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Extracellular enzyme production during anamorphic growth in the edible mushroom, *Pleurotus sajor-caju*

Y.H. Tan* and M.N. Wahab

Cultivation of mushrooms on lignocellulosic wastes represents a cost-effective organic recycling process. *Pleurotus sajor-caju* grown on cotton-waste produced relatively low levels of three components of the cellulase complex namely cellobiohydrolase (EC 3.2.1.91), CMCase (EC 3.2.1.4) and *β*-glucosidase (EC 3.2.1.21) with specific activity values of 10.0, 71.4 and 21.6 U (mg protein)$^{-1}$ respectively after 15 days. Higher specific activity was registered in alkali-treated cotton with 15.6, 83.4 and 56.1 U (mg protein)$^{-1}$ respectively after 20 days. Lower levels were noted on rubber-tree sawdust substrate with specific activity values of 0.28, 0.62 and 0.75 U (mg protein)$^{-1}$ for the respective enzymes after 28–35 days growth. The maximum production of xylanase (EC 3.2.1.8) of 0.63 U (mg protein)$^{-1}$ occurred after 20 days while a relatively higher level of the phenoloxidase enzyme, laccase (EC 1.14.18.1) of 27.4 U (mg protein)$^{-1}$ (maximum) was found after 35 days. Laccase, the activity of which is associated with morphogenesis, increased with mycelial growth, peaked at maximum growth and thereafter decreased rapidly. This could prove important commercially in timing the end of spawn-run in preparation for initiation of fruiting.

Key words: Carboxymethylcellulase, cellobiase, cellobiohydrolase, laccase, *Pleurotus sajor-caju*, xylanase.

Voluminous amounts of organic wastes are generated annually in agro-industrial processes. These represent a potentially valuable nutrient resource which could, with appropriate bioconversion technology, be profitably recycled into useful products. Mushroom cultivation represents one such viable process.

The major components of lignocellulosic wastes used for mushroom cultivation are cellulose, hemicellulose and lignin. Growth and fruiting are dependent on the ability of the particular mushroom to attack these components as nutrient sources. This in turn depends on the fungus' ability to produce the necessary hydrolytic and oxidative enzymes required to degrade these materials into smaller molecules for assimilation.

The oyster mushrooms *Pleurotus* spp., in particular *P. sajor-caju*, are edible, easy to grow and cultivation has now been commercialized in Malaysia by utilizing agro-industrial wastes which contain a high quantity of lignocellulose. Although *P. sajor-caju* is not a high cellulase producer, its ease of growth on cotton waste and sawdust has been established (Tan *et al*. 1986; Wahab 1986). This paper presents results on the activity of components of the inducible cellulase complex namely exo-1,4-*β*-D-glucanase (cellobiohydrolase, EC 3.2.1.91), endo-1,4-*β*-D-glucanase (carboxymethylcellulase, CMCase, EC 3.2.1.4), *β*-D-glucosidase (cellobiase, EC 3.2.1.21), the hemicellulase, xylanase (EC 3.2.1.8), and the phenoloxidase, laccase (EC 1.14.18.1) during the anamorphic phase of growth of *P. sajor-caju* on cotton waste submerged in liquid medium, and on supplemented sawdust in plastic bags.

Materials and Methods

A strain of *P. sajor-caju*, PL 27 (obtained from the Microbiology Laboratory of the Department of Plant Protection, Universiti Pertanian Malaysia) was cultured on potato dextrose agar slants for 2 weeks at 25 °C. After 9 days of growth at 25 °C, when the

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slants were fully covered with mycelia, mycelial plugs measuring 9 mm in diameter were made and used as inocula for cotton and sawdust substrates.

Cotton waste (1 g, with and without NaOH treatment) was placed in 50 ml MSNS liquid medium (Halliwell & Griffin 1973) contained in a 250-ml conical flask. One mycelial plug was inoculated into this medium which was then incubated stationary at 25 °C for 60 days; destructive sampling was carried out throughout incubation. Culture filtrate was collected by filtering twice through porous sintered glass filters (sizes 1 and 4, Jobling Ltd, UK) under vacuum. The culture filtrate was centrifuged at 12,000 rev min^{-1} (22,100 × *g*) using a Beckman JA 14 high-speed centrifuge for 20 min at 4 °C. The supernatant was collected for enzyme assays.

