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Production of plant cell wall-degrading enzymes in *Lentinula edodes* and the important role of laccase in early stages of solid-state cultivation

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Abstract

We studied the production of plant cell wall-degrading enzymes in *Lentinula edodes* grown on sawdust medium. A mycelial mat was spotted onto the center of a medium plate, which was then incubated at 23℃ for 31 days. Laccase (EC 1.10.3.2) activity was detected before that of other enzymes. Laccase activity increased along with mycelial growth until the leading edge of the mycelium reached the edge of the plate, and then decreased. Decrease of laccase activity was followed by increased activity of other enzymes. Sawdust treated with purified laccase (Lcc1) was more sensitive to degradation by crude enzymes from *L. edodes*. These results suggest that laccase plays an important role during early stages of solid-state cultivation of *L. edodes*.

Key words: Laccase, *Lentinula edodes*, Lignin-degrading enzymes, Plant cell wall-degrading enzymes, Solid-state cultivation

Introduction

Edible mushrooms such as *Lentinula edodes* (shiitake mushroom) are commonly grown using mycelial block cultivation (solid-state cultivation), a technique in which the culture medium primarily consists of sawdust and nutrient supplements, such as rice or wheat bran. During mycelial block cultivation, wood decomposition takes place through the action of various enzymes secreted by the fungal mycelia. Various compounds derived from the enzyme-degraded plant cells are used as nutrient sources for fungal mycelium growth and as energy sources for fruit body generation. White rot fungi produce lignin-degrading enzymes such as laccase (Lcc, EC 1.10.3.2), manganese peroxidase (MnP, EC 1.11.1.13), and lignin peroxidase (LiP, EC 1.11.1.14), which have been reported to effectively degrade phenolic compounds, which are environmental pollutants. Lcc enzymes secreted from shiitake mushroom culture, including Lcc1, have been found to decolorize various pigments and decompose some endocrine disruptors.[1-4] Enzymes produced by edible basidiomycetes such as shiitake mushroom are very safe for humans and the environment, and can be obtained from mycelial blocks discarded after fruit body harvest. In this context, the time required for initiation of enzyme production and enzyme productivity during mycelial block cultivation are of interest.

Quantities of plant cell wall-degrading enzymes secreted from fungal mycelia differ depending on the fungal mycelium growth stage. Matsumoto[5] reported that during *L. edodes* mycelial block cultivation, Lcc activity increased temporarily after the first flush (fruit body formation), and then rapidly decreased; in contrast, cellulase and hemicellulase activity increased later, during fruit body growth. According to Leatham[6], when shiitake mushroom is grown using the mycelial block method with konara oak (*Quercus serrata*) as the substrate, Lcc and amylase activities are high during early stages of cultivation (days 15 to 45) and then decrease, whereas hemicellulase activity reaches a maximum during middle and late stages of cultivation (day 45 and beyond).[6] In these studies, whole mycelial blocks were homogenized in buffer solution to extract crude enzyme, and Lcc activity data was reported as enzyme activity per mycelial block. Enzyme activities are assumed to vary in different portions of the extracted mycelial block because fungal mycelia growth states differ between different regions of the mycelial block culture medium. It has been reported, for example, that some enzymes are abundantly secreted at the mycelium tip in filamentous fungi of the genus *Aspergillus*, including *Aspergillus oryzae*.[7] In this case, it is thought that the mycelial tip region differs from the area near the mycelial inoculation point with respect to functions and roles. The mycelial tip region is the mycelial growing point and many enzymes are excreted there;

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in contrast, mycelia near the inoculation point are growing slowly and excrete little or no enzyme.

In our study, we investigated the production of lignin-degrading and plant cell wall-degrading enzymes in the culture medium during early stages of *L. edodes* cultivation. In addition, we examined the role of Lcc, which is primarily produced during early cultivation stages.

