CHAPTER

GENERAL DISCUSSION AND CONCLUSION

7.0 GENERAL DISCUSSION

With the rapid advancement in the processing of sago starch, the amount of wastes generated is becoming alarmingly high. Alternative use of the wastes produced would not only minimize pollution of the receiving bodies, but also generate additional income for the sago industry. The sago 'hampas' which contributes to a large proportion of solid residue produced at the sago factory, represents a suitable material for fungal fermentation and mushroom cultivation (Shim, 1992). 'Hampas' contains about 66% starch. This reflects the inability of the current processing techniques to completely extract the trapped starch within the fibrous matrix of 'hampas'. The degradation of the fibrous lignocellulosic portion of 'hampas' was of interest in the present study as this would render 'hampas' useful for other applications, such as animal feed.

This study reports encouraging findings for potential enzyme production in SSF of 'hampas' with *P. sajor-caju*. The sago 'hampas' used in this experiment was obtained *in situ* at the factory. Due to the high moisture content, drying of 'hampas' within the

shortest time possible was crucial to arrest natural fermentation. Other than drying, sieving of the dried 'hampas' through a 2 mm sieve was necessary as the dried substrate tend to clump together.

The selected fungus, *P. sajor-caju*, a tropical species from India has been reported in many studies for bioconversion of plant residues. *Pleurotus sajor-caju* has been reported to have greater tolerance to phenolic monomers and tannin derivatives (Cai *et al.*, 1993) which are often present in lignocellulosic residues due to fungal degradation of the lignin component (Cherney *et al.*, 1989). This fungus, apart from being edible, selectively degrades lignin and can be grown easily on various plant waste materials (Roxon and Jong, 1977; Madan *et al.*, 1987). Due to the physico-chemical nature of 'hampas' determined in this study, the ligninolytic fungi appear to be the most promising microorganism for use employing simple and low technology methods.

The present study gives information on the methods used for production of different enzymes by SSF of 'hampas' with *P. sajor-caju* and utilization of spent 'hampas' for mushroom cultivation. Summary of the results obtained and the conclusion drawn are described below.

The studies reported in this thesis on enzyme activities of *P. sajor-caju* during SSF of 'hampas' on a laboratory scale showed positive results. In the process of studying the

utilization of sago 'hampas' for enzyme production, several possibilities for further research were revealed.

7.1 CONCLUSIONS

Cellulolytic and ligninolytic enzymes of *P. sajor-caju* during SSF of 'hampas'

Studies on characterization of 'hampas' used showed that it contained 14% of fiber of which about 25% is made up of lignin. The crude protein content was 1.15% with a high C:N ratio. It was found necessary to add urea to 'hampas' to adjust C:N to 35:1 for fungal colonization.

The main constituents of 'hampas' being starch and fibers, its utilization by the ligninolytic microorganism, *P. sajor-caju* was found to be possible. 'Hampas' was found to support the growth of *P. sajor-caju* favorably over a fermentation period of 21 days. By 10 to 11 days of SSF, the substrate was completely colonized with white mycelia of the fungus. At the end of 21 days fermentation, the weight loss was found to be only 5% of the original substrate fresh weight. Abnormal fruit bodies appeared in cultures of *P. sajor-caju* after 17 days. Laboratory experiments carried out with mushroom cultivation on spent 'hampas' after SSF gave encouraging results.

Soluble protein content, which is used to measure fungal biomass indirectly, showed a rapid increase after day four with a maximum of 3.2 mg/g substrate on day 11. The

reducing sugars content increased by 62% from 26 mg/g to a maximum of 42 mg/g substrate by day nine of SSF.

After four days of lag phase, mycelial growth of *P. sajor-caju* was observed in the culture flasks and this corresponded with increases in soluble proteins, CMCase, β-glucosidase, xylanase and laccase activities in the culture extract. A wide spectrum of ligninolytic and cellulolytic enzymes were produced by *P. sajor-caju* during biodegradation of 'hampas'. Enzymes, particularly laccase which is a good indicator of mycelial biomass, was detected in the highest amounts in the culture extracts. The results of the screening process showed that the activities of enzymes quantitatively produced, were in the descending order of :

laccase > xylanase > endocellulase > FPase > β-glucosidase.