Sawdust cultures were prepared by inoculation of three mycelial plugs into 1 kg of supplemented rubber-tree sawdust medium packed tightly in 9 × 25 cm heat-resistant, polypropylene bags. These bags had been sterilized at 121 °C and 15 lbs in^{-2} for 1 h and stoppered with non-absorbent cotton filters. The sawdust substrate consisted of 94% sawdust, 5% rice-bran, 1% calcium carbonate and an equal volume of water. Measurements of the linear growth of the mycelia were made every 5 days until the mycelia had reached the bottom of the bags (35 days). Linear growth was recorded by measuring the distance (cm) of mycelial extension from the top of the sawdust substrate to the growing edge.

Enzymes were extracted in triplicate from 40 g samples of mycelium-penetrated sawdust with 200 ml of 0.05 M sodium acetate buffer (pH 6.5) by homogenization for 1 min; the homogenization vessel was placed in a beaker packed with ice. The homogenate was filtered and processed as described above.

The supernatants collected were assayed for enzyme activities either immediately or within 2 days of storage at 4 °C (there was no perceptible loss in activity). All spectrophotometric determinations were done in a Hitachi U 2000 spectrophotometer.

Cellulases were assayed strictly according to Halliwell (1962); hemicellulases were assayed using the method of Wood & Bhat (1988) without optimization of assay conditions; CMCase was assayed with the direct ferricyanide method; β-glucosidase was assayed with *p*-nitrophenyl β-D-glucopyranoside; and total cellulase (cellobiohydrolase) activity was measured using filter paper; the xylanase assay was carried out using beech wood xylan as the substrate and xylose was determined by dinitrosalicylic acid (Miller 1959); soluble protein was assayed according with the Folin–Lowry method. One unit of the cellulase and xylanase activity (U) is expressed as 1 μmol of glucose and xylose equivalent liberated per min respectively. Laccase activity was assayed polarographically using *p*-phenylenediamine as a substrate (Wood & Goodenough 1977). One unit of laccase activity is defined as the amount catalyzing the consumption of 1 μmol O_2 min^{-1}.

Results

The maximum amount of the cellulase complex was produced after 15–20 days growth on the cotton waste (recorded over a period of 55 days) for both the alkali-treated and non-treated cotton wastes. The specific activity for three of the components of the cellulase complex produced from alkali-treated cotton was maximum on day 15, at 15.6, 83.4 and 56.1 U (mg protein)$^{-1}$ for cellobiohydrolase, CMCase and β-glucosidase respectively. The enzymatic activities of the cellulases assayed were found to be proportional to protein concentration over the range 0.3–0.8 mg ml^{-1} (Figure 1). On the non-treated cotton, the highest activity for CMCase of 71.4 U (mg protein)$^{-1}$ was achieved on day 20, while the peaks for cellobiohydrolase and β-glucosidase at 10.0 and 21.6 U (mg protein)$^{-1}$ respectively were reached on day 15.

Production of cellulolytic enzymes on solid rubber-tree sawdust substrate in bags appeared to be more

