****

CHAPTER FOUR

****

OPTIMIZATION OF EXTRACTION AND RECOVERY OF ENZYMES FROM FERMENTED OPFPt.

4.0 INTRODUCTION

A knowledge of the methods used in producing commercial microbial enzymes is essential to an understanding of the character of the products. Cultivation of microorganisms which produce extracellular enzymes is a simple way of obtaining hydrolytic enzymes in large quantities. The isolation of extracellular enzymes is in general simpler than that of intracellular enzymes (Figure 4.1). The type of enzyme isolation techniques used also depends on the type of fermentation that is, whether it is SSF or submerged fermentation.

Industrial enzymes are mainly sold as crude preparations and in general, contain more than a single protein. Therefore, only partial purification of the crude extract of enzymes is needed. The first stage is the separation of the cells and particulate material from the extracellular fluid, usually achieved by filtration, centrifugation or various precipitation or extraction techniques (Arbige and Pitcer, 1989). Extraction technique is of great importance especially when solid substrate fermentation is used to produce value-added products such as enzymes. Consideration must also be given to the pH at which the enzyme is to be used. The effect of pH on an enzyme's operational activity is as important as the effect on the enzyme's stability. The alteration of pH is also attractive as an industrial method of causing fractional precipitation since the reagent costs are low. The principal difficulty in using differential pH precipitation is that the pH range for stability of many enzymes is limited.

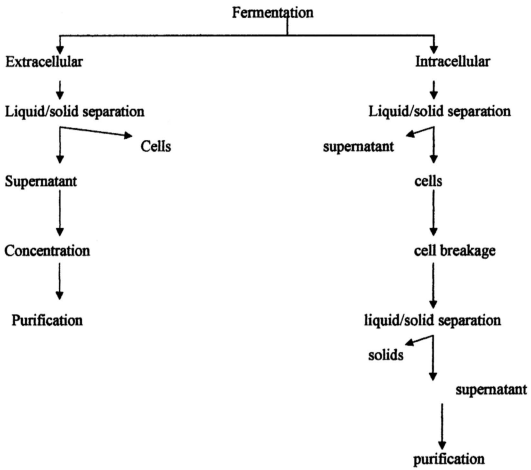


Figure 4.1: Comparison of the processing steps involved in the extraction of intra- and extracellular enzymes (Adapted and modified from Hacking, 1986)

Laccase from various fungal cultures differed markedly in their number of enzyme forms, molecular weight and also pH optimum for stability. Studies have been done to identify an ideal and optimal physical and chemical conditions such as extraction buffer and pH of the extraction medium as well as the optimal temperature to obtain an optimal enzyme activity (Youn *et al.*, 1995; Eggert *et al.*, 1996; Shin *et al.*, 1997; Hublik and Schinner, 2000).

In the previous study, crude enzyme filtrate was extracted from the solid culture with sodium acetate buffer at pH of 4.8 and shaken at 200 rpm at 4° C for 18 h.

The objectives of this investigation were:

a) to compare the effect of changes in temperature and the type of medium used in extraction of laccase and xylanase from the fermented OPFPt.
b) to compare the effect of changes in pH of extraction medium on activity of laccase and xylanase from the fermented OPFPt.

4.1 Materials and Methods

4.1.1 Substrate and Fermentation Condition

Solid substrate fermentation of OPFPt was carried out using 10g of sterilized substrate. Culture conditions for bioconversion of OPFPt was according to Ling (1994). This substrate was supplemented with 1% (w/w) calcium carbonate and urea containing 0.46% N. 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) ($\approx 2.35 \pm 0.05$g) of *Pyc. sanguineus* wheat grain inoculum and incubated at 25 ± 2°C in static condition. The fermentation was carried out for 10 days and at suitable intervals, three culture flasks were randomly sampled for each parameter tested. Assays were performed in triplicates using three culture flasks.

4.1.2 Optimization of Extraction of Crude Extracellular Enzymes

4.1.2.1 Effect of Temperature and Extraction Medium On Extraction and Recovery of Enzymes From Fermented OPFPt.

Optimization of extraction of crude extracellular enzyme was done with changes in the following parameters:

1. temperature of incubator shaker : 4° C and 25± 2°C
2. extraction medium : sodium acetate buffer (pH 4.8) and tap water (pH 8.0)

At suitable intervals of the fermentation period, sampling was done at random in triplicates for enzyme assay. The total number of culture flasks set up was 72 where four different combination of parameters were analysed (Table 4.1). The methods used

for the experimental procedures are summarised in Figure 4.2. Culture flasks with the respective extraction parameter was shaken at 200 rpm for approximately 18 hours. After shaking, the contents of each flasks were centrifuged at 9000 rpm for 20 min. The crude culture filtrate containing fungal enzymes was stored at - 20°C for 24 h prior to enzyme assays.

