Malaysian Journal of Science **22**: 35-42 (2003)

Optimization of laccase productivity during solid substrate fermentation of sago *hampas* by *Pycnoporus sanguineus*

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ABSTRACT *Pycnoporus sanguineus* showed good growth during solid substrate fermentation (SSF) of sago pith residue known as *hampas*, which was supplemented with 0.38% (w/v) (63.3mM) urea as nitrogen source, 0.2% (w/v) KH_2PO_4 and 0.05% (w/v) $MgSO_4.7H_2O$, over a period of 30 days. In unoptimized conditions using a two-week old, 10% (w/w) inoculum, maximum laccase productivity of 14.35 U/g of substrate was obtained on day 15. The productivity of laccase increased to 15.20 U/g of substrate when sago *hampas* was supplemented with 0.76% (w/v) (126.7mM) of urea. Further, laccase productivity was influenced by the inoculum age and density (weight of inoculum/weight of substrate). With a 3-week old inoculum at 10% density (w/w) the laccase productivity increased to 18.05 U/g of substrate after 15 days of SSF. This is an increase of 1.3 fold compared to the unoptimized value. The maximum laccase productivity obtained with the optimized conditions was 18.35 U/g of substrate on day 10 of SSF. A pH range of 4.0 – 5.0 seems to be suitable for the SSF. Laccase productivity was growth associated which correlated well with the soluble protein content measured during the course of SSF.

ABSTRAK *Pycnoporus sanguineus* tumbuh dengan baik semasa penapaian substrat pepejal (SSF) menggunakan sisa empular sago yang dipanggil hampas dan ditambah dengan 0.38% (w/v) (63.3mM) urea sebagai sumber nitrogen, 0.2% (w/v) KH_2PO_4 dan 0.05% (w/v) $MgSO_4.7H_2O$, selama 30 hari. Dalam keadaan tidak optimum, menggunakan inokulum yang berumur 2 minggu dan 10% (w/w) inokulum, penghasilan maksimum lakase sebanyak 14.35 U/g substrat berlaku pada hari ke 15. Pengeluaran lakase meningkat kepada 15.20 U/g substrat apabila hampas sago ditambah dengan 0.76% (w/v) (126.7 mM) urea. Pengeluaran lakase meningkat kepada 18.05 U/g substrat selepas 15 hari SSF dengan menggunakan inokulum berumur 3 minggu pada kepadatan 10%. Ini merupakan peningkatan 1.3 kali ganda berbanding dengan nilai lakase semasa penapaian dalam keadaan tidak optimum. Pengeluaran maksimum lakase dalam keadaan optimum ialah 18.35 U/g substrat pada hari ke 10 SSF. Julat pH dari 4.0 –5.0 adalah sesuai untuk SSF. Pengeluaran lakase mempunyai kaitan dengan pertumbuhan dan mempunyai korelasi dengan protein terlarut yang diukur sepanjang penapaian (SSF).

(Laccase, white-rot fungus, solid substrate fermentation, sago *hampas*)

INTRODUCTION

Lignocellulosic waste represents a huge amount of renewable resource for the production of paper products, feeds, chemicals and fuels. Therefore, there has been an increasing emphasis on the research of fungal degradation of lignin [1]. White-rot fungi are believed to be the most effective lignin-degrading microbes in nature. The majority of previous studies have focused on the lignin-degrading enzymes of *Phanerochaete chrysosporium* and *Trametes versicolor* (2). Recently, there has been a growing interest in studying the lignin-modifying enzymes of a wide variety of white-rot fungi, not only from the stand point of comparative biology but also with the expectation of finding better lignin-degrading systems for use in various biotechnological applications [2, 3].

Several studies have been done on the utilization of agro-residues to produce enzymes. The sago

starch processing industry produces three major types of by-products, viz bark of sago trunk, fibrous pith residue, commonly known as *hampas* and wastewater. As of date, utilization of *hampas* for enzyme production through solid substrate fermentation (SSF) has been reported by Vikineswary and Shim [4] and Kumaran *et al.*[5]. Exploitation of lignocellulosic material must be such that all, if not most of their major components, viz cellulose, hemicellulose and lignin are utilized.

Further, fungal enzymes and in particular lignin-modifying enzymes (LME) have gained attention in the field of waste management because of their ability to breakdown organic pollutants, recalcitrant wastes and bleach plant effluents [6]. Laccase (EC 1.10.3.2), an enzyme present in many white-rot fungi, catalyzes the oxidation of both phenolic and non-phenolic compounds [7]. It has been studied and identified as having an important role in fungal biotreatment.