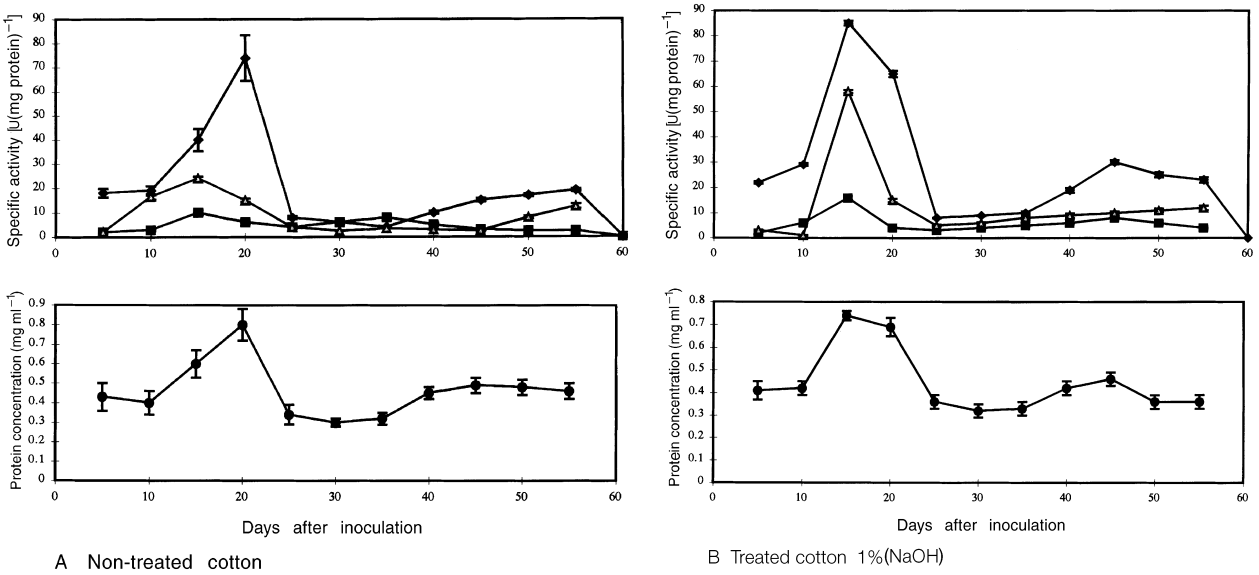

Figure 1. Cellulase complex activity from *Pleurotus sajor-caju* cultivated on: (A) non-treated cotton; (B) alkaline (1%) treated cotton. ■ – Carboxymethyl cellulase; ◆ – cellobiohydrolase; ▲ – β-glucosidase; ● – protein; I – standard error.

Table 1. Mycelial growth and enzyme production of *Pleurotus sajor-caju* grown on rubber-tree sawdust.

Time (days)	Mycelial growth (cm)*	Enzyme specific activity × 10^2 [U (mg protein)$^{-1}$]				
		Laccase	Xylanase	CM-Cellulase	β-Glucosidase	Cellobiohydrolase
5	1.17 ± 0.13	490 ± 8.20	0	2.0 ± 0.23	70.0 ± 4.22	165.0 ± 17.21
10	5.08 ± 0.42	791 ± 9.90	0	6.0 ± 0.62	6.0 ± 0.45	0
15	7.58 ± 0.53	1304 ± 14.56	5.0 ± 0.21	0	58.0 ± 6.53	0
20	10.75 ± 0.51	1595 ± 16.20	63.0 ± 0.82	49.0 ± 0.83	11.0 ± 0.58	5.1 ± 0.31
25	14.42 ± 0.81	1655 ± 18.30	5.0 ± 0.41	12.0 ± 0.63	29.0 ± 0.65	28.0 ± 0.93
30	17.25 ± 0.82	2690 ± 18.10	0	58.0 ± 0.94	75.0 ± 4.03	0.8 ± 0.04
35	18.0 ± 0.91	2740 ± 29.21	6.4 ± 0.42	62.0 ± 0.84	26.0 ± 0.64	11.0 ± 0.55
40	18.0 ± 0.93	742 ± 14.21	44.0 ± 0.83	35.0 ± 0.63	4.0 ± 0.39	0
45	18.0 ± 0.94	580 ± 16.21	0	16.0 ± 0.73	35.0 ± 0.74	34.0 ± 0.92
50	18.0 ± 0.93	715 ± 14.31	0	14.0 ± 0.83	42.0 ± 4.93	0
55	18.0 ± 0.91	815 ± 14.21	16.0 ± 0.60	12.0 ± 0.72	34.0 ± 0.83	13.9 ± 0.65
60	18.0 ± 0.94	493 ± 14.09	0	0	23.0 ± 0.86	16.8 ± 0.89

*Mycelial growth represents the linear extension from the top of the bag to the growing edge.
Data represent the mean ± SE of three replicates.

erratic than that on cotton waste. A number of peaks were recorded in some instances (Table 1, Figure 2). CMCase production peaked at a specific activity of 0.62 U (mg protein)$^{-1}$ after 35 days incubation which also corresponded with maximum growth of mycelia. β-Glucosidase output peaked at a specific activity of 0.75 U (mg protein)$^{-1}$ at about the same time (30 days after inoculation). Cellobiohydrolase levels on sawdust, like those observed on cotton waste, were very low, reaching a maximum of only 0.28 U (mg protein)$^{-1}$ after 28 days of growth (just before the peak). Maximum production of xylanase [0.63 U (mg protein)$^{-1}$] occurred at 20 days post inoculation and showed another lower peak after 40 days. The phenoloxidase enzyme, laccase, was

