CHAPTER

EFFECT OF INOCULUM AGE AND DENSITY ON SELECTED ENZYMES DURING SSF OF 'HAMPAS'

4.0 INTRODUCTION

In the previous study of SSF of 'hampas' using *Pleurotus sajor-caju*, laccase and xylanase enzymes were detected in significant amounts within the first 12 days of fermentation. Solid substrate fermentation was found to be suitable for producing enzymes because of the favorable energetics of the system. The simple SSF technology can also produce enzymes at a higher concentrations than submerged systems although growth rates in SSF are much lower compared to those in liquid fermentation. However, to harness SSF for increased enzyme production, a good quality and optimum inoculum is necessary (Mudgett, 1986). Too low a density may give insufficient biomass and allow the growth of other undesirable organisms. Too high densities may accelerate growth and deplete the substrate of nutrients necessary for product formation. The optimization of the inoculum parameters would be a priority investigation in bioconversion of sago 'hampas' for enzyme production.

Pleurotus spp. has been known to selectively degrade the lignin component of lignocellulosic residues (Zadrazil, 1984). The upgraded substrate then, with lower lignin content would be more suitable for ruminant feed. The starch trapped in the three-dimensional matrix of 'hampas' was also a point of interest in this study. To investigate whether *P. sajor-caju* utilized the starch component of 'hampas', activities of α-amylase and glucoamylase activities were assayed in this experiment together with laccase and xylanase.

The aims of the study were to:

a. improve the koji inoculum for *P. sajor-caju* using wheat grains
b. determine the optimum inoculum age and density of *P. sajor-caju* for further SSF of 'hampas'
c. study the fermentation pattern and utilization of the substrate, through estimation of pH, soluble protein, reducing sugars, and the enzyme activities during degradation of 'hampas'.

4.1 MATERIALS AND METHODS

4.1.1 Koji development

In this experiment, modifications were made in the koji development process as outlined in Fig. 5 (pp. 58), using wheat grains as substrate. Changes were made in step 3 of the procedure. Instead of adding 100 ml distilled water per flask, only 50 ml were added. The wheat grains were boiled in a water bath for a shorter period of five minutes.

4.1.2 Fermentation conditions and analysis

SSF cultures were grown as described in Chapter 3 (pp. 56), except for changes of the following parameters:

1. Inoculum (koji) age : 2-week and 4-weeks
2. Inoculum density : 10%, 20% and 30% (w/w)
3. Fermentation period : 12 days

Approximately, 1.0 ± 0.02 g, 2.0 ± 0.04 g, 3.0 ± 0.04 g (fresh weight) to obtain 10%, 20% and 30% (w/w) inoculum densities, respectively of 2- and 4-week old inoculum *P. sajor-caju* koji were inoculated into the flasks. The culture flasks were incubated at 25 ± 2°C in static conditions for 12 days. The total number of culture flasks were 126 (2 ages x 3 densities x 7 samplings x 3 replicates) and they were arranged as a 2 x 3

factorial experiment conducted in a Completely Randomized Design (CRD). The number of random samples analyzed in this study and the methods used for the experimental procedures are summarized in Table 11 and Fig. 12, respectively.

Table 11. Experimental design and the number of samples analyzed for the SSF of 'hampas' with *P. sajor-caju*

Inoculum age (week)	*Inoculum density (% w/w)*	*Duration of SSF (days)* Number of samples analyzed						
		0	*2*	*4*	*6*	*8*	*10*	*12*
2	10	3	-	3	3	3	3	3
	20	3	-	3	3	3	3	3
	30	3	-	3	3	3	3	3
4	10	3	3	3	3	3	3	3
	20	3	3	3	3	3	3	3
	30	3	3	3	3	3	3	3

Since very little fungal growth in the culture flasks was observed on day two with the 2-week old inoculum, sampling was not done.

All assays were performed in triplicate for each of the three culture flasks sampled. The results for all values are expressed as mean of triplicate. The crude culture filtrate was used to determine the protein content and sugars using the methods as described in Appendix A11 and A12. Moisture content and pH of the spent substrate were determined as described in Appendix A2 and A10.

