

Indoor Cultivation and Cultural Characteristics of *Wolfiporia cocos* Sclerotia Using Mushroom Culture Bottles

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We newly developed an indoor cultivation technique for *Wolfiporia cocos* (WOLF) RYVARDEN *et* GILBERTSON (Syn. *Poria cocos* WOLF), not with soil, but using mushroom culture bottles with pine logs, and clarified some cultural characteristics of sclerotia in the laboratory. To determine the optimum conditions for sclerotia growth, the weight of sclerotia and concentration of CO₂ in three different air filters; cloth, paper and urethane resin, and closed bottles were tested. When the cloth air filter was used, the growth rate was the fastest and the yield was maximal. These results suggested that the aeration was an important environmental factor for cultivation. To clarify the characteristics of culture in the cloth air filtered and closed bottles, the weight of sclerotia, the compositions of pine logs and the contents of pachymic acid and dehydropachymic acid were examined during 24 weeks. The growth of sclerotia and the wood decaying efficiency in the cloth air filtered bottles were better than those in the closed bottles. Also, it was found that *W. cocos* was a brown rot fungus due to the alkaline solubility of pine logs in the wood decay process. In addition, the contents of pachymic acid and dehydropachymic acid and the TLC pattern between the cultivated and commercial sclerotia did not differ remarkably.

Key words *Wolfiporia cocos* (Syn. *Poria cocos*); sclerotia; indoor cultivation; cultural characteristic; pine log; mushroom culture bottle

Wolfiporia cocos (WOLF) RYVARDEN *et* GILBERTSON (Syn. *Poria cocos* WOLF), belonging to the Polyporaceae in basidiomycetes,¹⁾ forms sclerotia on the roots of such trees as pine, cedar, fir and oak.²⁾ The major host plant of this fungus in Japan, China and Korea is the pine and the dried sclerotium of *W. cocos* is called “Bukuryo” in Japanese. In the Japanese Pharmacopoeia, the external layer is usually mostly removed.³⁾

It is one of the most important crude drugs and is used in many Kampo formulae, for example, keishibukuryogan, goshajinkigan, goreisan and saireito. Recently, it has been reported that the triterpenoids of sclerotia have anti-inflammatory and anti-tumor promotion effects,⁴⁾ anti-emetic activity⁵⁾ and an efferent activity of the gastric vagus nerve.⁶⁾

In Japan, there are few reports on the cultivation and biological studies of *W. cocos*. In the field, cultivation has obtained about 1 kg of sclerotium, which was unfortunately contaminated with a large amount of soil,⁷⁾ and obtained only a small amount of sclerotium, about 25 g, by dry weight.⁸⁾ In the laboratory, the formation of the fruit body has been obtained on agar or liquid media and sawdust media.^{2,7,8)} However, there have been no studies as to the indoor cultivation and biological characteristics necessary for the formation of sclerotia.

In the studies on cultivation with pine logs, we have shown cultivation in the field. The strain formed large sclerotia that were not contaminated with soil. The chemical qualities of the cultivated sclerotia were almost the same as those from commercial sources.⁹⁾ On the other hand, we attempted a culture method of sclerotia using closed plastic bottles without soil in the laboratory. Generally, soil has been considered necessary to cultivate *W. cocos* sclerotia, but the formation of

sclerotia was recognized in the bottles as well as in the cultivation in the field. The optimum temperature for sclerotia growth was 25 °C. The results suggested that this culture method may be used for the efficient production of sclerotia.¹⁰⁾

Here, in order to establish a suitable method of indoor cultivation for production, we report on the improved culture method using mushroom culture bottles and some cultural characteristics of sclerotia in the laboratory.

It was reported that a proper amount of moisture and supply of oxygen appeared to be important for the growth of sclerotia for cultivation in the field.⁸⁾ Therefore, in this report, to determine the optimum conditions for sclerotia growth, three different air filtered and closed bottles were tested. Next, to clarify the cultural characteristics, the growth of sclerotia, the wood compositions and the contents of pachymic acid and dehydropachymic acid were measured. Additionally, the contents of these compounds and the TLC pattern of the cultivated samples were compared with those of the commercial samples.

