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Production of Laccase by Immobilized Cells of *Agaricus* sp.

Induction Effect of Xylan and Lignin Derivatives

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Abstract

Laccase was produced in the supernatant of culture of a local isolate of *Agaricus* sp. obtained from decaying *Ficus religiosa* wood. The enzyme was produced at a constitutive level when growing the fungus in a nitrogen-limited medium supplemented with either glycerol, glucose, fructose, mannitol, arabinose, maltose, saccharose, cellulose, or cellobiose. A two- to sixfold increase in enzyme specific activity was observed when growing the strain in the presence of straw, xylan, xylose, lignosulfonate, veratryl alcohol, and ferulic and veratric acid. Experiments are consistent with the existence of an induction control on laccase and the absence of a form of carbon catabolite repression mediated by noninducing carbon sources. Immobilization of the *Agaricus* sp. on several supports, including polyurethane foam, textile strips, and straw, resulted in an increase of enzyme production as compared to cultivation in liquid medium.

Index Entries: *Agaricus*; white-rot fungi; laccase; polyphenol oxidase; cell immobilization; paper industry application.

Introduction

Lignin is a structurally elaborate biopolymer and constitutes one of the most abundant materials in the biosphere. Lignin is quite recalcitrant to degradation, but its mineralization is an important step in the global carbon

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cycle *(1)*. Studies on white-rot fungi, especially *Phanerochaete chrysosporium*, have opened the field for understanding the enzymology and molecular biology of lignin biodegradation *(2)*. White-rot fungi produce extracellular enzymes involved in lignin degradation. These extracellular enzymes include lignin peroxidases, manganese peroxidases, and laccases. Laccase is a polyphenol oxidase (*p*-diphenol oxidase; E.C. 1.10.3.2) that reduces oxygen to water and simultaneously performs one-electron oxidation of many aromatic substrates *(3)*. Laccases have been characterized in several nonlignolytic and lignolytic fungi and, besides a role in delignification, appear to be involved in sporulation, pigment production, and plant pathogenesis *(4)*. Laccase was reported to be produced constitutively by the edible button mushroom *Agaricus bisporus* and was secreted very abundantly, constituting 2% of mycelial protein during vegetative growth *(5,6)*.

Because of possible applications in the pulp and paper industry *(7)*, researchers became interested in improving laccase production. This article reports that xylan and lignin derivatives are possible inducers for laccase of a local isolate of *Agaricus* sp. Enzyme production by the fungus cultivated under immobilized form was better than in submerged culture, a property that could serve for future industrial production of the biocatalyst.

Materials and Methods

Microorganism

The *Agaricus* sp. strain used in this study was isolated from decaying *F. religiosa* wood. The strain was determined by following Ainsworth *(8)*. The culture was maintained on 2% (wt/vol) malt extract agar plates grown at 25°C and stored at 4°C.

Cultivation Conditions

For laccase production and induction studies, three agar cubes ($2 \times 2 \times 2$ mm) cut from colonized malt extract agar plates were inoculated in 200 mL of culture medium contained in 500-mL Erlenmeyer flasks. The basal liquid medium contained the following (per litre): 1.0 g KH_2PO_4; 0.26 g NaH_2PO_4; 0.317 g $(NH_4)_2SO_4$; 0.5 g $MgSO_4 \cdot 7H_2O$; 0.5 mg $CuSO_4 \cdot 7H_2O$; 2.2 g 2,2-dimethylsuccinic acid; 74 mg $CaCl_2 \cdot 2H_2O$; 6 mg $ZnSO_4 \cdot 7H_2O$; 5 mg $FeSO_4 \cdot 7H_2O$; 5 mg $MnSO_4 \cdot 4H_2O$; 1 mg $CoCl_2 \cdot 6H_2O$; 0.5 mL vitamin solution *(9)*. Carbon source was added at 1% (w/v). The pH of the medium was adjusted to 4.5 with NaOH. Cultures were incubated at 25°C either static or on a Bühler KS10 (TCPS, Belgium) rotary shaker (150 rpm). Triplicate cultures were removed at suitable intervals to follow growth and enzyme production. Culture filtrates and mycelium were harvested by filtration through Whatman GF/C glass-fiber discs (9 cm diameter, Merck, Belgium). Mycelial dry weights were estimated after oven drying at 105°C for 24 h.

