CHAPTER

3

CELLULOLYTIC AND LIGNINOLYTIC ENZYME ACTIVITIES OF *Pleurotus sajor-caju* DURING SSF OF 'HAMPAS'

3.0 INTRODUCTION

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' have been reported by Shim (1992), Vikineswary and Nadaraj (1992) and Vikineswary *et al.* (1994). Exploitation of lignocellulosic materials must be such that all, if not most of their major components, viz. cellulose, hemicellulose and lignin are utilized.

In the utilization of agricultural lignocellulosic materials by SSF, process variables such as pretreatment, nutrient supplementation, sterilization, selection of organism, inoculum density, temperature and pH, are of utmost importance.

In this study, an edible mushroom fungi, *Pleurotus sajor-caju* was used. *Pleurotus sajor-caju,* classified as a white rot fungi, is well known for its remarkable efficiency to grow on untreated or partially treated lignocellulosic residues and to decompose all the major components including lignin. The degradation of lignocellulosic residues

containing cellulose, hemicellulose and lignin requires the participation of a host of enzymes. Therefore, it is necessary to study the enzyme profiles of *P. sajor-caju* during SSF of 'hampas' to obtain an insight into the pattern of fungal-substrate interaction leading to the degradation of the residue. A total of five enzymes of interest were studied. The enzymes were endocellulase (EC. 3.2.1.4.), filter paper hydrolysis activity (EC. 3.2.1..) and β-D-glucosidase (EC. 3.2.1.21.) which act synergistically to solubilize crystalline cellulose; xylanase (EC. 3.2.1.8.) and laccase (EC. 1.10.3.2) which are involved in the degradation of hemicellulose and lignin, respectively.

The objectives of this study include:

a. characterizing the 'hampas' used in this study
b. developing koji inoculum for *P. sajor-caju* using wheat grain
c. assessing the growth of *P. sajor-caju* on 'hampas' with adjusted C:N ratio
d. studying the enzyme activities during fungal degradation and utilization of 'hampas'

3.1 MATERIALS AND METHODS

3.1.1 'Hampas' Collection, Preparation and Characterization

Sago 'hampas' was obtained from Hup Guan Sago Factory, Minyak Beku, Batu Pahat, Johor Darul Takzim. The 'hampas' from the starch extraction process was collected using plastic bags at the collection ram. The raw substrate had 90.86 ± 0.17% moisture and was observed to undergo rapid fermentation when left untreated in a heap. The samples were promptly air-dried on clean plastic groundsheets (Plate 2) with continuous mixing for about 18 h over a period of 5 days. The air-dried material was sieved through a 2.0 mm sieve and stored in plastic bags at room temperature. The 'hampas' was characterized and used as a substrate in the SSF studies.

3.1.2 Analytical Techniques

'Hampas' was ground to pass through a 1.0 mm mesh sieve using a Cyclotec laboratory mill. The milled samples were stored in air-tight plastic vials prior to analysis. For all tests, hampas samples were analyzed in triplicate. The determination of crude fiber, moisture, crude fat, total nitrogen and crude protein were carried out by the AOAC methods (1990) (Appendix A1 - A4). The total ash was determined using the same sample used in the dry matter determination (Appendix A5). The Acid Detergent Fiber (ADF), lignin, cellulose and insoluble ash contents were determined by the method of Van Soest and Wine (1968) (Appendix A6 - A8). The total carbon content was

determined by the rapid titration, wet oxidation method of Allen (1987) (Appendix A9). The Carbon:Nitrogen (C:N) ratio was calculated based on the total carbon and total nitrogen contents. All results are expressed as percentage on dry weight basis except for pH values (Appendix A10).

3.1.3 Inoculum Development

The spawn culture of the edible mushroom, *Pleurotus sajor-caju*, obtained from a local mushroom farmer was used in the experiments. The stock cultures of *P. sajor-caju* (Plate 3) were maintained on potato dextrose agar (PDA) slants at 4°C. The koji was prepared on autoclaved wheat grains in conical flasks incubated at 27°C for 18 days. The process is, schematically shown in Fig.5.