Maximum activities of the various enzymes obtained during fermentation of 'hampas' were as follows: laccase, xylanase and β-glucosidase with activities of 10.6U/g, 10.1U/g and 0.27U/g, respectively on day nine of SSF, while CMCase activity was 2.85U/g 'hampas' on day seven. In this study, it was noted that maximum activities of all the enzymes were attained within 10 days of fermentation. The enzyme activities correlated well with the age of the fermentation culture. The β-glucosidase and FPase showed almost similar activities with slight variation throughout the fermentation period, in spite of significant increase in fungal biomass measured as soluble protein,

presumably resulting from nutrient regulation. Overall, *P. sajor-caju* produced laccase and xylanase in higher quantities than the cellulolytic enzymes during SSF of 'hampas'.

Effect of inoculum age and density on enzymes during SSF of 'hampas'

The optimization of the fungal inoculum parameters for efficient SSF of 'hampas' with *P. sajor-caju* were carried out and encouraging results were obtained. Modifications were made in the koji development process. The improved koji inoculum developed in this study proved superior in terms of handling ease, enhanced fermentation and enzyme activities. With an improved koji inoculum, the laccase and xylanase enzyme activities were the highest within four to eight days of fermentation. Together with the improved granular-form koji, the age and density of the inoculum strongly influenced the SSF of 'hampas'. Generally, a high inoculum of 30% (w/w) gave rapid growth initially, but not later as fermentation progressed.

The soluble protein content in the crude culture filtrate obtained using a 4-week old inoculum was higher than the protein content obtained using 2-week old inoculum. It was observed that SSF with 10% inoculum density had the lowest reducing sugars while 30% inoculum had the highest. The moisture content of 'hampas' in the culture flasks increased slightly till the end of fermentation.

In this study, a 4-week old inoculum with 10% (w/w) density provided maximum laccase activity of 17.7 U/g 'hampas' within six days of SSF. This laccase activity is calculated to be about 60% more than that obtained in the earlier experiment of this study. With the mature 4-week old inoculum, laccase activity was observed to increase significantly by about 3-12 fold throughout the fermentation, compared to the activity with 2-week old inoculum. However, the increase in xylanase activity with the 4-week old inoculum compared to the 2-week old inoculum was less pronounced, with only a 1-2 fold increase throughout the SSF. Maximum xylanase activity of 13.5 U/g was noted with a 4-week old inoculum at 30% (w/w) density after four days of SSF. The 2-week old immature inoculum was found to be incapable of producing significant xylanase and laccase yields.

The starch degrading enzymes, α-amylase and glucoamylase activities were insignificant and did not show any noticeable trends. The variation in pH during SSF of 'hampas' using the two inoculum ages and three densities tested, were almost identical. The changes in pH correlated with the growth of fungus which in turn could be responsible for maintaining the pH of the substrate at a comfortable range for fungal colonization. As the fermentation continued with rapid growth of the fungus, ammonium uptake might have exceeded the hydrolysis rate of urea and might be responsible for the drop in pH. In the present study, the pH of 'hampas' was found to be between 5 and 6 throughout the SSF. The soluble protein and reducing sugars content of the culture extract gradually increased and stabilized towards the end of the

SSF. It can be concluded that within the limits of the present SSF system, the duration of six days was sufficient for the mycelium of *P. sajor-caju* to colonize and utilize 10 g of 'hampas' per flask and satisfactorily degrade it.

Effect of phenolic monomers on growth of *P. sajor-caju* and on laccase production

Ferulic acid, vanillin and 2,5 xylidine were used as inducers in this study. When the three different phenolic monomers were tested, growth of *P. sajor-caju* on potato dextrose agar (PDA) medium was prominent in the presence of vanillin or ferulic acid but was not significant in the presence of 2,5 xylidine. 2,5-xylidine at 0.8 mM suppressed mycelial growth by 16% compared to its positive controls. Supplementation of culture medium with 0.2 to 0.8 mM ferulic acid stimulated growth by 22 to 45%.

The aerial and sparse mycelium in the culture plates with irregular peripheral growth produced by *P. sajor-caju* on PDA in the presence of higher concentrations of more than 0.4 mM phenolic monomers, indicated the sensitivity and/or tolerance of this fungi to the phenolics.

The growth of *P. sajor-caju* on 'hampas' was more pronounced in the presence of ferulic acid and vanillin, as revealed by the sudden increase in laccase activity between days six and eight of SSF. Addition of 0.2 mM of either vanillin or ferulic acid doubled the laccase activity of *P. sajor-caju* to about 31-34 U/g 'hampas' within six

days of SSF with a 4-week old 10% (w/w) inoculum density. Rapid growth of *P. sajor-caju* on 'hampas' in the presence of the inducers caused thick white mycelium to completely colonize the substrate within eight days of SSF.