MATERIALS AND METHODS

The Strain of *W. cocos* This strain, which formed large sclerotia in the field cultivation, was maintained on a potato dextrose agar in our laboratory.⁹⁾

Mushroom Culture Bottle We used to Shiitake mushroom (*Lentinula edodes*) culture bottle with a cap equipped with an air filter. It consisted of three jointly combined parts, which were the cap, the upper part and the lower part, were autoclavable, 2300 cm³ in volume, approximately 15 cm diameter and 16 cm height. The three different air filters were

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commercially available and made of cloth, paper and urethane resin (the following is urethane). In addition, the closed bottle, where the cap was covered, was used as a control.

Inoculum and Indoor Cultivation The mycelia of the strain were inoculated on the sawdust rice bran (3:1 V/V) media with a moisture content of 70% and were cultured for one month at 30 °C.⁹⁾ Three pine logs of *Pinus densiflora* SIEB. *et* ZUCC., which were approximately 5 cm diameter and 10 cm in length, were soaked in water overnight, then placed in each bottle, sterilized by autoclave for 30 min, and cooled to room temperature. This spawn was inoculated to logs, and the bottles were incubated in the dark at 25 °C, the optimum temperature for sclerotia formation.¹⁰⁾

Harvest of Sclerotia The sclerotia formed on pine logs were harvested. The external layer was removed and dried at 50 °C for 18 h. The yields were determined by the fresh and dry weight on a wood volume (m³) basis.

The Concentration of CO₂ in Bottle The concentration of CO₂ was measured by a gas detector tube connected with silicon tubing to the bottle.

The Weight Loss Due to Wood Decay¹¹⁾ At first, dried weight of the initial pine logs were calculated from wet weight and mean moisture contents of soaked pine logs. After sclerotia formed on pine logs were harvested, cultured wood was dried at 105 °C for 18 h, and dried weight was measured. The weight loss due to wood decay was determined by the difference between the dry weight of pine logs before and after culture.

The Alkaline Solubility in 1% Aqueous NaOH Based on Weight of Decayed Wood^{11,12)} The sapwood of dried pine logs were pulverized, and about 1 g of the powder was extracted under reflux with 50 ml of 1% NaOH in boiling water for 1 h. The solution was passed through a glass filter (1G3) and rinsed with 150 ml of hot water. The residue was added to 25 ml of 10% CH₃COOH and rinsed with 150 ml of hot water. Then, the glass filter was dried at 105 °C for 18 h. The alkaline solubility based on weight of decayed wood was determined by the difference between the dried weight of sample and filtered residue.

pH of Wood¹³⁾ The log chips, cubes of approximately 2×2×2 cm, were cut from the sapwood of dried pine logs. The wood pH was measured using 17 ml of distilled water in which each chip was soaked for 5 d.

Analysis of Pachymic Acid and Dehydropachymic Acid⁹⁾ The powder of dried sclerotia was accurately weighed at 2 g, extracted with 20 ml of CH₃OH for 20 min under sonication, and then centrifuged at 3000 rpm for 10 min. The residue was similarly extracted by the same method. Both supernatants were combined and made to 50 ml with CH₃OH. The pachymic acid and dehydropachymic acid were measured by HPLC. The HPLC system consisted of ODS-80TM column (150×4.6 I.D. mm) at 40 °C and an injection volume of 20 μl. The mobile phase was CH₃CN, H₂O and CH₃COOH (700:300:1). The flow rate was 1.0 ml/min. The peaks were detected at 210 nm for pachymic acid and 240 nm for dehydropachymic acid. After the powder was dried at 105 °C for 5 h, these contents of pachymic acid and dehydropachymic acid were calculated on the basis of the dry weight of sclerotia.

Chemical Comparison of the Contents by HPLC and TLC Pattern in the Cultivated and Commercial Samples

The cultivated and commercial samples were measured by HPLC with the former method for the contents of pachymic acid and dehydropachymic acid. We examined the one sample of indoor cultivation in the laboratory and the 5 commercial samples of Chinese products (Locality: Fujian, Hubei, Anhui, Guizhou and Henan).

