FEMS Microbiology Letters 34 (1986) 65–68
Published by Elsevier

FEM 02370

Regulation of extracellular laccase production of *Agaricus bisporus* by nitrogen sources in the medium

(*Agaricus*; *Coprinus*; *Volvariella*; laccase; regulation)

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Received 14 October 1985
Revision received 15 November 1985
Accepted 16 November 1985

1. SUMMARY

Agaricus bisporus was grown on defined liquid media with protein as sole source of carbon, nitrogen or sulphur and with these nutrients supplied in the form of glucose, ammonium or sulphate. Cultures were incubated at 25°C in the dark in static conditions. Mycelia were harvested at suitable intervals, biomass yields determined and culture filtrates tested for extracellular laccase activity. Constitutive laccase production was observed under all conditions tested. However, *Agaricus* laccase, though constitutive, was induced by protein and repressed by ammonium. No detectable extracellular laccase activity was found in similarly tested cultures of *Coprinus cinereus* or *Volvariella volvacea.*

2. INTRODUCTION

The edible mushroom, *Agaricus bisporus,* is cultivated commercially on composted wheat straw [1]. The bulk of compost consists of macromolecules, such as lignin, cellulose, hemicellulose and protein, as well as microbial biomass [2]. Ligno-protein polymers constitute the fraction degraded most rapidly during mycelial growth in compost, and cellulose and hemicellulose during fruiting [3,4]. These nutritional changes are correlated with changes in extracellular enzyme activities in the compost. During mycelial colonisation a high level of laccase enzyme accumulates [5], which then decreases rapidly at the time of fruiting. In contrast, cellulase activity remains low during mycelial growth, increasing rapidly during fruit body development [4,6]. The rapid decrease in laccase activity during the appearance of fruit bodies is thought to be due to both inactivation and proteolysis [7]. Both the active and inactive forms exhibit identical pH optima, molecular weights, substrate specificities and share some immunological properties, differing only in the electrophoretic profiles of native and denatured forms of the enzyme [7]. Enzyme inactivation may occur because the products of enzyme activity are inhibitory to fruit body development, or because a nitrogen recycling mechanism is necessary, involving the reassimilation of the degraded enzyme product [2,7]. Laccase is the major excreted protein of *Agaricus* spp. accounting for 2% of the mycelial protein, or 0.7% of the fungal biomass [8]. The enzyme is produced

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constitutively in *A. bisporus,* as it is in *Polyporus versicolor* [9] and *Podospora anserina* [10]. Its activity can be used as an estimate of mycelial biomass of *Agaricus* in non-sterile solid substrates, such as composted wheat straw, since its activity is proportional to fungal biomass during the colonisation stage [11]. As part of a general investigation of resource utilisation by the two commercially cultivated mushrooms, *A. bisporus* and *V. volvacea,* in comparison with *C. cinereus,* a common weed on commercial mushroom beds, we have examined laccase production in various experimental circumstances.

3. MATERIALS AND METHODS

Stock cultures of *A. bisporus* strain D791 and of *V. volvacea* Indonesia strain were maintained on 2% (w/v) malt extract agar (slopes and plates). Stocks of *C. cinereus* Birmingham dikaryon strain (ATCC42721) were maintained on complete medium agar. The cultures were incubated at 25°C, and 7–10-day-old cultures of *C. cinereus* and *V. volvacea,* and 3–4-week-old cultures of *A. bisporus* were transferred to liquid media prior to inoculum preparation. *C. cinereus* was grown on a defined basal medium, designated SNC [12], containing 1% (w/v) D-glucose (= 56 mM), 30 mM NH_4Cl, and 1% (w/v) Bacto casamino acids (Difco). *A. bisporus* was grown on a basal medium (here called Treschow) as determined by Treschow [13] and described by Fermor and Wood [14]. To the medium was added 1% (w/v) glucose and 2 mM L-glutamate (Sigma). *V. volvacea* was grown on a 2% Bacto malt extract (Difco) liquid medium (50 ml in a 250-ml flask). The SNC basal medium contained (per l): 1.45 g Na_2HPO_4, 1.35 g KH_2PO_4; 0.5 g $MgSO_4 \cdot 7H_2O$. The Treschow basal solution contained (per l): 0.2 g KCl; 0.5 g $MgSO_4 \cdot 7H_2O$; 0.2 g $CaCl_2$; 10 mg $FeCl_3 \cdot 6H_2O$; 1.125 g $Na_2HPO_4 \cdot 2H_2O$; 0.135 g KH_2PO_4. All media were supplemented (1 $ml \cdot l^{-1}$) with a vitamin solution (20 mg thiamin-HCl + 2 mg biotin in 100 ml water), and with 1 $ml \cdot l^{-1}$ of a trace element solution containing (in 100 ml) 30 mg H_3BO_3 + 25 mg $CuSO_4 \cdot 5H_2O$ + 200 mg $MnCl_3 \cdot 4H_2O$ + 40 mg $Na_2MoO_4 \cdot 7H_2O$ + 20 mg $ZnSO_4 \cdot 7H_2O$ + 70 mg $CoCl_2 \cdot 6H_2O$. All the media were sterilised by autoclaving at 15 psi for 15 min. All three species were incubated in the dark at 25°C in static culture.

