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Lignocellulolytic enzyme profiles of edible mushroom fungi

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One of the most economically-viable processes for the bioconversion of many types of lignocellulosic wastes is represented by edible mushroom cultivation. *Lentinula edodes, Volvariella volvacea* and *Pleurotus sajor-caju* are three important commercially cultivated mushrooms which exhibit varying abilities to utilise different lignocellulosics as growth substrate. Examination of the lignocellulolytic enzyme profiles of the three species show this diversity to be reflected in qualitative variations in the major enzymic determinants (i.e. cellulases, ligninases) required for substrate bioconversion. For example, *L. edodes,* which is cultivated on highly lignified substrates such as wood or sawdust, produces two extracellular enzymes which have been associated with lignin depolymerisation in other fungi, (manganese peroxidase and laccase). Conversely, *V. volvacea,* which prefers high cellulose-, low lignin-containing substrates produces a family of cellulolytic enzymes including at least five endoglucanases, five cellobiohydrolases and two β-glucosidases, but none of the recognised lignin-degrading enzymes.

Key words: Cellulases, *Lentinula edodes,* ligninases, mushrooms, *Pleurotus sajor-caju, Volvariella volvacea.*

Various strategies have been developed to utilize part of the vast quantities of waste lignocellulose generated annually through the activities of the agricultural, forestry and food processing industries. One of the most significant, in terms of producing a higher value product from the waste, is the cultivation of edible mushrooms by solid state fermentation (Chang & Miles 1991). These mushrooms, a wide variety of which are cultivated in Southeast Asia, constitute a highly nutritious source of food (Buswell & Chang 1993). More recently, attention has focused on a second area of exploitation following the discovery that many of these fungi produce a range of metabolites of intense interest to the pharmaceutical (e.g. anti-tumour, immunomodulation agents and hypocholesterolaemic agents) (Mizuno *et al.* 1995) and food (e.g. flavour compounds) industries (Jong & Birmingham 1993).

Utilization of lignocellulosic substrates by mushroom fungi is dependent upon their ability to synthesize the relevant hydrolytic and oxidative enzymes which convert the individual components (cellulose, hemicellulose, lignin) into low molecular weight compounds that can be assimilated for nutrition (Buswell *et al.* 1993). Production of these enzymes by the fungal mycelium is a crucial part of the colonization process and an important determinant of mushroom yields. In this report, we describe the lignocellulolytic enzyme profiles of three major cultivated mushrooms, *Volvariella volvacea, Lentinula edodes* and *Pleurotus sajor-caju,* and the effects of various culture parameters on enzyme production.

Materials and Methods

Organism and Culture Conditions

Lentinula edodes, (Berk.) Pegler, strain L54, *Pleurotus sajor-caju,* strain Pl-27, and *V. volvacea* (Bull ex Fr.) Sing., strain V14, were obtained from the culture collection of the Department of Biology, The Chinese University of Hong Kong and maintained at 4°C (15 °C in the case of *V. volvacea*) on Potato Dextrose Agar (PDA) slants with periodic transfer. To determine ligninolytic enzyme production by *L. edodes* and *P. sajor-caju,* the fungi were cultivated in stationary 250-ml Erlenmeyer flasks containing 50ml of a defined medium containing (g/l): KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.05; $CaCl_2.2H_2O$, 0.013; glucose,10; 2,2′-dimethylsuccinate, 1.46; vitamins (Ohta *et al.* 1990); and 10 ml of a trace element solution

