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Enhanced Formation of Extracellular Laccase Activity by the White-Rot Fungus *Trametes multicolor*

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Abstract

The white-rot fungus *Trametes multicolor* MB 49 has been identified as an excellent producer of the industrially important enzyme laccase. The formation of extracellular laccase could be considerably stimulated by the addition of Cu(II) to a simple, glycerol-based culture medium. In this study, optimal concentrations of copper were found to be 0.5–1 m*M*, which were added during the growth phase of the fungus. Other medium components important for laccase production are the carbon and nitrogen sources employed. When using an optimized medium containing glycerol (40 g/L), peptone from meat (15 g/L), and $MgSO_4 \cdot 7H_2O$ and stimulating enzyme formation by the addition of 1.0 m*M* Cu, maximal laccase activities obtained in shake-flask cultures were approx 85 U/mL. These results, however, could not be scaled up to a laboratory fermentor cultivation. Laccase production by *T. multicolor* decreased considerably when the fungus was grown in a stirred-tank reactor, presumably because of damage of the mycelia caused by shear stress and/or changes in the morphology of the fungus.

Index Entries: *Trametes multicolor*; laccase; polyphenol oxidase; culture medium development; morphology.

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Introduction

Laccases (benzenediol:oxygen oxidoreductases; EC 1.10.3.2.), multicopper-containing enzymes belonging to the blue oxidases, catalyze the one-electron oxidation of a wide variety of inorganic and aromatic substrates, particularly phenols, with the concomitant four-electron reduction of oxygen to water *(1–4)*. Laccases are produced by the majority of white-rot fungi described to date as well as by other types of fungi, some bacteria, insects, and plants *(5–7)*. Fungal laccases are believed to be involved in the degradation of lignin and/or in the removal of potentially toxic phenols arising during this degradation *(2,8)*. In addition, fungal laccases are hypothesized to take part in the synthesis of dihydroxynaphthalene melanins *(9)*, fungal morphogenesis *(10,11)*, and phytopathogenesis and fungal virulence *(12,13)*.

In white-rot fungi, laccases are typically produced as multiple isoenzymes *(3,4,14)*. In several organisms, extracellular laccases are constitutively produced in small amounts *(14)*. However, their production can be considerably stimulated by the presence of a wide variety of inducing substances, mainly aromatic or phenolic compounds related to lignin or lignin derivatives, such as ferulic acid, 2,5-xylidine, p-anisidine, or veratryl alcohol *(7,15,16)*; aliphatic alcohols *(17)*; or polysaccharides *(18)*; as well as by aqueous plant extracts *(19,20)*. In addition, the production of laccase can be influenced by the nitrogen (N) concentration in the culture medium. Typically, higher N levels are required to increase laccase formation *(7)*, but with certain organisms such as *Pycnoporus cinnabarinus* or *P. sanguineus* N-limited culture conditions enhance the production of this enzyme *(15,21)*. Whereas the increased production of laccase by fungi in response to aromatic and phenolic substances is well documented, an important effect of copper on laccase formation has only recently been reported *(22,23)*. This stimulating effect of low amounts of Cu was found to be especially effective in several *Trametes* spp. including *T. multicolor (24)*. Copper is an essential micronutrient for most living organisms *(25)*, and copper requirements by microorganisms are usually satisfied by very low concentrations of the metal in the order of 1–10 μM. Yet, copper present in higher concentrations of its free, cupric form is extremely toxic to microbial cells, and, thus, its uptake, binding, and concentration in the fungal cell is well regulated *(26)*.

Laccases have became important, industrially relevant enzymes because of a number of diverse applications—e.g., for biocatalytic purposes such as delignification of lignocellulosics and crosslinking of polysaccharides, for bioremediation applications such as waste detoxification and textile dye transformation, for food technologic uses, for personal and medical care applications, and for biosensor and analytical applications *(27)*. To utilize laccases more efficiently for these biotechnologic and environmental applications and to understand better the properties of these important enzymes at a molecular and kinetic level, large amounts of crude and purified laccases are required *(7)*. At present, research and application

are sometimes hindered by the rather low yields of the enzyme formed by wild-type organisms, but also by the difficulties in efficiently overexpressing laccases heterologously in active form *(28)*. In the present study, laccase formation by the white-rot basidiomycete *T. multicolor*, which only recently has been identified as an excellent source of this enzyme *(24)*, was studied in detail, and several components of the culture medium important for laccase production were identified.

