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CHAPTER THREE

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CELLULOLYTIC AND LIGNINOLYTIC ENZYME PRODUCTIVITY PROFILES OF *Pycnoporus sanguineus* GROWING IN SSF ON SELECTED AGRO-RESIDUES

3.0 INTRODUCTION

Lignocellulosic wastes represent large amounts of unutilized renewable resources. Bioconversion of agricultural residues into value-added products is gaining attention of many researchers and industrialists because of its promising means of waste disposal as well as for the production of valuable commodities (Bisaria *et al.*, 1990).

Several studies have already been done on the utilisation of agro-residues to produce enzymes through SSF (Ortega *et al.*, 1993; Kumaran, 1996; Pandey *et al.*, 2000). A significant element in any fermentation process is the micro-organism itself. The identification and selection of high-yielding strain of producing organisms is a major area of research investment for the biotechnology industries. The white rot fungi which can degrade all the major components of wood are generally considered to be the main agents of lignin degradation in nature (Buswell and Odier, 1987). One of the well studied organism of this group is *Pha. chrysosporium* (Kirk and Farrell, 1987; Schoemaker and Leisola, 1990). Besides this, edible mushroom such as selected *Pleurotus* spp., which have been successfully cultivated on a commercial level worldwide, have also been reported to produce these enzymes (Kerem *et al.*, 1992; Kumaran , 1996; Hublik and Schinner, 2000).

Fungal enzymes, particularly the lignin modifying enzymes (LME) have gained attention in the field of waste management because of their ability to breakdown complex organic pollutants, recalcitrant wastes and bleach plant effluents (Shin *et al.*, 1997; Hublik and Schinner, 2000; Schliephake *et al.*, 2000). Laccase, an enzyme present in many white rot fungi has been studied and identified as having an important role in fungal biotreatment (Hublik and Schinner, 2000; Schliephake *et al.*, 2000). Further, *Pyc. sanguineus*, a white rot fungus has been reported to produce laccase as the sole ligninolytic enzyme in defined liquid growth medium and this enzyme play a role in dye decolorization (Pointing *et al.*, 2001).

The objectives of this study were :

a) to identify the potential of *Pyc. sanguineus* in producing enzymes during solid substrate fermentation of three selected agro-residues namely sago 'hampas', oil palm frond parenchyma tissue (OPFPt) and rubberwood sawdust.

b) to study the profiles of enzymes involved in degradation of hemicellulose such as xylanase (EC. 3.2.1.8) and endocellulase (EC.3.2.1.4) as well as laccase (EC.1.10.3.2) during SSF.

3.1 MATERIALS AND METHODS

3.1.1 Substrate Collection and Preparation

Three types of agro-residues were chosen as substrates in this study, namely sago 'hampas', oil palm frond parenchyma tissue (OPFPt) and rubberwood sawdust.

a) Sago *'hampas'* (Plate 1) was collected from Hup Guan Sago Factory in Johor Darul Takzim, Malaysia. The substrate was air-dried and sieved through a 2.0mm sieve and stored at room temperature prior to usage.

b) Rubberwood sawdust (Plate 2) was collected from a mushroom farm in Semenyih, Malaysia which uses this material as substrate for cultivation of *Plerotus sajor-caju* and *Plerotus hungarian* (Kuan, 1999).

c) Oil palm frond parenchyma tissue (Plate 3) was obtained from United Plantations Sdn. Bhd., Teluk Intan, Perak. Fresh OPF with leaflets intact, were processed by shredding, hammer milling and sieving followed by drying. The portion that passed through a 2.0 mm size sieve was defined the OPFPt.

3.1.2 Analytical Techniques For Characterization of Substrates

For all tests, the various substrates were analyzed in triplicates. Moisture content was determined for each substrate (Appendix A1). Total nitrogen was determined according to AOAC methods (1990) (Appendix A2). The total carbon content was analyzed by the rapid titration, wet oxidation method of Allen (1987) (Appendix A3). Carbon: Nitrogen (C:N) ratio was calculated for each substrate based on the total carbon and total nitrogen contents.