3.1.4 Fermentation conditions

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) $MgSO_4.7H_2O$ (Shim, 1992). Filter sterilized urea as nitrogen supplement of 0.38% (w/v) was added to the contents of the autoclaved flasks. The moisture content in each flask was 83.3%(w/w). The C:N ratio was about 35:1, which was reported by Moo-Young *et al.* (1983) to be the optimum C:N ratio for SSF. The contents of the flasks were then thoroughly mixed with a sterile spatula and allowed to stand for about 1 h.

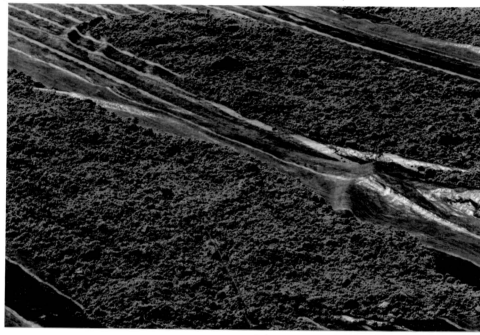

Plate 2. Air-drying 'hampas' on plastic sheets

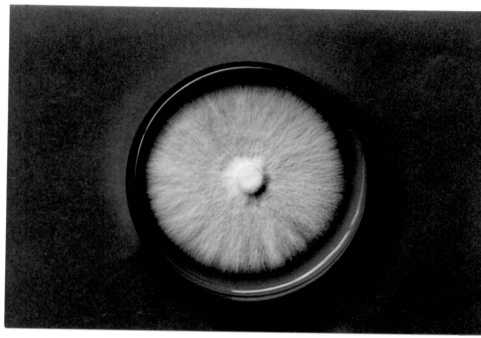

Plate 3. *Pleurotus sajor-caju* on PDA plate (Seven day old culture)

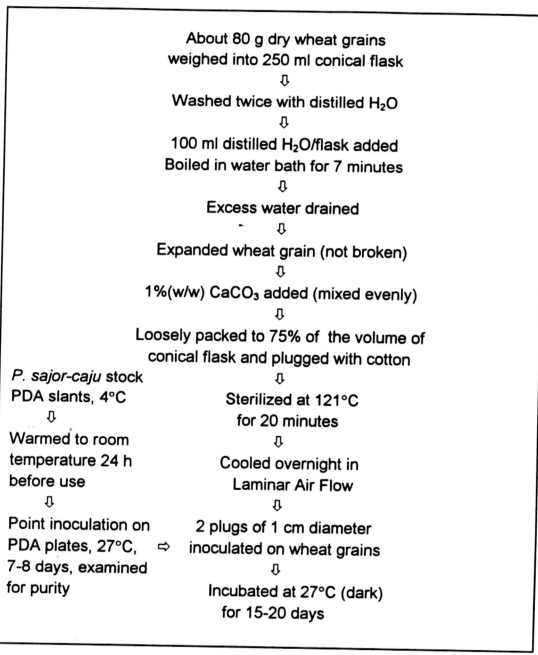

Figure 5. Flow Chart of the *Pleurotus sajor-caju* Koji Development using Wheat Grains (adapted from Quimio *et al.*, 1990)

Each flask was aseptically inoculated with average 23.5% (w/w) of 18 day old *P. sajor-caju* koji. The initial contents of the culture flasks were maintained as shown in Table 9. The cultures were incubated at 25 ± 2°C in static conditions for 21 days. The SSF experiment included flasks with uninoculated 'hampas', which were analyzed as controls.

