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# *CHAPTER FIVE*

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# OPTIMIZATION OF ENZYME PRODUCTIVITY IN SOLID SUBSTRATE FERMENTATION OF OPFPt BY *Pyc. sanguineus*

## 5.0 INTRODUCTION

One of the many factors involved in fungal enzyme productivity through SSF is the cultivation system. The quality, age and optimum inoculum size or density is of great importance in improving the productivity of enzyme through SSF (Mudgett, 1986). Solid substrate fermentation using inoculum of low density may produce insufficient biomass and also allow the growth of other undesirable organims. Further, too high a density of inoculum may cause rapid depletion in substrate nutrients and thus retard the growth of organism and affect the enzyme production. Thus, inoculum age and density is regarded as one of the important physical parameter to be tested in this study to optimize enzyme productivity through SSF.

Besides choosing the suitable microorganism and the optimum cultivation conditions, the properties of substrate used in SSF is also of great importance in the productivity of enzymes. One of the factor that affects the productivity of enzymes is the availability of substrate nutrients to allow good fungal growth. The relevance of the carbon to nitrogen (C:N) ratio as one measure of resource supply and the effect of it on fungal growth has been critically reviewed (Rayner and Boddy, 1988). The C:N ratio of culture medium has also been reported to affect ligninolytic peroxidase enzyme production (Reddy and D'Souza, 1994). *Microporus* sp., *Pyc. cinnabarinus* and *Pyc. sanguineus* has been reported to produce laccase as the sole ligninolytic enzyme whilst utilising glucose under conditions of low nitrogen level (Pointing *et al.*, 2000). Studies has been done to regulate C:N ratio of substrates using various nitrogen sources such as

organic nitrogen, ammonium nitrogen and nitrate nitrogen (Gutierrez-Correa and Tengerdy, 1998; Pointing *et al.*, 2000). Testing different nitrogen levels have been suggested to be important not just because of the emphasis usually placed on C:N ratios but it has been repeatedly shown that production of laccase and peroxidase in pure culture depend on nitrogen availability (Freitag and Morrell, 1992; Gold and Glenn, 1988).

The objectives of this investigation were:

a) to compare the effect of inoculum age on laccase and xylanase productivity through SSF of OPFPt by *Pyc. sanguineus.*

b) to compare the effect of inoculum density on laccase and xylanase productivity through SSF of OPFPt by *Pyc. sanguineus.*

c) to compare the effect of nitrogen supplementation of substrate on laccase and xylanase productivity through SSF of OPFPt by *Pyc. sanguineus.*

## 5.1 MATERIALS AND METHODS

### 5.1.1 Fermentation conditions and Substrate

Solid substrate fermentation of OPFPt was carried out as described in Chapter 4 with the following changes (Figure 5.1):

1.) inoculum age : - 2 week, 4 week and 6 week

2.) inoculum densities :- 10%, 20%, 30% (w/w)

3.) nitrogen supplementation (w/v): - 0.46% N, 0.69% N, 0.92% N and 1.15% N.

#### 5.1.1.1 Effect of Inoculum Age On Laccase and Xylanase Productivity

Solid substrate fermentation of OPFPt for 10 days was done to determine optimum inoculum age for enzyme productivity. A total number of 54 flasks which contained substrate supplemented with 0.46% N were inoculated with 10% of 2, 4 and 6 weeks old inoculum (Table 5.1). Sampling was done at suitable intervals in triplicates. The enzymes were extracted with tap water at pH 5.0 and shaken at 200 rpm for 18 h at 25 ± 2°C . Crude enzyme filtrate was then assayed for laccase and xylanase activity. Experimental procedures were as summarized in Figure 5.1.