Table 4.1 : Effect of Various Temperature and Extraction Medium on Recovery of Enzymes From Fermented OPFPt.

	Sampling days					
Incubator shaker temperature and type of extraction medium	**0**	**2**	**4**	**6**	**8**	**10**
4° C and buffer	3	3	3	3	3	3
4° C and tap water	3	3	3	3	3	3
25± 2°C and buffer	3	3	3	3	3	3
25± 2°C and tap water	3	3	3	3	3	3

* Triplicate flasks were sampled randomly for each experiment on day 0,2,4,6,8,10 respectively.

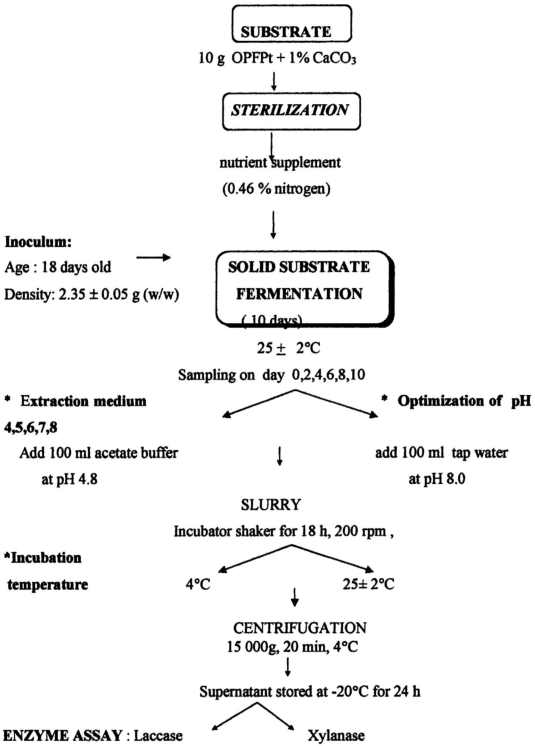


Figure 4.2: Procedure for optimization of extraction and recovery of enzymes from fermented OPFPt (Modified from Kumaran, 1996 and Ling, 1994).

4.1.2.2 Effect of pH of Extraction Medium on Extraction and Recovery of Enzymes From Fermented OPFPt.

The incubation temperature and the type of extraction medium which gave highest enzyme activity in 4.1.2.1 was chosen to run another 10 days of SSF with changes in pH of the chosen extraction medium:

1. pH of extraction medium - pH 4.0, pH 5.0 pH 6.0 , pH 7.0and pH 8.0

A total number of 90 culture flasks were prepared according to Table 4.2. Sampling was done randomly in triplicates for enzyme assay. Experimental procedures were followed according to Figure 4.2.

Table 4.2: Effect of Varying pH of Tap Water on Recovery of Enzymes From Fermented OPFPt.

	Sampling days					
Tap water with adjusted pH	**0**	**2**	**4**	**6**	**8**	**10**
4.0	3	3	3	3	3	3
5.0	3	3	3	3	3	3
6.0	3	3	3	3	3	3
7.0	3	3	3	3	3	3
8.0	3	3	3	3	3	3

Triplicate flasks were sampled randomly for each experiment on day 0,2,4,6,8 and 10 respectively.

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 the product per minute and are reported on the basis of per gram of substrate used in the SSF, under the conditions of assay.

4.1.3.1 Laccase and Xylanase

Laccase and xylanase activities of the crude culture filtrate were assayed using the standard methods described in Appendix A4 and A5.