Pycnoporus sanguineus (Fr.) Karst, a white-rot fungus, has been reported to produce laccase as sole ligninolytic enzyme in defined liquid growth medium and has dye decolorization potential [6]. The aim of this study was to investigate the growth and laccase productivity of *Pyc. sanguineus* in solid substrate fermentation of sago *hampas*. The effect of nitrogen supplementation and inoculum age as well as density on laccase production was studied. The productivity of laccase in this medium was optimized.

MATERIALS AND METHODS

Fungal strain

Pycnoporus sanguineus strain CY788 (courtesy of Professor Gareth Jones, BIOTEC, Thailand) was maintained on Potato Dextrose Agar (PDA-Difco) slants at 4 $\pm$ 2° C.

Substrate

Sago *hampas* was collected from Hup Guan Sago factory in Johor Darul Takzim, Malaysia. The substrate was air-dried and sieved through a 2.0mm sieve and stored at 28 $\pm$ 2 ° C prior to use.

Inoculum preparation

The *koji* of *Pyc. sanguineus* was prepared in autoclaved wheat grains inoculated with five plugs of 0.5mm diameter of mycelia from a seven day old culture on PDA plates (5).

Solid Substrate Fermentation (SSF)

Solid substrate fermentation was developed in 250 mL conical flasks. Each flask containing 10g of sago *hampas* was autoclaved at 121 ° C at 15 psi for 20 min. Filter sterilized urea as nitrogen supplement at 0.38% (w/v) (63.3mM) and 50mL nutrient solution containing 0.2% (w/v) KH_2PO_4 and 0.05% (w/v) $MgSO_4.7H_2O$ was added to the contents of the autoclaved flasks. The contents of the flask were then thoroughly mixed with a sterile spatula and allowed to stand for one hour (5). Each flask was then aseptically inoculated with 10% (w/w) of a 2 weeks old *Pycsanguineus* wheat grain inoculum and incubated at 25 $\pm$ 2 ° C in dark and in static condition. The flasks were set up in triplicates for each sampling day and fermentation was carried out for 30 days. The SSF included flasks with uninoculated *hampas* which were analyzed as controls.

Effect of nitrogen concentration on laccase productivity during SSF

As sago *hampas* was found to be deficient in nitrogen (5), the effect of different levels of 0.19% (w/v) (31.6mM), 0.38% (w/v) (63.3mM), 0.57% (w/v) (95mM) and 0.76% (w/v) (126.7mM) filter sterilized urea as nitrogen supplement on laccase production was studied. Ten percent of a two weeks old inoculum was added to the contents of the autoclaved flasks. Fermentation was carried out at 25 $\pm$ 2 ° C and triplicate flasks were sampled on day 15 of incubation.

Effect of inoculum age and density on laccase productivity in SSF

The effect of inoculum age:- 2-week, 3-week and 4-week old *koji* and inoculum density of 5% (w/w) and10% (w/w) of each inoculum age on laccase production was studied. The fermentation was carried out as described above at 25 $\pm$ 2 ° C. Triplicate flasks were sampled for analysis on day 15 of incubation.

Solid substrate fermentation with optimum conditions

SSF was carried out as described above except with the following changes:

(i) Inoculum age: 10% (w/w) 3-week old *koji* culture
(ii) Nitrogen level: filter sterilized urea at 0.76% (w/v) (126.7mM)
(iii) Fermentation period: 20 days Triplicate flasks were sampled on day 0, 5, 10, 15 and 20.

Extraction
The contents of each flask were extracted with 200 mL of tap water at pH 4.0 [8]. The solid culture was broken down into smaller particles and then homogenized at 8000 rpm for 8 min at 28 $\pm$ 2 ° C. The contents of each flask were then filtered through a double-layer of nylon cloth. The crude filtrate containing the fungal enzyme was stored in 1.5 mL microfuge tubes at –20°C for 24 hours prior to enzyme assay. Assays were performed in triplicates and the results for all values were expressed as a mean of triplicate values.

Analytical techniques

pH
The pH of the culture extract was measured using a digital pH meter.

Protein
The extracellular soluble protein was quantified using the dye-binding method of Bradford [9] with crystalline bovine albumin as standard.