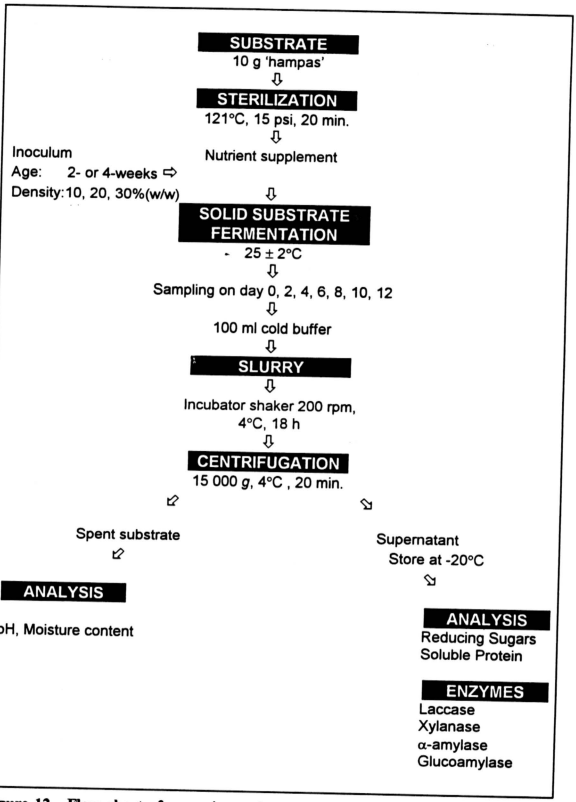

Figure 12. Flow chart of experimental procedures for sampling, analysis and enzyme assays during SSF of 'hampas'

The data obtained were statistically analyzed using two-way analysis of variance (ANOVA) to test the significance of inoculum age, inoculum density and the interaction between the parameters tested during SSF of 'hampas'. Mean separation was carried out using the Student-Newman-Keuls' (SNK) test. This test is referred to as the 'means test' throughout this study.

4.1.3 Enzyme assays

All enzyme activities are expressed in international units (U), defined as the quantity of enzyme required to produce one micromole of product per minute and are reported on the basis of per gram of substrate used in the SSF, under the conditions of assay, unless otherwise stated.

4.1.3.1 Xylanase and laccase

Xylanase and laccase activities of the crude culture filtrate were assayed using the standard methods described in Appendix A16 and A17.

4.1.3.2 α-Amylase and glucoamylase

Assays for α-amylase and glucoamylase were carried out in order to determine whether *P. sajor-caju* produced these enzymes to utilize the starch component of

'hampas'. The method of JICA (1986) was used with raw and cooked sago starch, as the substrate in the assay of α-amylase and glucoamylase, respectively (Appendix A18). The amount of reducing sugars liberated was determined by the DNS method of Miller (1959) with maltose or glucose as the standard for α-amylase and glucoamylase enzyme assays, respectively. One unit of α-amylase and glucoamylase activity is taken as the amount which catalyzed the formation and release of one μmole maltose or glucose/min/g substrate.

4.2 RESULTS AND DISCUSSION

4.2.1 Koji development

The modified method of koji development used in these studies produced a better inoculum. The modified procedure did not result in large sticky pellets of koji, as in the previous experiment. By reducing both the amount of water and boiling time, a granular-like inoculum was obtained (Plate 5). The granular koji had more air spaces within the wheat grains and permitted better aeration for aerobic growth. The improved koji was easier to handle and could be evenly distributed on 'hampas' in the culture flasks, resulting in a more efficient colonization of 'hampas'.

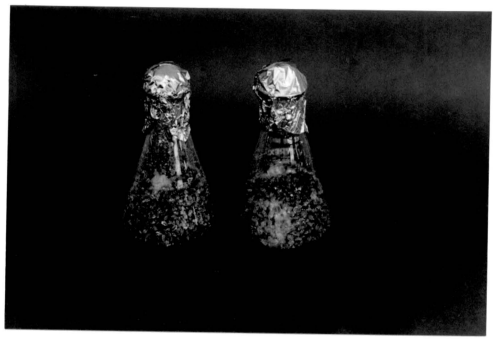

Plate 5. Improved *P. sajor-caju* koji using wheat grain as the carrier substrate [3 days old with initial moisture content of 37.8%]

In the previous experiment, sticky pellet-like koji resulted in the formation of areas inside the culture flasks where the fungus developed preferentially, and as a consequence fermentation was less effective and not uniform. This problem was eliminated with the improved koji as it was noted to colonize 'hampas' in a more rapid and uniform manner (Plate 6).

