CHAPTER THREE

ENZYME ACTIVITIES DURING LIGNOCELLULOSE DEGRADATION

INTRODUCTION

Lignocellulosic waste represents a huge amount of renewable resource for the production of paper products, feeds, chemicals and fuels, therefore there has been an increasing research emphasis of the fungal degradation of lignin (Boominathan and Reddy, 1992).

The ability of white-rot fungi to grow on a wide spectrum of lignocellulosic materials reflects their ability to secrete a range of degradatory enzymes. White - rot fungi are believed to be the most effective lignin-degrading microbes in nature. The majority of previous studies have focused on the lignin-degrading enzymes of *Phanerochaete chrysosporium* and *Tramites versicolor* (Gold and Alic, 1993; Reddy and D'Souza, 1994). Recently, there has been a growing interest in studying the lignin modifying - enzymes of a wide array of white- rot fungi, not only from the stand point of comparative biology but also with the expectation of finding better lignin-degrading systems for use in various biotechnological applications (Orth *et al.*, 1993; Hatakka, 1994; Pelaez *et al.*, 1995 and D'Souza *et al.*, 1996).

Several studies have been done on the utilization of agro-residues to produce enzymes. Research efforts on the utilization of the starchy lignocellulosic residue *hampas*, are still in the early stages. As of date, several utilization strategies of *hampas* for enzyme production through SSF have been

reported by Vikineswary and Shim (1996) and Kumaran *et al.*, (1997). Exploitation of lignocellulosic material must be such that all, if not most of their major components, viz. cellulose, hemicellulose and lignin are utilized.

Fungal enzymes have gained attention in the field of waste management because of their ability to breakdown complex organic pollutants, recalcitrant wastes and bleach plant effluents (Schliephake *et al.*, 1993). Laccase an enzyme present in many white rot fungi has been studied and identified as having an important role in fungal biotreatment. *Pycnoporus sanguineus* a white-rot fungi has been reported to produce laccase as sole ligninolytic enzyme in defined liquid growth medium and has dye decolorization potential (Pointing and Vrijmoed, 2000).

The objectives of this study were to:

(a) obtain a fermentation profile of pH, soluble protein, and the activities of various enzymes such as laccase, xylanase and lignin peroxidase during SSF by *Pycnoporus sanguineus* on *hampas*.

(b) identify the potential enzymes for further investigations.

MATERIALS AND METHODS

A time course study of 30 days was performed to study the solid-state fermentation of sago *hampas* using *Pycnoporus sanguineus*.

Substrate

Sago '*hampas*' was collected from Hup Guan Sago factory in Johor Darul Takzim, Malaysia. The substrate was air-dried and sieved through a 2.0 mm sieve and stored at room temperature prior to usage. (Plate. 3.1)

Inoculum Development

Pycnoporus sanguineus strain CY788 was provided by Professor Gareth Jones, City University, Hong Kong. The stock cultures of *Pycnosporus sanguineus* were maintained on the Potato Dextrose Agar (PDA) slants at 4° C. The fungus was transferred to PDA plates and incubated for 7 days at 27° C. (Plate. 3.2) The koji of *Pycnoporus sanguineus* was prepared using autoclaved wheat grains obtained from Mr. Kuan (Mushroom Farm, Semenyih). The process is, schematically shown in Fig. 3.1.

Solid Substrate Fermentation

Solid Substrate Fermentation cultures were developed in 250 ml conical flasks, each containing about 10 g of sago *hampas* autoclaved at 121° C at 15 psi for 20 minutes and 50 ml nutrient solution containing 0.2% (w/v) KH_2PO_4 and 0.05% (w/v) $MgSO_4.7H_2O$. Filter sterilized urea as nitrogen supplement of 0.38% (w/v) of urea was added to the contents of the autoclaved flasks. The contents of the flask were then thoroughly mixed with a sterile spatula and allowed to stand for 1 h (Kumaran *et al.*, 1997). Each flask was aseptically inoculated with 10% (w/w) of 14 days old *P. sanguineus* wheat grain inoculum and incubated at

