World J Microbiol Biotechnol (2006) 22:1215–1221
DOI 10.1007/s11274-006-9163-z

ORIGINAL PAPER

Effect of nutritional parameters on laccase production by the culinary and medicinal mushroom, *Grifola frondosa*

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Received: 27 June 2005/Accepted: 17 December 2005 / Published online: 20 April 2006

Abstract Extracellular laccase in cultures of *Grifola frondosa* grown in liquid culture on a defined medium was first detectable in the early/middle stages of primary growth, and enzyme activity continued to increase even after fungal biomass production had peaked. Laccase production was significantly increased by supplementing cultures with 100–500 μM Cu over the basal level (1.6 μM Cu) and peak levels observed at 300 μM Cu were ~7-fold higher than in unsupplemented controls. Decreased laccase activity similar to levels detected in unsupplemented controls, as well as an adverse effect on fungal growth, occurred with further supplementation up to and including 0.9 mM Cu, but higher enzyme titres (2- to 16-fold compared with controls) were induced in cultures supplemented with 1–2 mM Cu^{2+}. SDS-PAGE combined with activity staining revealed the presence of a single protein band (M_r ~70 kDa) exhibiting laccase activity in control culture fluids, whereas an additional distinct laccase protein band (M_r ~45 kDa) was observed in cultures supplemented with 1–2 mM Cu. Increased levels of extracellular laccase activity, and both laccase isozymes, were also detected in cultures of *G. frondosa* supplemented with ferulic, vanillic, veratric and 4-hydroxybenzoic acids, and 4-hydroxybenzaldehyde. Using 2,2′-azino-bis(ethylbenzothiazoline-6-sulfonate) (ABTS) as substrate, the optimal temperature and pH values for laccase activity were 65°C and pH 2.2, respectively, and the enzyme was relatively heat stable. In solid-state cultures of *G. frondosa* grown under conditions adopted for industrial-scale mushroom production, extracellular laccase levels increased during the substrate colonization phase, peaked when the substrate was fully colonized, and then decreased sharply during fruit body development.

Keywords Edible and medicinal mushrooms · Enzyme regulation · Extracellular laccase · *Grifola frondosa* · Liquid and solid-state cultures · Mushroom development cycle

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Introduction

The edible and medicinal mushroom, *Grifola frondosa*, (‘‘Hui Shu Hua’’ in Chinese, ‘maitake’ in Japanese) is a highly nutritious food source that is also reported to contain bioactive metabolites that exhibit various medicinal effects including antitumour (Ohno et al. 1986; Kodama et al. 2003), hypocholesterolemic (Kubo and Nanba 1997; Fukushima et al. 2001), antioxidant (Zhang et al. 2002) and antidiabetic activity (Kubo et al. 1994; Horio and Ohtsuru 2001). In 2003 (the latest figures available), an estimated 24,900 tonnes were grown in China, the main producing country, and the mushroom ranked 11th overall among all cultivated species in terms of worldwide annual output (Chang 2005). The fungus is cultivated in China using sawdust/cotton seed hull-based ‘‘composts’’ and, in order to obtain the nutrients required for growth and fruiting, is assumed to secrete the hydrolytic/oxidative enzymes that catalyse the degradation of the major macromolecular components (cellulose, hemicellulose, lignin) of the growth substrate (Buswell et al. 1996). However, compared with

other major cultivated mushrooms, very little is known about the nature of the lignocellulolytic enzymes produced by *G. frondosa*, the parameters affecting their production, and enzyme activity profiles during different stages of the developmental cycle. Therefore, a major study has been initiated in our laboratory in order to rectify these deficiencies, and to design strategies for increasing the relatively low biological efficiency (conversion of growth substrate into fruit bodies) of *G. frondosa*. Such strategies would be based in part on improved substrate utilization achieved by manipulating the expression of those genes controlling lignocellulolytic enzyme production. One part of this investigation has focused on laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2), an enzyme that has variously been assigned several physiological functions of relevance to mushroom cultivation (Thurston 1994). These include roles in lignin degradation (Archibald and Roy 1992; Ardon et al. 1988; Eggert et al. 1997), detoxification of phenolic compounds inhibitory to fungal growth and cellulolytic enzyme activity (Bollag et al. 1988), and sporophore development (De Vries et al. 1986; Wood 1980; Chen et al. 2004a).