Materials and Methods

Chemicals

Unless otherwise stated chemicals were obtained from Sigma (St. Louis, MO) and were of the highest purity available. Glycerol, peptone from soybean (enzymatic digest; 9.3% total N), and meat extract (12% total N) were from Fluka (Buchs, CH), and peptone from meat (12% total N), peptone from chicken (12% total N), and yeast extract (11% total N) were from Merck (Darmstadt, Germany). Peptone from casein (pancreatic digest) was from three different suppliers (Sigma: tryptone, 13% total N; Fluka: 13% total N; Merck: 13.5% total N).

Microbial Strain and Culture Conditions

The wild-type strain of the basidiomycete *T. multicolor* MB 49 was isolated from hardwood in Southern Germany and was obtained from the culture collection of the Institute of Applied Microbiology, University of Agricultural Sciences Vienna, where it is deposited under the indicated strain number. Stock cultures were maintained through periodic transfer at 25°C on potato dextrose agar plates (Difco, Detroit, MI). Shake-flask cultures of the organism were grown at 25°C with continuous agitation at 110 rpm in baffled 1000-mL Erlenmeyer flasks containing 200 mL of medium. Unless otherwise indicated, the basal medium used for experimental cultures contained 20 g/L of glucose, 5 g/L of yeast extract, 5 g/L of peptone from casein (Fluka), and 1 g/L of $MgSO_4 \cdot 7H_2O$ *(24)*. The pH was adjusted to 5.0 with H_3PO_4 prior to sterilization. Several agar plugs cut from the actively growing, outer circumference of a fungal colony growing on potato dextrose plates was used as inoculum. Inoculated flasks were cultivated for up to 34 d. Unless otherwise stated, a sterile stock solution of $CuSO_4 \cdot 5H_2O$ (1 *M* in water) was added to the actively growing culture after 10 d of incubation so that the final concentration in the medium was 1.0 m*M (24)*. Samples from duplicate flasks were taken periodically, centrifuged, and the clear supernatant was used for the determination of enzyme activity.

Bioreactor Cultivation

Cultivation was carried out in a 20-L stirred tank bioreactor (MBR Bioreactor, Wetzikon, Switzerland) with a working volume of 15 L. The

bioreactor was equipped with instrumentation for measurement and/or control of agitation, temperature, pH, dissolved oxygen concentration (P_{O_2}) and foam. The agitator was equipped with four standard disk turbine impellers, each with six flat blades. The culture medium contained 40 g/L of glycerol, 15 g/L of peptone from meat, and 1.0 g/L of $MgSO_4.7H_2O$ and was sterilized *in situ* at 121°C for 30 min. A 1*M* stock solution of $CuSO_4$ in water was added after 4 days so that the final Cu(II) concentration in the medium was 1.0 m*M*. During the cultivation, the temperature was maintained at 25°C and the pH, which was initially adjusted to 5.0 using phosphoric acid, was allowed to float. Agitation was constant at 100 rpm, and aeration was automatically varied from 0.1 to 1.25 vvm to maintain a P_{O_2} of 40% of air saturation. The preculture (7.5% [v/v]) was a 14-d-old shake-flask culture grown on the same basal medium as used for the cultivation in the bioreactor but without the addition of Cu.

Enzyme Activity Assay

Laccase activity was determined with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) as the substrate *(29)*. The assay mixture contained 1 m*M* ABTS, 20 m*M* sodium citrate buffer (pH 3.5), and 10 µL aliquots of appropriately diluted culture fluid. Oxidation of ABTS was monitored by following the increase in A_{436} ($\varepsilon = 29.3\ mM^{-1}\ cm^{-1}$). One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS/min at 25°C.

Other Analyses

Mycelial dry wt was determined by filtration through tared filter disks (ME 25/21, 0.45 µm; Schleicher and Schüll, Dassel, Germany), dried at 105°C until constant, and weighed. Glycerol concentration in the fermentation samples was determined by high-performance liquid chromatography on an Aminex HPX-87 H column (7.8 × 300 m*M*, Bio-Rad, Hercules, CA) operated at 65°C, using refractive index detection and an eluent of 0.01 *N* sulfuric acid at 0.6 mL/min.