On the other hand, the cultivated and commercial samples were measured by TLC pattern. The powder of dried sclerotia was weighed at 2 g, extracted with 20 ml of C₂H₅OC₂H₅ for 30 min under sonication, and then centrifuged at 3000 rpm for 10 min. The supernatant was evaporated and added to 0.5 ml of CH₃OH. The solution was analyzed by Silica gel F₂₅₄-TLC with CHCl₃-CH₃OH (90:10). The spots were detected under UV (254 nm). Then, the plate was sprayed with a vanillaldehyde-sulfuric acid solution and heated. We then examined the one sample of indoor cultivation in the laboratory and the 2 commercial samples of Chinese products (Locality: Fujian, Anhui).

RESULTS AND DISCUSSION

Effects of Aeration on Sclerotia Growth of *W. cocos* Using Mushroom Culture Bottles To determine the optimum conditions for sclerotia growth, the bottles with three different air filters; cloth, paper and urethane, which were commercially available, and the closed bottles as a control were tested.

The yields of cultivation incubated for 14 weeks under various air filters are shown in Table 1. The order of sclerotia weight was cloth>urethane>closed (control)>paper. In the yields, a significant difference was recognized between the cloth air filter and the others. Namely, when the cloth air filter was used, sclerotia weight was maximal. With the paper air filter, the ratio of dry/fresh weight was 72±4%, because sclerotia were dried out.

Indoor cultivation using mushroom culture bottles are shown in Fig. 1. Sclerotia formed on pine logs, and the length of each sclerotium was 15 cm or less. The external layers were dark brown, thin and soft. The internal ones were white in color and slightly soft. When the cloth air filter was used, the amount of sclerotia from which the external layers were removed weighed about 130 g (fresh) and 65 g (dry) per bottle.

Next, the growth of sclerotia and the concentration of CO₂, as an indication of the aeration in the bottles, were measured during 20 weeks. The effects of air filters on growth are shown in Fig. 2. When the cloth air filter was used, the

Table 1. Yields of Sclerotia Cultivation of *W. cocos* Incubated for 14 Weeks under Various Air Filters of Mushroom Culture Bottles in the Laboratory

Air filters of the bottles	Sclerotia weight of <i>W. cocos</i> (kg/m ³)		Dry/fresh ratio (%)
	Fresh	Dry	
Cloth	207±13	123±6	59±2
Paper	62±27**	44±19**	72±4
Urethane	180±17*	99±9**	55±2
Closed	104±34**	52±20**	50±4

Each value represents the mean±standard deviation (S.D.), n=7. * and **: Statistical significance levels are at p<0.05 and p<0.01 respectively when compared with the weight of sclerotia on the cloth air filter by the least significant difference test.

growth rate was the fastest. For the concentration of CO₂ in the bottles, the order of CO₂ levels was closed (control)>urethane>cloth>paper. The growth of sclerotia was influenced by the aeration in the bottles. Indeed, the aeration with the paper air filter was better than that with the cloth air filter, but it showed an excessive aeration, resulting in the sclerotia being dried out.

Therefore, the results obtained from these experiments suggested that the aeration, which was controlled by air filters, was an important environmental factor for the cultivation of *W. cocos*. In these studies, the cloth air filtered bottles were the optimum factor for indoor cultivation of *W. cocos* sclerotia.