4.2.2 Fermentation conditions

4.2.2.1 Soluble protein during SSF

The soluble protein values of the crude culture filtrate using the 2-week old and 4-week old inoculum cultures are shown in Fig. 13a and 13b, respectively. Generally, the two inoculum ages caused a significant difference ($P < 0.001$) in the soluble protein content with incubation time. However, no difference ($P = 0.2601$) was recorded in soluble protein content between the three inoculum densities tested (Appendix C1). On the whole, the soluble protein content in the crude culture filtrate using the 4-week old inoculum, however, was much higher than in filtrates obtained using the 2-week old inoculum.

With the 2-week old inoculum, *P. sajor-caju* produced significantly ($P<0.001$) varying quantities of soluble protein with the three levels of inoculum densities tested (Appendix C2). However, the means test revealed no significant difference in the soluble protein content between the three inoculum levels on days six, eight and ten of SSF. Generally, the fungal growth obtained with 30% inoculum density produced

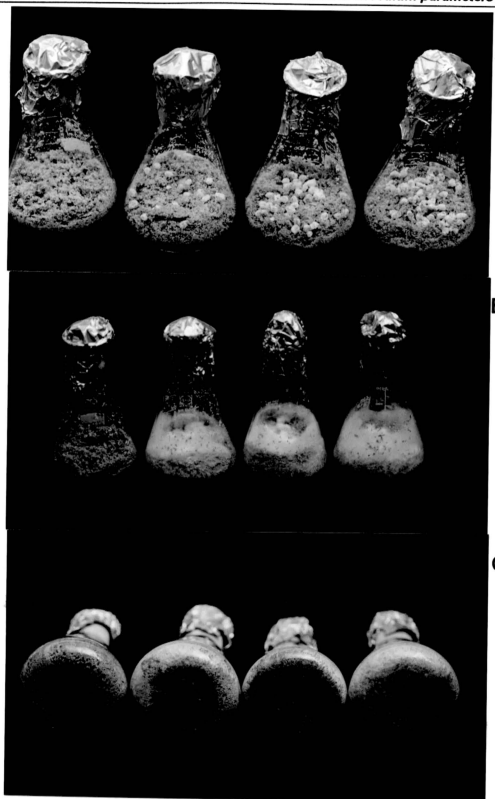

Plate 6. Growth of *P. sajor-caju* on 'hampas' (A = Day 0, B & C = Day 6)
a = Control b = 10% inoculum c = 20% inoculum d = 30% inoculum

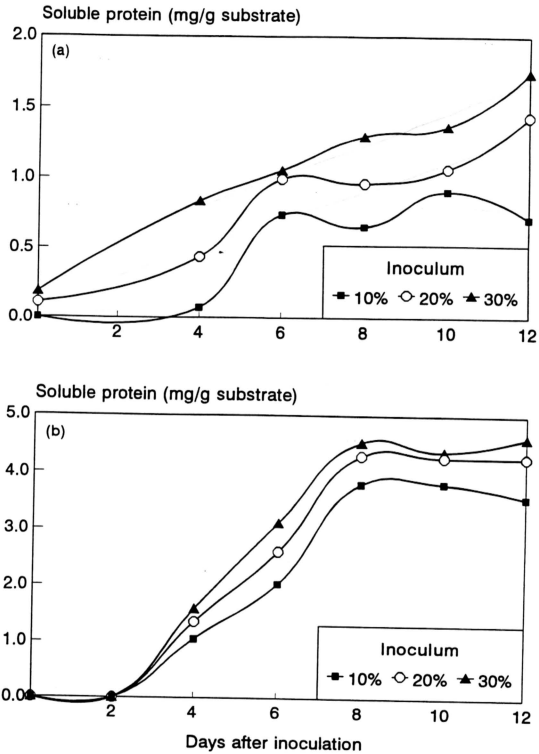

Figure 13. Soluble protein content of culture extract during SSF of 'hampas'
a = 2-week old inoculum b = 4-week old inoculum

higher quantities of soluble protein while the 10% inoculum density produced the lowest.

With the 4-week old inoculum, there was a significant difference ($P<0.001$) in protein content between the three inoculum densities tested and a significant interaction ($P<0.05$) between sampling day and inoculum density (Appendix C3).

As can be seen from Fig. 13b, after two days of SSF, the soluble protein of the supernatant increased at a steady rate till day eight. This linear trend corresponded to the increasing amount of enzymes produced by *P. sajor-caju* during biodegradation of 'hampas'. Following this, the means test revealed that the difference in the soluble protein content was negligible (Appendix C4). With the 4-week old *P. sajor-caju* inoculum, the soluble protein content of 'hampas' was maximum after eight days of SSF with an average value of 4.21 mg/g substrate. Valmaseda *et al.* (1991) observed a similar trend with SSF of wheat straw over 60 days using *Pleurotus ostreatus* and found that the fungal biomass content was related to protein content.

