

## Selection of Laccase Over-secreting Mutant in *Coprinus congregatus*

Soon-Ja Kim and Hyoung Tae Choi\*

Department of Microbiology, Kangwon National University, Chuncheon 200-701,  
and Research Center for Molecular Microbiology, Seoul National University,  
Seoul 151-742, Korea

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***Coprinus congregatus* has a membrane-associated laccase which is not secreted into culture media. A mutant monokaryon obtained, by U.V. irradiation followed by protoplast generation and regeneration method, was successfully isolated. When the mutant was grown on an agar plate or in a liquid medium, it secreted laccase while the wild type did not under the same growth conditions. The laccase of the mutant was compared with that of wild type by native PAGE analysis, and showed identical mobility.**

**Key words:** Laccase, fungal mutation, *Coprinus congregatus*

*Coprinus congregatus* Fries produces several laccase isozymes during its growth and development, including hyphal tip isozyme (3,5) and primordial isozyme (1). These show different banding patterns when examined by native PAGE analysis (1). The hyphal tip laccase is very important in the development of *C. congregatus*. The enzyme level is highest in the youngest growth zone where the mushroom formation occurs when this zone is illuminated (5). For normal development, the laccase level should be higher than a certain threshold level (5,6). Since the isozyme of mycelial pellets produced in liquid shake culture has identical electrophoretic mobility with that of the primordium (1), and since all these isozymes are associated with the cell membrane (5,2), it has been difficult to purify large amount of the hyphal tip isozyme in order to examine the roles of the enzyme.

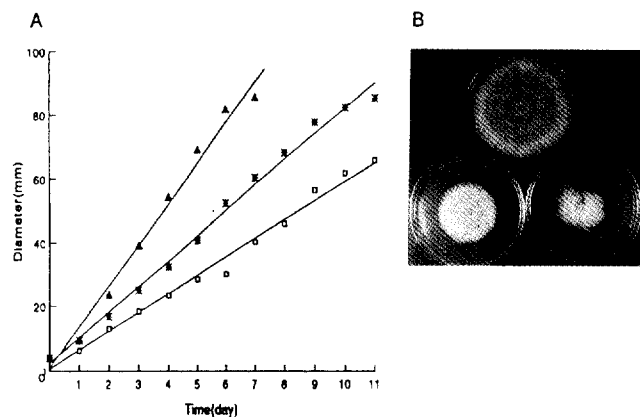
When *C. congregatus* dikaryon is transferred to yeast protein soluble starch (YpSs) low pH liquid medium (pH 4.0~4.5), the hyphal tip isozyme is secreted into the culture supernatant (4). Since *C. congregatus* can not develop into a mushroom at such a low pH agar medium, the possible roles of the hyphal tip isozyme in development (6) must be examined under normal growth conditions. In order to examine the secretion mechanism of the membrane-associated laccase, a mutant which can

secrete laccase in any culture has been generated, and its physiological characteristics have been determined.

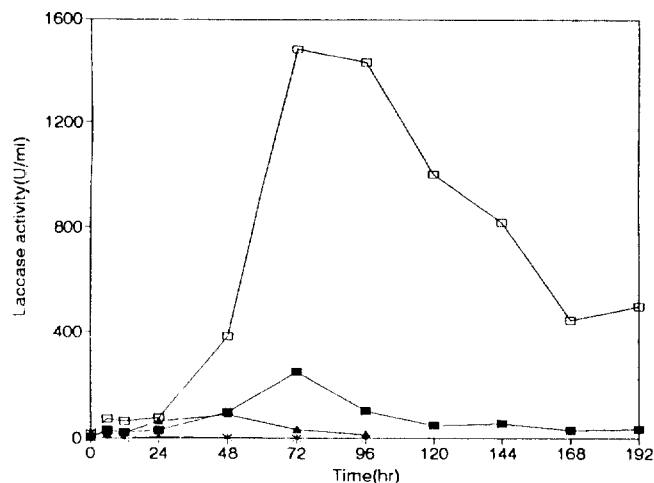
*C. congregatus* monokaryon (a1) was grown in YpSs liquid medium (pH 7.0) for 5 days in a shaker (120 rpm), then homogenized briefly in a Waring blender. The homogenate was transferred to a fresh medium (10 % v/v) and grown for 1 day in the same condition. The culture was homogenized 4 times (1 s/cycle) and washed twice with d-H<sub>2</sub>O. The fungal suspension (10 ml) in a Petri dish was exposed to a U.V. light (15 W) for 15 min at 15 cm distance, and then transferred to YpSs agar medium. Colonies which showed a rapid rate of pigmentation were transferred to two YpSs plates, a master plate and an enzyme assay plate. When the assay plate had colonial growth, *o*-tolidine (5) was added to determine laccase activity. The high laccase mutants were selected and cultivated in YpSs liquid medium, and then cell wall degrading glucanase (2) was applied to get protoplasts. Protoplasts were isolated by density gradient centrifugation (2). After the protoplasts were regenerated on YpSs plate with 0.55 M sorbitol as an osmotic stabilizer, every single colony was grown on YpSs agar plate