Studies on the Characteristics of Culture of *W. cocos*

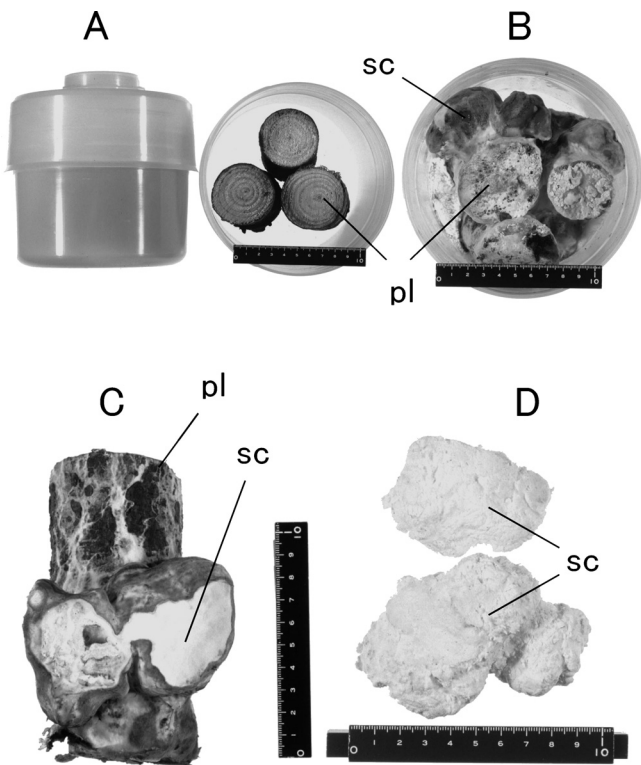


Fig. 1. Sclerotia Cultivation of *W. cocos* in Mushroom Culture Bottles with the Cloth Air Filter

(A) Diagram of mushroom culture bottle in three pine logs at 0 weeks. (B) Sclerotia cultivation of *W. cocos* incubated for 14 weeks in mushroom culture bottle. (C) Sclerotia were formed on pine log incubated for 14 weeks. (D) The external layer of fresh sclerotia was removed. pl, Pine logs; sc, Sclerotia of *W. cocos*.

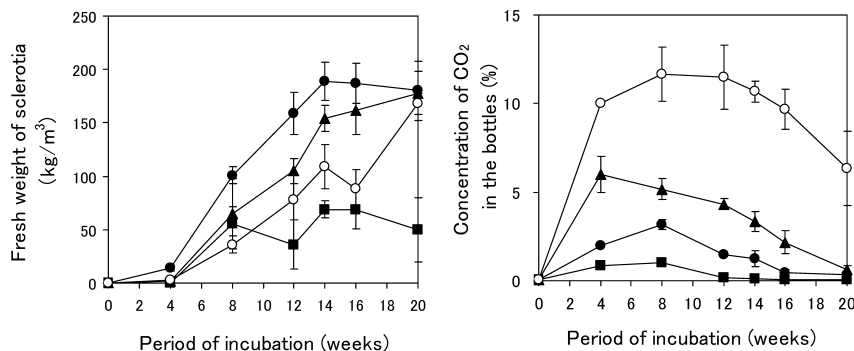


Fig. 2. Effects of Air Filters on Sclerotia Growth (Fresh Weight) of *W. cocos* and the Concentration of CO₂ in Mushroom Culture Bottles

Each point represents the mean ± S.D., n=3. ●, cloth; ■, paper; ▲, urethane; ○, closed.

with the Cloth Air Filtered and the Closed Bottles To clarify the cultural characteristics of the sclerotia of *W. cocos*, the growth during a period of 24 weeks was examined as to the character of sclerotia formed (the weight, the contents of pachymic acid and dehydropachymic acid) and the wood compositions of logs (the weight loss due to decay, the alkaline solubility based on weight of decayed wood and the pH of wood) in the bottles with cloth air filters and the closed bottles as a control.

The time course of sclerotia growth in both bottles are shown in Fig. 3. There was a remarkable difference in the growth patterns between in the cloth air filtered bottles and the closed bottles. The growth of sclerotia in the cloth air filtered bottles was faster and the sclerotia weight was maximum at the 14th week. On the other hand, that in the closed bottles slowly increased during 24 weeks. At the 14th week, fresh weight of the former ($224 \pm 2 \text{ kg/m}^3$) was about 1.6 times that of the latter ($144 \pm 28 \text{ kg/m}^3$). However, these were equivalent to the weight after the 20th week. Further, the growth curves of sclerotia on the cloth air filtered bottles were similar to the general growth pattern of microorganisms. The patterns showed that after an early lag phase, an exponential growth phase occurred and a stationary phase was sustained.