4.2.2.2 Reducing sugars content during SSF

For both the 2-week old and 4-week old inoculum cultures, the reducing sugar content of the substrate generally increased after two days of SSF (Fig. 14). In the SSF with *P. sajor-caju*, it was observed that the 10% inoculum density had the lowest reducing

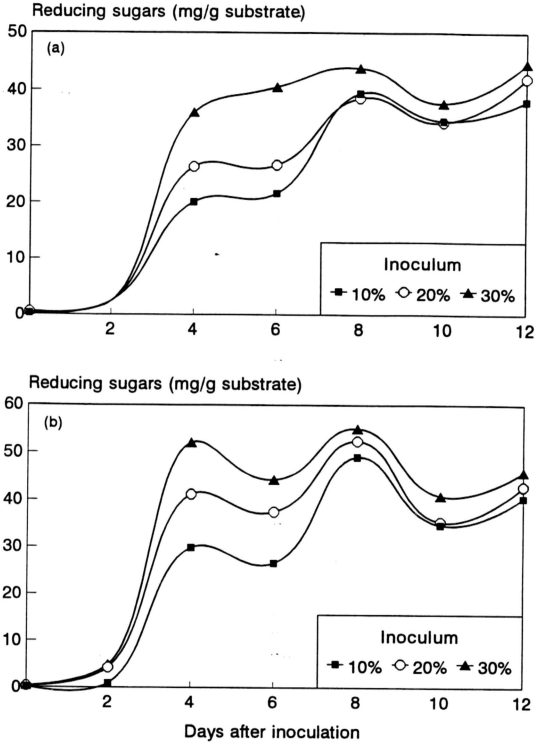

Figure 14. Reducing sugars content of culture extract during SSF of 'hampas'
a = 2-week old inoculum b = 4-week old inoculum

sugar contents while the 30% inoculum had the highest. However, overall statistical analysis revealed no significant ($P>0.05$) difference between the two inoculum ages, three inoculum densities and the interaction between both the parameters (Appendix C5). As seen in Fig. 14, the differences, generally became less distinct in the reducing sugar contents between the three densities of inoculum tested as fermentation progressed, stabilizing at an average value in the range of 42-43 mg/g substrate towards the end SSF.

In a comparative study of the lignocellulose degradation of cotton stalks during SSF using *Pleurotus ostreatus* and *Phanerochaete chrysosporium*, Kerem *et al.* (1992) reported that the sugar concentrations in the *P. ostreatus* culture increased over the first four days and then stabilized till the end of the fermentation of 30 days. A similar behavior was noted by Giovannozzi *et al.* (1986) working on SSF of wheat straw over 20 days using *P. ostreatus*.

4.2.2.3 pH variation during SSF

The pH changes during SSF of 'hampas' using the two inoculum ages were almost identical throughout the fermentation period (Fig. 15). As the fungus grew rapidly throughout the SSF, the fungal metabolism might have caused the hydrolysis of urea. This might have resulted in the liberation of ammonium ions thereby leading to an

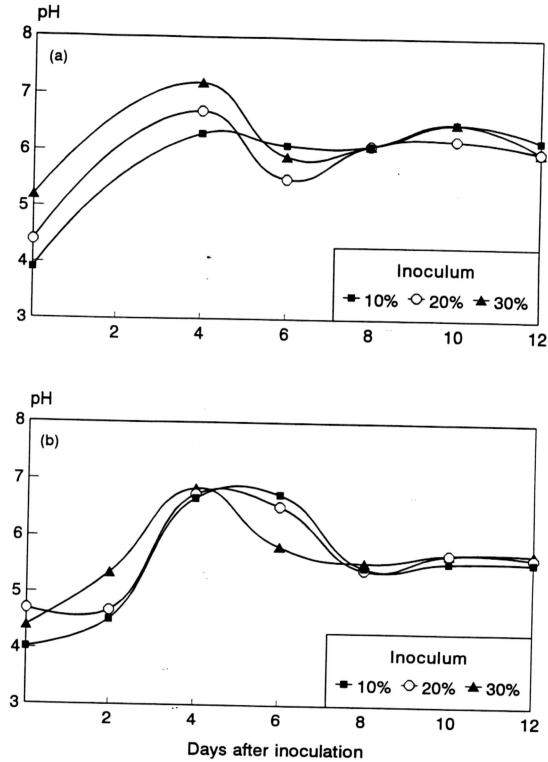

Figure 15. Variation of pH during SSF of 'hampas'
a = 2-week old inoculum b = 4-week old inoculum