Laccase Assay
Laccase activity was assayed by the increase in absorbance due to the formation of the tetramethoxy-azo-bis-methylenequinone resulting from the reaction of laccase with syringaldazine [10, 11]. The initial rate of colour change was measured spectrophotometrically at 525 nm. One unit of activity was defined as the enzyme producing one unit of absorbance change / min. In this study, laccase productivity was expressed as unit (U) / g of substrate.

Statistical Analysis
Analysis of variance was done with data of day 10 when maximum laccase activity was recorded to compare the fermentation profile of SSF after optimization with the fermentation profile before optimization.

RESULTS AND DISCUSSION

Solid Substrate Fermentation (SSF) (Unoptimized)
Good growth of *Pyc. sanguineus* was observed on sago *hampas*. The first sign of growth was seen two to three days after inoculation. As the culture grew older the colour of mycelia changed from white to reddish orange. By 10 to 11 days of fermentation complete colonization by the fungus was observed.

The initial pH of the supplemented culture on day 0 was 4.09 Figure 1a. There was not much variation of the pH in the crude culture extract during the fermentation period. The pH range of 3.9 – 5.0 (mean = 4.4) was almost similar to that reported by Kumaran *et al.*(5) during the SSF of sago *hampas* using *Pleurotus sajor-caju.*

The fungal metabolism during growth might have caused the hydrolysis of urea and the liberation of ammonium ions leading to an increase in pH values as seen in the first 15 days of SSF. As the growth of fungus and fermentation continued ammonium uptake might have exceeded the hydrolysis rate of urea and the pH dropped. Changes in pH were considerably smaller after 20 days of fermentation. The decrease in pH of the substrate may be correlated directly with the decomposition activity of the fungus [12]. In the present experiment, a pH range between 4 and 5 was found to be suitable for the growth of *Pyc. sanguineus* on *hampas.*

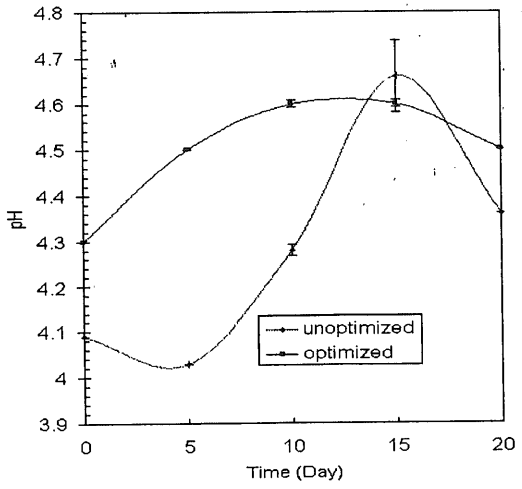

Figure 1a. Variation of pH in crude culture extract during SSF of sago *hampas* with *P. sanguineus.*

The concentration of soluble protein of the day 0 sample was 0.002mg/mL and the extractable protein reached 0.065mg/mL after the first 15 days of fermentation Figure 1b. The increase in protein content in the culture extract was not only due to the secretion of laccase but also the other extracellular enzymes responsible for the degradation of *hampas* [13]. On day 20 the soluble protein content dropped to 0.044mg/mL. The soluble protein content can be used to relate to the growth of fungus. The soluble protein content was used as an indirect assessment of fungal biomass [14]. Ling *et al* (15) reported that changes in soluble protein correlated with the morphogenesis of the fungus. After 20-25 days of fermentation by *Ple. sajor-caju* it was observed that primordia were formed. Protein is required for this phase of development [15].

Laccase productivity during SSF increased rapidly during the initial 15 days as shown in Figure 1c. Laccase productivity was 14.35 U/g (14350 U/kg) of substrate on day 15. The increase in laccase productivity in the initial stages of incubation showed that laccase productivity was growth associated. The laccase productivity of 14.35 U/g of substrate was about 1.4 fold compared to the maximum value of 10.6 U/g produced on day 9 of SSF of *hampas* with *Ple. sajor-caju* (5). Further, the laccase productivity of 14.35 U/g of substrate was 1.9 fold that of the maximum laccase productivity of 7.6 U/g produced on day 11 by the same strain during unoptimized SSF of oil palm frond parenchyma tissue (OPFPt) [16].