Results

Production of Laccase in the Presence of Copper

We selected *T. multicolor* as the source of laccase following a screening of a number of basidiomycetes for an efficient producer of this industrially relevant enzyme *(24)*. To study laccase formation by *T. multicolor* in more detail, the synthesis of laccase activity by this organism grown on a complex, glucose-based medium supplemented with various concentrations of $CuSO_4$, which was added after 10 d of cultivation to the actively growing culture, was followed over a time course of 30 d. Figure 1 presents the results of these shake-flask experiments obtained after 20 d of growth. Formation of laccase in the control experiment in which the addition of

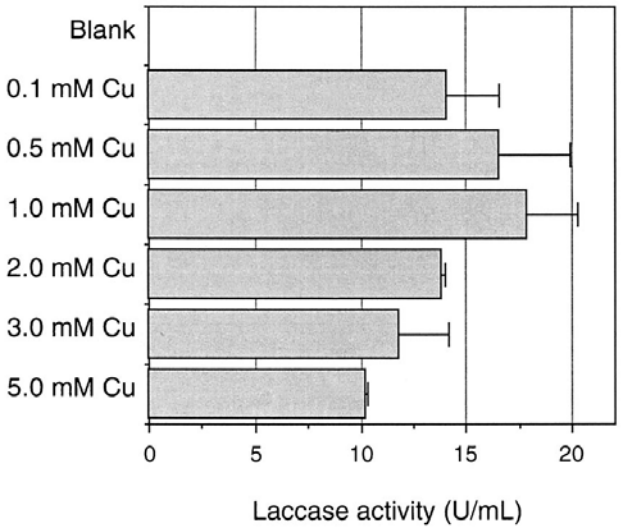

Fig. 1. Effect of various concentrations of $CuSO_4$ on laccase formation by *T. multicolor* MB 49. The organism was grown on a complex, glucose-based medium, to which Cu was added after 10 d of cultivation. Data given were obtained after 20 d of growth at 25°C and 110 rpm. Values shown are the mean of duplicate experiments and error bars represent the SD.

CuSO4 to the growth medium was omitted was negligible (<0.02 U/mL), while the supplementation of Cu, even in low concentrations, resulted in a marked increase in laccase activity formed. The optimal copper concentration for laccase synthesis by *T. multicolor* was found to be 0.5–1.0 m*M*. By using these concentrations, approx. 16.5–18.0 U/mL of laccase activity was obtained (Fig. 1). Interestingly, even high Cu concentrations of 5 m*M* when added after 10 d of growth stimulated laccase formation significantly, reaching comparably high laccase activity (18.7 U/mL). These values, however, were only obtained after 26 d of cultivation, while the enzyme activity after 20 d was significantly lower (Fig. 1). Apparently the high copper content in the medium inhibits growth of *T. multicolor* to a certain extent *(30)*.

Development of Culture Medium

To investigate the effect of the carbon (C) source on laccase production by *T. multicolor*, various carbohydrate substrates were added at a constant concentration of 20 g/L to a basal medium containing yeast extract (5 g/L), peptone from casein (5 g/L), and $MgSO_4 \cdot 7H_2O$ (1 g/L). In addition, different concentrations of glucose were evaluated pertaining to their effect on enzyme formation. Laccase synthesis was stimulated by the addition of Cu, which was added after 10 d of growth so that the final concentration in the medium was 1.0 m*M*. Growth of *T. multicolor* was good on most of the substrate tested; one exception was α-cellulose, which sustained only poor growth during the time investigated. Laccase was produced on all the C sources employed, albeit to a greatly varying extent (Table 1). In general,

Table 1
Effect of Various Carbon Sources on
Growth and Formation of Laccase by *T. multicolor* [a]

C source (concentration)	Biomass (g/L) [b]	Laccase activity (U/mL)
Glucose (10 g/L)	9.2	7.3
Glucose (20 g/L)	13.1	14.0
Glucose (40 g/L)	24.8	20.8
Cellobiose (20 g/L)	11.6	7.9
α-Cellulose (20 g/L)	ND	0.8
Glycerol (20 g/L)	36.2	33.2
Lactose (20 g/L)	18.2	16.0

[a]Substrates were added in the concentrations indicated to a medium containing yeast extract (5 g/L), peptone from casein (5 g/L), and $MgSO_4 \cdot 7H_2O$ (1 g/L). Copper was added after 10 d of growth so that the final concentration in the medium was 1 m*M*. Growth conditions: 20 d, 25°C, 110 rpm.