4.2 RESULTS AND DISCUSSION

4.2.1 Effect of Temperature and Extraction Medium on Recovery of Laccase and Xylanase From Fermented OPFPt.

Laccase and xylanase activity from fermented OPFPt using different temperature and extraction medium is shown in Figure 4.3 and 4.4 respectively. On day 10, the recovery of laccase was four times higher at 30.6 U/g substrate using tap water (pH 8.0) as extraction medium at 25± 2°C compared to recovery of 7.46 U/g substrate using sodium acetate buffer (pH 4.8) at 4° C as extraction medium. However, there was no significant difference in laccase recovery by extraction with tap water (pH 8.0) and sodium acetate buffer (pH 4.8) at 25± 2°C ($p< 0.001$). The four types of extraction techniques tested had significant effect ($p< 0.001$) on laccase recovery with

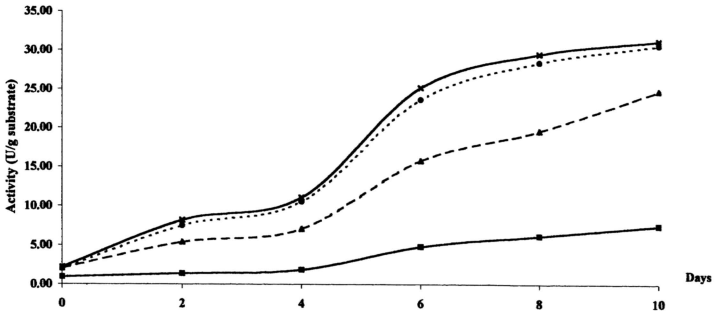
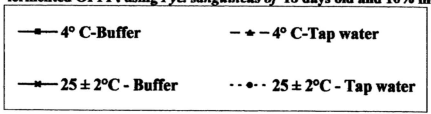


Figure 4.3 : Effect of various temperature and extraction medium on laccase activity from fermented OPFPt using *Pyc. sanguineus of* 18 days old and 10% inoculum



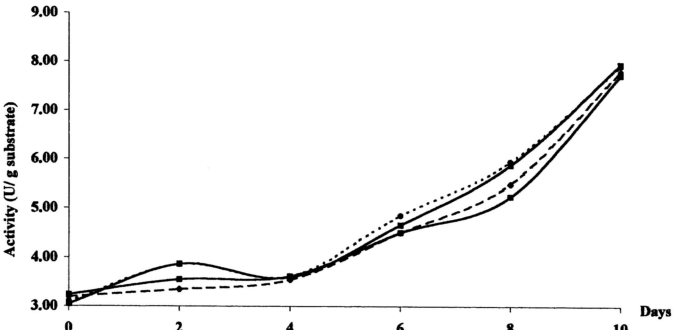


Figure 4.4: Effect of various temperature and extraction medium on xylanase activity from fermented OPFPt using *Pyc. sanguineus* of 18 days old and 10% inoculum

varying incubation time. However, there was no significant difference ($p>0.05$) in xylanase recovery using different temperature and extraction medium.

Studies show that laccase of *Pyc. sanguineus* in liquid growth medium was very stable at 4°C compared to 35°C (Pointing *et al.*, 2000). On the other hand, optimum temperature for laccase activity of *Ple. ostreatus* is 30-35°C (Youn *et al.*, 1995). Similarly, optimum laccase production by *Polyporus sanguineus* (Sandhu and Arora, 1984) and *Pyc. cinnabarinus* (Schliephake *et al.*, 2000) was carried out at 37°C. Laccase production from *Tra. versicolor* was also succesfully enhanced in a study done at 27°C (Lee *et al.*, 1999). Most proteins exhibit normal increased solubility with temperature elevation. Because enzymes generally show normal temperature dependence, temperature change is not often used to fractionally precipitate them. However, the differences in enzyme stability at higher temperatures are quite marked.

Laccase from *Pyc. sanguineus* was reported to have higher activity in glycine-HCL buffer (pH 3.0) compared to sodium tartrate or sodium acetate buffers at the same pH (Pointing *et al.*, 2000). Stability of laccase activity in buffers with variable pH values showed laccase activity decreasing in the order; phosphate-buffer > sodium-acetate-buffer > citratre-buffer in a study done using *Ple. ostreatus* (Hublik and Schinner, 2000). This study showed that extraction of crude enzyme using tap water at 25 ± 2°C gave higher yields of laccase although this factor did not seem to affect the extraction of xylanase. It is of economic interest that extraction with tap water had no significant difference in enzyme yield compared to sodium acetate buffer. Thus, extraction with tap water at 25 ± 2°C was chosen as the extraction method for further optimization studies to investigate the effect of pH of tap water in enzyme recovery.

4.2.2 Effect of pH of Tap Water on Laccase and Xylanase Recovery

Laccase and xylanase recovery during extraction of crude enzymes using different pH of tap water is shown in Figure 4.5 and 4.6, respectively. On day 10, the recovery of laccase was 50% higher at 46.5 U/g substrate using tap water at pH 5.0 as extraction medium at 25 ± 2°C compared to the recovery of 30.6 U/g substrate obtained using tap water at pH 8.0. There was however, no significant difference in laccase recovery between pH 5.0 and pH 4.0 ($p< 0.001$). However, the various level of pH tested caused significant difference ($p<0.001$) in laccase recovery with incubation time. Further, changes in pH of tap water did not show significant effect on xylanase recovery ($p>0.05$).