Materials and Methods

1. Organisms and culture conditions

In this study, we used a commercial dikaryotic strain of *Lentinula edodes*, Hokken No. 600 (Hokken, Tochigi, Japan). Mycelia were maintained on 1.5% agar plates with 0.25 × MYPG medium containing 0.25% Bacto malt extract (Difco, Detroit, MI, USA), 0.1% Bacto yeast extract (Difco), 0.1% tryptone peptone (Difco), and 0.5% glucose. For production of *L. edodes* fruit bodies, mycelia were cultivated on sawdust-based medium. Sawdust medium, containing 2.5 kg *Quercus serrata* (konara oak) sawdust (filtered through 30 mesh/0.5mm and 10 mesh / 1.7mm sieves), 370 g commercial nutrients named Baideru (Hokken Co., Japan) containing wheat bran etc., and 4.8 L distilled water, was prepared and the moisture adjusted to about 65%. The sawdust medium (20 g) was then placed in a glass Petri dish (90 mm diameter) and autoclaved at 121℃ for 60 min.

Lentinula edodes pre-cultured on a 1.5% agar plate with 0.25 × MYPG was cultivated at 23℃ for 15 d. A 7-mm-diameter portion of the culture medium was then inoculated onto the center of sawdust-based medium described above, and incubated at 23℃.

2. Preparation of crude extract from sawdust-based culture medium

To detect total enzyme activity in the glass Petri dish, an entire mycelial block (about 20 g) was placed in a beaker, and 100 mL of 10 mM phosphate buffer adjusted to pH 6.0 (PB) was added. The mycelial block was suspended and stirred at 4℃ for 12 h. The suspension was then centrifuged at 12,000 × *g* for 15 min, and the supernatant was assayed for enzyme activity using the method described in the next sub-section.

Alternatively, to detect activity in specific portions of the mycelial block, square sections (5 mm × 5 mm) were excised every 5 mm from the center of the block as shown in **Fig. 1** and weighed. Crude enzyme was extracted from each excised block using 10 × PB followed by shaking at 4℃ for 3 h in an EM-36 Microtube Mixer (Taitec, Japan). The suspension was then centrifuged at 12,000 × *g* for 15 min. Enzyme activities in the supernatant were measured using the method described below.

3. Enzyme activity measurements

Lcc activity was measured following the method of Nagai et al.[1] One unit of Lcc activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, ε = 36,000 $M^{-1} cm^{-1}$ at 420 nm) in a 100-μL reaction mixture for 1 min at 30℃. MnP activity was measured according to Nagai et al.[3] using 2,6-dimethoxyphenol (DMP, ε = 35,645 $M^{-1} cm^{-1}$ at 465 nm) instead of guaiacol.[8] One unit of MnP activity was defined as the amount of enzyme needed to oxidize 1 μmol DMP in a 200-μL reaction mixture for 1 min at 30℃.

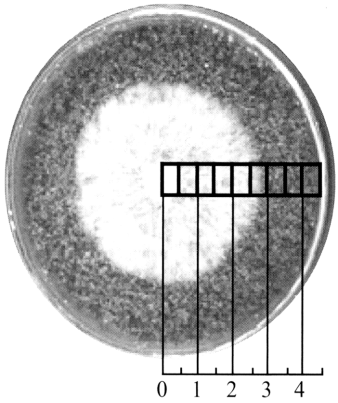

Fig. 1. Photograph of mycelia grown on konara oak sawdust medium for 10 d.
Boxes indicate the position of sections excised for crude enzyme extraction.

Carboxymethylcellulase (CMCase), endoxylanase, and amylase activities were measured using carboxymethylcellulose (CMC), xylan, and soluble starch, respectively, as substrates. Reaction mixtures for each assay, containing 10 μL of 0.1% substrate solution and 90 μL McIlvaine buffer (pH5.0), were incubated at 37℃. The formation of reducing sugar was detected using the Somogyi-Nelson method.[9] One unit of enzyme activity was defined as the amount of enzyme needed to produce 1 μmol reducing sugar per 1 h. Acid protease activity was measured according to Terashita et al.[10] Reaction mixtures consisted of 500 μL volumes with casein (Hammerstein grade) as a substrate. One unit of acid protease activity was defined as the amount of enzyme needed to produce 1 μg of tyrosine.

4. Measurements of free reducing sugar and free glucose concentrations

Reducing sugar concentrations in crude extracts were detected using the Somogyi-Nelson method.[9] Glucose concentrations were measured with a Wako glucose test kit (Wako, Osaka, Japan).

5. Lcc1 sawdust-degradation experiment

Lcc1 was purified from MYPG liquid culture broth after two-weeks cultivation with shaking according to Nagai et al.[1] In this experiment, the crude extract was derived from a 1.5- to 2.0-cm region from the center of a mycelial block cultivated in a dish with sawdust-based medium for 13 d as described earlier (**Fig. 3**, open circle region). The crude extract was used as the enzyme solution.