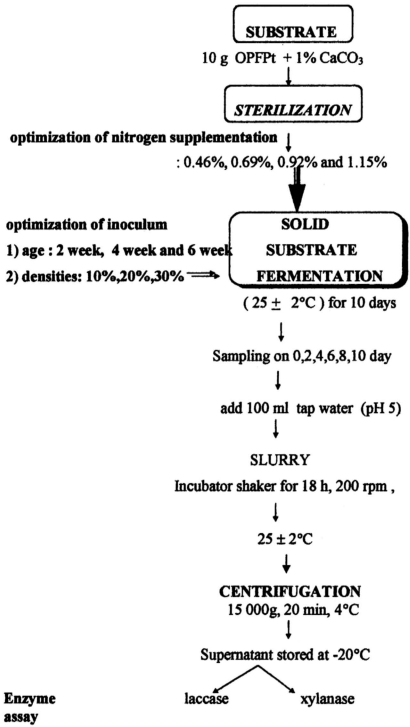


Figure 5.1 : Procedure for optimization of inoculum age, densities and nitrogen supplementation of substrate for laccase and xylanase productivity (Modified from Kumaran, 1996 and Ling, 1994).

**Table 5.1: Effect of Inoculum Age On Laccase and Xylanase Productivity**

| Inoculum age | Sampling days | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 6 | 8 | 10 |
| 2 week | 3 | 3 | 3 | 3 | 3 | 3 |
| 4 week | 3 | 3 | 3 | 3 | 3 | 3 |
| 6 week | 3 | 3 | 3 | 3 | 3 | 3 |

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

### 5.1.1.2 Effect of Inoculum Density On Laccase and Xylanase Productivity

Solid substrate fermentation of OPFPt was done for another 10. The inoculum age which gave the highest enzyme yield in 5.1.1.1 was chosen to determine the inoculum density for enzyme productivity. A total number of 54 flasks which contained substrate supplemented with 0.46% N were inoculated with different inoculum densities which was approximately 1.0 ± 0.03 g , 2.0 ± 0.02 g , 3.0 ± 0.04 g to obtain densities of 10%, 20% and 30% (w/w) as summarized in Table 5.2. Sampling was done at suitable intervals in triplicates. The contents of each flask was extracted with tap water at pH 5.0 and shaken at 200 rpm for 18 h at 25 ± 2°C. Crude enzyme filtrate was then assayed for laccase and xylanase productivity. Experimental procedures are shown in Figure 5.1.

**Table 5.2: Effect of Inoculum Density On Laccase and Xylanase Productivity**

| Inoculum Density | Sampling days | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 6 | 8 | 10 |
| 10 % | 3 | 3 | 3 | 3 | 3 | 3 |
| 20 % | 3 | 3 | 3 | 3 | 3 | 3 |
| 30 % | 3 | 3 | 3 | 3 | 3 | 3 |

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

### 5.1.1.3 Effect of Nitrogen Level of Supplementation on Laccase and Xylanase Productivity

Solid substrate fermentation of OPFPt was done for another 10 days. The inoculum age and density which gave the highest enzyme yield in 5.1.1.1 and 5.1.1.2 was chosen to determine the optimum level of nitrogen supplementation of the substrate for laccase and xylanase productivity. A total number of 72 flasks were prepared using different supplementation level of nitrogen which was approximately 0.46% N, 0.69% N, 0.92% N and 1.15% N by using filter sterilized urea (Table 5.3). Extraction of enzymes was done at suitable intervals with tap water at pH 5.0 and shaken at 200 rpm for 18 h at 25 ± 2°C. Crude enzyme filtrate was then assayed for laccase and xylanase productivity. Experimental procedures were as summarized in Figure 5.1.

