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Lignin-degrading activity of edible mushroom *Strobilurus ohshimae* that forms fruiting bodies on buried *sugi* (*Cryptomeria japonica*) twigs

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Abstract *Strobilurus ohshimae* is an edible mushroom, and it specifically forms its fruiting bodies on buried *sugi* (*Cryptomeria japonica*) twigs. In this research, we studied lignin-degrading activity of *S. ohshimae*. We isolated 18 strains of *S. ohshimae* from various regions of Japan, and determined their lignin degradation rates on *sugi* wood meal medium. All the strains of *S. ohshimae* degraded approximately 6%–12% of *sugi* lignin in 30 days, and these lignin degradation rates were 1.5–3 times higher than those of *Trametes versicolor*, which is a typical lignin-degrading fungus. Among the three main lignin-degrading enzymes, activity of lignin peroxidase and manganese peroxidase was not observed, while 4340 U/g of laccase was produced in 30 days. To investigate the effect of wood species on lignin degradation by *S. ohshimae*, the lignin degradation rate and laccase productivity on *sugi* wood meal medium were compared with those on beech (*Fagus crenata*). In *T. versicolor*, both lignin degradation rate and laccase productivity were higher on beech than on *sugi*. Conversely, in *S. ohshimae*, lignin degradation rate and laccase productivity were higher on *sugi* than on beech. Therefore, it was suggested that coniferous lignin is not always difficult to degrade for the fungi that inhabit softwood.

Key words White-rot fungi · Basidiomycete · Softwood · Laccase · Specificity of wood species

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Introduction

Sugi (*Cryptomeria japonica*) is a major conifer in Japan. While its total biomass exceeds 1×10^9 m^3, utilization of *sugi* wood, in particular the wood that is produced by thinning or pruning, is insufficient.[1] In order to use *sugi* wood more effectively, mushroom cultivation has been attempted using *sugi* wood meal. However, studies on some fungi have reported that *sugi* wood is unsuitable for mushroom cultivation.[2–7] For example, the mycelial growth of *Lentinula edodes, Flammulina veltipes,* and *Pleurotus ostreatus* were inhibited by antifungal substances contained in *sugi* wood, such as ferruginol and sandaracopimarinol.[3,4] Thus, it is necessary to remove such antifungal substances by a seasoning treatment to use *sugi* wood meal as a medium of mushroom cultivation.[6] On the other hand, there are also a few fungi such as *Strobilurus ohshimae*, which mainly develops its fruiting bodies on *sugi* wood in nature. *Strobilurus ohshimae* is an edible mushroom, and it predominantly forms its fruiting bodies on buried *sugi* twigs. Previously, we studied the cultivation properties of *S. ohshimae* and succeeded in fruiting body formation on the solid medium of *sugi* wood meal.[8–10] Based on these observations, it was assumed that *S. ohshimae* had a high capability on decaying *sugi* wood. However, it is still unclear whether *S. ohshimae* is a white-rot fungus or a brown-rot fungus.

It is widely known that many of the white-rot fungi mainly decay hardwood, while the brown-rot fungi mainly decay softwood.[11–13] Coniferous lignin is mainly composed of guaiacyl lignin, and that of hardwood is composed of both guaiacyl lignin and syringyl lignin.[11–14] For white-rot fungi, the degradation of guaiacyl lignin is difficult compared with that of syringyl lignin.[12,13] Thus, it is generally accepted that coniferous lignin is difficult to decay. Lignin degradation by white-rot fungi has been studied well in fungi such as *Trametes versicolor* and *Phanerochaete chrysosporium*.[15–18] However, these fungi mainly inhabit hardwood. Lignin degradation by fungi that mainly inhabit softwood, particularly *sugi*, has rarely been studied. Therefore, in the event that *S. ohshimae* is a white-rot fungus, it

would be interesting to study the lignin degradation by *S. ohshimae*. In this study, we report that the lignin-degrading activity and laccase productivity by *S. ohshimae* were higher on *sugi* wood than on beech wood.