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containing (g/l): nitrilotriacetate, 1.5; $MnSO_4.5H_2O$, 0.5; NaCl, 1.0; $FeSO_4.7H_2O$, 1.0; $CoSO_4$, 0.1; $ZnSO_4.7H_2O$, 0.1; $CuSO_4.5H_2O$, 0.01; $AlK(SO_4)_2$, 0.01; H_3BO_3, 0.01 and $NaMoO_4.2H_2O$, 0.01. In culture medium for *V. volvacea* the vitamin solution was replaced by 0.1 g yeast extract/l. Nitrogen was added as NH_4NO_3 and L-asparagine at concentrations of 2.6 mM-N and 26 mM-N for low nitrogen (LN) and high nitrogen (HN) media, respectively (Buswell *et al.* 1984). To determine cellulolytic enzyme activities, *P. sajor-caju* and *V. volvacea* were grown in gyratory shaken 250-ml flasks (150 rev/min) containing 50 ml of a culture medium containing (g/l): KH_2PO_4, 1.0; K_2HPO_4, 0.4; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.013; yeast extract (Difco), 0.1; L-asparagine, 1.5; NH_4NO_3, 0.5; thiamine.HCl, 0.0025; and 1 ml of a trace element solution consisting of (g/l): ferric citrate, 4.8; $ZnSO_4.7H_2O$, 2.64; $MnCl_2.4H_2O$, 2.0; $CoCl_2.6H_2O$, 0.4; and $CuSO_4.5H_2O$, 0.4. Cellulose (1%) (Sigmacell, Sigma Chemicals) or carboxymethylcellulose (1%) served as carbon source as indicated. Media were adjusted to pH 5.0 for *L. edodes*, and pH 6.0 for *P. sajor-caju* and *V. volvacea*. Growth temperatures were 25°C for *L. edodes*, 28°C for *P. sajor-caju*, and 32°C for *V. volvacea*. An aliquot (1 ml) of a hyphal suspension, prepared by blending mycelium from actively growing cultures of the three species grown on Potato Dextrose Broth (Difco) for 45 seconds in a Waring blender, was used as inoculum. In the case of *P. sajor-caju* cultures used to determine the production of lignin degrading enzymes, flasks were inoculated instead with a 4mm plug taken from the growth margin of a 4–5 day old Potato Dextrose Agar culture.

Enzyme Assays

Endoglucanase (carboxymethylcellulase, CMCase) activity was determined by measuring the amount of reducing sugar (as glucose) released in reaction mixtures containing: 1.7 ml 50 mM KOH-KH_2PO_4 (pH 6.2), 0.8 ml 2% CMC (Sigma) solution and 0.5 ml culture supernatant. Mixtures were maintained at 50°C for 30 min and the reducing sugar determined by the Somogyi-Nelson method (Somogyi 1954) using 520 nm wavelength and glucose as a standard. Exoglucanase (Avicelase) was measured using the same procedure except that 1% (w/v) Avicel (Sigma) replaced carboxmethylcellulose (CMC) as the substrate. Intra- and extracellular β-glucosidase was determined by measuring the amount of *p*-nitrophenol released in reaction mixtures containing: 0.9 ml 50 mM KOH-KH_2PO_4 (pH 6.2), 0.05 ml 40 mM *p*-nitrophenyl-β-D-glucopyranoside, and 0.05 ml fungal extract or 0.05 ml culture supernatant, respectively (Cai *et al.* 1994). Mixtures were maintained at 45°C for 30 min and, after addition of 3 ml 1M sodium carbonate, the *p*-nitrophenol released was measured spectrophotometrically at 400 nm. All assays were performed in duplicate. Enzyme activities are expressed in units defined as the amount of enzyme required to form 1 μmol of product (reducing sugar or *p*-nitrophenol)/min under the assay conditions. Laccase activity was determined at 37°C using 2,2′-azino-*bis*-ethylbenthiazoline (ABTS) in reaction mixtures (3 ml) containing 0.1 M sodium acetate buffer (pH 5), 0.03% (w/v) ABTS, and an appropriate amount of culture supernatant (Buswell *et al.* 1995). Oxidation of ABTS was measured by monitoring the increase in absorbance at 420 nm. One unit of enzyme activity is defined as the amount of enzyme required to oxidise 1 μmole ABTS per min using an Σ_{420} value for oxidised ABTS of 3.6 × 10^4/mol/cm (Bourbonnais & Paice 1988). Manganese peroxidase (Mn-P) was assayed at 37°C in reaction mixtures (1.0 ml) containing 50 mM Na lactate (pH 4.5), 40 mM manganese sulphate and culture supernatant (Glenn *et al.* 1986). The reaction was initiated by addition of hydrogen peroxide (40 μM) and the increase in absorbance measured at 240 nm. One unit of enzyme activity is defined as the amount of enzyme required to form 1 μmol of Mn^{3+}/min using an Σ_{240} value for Mn^{3+} of 8.1 × 10^3/mol/cm (Aitken & Irvine 1990). LiP was determined at 37°C in reaction mixtures (1ml) containing 0.1 M Na tartrate (pH 3.0), 0.4 mM veratryl alcohol and culture supernatant (Tien & Kirk 1984). The reaction was initiated by addition of 0.2 mM H_2O_2 and the increase in absorbance due to the oxidation of veratryl alcohol to veratraldehyde measured at 310 nm. One unit of enzyme activity is defined as the amount of enzyme required to form 1 μmol of veratraldehyde per min using an Σ_{310} value for veratraldehyde of 9300 mol/cm (Tien & Kirk 1984).