In this paper, we report for the first time the effect of various nutritional parameters on laccase production by this commercially important mushroom both during growth in submerged culture on a defined medium, and on a solid-state substrate under controlled environmental conditions representative of those adopted for large-scale cultivation.

Materials and methods

Organism and culture conditions

Grifola frondosa (accession no. FGF-1) was obtained from the Edible Fungi Culture Collection Center, China Culture Collection Center of Agricultural Microorganisms, and maintained on potato dextrose agar (PDA) at 4°C with periodic transfer.

For liquid culture, the fungus was grown at 28°C in stationary 250-ml Erlenmeyer flasks containing 50 ml basal medium (pH 6.0) consisting of (per litre): glucose, 10 g; Na_2HPO_4, 0.475 g; KH_2PO_4, 1.453 g; asparagine monohydrate, 1 g; NH_4NO_3, 0.5 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; yeast extract (Difco), 1.0 g; $CaCl_2{\cdot}2H_2O$, 0.013 g; thiamine, 25 mg and trace elements solution (Cai et al. 1999), 1 ml. Flasks were inoculated with 1.0 ml of a mycelial suspension prepared by homogenizing a 10-day PDA culture in 100 ml sterile distilled water using a Waring blender operated at half power for two 15 s periods. The effect of copper on laccase production was determined by supplementing basal medium with copper (as $CuSO_4$) to the concentrations indicated prior to inoculation and measuring enzyme activity after incubation for a further 35–48 days. To determine the effect of aromatic compounds on laccase production, each compound (2 mM final concentration) was added to 10-day-old cultures and laccase activity measured after a further 30 and 35 days incubation. Solid-state cultures were grown under controlled environmental conditions (vegetative growth: 25°C, 70% relative humidity, CO_2 concentration $<2{,}500$ ppm, no light; fruiting: 20°C, 98% R.H., CO_2 400–500 ppm, light 200–300 lux for 10 min every hour) in a modern mushroom factory. The substrate (200 g, pH 6.0–6.5), consisting of 70% beech sawdust, 25% wheat bran and 5% corn meal, was distributed into 850 ml plastic bottles, the moisture content adjusted to 62%, and the opening to the bottle covered with a polystyrene foam cap. After sterilisation (121°C, 65 min), each bottle was inoculated with 5–7.5% mushroom spawn (grown for 30 days on sawdust), and incubated at 25°C for 45 days prior to removal of the cap to induce pinning and fruit body development.

Sampling procedures

Liquid cultures (three replicates) were harvested over time-courses and laccase activity in the culture supernatants was assayed. Fungal biomass was determined by filtering mycelia through nylon mesh, washing with distilled water and drying to constant weight at 80°C.

Samples (20 g) (five replicates) from solid-state cultures were taken at different stages of the 65-day developmental cycle: half substrate colonization (day 20), full substrate colonization (day 33), prior to removal of bottle cap (day 45), after removal of bottle cap (day 48), primordia formation stage (day 53), small fruit bodies stage (day 59) and mature fruit bodies stage (day 65). Laccase activity in primordia, small fruit bodies and mature fruit bodies was also determined. Substrate and fruit body samples were suspended in 10 volumes (w/v) 50 mM sodium phosphate buffer (pH 7.0) and homogenized in a Waring blender (half power for two 30 s periods). Homogenates were transferred to 250 ml flasks, shaken (100 rpm) for 2 h at 25°C, filtered through nylon mesh and excess liquid collected by gentle hand squeezing. Filtrates were further clarified by centrifugation (5,000g for 10 min at 4°C) and the supernatant retained for enzyme assay.

Enzyme assay

Laccase activity was determined at 30°C using 2,2′-azino-bis(ethylbenzothiazoline-6-sulfonate) (ABTS) in reaction mixtures (1 ml) containing citrate–phosphate buffer (pH 2.2), 0.03% (w/v) ABTS, and an appropriate amount of culture supernatant. The reaction was initiated by addition of ABTS, oxidation of which was measured for 2 min by monitoring the linear increase in absorbance at 420 nm.