Materials and methods

Organisms

Strains of *Strobilurus ohshimae* (Hongo et Matsuda) Hongo used in this study were isolated from *sugi* woods from seven regions across Japan. The names of strains and the locations from where they were isolated are as follows: Kamogawa (Kamogawa, Chiba); Nagaoka 1-1, 2-1, and 3 (Nagaoka, Niigata); Ani 1, 2-1, 2-3, 1S-1, 1S-2, and 2S-1 (Ani, Akita); Kita-Ibaraki (Takahagi, Ibaraki); Kita 1 and 3 (Kyoto); Kuma ks 1-1-2, ks 1-2-1, and ks 1-2-2 (Kumamoto); Kwi 1 and S-1 (Shizukuishi, Iwate).

Fomitopsis palustris (Berk. et Curt.) Gilbn. & Ryv. strain MAFF 420001 and *Trametes versicolor* (L.:Fr.) Pilat strain MAFF 420002 were obtained from the Genebank of the National Institute of Agrobiological Sciences (NIAS). *Pestalotiopsis* sp. strain ST was isolated from *sugi* (*Cryptomeria japonica* D. Don) leaf collected from Akita Prefecture. We also used *Xeromphalina* sp. strain M-1 and *Mycena* sp. strain G-1, which had been used in previous studies.[8,9] All the fungi species used in this study were maintained on potato dextrose agar (PDA) medium and subcultured every 3 months.

Culture conditions

The cultures were incubated at 25°C under fluorescent light (approximately 800 lux). For preparation of the inoculum, *Strobilurus ohshimae* was cultivated on *sugi*-PDA plate medium (see below), and the tip of the fungal colony was cut out from the agar medium by using an 8-mm cork borer. This agar plug was inoculated onto every culture. For the preparation of *sugi*-PDA medium, *sugi* wood meal was added to PDA medium until the final concentration was 2.0% (w/v), and its supernatant was poured into separate petri dishes after autoclaving at 121°C for 40 min.

Determination of lignin-degrading activity

Bavendamm reaction was performed by following the method of Bavendamm.[19] Each fungal strain was inoculated onto the PDA medium containing 0.01% (w/v) guaiacol, 0.1% (w/v) gallic acid, or 0.05% (w/v) Remazol brilliant blue R (RBBR). After cultivation for 2 weeks, the ligninolytic activities of the fungi were qualitatively evaluated by observing the color changes of the media. Lignin degradation rates were determined by the Klason lignin method.[20] In order to prepare the solid medium of wood meal, *sugi* wood meal and beech (*Fagus crenata* Blume) wood meal, which could pass through a 1.0-mm mesh sieve and could not pass through 0.5-mm mesh sieve, were dried in a desiccator. Next, 1 g of this dried wood meal, 0.1 g of rice bran, and 5.0 ml of distilled water were mixed in a 100-ml Erlenmeyer flask and autoclaved at 121°C for 40 min. Then, each strain of fungi was cultivated on this solid wood meal medium for 30 days. After cultivation, the Klason lignin content of wood meal medium was determined.[19]

Enzyme assays

For preparation of the wood meal medium, 5.0 g of wood powder, 1.0 g of rice bran, and 25 ml of distilled water were mixed in a 100-ml Erlenmeyer flask and autoclaved (121°C, 40 min). The fungal strains were inoculated on this medium and cultivated for 30 days. After cultivation, 15 ml of potassium phosphate buffer (0.1 M, pH 7.0) was added to the medium, stirred, and left overnight at 5°C. The following day, the medium was sieved through a gauze and centrifuged (8000 g, 30 min), and the supernatant was used as the enzyme solution.

Lignin peroxidase (LiP) activity was determined using 3,4-dimethoxybenzyl alcohol (veratryl alcohol) as the substrate by measuring the increase in absorbance at 310 nm.[21] The reaction mixture contained 10 mM veratryl alcohol, 0.1 mM H_2O_2, and 20 mM sodium succinate buffer (pH 3.0). Manganese peroxidase (MnP) activity was measured by monitoring the oxidation of 2,2-azinnobis-(3-ethylbenzothialine-6-sulfonic acid) (ABTS) at 415 nm.[20] The reaction mixture contained 0.05 mM ABTS, 0.1 mM $MnSO_4$, 0.1 mM H_2O_2, 50 mM sodium lactate, and 20 mM succinate buffer (pH 4.5). We also measured MnP activity by monitoring the oxidation of 2,6-dimethoxyphenol (2,6-DMP) at 468 nm.[22] The reaction mixture contained 1.0 mM 2,6-DMP, 0.4 mM H_2O_2, 1.0 mM $MnSO_4$, and 0.1 M sodium acetate buffer (pH 4.0). Laccase activity was determined by using ABTS as the substrate.[21] Oxidation of ABTS was monitored by measuring the increase in absorbance at 415 nm. The reaction mixtures contained 0.05 mM ABTS and 0.1 M sodium acetate buffer (pH 4.0). One unit of enzyme activity was defined as the amount of enzyme required to increase 0.1 unit of absorbance per 1 min under the above conditions.[21,22] For comparison, the enzyme activity was indicated per 1 g of wood meal, because the enzyme solution was extracted from the solid medium.