PAGE and Activity Staining of Gels

Native PAGE was performed on aliquots of freeze-dried culture supernatants using the Mini-Protean II system (Bio-Rad). Protein bands exhibiting laccase activity stained green with ABTS (0.03% w/v) in 0.125 M acetate buffer, pH 5.0. Mn-P bands were detected using ABTS (0.075% w/v) in 55.6 mM acetate buffer, pH 4.5, containing 40 mM $MnSO_4$ and 0.04 mM H_2O_2. Endoglucanase (CMCase) and exoglucanase(Avicelase) activities were detected with Congo red (Teather & Wood 1982), and β-glucosidase with 40 mM *p*-nitrophenyl-β-D-glucopyranoside solution.

Protein Determination

Protein in culture supernatants and fungal extracts was determined by the Lowry method using bovine serum albumin as standard (Lowry *et al.* 1951).

Growth Yields

Mycelia were filtered through tared Whatman No 1 filter paper, washed with distilled water and dried at 100°C to constant weight.

Results and Discussion

Production of Cellulases

Endoglucanase (CMCase), exoglucanase (Avicelase) and β-glucosidase activities in culture supernatants of *P. sajor-caju* and *V. volvacea* grown in submerged culture over time courses, using either crystalline cellulose (Avicel) or CMC as carbon source, are shown in Figures 1 and 2. Both fungi were active cellulase producers although enzyme titres detected in culture supernatants of *P. sajor-caju* were considerably higher than those for *V. volvacea*.

Endoglucanase activity observed in culture supernatants of *P. sajor-caju* grown on Avicel peaked about 24 hours later, and reached maximum levels approximately 30% higher, compared to the corresponding CMC-grown samples (Figure 1). Conversely, exoglucanase activity was still increasing in CMC-grown cultures when the experiment was terminated after approximately 6 days incubation whereas, in Avicel cultures, enzyme levels peaked around 4 days and then decreased sharply (Figure 1). Extracellular β-glucosidase was readily detected in culture supernatants of *P. sajor-caju* grown on either substrate (Figure 1) although most of the enzyme activity has been reported to reside in extracts of fungal hyphae (Buswell *et al.* 1996). Rai & Saxena (1990) also investigated cellulase production by *P. sajor-caju* during growth on rice straw. These authors