Dried sawdust (100 mg) of *Q. serrata* (konara oak) was transferred to a 1.5-mL disposable plastic (micro-

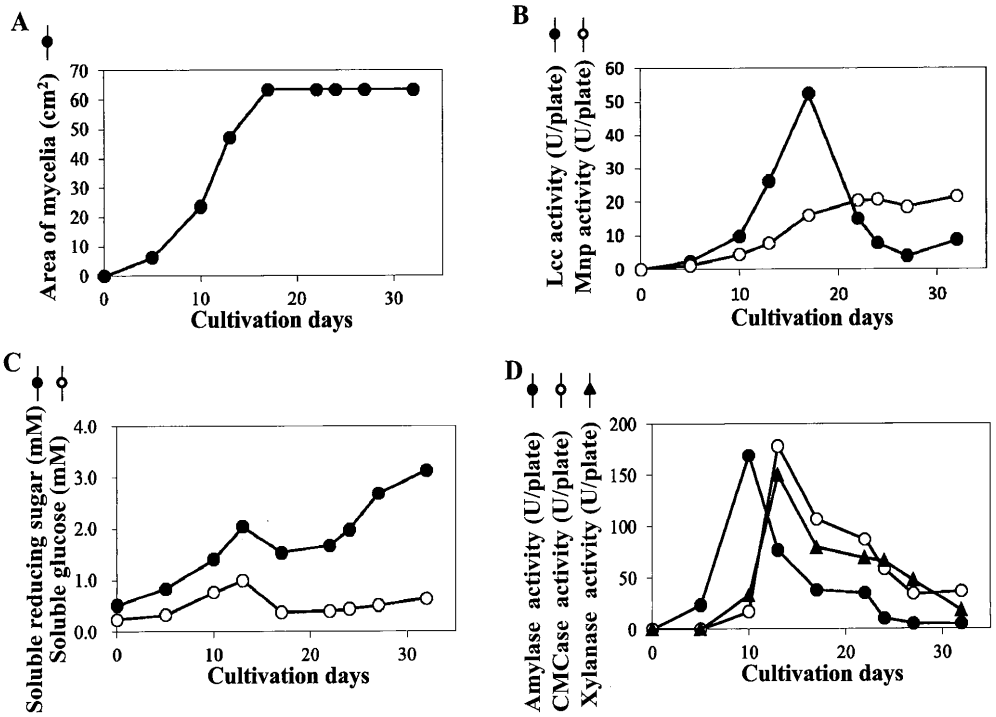

Fig. 2. Time course of some parameters and enzyme activities during solid-state culture of *Lentinula edodes*.
A, Growth of mycelia. B, Activities of lignin-degrading enzymes. C, Changes of soluble sugar concentrations. D, Activities of cell wall hydrolases. Detailed methods were described in Materials and Methods.

centrifuge) tube, and suspended in 1 mL McIlvaine buffer (pH 5.0). Purified Lcc1 (1 U/mL final concentration) without reducing sugar was added, and the tube was incubated at 37℃ for 12 h with shaking at 120 rpm. Following the reaction, the mixture was centrifuged at 15,000×*g*. Reducing sugar concentration was measured in the resultant supernatant according to the Somogii-Nelson method.[9] A blank (control) reaction mixture was composed using MQ (ultrapure) water in place of Lcc1 and the crude extract. Three replicates were performed, and results were taken from the average value.