Determination of glucosamine content in wood meal medium

We evaluated the quantity of mycelial growth on wood meal medium by measurement of glucosamine content, because it was reported that glucosamine content and mycelial growth show a proportional relationship.[23] The glucosamine content in wood meal medium was estimated by following the method of Tokimoto and Fukuda.[23] The wood meal medium was prepared by the same method mentioned for the determination of lignin-degrading activity. After cultivation for 30 days, the medium was dried at 105°C for 24 h.

Then, it was hydrolyzed with 72% sulfuric acid (26.5°C, 24 h) and then 4.5% sulfuric acid (100°C, 24 h) in a sealed bottle. The hydrolyzate was neutralized with barium hydroxide to pH 6.0, and the pH was stabilized by the addition of 2 ml of 1 N sodium acetate buffer (pH 6.0). After filtering the mixture through a filter paper, the filtrate was loaded on an ion exchange column (1 cm^2 × 15 cm) of Amberlite-IR 120B that had been converted to the Na^+ form with 2 M sodium chloride solution containing 0.1 N sodium hydroxide. Then the column was washed with 0.01 M sodium acetate (pH 6.0) and distilled water. Glucosamine was eluted from the column with 0.05 M sodium carbonate buffer (pH 9.0). The glucosamine content of the eluate was determined by the Elson-Morgan method.[24]

Results and discussion

Lignin degradation

The Bavendamm test was carried out as a preliminary test, and it was qualitatively shown that *Strobilurus ohshimae* possesses the capability to degrade lignin. Next, the lignin degradation rates of *S. ohshimae* were quantitatively measured on *sugi* wood meal medium by applying the Klason lignin method.[20] All the strains of *S. ohshimae* tested were found to degrade *sugi* lignin (Fig. 1a). In particular, the strain Nagaoka 2-1 degraded approximately 12% of the lignin. Under this condition, the lignin-degrading activities of *S. ohshimae* were higher than those of *Mycena* sp., *Pestalotiopsis* sp., *Xeromphalina* sp. (wood-decay fungi that were originally isolated from *sugi* woods), *Fomitopsis palustris* (a typical brown-rot fungus), and *Trametes versicolor* (a typical white-rot fungus). Therefore, *S. ohshimae* appeared to be preferable as a lignin-degrading fungus on *sugi* wood. In addition, we measured the sequential lignin degradation rate of *S. ohshimae*. As shown in Fig. 1b, the lignin content of *sugi* wood meal medium was reduced linearly, and the lignin degradation rate was approximately 28% in 90 days.

Takahashi reported that 77% of the white-rot fungi inhabit hardwood, 13% inhabit softwood, and 10% inhabit both hardwood and softwood in Japan.[12] Therefore, it is reasonable to consider that *S. ohshimae* is a comparatively minor white-rot fungus that inhabits softwood.

Productivity of lignin-degrading enzyme

Lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase productivity of *S. ohshimae* were determined. The strains Ani 2-3, Kita-Ibaraki, Nagaoka 1-1, and Nagaoka 2-1 were used in this experiment because these strains showed different lignin degradation rates in the above experiment. The production of LiP and MnP was not observed in all the strains under this condition (Table 1). On the other hand, laccase activity was detected in all the strains. The strain Nagaoka 1-1 showed the highest laccase activity of 4340 U/g.