One unit of laccase activity is defined as the amount of enzyme required to oxidize 1 μM ABTS per min using an ϵ_{420} value for oxidized ABTS of 3.6×10^4 mol^{-1} cm^{-1} (Bourbonnais and Paice 1988). Since peroxidases in the presence of H_2O_2 (both of which may have been present in the culture fluids) also oxidize ABTS, assays were repeated with catalase-treated extracellular fluid to confirm the presence of laccase activity. In cases where differences were observed, ABTS-oxidizing activity in treated samples was >90% that of untreated samples.

The standard assay conducted over the range 20–80°C was used to determine the optimal temperature for total laccase activity, and the optimal pH was established using 0.1 M sodium chloride and hydrochloric acid (pH 1.0–2.2) and citrate–phosphate (pH 2.2–8.0) buffer systems.

SDS-PAGE and activity staining of gels

SDS-PAGE was performed on aliquots of filtered (0.45 μm Millipore filter) culture fluids using the Mini-Protean II system (Bio-Rad) operated at 100 mV for 2 h. Samples (15 μl) were first mixed with 15 μl SDS sample buffer (1.0 ml glycerol, 2.0 ml 10%(w/v) SDS, 2.0 ml 0.01% bromophenol blue 1.25 ml stacking buffer [see below] and 4 ml β-mercaptoethanol) and then 15 μl of the mixture loaded into each well. The separating and stacking gels contained 12% and 4% (w/v) acrylamide, respectively. Buffer solutions consisted of 155 mM Tris (pH 8.8) and 0.4% (w/v) SDS for the separating gel, and 50 mM Tris (pH 6.8) and 0.4% (w/v) SDS for the stacking gel. The electrode reservoir solution (pH 8.3) consisted of Tris buffer (25 mM) containing 190 mM glycine. Gels were rinsed twice with 2.5% (w/v) Triton X-100 for 10 min to restore laccase activity after electrophoresis, and then rinsed twice with citrate-phosphate buffer (pH 2.2) prior to staining with ABTS (0.03%) in the same buffer. Protein bands exhibiting laccase activity stained green. Molecular weight determinations were carried out on other sections of the same gel which were stained instead with 0.1% (w/v) Coumassie Brilliant Blue R-250 in distilled water:methanol:acetic acid mixture (5:4:1) at room temperature for 20–30 min and destained with the same mixture without Coumassie Blue. Phosphorylase B (M_r 97,400), bovine serum albumin (M_r 66,200), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000), trypsin inhibitor (M_r 21,500) and lysozyme (M_r 14,400) were used as molecular markers.

Results and discussion

Laccase production in submerged culture

Figure 1 shows the time-courses of biomass and extracellular laccase production by *G. frondosa* grown in submerged culture. Fungal biomass increased steadily for the first 27 days of the 52-day culture period to a maximum of 3.2 mg/ml and then gradually declined. Laccase activity was detectable after 12 days, reached an initial peak of 61 U/l after 42 days, declined by approximately 50% after 45 days before continuing to increase again to reach levels of ~70 U/l when the experiment was terminated. SDS-PAGE analysis of culture fluids and activity staining of the gels revealed the presence of a single protein band exhibiting laccase activity throughout the incubation period.

The laccase production profile in liquid cultures of *G. frondosa* is unusual since the enzyme was detectable in culture fluids only after 12 days of primary growth during which considerable fungal biomass had been generated. In other basidiomycetes, enzyme synthesis is more closely associated with trophophase, for example in *Trametes versicolor* (Collins and Dobson 1997), *Pleurotus sajor-caju* (Soden and Dobson 2001; Fu et al. 1997) and *Pycnoporus cinnabarinus* (Eggert et al. 1997), or detectable only in the later stages of primary growth when fungal biomass production had reached a maximum as in the case of *Volvariella volvacea* (Chen et al. 2003).

pH and temperature optima, and heat stability, of laccase activity in culture fluids