Laccase recovery seems to be more enhanced in an acidic condition compared to alkaline medium (pH 7-8). Several workers have also reported that pH optimum for laccase activity is in the acidic region (Bollag and Leonowicz, 1984; Pointing *et al.*, 2000). Elsewhere, optimum laccase activity from *Pyc. cinnabarinus* was achieved between pH 4.4 and 5 (Eggert *et al.*, 1996; Schliephake *et al.*, 2000). The low pH value has also been reported to be favorable for the release of laccase by *Pol. sanguineus* (Sandhu and Arora, 1984). It was suggested that the recovery at low pH may be physiologically more significant and depends on the natural habitats of the laccase producing fungi. Fungi growing in acidic environments come in contact with various acidic plant phenols or pesticides, and the lower pH optima of the laccase allows a more effective oxidation of toxic compounds (Leonowicz *et al.*, 1978). However, maximum laccase activity of *Ple. ostreatus* was obtained at pH 5.8 and this activity has been

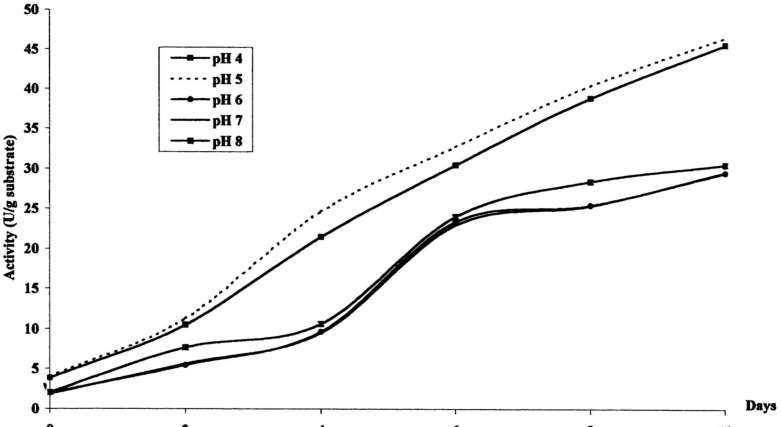


Figure 4.5: Effect of various pH level of extraction medium on laccase activity from fermented OPFPt using *Pyc. sanguineus* of 18 days old and 10% inoculum

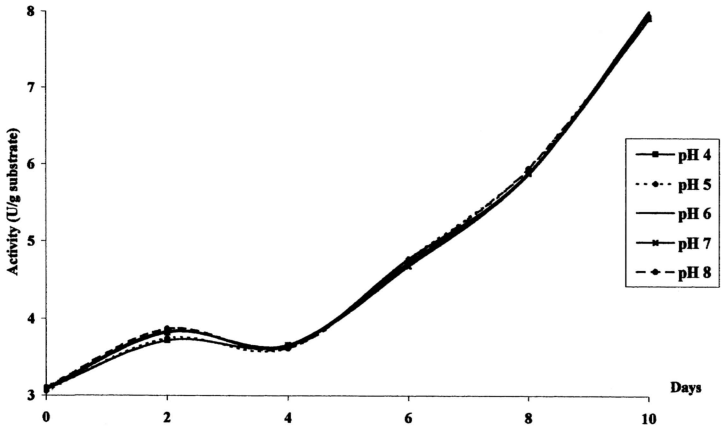


Figure 4.6: Effect of various pH level of extraction medium on xylanase activity from fermented OPFPt using *Pyc. sanguineus* of 18 days old and 10% inoculum

preserved in extreme alkaline environment of pH 10 which is very unusual and has not yet described for other laccases (Hublik and Schinner, 2000).

CONCLUDING REMARKS

Based on the enzyme recovery profile of laccase from fermented OPFPt by *Pyc. sanguineus*, four to six times more laccase was recovered using extraction with tap water at pH 5.0 and incubated at 25 ± 2°C for 18 h compared to extraction using buffer at pH 4.8 at 4° C for 18h . Changes in pH of tap water, however, did not seem to affect the recovery of xylanase. Thus, tap water at pH 5.0 at 25 ± 2°C was used as an enzyme extraction method in further studies to optimize the enzyme production.