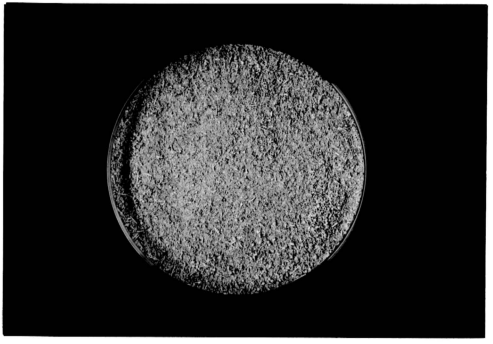

Plate 3.1: Sago 'hampas' used in the experiment

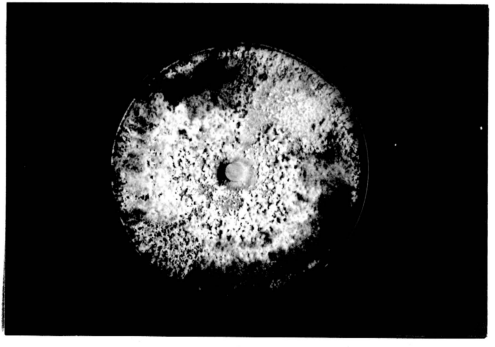

Plate 3.2: *Pycnoporus sanguineus* on PDA plate (seven day old culture)

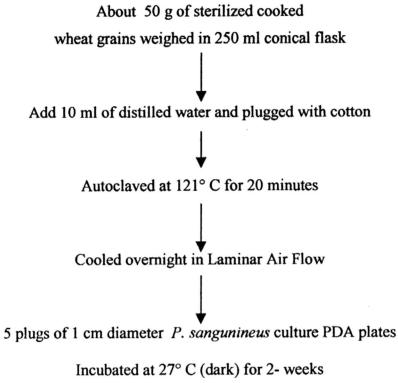

Fig 3.1: Flow chart of the *P. sanguineus* k oji development using sterilized Wheat Grains (adapted from Renuvathani 2002)

25 ± 2°C in static condition. The initial contents of the culture flasks were as shown in Table 3.1. The fermentation was carried out for 30 days. The SSF included flasks with uninoculated '*hampas*' which were analyzed as controls.

Table 3.1: Initial Contents of flask

Substrate	**Mineral solution and urea**	**Inoculum**	**Moisture**
Dried sago *hampas* 10g	0.2% (w/w) KH_2PO_4 0.5% (w/w) $MgSO_4.7H_2O$ 0.38% (w/v) urea	*P. sanguineus* 14 days old, 10% (w/w)	Mineral solution and urea (50 ml) 83% (w/v)

Extraction of Crude Extracellular Enzymes

Three culture flasks were randomly sampled on day 0,5,10,15,20,25,30 of SSF. The content of each flask was extracted with 200 ml of tap water at pH 4.0. A spatula was used to break the solid culture into smaller particles. The flasks were then homogenized at 8000 rpm for 8 minutes at room temperature (Avneesh *et al.*, 2000). The contents of each flask were filtered through a double-layered nylon cloth. The crude filtrate containing the fungal enzymes was stored in 1.5 ml microfuge tubes for 24 h prior to enzyme assays. Assays were performed in triplicates and the results for all values were expressed as mean of triplicates. The outline of the experimental procedure is shown in Fig 3.2.

pH

The pH of the crude culture extract was measured using a digital pH meter.

Protein

The extracellular soluble protein was quantified using the dye-binding method of Bradford (1976) with crystalline bovine serum albumin as standard (Appendix A, pp. 123).

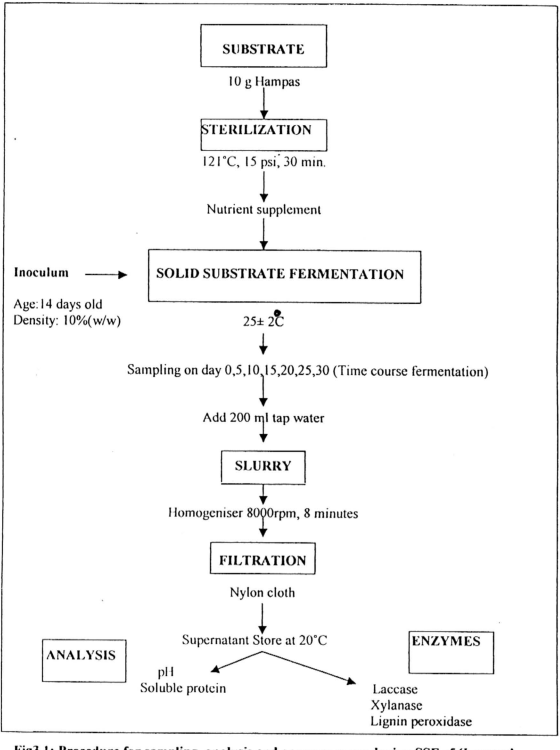

Fig3.1: Procedure for sampling, analysis and enzymes assay during SSF of *'hampas'*

Enzyme assays

(a) Xylanase

Xylanase activity was determined by the method of Bailey *et al.* (1992) (Appendix A, pp. 125). Xylan from oat spelts (Sigma) was used as the substrate and the amount of reducing sugars released was determined. Xylose was used as the standard and the unit of enzyme activity (U) was expressed as the enzyme needed to liberate 1 μ mole of xylose/min/g substrate.

