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CHAPTER SIX

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6.0 GENERAL DISCUSSION

While scientific and technological developments are undoubtly important, developments in environmental management are likely to be equally significant in waste treatment applications. The last few years have seen a major change in industrial emphasis from waste treatment to waste reduction or minimization (Bisaria, 1999). The driving pressure to eliminate wastes at source has already had significant impacts on traditional waste treatment industries, for example incineration, and will influence the development of enzyme-based processes. While pollution prevention and clean technology programmes have and will continue to reduce the volume to be treated, it is equally clear that waste cannot be completely eliminated. The tenets of waste minimization require that, where waste cannot be avoided, its effects should be mitigated by recycling or by conversion into useful and/or valuable by-products (Bisaria, 1999). It is likely that these trends will lead to smaller volumes of more concentrated and possibly more toxic wastes such that niche processes will be needed for their treatment which are capable of maximizing the recovery or generation of valuable products. Bearing this in mind, this preliminary study has been done to compare the potential of a few agricultural residues which is abundant in Malaysia to produce bulk enzymes through SSF using a white rot fungus, *Pyc. sanguineus*. Results showed that OPFPt gave highest productivity of all enzymes studied at day 10 compared to sawdust and sago 'hampas'. However, in any experimental procedures to the eventual results, there are weakness which must be discussed and improvements must be made.

One important criteria to consider in choosing the suitable microorganism for SSF is whether the microorganism produces toxic compounds or not. So far, there has been no reports on the toxicity profile of *Pyc. sanguineus.* Thus it would be essential to carry out some screening tests for toxins such as aflatoxin during SSF.

Pycnoporus sanguineus was the chosen micro-oganism in this study as suggested and supported by the study done by Pointing *et al.* (2000). This study revealed high laccase productivity during SSF of OPFPt compared to sawdust and 'hampas'. Laccase plays an important role during lignin degradation (Hatakka, 1994). Lignin is generally considered to be the major biodegradation obstacle. The chemical nature of lignins and how they are linked with other cell wall polymers is as important as the total amount of lignin present. Due to its complex and heterogenous structure, lignin degradation is slow and limited to a relatively small number of microorganisms. The white rot fungi, are known to be the main agents of lignin degradation in nature (Buswell and Odier, 1987). Due to time constraint and lack of facility, the lignin degradation pattern for OPFPt using *Pyc. sanguineus* was not done. However, it is essential to determine the capability of this species to degrade lignin efficiently and is strongly suggested for future studies.

This preliminary study was aimed to determine the enzyme productivity profile of *Pyc. sanguineus* through SSF. Although good enzyme productivity was observed, the growth of the fungus was not determined. The growth rate of the fungus, however, may affect the fermentation time for producing enzyme. Elsewhere *Pol. sanguineus* proved to be better than *Pol. versicolor* and *Tra. hirsuta* in laccase production but comparatively poor growth was observed in lignin medium which was explained as

poor utilization in the absence of some easily metabolizable carbon source (Sandhu and Arora, 1985). However, no correlation existed between the enzyme and the fungal mass produced. Not much difference in growth was observed at different pH values when optimising laccase productivity (Sandhu and Arora, 1985). This finding proved that enzyme productivity may not necessarily be associated with fungal growth. However, it is important to determine if *Pyc. sanguineus* shows similar pattern.

Separating the end-product from the process of fermentation is one of the major problems encountered in the bioconversions. The extractive bioconversion (extracting the end-product from a fermenting mixture of substrates) must be applied for optimizing the industrial-scale to produce value-added products. From this laboratory scale experiment, laccase recovery was four times higher using extraction with tap water at 25 ± 2°C compared to extraction with sodium acetate buffer at 4°C. Although extraction using tap water with adjusted pH of 5 gave better enzyme yield, the stability of the enzyme was yet to be studied. Processes and conditions that was used in SSF should be generally able to minimise product degradation due to heat, pH or oxidation. Although for economical purposes, tap water which gave comparable results with acetate buffer seems to be more appealing, but it is of no use if the stability of the enzyme was not maintained in such solvents. Thus, it is essential in future to study the stability of enzymes in the extraction solvent or medium.

In this study, enzyme extraction was done by adding the buffer/tap water into the solid culture and mixing it manually with a spatula. After centrifugation, only 70% of the extraction fluid could be recovered as crude filtrate This proves the inefficiency of the manual method as it may affect enzyme extraction as it was important to break up he

fungal mycelia which is intricately interwoven with the substrate. Future experiments can be done using a homogenizer to improve enzyme extraction. Besides this, a simple filtration using a nylon cloth to separate the solids and filtrate have been commonly carried out prior to centrifugation. This method could be adapted to improve the recovery of crude filtrate from the solid culture.

In this study, optimisation of the cultivation was done by testing various parameters such age and density of inoculum and also the nitrogen content of the substrate. About 8 times higher yield of laccase was obtained using 30% of 4 weeks old inoculum with 0.92% (w/w) of nitrogen supplementation of the substrate compared to the initial cultivation conditions of 10% of 18 days old inoculum and 0.46% N level of substrate. One other key element for regulating and optimizing SSF processes is the moisture level (Laukevics *et al.*, 1984). Too much moisture compacts the substrate, prevents O_2 penetration, and facilitates contamination by fast growing bacteria (Black, 1996). Too little moisture inhibits growth, enzyme activity, and accessibility to nutrients. Moisture also can be used for evaporative heat removal, perhaps the most crucial factor in large-scale SSF processes. Thus further optimization of the current SSF process can be done by determining the optimal moisture level for fungal growth and enzyme production.