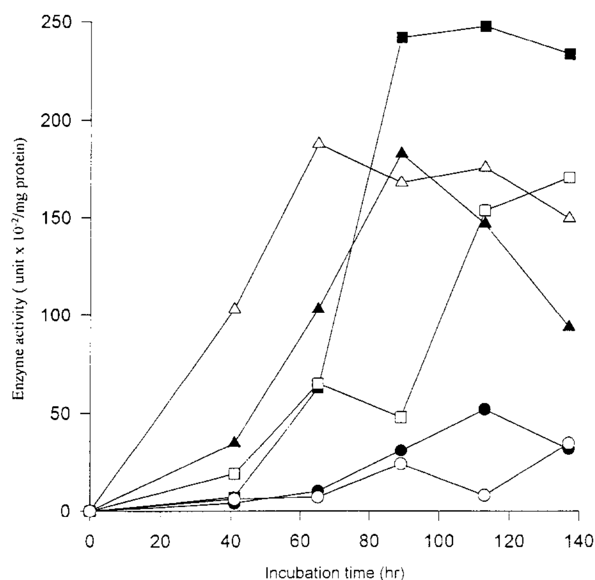

Figure 1. Production of endoglucanase (CMCase), exoglucanase (Avicelase) β-glucosidase by *P. sajor-caju* grown on Avicel and on carboxymethylcellulose. Open symbols—Avicel-grown cultures; Closed symbols—CMC-grown cultures; □,■— CMCase; ○,●—Avicelase; △,▲—β-glucosidase. Values represent the mean of three replicate cultures.

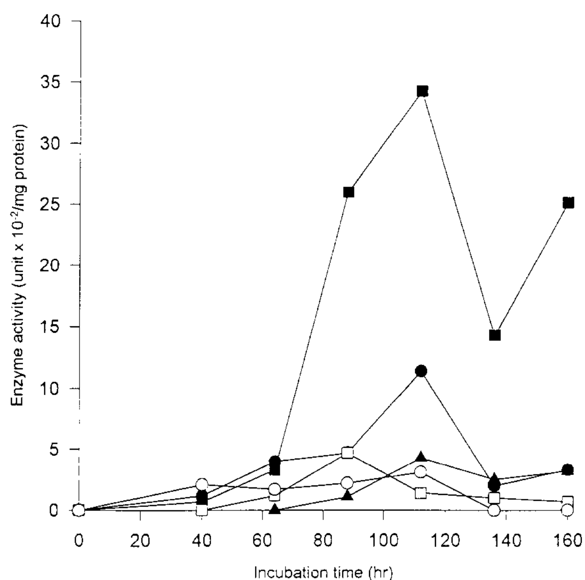

Figure 2. Production of endoglucanase (CMCase), exoglucanase (Avicelase) β-glucosidase by *V. volvacea* grown on Avicel and on carboxymethylcellulose. Open symbols—Avicel-grown cultures; Closed symbols—CMC-grown cultures; □,■— CMCase; ○,●—Avicelase; △,▲—β-glucosidase. Values represent the mean of three replicate cultures.

reported no activity towards filter paper but very low levels of endoglucanase and β-glucosidase appeared late in the cultivations.

Maximum CMCase and Avicelase levels were approximately seven- and four-fold higher, respectively, in Avicel-grown cultures of *V. volvacea* compared with peak enzyme titres observed when CMC served as the carbon source (Figure 2). Extracellular β-glucosidase activity remained extremely low in CMC-grown cultures (< 0.01 U/mg protein) throughout the experimental period. *V. volvacea* V14, has a complex cellulolytic enzyme system consisting of at least five endoglucanases, five cellobiohydrolases and two β-glucosidases (Buswell *et al.* 1996). It is not yet clear to what extent these represent separate gene products or simply post-translational modification. However, genes encoding individual components of the mushroom's cellulase complex have now been isolated using the heterologous probes *cbh-1* and *cbh-2* from *Phanerochaete chrysosporium* (J. Jia, J.A. Buswell & J.F. Peberdy, unpublished work).

L. edodes grew very poorly in agitated submerged culture on crystalline cellulose (Avicel). This poor growth is reflected by the absence of detectable cellulolytic activity (endoglucanase, exoglucanase and β-glucosidase) in culture supernatants. However, endoglucanase activity is induced in other strains of *L. edodes* during growth on this substrate (H.L. Yie, C.H. Wang & J.A. Buswell unpublished work). Mishra and Leatham (1990) reported endoglucanase, exoglucanase and β-glucosidase activity in *L. edodes* grown in solid-state cultures with red oak wood (*Quercus rubra*) as substrate. An inability by these authors to detect cellulolytic activity in sawdust-wheat bran cultures may have been due to enzyme repression as a result of the high levels of reducing sugar encountered in the growth medium.

