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Productivity of hydrolytic enzymes by mycorrhizal mushrooms

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To survey the potential for production of extracellular hydrolytic enzymes by mycorrhizal mushrooms, productivities of these exo-enzymes from mycelia on potato-dextrose liquid medium were determined. *Tricholoma matsutake* produced relatively high levels of CM-cellulase and avicelase activities in all test strains. It also produced higher activity of acid proteinase than neutral proteinase. Its xylanase activities seemed to be higher than those of the other carbohydrases. The productivities of *Lyophyllum shimeji* strains were at similar levels to those of *T. matsutake* strains. CM-cellulase and avicelase activities of *L. shimeji* were higher than those of *T. matsutake*. Neutral proteinase in *L. shimeji* strains showed higher activity levels than acid proteinase. The relative productivities of hydrolytic enzymes between the groups of mycorrhizal mushrooms and wood-rotting mushrooms were also examined. *T. matsutake* and *L. shimeji* both produce many kinds of hydrolytic enzymes in their culture broth, and the potential for production of hydrolytic enzymes by mycorrhizal mushrooms was judged to be relatively high.

Key Words——extracellular hydrolytic enzyme; *Lyophyllum shimeji*; mycorrhizal mushroom; *Tricholoma matsutake*

Since the growth of the ectomycorrhizal mushrooms *Tricholoma matsutake* (S. Ito et Imai) Singer and *Lyophyllum shimeji* (Kawamura) Hongo depends on living plants, they grow slowly in vitro and scarcely produce fruit-bodies in artificial cultivation. These mushrooms thus seem to differ in their physiological and nutritional traits from wood-rotting mushrooms such as *Lentinus edodes* (Berkeley) Singer (Leathum, 1985; Azuma and Kitamoto, 1994). The nutritional requirements of *T. matsutake* were precisely investigated by Kawai and Abe (1976) and Kawai and Terada (1976). Ohta (1990) and Inaba et al. (1993) proposed suitable ingredients of culture media for mycorrhizal mushrooms. Recently, Yoshida et al. (1994) reported the changes of chemical components in mycelia during the vegetative growth of *L. shimeji* and *L. fumosa* (Pers.: Fr.) P. D. Orton.

Little research has been conducted on hydrolytic enzymes of mycorrhizal mushrooms. Nakazawa et al. (1974) and Enokibara et al. (1993) have reported the productivities of cellulolytic enzymes of this kind of mushrooms. Terashita and Kono (1987) reported the purification and properties of carboxyl proteinases of mycorrhizal mushrooms, *T. matsutake* and related species. The carboxyl proteinase was found to be common to all 17 strains tested (Terashita and Kono, 1989). Zhu et al. (1990) have reported the production, purification and characterization of extracellular acid proteinase from the ectomycorrhizal mushroom *Hebeloma crustuliniforme* (Bull.: St.-Amans) Quél. However, other hydrolytic enzymes of mycorrhizal mushrooms have been not yet investigated, though they may play an important role in the degradation of substrate for the growth of the mushroom.

In this paper, to examine the potential for production of hydrolytic enzymes by mycorrhizal mushrooms such as *T. matsutake, L. shimeji, Suillus bovinus* (Fr.) Kuntze and *Lactarius hatsudake* Tanaka, the productivity of the enzymes was studied on potato-dextrose liquid medium.

Materials and Methods

Microorganisms The strains of mycorrhizal mushrooms used were *T. matsutake, L. shimeji, S. bovinus* and *L. hatsudake* (Table 1). For comparison with these mycorrhizal mushrooms, strains of the wood-rotting mushrooms *Lentinus edodes* (Berkeley) Singer, *Flammulina velutipes* (Curtis: Fr.) Singer and *Pleurotus ostreatus* (Jacquin: Fr.) Kummer were also used (Table 1). These strains were stocked in our laboratory.