The spent 'hampas' obtained when inducers were used, had on an average 14% (w/w) less lignin and a significantly higher cellulose/lignin ratio of 3.3 compared to control cultures. The cellulose component of 'hampas' was not utilized during SSF indicating the preference of *P. sajor-caju* for lignin and hemicellulose as carbon source. The spent 'hampas' having a lower lignin content and considerable amounts of starch is more favorable for mushroom cultivation as shown in this study or as a supplement for animal feed.

Characterization and purification of laccase produced during SSF of 'hampas'

Laccase which was consistently detected in large quantities during the SSF of 'hampas', was then chosen for characterization and purification. The K_m and V_{max} values of crude laccase were found to be 0.073 mM for syringaldazine and 0.962U/min., respectively.

The pH and temperature optima of crude laccase enzyme were found to be 6.0 and 50°C, respectively. The enzyme was completely thermostable for 2 h between

30-55°C and a pH of 4.5 and 9.5 at 30°C. Partially purified laccase showed wider pH optima and temperature stability.

A three-step purification of the induced crude extract gave a brown precipitate with 81% laccase yield and a purification of 3-fold. The precipitate had laccase activity of about 380 U/g and was twelve fold higher than the activity in the crude (induced) culture filtrate.

The partially purified laccase produced by *P. sajor-caju* during degradation of 'hampas' showed varied and wide tolerance to potential inhibitors. The inhibition effect of inhibitors on laccase activity can be categorized in the ascending order of :

CTAB > EDTA > acetone > methanol > sodium azide.

Mushroom cultivation using 'hampas'

The starchy-fibrous nature of 'hampas' together with the rapid growth of *P. sajor-caju* during SSF in this study demonstrates the potential for utilization of spent 'hampas' after SSF as a substrate for mushroom cultivation. The incidental formation of primordia in the culture flasks (Plate 4) towards the end of the SSF led to studies to investigate the possibility of using spent 'hampas' after enzyme extraction for mushroom cultivation. The spent 'hampas' supplemented with Palm Oil Sludge Solids (POSS) and shredded paper was found to support mushroom (*P. sajor-caju*) growth

with a biological efficiency of 40 to 60%. A detailed account of these studies is given in Appendix D.

The cultivation of mushrooms provides an opportunity to convert waste materials into protein-rich food and help minimize pollution. Currently in Sarawak, huge quantities (in the range of 39,000 tonnes/year) of 'hampas' is generated through the activity of sago starch processing. 'Hampas', with physico-chemical characteristics favorable for fungal growth, as noted in the present study, indicate strong potential of its utilization for crude enzyme production through SSF. Therefore, there is tremendous scope for SSF of sago 'hampas' and cultivation of mushroom on spent 'hampas' for the sago farmer. Simple economics of the SSF system of 'hampas' and mushroom cultivation worked out based on findings of the present study offers additional income generation for sago growers (Table 18). Based on these studies, it was calculated that an estimated net income of $16,000 (Ringgit Malaysia) per SSF/mushroom cycle, could be generated from 100 kg of dry 'hampas' at farm level with minimal inputs.

Biodegradation of lignocellulosics by *P. sajor-caju* were found to enhance the *in vitro* digestibility compared to that of the undegraded residues (Zadrazil, 1977; Kamra and Zadrazil, 1986). The fungal mycelia in the upgraded substrate could also provide valuable compounds such as amino acids, vitamins, and fats (Rajarathnam *et al.*, 1992). The upgraded 'hampas' having less lignin and high starch content after SSF by *P. sajor-caju*, may be a potentially economical source of ruminant feed.

Table 18. Economics of SSF of sago 'hampas' and mushroom (*Pleurotus sajor-caju*) cultivation as an income generating activity for sago farmers