For the wood compositions of logs, the time course of weight loss of pine logs due to wood decay are shown in Fig. 4. The level of wood decay in the cloth air filtered bottles

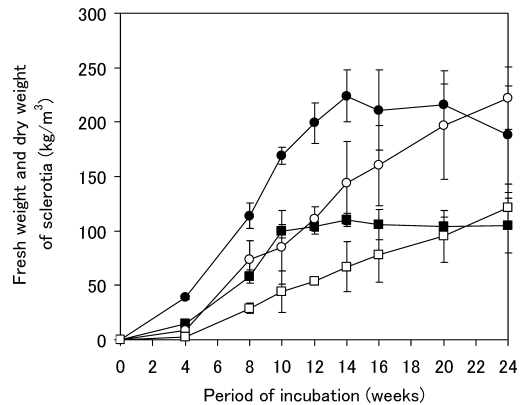


Fig. 3. Time Course of Sclerotia Growth of *W. cocos* in Mushroom Culture Bottles with the Cloth Air Filter and the Closed Bottles

Each point represents the mean ± S.D., n=5. ●, fresh weight in cloth air filtered bottles; ■, dry weight in cloth air filtered bottles; ○, fresh weight in closed bottles; □, dry weight in closed bottles.

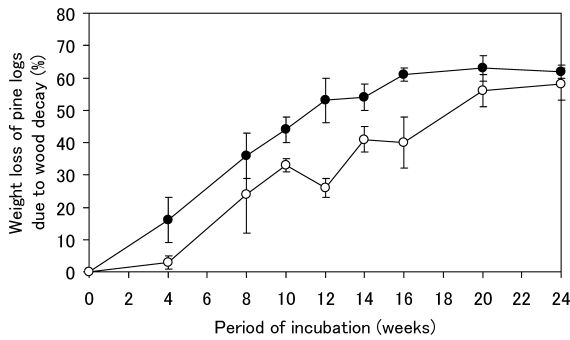


Fig. 4. Time Course of Weight Loss of Pine Logs due to Wood Decay by *W. cocos* in Mushroom Culture Bottles with the Cloth Air Filter and the Closed Bottles

Each point represents the mean \pm S.D., $n=5$. ●, cloth air filtered bottles; ○, closed bottles.

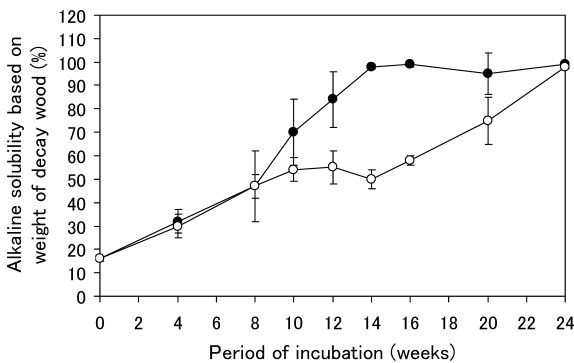


Fig. 5. Time Course of Alkaline Solubility in 1% Aqueous NaOH Based on Weight of Decayed Pine Logs by *W. cocos* in Mushroom Culture Bottles with the Cloth Air Filter and the Closed Bottles

Each point represents the mean \pm S.D., $n=5$. ●, cloth air filtered bottles; ○, closed bottles.

was higher than those in the closed bottles during culture. The wood decay curves were similar to the growth patterns of sclerotia. This method of cultivation was found to effect faster growth of sclerotia and wood decay of pine logs by aeration.

The time course of alkaline solubility in 1% aqueous NaOH based on weight of decayed pine logs are shown in Fig. 5. The alkaline solubility generally involves the contents of lignin, tannins, lipids and a part of hemicelluloses in the wood composition.^{11,14} The alkaline solubility rapidly increased after the 8th week, and the level was about 100% at the 14th week in the cloth air filtered bottles. It seems that *W. cocos* degrades most of the other wood constituents (cellulose and hemicelluloses) at the 14th week, the growth of sclerotia stops due to exhaustion of these substances, cellulose and hemicelluloses. On the other hand, the level in the closed bottles was 100% at the 24th week. Therefore, the growth of sclerotia and the wood decaying efficiency in the cloth air filtered bottles were better than those in the closed bottles.