Culture conditions As inoculum, a mycelial block (5 × 5 mm) was cut from a mycelial colony grown on potato-dextrose agar medium (PDA, Nissui Co.) for 10 days for wood-rotting mushrooms or 30 days for mycorrhizal mushrooms at 24°C on a Petri dish (90 mm in diam). It was planted in a 300-ml Erlenmeyer flask containing 30 ml of potato-dextrose liquid medium (400 g potato extract and 20 g glucose per liter) according to Kitamoto and Gruen (1976). The initial pH of medium was adjusted to 5.1 before sterilization at 119°C for 8 min.

The mycelia of *T. matsutake* were stationarily cultured at 24°C for 120 days and those of *L. shimeji* and other mycorrhizal mushrooms at 24°C for 75 days under 50–100 lx of fluorescent light. Wood-rotting mushrooms, *L. edodes, F. velutipes* and *P. ostreatus*, were cul-

Table 1. Mushroom test stocks.

Tricholoma matsutake KBM-01	*Lyophyllum shimeji* Yamada-2
Tricholoma matsutake KBM-08	
Tricholoma matsutake Ichikawa	*Suillus bovinus* Ichikawa
Tricholoma matsutake ATCC 64715 (KUM-01)	*Suillus bovinus* Kasai
Tricholoma matsutake Kyoto M-48	
Tricholoma matsutake Okayama Tetsuta	*Lactarius hatsudake* Kasai
Tricholoma matsutake Yamaguchi	
Tricholoma matsutake Shiga Tm-18	
	Lentinus edodes Shohatsu
Lyophyllum shimeji Kamiakizato-1	*Lentinus edodes* KB-10
Lyophyllum shimeji Kamiakizato-3	*Lentinus edodes* Niigata
Lyophyllum shimeji Asako	*Flammulina velutipes* Iisui
Lyophyllum shimeji Yamada-1	*Pleurotus ostreatus* Kitamura

tured on the same medium at 24°C for 25 days.

Sampling and assays of hydrolytic enzymes Culture broths of each strain were obtained by filtration with No. 2 filter paper and assayed for each of the enzyme activities described below. Each assay was done twice.

Acid proteinase activity was assayed at pH 3.0 and neutral proteinase activity at pH 7.0 (Terashita et al., 1993a). The enzyme reaction was carried out at 37°C for 30 min with Hammarsten casein (Wako Pure Chem. Indust. Co.) in 0.1 M McIlvaine buffer (pH 2.8) or Bacto hemoglobin (Difco Lab.) in 0.1 M Kolthoff buffer (pH 7.2) as a substrate. One unit of enzyme activity was defined as the quantity of enzyme that liberates 1 μg of tyrosine per ml of reaction mixture per min.

CM-cellulase and avicelase were assayed by determining the amounts of reducing sugar liberated from, respectively, carboxymethyl cellulose (CMC-Na, Wako Pure Chem. Indust. Co.) in 0.1 M sodium acetate buffer (pH 5.0) and avicel-SF (Asahi Kasei Co.) in the same buffer (Terashita et al., 1993b). Chitinase activity was determined by the method reported previously (Terashita et al., 1992): the reaction mixture consisting of 0.5 ml of crude enzyme solution and 0.5 ml of colloidal chitin in 0.2 M sodium acetate buffer (pH 5.0) was incubated at 37°C for 60 min, and the reaction was stopped by boiling at 100°C for 2 min. The reducing sugars produced in the mixture were measured by the method of Somogyi-Nelson (Somogyi, 1952). For the substrates of β-1,3-