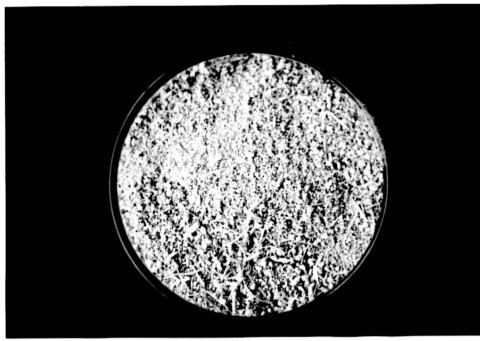

Plate 1 : Sago pith residue (hampas)

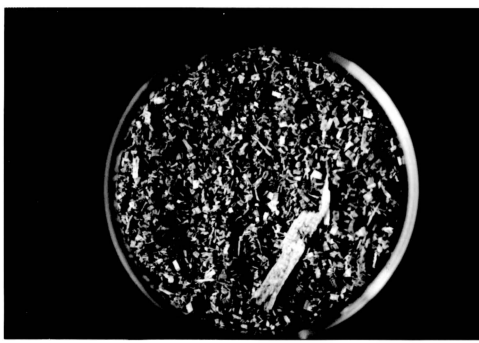

Plate 2: Rubberwood sawdust

Plate 3: Oil palm frond parenchyma tissue (OPFPT)

3.1.3 Inoculum Development

Pycnoporus sanguineus strain CY788 was given by Professor E.B. Gareth Jones, City University, Hong Kong. The stock cultures of *Pyc. sanguineus* were maintained on Potato dextrose agar (PDA) slants at 4° C (Plate 4). The fungus was transferred to PDA plates and incubated for 7 days at 27° C. The koji development (Figure 3.1) of *Pyc. sanguineus* (Plate 5) was done using autoclaved wheat grains obtained from Mr.Kuan (Mushroom Farm, Semenyih).

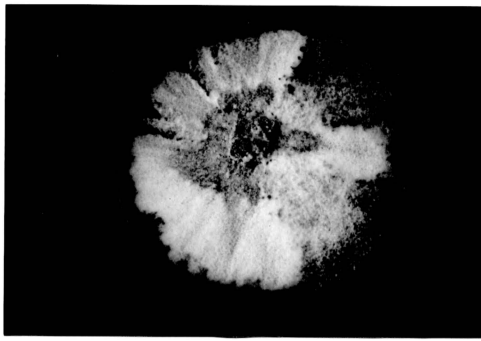

Plate 4: *Pyc. sanguineus* on PDA plate (Seven day old culture)

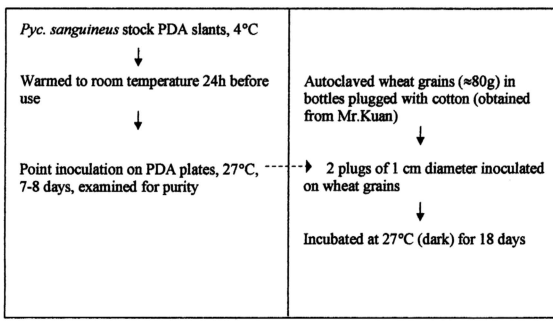

Figure 3.1: The koji development of *Pyc. sanguineus* in autoclaved wheat grains

Plate 5 : The koji development of *Pyc. sanguineus*

3.1.4 Solid Substrate Fermentation

Solid substrate fermentation cultures were done according to Kumaran *et al.* (1997) using sago 'hampas' and rubberwood sawdust as substrate (Table 3.1). About 10g of each of the sterilized substrate was supplemented with nutrient solution containing (w/v): 0.2% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ and 0.38% nitrogen in the form of filter sterilized urea. Culture conditions for bioconversion of OPFPt was according to Ling (1994) (Table 3.1). This substrate was supplemented with 1% (w/v) calcium carbonate and urea containing 0.46% (w/v) nitrogen. The moisture content for each substrate was adjusted within the range of 75-85% (v/w). The contents of the flasks were inoculated with 10% w/w (≈ 2.35 ± 0.05g) of *Pyc. sanguineus* wheat grain inoculum. The flasks were incubated at 25 ± 2°C in static and dark condition. The

fermentation was carried out for 21 days and at suitable intervals, three culture flasks were randomly sampled for each type of substrate tested. Assays were performed in triplicates using the three culture flasks.