In growth experiments on insoluble substrates, the production of cell biomass was determined indirectly by estimation of total nitrogen in the

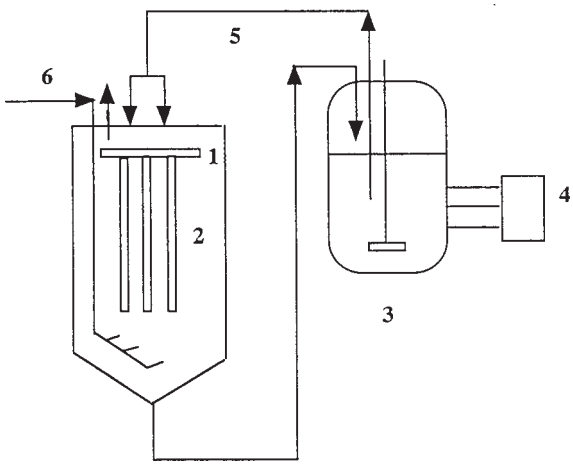

Fig. 1. The immobilization bioreactor. 1, Liquid distribution module; 2, fiber strips or sandwiched straw support; 3, appended 3 L Applikon bioreactor (Applitek, Deinze, Belgium); 4, regulatory module (pH, T°, and pO_2); 5, recycling loop; 6, air in and out.

solids from the culture using the Kjeldahl procedure with a Büchi apparatus. A conversion factor between total cellular nitrogen and mycelium dry weight was determined for cells of the *Agaricus* sp. growing exponentially on different soluble substrates. The culture was divided into two parts and filtered on glass-fiber discs. One part served for determination of cellular nitrogen and the other was used for determination of dry weight. The preweighed discs were washed twice with distilled water and dried at 105°C to constant weight (24 h). From five independent experiments, 1 mg of cellular nitrogen was equivalent to 17.85 ± 0.76 mg dry wt. This mean value was used for the estimation of enzyme specific activity.

For cultivation on polyurethane foam, four cubes (3 × 3 × 3 cm) cut from a polymer sheet were immersed in 200 mL of culture medium contained in a 1-L Erlenmeyer flask. The medium was sterilized at 121°C for 10 min. Fifty milliliters of a 7-d liquid culture were used for inoculation. Cultures were incubated at 25°C on a Bühler KS10 rotary shaker (150 rpm). Laccase activity was estimated on the culture supernatant. Dry weight of mycelium fixed on the foam cubes was estimated after gentle washing of the cubes with distilled water and oven drying for 24 h at 105°C.

Figure 1 illustrates the characteristics of the textile strip immobilization bioreactor *(10)*. A liquid film of the culture medium flowed down on two parallel strips of 3 cm width and 42 cm length. The strips were colonized with the fungal biomass produced in an appended bioreactor connected to the immobilization module. A flat cake (2 cm thickness) of finely chopped wheat straw, sandwiched in a wire netting, was also used for immobilization. Colonization was rapidly effective with the *Agaricus* sp. strain, and the circulating medium remained free of mycelium during the experiments with the textile strip and straw. The reactor was operated in a

discontinuous (batch) mode of production. The culture medium was continuously recycled in the device and eventually replaced after collection.

Determination of Enzyme Activity

Laccase-mediated oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was monitored by measuring the increase in A_{420} ($\varepsilon = 36{,}400M^{-1} \cdot cm^{-1}$) *(11)*. The reaction mixture contained 0.5 m*M* ABTS, 0.1*M* sodium acetate (pH 5.2), and a suitable amount of enzyme. One unit of enzyme activity was defined as 1 µmol of ABTS oxidized per minute. In control experiments, the culture supernatants were incubated in the presence of 1000 U/mL catalase (Sigma-Aldrich, Bornem, Belgium) in order to destroy any hydrogen peroxide that could have been produced by the fungus. H_2O_2 is in fact a cosubstrate for lignin and manganese peroxidases, both enzymes catalyzing the oxidation of ABTS *(12)*. No effect of catalase was observed. We concluded from these experiments that neither lignin peroxidase nor manganese peroxidase were produced, and that ABTS oxidation is most probably catalyzed by a typical laccase activity. Enzyme specific activity was expressed in terms of U/(g dry wt mycelium)$^{-1}$.