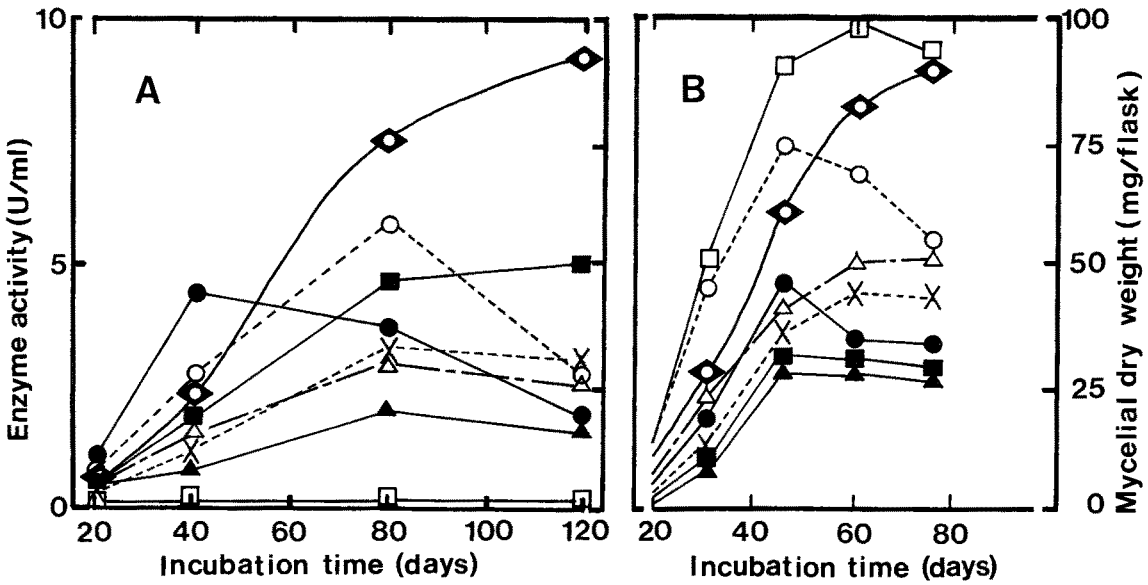

Fig. 1. Time course of hydrolytic enzyme production of *Tricholoma matsutake* and *Lyophyllum shimeji* in potato-dextrose liquid medium by stationary culture.
A: *Tricholoma matsutake* ATCC 64715 (KUM-01).
B: *Lyophyllum shimeji* Kamiakizato-1.
—●—: CM-Cellulase, ···○···: Avicelase, —■—: β-1,3-Glucanase, ---△---: Xylanase,
—▲—: Chitinase, ···×···: Acid proteinase, —□—: Neutral proteinase, —◇—: Mycelial dry weight.

glucanase and xylanase (Ohga, 1992), laminarin (Nakarai Chem. Co.) in 0.1 M McIlvaine buffer (pH 5.0) and xylan (Sigma Chem. Co.) in distilled water were used, respectively. These reactions were carried out at pH 5.0, and the activities were estimated by determining the liberated amount of reducing sugar by the method of Somogyi-Nelson (Somogyi, 1952) using D-glucose as a standard.

Determination of mycelial dry weight The harvested mycelia were washed with distilled water to remove the culture broth, dried in an oven at 110°C to constant weight, then weighed after cooling in a vacuum desiccator. The average value of five replicates is shown in the results.

Results

Time course of production of extracellular hydrolytic enzymes by *T. matsutake* and *L. shimeji* In a preliminary experiment to determine the optimal conditions for production of extracellular hydrolytic enzymes from mycorrhizal mushrooms, the time course of production of CM-cellulase, avicelase, β-1,3-glucanase, xylanase, chitinase and proteinases (acid and neutral) during the mycelial growth was studied for each one strain of *T. matsutake* and *L. shimeji*. Figure 1 shows the relation between their extracellular enzyme activities and mycelial dry weights. The mycelial dry weights of both organisms increased during 80 days of cultivation. *T. matsutake* produced all of the extracellular hydrolytic enzymes except for neutral proteinase, and most of the enzyme activities reached a maximum activity at 80 days (Fig. 1-A). *L. shimeji* produced all of the hydrolytic enzymes, and their maximum levels were attained 45-60 days after inoculation (Fig. 1-B).

These results demonstrated that *T. matsutake* and *L. shimeji* produced weak activities of various hydrolytic enzymes including cellulases.