**Table 5.3: Effect of Nitrogen Level of Supplementation on Laccase and Xylanase Productivity**

| % of nitrogen supplementation | Sampling days 0 | 2 | 4 | 6 | 8 | 10 |
|---|---|---|---|---|---|---|
| 0.46 | 3 | 3 | 3 | 3 | 3 | 3 |
| 0.69 | 3 | 3 | 3 | 3 | 3 | 3 |
| 0.92 | 3 | 3 | 3 | 3 | 3 | 3 |
| 1.15 | 3 | 3 | 3 | 3 | 3 | 3 |

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

### 5.1.3. Laccase and Xylanase Assays

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

## 5.2 RESULTS AND DISCUSSION

### 5.2.1 Effect of Inoculum Age on Laccase and Xylanase Productivity

Laccase and xylanase productivity during SSF of OPFPt using different inoculum age is shown in Figure 5.2 and 5.3. The inoculum age had a significant effect on the laccase productivity ($p<0.05$). A similar trend was observed for all levels of inoculum tested. There was a gradual rise in laccase productivity which peaked at day 10 of fermentation for all the three different ages of inoculum. However, SSF using 4 weeks old inoculum had highest laccase productivity of 46.6U/g substrate at day 10 compared to 2 weeks and 6 weeks old inoculum. This maximum laccase activity produced, however, was not different from the previous SSF done using 18 days old

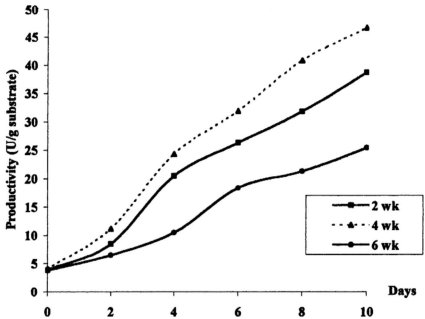


**Figure 5.2: Effect of various ages of inoculum on laccase productivity during SSF of OPFPt using *Pyc. sanguineus***

***SSF condition: 10% inoculum density, 0.46% N supplementation of substrate and enzyme extraction with tap water (pH 5.0)**

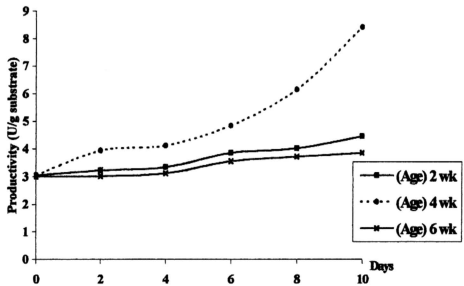


**Figure 5.3: Effect of various ages of inoculum on xylanase productivity during SSF of OPFPt using *Pyc. sanguineus***
*** SSF condition: 10% inoculum density, 0.46% N supplementation of substrate and enzyme extraction with tap water (pH 5.0)**

inoculum with optimised extraction technique at day 10. On the other hand, when a 6 weeks old inoculum was used, the laccase productivity was 45% lower at day 10 compared to 4 weeks old inoculum. Optimum laccase productivity using a 4 weeks old inoculum was also reported by Kumaran *et al.*, (1997) and Ling (1994) using *Ple. sajor-caju* from SSF of sago 'hampas' and OPFPt respectively.

As for xylanase productivity during SSF of OPFPt, there was a significant effect of the age of inoculum on the productivity of xylanase. It was observed that when either 2 weeks or 6 weeks old inoculum was used in SSF, the productivity of xylanase was significantly lower (46% -56%) when compared to the 4 week old inoculum in SSF. Solid substrate fermentation using 4 weeks old inoculum showed a gradual increase in xylanase productivity during the 10 days of fermentation unlike the 2 and 6 weeks old inoculum. Similar findings was reported by Kumaran *et al.*, (1997) using 4 weeks old inoculum of *Ple. sajor-caju* during SSF of 'hampas'.

From this study, it was observed that a 4 weeks old inoculum produced higher enzyme yield compared to the other inoculum ages tested. Thus, this inoculum age was chosen for further studies for the optimization of enzyme productivity through solid substrate fermentation.