Analysis of Polysaccharide Slime

The slime layer attached to the surface of the mycelial mat was scraped off gently and washed with absolute ethanol. Total carbohydrate and reducing sugars were determined by the anthrone *(13)* and dinitrosalycilate (DNS) procedures *(14)*, respectively. The ethanol-washed slime was suspended in 10 mL N HCl in a screw-cap bottle and heated in a water bath for 1 h at 60°C.

The solution obtained was neutralized with solid Na_2CO_3 and adjusted to 25 mL with double-distilled water. High-performance liquid chromatography (HPLC) analysis of the hydrolysate was done with an Aminex Carbohydrate HPX-87P column (Waters, Brussels, Belgium) eluted with double-distilled water. Peaks were detected by differential refractometry using standards to identify the sugars *(15)*.

Results

Growth Curves and Laccase Production in Batch Culture

As shown in experiments of differential centrifugation not illustrated here, extracellular laccase accounted for more than 95% of the total activity produced by this strain. This was also observed with other *Agaricus* bisporus strains *(5)*.

Production of the enzyme in relation with the carbon source in a nitrogen-limited medium was first examined in batch culture that was conventionally. In a first series of experiments, a range of carbon sources was examined. Figure 2A shows the growth curve of the *Agaricus* sp. on glucose and 1% ball-milled straw, respectively. Straw is in fact one of the natural