Table 3.1 : Summary of contents of fermentation flask (Initial content)

Substrate (10 g)	**Sago *'hampas'* (Kumaran *et al.*, 1997)**	**Rubbetrwood Sawdust (Kumaran *et al.*, 1997)**	**OPFPt (Ling, 1994)**
Mineral solution and urea (w/v) • **urea stock solution = 0.0986 g/ml**	0.2% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ 0.38% N	0.2% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ 0.38% N	1% $CaCO_3$ 0.46% N
Inoculum (w/w) ***(Pyc. sanguineus)*** ***(18 days old)***	23.5% (2.35g)	23.5% (2.35g)	23.5% (2.35g)
Moisture content (mineral and urea solution)	83% (v/w) 50 ml	75% (v/w) 20 ml	80% (v/w) 40 ml

3.1.5 Extraction of Crude Extracellular Enzymes

At suitable intervals of the fermentation period, sampling was done at random and in triplicates. The contents of each flask was extracted with 100 ml of cold 50mM sodium citrate buffer at pH 4.8 (Figure 3.1) (Appendix B1). A spatula was used to break up the solid culture into smaller bits. The flasks were then transferred to an incubator shaker at 200 rpm and at 4° C for approximately 18 h (Kumaran *et al.*, 1997). After shaking, the contents of each flask was centrifuged at 9000 rpm for 20 min. The

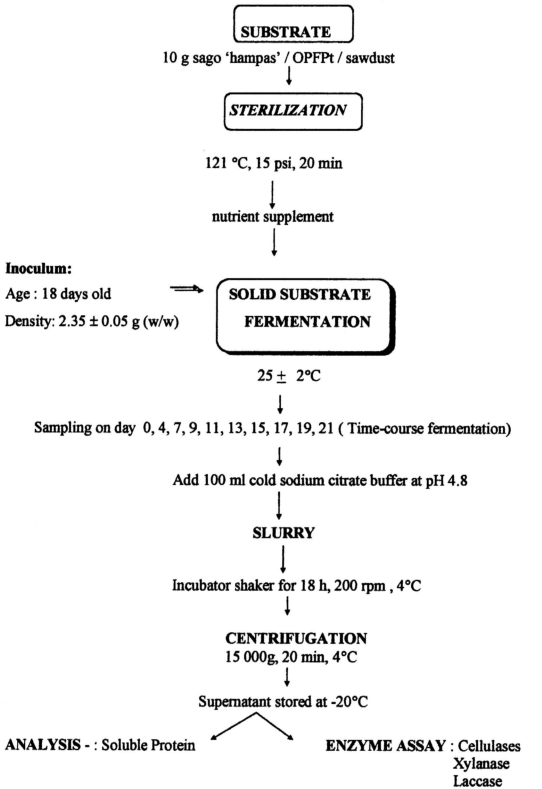

Figure 3.1 : Procedure for inoculation sampling, analysis and enzyme assay during SSF of the various substrates studied (Kumaran, 1996; Ling, 1994).

crude culture filtrate containing 'cocktail' fungal enzymes was stored at - 20°C for 24 h prior to enzyme assays.

3.1.6 Analytical Techniques

3.1.6.1 Enzyme assays

Laccase

Laccase activity (Appendix A4) was determined by the increase in the absorbance due to the formation of tetramethoxy-azo-bis methylenequinone from the reaction of laccase with syringaldazine (Harkin and Obst, 1973; Leonowicz and Grzywnowicz, 1981). One unit (U) was defined as the amount of enzyme producing one unit change in absorbance/min at $\lambda = 525$nm.

Xylanase

Xylanase activity (Appendix A5) was measured using xylan from oat spelts as a substrate in 50mM sodium citrate buffer at pH 4.8. Xylose was used as the standard and absorbance was read at $\lambda = 575$ nm (Bailey *et al.*, 1992). Reducing sugar was measured using dinitrosalicylic acid (DNS) reagent (Miller, 1959). One unit of enzyme productivity (U) was defined as one μmol of xylose liberated/ min/ g substrate.

Endocellulase (CMCASE)

Endocellulase activity (Appendix A5) as carboxymethyl cellulase (CMCase) was estimated by the DNS method (Miller, 1959) using 1% sodium salt carboxymethylcellulose (medium viscosity) as the substrate and absorbance was read at

λ = 575 nm (Dong *et al.*, 1992). One unit of enzyme productivity (U) is expressed as one µmol of glucose released /min/ g substrate.

3.1.6.2 Soluble Protein

The extracellular soluble protein (Appendix A6) was quantified using Bradford dye-binding method. Crysalline bovine serum albumin (BSA) was used as the standard (Bradford, 1976).