Table 9: Contents of fermentation flask (Initial)

Substrate	*Mineral solution and urea*	*Inoculum*	*Moisture*
Dried sago 'hampas'.	0.2% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4.7H_2O$, 0.38% (w/v) N source (urea).	*Pleurotus sajor-caju*, 18 days old, 23.5% (w/w).	Mineral solution and urea (50 ml).
10 g	50 ml	2.35 g	83% (v/w)

3.1.5 Extraction of Extracellular Enzymes

At suitable intervals of time, flasks were sampled in triplicate at random and weighed. About 100 ml of cold 50 mM sodium citrate buffer (pH 4.8) was added to the contents of each flask. A spatula was used to break the solid culture into smaller particles. The flasks were transferred to an incubator shaker at 4°C and 200 rpm for approximately 18 h. The culture flasks were removed from the shaker and the solids were separated by centrifugation at 9000 rpm (15 000 *g*) for 20 min. The crude culture filtrate containing

the fungal enzymes were kept in 1.5 ml microcentrifuge tubes at -20°C for 24 h prior to enzyme assays. The experimental procedures used are outlined in Fig. 6.

3.1.6 Analysis

All assays were carried out in triplicate using three culture flasks at room temperature. The results for all values are expressed as mean of triplicates.

3.1.6.1 *Growth Profile Estimation*

The growth of *P. sajor-caju* was determined at each harvest by fresh weight measurements of the whole flask which included 'hampas' and fungal mycelium. Weight loss (% wet weight) at the end of the fermentation was determined.

3.1.6.2 *Soluble Protein*

The extracellular soluble protein was quantified using the dye-binding method of Bradford (1976) with crystalline bovine serum albumin as standard (Appendix A12).

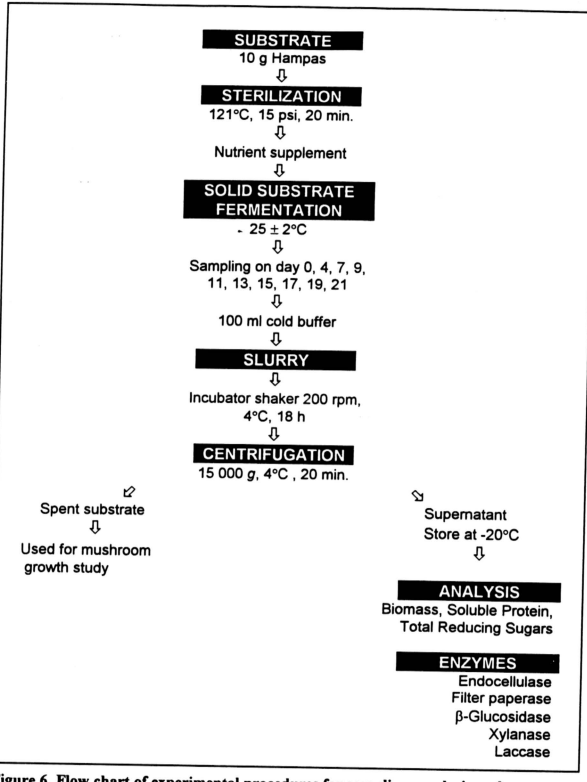

Figure 6. Flow chart of experimental procedures for sampling, analysis and enzyme assays during SSF of 'hampas'

3.1.6.3 *Total Reducing Sugars*

The reducing sugars present in the crude culture filtrate were determined by the dinitrosalicylic acid (DNS) method of Miller (1959) using glucose as the standard (Appendix A11).

3.1.7 Enzyme Assays

Enzyme activities are expressed in units defined as the amount of enzyme required to form one µmole product/g substrate under the standard conditions of assay.

3.1.7.1 *Carboxymethyl Cellulase Activity*

Carboxymethyl cellulase (CMCase) activity was determined by the method of Dong *et al.* (1992) (Appendix A13). Sodium salt carboxymethylcellulose (Medium viscosity) was used as the assay substrate. The amount of reducing sugars liberated was determined by the DNS method of Miller (1959) with glucose as the standard. One unit of enzyme activity (U) is expressed as one µmole glucose equivalent released/min./g substrate.

3.1.7.2 *Filter Paper Hydrolysis Activity (FPase)*

Filter paper hydrolysis activity was determined by the method of Elshafei *et al.* (1990) (Appendix A14). Whatman No. 1 filter paper strips of 1 cm x 3 cm, weighing 25 mg was used as the substrate. The amount of reducing sugars liberated was determined as described for the CMCase assay.