Also, the time course of wood pH of pine logs are shown in Fig. 6. The wood pH remained constant at about pH 3.0 after the 4th week. It was reported that brown rot fungi produced oxalic acid in culture, and that both iron and oxalic acid were involved in cellulose depolymerization by these fungi, and oxalic acid production by *W. cocos* was enhanced

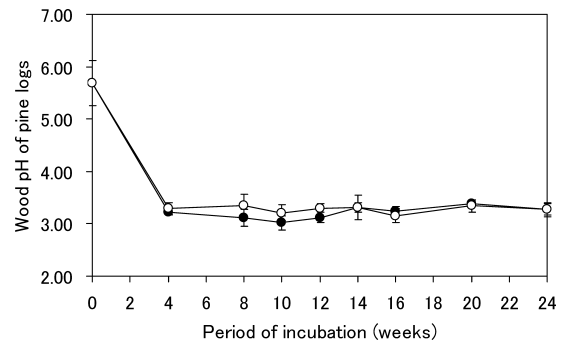


Fig. 6. Time Course of Wood pH of Pine Logs in Mushroom Culture Bottles with the Cloth Air Filter and the Closed Bottles

Each point represents the mean \pm S.D., $n=5$. ●, cloth air filtered bottles; ○, closed bottles.

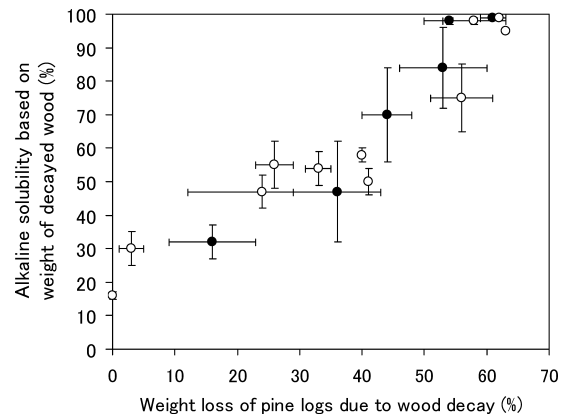


Fig. 7. Alkaline Solubility in 1% Aqueous NaOH of Pine Logs in Progressive Stages of Decay by *W. cocos* in Mushroom Culture Bottles with the Cloth Air Filter and the Closed Bottles

Each point represents the mean \pm S.D., $n=5$. ●, cloth air filtered bottles; ○, closed bottles.

on wood compared to liquid medium.^{15,16} Therefore, it seemed that wood pH was acidic due to oxalic acid.

This indoor cultivation was compared with our cultivation in the field as to the yields of dried sclerotia and cultivation periods. The former was $110 \pm 6 \text{ kg/m}^3$ on incubation for 14 weeks. The latter was about 21 kg/m^3 after removing the crust and drying on cultivation for 21 months.⁹ According to the yields and periods, it seems that the efficiency of productivity of this indoor method is better than that in the field method.

The types of wood decaying fungi are generally classified into brown rot fungi and white rot fungi. It is known that brown rot fungi mainly decay cellulose and hemicelluloses, whereas white rot fungi simultaneously decay cellulose, hemicelluloses and lignin.¹⁴ The classification of both rot fungi can be presumed from the Bavendamm reaction¹⁷ or from the relation between the alkaline solubility based on weight of decayed wood and the weight loss due to wood decay. It was reported that *W. cocos* was a brown rot fungus using the Bavendamm reaction test.^{8,18}

In our studies, the wood decay due to *W. cocos* was examined by the alkaline solubility based on weight of decayed wood and the weight loss due to wood decay. The progressive changes in wood during decay are shown in Fig. 7. As alkaline solubility in 1% aqueous NaOH of pine logs in the progressive stages of decay by fungus, brown rot fungi markedly