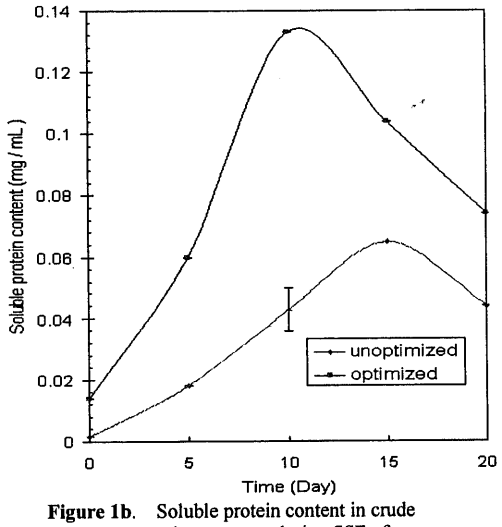

Figure 1b. Soluble protein content in crude culture extract during SSF of sago *hampas* with *Pyc. sanguineus.*

However, a rapid decline in laccase productivity was observed after day 15 of SSF of *hampas.* The activity was reduced to 5.85 U/g of substrate at the end of 30 days of fermentation. The loss in productivity might be due to the inhibition of enzyme synthesis or enzyme degradation [17].

This strain has been reported to produce 1368 U/L of laccase in a defined liquid growth medium containing inducers [18]. Further, Eggert *et al* [19] had reported that *Pyc. cinnabarinus* growing in liquid culture produced 9600 U/L of laccase. Thus, *Pyc. sanguineus* not only grew well during SSF of sago *hampas* but produced large quantities of laccase during the growth.

Effect of nitrogen concentration on laccase production during SSF

The growth of *Pyc. sanguineus* on sago *hampas* supplemented with different concentrations 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. *Pyc. sanguineus*, a slow growing fungus, was observed to grow rapidly on sago *hampas* supplemented with 0.38% (w/v) (63.3mM), 0.57% (w/v) (95mM) and 0.76% (w/v) (126.7mM) urea. However, in culture flasks supplemented with 0.19% (w/v) (31.6mM) urea very little mycelial growth was observed. It was presumed that the lower levels of urea supplementation did not support rapid mycelial growth. For fungal growth, optimum C: N ratio is a pre-requisite. *Pyc. cinnabarinus* showed optimum growth and laccase production in a liquid medium with a C : N ratio of about 15 (3g of glucose per liter, 2.4mM ($(NH_4)_2$ SO_4) (19). The amounts of nitrogen that occur naturally in *hampas* were less than optimal for growth of *Pyc. sanguineus.* Adequate supplementation with 0.76% (w/v) (126.7mM) of urea as nitrogen in the present study was found suitable for maximum laccase production. Due to facility constraints the C: N ratio of the supplemented substrate as well as the C: N ratio change during SSF was not monitored. In our laboratory, urea has been used as the preferred nitrogen source for this fungus. Growth on other nitrogen sources such as ammonium and nitrate was not determined.

There was not much variation in pH at all the nitrogen levels tested. In the supplemented cultures the pH ranged from 4.5 to 4.9 (Table 1). However, the different nitrogen levels tested had

a significant ($P < 0.05$) effect on the pH of the crude culture extract. The soluble protein content increased with the increase in nitrogen levels. In the culture flasks supplemented with 0.19% (w/v) (31.6mM) of urea the soluble protein content was very low (0.02mg/mL) while when supplemented with 0.38% (w/v) (63.3mM) of urea the protein content increased by twofold. The soluble protein content of cultures supplemented with 0.57% (w/v) (95mM) of urea was about the same as that with 0.38% (w/v) (63.3mM) of urea. The highest soluble protein content of 0.108 mg/mL was recorded in cultures supplemented with 0.76% (w/v) (126.7mM) of urea (Table 1). This maximum value recorded was 4.6 fold that of the soluble protein recorded at 0.19% (w/v) (31.6mM). Further, the maximum soluble protein content recorded in this study was 1.5 fold more when compared to the maximum value of 0.071 mg/mL recorded on day 25 of unoptimized study. The supplementation of sago *hampas*with different nitrogen levels had a significant ($P < 0.05$) effect on the production of soluble protein content and thus the enzyme concentration.