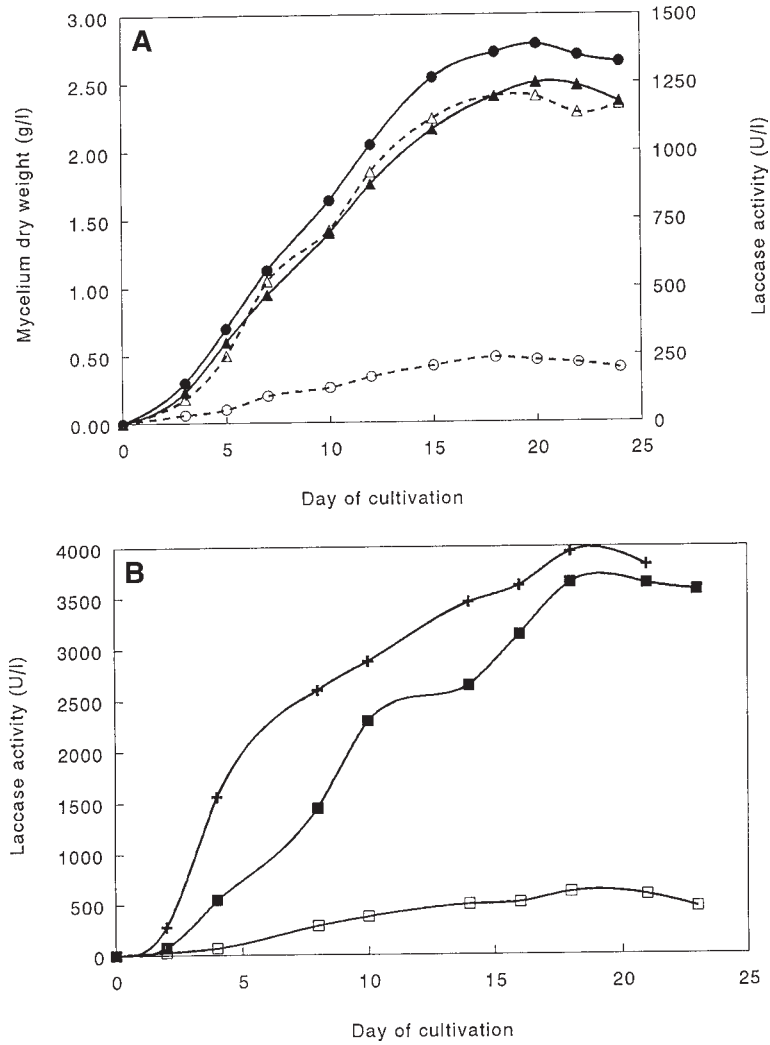

Fig. 2. Laccase production of *Agaricus* sp. in submerged shaken culture and under immobilized form. **(A)** Submerged cultivation on glucose (open symbols) and straw (solid symbols); (△, ▲) mycelium biomass; (○, ●) laccase activity. **(B)** Production of laccase by immobilized cells on polyurethane foam and on the textile strip. The fungus was immobilized on the polyurethane foam and cultivated on glucose (□) or xylan (■). Production of laccase in the immobilization bioreactor operated in batch mode of cultivation on xylan (+).

lignocellulose substrates of *Agaricus* sp. when the fungus is produced as an edible mushroom in solid-state fermentation *(16)*. Laccase production paralleled fungal growth, and the stationary phase of growth was attained after about 20 d. Similar patterns were observed with the other carbon sources (not illustrated).

Expression of laccase activity in relation to the carbon source was studied in more detail by examining the kinetics of enzyme production.

Table 1
Laccase Induction in the *Agaricus* sp.

Carbon source (1%, w/v)	Laccase (U/[g dry wt mycelium]$^{-1}$)
Glycerol	91
Glucose	93
Glucose + 2,5-xylidine	89
Glucose + veratryl alcohol	201
Glucose + ferulic acid	198
Glucose + veratric acid	206
Fructose	94
Mannitol	90
Arabinose	96
Xylose	177
Maltose	89
Saccharose	88
Cellobiose	92
Cellulose (Avicel)	91
Xylan (oat spelt—soluble fraction)	482
Wheat straw (ball milled)	582
Straw + glucose	579
Straw + glycerol	587
Lignosulfonate	247

Note: Cells of the fungus pregrown 7 d on glycerol as carbon source were transferred after sterile filtration to the different media as described in Materials and Methods. Effects of xylidine, veratryl alcohol, and veratric and ferulic acid were tested in the presence of glucose, a metabolizable substrate. Laccase activity was determined in the culture supernatant after 10 d of cultivation and expressed as U/(g dry wt mycelium)$^{-1}$. Experiments were done in triplicate; data are mean values. Standard deviations were all within 7% of the mean values presented.

Cells were pregrown on glycerol to inoculate media containing various carbon sources. Glycerol was used because it is a good carbon substrate for the fungus and is not related to lignocellulose and its components. Laccase production was not significantly affected when *Agaricus* sp. was transferred from the glycerol medium into media containing either glycerol, glucose, fructose, mannitol, arabinose, maltose, saccharose, cellulose, or cellobiose (Table 1). By contrast, straw, xylan, xylose, lignosulfonate, veratryl alcohol, and ferulic and veratric acid increased apparently from two- to sixfold the enzyme specific activity. The highest specific activity of laccase was attained on straw (Table 1). When straw was added to a glucose- or xylan-growing culture, the specific activity of laccase increased to attain the value observed on straw alone (Fig. 3A). Addition of xylan to the straw medium did not affect laccase production (Fig. 3B). When glycerol or glucose was present in the media supplemented with an inducer, the laccase specific activity was not lower than in the media without glycerol or glucose (Table 1).

