Induction of laccase activity in the edible straw mushroom, *Volvariella volvacea*

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Received 19 September 2002; accepted 4 November 2002
First published online 29 November 2002

Abstract

*Volvariella volvacea*, strain V14, produces multiple forms of extracellular laccase when grown in submerged culture in a defined medium with glucose as sole carbon source, and on cotton waste ‘compost’ representative of the conditions used for industrial-scale mushroom cultivation. In liquid culture, enzyme synthesis is associated with the onset of secondary growth, and is positively regulated by copper (up to 200 μM CuSO4) and by various aromatic compounds. In solid-state systems, only low levels of laccase are detectable during the vegetative growth phase but enzyme activity increases sharply at the onset of fruiting and during sporophore development.

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Keywords: Laccase; Mushroom; Secondary metabolism; Enzyme induction; *Volvariella volvacea*

1. Introduction

The edible straw mushroom, *Volvariella volvacea*, is among the most extensively cultivated mushrooms in tropical and sub-tropical regions. Although the mushroom currently ranks fifth in terms of annual production worldwide [1], biological efficiency (i.e. conversion of growth substrate into mushroom fruit bodies) is considerably lower compared with the other major cultivated species. It has been suggested that the relatively low growth yields may be linked, in part, to an apparent inability of *V. volvacea* to produce laccase (p-diphenol oxidase, EC 1.10.3.2) [2]. This enzyme is widely distributed among fungi where it has been assigned roles in lignin degradation [3–5], in rendering phenolic compounds less toxic via oxidative coupling and polymerisation [6], and in mushroom fruit body morphogenesis [3,7,8]. Since all these three functions are of fundamental importance for the colonisation of the various lignocellulosic substrates used in mushroom cultivation systems and for sporophore development, we have re-assessed earlier studies that failed to detect laccase production by *V. volvacea* [2]. We now show that this commercially important mushroom produces multiple forms of the enzyme, and have identified various factors affecting laccase induction during growth of the fungus in submerged culture. We also report some novel features associated with laccase production in both liquid culture and in miniaturised solid-state systems representative of the conditions used for industrial cultivation.

2. Materials and methods

2.1. Organism and growth conditions

*V. volvacea*, strain V14, was obtained from the culture collection of the Centre for International Services to Mushroom Biotechnology located at The Chinese University of Hong Kong (accession no. CMB 002) and maintained on potato dextrose agar at room temperature with periodic transfer. The fungus was cultivated in stationary 250-ml Erlenmeyer flasks containing 50 ml of a defined medium, pH 6.0, as described previously [9]. This medium was supplemented with different concentrations of CuSO4 as shown, and nitrogen was added as NH4NO3 and l-asparagine at concentrations of 2.6 mM-N and 26 mM-N for low nitrogen (LN) and high nitrogen (HN) media.
respectively [10]. In experiments to examine the effect of Cu on laccase production, CuSO4 was omitted from the trace element solution used to prepare the basal medium described in [9].

The effect of aromatic compounds on laccase production was determined using basal medium with HN as described above. Cultures were grown for 3 days before the addition of inducer to a final concentration of 2 mM (0.02 M in the case of 2,5-xylidine), and laccase activity was determined after a further 11 days incubation.

In order to study laccase production during the different stages of fungal development, we have developed a miniaturised solid-state growth system to mimic the cultivation conditions used in commercial operations. Cylindrical-shaped (6 cm diameter, 6 cm long), miniaturised compost cultures were prepared as follows: compost mixture consisting of 30 g (dry weight) cotton waste, 0.3 g lime and 60 ml distilled water was placed into a plastic bag which was then fitted with a stainless-steel neck ring and plugged with a paper plug. Bags were autoclaved at 121°C for 1 h and, after cooling to room temperature, the centre of the upper surface of the compost was inoculated with 2 cm² pieces of fungal mycelium taken from a 7-day-old culture grown on potato dextrose agar (Difco) at 32°C. Compost cultures were incubated at 32°C for 12 days and then transferred to an environmental chamber maintained at 30°C, 85% humidity, and with alternate 12 h periods, the compost cultures (three replicates) were cut longitudinally through the centre of the cylinder and one portion (half the total weight of the compost culture) suspended in 10 volumes (v/dry weight) 50 mM KH2PO4 buffer, pH 6.5, and shaken (150 rpm) for 2 h at 30°C. In older cultures, fruit bodies at various stages of development were removed beforehand. The mixture was squeezed through nylon mesh, the filtrate further clarified by centrifugation at 5000 × g for 30 min at 4°C, and the supernatant retained. This extraction procedure appears to be highly effective since laccase levels detected in reaction mixtures containing samples of treated compost in place of buffer extract were always less than 5% of values recorded for corresponding volumes of extract supernatant.

2.2. Sampling procedures

Liquid cultures were harvested at specified time intervals and laccase activity was assayed in the culture supernatants. Fungal biomass was determined by filtering mycelia through tared Whatman No. 1 filter paper, washing with distilled water and drying to constant weight at 95°C.