Preliminary characterization revealed that laccase activity in culture fluids was detectable only when the pH of reaction mixtures was adjusted to 5.5 or below and, for ABTS, an activity peak was observed at pH 2.2. Similar low pH optima for ABTS oxidation have been reported for laccases from the basidiomycetes *Trametes villosa* (Yaver et al. 1996), *Pleurotus sajor-caju* (Lo et al. 2001) and *Volvariella volvacea* (Chen et al. 2003). In standard assay mixtures, the velocity of ABTS oxidation was maximal at 60–65°C. Furthermore, enzyme activity was relatively heat stable with 90% and 60% remaining after 1 h exposure to

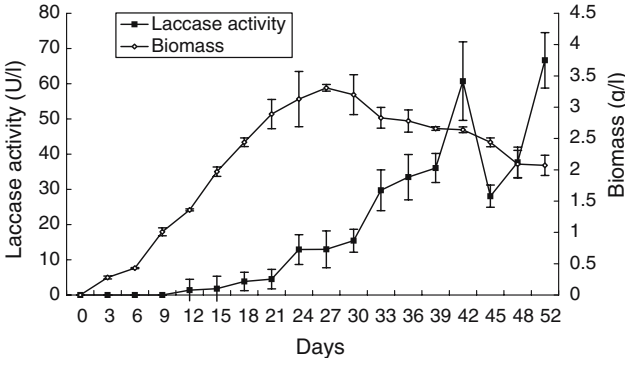

Fig. 1 Time courses of extracellular laccase and fungal biomass production by *G. frondosa* grown in liquid cultures on a defined medium. Values represent the mean ± SD of triplicate samples

60°C and 70°C, respectively (data not shown). However, rapidly denaturation of the enzyme occurred above 70°C.

Effect of copper on laccase production by *G. frondosa*

Since laccase is a copper-containing protein (Messerschmidt and Huber 1990), and copper supplementation of the culture medium is reported to enhance enzyme production in many basidiomycetes (Collins and Dobson 1997; Palmieri et al. 2000; Soden and Dobson 2001; Chen et al. 2003), we examined the effect of added copper on laccase synthesis by *G. frondosa*.

Figure 2 shows the effect on extracellular laccase activity of adding 0.1–1.0 mM (as copper sulphate) to basal medium containing 1.6 μM Cu^{2+}. After 35 days incubation, supplementation over the range 0.1–0.5 mM Cu^{2+} resulted in approximately 3- to 6-fold increases in laccase activity over the basal level of 34 U/l detected in unsupplemented (1.6 μM Cu^{2+}) culture fluids, with the highest enzyme titre of 227 U/l recorded at concentrations of 0.3 mM $CuSO_4$. Supplemention over this range had no significant effect on fungal biomass production. However, additional supplementation up to and including 0.9 mM $CuSO_4$ resulted in decreased laccase activity to levels similar to those detected in unsupplemented controls, as well as an adverse effect on fungal growth (Fig. 2). However, enzyme titres in cultures supplemented with 1 mM Cu^{2+} were approximately 2-fold higher than control values (Fig. 2)). Interestingly, although only one laccase protein with an apparent M_r of ~70 kDa was detected in cultures with added Cu^{2+} concentrations up to 0.9 M, an additional distinct second band with an apparent M_r of ~45 kDa was observed at 1.0 M Cu^{2+}. Both these molecular masses are within the range of those reported for laccases from other basidiomycetes including *Coriolus versicolor* (64.5 kDa) (Fåhraeus and Reinhammar 1967), *Dichomitus squalens* (66 kDa) (Périé et al. 1998), *Lentinus edodes* (66 kDa) (Kofujita et al. 1991), *Phanerochaete chrysosporium* (46.5 kDa) (Srinivasan et al. 1995), *Pleurotus ostreatus* (59 kDa) (Sannia et al. 1986), *Pyconoporus cinnabarinus* (76–81 kDa) (Eggert et al. 1996), *Rigidoporous lignosus* (52–55 kDa) (Galliano et al. 1991), *Trametes pubescens* (65 kDa) (Galhaup et al. 2002) and *Volvariella volvacea* (58 kDa) (Chen et al. 2004a).