3.2 RESULTS AND DISCUSSION

3.2.1 Carbon and Nitrogen ratio of substrates

Carbon and nitrogen content of each substrate used in this study were analysed and carbon and nitrogen ratios (C:N) were calculated accordingly (Table 3.2). The analysis revealed that the C:N ratio of 'hampas' and sawdust was very high mainly due to the low nitrogen levels present in it. Adequate supplementation with urea as a nitrogen to adjust the C:N ratio to about 35:1 rendered 'hampas' suitable for fungal colonization (Moo-Young *et al.*, 1983). Nitrogen supplementation with urea at 0.46% N/ 10 g of OPFPt was used for SSF (Ling, 1994) which showed optimal requirement for fungal growth and enzyme production.

Good growth of *Pyc. sanguineus* was observed on all the three substrates tested. The first signs of growth was seen two to three days after inoculation. As the culture grew older, the colour of the mycelia changed from white to reddish orange and

complete colonization of the fungus was seen within 11 days of fermentation (Plate 6,7 and 8).

Table 3.2 : Carbon and Nitrogen Content of Substrates

Substrate (10 g)	Carbon (%)	Nitrogen (%)	C : N ratio of raw substrate
Sago 'hampas'	37.14	0.18	206
OPFPt	35.24	0.92	38
Sawdust	37.85	0.24	158

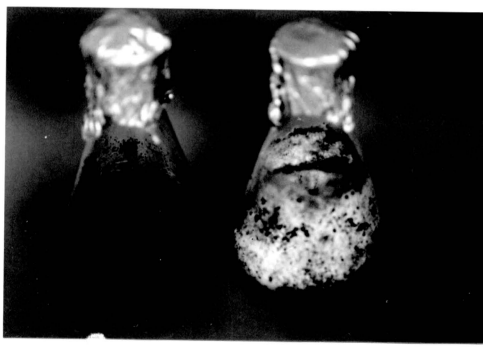

Plate 6 : Colonization of sago 'hampas' by *Pyc. sanguineus* at day 11 of SSF (Left flask : Inoculated substrate at day 0 of SSF; Right flask : Colonized substrate at day 11 of SSF)

Plate 7 : Colonization of OPFPt by *Pyc. sanguineus* at day 11 of SSF

(Left flask : Inoculated substrate at day 0 of SSF; Right flask : Colonized substrate at day 11 of SSF)

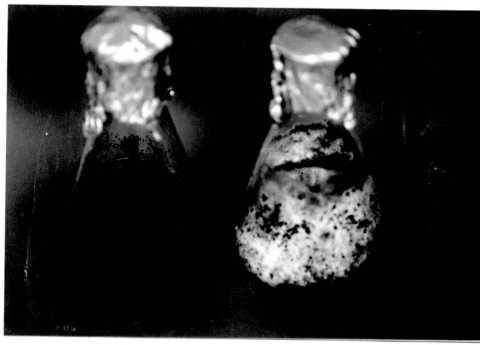

Plate 8 : Colonization of rubberwood sawdust by *Pyc. sanguineus* at day 11 of SSF

(Left flask : Inoculated substrate at day 0 of SSF; Right flask : Colonized substrate at day 11 of SSF)

3.2.2 Soluble Protein

The soluble protein content of OPFPt which had an initial level of 0.5 mg/g substrate increased rapidly during the SSF and peaked at 4.3 mg/g substrate on day 11 of SSF (Figure 3.2). This value was higher than reported value of 3.2 mg/g of 'hampas' on day 11 using *Ple. sajor-caju* for SSF of 'hampas' (Kumaran *et al.*, 1997). This value was, however, comparable to the reported value of 4.5 mg/g OPFPt on day 10 of SSF of OPFPt with *Ple. sajor-caju.* As for sawdust and 'hampas', the soluble protein content was in the range of 0- 0.9 mg/g substrate throughout the fermentation. The increase in soluble protein content may be due to the secretion of enzymes such as laccase, xylanase and cellulase during degradation of the substrate. The soluble protein content was used as an indirect assessment of fungal biomass (Moo-Young *et al.*, 1983) and also as an indicator of the fungal enzyme content. Thus, based on this profile, *Pyc. sanguineus* may be a suitable fungi for the production of lignocellulose degrading enzymes compared to *Ple. sajor caju.* Further, OPFPt produced a maximum protein concentration of 4.3 mg/g substrate on day 11 of SSF which was higher compared to 'hampas' and sawdust. It was thus selected for further studies.

