CHAPTER FOUR

EFFECT OF (a) NITROGEN CONCENTRATION AND (b) INOCULUM AGE AND DENSITY (W/W) ON LACCASE PRODUCTIVITY DURING SSF OF 'HAMPAS'

INTRODUCTION

Sago *hampas* represents a rich and abundant source of starchy lignocellulose material. It has been shown to be degraded by *Myceliophthora thermophila, Trichoderma harzianum* and *Pleurotus sajor-caju* (Shim, 1992; Vikineswary and Nadaraj, 1992; Kumaran *et al.*, 1997). They reported the presence of appreciable amounts of cellulase, α-amylase, xylanase and laccase during the growth of those organisms and proposed that sago *hampas* can be used as a cheap substrate for enzyme production. Further studies were found to be necessary on optimization of enzyme production using sago *hampas* as substrate.

To harness SSF for increased enzyme production, a good quality and optimum inoculum is necessary (Mudgett, 1986). Too low a density (w/w) may give insufficient inoculum and allow the growth of other undesirable organisms. Too high densities (w/w) may accelerate growth and deplete the substrate of nutrients that is necessary for product formation. The optimization of the inoculum parameters would be a priority investigation in bioconversion of sago 'hampas' for enzyme production.

The general composition of sago *hampas* (Table 4.1) would support good fungal growth, though requiring sufficient nitrogen levels to maximize the enzyme yield.

Table 4.1. Proximate analyses of sago 'hampas'

Component	*% Dry matter*
Dry matter	89.90 ± 0.10
Crude fat	nd
Crude protein	1.15 ± 0.12
Crude fiber	14.45 ± 0.25
Cellulose	71.18 ± 1.32
Lignin	24.79 ± 0.82
Residual ash	5.48 ± 0.24
Acid detergent fiber	25.28 ± 0.51
Total carbon	37.74 ± 0.18
Total nitrogen	0.18 ± 0.02
pH	5.36
C: N ratio	205

nd = not detectable.
All units are in % dry matter except pH and C: N ratio.

Summarised from: Kumaran (1996)

The objectives of this study were to:

1. study the effect of nitrogen concentration on growth and enzyme production in SSF of sago *hampas*.
2. study the effect of inoculum size and age on growth and enzyme production by *Pycnoporus sanguineus*.

MATERIALS AND METHODS

Substrate

Sago 'hampas' obtained as described in (Chapter 3. pp. 47) was used in this experiment.

Inoculum

Pycnoporus sanguineus as prepared (Chapter 3. pp. 47) and was used as the spawn culture in the experiment.

CULTURING CONDITIONS

Solid substrate fermentation cultures were developed in 250 ml conical flasks, each containing about 10 g of sago *hampas* autoclaved at 121° C at 15 psi for 20 minutes. The flasks were cooled overnight and about 50 ml nutrient solution containing 0.2% (w/v) KH_2PO_4 and 0.05% (w/v) $MgSO_4.7H_2O$ was added. The contents of the flask were then thoroughly mixed with a sterile spatula and allowed to stand for 1 h. Each flask was aseptically inoculated with

10% of 14 days old *P. sanguineus* wheat grain inoculum and incubated at 25 ± 2°C in static condition. The fermentation was carried out for 15 days. Triplicate flasks were sampled for analyses on 15 day of incubation. The outline of experimental design for SSF is shown in Table 4.3

Effect of nitrogen concentration on enzyme production during SSF.

Different levels of filter sterilized urea as nitrogen supplement (Table 4.2) and 10% 2 –week old inoculum were added to the contents of the autoclaved flasks.

Table 4.2: Nitrogen levels tested in the experiment

N source	% (w/v)
Urea	0.19
	0.38
	0.57
	0.76

Further, studies of SSF was done using the optimum nitrogen level chosen from this run of SSF.

Effect of inoculum age and size on enzyme production in SSF.

The parameter tested was:

i. Inoculum age: 2- week, 3-week and 4-week old koji.

ii. Size: 5% and 10% of 2- week, 3-week and 4-week old koji.

Table 4.3: Experimental design for the SSF of sago *hampas* with *Pycnoporus sanguineus*.

Inoculum age flask (week)	Inoculum size (% w/w)	% urea (w/v)	No of sample
2	5 10	0.76	3
3	5 10		
4	5 10		

Extraction of crude culture extract

The cultures were extracted as described in Chapter 3 (pp. 51).