(b) Lignin peoxidase

Lignin peroxidase activity was measured by recording the increase in absorbance at 310 nm (Tien and Kirk 1984). A vertaldehyde standard curve was used to give the concentration of vertaldehyde produced by oxidation of vertyl alcohol (Appendix A, pp. 128). One unit of enzyme (U) was defined as 1μmol vertaldehyde released/min/g/ of substrate.

(c) Laccase

Laccase activity was assayed by the increase in the absorbance due to the formation of the tetramethoxy-azo-bis-methylenequinone resulting from the reaction of laccase with syringaldazine (Harkin and Obst, 1973; Leonovicz and Grzywmowicz, 1981). The initial rate of color change was measured spectrophotometerically at λ=525nm. One unit of activity was defined as the enzyme producing one unit of absorbance change/min/g substrate (Appendix A, pp. 127).

RESULTS AND DISCUSSION

Good growth of *Pycnoporous sanguineus* was observed on sago *hampas*. The first sign of growth was seen two to three days after inoculation. As the culture grew older, the color of mycelia changed from white to reddish orange. By 10 to 11 days of fermentation, complete colonization by the fungus was observed (Plate 3.3).

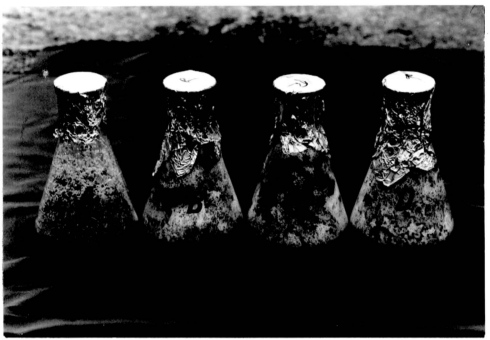

Plate 3.3: Colonization of 'hampas' by *Pycnoporous sanguineus* after 11 days of SSF. A= Control B, C & D = (triplicate flasks)

pH Variation

The pH changes during SSF of 'hampas' are shown in Fig.3.3. (Appendix C. Table 17) The initial pH of the supplemented culture on 0 day was 4.03. There was not much variation of pH in the crude culture extract during the fermentation period. This could be due to the use of tap water of pH 4 for enzyme extraction. The pH profile was in the range of 3.9-5.0 (mean = 4.4) and it was almost similar to that reported by Kumaran (1997) during the SSF of sago *hampas* using *Pleurotus sajor-caju.*

As the fungus grew throughout the SSF, the fungal metabolism might have caused the hydrolysis of urea. This might have resulted in the liberation of ammonium ions and thereby leading to an increase in pH values as seen in the first fifteen days of SSF. As the growth of fungi and fermentation continued ammonium uptake might have exceeded the hydrolysis rate of urea and the pH dropped. Changes in pH were considerably smaller after 20 days of fermentation. The decrease in pH in the substrate may be correlated directly with the decomposition activity of the fungus (Agosin and Odier, 1985). In the present experiment, a pH range between 4 and 5 was found to be suitable for the growth of *Pycnoporus sanguineus* on *hampas.*

Soluble protein

The soluble protein content was used as an indirect assessment of fungal biomass (Moo -Young *et al.*, 1983). The initial concentration of soluble protein culture extract was 0.002mg/ml and the extractable protein reached

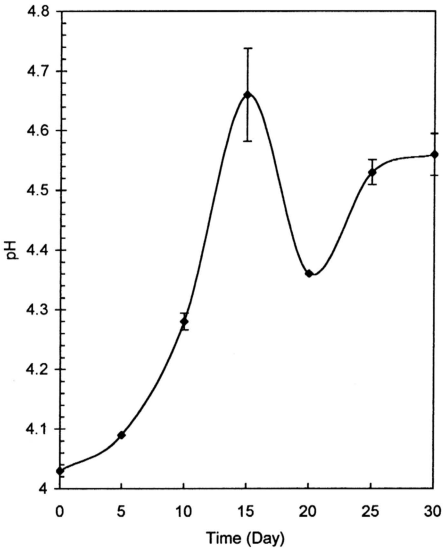

Fig 3.3: Variation of pH in crude culture extract during SSF of sago 'hampas' with *P. sanguineus*

0.065mg/ml after the first 15 days of fermentation as shown in Fig.3.4. (Appendix C. Table 18).