3.1.7.3 *β-D-Glucosidase Activity*

The β-D-glucosidase activity was determined by the method of Dong *et al.* (1992) (Appendix A15). The substrate used for the determination of β-D-Glucosidase activity was *p*-nitrophenyl-β-D-glucopyranoside (Sigma). The amount of *p*-nitrophenol released was determined spectrophotometrically at λ = 400 nm with *p*-nitrophenol as the standard. A unit of enzyme activity (U) was defined as the amount of enzyme which liberated 1μmole of *p*-nitrophenol/min./g substrate.

3.1.7.4 *Xylanase Activity*

Xylanase activity was determined by the method of Bailey *et al.* (1992) (Appendix A16). 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) is expressed as the amount of enzyme needed to liberate 1μmole of xylose/min./g substrate.

3.1.7.5 *Laccase Activity*

Laccase activity was assayed by the increase in the absorbance due to the formation of tetramethoxy-azo-*bis*-methylenequinone resulting from the reaction of laccase with syringaldazine (Harkin and Obst, 1973; Leonowicz and Grzywnowicz, 1981) (Appendix A17). The initial rate of color change was measured spectrophotometrically at a λ = 525 nm. One unit of activity was defined as the enzyme producing one unit of absorbance change/min./g substrate.

3.2 RESULTS AND DISCUSSION

3.2.1 'Hampas' characterization

The results of proximate analysis of sago 'hampas' are presented in Table 10. Wet 'hampas' was pinkish beige in color and turned to light brown and fluffy in nature, upon drying. Observation under the microscope revealed that large quantities of starch granules are trapped within the fibrous matrix of 'hampas'. This is not surprising as several authors have earlier reported high quantities of starch in 'hampas', varying from about 66% (Cecil *et al.*, 1982; Shim, 1992) to more than 90% (Lim, 1967; Horigome *et al.*, 1991) on a dry weight basis. The high quantities of starch in 'hampas' reflects the

inability of the current methods employed in sago processing to extract starch completely, thereby leading to losses of starch and consequent pollution problems.

Hampas contained about 14% fiber of which about 25% was lignin. In a survey conducted by Shim (1992) of major sago-processing factories in the Sibu Division, Sarawak, the fiber content of 'hampas' was found to vary between 11 and 20%, and lignin content was found to be as high as 20% of the total fiber content.

Table 10. Proximate analyses of sago 'hampas'

Component	*% dry matter*
Dry matter	89.90 ± 0.10
Crude fat	nd
Crude protein	1.15 ± 0.12
Crude fiber	14.45 ± 0.25
Cellulose	71.18 ± 1.32
Lignin	24.79 ± 0.82
Residual ash	5.48 ± 0.24
Acid detergent fiber	25.28 ± 0.51
Total carbon	37.74 ± 0.18
Total nitrogen	0.18 ± 0.02
pH	5.36
C:N ratio	205

nd = not detectable.
All units are in % dry matter except pH and C:N ratio

Müller (1977) studying the nutritive value of sago and sago refuse observed that the protein content, lipids, minerals and vitamins in sago products is so insignificant that it had little or no importance in terms of quality. In the present study the crude protein content of 'hampas' was found to be 1.15%. The analysis revealed that the C:N ratio of 'hampas' was very high mainly due to the low nitrogen present in it. Adequate supplementation with urea as nitrogen to adjust the C:N ratio in the present study to about 35:1 as reported by Moo-Young *et al.* (1983) rendered 'hampas' suitable for fungal colonization.

Ozawa *et al.* (1991) reported the presence of phenolic compounds, polyphenol oxidases and peroxidases from sago pith which is responsible for the browning of the exposed pith. However, no attempts were made to quantify the presence of these compounds in this study.

3.2.2 Growth Profile and Primordiation

The first sign of *P. sajor-caju* mycelial growth on 'hampas' during SSF was seen two to three days after inoculation. Thereafter, *P. sajor-caju* grew rapidly on 'hampas'. By 10 to 11 days of fermentation, visual observation revealed that the substrate was completely colonized with white mycelia of the fungus.