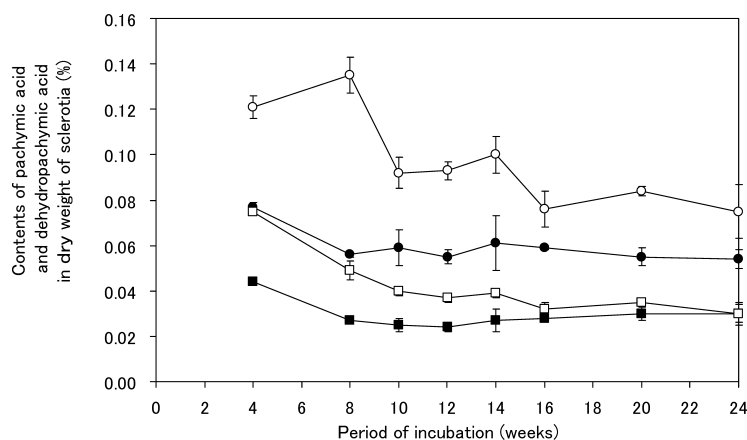


Fig. 8. Time Course of the Contents of Pachymic Acid and Dehydropachymic Acid in Sclerotia of *W. cocos* from the Cultivated Samples in the Laboratory

Each point represents the mean \pm S.D., $n=5$. ●, pachymic acid of cloth air filtered bottles; ■, dehydropachymic acid of cloth air filtered bottles; ○, pachymic acid of closed bottles; □, dehydropachymic acid of closed bottles.

increase the solubility of wood, on the other hand, white rot fungi do not greatly change the solubility of wood.¹¹⁾ From both progressive changes, it was found that *W. cocos* was a brown rot fungus from the results of alkaline solubility of pine logs in progressive stages of decay on an indoor cultivation using mushroom culture bottles. In addition, this result agrees with that obtained by the Bavendamm reaction test.^{8,18)}

The time course of the contents of pachymic acid and dehydropachymic acid in sclerotia are shown in Fig. 8. The changes in the contents of the cloth air filtered and closed bottles showed the same tendency. Both contents were contained from the early phase in culture. The former was gradually decreased during the period of culture, and the latter did not change much after the 8th week. However, the contents of pachymic acid and dehydropachymic acid in sclerotia with the closed bottles were higher than that with the cloth air filtered bottles.

Comparison between the Cultivated and Commercial Samples of These Compounds The comparison of the contents of pachymic acid and dehydropachymic acid are shown in Fig. 9. The contents of pachymic acid and dehydropachymic acid did not differ remarkably between the cultivated sample incubated for 14 weeks in the cloth air filtered bottles and the 5 commercial samples. Also, The TLC pattern under UV and a vanillaldehyde-sulfuric acid solution spraying did not differ remarkably between the cultivated sample and the 2 commercial samples (data not shown).

In the morphological comparison of internal sclerotia in the cultivated sample and the commercial samples, the cultivated sample was white in color and slightly soft. On the other hand, the commercial samples were white in color and hard in texture.

In conclusion, the indoor cultivation of *W. cocos* using mushroom culture bottles without soil forms sclerotia in high yield during a short period. Also, the cultural characteristics for the formation of sclerotia have been clarified by these studies. The results presented here suggest that it is possible to mass-produce the sclerotia of *W. cocos* using an indoor cultivation method.

Hereafter, we are going to evaluate the chemical qualities of the cultivated samples, except for the contents of

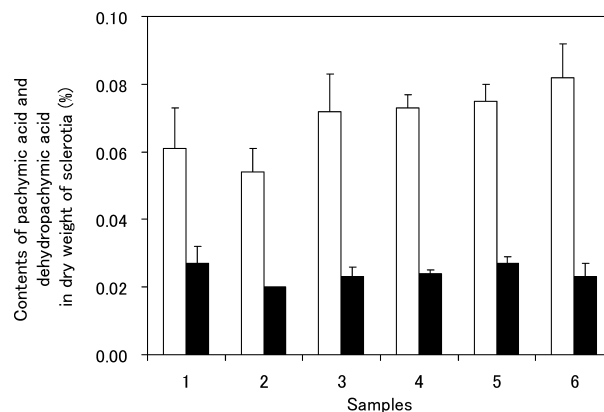


Fig. 9. Comparison of the Contents of Pachymic Acid and Dehydropachymic Acid in the Cultivated Sample and the Commercial Samples

Each point represents the mean \pm S.D., $n=3$. □, pachymic acid; ■, dehydropachymic acid. Cultivated sample 1, indoor cultivation. Commercial samples (Chinese products) 2, Fujian; 3, Hubei; 4, Anhui; 5, Guizhou; 6, Henan.

pachymic acid and dehydropachymic acid.

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