### 5.2.2 Effect of Inoculum Density on Laccase and Xylanase Productivity

Laccase and xylanase productivity profiles of *Pyc. sanguineus* in solid substrate fermentation of OPFPt are shown in Figure 5.4 and 5.5. Analysis of variance showed that the inoculum density had significant effect ($p< 0.01$) on both laccase and

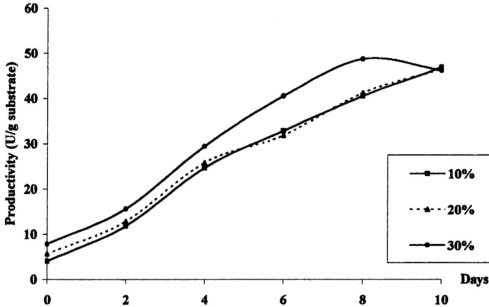


**Figure 5.4: Effect of various inoculum density on laccase productivity during SSF of OPFPt using *Pyc. sanguineus***
***SSF condition : 4 weeks old inoculum, 0.46% N supplementation and enzyme extraction with tap water (pH 5.0)**

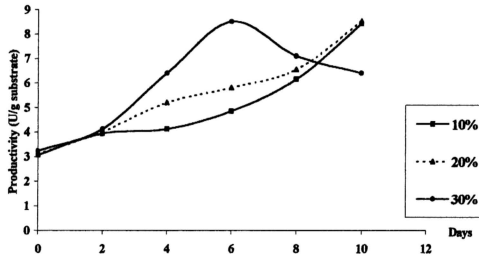


**Figure 5.5: Effect of various inoculum density on xylanase productivity during SSF of OPFPt using *Pyc. sanguineus***
***SSF condition : 4 weeks old inoculum, 0.46% N supplementation and enzyme extraction with tap water (pH 5.0)**

xylanase productivity. Laccase productivity increased rapidly with 30%(w/w) inoculum density and the activity peaked at day 8 with 48.7 U/g substrate compared to 10% and 20% densities which peaked at day 10 (Figure 5.4). However, there was a decline in laccase productivity after day 8 with 30% inoculum density and this may be due to enzyme inhibition (Bastawde, 1992). Similar trend was reported by Kumaran *et al.*, (1997) using *Ple. sajor-caju* on 'hampas'.

Xylanase productivity showed an increasing trend for the 10 and 20% inoculum density although maximum xylanase activity of 8.5 U/g substrate was observed with a 30% inoculum density at day 6 of SSF and decreased after that. This could also be attributed to enzyme inhibition (Bastawde, 1992).

This study showed that enzyme productivity was influenced by the inoculum density and maximum enzyme yield was obtained using 4 weeks old and 30% inoculum density. Laccase and xylanase productivity also peaked much earlier at day 8 and day 6 of SSF respectively.

Based on the enzyme productivity profiles of laccase and xylanase during 10 days of solid substrate fermentation of OPFPt by *Pyc. sanguineus*, maximum enzyme yield of both laccase and xylanase was produced using 4 weeks old and 30% inoculum density with extraction using tap water at pH 5.0 and shaken at 200 rpm for 18 h at 25 ± 2°C. These optimised parameters of inoculum age and density were used for further studies to increase enzyme productivity.

### 5.2.3 Effect of Nitrogen Content of Substrate on Laccase and Xylanase Productivity

Laccase and xylanase productivity during SSF of OPFPt using various levels of nitrogen supplementation of substrate is shown in Figure 5.6 and 5.7. Different nitrogen levels had a significant effect on the productivity of these enzymes ($p<0.01$). Gradual increase in laccase productivity was noticed with all the four levels of nitrogen tested during the first 4 days of fermentation. The peak in laccase productivity was observed much earlier in fermentation using 0.92% N and 1.15% N on day 6 compared to day 8 of SSF for 0.46% N and 0.69% N. However, maximum laccase productivity of 46.5 U/g substrate was obtained with 0.92% N which was maintained till day 10. There was, however, a decline in laccase productivity with supplementation of 1.15% N which dropped to 37.9 U/g substrate after day 6 of fermentation.