Analysis

Enzyme assays were performed in triplicates (using standard methods as described in (Appendix A) and the results are expressed as mean of triplicates.

RESULTS AND DISCUSSIONS

Effect of Nitrogen

The growth of *P.sanguineus* on sago 'hampas' supplemented with different conc. of nitrogen was observed two to three days after incubation. Fungal growth on sago *hampas* was improved by the addition of suitable levels of nitrogen. *Pycnoporus sanguineus* is a slow growing fungus but the growth was observed to be rapid on sago *hampas* supplemented with 0.19%, 0.38%, 0.57% and 0.76% urea. As the culture grew older the color of mycelia changed from white to reddish orange and complete colonization by the fungus was seen within 10 days of fermentation. However, in culture flask supplemented with 0.19% urea (w/v) very little mycelial growth was observed (Plate 4.1). It is presumed that the lower levels of urea supplementation did not support rapid mycelial growth. For fungal growth optimum C: N ratio is pre-requisite.

Plate 4.1. Growth of *Pycnoporus sanguineus* on day 15 of SSF of 'hampas' (A=Control, B=0.19% (w/v) of urea, C=0.38%(w/v) of urea, D=0.57%(w/v) of urea, E=0.76%(w/v) of urea)

pH variation

The pH during SSF of 'hampas' using various levels of nitrogen supplementation is shown in Fig 4.1. (Appendix C. Table 22).

There was not much variation in pH at all the nitrogen levels tested. In the cultures supplemented with 0.19% (w/v) of urea the pH was 4.4. In the cultures supplemented with 0.38% and 0.57% (w/v) of urea the pH ranges from 4.41-4.59 (mean =4.5). In the cultures with 0.76% (w/v) of urea supplementation the pH increased to 4.9.

Different nitrogen content showed significant ($P < 0.05$) effect on the pH of the crude culture extract.

Soluble Protein

The soluble protein content during SSF 'hampas' using various level of nitrogen is shown in Fig 4.2. (Appendix C. Table 23).

The soluble protein content increases with the increase in nitrogen levels. In the culture flasks supplemented with 0.19 % (w/v) of urea the soluble protein content was very low (0.02 mg/ml) while if supplemented with 0.38 % (w/v) of urea the protein production was about 2 times higher. The soluble protein content of cultures supplemented with 0.57 % (w/v) of urea was same as that with 0.38 % (w/v) of urea. The highest soluble protein content of 0.108 mg/ml was recorded in cultures supplemented with 0.76 % (w/v) of urea. This maximum value recorded was 4.6 times higher when compared to the soluble protein content recorded at 0.19% (w/v) Fig 4.3. The maximum soluble protein content

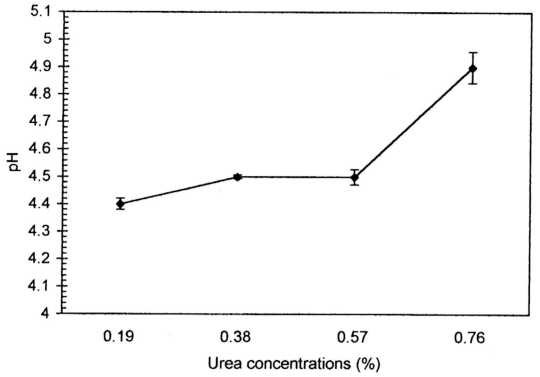

Fig 4.1: pH variation in the crude culture extract using 10% 2-week old inoculum (on 15 day of SSF)

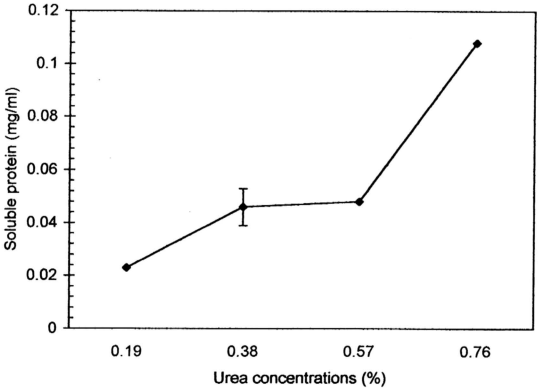

Fig 4.2: Soluble protein content in the crude culture extract using 10% 2-week old inoculum (on day 15 of SSF)

recorded in this study was 1.5 times more when compared to the maximum value of 0.071 mg/ml recorded on day 25 of initial study (Appendix C. Table 1).