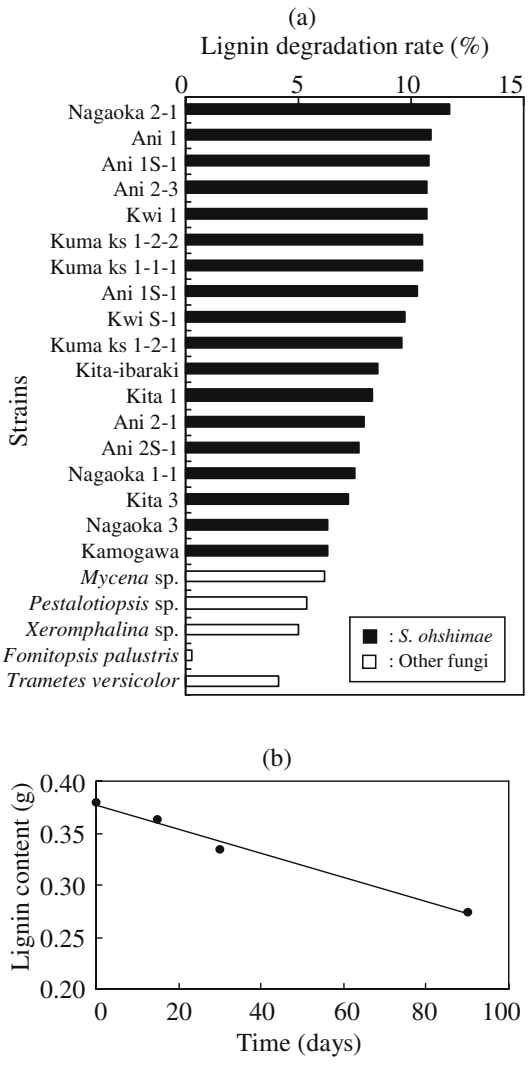

Fig. 1a,b. Lignin degradation by *Strobilurus ohshimae* on the *sugi* wood meal medium as measured by the Klason lignin method. **a** Comparison of lignin degradation rate for each strain (after 30 days of cultivation). **b** Sequential change in the lignin content of *sugi* wood meal medium on which the *S. ohshimae* Nagaoka 2-1 strain was cultivated. Lignin content is indicated per gram of *sugi* wood meal

Table 1. Productivity of lignin-degrading enzymes for each strain of *Strobilurus ohshimae*

Strain	Lignin peroxidase (U/g)[a]	Mn peroxidase (U/g)[a]	Laccase (U/g)[a]
Nagaoka 1-1	nd	nd	4340
Nagaoka 2-1	nd	nd	3240
Kita-Ibaraki	nd	nd	1680
Ani 2-3	nd	nd	1610

Each strain was cultivated on *sugi* wood meal medium for 30 days
nd, Not detected
[a] Enzyme activity per gram of wood meal

It is well known that LiP, MnP, and laccase are the main enzymes that are involved in lignin degradation by white-rot fungi and that various enzyme production patterns exist in white-rot fungi.[25] For example, *Phanerochaete*

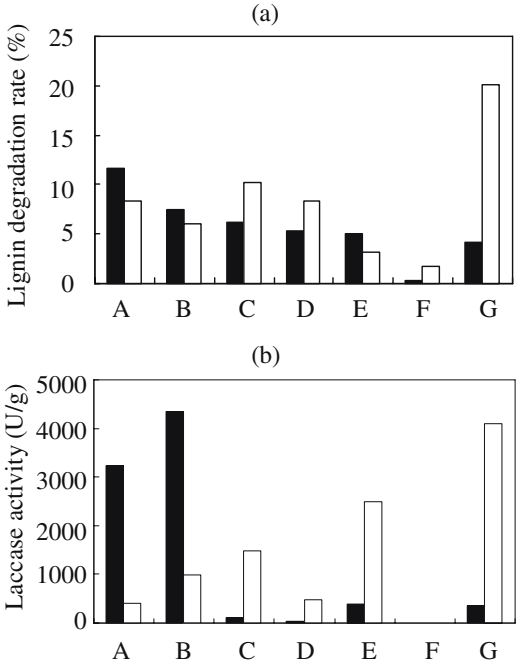

Fig. 2a,b. Comparison of *sugi* wood and beech wood in terms of lignin degradation and laccase production by *S. ohshimae* (after 30 days of cultivation). **a** Klason lignin degradation rate. **b** Laccase productivity. *Filled bars*, *sugi* wood meal medium; *open bars*, beech wood meal medium. A, *S. ohshimae* Nagaoka 2-1; B, *S. ohshimae* Nagaoka 1-1; C, *Mycena* sp.; D, *Pestalotiopsis* sp.; E, *Xeromphalina* sp.; F, *Fomitopsis palustris*; G, *Trametes versicolor*. Laccase activity is indicated per gram of wood meal