Biomass estimation in SSF is very difficult, where the fungal mycelia are intricately interwoven with the substrate (Moo-Young *et al.*, 1983; Mudgett, 1986). Therefore, an alternative method of measuring the growth profile of *P. sajor-caju* by weighing the fresh weight of the spent substrate gave an indication of the biomass produced. The growth profile of *P. sajor-caju* on 'hampas' showed a gradual decline in weight (Fig. 7). At the end of the fermentation period of 21 days, the weight loss was calculated as 5% of the original substrate fresh weight. Water condensation was also observed in all the culture flasks. This suggested that 'hampas' might have been biodegraded to CO_2, H_2O and humic substances (Giovannozzi *et al.*, 1986). As the negligible loss of material of 5% is favorable for product recovery, all analytical values are expressed in this study without taking into account this insignificant weight loss. Shim (1992) working with SSF of 'hampas' using *Myceliopthhora thermophila*, a thermophilous fungus, noted a higher weight loss of 21.5% after 60 h of fermentation. The larger portion of the substrate lost during the fermentation seemed to be the fibrous part which made up the bulk of 'hampas' but was of a smaller percentage on weight basis. Ortega *et al.* (1992) working with four strains of *Pleurotus* on sugar cane residues recorded up to 33% weight loss in the substrate after 30 days SSF.

In the present study, abnormal fruit body (Plate 4) appeared in cultures of *P. sajor-caju* after 17 days. The accumulation of CO_2 in the culture flask might have caused the abnormal primordia formation towards the end of the fermentation (Zadrazil, 1978).

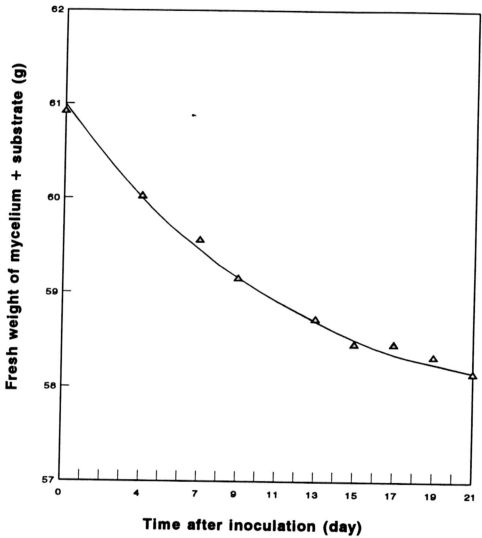

Figure 7: Weight loss of *Pleurotus sajor-caju* culture during SSF of hampas

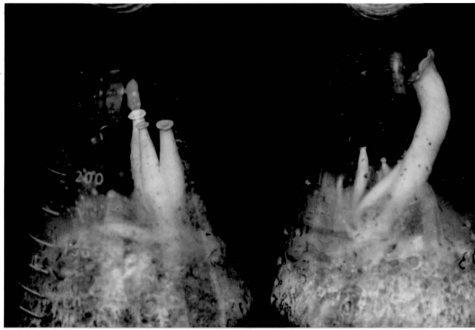

Plate 4. Abnormal fruit body formation in SSF culture flask

In a study on wheat straw, Zadrazil (1977) observed fruit body formation of various white rot fungi after 25 days of SSF. Ortega *et al.* (1992) reported fructification in *Pleurotus* sp. after 28 days on sugar cane crop residues.

The above findings led to the belief that 'hampas' could be possibly exploited for mushroom production under more suitable conditions. Therefore, a laboratory scale experiment was carried out to observe mushroom growth on the spent substrate of SSF (Fig. 6). The findings of this study are provided in Appendix D.