3.2.3Cellulolytic productivity (CMCase)

Maximum CMCase productivity was 6.6 U/g substrate on day 9 during SSF of OPFPt with *Pyc. sanguineus,* which did not show significant difference from the reported value of 6.3 U/g substrate at day 9 during fermentation of 'hampas' with *Ple. sajor-caju* (Kumaran *et al.*, 1997). During SSF of rubberwood sawdust, CMCase productivity peaked at day 11 at about 6.3 U/g substrate (Figure 3.4). High values of

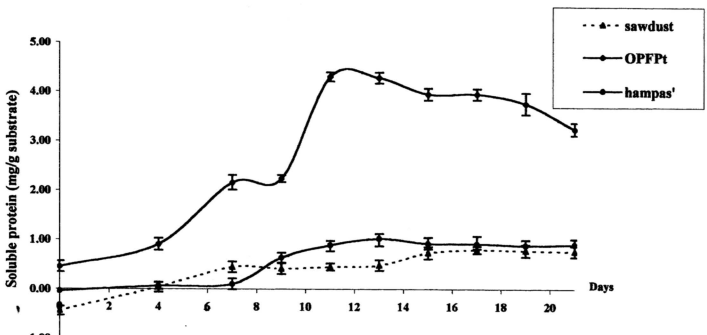

Figure 3.3: Soluble protein content (mg/g substrate) during SSF of various substrates using *Pyc. sanguineus* (incubation at 25 ± 2°C, 18 days old and 10% inoculum)

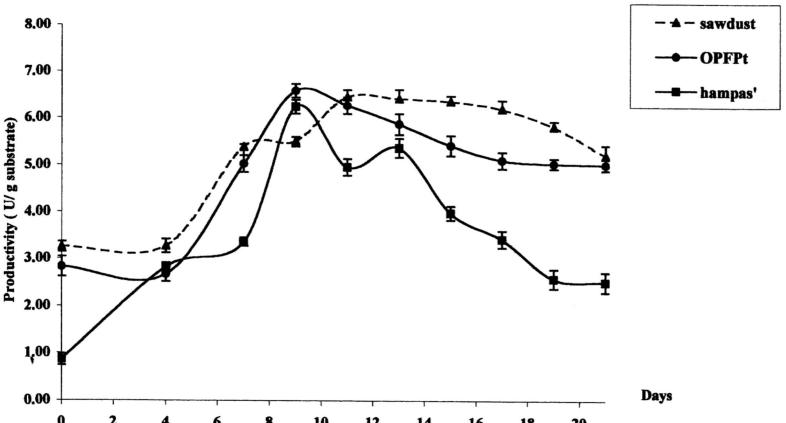

Figure 3.4: Endocellulase (CMCase) productivity during SSF of various substrates using *Pyc. sanguineus* (incubation at 25 ± 2°C, 18 days old and 10% inoculum)

cellulase productivity was also observed for the substrates when tested at day 0. This could be because the koji used was 18 days old and the inoculum size used was 10% (≈2g) which may be the cause of having cellulose activity at day 0. Further, there was a similar pattern in the productivity of CMCase on the various substrates which increased during the first 12 days of SSF and decreased towards the end of the fermentation. This may be due to induction and repression of cellulase enzymes as suggested by Wang *et al.* (1979) and Frost and Moss (1987).

Carboxymethyl cellulase (CMCase) productivity by *Pyc. sanguineus* during SSF of OPFPt was the highest of the three agro-residues tested. This value was also almost 50% higher than CMCase productivity of *Ple. sajor-caju* on OPFPt as reported by Ling (1994). Cellulase (CMCase) from degradation of 'hampas' with *Pyc. sanguineus* was about 120% higher in yield compared to degradation of 'hampas' with *Ple. sajor-caju* (Kumaran *et al.*, 1997). At present, cellulases are used in food, brewery and wine, animal feed, textile and laundry as well as pulp and paper industries (Bhat, 2000). With this increasing demand of this enzyme in the field of biotechnology, it is essential to screen potential microorganisms which can produce this enzyme in high titre. Therefore, *Pyc. sanguineus* which showed comparable results during degradation of OPFPt was a suitable choice for further studies to optimise the production of bulk enzymes.

3.2.4 Xylanase productivity

Maximum xylanase productivity of 9.1 U/g substrate was observed on day 11 during SSF of OPFPt with *Pyc. sanguineus*. This level sustained till day 15 and then

reduced to 6.1 U/ g substrate at the end of SSF (Figure 3.5). There was a significant difference ($p< 0.001$) between maximum yield of xylanase produced during SSF of OPFPt compared to SSF of 'hampas' with *Ple. sajor-caju* which yielded maximum of 7.6 U of xylanase per gram of substrate on day 11 of SSF (Kumaran *et al.*, 1997). Further, maximum xylanase productivity of 8.6 U/g substrate was obtained during SSF of rubberwood sawdust on day 17.