increase in pH values (Raimbault and Alazard, 1980) as seen in the first four days of SSF. As the fermentation continued with rapid growth of the fungi, ammonium uptake exceeded the hydrolysis rate of urea and the pH dropped. Changes in pH were considerably smaller after seven 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). Freitag and Morrell (1992) studying enzyme activities of a white-rot fungus reported that the complex enzyme systems involved in substrate degradation could also be responsible for maintaining the pH of the substrate at a range suitable for fungal colonization. The optimization and maintenance of pH may be difficult because it can change during the course of the fermentation as seen in this study.

The original pH value of the unbuffered 'hampas' substrate was 5.36. The initial value of pH for urea supplemented culture was lower, in the range of 3.9-5.0 (mean = 4.4). The addition of mineral nutrients with urea supplement was found to reduce the pH of 'hampas' slightly. This resembled the natural growing conditions of white-rot fungi where the pH is normally acidic. In the present experiment, a pH range between 5 and 6 was found to be suitable for the growth of *P. sajor-caju* on 'hampas'.

4.2.2.4 Variation in moisture content during SSF

The moisture content of 'hampas' during SSF with *P. sajor-caju* are shown in Fig. 16. The moisture content of 'hampas' in the culture flasks was found to increase slightly in a linear manner till the end of the fermentation. Very little difference was observed in moisture content in 'hampas' during SSF using 10, 20 and 30% inoculum density throughout the fermentation. At the end of the 12 day fermentation, the moisture content increased by about 3-4% of the original moisture content of 'hampas'. As suggested by Rajarathnam *et al.* (1992) and Giovannozzi *et al.* (1986), H_2O together with CO_2 is normally produced during lignocellulosic biodegradation. This negligible increase in moisture content of 'hampas' would not have altered the water to air ratio of the substrate to affect aeration and fungal growth significantly. Zadrazil and Brunnert (1981) noted that slow growing fungus such as *Pleurotus* sp. produced small amounts of metabolic water and practically this amount can be neglected. Raimbault and Alazard (1980) found that the constitutive water of the mycelium which represented about 80% of the wet biomass did not limit fungal growth on cassava meal in the solid state. They also noted that moisture content increased during fermentation because of the loss of dry matter and increase in metabolic water during carbohydrate oxidation.

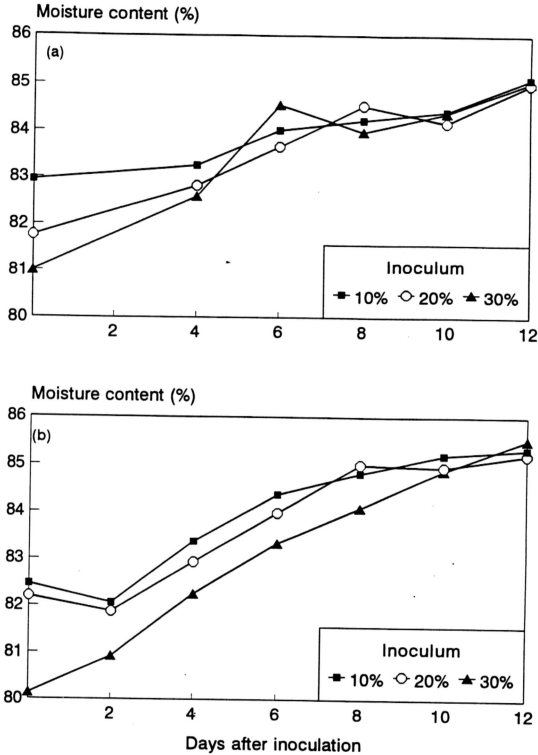

Figure 16. Variation in moisture content of 'hampas' during SSF
a = 2-week old inoculum b = 4-week old inoculum

Aidoo *et al.* (1982) reviewing the physiological aspects of microbial growth on solid substrates, noted a substantial loss of moisture from the solid substrate (for instance in composting, which is an open system) and little or no moisture change in the substrate in a closed system as in mushroom cultivation. Moisture content as high as 70% was reported to be produced by *Pleurotus florida* when cultured on wheat straw substrate (Rajarathnam *et al.*, 1992).

Moisture content of the substrate is an important factor during fungal growth in a SSF system (Aidoo *et al.*, 1982) and the secret to successful SSF is having the substrate moist enough for fungal growth, but not so wet as to promote bacterial growth (Cannel and Moo-Young, 1980).