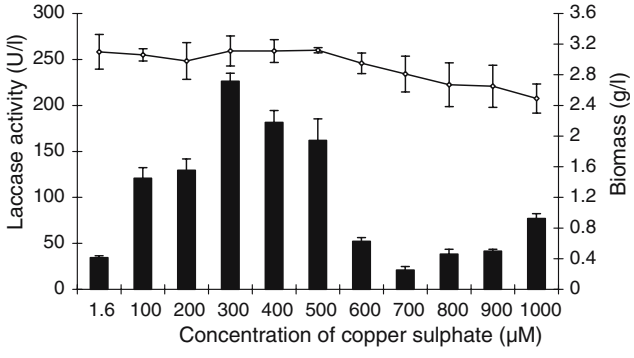

Fig. 2 Effect of copper on fungal growth (line graph) and extracellular laccase production (bars). Values represent the mean ± SD of triplicate samples

In a separate experiment in which cultures containing the basal level of 1.6 μM Cu^{2+} were supplemented with 0.6–2.0 mM copper, the low molecular weight band was detected in culture supernatants containing Cu^{2+} concentrations of 1.0 mM and higher (Fig. 3a). However, both band and enzyme activity patterns also varied with sampling time (see also next section) and although both bands were detectable in 42-day-old cultures supplemented with 1.0–2.0 mM Cu^{2+} (Fig. 3a), the 45 kDa band was clearly evident only in cultures with 2.0 mM Cu^{2+} after 48 days (Fig. 3b). Laccase activity in cultures supplemented with 1.0, 1.25, 1.5 and 2.0 mM Cu were approximately 7-, 16-, 13- and 9-fold higher than unsupplemented controls, respectively, after 48 days incubation. Higher Cu^{2+} concentrations (1.5 and 2.0 mM) resulted in significant fungal growth inhibition (43% and 57%, respectively) compared with unsupplemented controls (3.6–3.8 g/l). Furthermore, fungal growth in cultures with 2.0 mM added Cu was in the form of discrete surface pellets of variable size instead of the continuous fungal mat produced at all lower Cu^{2+} concentrations.

Our data suggest that low and high Cu may control the expression of separate laccase genes in *G. frondosa*. Positive regulation of laccase protein synthesis and laccase gene transcription has previously been reported in numerous basidiomycetes including *Pleurotus* spp (Collins and Dobson 1997; Palmieri et al. 2000; Baldrian and Gabriel 2002), *Trametes* spp. (Yaver et al. 1996; Soden and

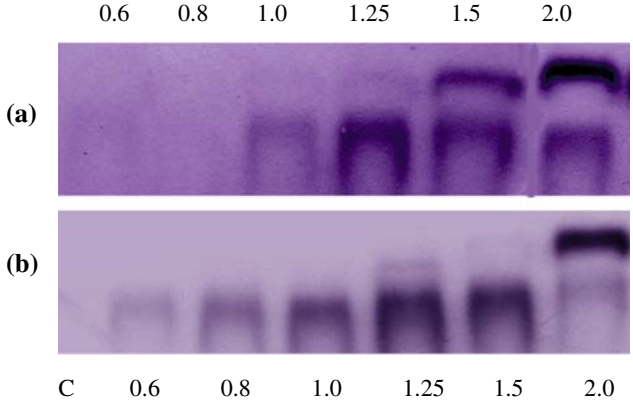

Fig. 3 Laccase protein band patterns in cultures of *G. frondosa* supplemented with different Cu concentrations. Bands representing laccase proteins were separated by SDS-PAGE and visualized by activity staining with ABTS. (**a**): 42 days incubation; (**b**): 48 days incubation. Lanes: Control (1.6 μM), 0.6, 0.8, 1.0, 1.25, 1.5 and 2.0 mM Cu, respectively

Dobson 2001) and *Volvariella volvacea* (Chen et al. 2003). The observed changes in band and enzyme activity patterns over time may also result from different copper concentrations affecting isozyme stability to varying degrees. A positive affect on the stability of laccase by Cu has been previously reported (Baldrian and Gabriel 2002), which may be due in one case at least to Cu-mediated inhibition of an extracellular protease that degrades the laccase protein (Palmieri et al. 2001).