Results

1. Changes in lignin-degrading enzyme, cell wall-degrading enzymes, and amylase activities in whole mycelial blocks

To investigate production of different enzymes in early stages of shiitake mushroom cultivation, shiitake mushroom mycelium was inoculated into sawdust medium in a glass Petri dish and cultivated at 23℃ for 31 d. Enzymes were extracted from the sawdust medium for several days using 100 mL of PB, and enzyme activities and amounts of reducing sugar in the crude extracts were measured. Average results obtained from three dishes are shown in Fig. 2. Growth of *L. edodes* was plotted as total area occupied by the fungal mycelia (Fig. 2A). Lateral mycelial growth increased until day 17. Although lateral growth was completed on day 17 (Fig. 2A), mycelial growth continued through day 31 in the form of aerial mycelia. Lcc activity increased along with mycelial growth, reaching a maximum on day 17 (Fig. 2B). When the fungal mycelia had spread to the edge of the dish, Lcc activity promptly decreased. In contrast, MnP production began later than Lcc, with activity generally peaking on day 21 of cultivation and then leveling off (Fig. 2B). The amount of reducing sugar detected in the extract increased until day 13 of cultivation, decreased once, and then increased again (Fig. 2C). Glucose concentration followed the same general trend, except, compared with reducing sugar, little increase was observed after day 17. Amylase activity reached its maximum during early stages of cultivation (day 10) and then decreased (Fig. 2D). CMCase and xylanase activities peaked on day 13 and then decreased gradually (Fig. 2D).

2. Changes in lignin-degrading enzyme, cell wall-degrading enzymes hydrolase activities in mycelial block regions

Lignin-degrading enzyme, cell wall-degrading enzymes, and amylase activities were measured in crude extracts of regions excised from mycelial blocks in sawdust cultures (Fig. 1). Enzymes were extracted from two portions of each mycelial block in a glass Petri dish, with three dishes used for each sample, so that measured enzyme activity was represented by the average of six replicates. The amount of reducing sugar produced near the tip (growing point) of the fungal mycelial mat increased during early stages of mycelium growth, then decreased, and finally increased again (Fig. 3). Free glucose concentration showed a similar trend. Lcc activity in the mycelial block was strongest in the growing point portion of the fungal mycelia, with its activity spreading to areas outside the active growth regions. After the fungal myc-

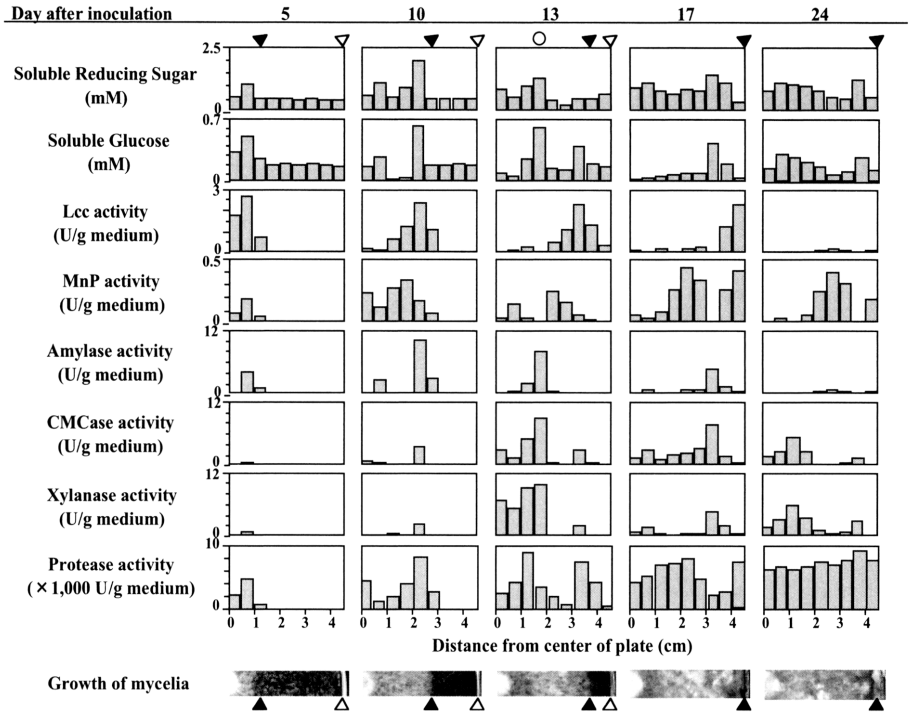

Fig. 3. Changes of enzyme activities of *L. edodes* on solid-state medium.
Open arrows indicate the edge of the plates and closed arrows indicate tips of hyphae. The open circle indicates the crude enzyme used for the sawdust degradation experiment (Fig. 4). Detailed methods were described in Materials and Methods.

elia reached the edge of a dish, activity decreased. Although changes in MnP activity were generally similar to the Lcc activity trend, MnP activity peaked twice (Fig. 3). When free glucose concentration was high, MnP activity was low. As with Lcc and MnP, high amylase activity was observed during earlier stages of cultivation (up to day 10) at the mycelial growing point (Fig. 3). After day 13, however, amylase activity gradually decreased. CMCase and xylanase activities were detected later than amylase activity (Fig. 3). For both enzymes, the greatest activity was detected at day 13. Although both activities in other region (3-3.5 cm from the center) increased at day 17, the whole activities were decreased from day 13 (Fig. 2, 3). Just as with Lcc activity, high acid protease activity was detected near the mycelial growing point at day 13 (Fig. 3). Strong acid protease activity was also detected near the center of the dish, similar to CMCase and xylanase, which then spread outwards and stayed at high levels throughout the cultivation period.