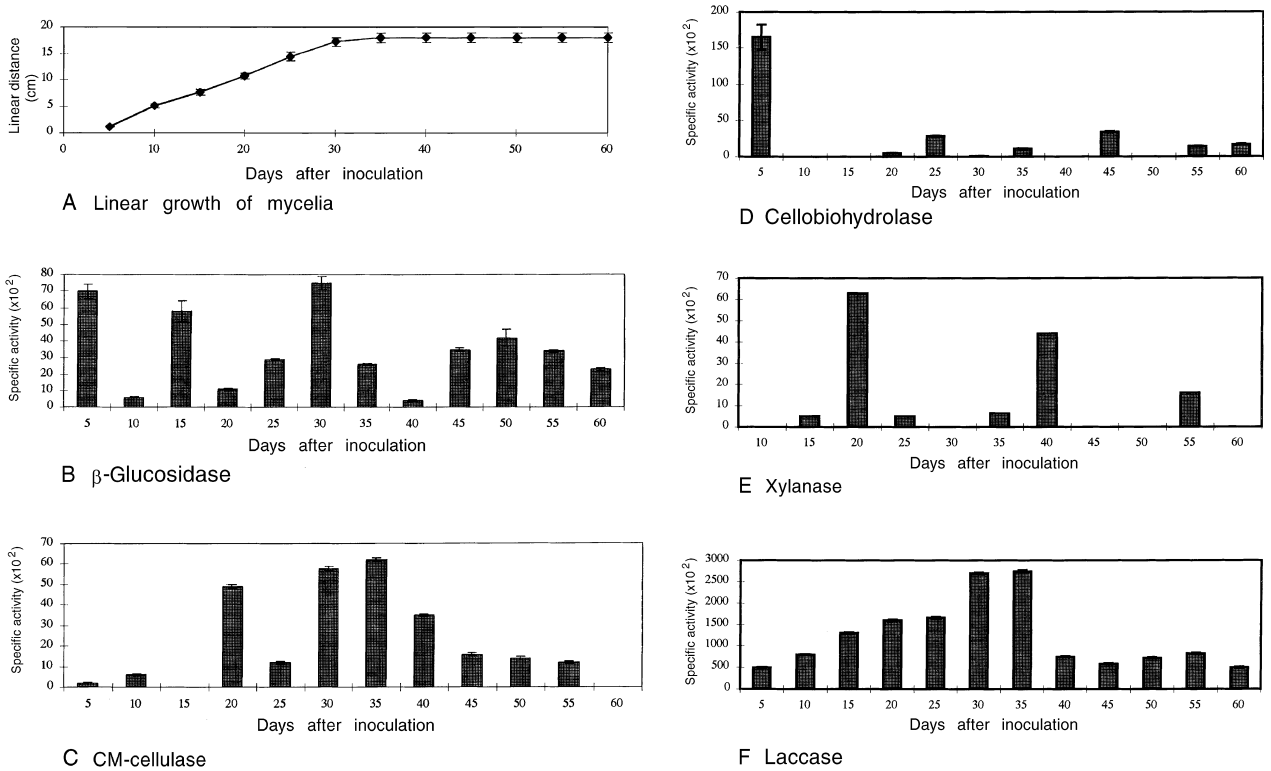

Figure 2. Mycelial growth and lignocellulolytic enzyme activities of *Pleurotus sajor-caju* cultivated on rubber-tree sawdust substrate. I – Standard error.

produced at levels much higher than that of the cellulases, reaching 27.4 U (mg protein)$^{-1}$ after 35 days.

Discussion

Many basidiomycetes have the capability to produce simultaneously the hydrolytic and oxidative enzymes which are needed to degrade complex lignocellulosic substrates such as wood (Kirk 1983; Wood 1984). These enzymes are extracellular and inducible, and most white-rot fungi exhibit low cellulolytic activity in the anamorphic stage; these fungi attack mainly highly lignified substrates such as wood and sawdust and produce enzymes targetted at lignin polymerization. Conversely, fungi such as the rice straw mushroom, *Volvariella volvacea* prefer high-cellulose, low-lignin materials and accordingly produce appreciable amounts of cellulases but no ligninases. *P. sajor-caju*, a white rot fungus, grows on a wide range of agricultural wastes with varying ratios of polysaccharide to lignin (Buswell *et al*. 1996).