4.2.3 Enzyme activity

4.2.3.1 Xylanase activity

The results of xylanase activities of *P. sajor-caju* during the SSF of 'hampas' are shown in Fig. 17. The inoculum age significantly ($P<0.001$) influenced xylanase activity of *P. sajor-caju* during SSF of 'hampas' (Appendix C6) while, inoculum densities did not affect xylanase activity ($P = 0.1406$). Throughout the fermentation period, xylanase activity with the 4-week old inoculum was higher when compared to the activity with 2-week old inoculum.

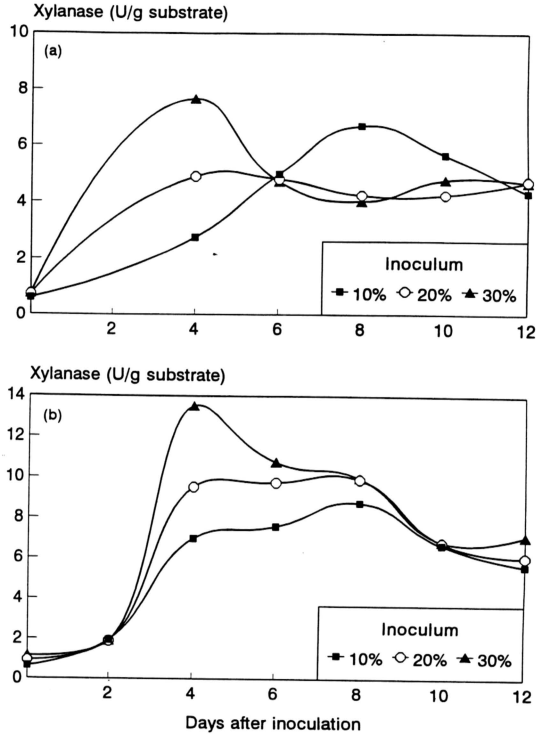

Figure 17. Xylanase activities of culture extract during SSF of 'hampas'
a = 2-week old inoculum b = 4-week old inoculum

Statistical analysis revealed that there were no significant difference (P = 0.4315) in the xylanase activity when the 2-week old inoculum was used with three densities in SSF of 'hampas' (Appendix C7). This could be attributed to the low biomass resulting in lower enzyme activities produced by *P. sajor-caju*. As seen in Fig. 17a, the xylanase activity was maximum on day four when 20 and 30% inoculum densities were used, and on day eight when 10% inoculum density was used. The decline in xylanase activity thereafter indicated that the enzyme activity could have been inhibited, as observed in previous study (Chapter 3, pp. 75 - 76). Means test analysis showed no difference in xylanase activity after day four of fermentation (Appendix C8). With the 2-week old inoculum of *P. sajor-caju*, highest xylanase activity obtained was 7.7 U/g 'hampas' with 30% inoculum after four days fermentation.

The mature 4-week old inoculum tested was more favorable for xylanase activity (Fig. 17b). With the 30% inoculum density of *P. sajor-caju* using the 4-week old inoculum, a short lag phase of two days was followed by a rapid increase and maximum xylanase activity of 13.5 U/g 'hampas' on day four of SSF. On the other hand, for the 10 and 20% inoculum, the activity increased gradually and leveled off between day four and eight of SSF. After eight days, the xylanase activity, when 'hampas' was fermented with all the three densities, gradually decreased, possibly due to inhibition (Bastawde, 1992).

Overall, the 30% inoculum density produced the highest xylanase activity with both the inoculum ages after four days of SSF. However, the increase in xylanase activity with the 4-week old inoculum when compared to the 2-week old inoculum was less pronounced with an increase of 1.1 to 2.5 fold only throughout the fermentation.

4.2.3.2 Laccase activity

The laccase activities of *P. sajor-caju* during SSF of 'hampas' are shown in Fig. 18. The inoculum age had a significant ($P < 0.001$) effect on laccase activity of *P. sajor-caju* during SSF of 'hampas' (Appendix C9). The 4-week old inoculum regardless of the inoculum density gave higher enzyme activity throughout the fermentation compared to the 2-week old inoculum.

With the 2-week old inoculum, a gradual rise of laccase activity with a maximum of 5.4 U/g 'hampas' was recorded on day eight with no distinct lag phase. However, the means test showed no significant difference ($P > 0.05$) in laccase activity from day six to day twelve (Appendix C10). Statistical analysis showed that the interaction between the inoculum densities and sampling day was significant ($P<0.001$)(Appendix C11). The laccase activity declined slightly towards the end of SSF with 20% and 30% inoculum densities (Fig. 18a).