[b]Mycelial wet wt.

ND, not determined.

increased biomass formation also led to increased laccase formation in this experiment. This is among others evident from the cultivations using different glucose concentrations, in which increasing substrate concentrations resulted in elevated biomass and laccase production. Best results pertaining both to growth of *T. multicolor* and to laccase formation were obtained when using glycerol, which was selected as the substrate for further experiments. Glycerol as the C source also influenced the growth morphology of *T. multicolor* distinctly. When cultivated on this substrate the fungus formed small pellets with a diameter of less than 1 mm. By contrast, the pellets formed during cultivation on glucose were significantly larger (average diameter of approx 5 mm).

Since one of the objectives of this work was to identify medium components that positively affect laccase formation in *T. multicolor*, the effects of various, mainly complex N sources were studied subsequently. Both the nature and the concentration of the N source employed were reported to be of considerable importance for laccase synthesis in different fungi *(7,15,22,31,32)*. The indicated N sources were added in the concentrations stated (Table 2) to a medium containing glycerol (20 g/L) and $MgSO_4 \cdot 7H_2O$ (1 g/L). Cu was again added after 10 d of growth to a final concentration of 1.0 m*M*. Table 2 gives the results for growth and laccase formation. All of the complex, organic nutrients tested sustained good growth of the fungus.The addition of these organic N sources seems to be necessary for laccase production, since they could not be substituted by $(NH_4)_2SO_4$ or urea, which gave only low yields of laccase activity or resulted in no growth of the fungus, respectively. Distinct differences pertaining to laccase pro-

Table 2
Effect of Various Nitrogen Sources on Formation of Laccase by *T. multicolor* When Grown in Shake-Flask Cultures[a]

N source (concentration)	Biomass (g/L)[b]	Laccase activity (U/mL)
Yeast extract (5 g/L) + peptone/casein (Fluka; 5 g/L)	34.4	33.2
Peptone/casein (Fluka; 10 g/L)	8.5	1.6
Peptone/casein (Merck; 10 g/L)	7.9	8.0
Peptone/casein (Sigma; 10 g/L)	15.2	32.3
Peptone/chicken (10 g/L)	8.5	25.2
Peptone/meat (10 g/L)	26.3	42.9
Peptone/soybean (10 g/L)	40.4	12.3
Meat extract (10 g/L)	30.1	17.6
Yeast extract (10 g/L)	19.6	14.4
$(NH_4)_2SO_4$ (4.7 g/L) + yeast extract (1 g/L)	4.6	0.43
Urea (2.15 g/L) + yeast extract (1 g/L)	No growth	—

[a]The growth substrate was glycerol (20 g/L). Urea and $(NH_4)2SO_4$ were added on the basis of an equivalent N concentration. Copper was supplemented after 10 d of growth so that the final concentration in the medium was 1 m*M*. Growth conditions: 20 d, 25°C, 110 rpm.

[b]Mycelial wet wt.

duction by *T. multicolor* are obvious for the different N sources examined in this experiment. Especially peptone from meat stimulated the formation of extracellular laccase activity considerably, giving the highest result of 42.9 U/mL. Interestingly, the results obtained when using several preparations of peptone from casein (pancreatic digest), which were purchased from different suppliers, were quite ambiguous, with laccase activities ranging from 1.6 to 32.3 U/mL.