The supplementation of sago *hampas* with different nitrogen levels had significant ($P < 0.05$) effects on the production of soluble protein content (Appendix C. Table 1).

Xylanase

The xylanase productivity during SSF 'hampas' using various level of nitrogen supplementation is shown in Fig 4.3. (Appendix C. Table 24).

The xylanase productivity of 3.4 U/g of substrate was recorded in cultures with 0.19 % (w/v) of urea supplementation. In the cultures supplemented with 0.38% (w/v) of urea very little increase in xylanase production was recorded, with a value of 4.9 U/g. The highest xylanase productivity of 6.01 U/g of substrate was recorded in cultures supplemented with 0.57 % (w/v) of urea. This maximum productivity was almost 1.8 times more when compared to the value recorded at 0.19 % (w/v) of urea. But there was a sharp decline in xylanase productivity from 0.76% (w/v), which dropped to 2.82 U/g of substrate. This may be due to inhibition caused by higher concentration of urea, which suggested the existence of an optimum nitrogen level for enzyme production by *Pycnoporus sanguineus*. Urea has been reported to strongly inhibit an endoxylanase from *F. avenaceum* (Zalewska and Urbanek 1981), Repression in laccase activity has also been reported in fermentation with high carbon and high nitrogen growth medium (Pointing *et al.*, 2000). Further, maximum xylanase productivity of 9.12

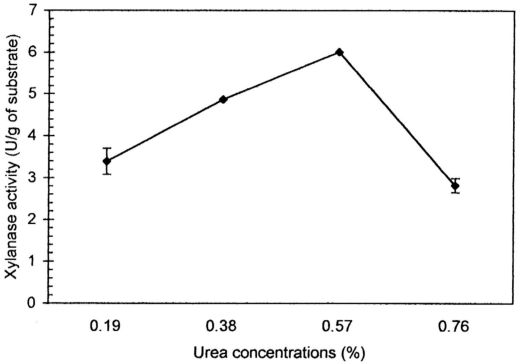

Fig 4.3: Xylanase activity of the crude culture extract using 10% 2-week old inoculum (on 15 day of SSF)

U/g of substrate was recorded on day 6 during SSF of OPFPt using 4-week old 30% *Pycnoporus sanguineus* inoculum (Renuvathani, 2002)

Different levels of nitrogen supplementation had significant ($P < 0.05$) effects on the xylanase productivity. (Appendix C. Table 6).

Lignin peroxidase

The results of the effect of nitrogen levels on the lignin peroxidase productivity are given in Fig 4.4. (Appendix C. Table 25). For all the nitrogen levels tested lignin peroxidase productivity was low reaching a maximum of 1.05 U/g of substrate at 0.57% (w/v) of urea.

There was no significant ($P > 0.05$) effect for all nitrogen levels tested on the lignin peroxidase activity in the crude culture extract (Appendix C. Table 7).

Laccase

The laccase productivity during SSF 'hampas' using various level of nitrogen supplementation is shown in Fig 4.5. (Appendix C. Table 26).

The laccase productivity of 0.65U/g of substrate was recorded at 0.19% (w/v) of urea. Rapid increase in laccase productivity was observed in cultures supplemented with 0.38% (w/v) of urea with a laccase productivity of 14.35 U/g of substrate. The increase in laccase productivity was 22 times higher when compared to the activity recorded at 0.19% (w/v) of urea. However, there was not much increase in the laccase productivity when 0.57% and 0.76% (w/v) of urea was supplied in the cultures.

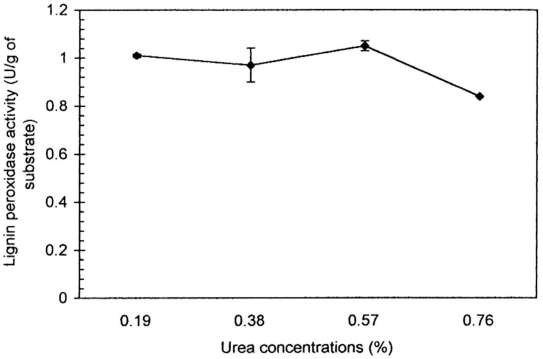

Fig 4.4: Lignin peroxidase activity of the crude culture extract using 10% 2-week old inoculum (on 15 day of SSF)