Production of Ligninolytic Enzymes

The activities of three recognized lignin-modifying extracellular oxido-reductases were examined in this study, namely lignin peroxidase (LiP) (Glenn *et al.* 1983; Tien & Kirk 1983), manganese-dependent peroxidase (MnP) (Kuwahara *et al.* 1984; Glenn & Gold 1985) and laccase (Reinhammar 1984). Time courses for Mn-P production by *L. edodes* L54, and *P. sajor caju* PI-27, grown in stationary culture on a defined medium with glucose as the sole carbon source and under low nitrogen conditions are shown in Figure 3. As reported previously (Buswell *et al.* 1995), MnP production by *L. edodes* was dependent upon nutrient nitrogen levels in the culture medium and, under LN conditions (2.6 mM-N), enzyme activity was detected after 5 weeks and reached a maximum of 300 U/ml after 12 weeks. No Mn-P was recorded in HN cultures (26 mM-N) over the 13 week experimental period (Buswell *et al.* 1995). By comparison, relatively low levels of Mn-P were produced by the faster growing *P. sajor-caju* grown under LN conditions (Figure 3). Enzyme activity was also detectable in HN cultures of *P. sajor-caju* although specific activities (U/mg mycelium) were much lower (data not shown).

Lignin degradation by *L. edodes* is Mn dependent (Leatham 1986) and the concentration of Mn in the culture

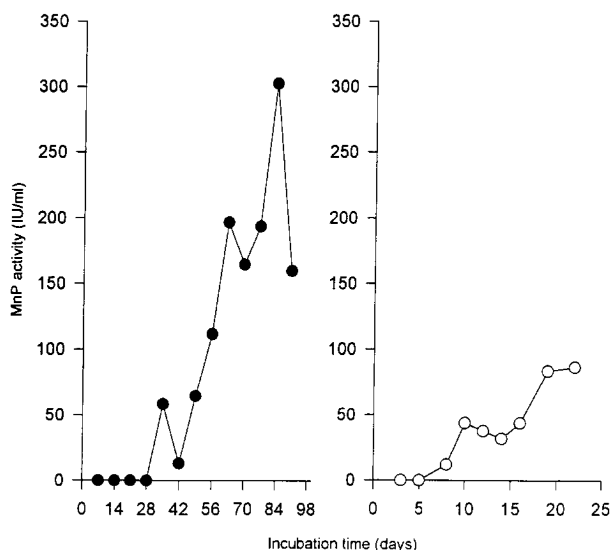

Figure 3. Mn-peroxidase production by *L. edodes* and *P. sajor-caju*. ○—*L. edodes*; ●—*P. sajor-caju*. Values represent the mean of three replicate cultures.

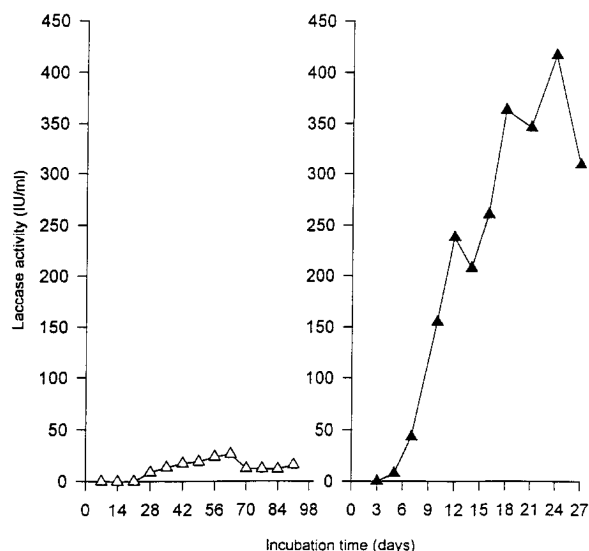

Figure 4. Laccase production by *L. edodes* and *P. sajor-caju*. ○—*L. edodes*; ●—*P. sajor-caju*. Values represent the mean of three replicate cultures.

medium also influences both the titre and the time of appearence of Mn-P in LN cultures of *L. edodes* (Buswell *et al.* 1995). Highest enzyme titres were recorded in cultures containing 1.1 ppm added Mn. Since manganese supplementation at concentrations over the range 0.3–15 ppm only marginally affected fungal growth, the effect of Mn on MnP production was not due to increased biomass production. This response to manganese may result from Mn induction of MnP expression. In the white-rot fungus *P. chrysosporium*, Mn regulates the expression of MnP by activating the transcription of the *mnp* gene via a growth-stage-specific and concentration-dependent mechanism (Brown *et al.* 1991).