Subsequently, the optimal concentrations of the best C and N source, (i.e., glycerol and peptone from meat), were determined. The glycerol concentration was varied over a range of 20–60 g/L with a fixed concentration of 10 g/L of peptone from meat. Figure 2 presents the results of this experiment. Raising the glycerol concentration from 20 to 40 g/L almost doubled the yield of laccase. A further increase in this substrate's concentration resulted only in a slight increase in laccase activity; however, the lag phase until the start of laccase excretion was prolonged by more than 5 d. As is obvious from Fig. 2, laccase production only commenced when glycerol was consumed from the medium. Apparently, high glycerol concentrations inhibit laccase synthesis in *T. multicolor*. In a similar way, the pH, which initially dropped to approx 3.5 during consumption of the substrate, increased sharply once glycerol was completely depleted (Fig. 2).

A variation in the concentration of peptone from meat (5–40 g/L) affected laccase production markedly. Highest laccase formation was observed when using 15 g/L of this complex N source and 40 g/L of glycerol

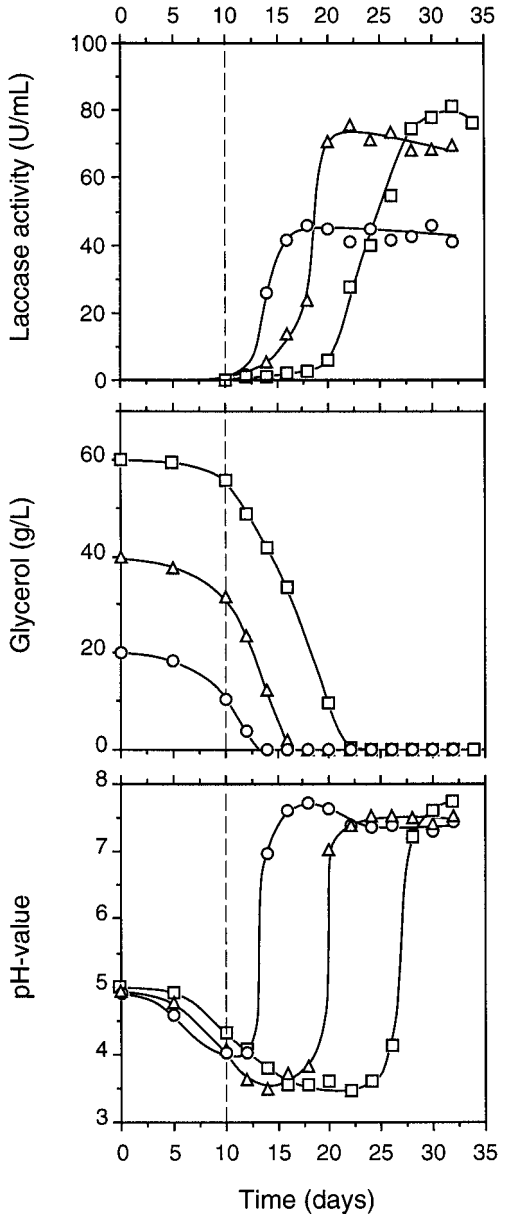

Fig. 2. Effect of varying glycerol concentrations (20–60 g/L) on laccase formation by *T. multicolor* MB 49. In addition to the carbon source, the growth medium contained 10 g/L of peptone from meat, and 1 g/L of $MgSO_4 \cdot 7H_2O$; $CuSO_4 \cdot 5H_2O$ was added after 10 d (dashed line) so that the final concentration was 1.0 m*M*. Values shown are the mean of duplicate experiments. (—○—), 20 g/L; (—△—), 40 g/L; (—□—), 60 g/L.

(86.4 U/mL). A further increase in the concentration of the peptone under otherwise identical growth conditions resulted in a dramatic decrease in laccase production. Laccase activities obtained when using 25 and 40 g/L of this complex N source were 18.0 and 12.5 U/mL, respectively. This sig-