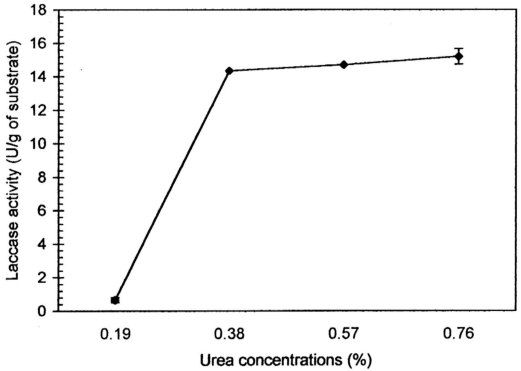

Fig 4.5: Laccase activity in the crude culture extract using 10% 2-week old inoculum (on 15 day of SSF)

The laccase productivity reached a maximum of 15.20 U/g of substrate in the cultures supplemented with 0.76% (w/v) of urea. This value was 1.1 times higher when compared to the maximum laccase productivity of 13.92 U/g of substrate recorded by Ling (1994) on day 5 of SSF of OPFPt using 3-week old 10% *Pleurotus sajor-caju* inoculum. However, maximum laccase activity of 46.5 U/g of OPFPt was recorded by Renuvathani (2002) on day 6 of SSF of using 4-week old 30% *Pycnoporus sanguineus* inoculum.

The different nitrogen levels tested had significant ($P < 0.05$) effects on the laccase productivity. (Appendix C. Table 8).

Effect of Inoculum Age and Density (w/w)

pH variation

The pH values during SSF of hampas using 2, 3 and 4-week old inoculum are shown in Fig 4.6. (Appendix C. Table 27). Statistical analysis revealed that there was no significant effect on the pH when 2, 3 and 4-week old inoculum ages were used at 5% and 10% inoculum densities (w/w). The pH changes during SSF of 'hampas' using three inoculum ranges from pH 4.7-pH 5.1. A maximum pH of 5.1 was recorded in extracts using 10% 2-week old inoculum.

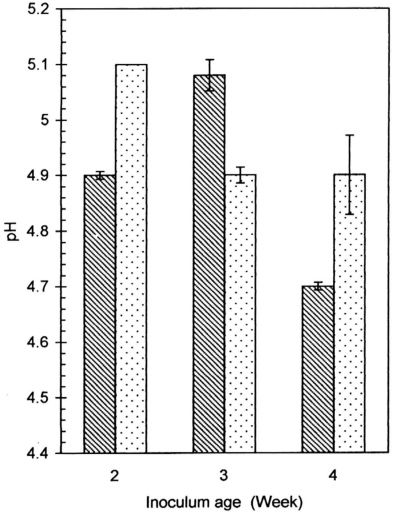

Fig 4.6 : Variation of pH in crude culture extract during SSF of sago 'hampas' with *P. sanguineus* (on day 15 of SSF)

Soluble Protein

The soluble protein values of the crude culture extract using the 2-week, 3-week and 4-week old inoculum are shown in Fig 4.7. (Appendix C. Table 28). There was no significant effect for the 5% inoculum density (w/w) of the three-inoculum ages tested. However, a significant ($P < 0.05$) effect in the soluble protein content of the 10% inoculum density (w/w) of the three-inoculum ages was recorded (Appendix C. Table 9).

With the 2 – week old 10% inoculum the soluble protein content recorded with a value of 0.12 mg/ml. The soluble protein content of the 4-week old 10% inoculum was 0.160 mg/ml. The soluble protein content of 0.211 mg/ml in the crude culture extract using 3-week old 10% inoculum was 1.8 times and 1.3 times higher than in extracts using a 2-week and 3-week old 10% inoculum. The max of 0.211mg/ml soluble protein content recorded in this study was two times higher when compared to the 2-week old inoculum of the same density (w/w). However, this soluble protein content obtained was less when compared to the soluble protein content of 4.21 mg/g obtained with a 30% 4-week old *Pleurotus sajor-caju* inoculum during SSF of *hampas* on day 8 (Kumaran, 1996).

Xylanase

The result of xylanase productivity of *Pycnoporus sanguineus* during the SSF of 'hampas' is shown in Fig 4.8. (Appendix C. Table 29).