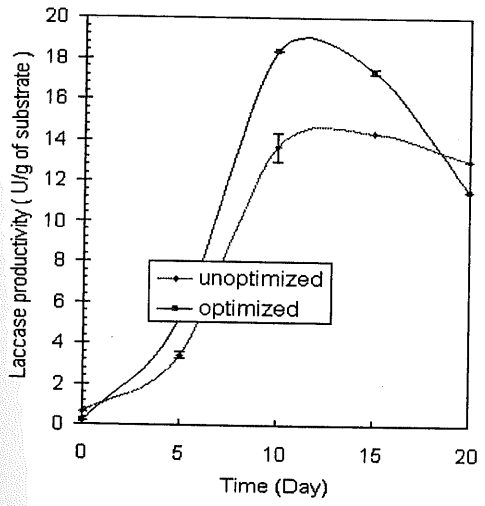

Figure 1c. Laccase productivity in crude culture extract during SSF of sago '*hampas*' with *Pyc. sanguineus.*

The laccase productivity during SSF of sago *hampas* using various levels of nitrogen supplementation is shown in (Table 1). The laccase productivity of 0.65 U/g of substrate was recorded at 0.19% (w/v) (31.6mM) of urea. Rapid increase in laccase productivity was observed in cultures supplemented with 0.38% (w/v) (63.3mM) of urea with a laccase productivity of 14.35 U/g of substrate. The increase in laccase productivity was 22 fold that of the productivity at 0.19% (w/v) (31.6mM) of urea. However, there was not much increase in the laccase productivity when 0.57% (95mM) and 0.76% (w/v) (126.7mM) of urea was supplemented in the cultures. 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 was 24 fold that of the productivity at 0.19% (w/v) (31.6mM) of urea. The different nitrogen levels tested had a significant ($P < 0.05$) effect on the laccase productivity.

In this respect, the results are in agreement with earlier findings that reported high levels of laccase productivity of *Rigidisporus lignosus* [20], *Ceriporiopsis subvermispora* [21], *Lentinula edodes* [22] and *Agaricus bisporus* [23] when grown in nitrogen rich conditions.

Effect of inoculum age and density on laccase production during SSF

Statistical analysis revealed that there was no significant effect on the pH when 2, 3 and 4-week old inoculum were used at 5% and 10% inoculum densities (w/w). The pH changes during SSF of *hampas* using the three inoculum range from 4.7-5.1. A maximum pH of 5.1 was recorded in extracts using 10% 2-week old inoculum [13].

There was no significant effect on soluble protein content for the 5% inoculum density (w/w) of the three inoculum ages tested. However, a significant ($P < 0.05$) effect on the soluble protein content of the 10% inoculum density (w/w) of the three inoculum ages was recorded. With the 2-week old 10% inoculum the soluble protein recorded was 0.12 mg/mL. The soluble protein content of 0.21mg/mL in the crude culture extract using 3-week old 10% inoculum was 1.8 fold and 1.3 fold more than in extracts using a 2-week and 4-week old 10% inoculum [13]. However, this soluble protein content obtained was less when compared to the soluble protein content of 4.21 mg/mL obtained with a 30% 4-week old *Ple. sajor-caju* inoculum during SSF of *hampas* on day 8 [5].

The results of laccase productivity of *Pyc. sanguineus* during the SSF of *hampas* are shown in Figure 2. With the 3-week old inoculum *Pyc.*

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 fold that of 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 was recorded. This productivity was approximately 1.2 fold that of the value obtained with the 2-week old inoculum with same inoculum density.

Table 1. Effect of nitrogen concentration on pH, soluble protein content and laccase production during SSF of *hampas* by *Pyc. sanguineus*.

Nitrogen (Urea)concentration (% w/v) (mM)	pH	Soluble protein content (mg/mL)	Laccase productivity (U/g)
0.19% (31.6mM)	4.4 ± 0.021	0.023 ± 0.000	0.65 ± 0.14
0.38% (63.3mM)	4.5 ± 0.007	0.046 ± 0.007	14.35 ± 0.00
0.57% (95mM)	4.5 ± 0.028	0.048 ± 0.000	14.70 ± 0.00
0.76% (126.7mM)	4.9 ± 0.057	0.108 ± 0.000	15.20 ± 0.46

The maximum laccase productivity recorded in this study was also more when compared to that of 17.7 U/g recorded with a 10% 4-week old *Ple. sajor-caju* inoculum after 6 days of SSF of *hampas*. However, *Pyc. sanguineus* has been reported to produce maximum laccase productivity of 46.5 U/g of OPFPt on day 6 of SSF using a 4-week old 30% (w/w) inoculum [16].The inoculum age and density significantly ($P < 0.05$) influenced laccase productivity of *Pyc. sanguineus* during SSF of *hampas*.