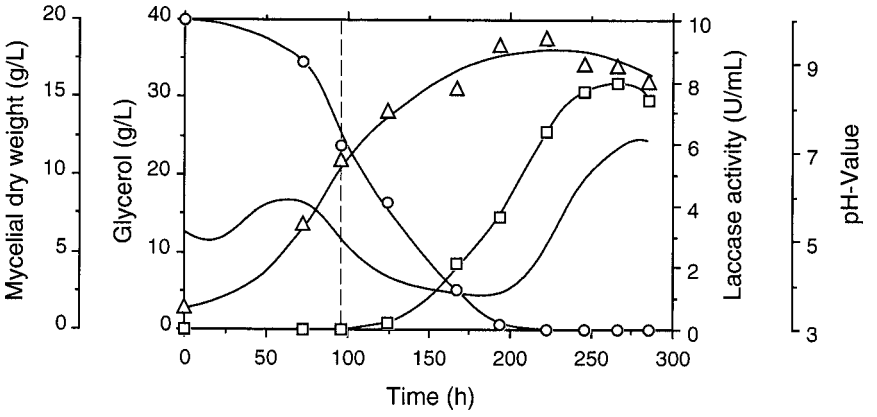

Fig. 3. Time course of laboratory cultivation of *T. multicolor* MB 49 in 20-L stirred tank reactor (working volume of 15 L) using medium containing 40 g/L of glycerol, 15 g/L of peptone from meat, and 1 g/L of $MgSO_4 \cdot 7H_2O$; $CuSO_4 \cdot 5H_2O$ was added after 96 h (dashed line) so that the final concentration was 1.0 m*M*. The temperature was controlled at 25°C and the pH, initially adjusted to 5.0, was allowed to float. Aeration was automatically varied from 0.1 to 1.25 vvm to maintain a P_{O2} 40% of air saturation, and agitation was constant at 100 rpm. (—○—), glycerol; (—△—), mycelial dry weight; (——), pH-value; (—□—),laccase activity.

nificant reduction in laccase formation seems to be caused by a decrease in growth of the fungus observed when using the higher concentrations of peptone *(30)*; the reason for this, however, is not known at present. A variation in the peptone concentration affected the time course of the culture pH during these cultivations to some extent. When using peptone in lower concentrations (5–25 g/L) the pH decreased to approx 3.5 during the phase of glycerol consumption and concomitantly with substrate depletion increased sharply to approx 7.5, similar to the time dependence shown in Fig. 2. A further increase in the peptone concentration to 40 g/L apparently had a certain buffering effect, and the increase in pH after glycerol consumption was much more gradual.

Bioreactor Cultivation

Production of laccase by *T. multicolor* was studied in a 20-L laboratory fermentor; the working volume was 15 L of a medium based on glycerol and peptone from meat, which was found to be optimal for enhanced laccase formation in the previous experiments. Figure 3 presents the time course of this bioprocess experiment. Laccase synthesis was stimulated by the addition of $CuSO_4 \cdot 5H_2O$ (1 m*M* final concentration) after 96 h of growth. The growth phase of the organism was accompanied by a characteristic decrease in the culture pH, which was not controlled during the fermentation, from 5.0 to 3.8. When glycerol was exhausted from the medium, which occurred after approx 200 h of cultivation, the pH increased sharply. The time of glycerol depletion also coincided with the maximum amount of

biomass formed (18.9 g/L of mycelial dry wt). Subsequently, the biomass concentration decreased slightly, presumably because of the utilization of intracellular storage compounds and/or autolysis. In contrast to the shake-flask cultivation, in which *T. multicolor* grew in small pellets, the growth form in the laboratory fermentor was as dispersed mycelia. Laccase synthesis only started after a lag phase (approx 25 h after the addition of Cu.). The maximum of extracellular laccase activity (8.0 U/mL) was found after 265 h of growth; thereafter, laccase activity decreased slightly. This value, however, is significantly lower than those obtained in shake-flask cultures when using an identical growth medium.

Discussion

Although laccase synthesis in a number of white-rot fungi is known to be influenced by various physiologic factors, most research efforts have been devoted to identifying suitable aromatic inducers and to optimizing their chemical nature, their concentration employed, and the time of their addition *(7,33–35)*. Here we report on some additional environmental factors that can be important for the formation of fungal laccases. Furthermore, the optimization of some of these factors for the production of extracellular laccase by the wood-degrading fungus *T. multicolor* MB 49, which only recently has been shown to be an excellent producer of this enzyme *(24)*, is described.