In the case of laccase, the position was reversed and maximum enzyme titres observed in HN cultures of *P. sajor-caju* were approximately 16-fold higher compared to *L. edodes* (Figure 4). Low nutrient nitrogen conditions are not required for laccase biosynthesis by either mushroom.

Unlike the multiple forms of MnP (Leisola *et al.* 1987; Niku-Paavola *et al.* 1988; Ruttimann-Johnson *et al.* 1994) and laccase (Morohoshi *et al.* 1987) reported in some other ligninolytic fungi, native PAGE of crude *L. edodes* culture supernatants combined with activity staining with ABTS in the presence and absence of H_2O_2 revealed only one MnP protein and one laccase protein, respectively. Activity staining with ABTS in the presence of H_2O_2 revealed a single band with MnP activity only in gels of LN culture supernatants (Buswell *et al.* 1995). Forrester *et al.* (1988) also detected only one major MnP by *L. edodes* when the fungus was grown on a commercial oak-wood substrate. Conversely, at least five proteins exhibiting laccase activity can be separated by PAGE from culture supernatants of *P. sajor-caju* (S.C. Lo & J.A. Buswell unpublished work).

No LiP activity was detected in the extracellular medium following growth of either *L. edodes* L54 or *P. sajor-caju* under the growth conditions used in this study. However, two veratryl alcohol oxidases have been reported in *P. sajor-caju* which may play a role in lignin degradation by this fungus (Bourbonnais & Paice 1988).

No laccase, Mn-P or lignin peroxidase production by *V. volvacea* V14, or by another strain of this fungus, was observed over a wide range of growth conditions.

Lentinus edodes, *Pleurotus sajor-caju* and *Volvariella volvacea*, exhibit quite different growth responses to different lignocellulosic wastes. *L. edodes* grows well on woody substrates and is traditionally cultivated on logs of *Fagaceae* species although this procedure has, to a considerable extent, been replaced by the 'bag' system using supplemented sawdust media. The natural substrate for *V. volvacea* is paddy straw which has a relatively low lignin content (Dale 1987). The preference of this mushroom for 'less-lignified' substrates is exemplified by the increased production yields obtained when *V. volvacea* is cultivated on cotton wastes. *P. sajor-caju* is the most adaptable of the three species and can be grown on a wide variety of agricultural waste materials of differing composition in terms of polysaccharide/lignin ratio. Although the cellulolytic capacity of *V. volvacea* is clearly evident, the mushroom's aversion to highly lignified substrates is undoubtably reflected in the apparent inability of the fungus to synthesize any of the recognized lignin-transforming enzymes. The cellulose and hemicellulose components of the plant cell wall are intimately associated with the lignin moiety which presents a barrier to the hydrolytic enzymes catalyzing the degradation of the polysaccharides. Since *V. volvacea* appears to lack a ligninolytic or lignin transforming system, this will restrict fungal

access to the polysaccharide components and will reduce the capacity of the fungus to grow and fruit in lignified substrates.

An alternative, or supplementary, explanation for the preference of *V. volvacea* for less-lignified substrates might also be found in a higher sensitivity to phenolic monomers and tannins, and in a more pronounced effect of these compounds on the production and/or activity of the straw mushroom's cellulases and hemicellulases (Martin & Akin 1988; Cai *et al.* 1993). Conferring to this fungus an ability to produce extracellular enzymes able to degrade lignin and/or to detoxify inhibitory phenolics may lead to increased substrate colonisation and improved fruit body yields during commercial cultivation.

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