3. Effect of laccase on generation of reducing sugar from sawdust using a mycelial block CMCase fraction

The sensitivity of laccase-treated sawdust to degradation from a highest CMCase activity fraction at day 13 was tested using the crude extract shown in Fig. 3 with low lignin-degrading enzyme activities (Lcc: 0.004 U/mL; MnP: 0.0008 U/mL) and high CMCase, xylanase, and amylase activities (CMCase: 0.98 U/mL; xylanase: 0.96 U/mL; amylase: 0.78 U/mL). A hundred μl of the crude extract was added to a suspension of *Q. serrata* (konara oak) sawdust in PB, and levels of reducing sugar

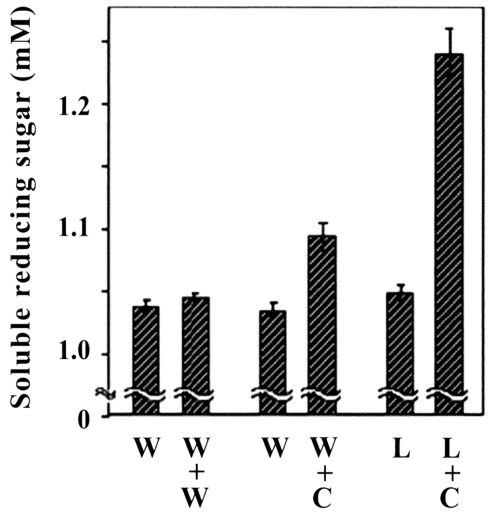

Fig. 4. Effect of laccase on sensitivity of sawdust against cellulases.
Sawdust was incubated for the indicated times with the following enzymes: W, incubated with water for 12 h. W+W, incubated with water for 12 h and afterwards with water for 12 h. W+C, incubated with water for 12 h and then with crude enzyme for 12 h. L, incubated with Lcc1 for 12 h. L+C, incubated with Lcc1 for 12 h and then with crude enzymefor 12 h. Other detailed methods were described in Materials and Methods.

generated in the suspension were measured using the Somogyi-Nelson method[9] (Fig. 4).

The reaction was performed in two steps. First, the sawdust suspension was incubated for 12 h with either MQ water or Lcc1 prepared by liquid culture broth, and then incubated an additional 12 h after addition of either

crude extract or more MQ water. In the case of MQ water alone (the control), a reducing sugar concentration of 1.04 mM was detected after the first 12-h incubation. No increase in the reducing sugar concentration was observed after further incubation for 12 h. In the case of the addition of crude extract to sawdust after 12-h incubation with MQ water, 1.09 mM reducing sugar was detected. In contrast, when sawdust was first incubated with Lcc1 before addition of the crude extract, a concentration of 1.25 mM reducing sugar was generated; concentrations of reducing sugar after the initial incubation with Lcc1 were similar to MQ water controls.

Discussion

In this study, we monitored changes in production of plant cell wall-degrading enzymes and lignin-degrading enzymes in *L. edodes* during solid-state cultivation, and demonstrated the important role of laccase in production of reducing sugar.

We first demonstrated that changes occurred in enzymes produced in the whole mycelial block during solid-state cultivation (Fig. 2). Amylase was detected first in the mycelial block; maximum activity was observed on day 10, followed by a gradual decrease (Fig. 2D). After amylase activity decreased, CMCase and xylanase activities increased followed by increases in reducing sugar and free glucose (Fig. 2C and D). Glucose amounts peaked on day 13 when CMCase activity reached a maximum, decreased on day 17, and then increased slowly (Fig. 2C). The amount of reducing sugar was at a maximum on day 13, decreased at day 17, and then gradually increased (Fig. 2C). These results suggest that *L. edodes* first utilizes starch derived from nutrients in the solid-state medium as a carbon source through the action of amylase; following consumption of starch as a carbon source for mycelial growth, it uses cellulose and xylan, a component of hemicellulose. At this point, a clear difference can be observed in the rate of increase of free glucose and reducing sugar concentrations. These results suggest that *L. edodes* preferentially digests hemicelluloses such as xylan rather than cellulose after consumption of starch, because the glucose concentration is lower than the reducing sugar concentration (Fig. 2C).