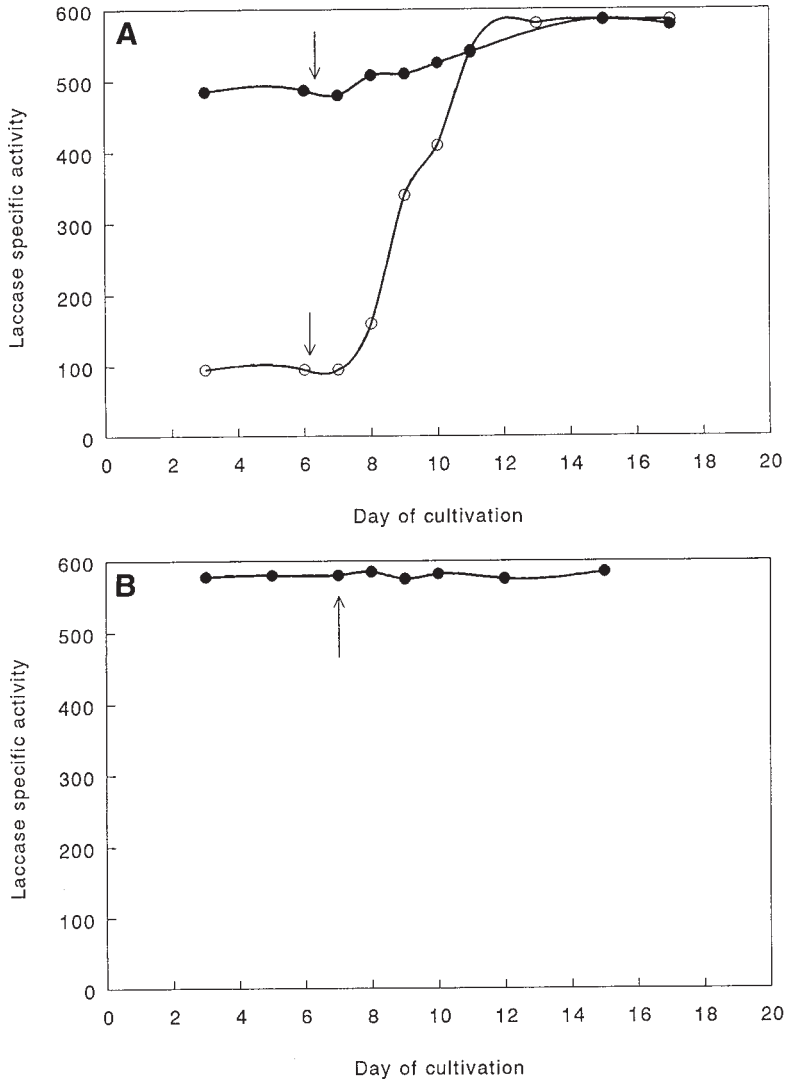

Fig. 3. Kinetics of laccase induction. *Agaricus* sp. was cultivated in submerged form as indicated in Materials and Methods. At the time indicated by the arrow, the tested substrate was added at 1% (w/v). **(A)** Effect of straw addition to a glucose- (○) or a xylan- (●) grown culture; **(B)** effect of xylan addition to a straw culture.

As also previously shown for *A. bisporus* by Wood and Goodenough *(5)*, 2,5-xylidine has no inducing effect on the laccase of the local strain (Table 1), even in the presence of glucose, a readily metabolizable cosubstrate.

These effects are consistent with the existence of an induction control on laccase and the absence of a form of carbon catabolite repression mediated by noninducing sources. In further studies, we have examined the production of laccase by immobilized forms of the *Agaricus* sp. strain.

Production of Laccase by Immobilized Agaricus *sp.*

Fungi, especially basidiomycetes, grow naturally on insoluble supports and this may strongly affect many physiological properties, in particular enzyme production *(17)*.

Cells of the *Agaricus* sp. were immobilized by adding premade cubes of porous polyurethane to suspension cultures of the fungus, as described in Materials and Methods. The cells invaded rapidly, and were strongly retained in the foam particles over a 21-d culture period. Laccase production started rapidly and culminated at 578 and 3650 U/(L culture)$^{-1}$ on glucose and xylan, respectively, after 21 d (Fig. 2B). The amounts of mycelium fixed on the foam after 21 d of cultivation were 2.75 and 3.70 g dry wt/L for glucose and xylan, respectively, as carbon source. Specific activities of laccase produced by the immobilized *Agaricus* were 176 and 985 U/(g dry wt mycelium)$^{-1}$ with glucose and xylan, respectively. For comparison, values obtained in conventional shaken culture were 93 and 482 U/(g dry wt mycelium)$^{-1}$ (Table 1). The immobilization on polyurethane thus results in a twofold increase in the biosynthetic activity of the fungal cells as compared to cultivation of the mushroom under the form of submerged pellets.