Rapid increase in xylanase productivity was observed in SSF using 0.92% N and 1.15% N for the first 4 days of fermentation. Maximum xylanase productivity of 9.1 U/g substrate was obtained from 0.92% N on day 6 of SSF while maximum xylanase productivity of 7.9 U/g substrate was obtained for 1.15% N on day 4 of fermentation which also showed a rapid reduction in productivity after day 4. The other two levels of nitrogen tested showed a lag phase initially but also showed maximum xylanase productivity on day 6.

Hence, increase in nitrogen levels had a significant effect in enzyme productivity. It was also reported that urea stimulated fungal growth when it is made up to 40-50% of total nitrogen in the substrate (Raimbault and Alazard, 1980). There was a

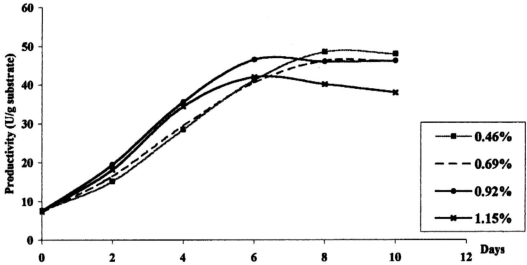


**Figure 5.6: Effect of various level of nitrogen supplementation of substrate on laccase productivity during SSF of OPFPt using *Pyc. sanguineus***

*** SSF condition: 4weeks old inoculum, 30% inoculum density and enzyme extraction using tap water (pH 5.0)**

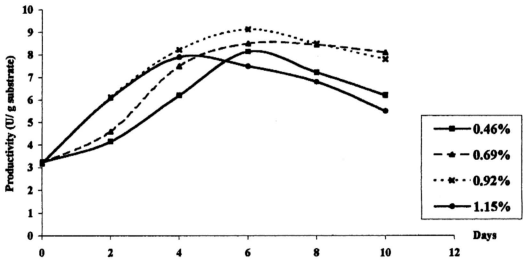


**Figure 5.7: Effect of various level of nitrogen supplementation of substrate on xylanase productivity during SSF of OPFPt using *Pyc. sanguineus***

*** SSF condition : 4 weeks old inoculum, 30% inoculum density and enzyme extraction with tap water (pH 5.0)**

reduction in xylanase and laccase productivity after the peak, and this may be due to inhibition which suggests the existence of an optimum nitrogen level for enzyme production by *Pyc. sanguineus*. Optimum laccase production of 1368 U/L by *Pyc. sanguineus* was reported by Pointing *et al.*, (2000) in a culture condition of high carbon and low nitrogen medium. Slight repression of laccase production was also noticed at high nitrogen levels. Similarly, laccase of *Pyc. cinnabarinus* was also reported to be slightly repressed by high nitrogen levels (24mM) (Eggert *et al.*, 1996). In contrast, laccase activity was only detectable in *Pha. chrysosporium* under high nitrogen level (24mM). This study also showed slight repression in laccase productivity at a high nitrogen level of 1.15%.

## CONCLUSION REMARKS

Based on the enzyme productivity from the series of optimization studies done, solid substrate fermentation of OPFPt by *Pyc. sanguineus* had maximum productivity of laccase of 46.5 U/g substrate and maximum xylanase productivity of 9.1 U/g substrate on day 6 of fermentation using 30% (w/w) of 4 weeks old inoculum and 0.92% of nitrogen content of substrate. Optimization processes had made laccase productivity reach its maximum value of 46.5 U/g substrate much earlier which is at day 6 compared to day 10 of SSF in unoptimized conditions although there was no increase in laccase productivity observed after optimization. Xylanase productivity reached its peak on day 6 of SSF which is an increase of 15% as compared to day 10 of SSF in unoptimized conditions.