Xylanase productivity with 10% 3-week old inoculum was higher when compared to the productivity obtained with 2-week and 4-week old inoculum of the same level tested. With a 10% (w/w) of the 3-week old inoculum maximum

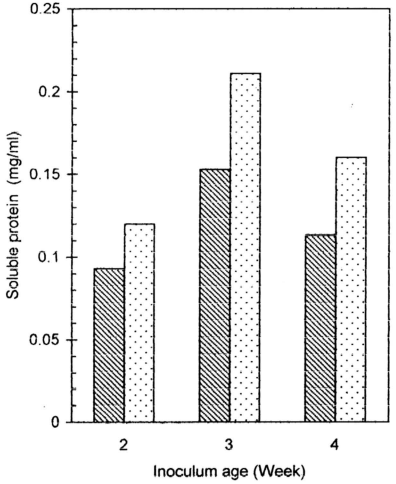

Fig 4.7 : Soluble protein content in crude culture extract during SSF of sago 'hampas' with *P. sanguineus* (on day 15 of SSF)

xylanase productivity of 5.06 U/g of substrate was obtained on day 15 of fermentation. This maximum value recorded was almost 1.7 times higher when compared to the xylanase productivity obtained with a 10% 2-week old inoculum. However, the maximum xylanase productivity of 8.5 U/g of substrate was recorded during SSF of OPFPt on day 6 with a 30% 4-week old inoculum of the same species (Renuvathani, 2002). Kumaran (1996) using a 30% 4-week old *Pleurotus sajor-caju* inoculum recorded a maximum xylanase productivity of 13.5 U/g of substrate during SSF of sago *hampas* on day 4.

The inoculum age and size significantly ($P < 0.05$) influenced xylanase productivity of *Pycnoporus sanguineus* during SSF of 'hampas' (Appendix C. Table 10).

Lignin peroxidase

The result of lignin peroxidase productivity of *Pycnoporus sanguineus* during the SSF of 'hampas' is shown in Fig 4.9 (Appendix C. Table 30). The ANOVA test revealed that there was no significant ($P > 0.05$) difference in the lignin peroxidase productivity by the three-inoculum levels tested on day 15 of SSF (Appendix C. Table 11) Generally with a 10% 3-week old inoculum density (w/w) the lignin peroxidase productivity was a maximum with a value of 1.77 U/g of substrate when compared to value recorded with a 10% 2-week and and 4-week old inoculum. This maximum lignin peroxidase productivity was 1.8 times higher when compared to value of 0.93 U/g of substrate recorded with a 10% 2-week old inoculum tested.

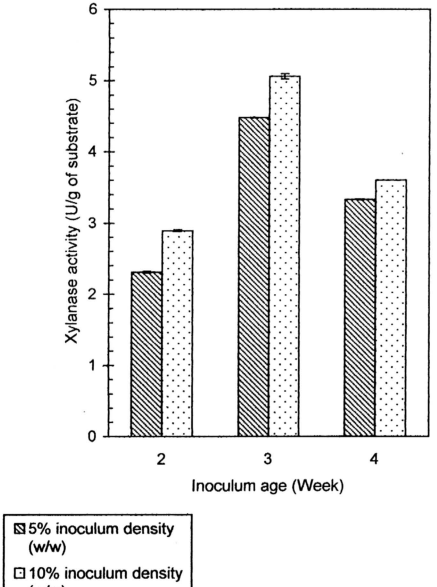

Fig 4.8 : Xylanase activity in crude culture extract during SSF of sago 'hampas' with *P. sanguineus* (on day 15 of SSF)

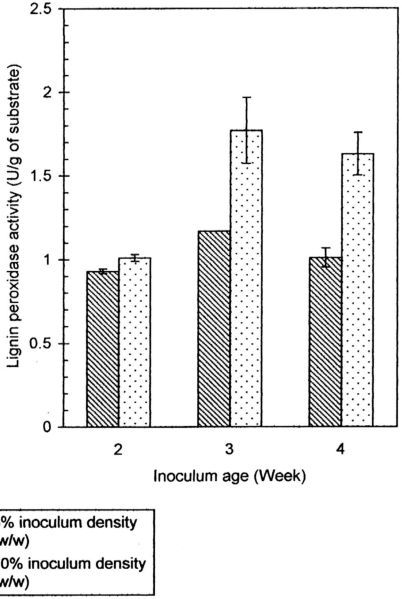

Fig 4.9 : Lignin peroxidase activity in crude culture extract during SSF of sago 'hampas' with *P. sanguineus* (on day 15 of SSF)

Laccase

The result of laccase productivity of *Pycnoporus sanguineus* during the SSF of 'hampas' is shown in Fig. 4.10. (Appendix C. Table 31).