The textile strip immobilization bioreactor described in Materials and Methods (Fig. 1) was operated in batch mode of laccase production with xylan as substrate. After inoculation, laccase activity increased in the supernatant to attain a maximal value of 3950 U/(L culture)$^{-1}$ after 18 d (Fig. 2B). The weight of the dry biomatter fixed on the textile strip was 3.9 g, and the maximal specific activity attained in the bioreactor was 1013 U/(g dry wt mycelium)$^{-1}$. This value was similar to the specific activity obtained by immobilization on polyurethane foam.

Complete removal of culture medium after 20 d of production and replacement with fresh medium was followed by a rapid restoring within 3 d of a stationary state of enzyme production similar to that in Fig. 2B (not illustrated).

Production of laccase on the sandwiched straw support described in Materials and Methods was also effective and attained a level of 2678 U/(L culture)$^{-1}$ when using the basal medium without carbon source in a batch mode (not illustrated). It was not possible to estimate the specific activity of laccase because consumption of the straw was supported by the fungus.

The *Agaricus* sp. secreted concomitantly to laccase production an extracellular slimy thick material. By visual inspection, it was estimated that the highest slime production corresponded to the highest enzyme production. Owing to a possible implication in the mechanism of enzyme activity or stability, the nature of this slime was investigated.

Nature of Slime Produced

The slime layer was scraped off gently, dried, and hydrolyzed in 1*N* HCl, as described in Materials and Methods. HPLC analysis revealed

the presence of the following sugar residues: maltose, glucose, mannitol, fructose, raffinose, and sorbitol. A calculation based on the amount of the different sugars obtained after hydrolysis showed that more than 97% of the slime can be accounted for by sugars, indicating its polysaccharide nature.

Discussion

Laccase is perceived to be produced constitutively by *A. bisporus (5)*, although it is induced at least in part in other fungal genera by lignin derivatives: lignosulfonate, veratryl alcohol and 2,5-xylidine *(11)*. We have found that xylan, a hemicellulose, and some lignin derivatives are inducers for an extra amount of laccase by our *Agaricus* isolate. Straw, one of the natural substrates for mushroom growth, also induced laccase. This is most probably through a combined effect of soluble nutrients and inducers released from (hemi)cellulose and lignin straw by biodegradation. Many fungi produce laccase in the form of isoenzymes *(18)*. Two laccase genes were, e.g., identified in *Trametes villosa (19)*, *A. bisporus (20)*, and *Pleurotus eryngii (18)*. In *T. villosa*, expression of *lcc1* is highly induced, whereas the expression of *lcc2* appeared to be constitutive *(19)*. No regulation of gene expression was apparently reported for *A. bisporus (20)*. In the case of the present *Agaricus* sp., a constitutive level of laccase was produced, but an enhanced level of enzyme production was observed in the presence of inducers (Table 1). If the genetic profile of this strain had some similarity to other strains producing laccase isoenzymes, then regulation might possibly affect a second gene, as in the case of *T. villosa (19)*. This remains hypothetical and needs to be investigated.

Traditional growth of the fungus on straw bundle is more adequate for biomass production than for enzyme production. This would necessitate an extraction procedure from the solid material. Production in liquid medium under immobilized form was shown to be a convenient procedure to obtain laccase. Enzyme production on xylan in relation to the cultivation mode was calculated. Yields of 24, 55, and 56 $U/(d \cdot g \text{ dry wt mycelium})^{-1}$ were obtained for the batch conventional cultivation, polyurethane foam immobilized cells, and textile strip operated under batch mode, respectively.

Production of a polysaccharide slime simultaneously with laccase could be related to a protection mechanism of the mycelium against toxic highly reactive molecular species produced by the fungus in a ligninolytic phase *(4)*. It has been proposed that laccase may play a role in scavenging toxic radicals produced by lignin degradation *(21)*, and that production of slime is an alternative protection mechanism in the absence of laccase. Considering the catalytic properties of laccase, it is also plausible that this enzyme may generate toxic radicals and that the production of slime is a protection mechanism against the products generated by laccase and ligninolytic peroxidases.

In conclusion, we have shown that it is possible to manipulate the growth medium and the form of cultivation of an *Agaricus* sp. in order to

increase laccase production, and that the technology of immobilization could be promising for future industrial development.

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