The above trends were corroborated by analysis of enzyme activity changes in portions of the mycelial block on solid-state medium (Fig. 3). High amylase activity was observed in the inner portion of the mycelial growth point in the solid-state culture from days 5 to 17. A small amount of glucose was detected in the extract derived from solid-state medium with no mycelium growth, suggesting that the glucose was obtained from nutrients in the solid-state medium. Although little amylase activity was detected surrounding the growth point on the mycelial block, amylase activity was observed in the inner portion of the mycelial growth point, where free glucose had been consumed. In addition, during this time, the fraction having strong amylase activity was also at a maximum glucose concentration. This suggests that catabolite repression of amylase by glucose was taking place. Amylase activity in the inner portion of the mycelial block was strong, and gradually decreased during late cultivation stages (from day 17 to day 24). On the other hands, CMCase and xylanase were produced after a delay of amylase, and further highly produced in the middle of the mycelial block than amylase. The activities of CMCase and xylanase increased in the very middle of the mycelial block by day 24.

Interestingly, patterns of CMCase and xylanase activity changes in each portion of the mycelial block closely mirrored each other (Fig. 3). Arai et al.[11] reported that amylase, CMCase, and xylanase activities in *L. edodes* were increased by the addition of hot water extracts of cornstarch; they also noted that during 70 d of monitoring, CMCase and xylanase activity in the *L. edodes* liquid medium culture followed similar patterns. Tsujiyama[12] found that CMCase and xylanase activities are induced by vaniline and cellobiose. These results suggest that same substances exited in the solid-state culture may regulate the both enzyme expression. In *A. niger*, expression of xylanase genes is induced by xylose, and most of these hemicellulase enzyme genes are also regulated by the same transcription factor, XlnR.[13] A similar transcription system, simultaneously controlling CMCase and hemicellulases as with XlnR in *Aspergillus*, may exist in basidiomycetes such as *L. edodes*. A molecular biological investigation addressing whether such a system exists in basidiomycetes would be useful.

We then carried out a similar experiment with respect to MnP and Lcc. We found that Lcc was heavily produced near a mycelial growth point, but scarcely elsewhere (Fig. 3). In contrast, MnP production occurred later than Lcc, then declined, and finally increased again (Fig. 3). As a result, Lcc production peaked when a mycelial growth point arrived at the edge of the dish, and decreased promptly thereafter (Fig. 2). These results are in agreement with the observations of Leatham[6] for whole mycelial blocks during solid-state cultivation. In that study, it was suggested that the prompt reduction of Lcc was caused by protease. In contrast, MnP activity was maintained even after 21 d in our study.

The results of anion exchange column chromatography reported by Nagai et al.[3] showed that at least two types of Lccs existed in the mycelial block on the sawdust solid-state medium. Analysis of chromatographic elution profiles[3], SDS-PAGE[3], and Western blotting by rabbit polyclonal peptide antiserum directed against Lcc1 (data not shown) suggested that one was identical to Lcc1 produced in liquid culture broth in previous studies.[1,14] We then demonstrated the effect of Lcc1 (purified from liquid culture broth) on the generation of reducing sugar due to sawdust degradation by a mycelial block CMCase extract (circular region at day 13 in Fig. 3) possessing low Lcc

activity and high CMCase and xylanase activities. Only a 0.05 mM increase in reducing sugar concentration was observed ([W+C] – W in **Fig. 4**) from an MQ water control treatment subjected to CMCase extract treatment, whereas a four-fold increase of 0.2 mM ([L+C] – L in **Fig. 4**) was observed from sawdust treated with Lcc1. A reducing sugar was recovered by 1.05 mM from MQ water treatments of the sawdust (**Fig. 4**, W and W+W). In this experiment, MQ water treatment (W and W+W in **Fig. 4**) can be regarded as low enzyme activity region around tips of mycelia in sawdust medium. Therefore, the reducing sugars released by MQ water (no enzyme) are probably quickly consumed by the mycelia, such as around tips of mycelia at day 17. These results indicate that after existing reducing sugars in sawdust have been consumed, Lcc is required for sawdust degradation. Consequently, after existing sugars have been consumed, Lcc appears to increase substrate susceptibility to glycoside hydrolases, such as CMCase and xylanase, that are active during lignin degradation of woody tissue.