Mycelial suspensions were prepared by centrifuging liquid cultures of the organisms on a bench centrifuge (5000 rev./min, 20 min) in sterile centrifuge tubes. The supernatant was decanted, the pellet rinsed with sterile distilled water and resuspended in approximately the same volume of distilled water, containing about 100 glass beads (4.5–5.5 mm diameter). The suspensions were shaken in an orbital shaker (300 rev./min, 20 min) to provide a fragmented mycelial suspension.

Each of the species was grown on Treschow medium, using identical quantities of trace elements and vitamins. The media contained different combinations of: 1.0% (w/v) insoluble casein (BDH No. 44018); 1.0% (w/v) glucose and/or 30 mM NH_4Cl. The different combinations used are described below. Basal media solutions and nutrient solutions were autoclaved separately. The constituents were subsequently mixed in suitable proportions and made up to 25-ml volumes in 250-ml flasks. These were inoculated with 0.5-ml volumes of mycelial suspensions. All test media were prepared at least in duplicate, and mostly in triplicate. The cultures were incubated in static conditions at 25°C, in the dark, and were harvested at suitable intervals. *C. cinereus* cultures were harvested at 3-day intervals up to day 15. *A. bisporus* cultures were harvested on day 9, and then every 8 days up to day 49. *V. volvacea* cultures were harvested after 4 days, and then every 5 days up to 24 days. The cells were harvested by filtration using Whatman GF/A filter discs. The mycelium, harvested by filtration, was dried to constant weight at 60°C.

Laccase was assayed polarographically [4] using an oxygen electrode. Each sample was assayed in duplicate. 1.0 ml of sample was mixed with 1.7 ml of 0.2 M sodium acetate-acetic acid buffer pH 5.6 and allowed to equilibrate at 25°C. The mixture was placed in an oxygen electrode chamber and the reaction initiated by the addition of 0.3 ml of 0.1 M *p*-phenylenediamine. Initial rates of oxygen consumption were recorded using a Vitatron chart recorder, and the amount of oxygen consumed

calculated assuming a solubility of oxygen of 7.72 $mg \cdot l^{-1}$. One unit of laccase was defined as the amount of enzyme catalysing the consumption of 1 μmol oxygen $\cdot$ min^{-1}.

4. RESULTS AND DISCUSSION

Results are shown from experiments with 5 different medium constitutions. In one the three major elements were supplied as 56 mM glucose, 30 mM NH_4Cl and 2 mM $MgSO_4 \cdot 7H_2O$ while each of the other mixtures contained protein (as 1% insoluble casein). In one this was the sole source of C, N and S; the others had additional carbon (56 mM glucose) and/or nitrogen (30 mM NH_4Cl) supplementation.

When either glucose or protein was supplied as sole carbon source biomass yields were broadly similar but there was a considerable synergistic response by all three species to media containing both carbon sources; growth being increased by a factor of 3–4 (Fig. 1). Although some differences in response to the media tested were observed these were rather minor and in general the three basidiomycetes behaved remarkably similarly.

No extracellular laccase activity was found in any culture of *C. cinereus* or *V. volvacea*. In the case of *Agaricus bisporus*, extracellular laccase activity was detected in all media examined. The same pattern of results was evident whether lac-

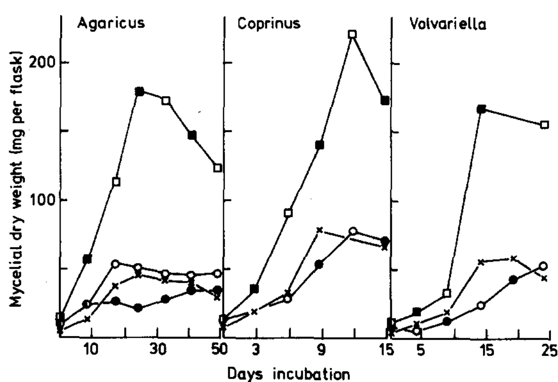

Fig. 1. Biomass yields of shake-flask cultures of 3 basidiomycetes grown in medium supplemented with: ●, protein only; ○, protein and ammonium; ■, protein and glucose; □, protein, ammonium and glucose; or ×, glucose, sulphate and ammonium. Data points are the means of 3 replicates; standard error being less than 10% of the mean; where the results for different media were not significantly different they have been combined in a single plot with alternating symbols. Thus, there was no significant difference with any of the three species tested between biomass yield on protein + glucose and on protein + ammonium + glucose, and for *C. cinereus* and *V. volvacea* there was no significant difference between growth on protein alone and on protein + ammonium.