Effect of aromatic compounds on laccase activity in submerged culture

The effect of different aromatic compounds (2 mM) on laccase activity and fungal biomass production in cultures of *G. frondosa* containing the basal level of Cu^{2+} (1.6 μM) is shown in Fig. 4. Incubation for 30 days in the presence of four of the five aromatic compounds tested resulted in higher extracellular laccase levels compared with controls: vanillic acid (258 U/l), 4-hydroxybenzaldehyde (193 U/l), 4-hydroxybenzoic acid (133 U/l) and veratric acid (122 U/l). Under these conditions, laccase levels in ferulic acid-supplemented cultures (70 U/l) were only slightly higher than the basal levels recorded in unsupplemented controls (58 U/l). As in the case of copper supplementation experiments (see above), relative laccase activities varied with sampling time (data not shown) although enzyme titres in supplemented cultures were consistently higher than in unsupplemented controls.

Under these conditions, SDS-PAGE and activity staining revealed only the 75 kDa laccase band in control cultures. However, in supplemented cultures, this band was much stronger and the 45 kDa band was also detectable at varying intensities (Fig. 4). None of the aromatic compounds tested had any significant effect on fungal biomass production.

There are numerous reports describing the different effects of aromatic compounds on laccase activity in basidiomycetous fungi. These vary widely and include the induction of new laccase isozymes (Bollag and Leonowicz 1984; Lo et al. 2001) as well as increased enzyme titres but without any accompanying changes in the isozyme pattern (Eggert et al. 1996). The various responses are thought to constitute a protective mechanism against toxic residues either indigenous to (Cherney et al. 1989) or generated during fungal degradation of the lignin component of the lignocellulosic growth substrate (Thurston 1994), or secreted as antimicrobial agents by microbial competitors (Eggert et al. 1996).

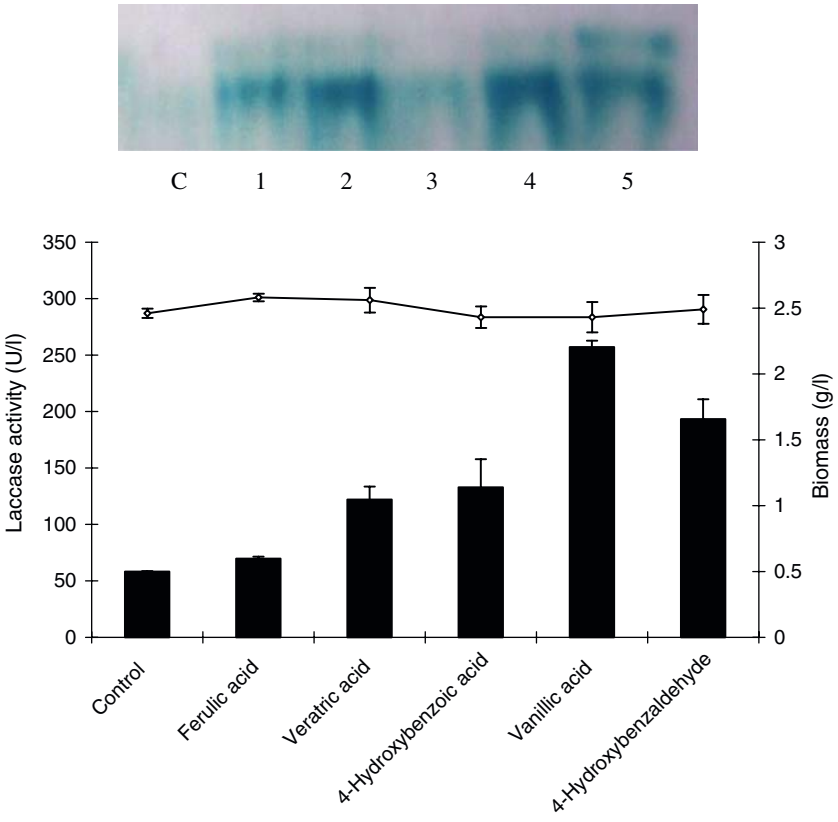

Fig. 4 Effect of aromatic compounds on fungal growth (line graph) and extracellular laccase induction (bars). Values represent the mean ± SD of triplicate samples. Bands representing laccase proteins were separated by SDS-PAGE and visualized by activity staining with ABTS. Lane numbers correspond to the aromatic compound sequences shown on bar graph