At the optimum nitrogen level of 0.76% (w/v) (126.7mM), the laccase productivities with the 3 inoculum ages at 10% (w/w) inoculum density were in the order of:

3 week old > 2 week old > 4 week old

It was concluded that a 2-week old inoculum (early exponential phase) may be immature and 4-week old inoculum (late exponential phase) may be too old. Maximum laccase productivity of 18.05 U/g was recorded with 10% (w/w) inoculum of a 3-week old culture after 15 days of SSF.

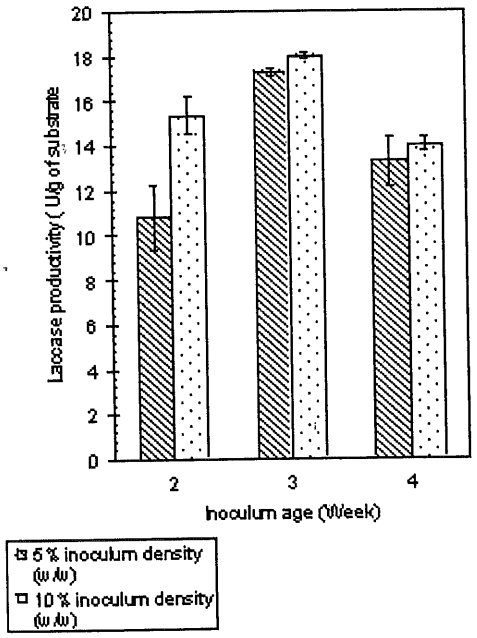

Figure 2. Laccase productivity in crude culture extract during SSF of sago *hampas* using different inoculum age and density of *Pyc.sanguineus* (on day 15 of SSF).

Solid substrate fermentation with optimum conditions

The initial value of pH of the substrate was 4.3 Figure 1a. There was a similar trend of gradual rise in pH which peaked at 4.6 on day 10 of fermentation compared to the same maximum pH on day 15 in unoptimized conditions. Thus, optimization had a significant ($P < 0.05$) effect on pH during SSF.

Freitag and Morell [24] studying enzyme activities of a white-rot fungus reported that the complex enzyme systems involved in the substrate degradation could be responsible for maintaining the pH of the substrate at a suitable range for fungal colonization. At the end of the fermentation a pH of 4.5 was recorded which was not significantly different from that recorded in the unoptimized study on day 20 of fermentation.

The soluble protein content was significantly ($P < 0.05$) affected under optimized parameters. Gradual rise in soluble protein content during the first 10 days of SSF was observed Figure 1b. The peak in soluble protein content was observed on day 10 whereas in the unoptimized fermentation it was observed on day 5. The maximum soluble protein content of 0.13 mg/mL was a 1.9 fold increase of that obtained on day 25 in the unoptimized study. There was, however, a decline in soluble protein content from day 10 onwards to 0.074 mg/mL at the end of fermentation Figure 1b. It was thus shown that optimized parameters had a significant ($P < 0.05$) effect on soluble protein and higher levels of soluble protein were produced.

The laccase profile of *Pyc. sanguineus* in SSF cultures of sago *hampas* are shown in Figure 1c. The optimized parameters had a significant effect ($P < 0.05$) on the productivity of laccase. Laccase productivity increased more rapidly during the SSF and peaked with a value of 18.35 U/g compared to the maximum value of 14.35 U/g of substrate recorded in the unoptimized study. The maximum laccase productivity had increased by approximately 1.3 fold in optimized conditions. Further, the peak in laccase productivity was observed much earlier in optimized conditions that is, at day 10 as compared to day 15 in unoptimized conditions. There was a rapid decline in the laccase productivity towards the end of optimized fermentation.

A general trend was seen where the enzyme productivity increased rapidly and peaked and then decreased slightly towards the end of the fermentation. Prolonging the SSF period did not result in any laccase increase. The laccase profile followed that of soluble protein content where the maximum was recorded on day 10 of fermentation. Laccase has been reported to be produced constitutively during primary metabolism by *Pyc. cinnabarinus* [19]. In this study it was found that the 10% (w/w) 3-week old inoculum density with 0.76% (w/v) (126.7mM) urea was optimum for laccase productivity during SSF of *hampas*.

To our knowledge, this is the first report of laccase production in SSF of sago *hampas* by *Pyc. sanguineus*. To date there has been no report of toxicity of *Pyc. sanguineus* and the *hampas* spent substrate may be considered for animal feed or as organic fertilizer.

Acknowledgements The authors wish to thank MOSTE (grant 09-02-03-0675) and University of Malaya for the Vote F.

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