3.2.3 Soluble Protein

The soluble protein content which is used to indirectly assess fungal biomass (Moo-Young *et al.* 1983) showed a rapid increase after day four, peaked on day 11 with a value of 3.2 mg/g substrate and leveled off towards the end of the fermentation period (Fig. 8). The protein levels were similar to those reported for *P. sajor-caju* during SSF of rice straw (Madan and Bisaria, 1983). This suggested that the increase in protein content in the culture extract was partly due to the secretion of enzymes such as laccase, xylanase and CMCase. Other extracellular enzymes responsible for the degradation of 'hampas' also contributed to protein content.

3.2.4 Reducing Sugars

The breakdown of carbohydrates during koji preparation has contributed to the high reducing sugars of about 26 mg/g 'hampas' in the culture flasks at the initiation of the fermentation (Fig. 9). The reducing sugars gradually increased by about 62% from 26 mg/g to 42 mg/g substrate within the initial nine days of SSF. On further incubation the reducing sugar level did not vary greatly except a slight decline towards the end of the fermentation (Giovannozzi *et al.*, 1986).

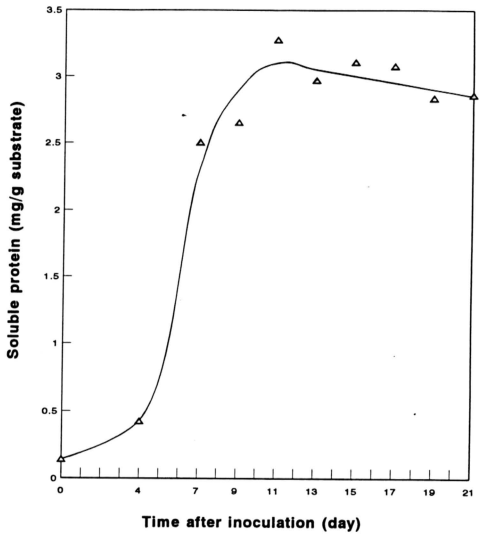

Figure 8: Soluble protein content of crude culture extract

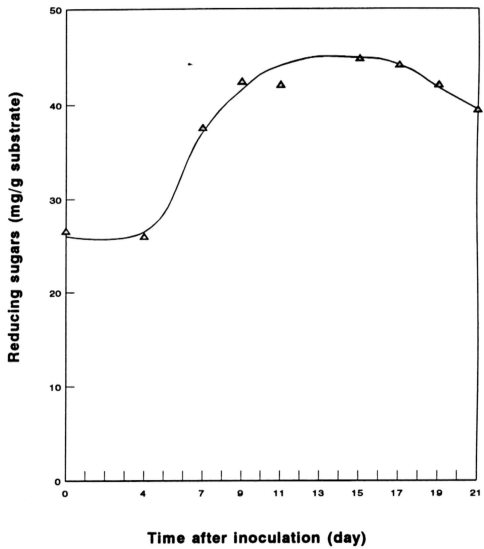

Figure 9: Reducing sugars content of crude culture extract

3.2.5 Cellulolytic activity

After a four day lag phase, the endocellulase (CMCase) activity of *P. sajor-caju* significantly increased and reached a maximum value of 2.85 U/g 'hampas' on day seven of fermentation (Fig. 10). Subsequently the endocellulase activity decreased when fermentation was continued up to 21 days. The increase and decrease in endocellulase activity may be due to induction and repression of cellulase enzymes. The decline in CMCase may be explained on the basis of 'catabolite repression' of cellulase enzyme system (Frost and Moss, 1987; Wang *et al.*, 1979). In the fermentation of sago 'hampas' by *P. sajor-caju*, there was a distinct trend of where the endocellulase activity appeared earlier, increased rapidly and then decreased. In relation to the increasing reducing sugars pattern, the end product inhibition by cellobiose or glucose may have occurred.

The FPase and β-glucosidase activity remained low and did not show any distinct pattern throughout the course of incubation. The FPase and β-glucosidase activity fluctuated between 0.84 - 0.97 U/g and 0.04 - 0.27 U/g respectively. Results comparable to these have been reported during SSF of rice straw with *P. sajor-caju* (Madan and Bisaria, 1983).