It is known that cultivation conditions influence activity (Ginterova *et al*. 1981). The present experiments have shown that production of cellulases on cotton waste by *P. sajor-caju* was much higher than that on sawdust although levels were low. Similar low levels of cellulolytic activity have been reported in *Pleurotus* spp. during growth on rice straw; no activity was reported towards filter paper but very low levels of endoglucanase and β-glucosidase appeared late in cultivation (Rai & Saxena 1990). Earlier studies have similarly reported that no cellulolytic enzyme activity was detected in culture supernatants of *P. sajor-caju* and *Lentinula edodes* grown on crystalline cellulose (Avicel) or in those cultivated on sawdust (Buswell *et al*. 1993). The much higher production of cellulases on our cellulosic cotton substrate compared with the lignified sawdust substrate reflects the inducibility of the cellulase–enzyme complex. The alkali-treated cotton used in the present experiments, which would have undergone some chemical hydrolysis, resulted in higher levels of the cellulase complex; some pre-treatment of substrate would, therefore, be beneficial for the degradation of its cellulose component by microorganisms cultivated on it.

As expected, the lowest activity among the cellulases was recorded in cellobiohydrolase. This exo-glucanase limits the rate at which white-rot fungi degrade native cellulose. Its action is absolutely necessary for the degradation of highly ordered (crystalline) forms of cellulose where it acts synergistically with the endoglucanases (MacKenzie *et al*. 1984). Leatham (1985) reported on the great difficulty encountered in growing *L. edodes* on native cellulose as a sole carbon source and further noted that, despite heavy mushroom production, logs colonized by *L. edodes* retained fibre integrity for many years. Degradation of lignin without loss of cellulose has also been previously reported in the white-rot fungus, *Pycnoporus cinnabarinus* (Ander & Eriksson 1977).

A low amount of xylanase was produced on the sawdust substrate; high activity of this hemicellulase has been reported in *L. edodes* and *P. sajor-caju* growing on birchwood, while no activity was detected in *Volvariella volvacea* (rice straw mushroom) growing on xylan (Buswell *et al*. 1993). Again, the nature of the substrate was shown to be important in the production of inducible enzymes.

Laccase is a lignin-modifying extracellular oxidoreductase. It is an enzyme whose activity appears to be regulated in association with morphogenesis and often with the development of fruiting bodies (Leonard 1971; Leonard & Philips 1973; Philips & Leonard 1976; Wood & Goodenough 1977; Wood 1980, 1984; Leatham & Stahmann 1981; Ross 1982). Predictably, laccase activity, in the present experiments, was very high in relation to the cellulases and hemicellulase, the substrate being a highly lignified sawdust substrate. It increased with mycelial growth and peak production coincided with maximum growth after which the level of enzyme declined rapidly. Increase in laccase activity during the vegetative phase until the appearance of fruiting bodies has also been reported in *Schizophyllum commune* (Leonard & Phillips 1973), *Agaricus bisporus* (Wood 1980), *L. edodes* (Leatham & Stahmann 1981) and *Coprinus congregatus* (Ross 1982). Copious amounts of laccase were found in the culture supernatants of *P. sajor-caju* (Buswell *et al*. 1996). Laccase acts as a developmental marker, the level of which is maintained until the culture begins to fruit; after this the level falls. It was reported that in strains of *A. bisporus* which failed to fruit, activity remained at a high level. In our *Pleurotus* cultures which have been maintained for up to 60 days in the anamorphic stage, the results show some variance with the *Agaricus* work. Both laccase activity and mycelial growth peaked simultaneously after 35 days. Thereafter laccase activity fell even though fruiting bodies were not produced. In this case it would seem that the exhaustion of particular nutrients or aging of the culture triggered the shutdown of laccase production. On a practical level, laccase could be used as a morphogenetic landmark, a rapid drop indicating that maximum anamorphic growth has been achieved. This would be useful to mushroom growers in timing the opening of their spawn-run bags for fruiting; maximum vegetative growth cannot be ascertained visually with a high degree of accuracy.

In reports on fruiting cultures, the decline in activity of laccase when fruiting bodies develop corresponds with an increase in cellulase activity which has remained at a low level during the vegetative phase (Turner *et al*. 1975; Wood & Goodenough 1977). Our work on *P. sajor-caju*

supported the fact that the cellulolytic activity was indeed low and the laccase activity high during the anamorphic phase of development in *P. sajor-caju*.

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