We have successfully isolated a laccase-secreting mutant (Fig. 1) by U.V. irradiation followed by protoplast generation and regeneration. Since no asexual sporulation had been reported in *C. congregatus* monokaryons (7), a suspension of hyphal fragments which consisted

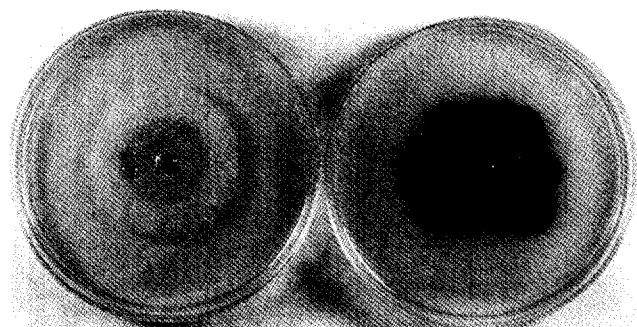
\*To whom correspondence should be addressed.



**Fig. 1.** Growth rates (A) and photographs of colonies of 6 day cultures (B) of dikaryon, a1, and CL 110. A) Growth rates of dikaryon (▲-▲), a1 (\*-\*), and CL 110 (□-□) on YpSs plate. Representative result of 5 replicates. B) Photograph of colonies of 6 day cultures: dikaryon (top), a1 (bottom left), and CL 110 (bottom right).



**Fig. 3.** Comparison of Laccase secretion in YpSs liquid medium. Dikaryon in pH 4.2 (▲-▲), a1 in pH 4.2 (\*-\*), CL 110 in pH 7.0 (□-□), CL 110 in pH 4.2 (■-■). Representative result of triplicate.



**Fig. 2.** Colonies stained with o-tolidine on day 6. a1 (left) and CL 110 (right).

of many cells was used to obtain mutants. During the U.V. treatment, many cells in a fragment which have genetically different nuclei are generated. That was why protoplasts were generated from the mutant culture to get a cell which had a genetically pure clone.

The mutant (CL 110) showed irregular colonial growth (Fig. 1), and slower growth rate than that of the wild type, a1 (72.7%, Fig. 1). CL 110 showed another interesting phenomenon. Laccase activity appeared outside of its colony margin when stained with o-tolidine while a1 had very poor color reaction (Fig. 2). Quantitative laccase assay was performed with the addition of 1.0 mM o-tolidine as the enzyme substrate in a 0.1 M sodium acetate buffer at pH 4.7 (5). The assay tubes were incubated at 25°C for 30 min. and read on a spectrophotometer at 590 nm. CL 110 secreted more laccase (2.5 fold) than dikaryon did in YpSs low pH liquid medium (Fig. 3). There was no laccase activity in a1 culture medium (Fig. 3). CL 110 secreted laccase not only in the low pH medium but also in the neutral pH YpSs medium, while



**Fig. 4.** Native PAGE analysis of laccase isozymes. a, hyphal tip laccase of dikaryon; b, secreted laccase of CL 110 in pH 4.2 medium; c, secreted laccase of CL 110 in pH 7.0 medium.

the dikaryon secreted laccase only in the low pH medium (4). Laccase activity in the neutral pH culture medium was 6 times higher than in the low pH medium (Fig. 3). When the secreted laccase was compared by native PAGE analysis with the hyphal tip laccase, they showed identical mobility (Fig. 4). Since the hyphal tip laccase of a monokaryon (a1) also showed identical mobility with that of the dikaryon (1), and since laccase from the culture supernatant of CL 110 showed identical

mobility with that of the dikaryon, CL 110 seems to secrete the membrane-associated laccase into culture media. Therefore, this mutant could give much information about the secretion mechanism of the membrane-associated enzyme and various functions of the laccase in *C. congregatus* morphogenesis.

### Acknowledgement

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