At periodic intervals throughout a 25-day experimental period, the compost cultures (three replicates) were cut longitudinally through the centre of the cylinder and one portion (half the total weight of the compost culture) suspended in 10 volumes (v/dry weight) 50 mM KH2PO4 buffer, pH 6.5, and shaken (150 rpm) for 2 h at 30°C. In older cultures, fruit bodies at various stages of development were removed beforehand. The mixture was squeezed through nylon mesh, the filtrate further clarified by centrifugation at 5000 × g for 30 min at 4°C, and the supernatant retained. This extraction procedure appears to be highly effective since laccase levels detected in reaction mixtures containing samples of treated compost in place of buffer extract were always less than 5% of values recorded for corresponding volumes of extract supernatant.

2.3. Enzyme assay

Laccase activity was determined at 32°C using 2,2′-azino-bis-ethylbenthiazoline (ABTS) in reaction mixtures (1 ml) containing 0.1 M sodium acetate buffer (pH 5), 0.03% (w/v) ABTS, and an appropriate amount of culture supernatant. One unit of enzyme activity is defined as the amount of enzyme required to oxidise 1 μmol ABTS per minute using an ε190 value for oxidised ABTS of 3.6 × 10^4 mol⁻¹ cm⁻¹ [11].

2.4. Polyacrylamide gel electrophoresis (PAGE) and activity staining of gels

Native PAGE (12% w/v gel) was performed on aliquots of culture fluids 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.

2.5. Protein determination

Protein was determined by the method of Bradford [12] with bovine serum albumin as standard.

3. Results and discussion

3.1. Effect of copper on laccase induction in submerged culture

In submerged cultures of V. volvacea, two laccase isoforms were induced, one more strongly than the other, in response to copper supplementation of the culture medium. The effect of different Cu concentrations on laccase production in 14-day HN cultures of V. volvacea V14 is shown in Fig. 1. Readily detectable levels of laccase were observed in cultures supplemented with 50–300 μM CuSO4 with the highest enzyme titres recorded at concentrations of 200 μM CuSO4. No laccase activity was detected in the absence of added Cu. Copper supplementation at concentrations over the range 0–300 μM only marginally affected fungal growth in HN cultures and the influence of Cu on laccase production was not due to increased biomass production. Native PAGE analysis of culture fluids and activity staining of the gels revealed at least two protein bands (a strong staining band and a fainter second band) with laccase activity (Fig. 1). Positive regulation of laccase protein and laccase gene transcription levels by Cu was also reported in species of Trametes [13,14] and Pleurotus [15–17]. Experiments with purified laccase showed that Cu not only induces laccase by the expression of laccase genes, but it also positively affects the activity and stability of the enzyme [17]. The effect on enzyme stability may be due to inhibition by Cu of an
extracellular protease shown to be responsible for the degradation of laccase [18].

3.2. Laccase production over time courses under HN and LN conditions

Time courses for laccase activity in the extracellular fluid of LN and HN *V. volvacea* cultures supplemented with 200 µM CuSO₄ are shown in Fig. 2. In LN cultures, laccase was detectable after 6 days but enzyme levels were relatively low (3.4 U l⁻¹) and increased only slightly to a maximum of 7.2 U l⁻¹ over the 16-day growth period. In HN cultures, laccase activity was detected after 8 days incubation and reached peak levels (31.8 U l⁻¹) at day 14. Native PAGE analysis of culture fluids and activity staining of the gels (Fig. 2) revealed the same band patterns observed in Cu-induced cultures.

Increased fungal biomass was not solely responsible for the higher laccase activities in HN cultures of *V. volvacea* since maximal mycelial growth yields were approximately twice those of LN cultures compared to a 4.4-fold increase in enzyme levels (Fig. 2). Enzyme levels were also higher in HN cultures of *Lentinula edodes* [19], and a direct correlation was observed between nutrient nitrogen and the expression level of a laccase gene in *Trametes versicolor* [14]. In *Pleurotus sajor-caju*, although peak levels of total laccase activity were considerably higher in HN compared to LN cultures, increased fungal biomass production accounted for this difference and enzyme specific activities (i.e. U mg⁻¹ biomass) were similar under both sets of growth conditions [20].

A major distinguishing feature of laccase production in liquid cultures of *V. volvacea* is that enzyme activity under both LN and HN conditions was detectable in culture fluids only in the later stages of primary growth when fungal biomass production had reached a maximum. This is in sharp contrast to the pattern of laccase production reported for other basidiomycetes where enzyme synthesis is associated with primary growth [14,16,20,21]. Lignin peroxidase and manganese peroxidase, two other enzymes generally considered to play key roles in lignin breakdown, were not detected in *V. volvacea* cultures under either growth condition. These results suggest that laccase production may be an important mechanism for lignin degradation in *V. volvacea*.