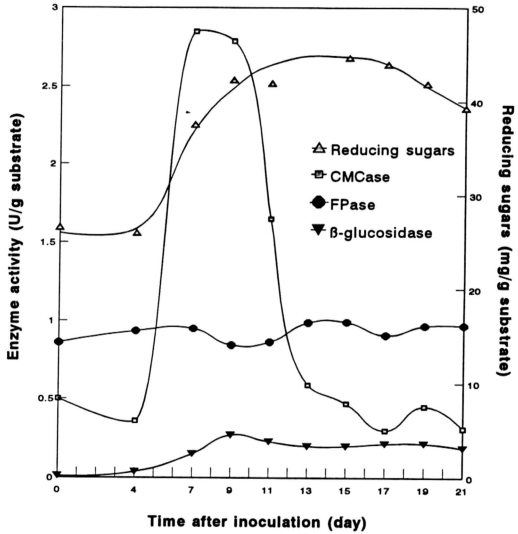

Figure 10: Cellulase activities of crude culture extract

3.2.6 Xylanase activity

The xylanase activity of the crude culture extract of *P. sajor-caju* during fermentation of 'hampas' is shown in Fig. 11. The xylanase production resembled that of CMCase with a maximum activity of 10.1 U/g 'hampas' on day nine of SSF. After 10 days, the xylanase activity dropped to 5 U/g 'hampas' and then finally reduced to below 4 U/g substrate towards the end of fermentation. A similar pattern of extracellular xylanase production has been reported by Ortega *et al.* (1993) studying the behavior of two strains of *Pleurotus* spp. on sugar cane residues. Madan and Bisaria (1983) in a study of the enzymes of *P. sajor-caju* during the growth on rice straw medium, detected similar xylanase profiles with maximum xylanase activity on days 8 to 10 with a decline later.

Xylanase activity regulation is similar to the activity of cellulase enzymes with xylan acting as inducer and xylose as inhibitor (Biely, 1985) and this could be one of the reasons for the xylanase profile obtained in the current study.

Xylanase catalyzes the random hydrolysis of 1,4-β-D-xylosidic linkages in xylans. (IUB, 1982). Xylanases are produced widely by many bacteria, yeast and fungi, although the latter have been extensively studied (Linko *et al.*, 1989; Wong *et al.*, 1988; Coughlan and Hazlewood, 1993). Xylanase has attracted increasing attention in biotechnical research during the past decade, largely because of its potential applications

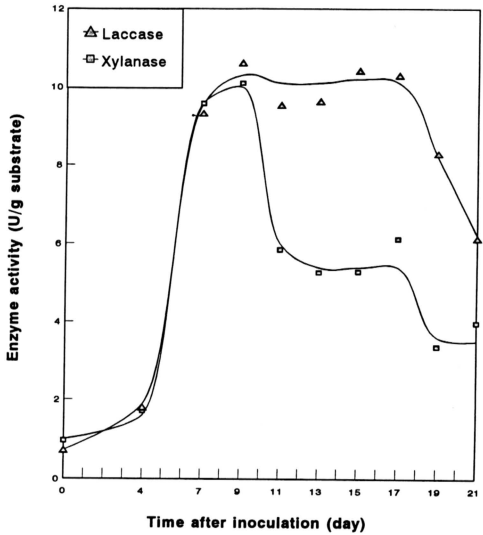

Figure 11: Laccase and xylanase activities of crude culture extract

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; Bajpai *et al.*, 1994; Buchert *et al.*, 1994; Royer and Nakas, 1989). In this study, xylanase production ranked second to laccase and was further studied in order to increase the production.