Using *Pleurotus ostreatus*, Taniguchi et al.[15] examined the effect of biological pretreatment of rice straw by evaluating quantitative and structural changes in the pretreated rice straw components as well as susceptibility to enzymatic hydrolysis. They found that *P. ostreatus* selectively degraded the lignin fraction of rice straw, and observed 2- to 2.5-fold enhancement of enzymatic solubilization of holocellulose and cellulose fractions of rice straw pretreated with *P. ostreatus*. Although we did not measure the lignin content of sawdust in our experiment, it is difficult to imagine that large-scale lignin degradation due to Lcc activity occurs only near the mycelial growth point. We propose that the three-dimensional structure of lignin in sawdust is degraded partially by Lcc, making cellulose and hemicellulose more susceptible to attack by glycoside hydrolases such as CMCase and xylanase. On the other hand, Ximenes et al.[16] reported that a lignin monomer such as vanilline etc. inhibited cellulose hydrolysis. Therefore, another possibility is that the recovered CMCase activity because of the disappearance of phenolic inhibitors by Lcc secreted by the *L. edodes* results in the increase of reducing sugar.

Lcc expression is reportedly induced by copper, tannic acid, ferulic acid, veratric acid, caffeic acid, and vaniline, which are plant cell wall components.[17-18] These findings suggest that Lcc production is triggered by such inducers existing in intact sawdust; once the inducers are digested by growing mycelia, Lcc production ceases. Kirk et al.[19] reported that in *Phanerochaete chrysosporium*, low nitrogen concentration in growth media promotes ligninolytic activity through decrease of free nitrogen in the sawdust medium followed by lignin degradation. Pointing et al.[20] reported that *Pycnoporus sanguineus* produced Lcc under the conditions such as high carbon and low nitrogen in submerged liquid culture. On the other hand, nitrogen repression response element (NIT2) activates the expression of many structural genes encoding nitrogen catabolic enzymes during conditions of nitrogen limitation.[21] We found that two site of NIT2 having consensus sequence TATCDH located at -964 to -959 and -162 to -157 in the promoter region of *lcc1* gene (DDBJ accession No. AB055157) cloned by Sakamoto et al.[14] The data imply that *lcc* gene expression in *L. edodes* may regulate by nitrogen content in the medium. Collectively, these results indicate that Lcc expression is induced by lignocellulose existing in the mycelial growth point of *L. edodes* mycelial blocks, and that Lcc produces partially-degraded lignin near the mycelial growth point in sawdust medium.

The results of our study suggest that Lcc production plays an important role in *L. edodes* growth on sawdust medium. In addition, on the basis of drastic changes observed in fungal mycelia growing conditions upon alteration of medium composition, we demonstrated that enzyme production differs greatly in every part of a mycelial block under solid-state cultivation. In the future, we hope to use these findings for large-scale production of lignin-degrading enzymes, with applications to environmental pollutant bioremediation and elucidation of lignin degradation mechanisms in wood.

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和 文 摘 要

シイタケ菌床栽培初期における植物細胞壁分解酵素活性の変動と菌床中ラッカーゼの役割

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菌床を模したシャーレ上のコナラの大鋸屑を含む固体培地で生育させたシイタケが生産するリグニン分解酵素活性と植物細胞壁分解酵素活性の変動を測定した．シイタケは，シャーレ上の固体培地で 23℃，31 日間培養した結果，菌

糸の成長点付近において強いラッカーゼ活性が認められ，ラッカーゼ活性は菌糸のシャーレへの蔓延とともに減少した．アミラーゼ，カルボキシメチルセルラーゼ，キシラナーゼはラッカーゼが減少した後に生産された．ラッカーゼにより処理したコナラの大鋸屑では未処理の大鋸屑に比べてセルラーゼを含む粗酵素液に対する感受性が高まった事から，シイタケの固体培養におけるラッカーゼの重要性が示唆された．

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