![Fig. 1. Effect of copper on fungal growth and laccase induction.](image1.png)

![Fig. 2. Time courses of laccase production by *V. volvacea* under low and high nutrient nitrogen conditions.](image2.png)

![Fig. 3. Effect of aromatic compounds on laccase production and isozyme distribution in *V. volvacea*.](image3.png)
3.3. Effect of aromatic compounds on laccase induction in submerged culture

The induction of laccase by different aromatic compounds in HN cultures of *V. volvacea* containing a reduced CuSO₄ content (1.6 μM) is shown in Fig. 3. After 11 days incubation in the presence of inducer, highest titres of laccase were observed in cultures supplemented with ferulic acid (52.2 U l⁻¹), 2,5-xylidine (43.5 U l⁻¹), veratic acid (43.2 U l⁻¹) and 4-hydroxybenzoic acid (33.6 U l⁻¹). 4-Hydroxybenzaldehyde also induced lower levels of laccase (12.3 U l⁻¹) but no enzyme activity was detected in cultures supplemented with p-coumaric acid, syringic acid, vanillic acid, homovanillin or catechol. Native PAGE analysis of laccase-induced culture fluids revealed two protein bands staining with ABTS. However, in this case, the bands stained with equal intensity. Basidiomycetes exhibit widely varying responses in terms of the effects elicited by aromatic compounds on laccase production. Thus, an individual aromatic compound may increase laccase titres without altering the isoform pattern [21], induce new laccase isoforms [24,25], or have no induction effect [26]. Induction is considered to be a protective response to toxic compounds produced either during the degradation of the lignin component of lignocellulosic residues which serve as the natural growth substrates of the fungi or as antimicrobial agents secreted by microbial competitors [14,21].

![Fig. 4. Extracellular laccase production during the developmental cycle of *V. volvacea*.](image)

**Fig. 4.** Extracellular laccase production during the developmental cycle of *V. volvacea*. • Laccase, total protein, Values represent the mean ± S.D. of triplicate samples. Development stages: 0–16 days, substrate colonisation phase; 17 days, pinheads; 22 days, button stage; 23 days, egg stage; 24 days, elongation stage; 25 days, mature fruit body.

3.4. Laccase production in compost cultures

Laccase activity in extracts of composts prepared at periodic intervals during mycelial growth and fruit body formation is shown in Fig. 4. Colonisation of these miniaturised compost cultures was normally complete after 10 days incubation at 32°C. Sporophore development occurred over 7–9 days with the first pinheads detected after 17 days, and buttons observed after 20–22 days, respectively. Once the button stage was reached, the fruit bodies grew very rapidly: the egg stage was evident on day 23, elongation took place on day 24, and formation of the mature fruit body was usually complete after 25 days. Only very low laccase levels were detectable throughout the substrate colonisation phase but enzyme activity increased markedly during the period from fruit body initiation up to the maturation stage. The increase in laccase activity was observed only in those composts that produced fully developed sporophores. Total protein in extracts of the compost increased throughout the substrate colonisation phase (10 days) and declined after the onset of fruiting (Fig. 4).

The pattern of laccase production by *V. volvacea* in the compost cultures is therefore completely different from that observed with other mushroom species such as *Agaricus bisporus* [7,27], *Pleurotus cornucopiae var. citrinopileatus* [28] and *L. edodes* [29,30]. High laccase activities were recorded during mycelial growth of *A. bisporus*, when a large proportion of the compost lignin was degraded [7]. Moreover, laccase gene expression measured as mRNA levels was maximal at the fully colonised stage prior to fruiting and then declined to very low levels during fruiting [31]. Similarly, the activity of laccase is strongly regulated during the development of *L. edodes* fruit bodies [29,30]. The level of laccase transcripts during growth of the fungus on a sawdust-based substrate was maximal during the mycelial growth stage and then declined rapidly at the fruiting stage. Laccase activity in straw cultures of *P. cornucopiae var. citrinopileatus* was also highest during the vegetative mycelial growth phase and declined sharply at the onset of fruiting [28].

The earlier fructification and increased growth yields achieved using cotton waste ‘composts’ compared with the traditional rice straw substrate [32] have led to suggestions that *V. volvacea* is poorly equipped to degrade the lignin component of lignocellulosic residues commonly used for mushroom cultivation [2]. Although we have now demonstrated that the fungus does produce laccase, the temporal correlation established here between laccase production and sporophore formation suggests that, rather than a lignin-degrading function, the enzyme instead plays an important role in the morphogenesis of the *V. volvacea* fruit body. The association of laccase activity with the onset of secondary metabolism in submerged cultures of *V. volvacea* (Fig. 3) lends further support for such a role.
De Vries and coworkers [8] reported that laccase was required for fruit body formation in Schizophyllum commune, and Bu’lock [33] has proposed that phenoloxidases such as laccase may crosslink hyphal walls into coherent aggregates during primordium initiation. It has also been proposed that laccase continues to act on the hyphal surfaces throughout fruit body development [34].

We have recently isolated and cloned four different laccase genes from Volvariella volvacea (Chen, Ge and Buswell, to be published) and are now examining the expression of these genes under different culture conditions in order to assign physiological functions more definitively to the gene products. A fuller understanding of enzyme expression during substrate colonisation and sporophore formation will facilitate the development of strategies aimed at improving mushroom yields.

Acknowledgements

We thank Ce Mun Tang for critical reading of this manuscript. This work was supported by a Grant (CUHK 4063/01M) from the Hong Kong Research Grants Council, and a Strategic Research Grant from The Chinese University of Hong Kong.

References