The increase in protein content in the culture extract was partly due to the secretion of enzymes such as laccase and xylanase. Further, other extracellular enzymes responsible for the degradation of *hampas* also contributed to protein content. On day 20 there was a decrease in soluble protein content. This could be due to the degradation of the polymers into monomers e.g. xylose. In the presence of easily assimilable monomers enzyme production drops (Shim, 1992).

The soluble protein can be used to relate the growth of fungus. First fungal growth occurred followed by exponential growth, deceleration and finally catabolic inhibition. The amount of soluble protein may correlate directly with the xylanase activity in the extract.

Xylanase activity

The xylanase activity of the crude culture extract using *Pycnoporous saguineus* during fermentation of sago 'hampas' is shown in Fig.3.5. (Appendix C. Table 19). The profile of xylanase activity resembled that of protein with a decrease on day 20 and increase after day 20 of SSF.The initial xylanase activity was 3.45 U/g substrate and it increased to 5.26 U/g substrate on day 10 of fermentation. The decrease in enzyme activity could be due to catabolic inhibition by increasing amounts of xylose. As xylose level goes down enzyme activity increased and the highest activity of 5.64 U/g substrate was recorded on day 30. This activity was 1.6 times lesser than the highest xylanase activities of 9.14 U/g OPFPt on day 15 by Renuvathani (2002) using *Pycnoporus sanguineus.*

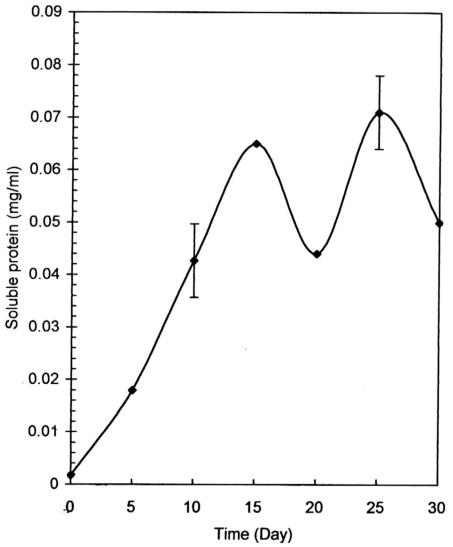

Fig 3.4: Soluble protein content in crude culture extract during SSF of sago 'hampas' with *P. sanguineus*

Vimala *et al.*, (2000) reported xylanase activities of 11.13 U/g OPFPt on day 20 using the same species. A xylanase activity of 2.85 U/g of OPFPt was also reported on day 30 by Ling (1994) using *Pleurotus sajor- caju* with Palm Oil Sludge Solids (POSS) supplementation.

Pycnoporus sanguineus has also been reported to produce xylanase in SmF using wheat bran as a carbon source (Almeida *et al.*, 1993). In this study xylanase ranked second to laccase. Xylanase has attracted increasing attention in biotechnological research during the past decade, largely because of its potential applications in improving the effectiveness of conventional bleaching chemicals in Kraft pulp bleaching, bioconversion of lignocellulose-derived sugars into fuels, bread-making and clarification of beer and juices (Paice *et al.*, 1988; Royer and Nakas, 1989; Bajpai *et al.*, 1994 and Buchert *et al.*, 1994).

Lignin peroxidase activity

The lignin peroxidase activity of the crude culture extract of *Pycnoporus sanguineus* during SSF of 'hampas' is shown in Fig 3.6. (Appendix C. Table 20). The initial lignin peroxidase activity was 0.83 U/g of substrate. The activity remained low and did not show any distinct increase during the first 5 days of fermentation. The activity showed a rapid increase of 1.31U/g substrate on day 10. This activity however, was less compared to the highest lignin peroxidase activity of 4.72 U/g of OPFPt at day 5 of SSF using *Pycnoporus sanguineus* (Vimala *et al.*, 2000). From day 10 onwards the lignin peroxidase activity decreased rapidly until the end of fermentation period of 30 days.