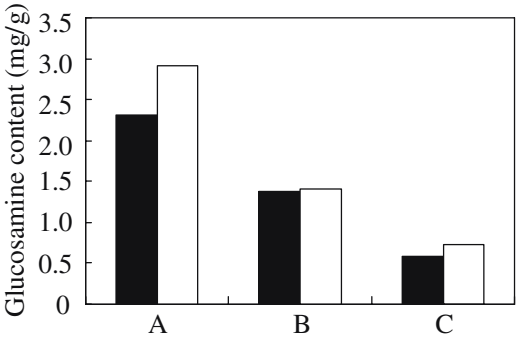

Fig. 3. Comparison of quantities of mycelial growth on *sugi* wood and beech wood determined by glucosamine content in wood meal medium (after 30 days of cultivation). *Filled bars*, *sugi* wood meal medium; *open bars*, beech wood medium. A, *S. ohshimae* Nagaoka 1-1; B, *Trametes versicolor*; C, *Fomitopsis palustris*. Glucosamine content is indicated per gram of wood meal

chrysosporium produces LiP and MnP, but lacks laccase activity.[15,16,26] *Trametes versicolor* produces all of these three enzymes,[17,18,25] and *Pycnoporus cinnabarinus* shows only laccase activity.[25–27] Therefore, *S. ohshimae* was also considered to be a lignin-degrading fungus, which mainly produced laccase, similar to *T. versicolor* or *P. cinnabarinus*.

Comparison of *sugi* wood and beech wood in terms of lignin degradation and laccase production

As described above, it is considered that *S. ohshimae* is a comparatively minor white-rot fungus that mainly decays softwood. Thus, to investigate whether it is difficult for *S. ohshimae* to degrade the lignin of *sugi*, a conifer, we determined the lignin degradation rate and laccase productivity both on the *sugi* wood meal medium (*sugi* medium) and on the beech wood meal medium (beech medium); the results were compared with those of other wood-decay fungi. The strain Nagaoka 2-1, which showed the highest lignin degradation rate, and Nagaoka 1-1, which showed the highest laccase productivity, were used in this experiment. The lignin degradation rates of *T. versicolor*, *F. palustris*, *Mycena* sp., and *Pestalotiopsis* sp. were higher on the beech medium than on the *sugi* medium (Fig. 2a). On the other hand, the lignin degradation rate of *S. ohshimae* was higher on the *sugi* medium than on the beech medium. Similarly, the laccase productivity of *S. ohshimae* differed from that of other fungi, i.e., laccase production by *S. ohshimae* on the *sugi* medium was higher than that on the beech medium (Fig. 2b). We also determined the quantity of mycelial growth on the *sugi* medium and on the beech medium by measurement of glucosamine content in the wood meal medium. The glucosamine content in the *sugi* and beech mediums was similar for *T. versicolor*, while the content was higher in the beech medium than in the *sugi* medium for *S. ohshimae* and *F. palustris* (Fig. 3). It might be considered that the growth is not necessarily the primary factor that influences lignin degradation; some other factors, such as the productivity of lignin-degrading enzymes, might influence lignin degradation more greatly. Furthermore, our results suggest that lignin of softwood is not always difficult to degrade for certain fungi, particularly for fungi that inhabit softwood such as *S. ohshimae*, although it is generally stated that the degradation of coniferous lignin is difficult in comparison with that of hardwood.[12,13]

Limited progress has been achieved in research on white-rot fungi, which mainly decay softwood. It is known that the lignin structure of softwood and hardwood differ widely.[11–14] Therefore, the lignin degradation system of *S. ohshimae* might be slightly different from that of fungi such as *T. versicolor*, which mainly decay hardwood. We are currently attempting to purify and characterize the laccase produced by *S. ohshimae* in order to clearly understand its lignin degradation system.

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