Laccase production using various fungal cultures differed markedly in their inducibility, number of enzyme forms, molecular weight, pH optimum, and substrate specificity. The laccases of various fungi show considerable heterogeneity regarding their molecular properties and substrate specificities (Hublik and Schinner, 2000). This could very well explain the varied data obtained for laccase production using different

micro-organisms. Thus, the molecular characterization of laccase obtained through SSF using *Pyc. sanguineus* should be studied to improve the process variables such as inducibility. The catalytic lifetime of an enzyme can be extended through optimization of process variables that significantly affect enzyme activity and stability (Schmid *et al.*, 2001). A simple immobilization method that prolonged the catalytic lifetime and improved the activity as well as stability of laccase can be carried out for optimizing the function of laccase in certain biotechnological applications such bioremediation.

Concentration is one of the principal isolation steps in extracellular enzymes since the sole concern is often potency rather than purity. For reasons which are not clearly understood, but may be due to protein aggregation, many enzymes are stabilized as their concentration is increased (Arbige and Pitcher, 1989). Enzyme concentration may use equipment for reduced temperature evaporation, such as has been developed for fruit juices and similar products. For this purpose, enzyme solutions must be of even consistency, well filtered, and free from air and large particles. For extracellular enzymes, total precipitation or complete adsorption may be used for recovery. However, for many extracellular industrial enzymes, the only justification for fractionation will be to gain potency per given weight, not to achieve purification. For full industrial-scale products the degree of processing must be minimal for economic reasons. Since the raw material and fermentation costs are relatively low, the overall percentage recovery will be crucial.

6.1 FUTURE WORK

The SSF of OPFPt using *Pyc. sanguineus* has shown good enzyme productivity. These results showed promising potentials and thus warrants for further studies :

1. Enzymes in Bioremediation

Extensive research have been successfully carried out to explore the potential of lignin degrading enzymes namely laccase in the area of bioremediation (Schliephake *et al.*, 1993; Pointing *et al.*, 2000 and Schliephake *et al.*, 2000). Preliminary studies showed decolorization of azo dyes and triphenylmethane dyes by enzymes produced by *Pyc. sanguineus* through SSF (Vimala *et al.*, 2001). This encourages future work to determine the function and capability of crude enzyme produced from SSF in dye decolorization and detoxification of the vast amount of effluents generated by industries such as the pulp and paper as well as textile industries. Reports also showed that laccases are capable of degrading highly toxic environmental pollutants such as xenobiotics (Field *et al.*, 1993 and Collins *et al.*, 1996). Polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbon (PAH) degradation using these crude enzymes can be done as these are some of the most common environmental pollutants.

2. Scale-up

Although active research have been ongoing for optimization of SSF process, but very fewe studies have been done on improving the volume of production through SSF. Apparently, there are few reports related to scale-up experiments in enzyme production except for the preliminary scale-up trials conducted for the production of alkaline proteases by *A. flavus* (Karanth and Lonsane, 1988). However, a large-scale enzyme

production facility has been establised by a company called Alltech in Nicholasville, USA (Alltech). This organization is claimed to be first in enzyme technology based on SSF and has initiated the way to explore and optimize the usage of SSF technology by future researchers. This lab-scale experiments should however take a step further to expand the volume of enzyme production through scale-up experiments to materialise the production of enzymes for industrial applications.

In the production of crude extracellular enzymes, the residual substrate after crude enzyme extraction could be used as animal feed ingredient or for soil abatement. This possibility, however, depends on the toxicity profile of the species and also the solvents used during the extraction process.

Biodegraded OPFPt was reported to have increased C:N ratio of 1:21 (Dinesh,1994). This condition can be subjected to fermentation by methanogenic bacteria to produce biogas. Similar application was suggested by Bisaria *et al.* (1990) who reported that the spent residues obtained after cultivation of edible mushroom *Ple. sajor-caju* were used in anaerobic digestors for biogas production.

The potential of bioconversion of lignocellulosic material has not been fully harnessed to bring biotechnology to a realistic level in the bioremediation of environmental impacts, and in producing fuels and valuable chemicals. Further research is needed to enhance the available data on enzymatic activities, feedstock pretreatment, bioreactors, immobilization of cells and enzymes, fuel production and enzyme application in bioremediation.

CONCLUSION:

1. Oil palm frond parenchyma tissue (OPFPt) produced maximum enzyme cocktail at day 11 of SSF compared to sago 'hampas' and rubberwood sawdust.
2. Optimization of enzyme recovery showed that extraction using tap water at pH 5.0 at 25 ± 2°C recovered 8 times more laccase activity compared to extraction with sodium acetate buffer at pH 4.8 at 4°C whereas xylanase recovery was not affected.
3. Age of inoculum had a significant effect on enzyme production and maximum laccase and xylanase productivity was with using 4 weeks old inoculum.
4. Density of inoculum had a significant effect on enzyme production and maximum laccase and xylanase productivity was with using 30% inoculum density.
5. Level of nitrogen supplementation of substrate had a significant effect on enzyme production and maximum laccase and xylanase productivity was with using 0.92% N (w/w).