	RM/kg dry 'hampas'
INPUT FOR SSF OF 'HAMPAS'	
RAW MATERIAL, KOJI, NUTRIENTS	
'Hampas' @ RM20/100kg dry	0.20
3 bottles Koji @ RM1.20/bottle	3.60
2.4g $CaCO_3$ @ RM60/500g 1%	0.30
9g KH_2PO_4 @ RM50/500g 0.2%	0.90
2.3g $MgSO_4$ @ RM150/500g 0.05%	0.70
17g Urea @ RM125/500g 0.38%	4.25
INCUDERS AND LACCASE ASSAY	
Vanillin @ RM207/500g	0.30
Syringaldazine @ RM86/g (assume 100ml used/kg 'hampas')	0.30
Misc. charges e.g. flasks, electricity, water (depreciation + interest) @30% of above	3.20
INPUT FOR MUSHROOM CULTIVATION	
Shredded paper @ Free of charge	
Polyethylene bag @ RM9.90/kg bags (Estimate at $0.10 for 1kg dry 'hampas')	0.10
Koji, Rice bran and $CaCO_3$	1.50
TOTAL INPUT FOR SSF AND MUSHROOM CULTIVATION	15.35
OUTPUT FOR SSF OF 'HAMPAS'	
Laccase @10 000U/ RM76 (Assume maximum laccase yield of 32U/g 'hampas' with inducer and 70% enzyme recovery inducer)	170.00
OUTPUT FOR MUSHROOM CULTIVATION	
500g (fresh) Mushroom yield @ 50% BE. Mushroom sold at @ RM1.25/100g	6.25
TOTAL OUTPUT FOR SSF AND MUSHROOM CULTIVATION	176.25
NET INCOME calculated per 100 kg 'hampas' (Laccase yield + Mushroom) - Total Input (RM17 000 + RM625) - RM1535	RM16 000/100 kg 'hampas'

About RM2.50 = US$1.00

BE = Biological Efficiency (refer Chang *et al.*, 1981)

8.0 RECOMMENDATION FOR FURTHER WORK

The studies on SSF of 'hampas' using *P. sajor-caju* showed excellent potential for further study as follows:

i. Natural selection of *P.sajor-caju* for finding the variant which will produce more laccase and/or xylanase.
ii. Studies with more inducers for selection of the best inducer for higher laccase production.
iii. Studies on more cost effective laccase purification methods.
iv. Pilot plant studies for commercial production of laccase and/or xylanase.
v. Use of immobilized laccase and xylanase for treatment of wastewaters containing phenolics or lignin.
vi. Studies on integrated system of utilization and treatment of sago starch processing wastes.

9.0 CONCLUDING REMARKS

More effective treatments for enhancing lignocellulosic byproducts using ligninolytic fungi are dependent on coordinated research aimed at optimization of the solid substrate fermentation processes involved.

The future prospects for the development of the utilization of sago by-products for recovering value-added by-products need to be studied with a multi dimensional approach. The complete bioconversion of sago 'hampas' with the main aim of minimizing pollution must fit into an integrated treatment system together with sago waste utilization.

The integrated system may incorporate, among others enzyme extraction, ruminant feed, mushroom cultivation, phototrophic bacteria, algae, and fish cultivation. A schematic representation of the proposed options heading towards a practical system for sustainable development is shown in Fig. 29.

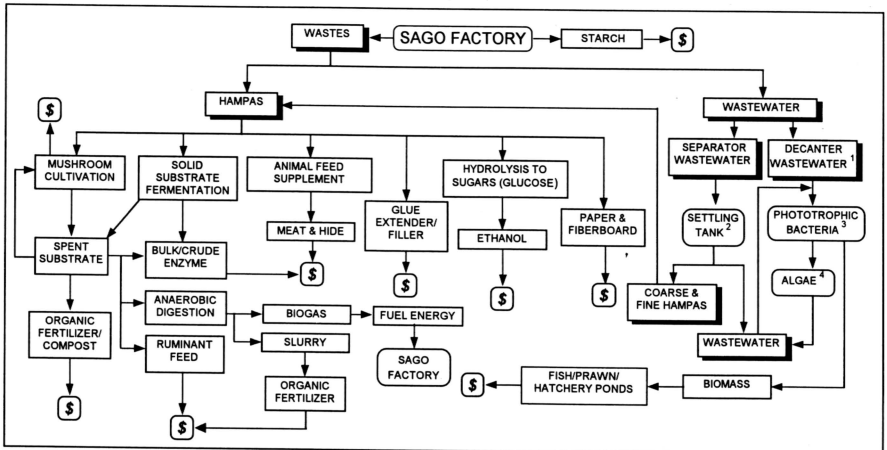

Fig. 29. Proposed integrated system for the utilization and treatment of sago starch processing wastes

= Product = By-product ($) = Revenue

[1]Getha (1995), [2]Simple settling technique, [3]Phototrophic bacteria treatment system, [4]Algal treatment ponds