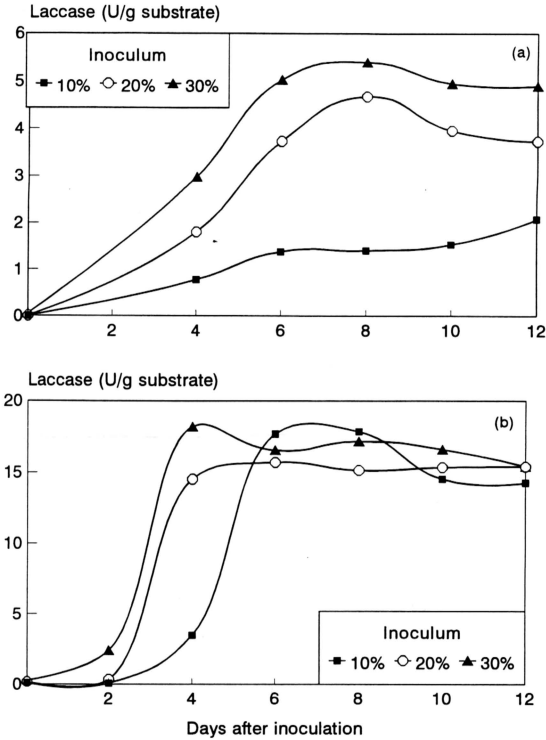

Figure 18. Laccase activities of culture extract during SSF of 'hampas'
a = 2-week old inoculum b = 4-week old inoculum

With the 4-week old inoculum, a lag phase of about two days was noted in the SSF cultures. The improved inoculum may have reduced the lag phase to about two days. This was followed by a general trend of rapid increase in laccase activity between days two and four (Fig. 18b). The laccase activity then remained same or decreased slightly towards the end of the fermentation period. A significant difference on laccase activity ($P<0.001$) was noted with the 4-week inoculum when the three densities of inoculum were used for SSF (Appendix C12). The higher densities of 20 and 30% gave significant and higher enzyme activities of 14.5 to 18.2 U/g 'hampas' during the first four days of SSF when compared to an enzyme activity of only 3.5 U/g 'hampas' with 10% inoculum. However, the 10% inoculum was able to produce laccase activity (17.7 U/g 'hampas') comparable to that produced by higher inoculum densities on day six of SSF. There was no significant difference in laccase activities with the three densities of the 4-week old inoculum on day six of SSF. Prolonging the SSF after six days did not result in a significant laccase increase (Appendix C13).

On the whole, the 2-week old immature inoculum was found to be incapable of producing significant xylanase and laccase yields. This is may be mainly due to the insufficient and visibly sparse fungal biomass produced during the SSF. The four week old inoculum with 10% density of *P. sajor-caju* produced highest laccase activity of 17.7 U/g 'hampas' after six days of fermentation while the xylanase activity was still on the increase. Significant laccase activity, as much as 3-12 fold increase was obtained with the 4-week old inoculum compared to the 2-week old inoculum during

the fermentation of 'hampas'. This showed that the use of a mature inoculum at a lower density with sufficient fermentation time produced satisfactory enzyme yields. The use of lower inoculum densities will result in the reduction of the overall cost of fermentation. While the use of higher inoculum densities speeded up growth and depleted the substrate with nutrients, considerable increase in product (e.g. enzyme) formation did not occur correspondingly.

4.2.3.3 α-Amylase and glucoamylase activities during SSF

The starch degrading enzymes, α-amylase and glucoamylase contents of *P. sajor-caju* during SSF of 'hampas' are shown in Figs. 19 and 20, respectively. Both the enzyme activities did not show any distinctly noticeable pattern and were generally low in activity as obtained by the assay methods employed. The α-amylase and glucoamylase activity fluctuated between 0.05-1.17 U/g and 0.01-1.17 U/g 'hampas' respectively for all the inoculum densities used. The insignificant and low activities of α-amylase and glucoamylase compared to the other enzymes assayed, indicated that these enzymes were either not produced by *P. sajor-caju*, inhibited during fungal growth or not adequately extracted from the substrate and thereby not detected by the assaying methods employed. Microscopic inspection of the fermented 'hampas' showed that large quantities of starch granules were still trapped within the fibrous matrix. This indicated that the starch component of 'hampas' was not utilized by *P. sajor-caju* during SSF as carbon source.