Based on the profile, OPFPt produces maximum xylanase on day 11 compared to 'hampas' and sawdust. This value was also higher than results reported by Ling (1994) using *Ple. sajor-caju*. However, the xylanase profile of the three substrates showed similar pattern which increased in the early stages and decreased at the end of fermentation. A similar pattern of extracellular xylanase production has been reported by Ortega *et al.* (1993) and Kumaran *et al.* (1997) using *Pleurotus* spp. This may be due to the similar activity as cellulase enzymes with xylan acting as inducer and xylose as inhibitor (Biely, 1985).

Xylanase has gained much attention lately mainly because of its potential applications in the areas such as bio-bleaching, bread-making and also in the clarification of beer and juices (Poutanen, 1997; Galante *et al.*, 1998a; Uhlig, 1998). From this study, solid substrate fermentation of OPFPt using *Pyc. sanguineus* produced maximum xylanase on day 11 of SSF which was comparable to xylanase productivity by *Ple. sajor caju* growing in SSF of sago 'hampas'. Thus further studies were done to optimise the production of this enzyme during degradation of OPFPt with *Pyc. sanguineus*.

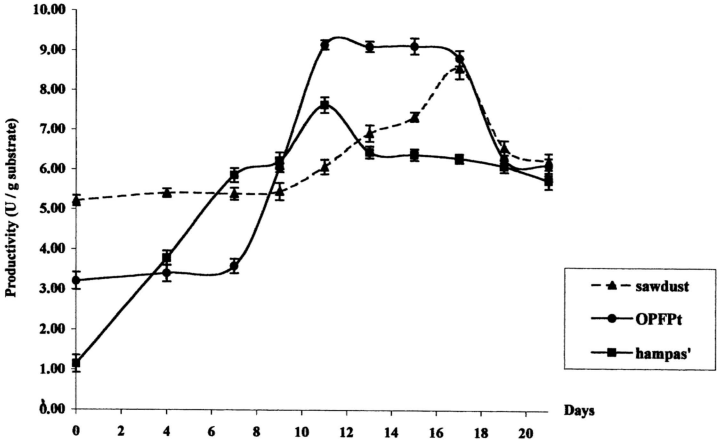

Figure 3.5: Xylanase productivity during SSF of various substrates using *Pyc. sanguineus* (incubation at 25 ± 2°C, 18 days old and 10% inoculum)

3.2.5 Laccase productivity

Figure 3.6 shows the profile of laccase productivity during degradation of various agro-residues by *Pyc. sanguineus*. There was rapid increase in laccase productivity during SSF of both 'hampas' and OPFPt in the first 11 days of fermentation with maximum laccase productivity of 7.6 U/g 'hampas'and 7.5 U/g OPFPt on day 11. However, a rapid decline in laccase productivity was observed during SSF of 'hampas' which reduced to 3.1 U/g substrate at the end of 21 days of fermentation. There was, however, sustained laccase productivity during SSF of OPFPt after day 11 till day 15. After day 15, laccase productivity reduced but at a lower rate which gave 5.4 U of laccase per gram of substrate on day 21. Degradation of rubberwood sawdust produced maximum laccase of only 5.7 U/g substrate on day 11.

3.2.6 Enzyme Profiles of *Pyc. sanguineus*

The enzyme profile during degradation of rubberwood sawdust (Figure3.7) showed vast difference in the increase of laccase within the first 4 days which was about 475% compared to laccase produced during SSF of 'hampas' and OPFPt. However, there were no significant increase in laccase productivity during SSF of sawdust after day 4. Xylanase and cellulase showed a significant rise only after day 4 of SSF of sawdust. This may indicate that laccase was produced to degrade the high lignin content of sawdust which makes the substrate more easily accessible for microbial growth. This pattern was more prominent in the enzyme profile during SSF of OPFPt (Figure 3.7) where laccase showed rapid increase between day 4 and day 11 while xylanase and cellulase only increased after day 9 by which time laccase content

