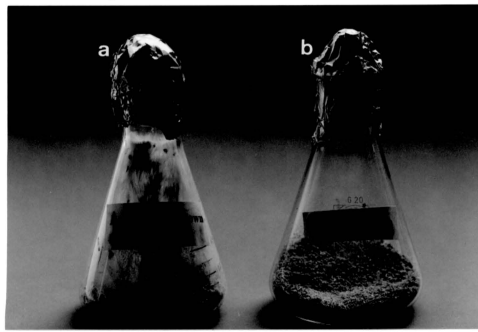


Plate 9. Bioconversion of 'hampas' using *Pleurotus sajor-caju*
a = upgraded 'hampas' b = raw sago 'hampas'
Note the side of culture flasks with mycelial growth

absence of phenolics with the 4-week old 10% inoculum density after six days of incubation. Supplementation of 'hampas' with 0.2 mM of either vanillin and ferulic acid, markedly ($P<0.001$) stimulated the production of laccase by about two-fold between days six and eight of SSF (Appendix C14 and C15). Trojanowski and Leonowicz (1969) working on wood-rotting fungi, *Coriolus versicolor* and *Pleurotus ostreatus* reported that laccase is active in the 'demethylation' process which is the first step in lignin degradation and is induced by lignin or lignin degradation products.

The induction of new forms of laccase by phenolic substrates such as ferulic acid has been observed by other workers (Leonowicz and Trojanowski, 1975a). The addition of various components of lignin into the growth medium displayed significant stimulation of intra- and extra-cellular laccase production by *Pleurotus ostreatus*. Further experiments therefore, are required to verify the possible *de novo* synthesis of laccase in this study.

In the present study, the induced laccase activity after eight days of SSF, decreased and stabilized towards the end of fermentation with values of 14.6 - 16.0 U/g 'hampas'. Similar findings have been reported with extra-cellular laccase of *Botrytis cinerea* in the presence of inducer, activity becoming maximum after 12 to 14 days and later declining (Gigi *et al.*, 1980). Interestingly, the laccase activities of *P. sajor-caju* in 'hampas' with the two phenolic monomers were almost identical as can be seen in Fig. 22. This was confirmed by the 'contrast' method of analysis which showed no

significant difference between vanillin and ferulic acid on laccase activity on day six and eight of fermentation (Appendix C16).

Analysis of the fibrous composition of the spent 'hampas' substrate revealed an average reduction of 14% (w/w) in lignin in cultures supplemented with the phenolic monomers as seen in Table 14. This reduction in the lignin component could be directly related to the increase in induced laccase activity as shown earlier. The cellulose component was not consumed by *P. sajor-caju* during SSF of 'hampas'. Somewhat similar findings by Moyson and Verachtert (1991) showed that *P. sajor-caju* had a preference for hemicellulose and lignin when grown on wheat straw.

Table 14. Fibrous composition of 'hampas', before(fresh) and after(spent) SSF for 12 days

Substrate type	*Lignin (%)*	*Cellulose (%)*	*Residual ash (%)*	*Cellulose/Lignin*
Fresh	24.79 ± 0.82 a	71.18 ± 1.32 a	5.48 ± 0.24 a	2.87 a
Spent-Control	25.24 ± 0.85 a	69.02 ± 1.11 a	6.67 ± 0.09 ab	2.74 a
Spent-Vanillin	21.67 ± 0.10 b	70.71 ± 0.31 a	7.62 ± 0.74 b	3.26 b
Spent-Ferulic acid	21.06 ± 0.27 b	70.02 ± 0.52 a	8.92 ± 0.14 c	3.33 b

Average ± std. error of three replicates. Mean separation in a column by LSD at 5% level. All units are in % dry weight basis except cellulose/lignin ratio.

The cellulose/lignin ratio increased during the 12 days of SSF from 2.74 in the control to 3.3 when 0.2 mM of either vanillin or ferulic acid was added to the medium. This proved higher ligninolytic activity of *P. sajor-caju* with vanillin or ferulic acid compared to that of *P. sajor-caju* without phenolics. These results were in agreement with those reported for sugar cane residues with four *Pleurotus* sp. strains (Ortega *et al.*, 1992) and SSF of cotton stalks by *P. ostreatus* (Kerem and Hadar, 1995).

Another evident observation was that the dried spent 'hampas' was more pale in color compared to the fresh samples and long fibers present in the fresh samples were not present in the spent 'hampas'. (Plate 10). From this study, it was observed that *P. sajor-caju* degraded lignin preferentially during growth on a lignocellulosic waste like 'hampas', and the degraded substrate after lignin degradation became more whiter in color possibly due to exposed cellulose. This fungal degradation phenomena is commonly known as white-rot decay. The brightening of the spent 'hampas' could have been due to a physical effect associated with the dilution of the brown colored 'hampas' by the relatively high levels of white fungal mycelium produced by *P. sajor-caju* as observed by Kirkpatrick *et al.* (1989) working on brightening of Kraft pulp by *Coriolus versicolor*.

In this study, vanillin and ferulic acid at 0.2 mM were found to be non-toxic to the growth of *P. sajor-caju*. Maximum laccase activity of *P. sajor-caju* doubled to about 31.2 - 34.8 U/g 'hampas' after six days of SSF by addition of 0.2 mM vanillin or

ferulic acid. As the magnitude of increase of laccase activity from day six to eight was only 8%, six days of SSF was sufficient to achieve satisfactory bioconversion of 'hampas' and laccase production. Further studies on characterization and purification of laccase would undoubtedly be useful in knowledge of its properties.

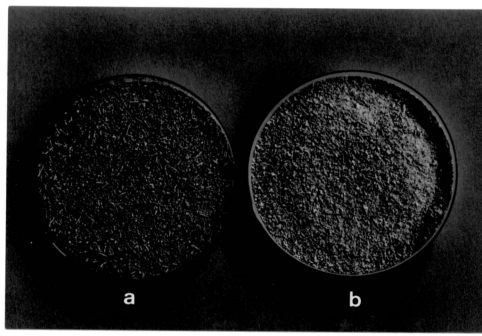


Plate 10. Difference in color of *P. sajor-caju* degraded 'hampas'
a = fresh 'hampas' b = spent substrate
Note the more pale color of spent 'hampas' and long fibers in the fresh samples