3.2.7 Laccase activity

Laccase activity in SSF increased rapidly and its production was almost identical to xylanase during the initial seven days of SSF (Fig. 11). Laccase was produced in high quantities with an activity of 10.6 U/g substrate on day nine and the activity remained almost constant after reaching peak production. on day nine. On the onset of primordiation, after 17 days of SSF, a decline in laccase activity was observed. This showed that the changes in laccase activity correlated with the morphogenesis of the fungus. This loss in activity after primordiation might be either due to cessation of laccase synthesis, enzyme inhibition or enzyme degradation (Wood, 1980a). Bonnen *et al.* (1994), Wood and Goodenough (1977) and Wood (1980a) reported that laccase concentration of the button mushroom, *Agaricus bisporus* was the highest during mycelial colonization and then declined rapidly at the onset of fruit body development. According to Wood (1980b), laccase was the major excreted protein and accounted for 2% of the mycelial cell protein of the mushroom in a defined medium. Wood also found that fruit body formation needed extra nitrogenous material over that supplied by the

mycelium and therefore extracellular proteins including laccase may be degraded and reassimilated as a source of nitrogen. Thus, one of the roles of laccase may be as an extracellular reservoir of nitrogen and being translocated to the new forming sporophores.

Studies with *P. sajor-caju* on paper-mill sludge carried out by Kannan *et al.* (1990) showed that laccase activity was growth associated, as its activity increased tremendously during the early stages of fermentation and was maximum on day 10. Platt *et al.* (1984) and Kerem *et al.* (1992) working on solid state fermentation of cotton stalks by *Pleurotus*, found that laccase activity was more prominent while peroxidase activity was not significant during lignin degradation. The maximum activities were recorded after six to eight days of growth. Valmaseda *et al.* (1991) studying the kinetics of wheat straw SSF with two white rot fungi, *Trametes versicolor* and *Pleurotus ostreatus*, detected relatively high levels of laccase within 20 days of growth on wheat straw.

Different strains of *Pleurotus* showed different levels of extracellular laccase activity and this could be due to different degrees of lignin and straw degradation. Laccase secretion by *Pleurotus ostreatus* on wood meal-rice bran increased during mycelial growth and then declined rapidly at the start of fruit body formation (Iwahara *et al.*, 1981).

Laccase is widely distributed in many microorganisms and plants, including potatoes, apples, cabbages, and several varieties of mushrooms. The biological function of laccase in fungi is less obvious and a possible clue comes from the common occurrence of the enzyme in lignin-decaying Basidiomycetes (Fåhraeus, 1952; Schubert, 1965; Mayer, 1987). It is a type of copper-containing glycoprotein and has been implicated in fungal degradation of lignin and its derivatives (Higuchi, 1982; Galliano *et al.*, 1991). Laccase has been reported to depolymerize lignin and lignin like compounds. Both inducible and constitutive laccases were reported to be present in many fungi (Bollag and Leonowicz, 1984; Leonowicz and Trojanowski, 1975a, 1975b, 1978).

Many workers suggested that laccase has considerable potential for detoxification of phenolic compounds through polymerization reactions although the natural function of laccase is still unclear (Bollag *et al.*, 1988; Shuttleworth and Bollag, 1986; Roy-Arcand and Archibald, 1991). In a study of decolorization of phenolic wastewater by Davis and Burns (1990), laccase was found to polymerize and precipitate high molecular mass phenolics from bleach plant effluents.

In these studies, it was found that the measurement of extracellular enzymes degrading various lignocellulosic components are greatly influenced, among others, by the assaying parameters and accessibility of the substrates. Besides, the specific assay conditions used may possibly be more appropriate for one enzyme than the other, which may in turn affect realistic comparisons between the enzymes produced. An increased

understanding of the utilization of lignocellulosic residues and the value of enzyme quantitation will be of considerable assistance in the development of suitable and practical methods for biological treatment of such residues.

In this study, it was noted that handling of the wet and sticky clumps of koji was difficult. The poor quality koji was therefore not uniformly spread on the 'hampas'. This resulted in uneven mycelial colonization of the substrate. Hence, the development of an improved inoculum for use in further studies of SSF was found necessary to achieve rapid mycelial colonization, enhanced fermentation and increased enzyme production.

In this study of SSF using *P. sajor-caju*, the major enzymes produced were laccase and xylanase with activities of 10.6 U/g and 10.1 U/g substrate, respectively after nine days of SSF. More studies are required to utilize the ligninolytic components of *P. sajor-caju* usefully by these enzymes.