With the 3-week old inoculum *Pycnoporus sanguineus* produced maximum laccase productivity with both the levels of inoculum densities (w/w) tested compared to the 2-week and 4-week old inoculum. The laccase productivity of 17.30 U/g of substrate was recorded with a 5% 3-week old inoculum. This productivity was 1.6 times higher when compared to the value obtained with 2-week old inoculum with same density. With the 3-week old inoculum at 10% inoculum density (w/w) the laccase productivity of 18.05 U/g of substrate was recorded. This productivity was approx.1.2 times higher when compared to the value obtained with 2-week old inoculum with same inoculum density (w/w).

The maximum laccase productivity recorded in this study was also more when compared to the maximum laccase productivity of 17.7 U/g of substrate recorded with a 10% 4-week old *Pleurotus sajor-caju* inoculum after 6 days of SSF of same substrate (Kumaran, 1996). However, *Pycnoporus sanguineus* produced a maximum laccase productivity of 46.6 U/g of substrate on day 10 during SSF of OPFPt (Renuvathani 2002).

The inoculum age and size significantly ($P < 0.05$) influenced laccase-productivity of *Pycnoporus sanguineus* during SSF of 'hampas' (Appendix C. Table 12)

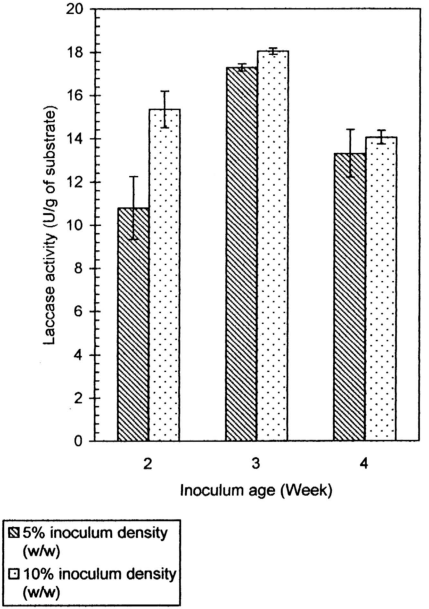

Fig 4.10 : Laccase activity in crude culture extract during SSF of sago 'hampas' with *P. sanguineus* (on day 15 of SSF)

CONCLUSION

Sago 'hampas' supplemented with 0.76% (w/v) of urea supported good fungal growth. Nitrogen concentration had significant effect ($P > 0.05$) on all the enzymes produced by *P . sanguineus* during SSF in the present study. Xylanase and lignin peroxidase productivity were higher at 0.57% (w/v) of urea. Productivity of these two enzymes, however, was repressed with 0.76% (w/v) of urea. The nitrogen concentration had significant effect ($P > 0.05$) on laccase productivity, too. Among the nitrogen levels tested a sharp increase in the laccase productivity was recorded at 0.38% (w/v) of urea. The highest laccase productivity of 15.2 U/g of substrate was recorded at 0.76% (w/v) of urea. This activity was 24 times higher compared to the activity of 0.65 U/g. of substrate with 0.19% (w/v) of urea.

In this respect, our results are in agreement with earlier findings that reported high levels of laccase productivity of *Rigidoporus lignosus* (Galliano *et al.*, 1991), *Ceriporiopsis subvermispora* (Lobos *et al.*, 1994), *Lentinula edodes* (Buswell *et al.*, 1995) and *Agaricus biosporus* (Perry *et al.*, 1993) when grown in nitrogen rich conditions.

Laccase activity increased with the increase in urea concentration. The significant effect of nitrogen supplementation was due to the suitable C: N ratio of supplemented 'hampas'. The amounts of nitrogen that occur naturally in 'hampas' are less then optimal for *P. sanguineus* growth. Adequate supplementation with 0.76% (w/v) of urea as nitrogen to adjust the C: N ratio in the present study was found suitable for maximum laccase production. In this study, due to facility constraints the C: N ratio was not monitored.

Using the optimum nitrogen level chosen from the above study soluble protein content lignin peroxidase and xylanase activities with 3 inoculum densities (w/w) were in the same order of:

3 week >4 week >2 week

while, the laccase activities with the 3 inoculum densities (w/w) were in the order of:

3 week > 2 week > 4 week

It was concluded that the 2- week old inoculum may be immature and 4-week-old inoculum may be too old. Thus according to the results obtained it was found that a 3 - week old 10% inoculum was favorable for maximum enzyme production. Laccase was the most prominent enzyme produced in this study. Maximum laccase activity of 18.05 U/g was recorded with 10% inoculum of 3week old culture on day 15 of incubation. Further studies for increased laccase production was done using these optimized parameters.