Laccase activity in solid-state cultures and in mushroom fruit bodies

A time-course for laccase production in extracts of the solid ''compost'' used to cultivate *G. frondosa* under conditions adopted for industrial-scale mushroom production is shown in Fig. 5. The normal cultivation period lasts for approximately 65 days and can be divided into seven discrete stages: half colonization of the growth substrate, full colonization, before cap removal, after cap removal, appearance of primordia, small fruit bodies and mature fruit bodies. As seen from Fig. 5, enzyme activity increases during the colonization phase and reached a peak (~6 U/ml of extract) when the substrate was fully colonized. Enzyme levels decreased sharply throughout the fruit body developmental stages, reaching the lowest levels recorded (0.3 U/ml extract) during the later stages of fruit body formation and maturation. Laccase activity was also readily detectable in extracts of primordia, and of small and mature fruit bodies (Fig. 5), with increasing enzyme levels observed as the primordia developed.

Laccase production, and the transcriptional regulation of laccase genes, has been examined during the developmental cycles of other mushroom fungi including *Agaricus bisporus* (Wood and Goodenough 1977; Ohga et al. 1999), *Lentinula edodes* (Ohga 1992; Ohga and Royse 2001), *Pleurotus abalonus* (Takayama et al. 1993) and *V. volvacea* (Chen et al. 2003, 2004b). In compost cultures of *A. bisporus* (Wood and Goodenough 1977), *L. edodes* (Ohga 1992) and *P. abalonus* (Takayama et al. 1993), laccase concentrations increased during mycelial growth and then declined rapidly at the onset of fruiting. Laccase transcription in compost-grown mycelia of *A. bisporus* attained maximum levels during the mycelial growth phase prior to the onset of fruiting, declined during the fruit body enlargement phase and increased again after harvesting and during the second flush of fruit body production (Ohga et al. 1999). Similarly, laccase transcription in *L. edodes* peaked during the vegetative growth phase and declined at the fruiting stage (Ohga and Royse 2001). Such profiles, and that observed for *G. frondosa* in this study, are compatible with the oft-assigned role of laccase in lignin degradation which is essential if the fungus is to gain access to the cellulose and hemicellulose components of the growth substrate. Hydrolysis of these polysaccharide materials provides the nutrients required for vegetative growth as a prelude to fruiting (Das et al. 1997). Laccase may also play a more direct role in the fruiting process (Chen et al. 2003, 2004a), possibly by polymerizing cell wall components to cross-link hyphal walls during primordium formation and further strengthening of cell-to-cell adhesion throughout fruit body development (Leatham and Stahmann 1981; Thurston 1994).

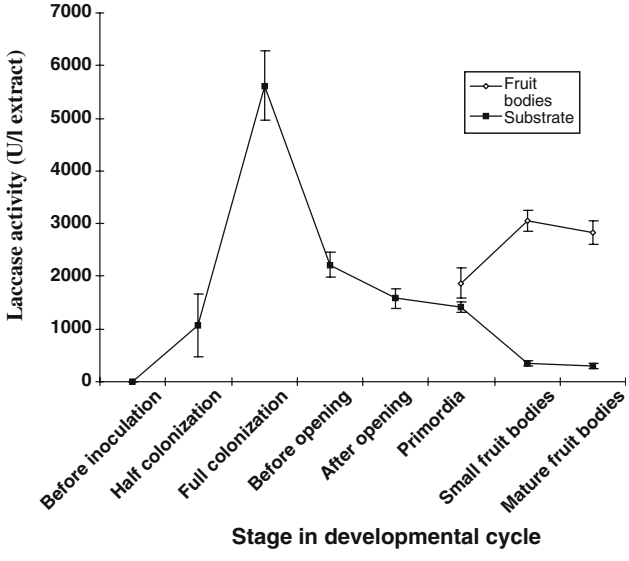

Fig. 5 Laccase activity in extracts of growth substrate, primordia and fruit bodies during the developmental cycle of *G. frondosa*. Values represent the mean ± SD of five samples

Further work is currently underway to purify and characterize the different laccase isozymes produced by *G. frondosa*, to isolate the encoding genes and to investigate parameters controlling gene expression.

Acknowledgements We thank Mr Yaosong Wang for technical assistance, and John Buswell for linguistic revision of the manuscript.

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