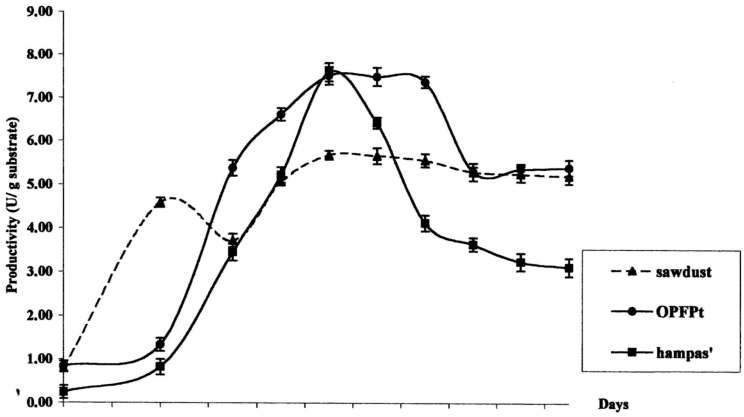

Figure 3.6: Laccase productivity during SSF of various substrates using *Pyc. sanguineus* (incubation at 25 ± 2°C, 18 days old and 10% inoculum)

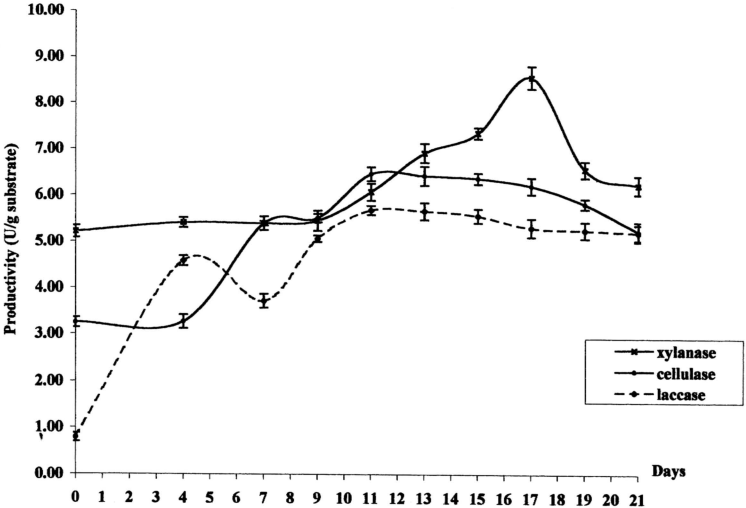

Figure 3.7: Enzyme profile during SSF of rubberwood sawdust using *Pyc. sanguineus* (incubation at 25 ± 2°C, 18 days old and 10% inoculum)

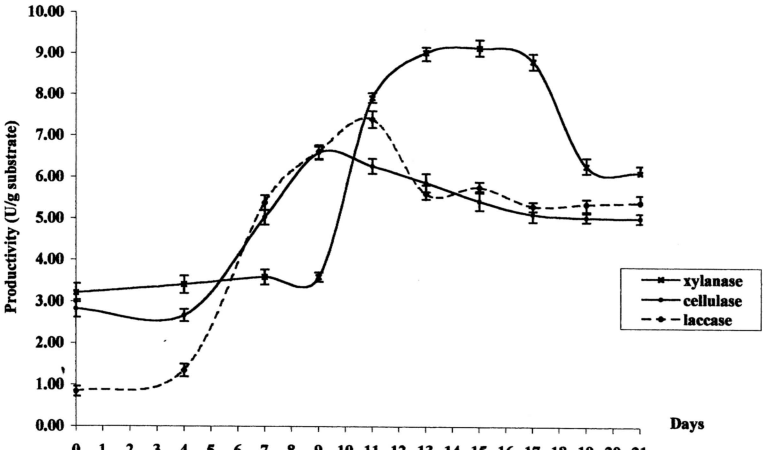

Figure 3.8: Enzyme profile during SSF of OPFPt using *Pyc. sanguineus* (incubation at 25 ± 2°C, 18 days old and 10% inoculum)

was reducing. Similar pattern was observed during SSF of 'hampas' (Figure 3.9) which showed the degradation pattern of these substrates by *Pyc. sanguineus* where lignin appeared to be degraded first, followed by cellulase and hemicellulase. Similar pattern of lignin biodegradation was also reported with *Ple. sajor caju* and *Ple. ostreatus* (Zadrazil, 1985).

The highest laccase productivity during SSF of *hampas* using *Pyc. sanguineus* was 40% lower compared to the reported value of 10.6 U/g *hampas* using *Ple. sajor caju* (Kumaran *et al.*, 1997). Maximum laccase productivity using *Pyc. sanguineus* was also 56% lower compared to degradation of OPFPt using *Ple. sajor-caju* which yielded maximum of 16.9 U/g substrate on day 10 as reported by Ling (1994).