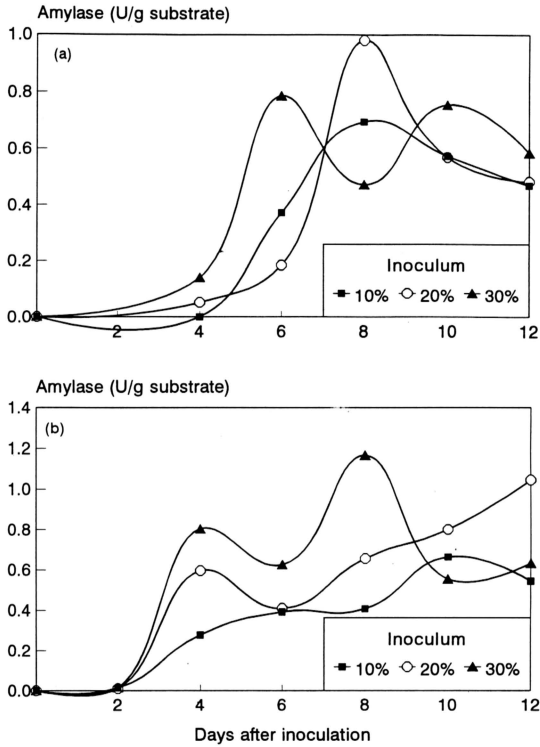

Figure 19. α-amylase activities of culture extract during SSF of 'hampas'
a = 2-week old inoculum b = 4-week old inoculum

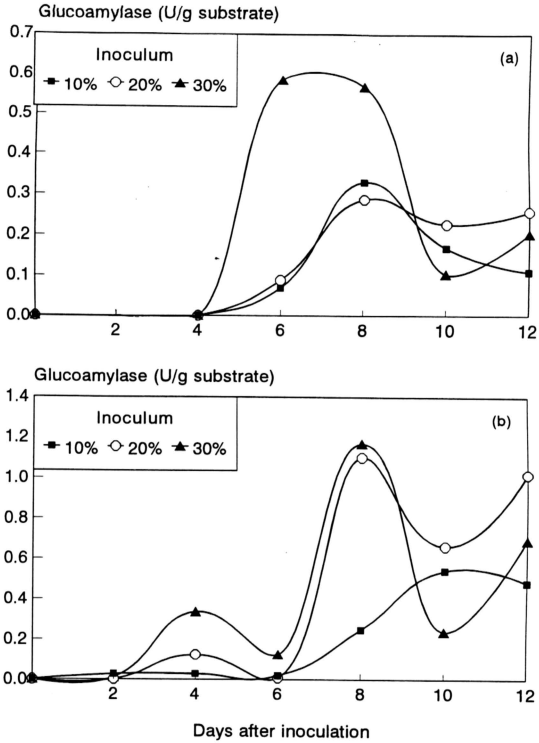

Figure 20. Glucoamylase activities of culture extract during SSF of 'hampas'
a = 2-week old inoculum b = 4-week old inoculum

The upgraded 'hampas' with less lignin but high starch content obtained after enzyme extraction makes it suitable as animal feed. Yahya *et al.* (1992) observed that 'hampas' which contained substantial amounts of digestible carbohydrates together with moderate amounts of fiber when supplemented with proper amounts of proteins and essential minerals was suitable for ruminants as a roughage source. Pongsapan *et al.* (1984) found that with grass and urea supplementation, sago 'hampas' could replace up to 45% of the diet of young cattle without affecting feed intake, live weight gain and feed efficiency.

In this study, the optimization of selected physiological parameters for SSF of sago 'hampas' was carried out. The information obtained showed that *P. sajor-caju* had an advantage in rapidly colonizing the substrate and produced higher quantities of laccase. The improved koji was more uniform, easier to handle in granular form and provided rapid colonization and as a consequence, enhanced fermentation. In this study of SSF of 'hampas' with the improved koji, laccase was the most prominent enzyme produced by *P. sajor-caju*. Maximum laccase activity of 17.7 U/g was noted with a 10% inoculum within six days SSF of 'hampas'. This was about 67% more than that found in the previous study (Chapter 3, pp. 77). The amount of laccase produced may vary tremendously as enzyme synthesis is regulated in both positive and negative directions by such control mechanisms as induction, end product repression and catabolite repression. More studies to induce *P. sajor-caju* for increased laccase production in the present system are needed.