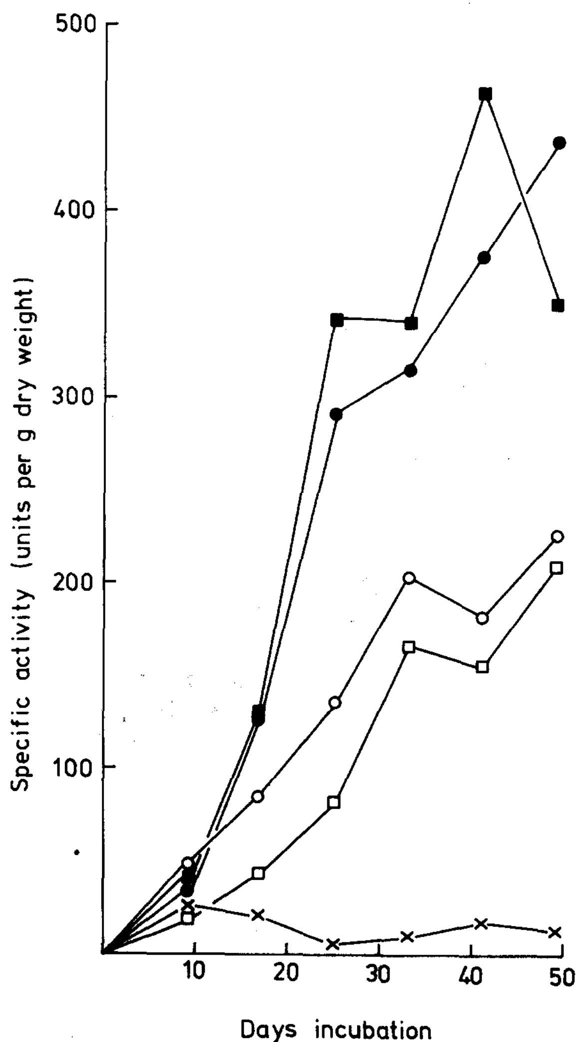

Fig. 2. Extracellular laccase activity produced by *A. bisporus*. The data show specific activity levels (units of activity per g mycelial dry wt.) recorded from cultures grown in medium supplemented with: ●, protein only; ○, protein and ammonium; ■, protein and glucose; □, protein, ammonium and glucose; or ×, glucose, sulphate and ammonium. Data points are the means of 3 replicates, standard error being less than 10% of the mean.

case activities were expressed as total activity (units per flask irrespective of the amount of mycelium) or as a specific activity (U/g dry wt. of mycelium) so only the latter are shown in Fig. 2. The greatest laccase activity was found in filtrates of media supplied with protein and glucose as carbon sources. In media containing protein plus glucose further supplementation with ammonium caused a 50% decrease in laccase activity. In the absence of supplied protein total laccase activity was low. A maximum specific activity of 430 U/g dry wt. was observed in cultures grown without ammonium supplementation; the maximum specific activity of cultures grown with protein plus ammonium was 210 U/g dry wt.

Demonstration of extracellular laccase activity only in the culture filtrates of *A. bisporus,* seems to confirm previous findings that this organism secretes laccase in an apparently constitutive manner [7] and that *C. cinereus,* like *C. congregatus* [15], and *V. volvacea* [16] do not produce this enzyme extracellularly. We take this to imply some sort of resource specialisation between organisms which otherwise appear to occupy the same habitat (composted plant remains). The *A. bisporus* laccase, though considered to be constitutive, is clearly strongly regulated in a positive manner by protein and is repressed by ammonium. The presence of protein effected a 6-fold increase in the laccase specific activity (as defined above). Supply of additional ammonium reduced the specific activity by 50%. In composts, *A. bisporus* degrades macromolecules, especially lignoproteins, and laccases are used to detoxify aromatic metabolites formed from lignin degradation [17]. Proteases induced by exogenous substrate proteins may therefore act to stimulate increased laccase secretion as part of an integrated nitrogen recycling mechanism providing for the most efficient utilisation of lignoprotein complexes. The adverse effect of ammonium on laccase production is difficult to account for without detailed knowledge of the precise metabolic role of this enzyme. Supplementation of these media with ammonium had no effect on the utilisation of protein substrates for growth and had a slightly stimulatory effect on the production of extracellular proteinases by *A. bisporus* (unpublished observations).

ACKNOWLEDGEMENTS

HMK thanks the Science and Engineering Research Council for the award of a research studentship which enabled this work to be carried out.

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