On the other hand, previous studies among species of *Pyc.* spp. in liquid fermentation are sparse and varied data has been reported. This strain has been reported to produce 22 U/L^{-1} (0.022 U/mL)in liquid growth medium without the inducer and optimum laccase production was 1368 U/L after induction with 20μM xylidine (Pointing *et al.*, 2000), which was higher than that reported for many other basidiomycetes cultured under similar conditions (Orth *et al.*, 1993; Kantelinen *et al.*, 1989; Srinivasan *et al.*, 1995). However, it was not clear if such culture conditions were optimal for each fungus. Conversely few strains are also reported to produce laccase at greater levels than those recorded for *Pyc. sanguineus* by Pointing *et al.* (2000) under similar growth conditions. Notable exceptions are *Tra. versicolor* (5000 U/L) (Collins and Dobson, 1997) and *Ple. sajor-caju* (4000 U/L) (Buswell *et* al., 1996). Further, *Pyc. cinnabarinus* has been reported to produce laccase at higher levels compared to *Pyc.*

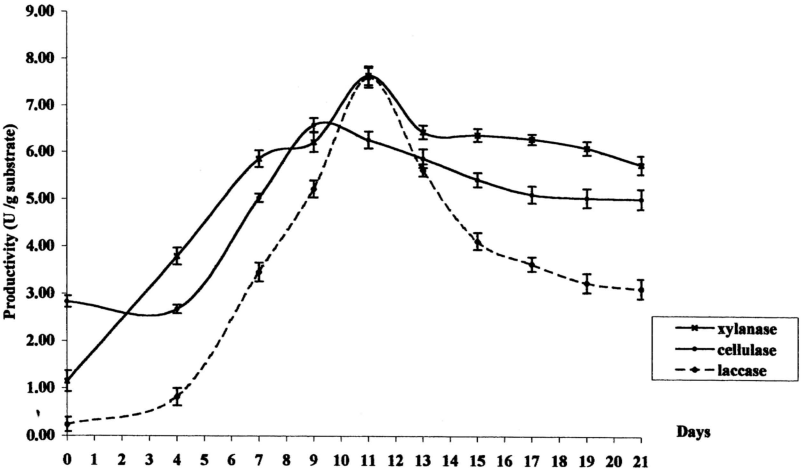

Figure 3.9: Enzyme profile during SSF of sago 'hampas' using *Pyc. sanguineus* (incubation at 25 ± 2°C, 18 days old and 10% inoculum)

sanguineus under similar growth conditions (Eggert *et al.*, 1996) but laccase from *Pyc. coccineus* showed relatively lower levels (Oda *et al.*, 1991).

Pointing *et al.* (2000) suggested that *Pyc. sanguineus* could be grown effectively on waste lignocellulose substrates. Laccase production was increased 12 fold by addition of wood fibres to liquid cultures without inducers and this value was also reported to be 3 fold higher than those reported for *Pyc. sanguineus* grown on *Eucalyptus grandis* wood chips. This preliminary study using *Pyc. sanguineus* on several common agro-residues in tropical countries showed favourable results as laccase production was higher than any reported study using this strain.

Solid substrate fermentation holds tremendous potential for the production of enzymes (Pandey *et al.*, 2000). It can be of special interest in those processes where the crude fermented products may be used directly as an enzyme source (Tendergy, 1998). One of this application is the decolorization of a number of phenol-azo dyes using a commercial, crude laccase preparation of *Pyrtcularia oryzae* (Chivukula and Renganathan, 1995). Research efforts has been taken to explore the potential of laccase in detoxification of phenolic compounds through polymerization reactions although the function of laccase is still unclear (Bollag *et al.*, 1988; Shuttleworth and Bollag, 1986). Elsewhere, laccase has been reported to decolorize dye (Pointing *et al.*, 2000) and also to decolorize wastewater from a pigment plant (Schliephake *et al.*, 1993). With the same interest, this comparative study of crude laccase production from three different agro-residues using *Pyc. sanguineus* was carried out. In this study, laccase was one the enzyme produced in high titres through SSF. Thus further studies were done to optimise the production of this enzyme.

CONCLUDING REMARKS

Based on the enzyme profiles of cellulase, laccase and xylanase for the various substrates tested, solid substrate fermentation of OPFPt by *Pyc. sanguineus* gave better enzyme yield compared to *hampas* and sawdust. Thus, OPFPt was chosen as substrate for further studies to optimise the production of the enzymes.