The addition of low amounts of Cu to the culture medium of some basidiomycete fungi can have a significant stimulatory effect on laccase synthesis as was demonstrated for, e.g., *T. versicolor, Ceriporiopsis subvermispora, Pleurotus ostreatus*, and *T. pubescens (22–24,36)*. In accordance with previous reports, the addition of Cu to the growth medium stimulated remarkably laccase formation by *T. multicolor*, even in the absence of typically used aromatic inducers. The Cu concentration optimal for laccase production by *T. multicolor* was found to be 0.5–1 m*M*. These Cu concentrations are much higher than the 2–600 µM used in typical cultivation media for the production of laccase by other fungal strains *(22,23,37)*. *T. multicolor* was able to grow at Cu concentrations as high as 5 m*M* when this trace metal was added after 10 d of cultivation. Even though growth was markedly reduced at this Cu concentration, maximal laccase activities were comparable with those obtained under optimal conditions.

Another medium component important for laccase production is the N source employed. Although formation of ligninolytic enzyme activities is favored by low N conditions, at least in *Phanerochaete chrysosporium*, the white-rot fungus most intensively studied with respect to its physiology *(38)*, high nitrogen levels seem to be required to obtain greater amounts of laccase *(7,39)*. This can, however, not be generalized, and some researchers have found that laccase activity increases under N-limiting conditions *(15,21)*. In *T. multicolor*, both the type and the concentration of the N source have a profound effect on the levels of laccase activity produced, with

nitrogen-rich media generally enhancing laccase activity. Complex organic N sources such as various peptones or yeast extract are required for the efficient formation of laccase and cannot be replaced by inorganic N sources such as NH_4^+. The optimal N source was found to be peptone from meat in a concentration of 15 g/L (corresponding to approx 125 m*M* peptone-N). A further increase in the concentration of the N source resulted in drastically decreased laccase activities. When using the optimal culture medium—40 g/L of glycerol, 15 g/L of peptone from meat, and 1 g/L of $MgSO_4 \cdot 7H_2O$, laccase activities obtained in shake-flask cultures were approximately 85 U/mL, which is remarkably high when compared to reports on laccase production by other fungi *(15,17,21 22,35,40)*.

Laccase production by *T. multicolor* decreased considerably when the organism was grown in a stirred-tank laboratory fermentor as compared to the cultivations in shake-flasks. A similar negative effect was also observed when comparing the production of a periplasmic enzyme, pyranose oxidase, by the same isolate of *T. multicolor* in shake-flasks and a stirred-tank reactor *(41)*. A possible explanation for this could be damage of the mycelium caused by high shear stress, which most likely will occur near the tips of the disk turbine impellers. These negative effects of shear stress and mechanical forces on filamentous fungi, which can cause breakage of the hyphae and leakage of intracellular material, have recently been reviewed *(42)*. Moreover, a characteristic change in the morphology was observed when comparing growth of *T. multicolor* in shake-flasks and stirred-tank reactors. The fungus formed small pellets in shake flask cultures, whereas it grew in filamentous form in the fermentor cultivations. Fungal morphology is often considered one of the key parameters in industrial fermentations. It is known that the growth form can influence the production of fungal metabolic products to a great extent, yet the desired morphology varies from product to product. For some fungal enzymes, pellet formation has been described as an important factor contributing to high yields. An increase of almost two orders of magnitude in polygalacturonase and α-galactosidase production between the diffuse mycelium and the pellet mycelium of *Aspergillus niger* and *Mortierella viniceae*, respectively, has been reported *(43)*. Possibly, growth conditions that can induce mycelial pellet formation in fermentation cultures, such as reduced shear (e.g., as characteristic for air lift fermentors), or the use of mild nonionic detergents *(43)*, will be favorable for a successful scale-up of the shake-flask experiments and the enhanced production of laccase in *T. multicolor* in fermentor cultivations.

In conclusion, the wild-type basidiomycete *T. multicolor* MB 49 is an extremely efficient producer of laccase activity, which can be obtained in very high yields on a simple, glycerol-based medium that does not contain toxic inducers such as the aromatic compounds routinely used for stimulating laccase synthesis. The addition of millimolar amounts of Cu is sufficient to boost laccase production. Therefore, this strain could be an attractive and alternative source of laccase, an enzyme that has recently

found widespread industrial applications, such as for biocatalytic purposes *(44)*, in the pulp and paper industry *(27)*, for biosensors *(27)* or for bioremediation *(7)*.

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