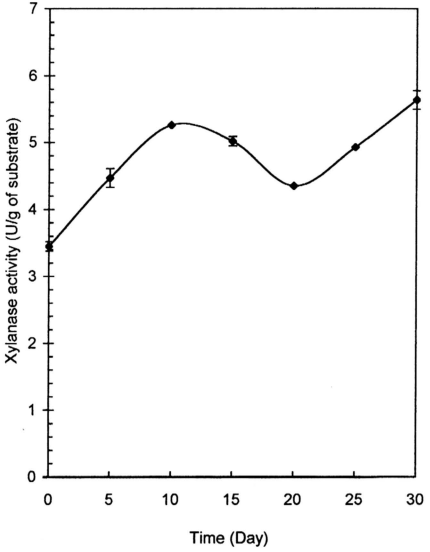

Fig 3.5: Xylanase activity in crude culture extract during SSF of sago 'hampas' with *P. sanguineus*

Since the discovery of lignin peroxidase from *Phanerochaete chrysosporium* (Tien and Kirk 1983) many white rot-fungi have been found to be efficient lignin degraders. But the production of lignin peroxidase, however, has been reported hitherto only from *Phlebia radiata* (Hatakka *et al.*, 1986), *Coriolus* (*Polyporus, Trametes*) *versicolor* (Waldner *et al.*, 1986; Dodson *et al.*, 1987; Jonsson *et al.*, 1987) and *Panus tigrinus* (Golovleva *et al.*, 1986).

The lignin peroxidase remained very low through out the fermentation period. It has been reported by many workers that lignin peroxidase alone is not able to degrade lignin but other enzymes such as laccase and manganese peroxidase are involved in the lignin degradation (Thurston, 1994). In this study *Pycnoporus saguineus* produced 13.4 U/g of substrate of laccase more then lignin peroxidases.

Laccase activity

Laccase activity during SSF increased rapidly during the initial 15 days as shown in Fig 3.7. (Appendix C. Table 21). Laccase was produced in high quantities with an activity of 14.35 U/g substrate on day 15. The tremendous increase of laccase activity in the initial stages of incubation shows that laccase activity was growth associated. The laccase activity of 14.35 U/g of substrate during current study was approx. 1.4 times more compared to the reported value of 10.6 U/g on day 9 during SSF of *hampas* with *Pleurotus sajor- caju* (Kumaran, 1997). The laccase activity of 14.35 U/g of substrate was 1.9 times more than the maximum laccase activity of 7.6 U/g of OPFPt at day 11 using *Pycnoporus sanguineus* (Renuvathani, 2002).

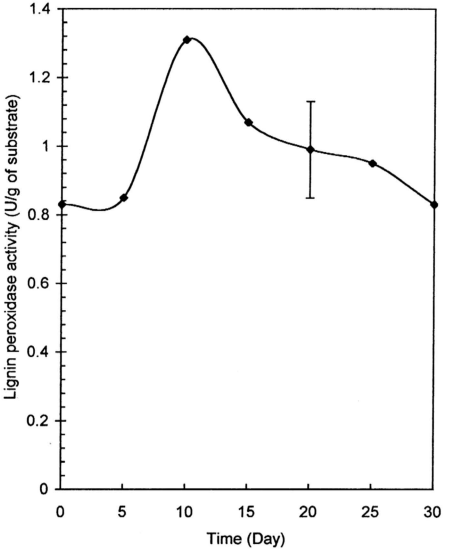

Fig 3.6: Lignin peroxidase activity in crude cultureextract during SSF of sago 'hampas' with*P. sanguineus*

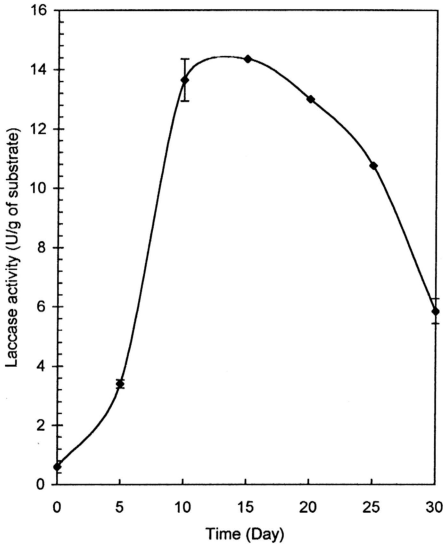

Fig 3.7: Laccase activity in crude culture extract during SSF of sago 'hampas' with *P. sanguineus*

CONCLUSION

In this study, laccase was one of the enzymes produced in high titers with an activity of 14.35 U/g of substrate through SSF. Thus, further studies were done to optimize the production of this enzyme.