Productivity of hydrolytic enzymes of mycorrhizal mushrooms

Hydrolytic enzymes of *T. matsutake* strains Variations in productivity of hydrolytic enzymes between strains of *T. matsutake* were examined. β-1,3-Glucanase activity was assayed at 120 days, while other activities were measured 80 days after inoculation. As shown in Table 2, *T. matsutake* produced relatively high values of CM-cellulase and avicelase activities in all strains. Of the proteinases, acid proteinase showed higher activity than neutral proteinase in each strain, but the acid proteinase activity varied widely among the test strains. Xylanase activities seemed to be higher than those of the other carbohydrolases, chitinase and β-1,3-glucanase.

Hydrolytic enzymes of *L. shimeji, S. bovinus* and *L. hatsudake* The activities of extracellular hydrolytic enzymes of *L. shimeji, S. bovinus* and *L. hatsudake* (Table 1) grown in potato-dextrose liquid medium were determined. CM-cellulase and avicelase were assayed at 45 days, while other activities were measured 60 days after inoculation (see Fig. 1B). The results are shown in Table 3. The productivity of extracellular hydrolytic enzymes of *L. shimeji* strains was similar to those of *T. matsutake* stains. CM-cellulase and avicelase activities of this mushroom seemed to be higher than those of *T. matsutake*. Neutral proteinase in *L. shimeji* strains showed higher activity levels than acid proteinase.

In *S. bovinus* and *L. hatsudake*, avicelase showed higher activities than CM-cellulase. The productivity of hydrolytic enzymes in *S. bovinus* and *L. hatsudake* was considerably lower than those in *L. shimeji* and *T. matsutake* strains.

Productivity of hydrolytic enzymes in wood-rotting mushrooms To compare the productivity of hydrolytic enzymes of the groups of mycorrhizal mushrooms with that of wood-rotting mushrooms, three strains of *L. edodes* and one strain each of *F. velutipes* and *P. ostreatus* were examined as wood-rotting mushrooms. The enzyme activity was assayed 25 days after inoculation.

As shown in Table 4, xylanase showed the highest activity (16-20 U/ml) among the enzymes tested in all strains of *L. edodes*, while the activities of chitinase and

Table 2. Productivity of hydrolytic enzymes of mycorrhizal mushrooms on potato-dextrose liquid medium.

Organism	Activity(U)*						
	Proteinase		Chitinase	β-1,3-Glucanase	Cellulase		Xylanase
	Acid	Neutral			CM-cellulase	Avicelase	
Tricholoma matsutake KBM-01	1.51±0.64	0.65±0.13	3.34±0.52	3.80±0.71	4.32±0.49	5.23±0.81	3.38±0.57
Tricholoma matsutake KBM-08	2.24±0.60	0.87±0.09	1.92±0.91	5.35±0.67	5.21±0.63	4.40±0.65	4.97±0.79
Tricholoma matsutake Ichikawa	2.95±0.43	0.66±0.07	4.18±0.87	4.52±0.75	9.46±0.87	5.02±1.00	2.00±0.25
Tricholoma matsutake ATCC 64715	1.66±0.43	0	1.12±0.25	1.51±0.22	5.13±0.48	7.11±0.49	1.50±0.33
Tricholoma matsutake Kyoto M-48	10.0±1.13	1.28±0.21	1.31±0.09	1.27±0.11	4.98±0.75	9.95±1.38	6.32±0.85
Tricholoma matsutake O. T.**	4.66±0.77	0.75±0.09	1.25±0.36	1.74±0.07	2.69±0.71	4.84±0.05	10.3±1.21
Tricholoma matsutake Yamaguchi	10.3±0.82	1.05±0.05	3.95±0.42	4.18±0.81	4.72±0.87	10.5±0.48	3.01±0.64
Tricholoma matsutake Shiga Tm-18	10.7±1.72	1.68±0.20	1.00±0.11	2.52±0.09	2.35±0.25	7.64±1.11	1.97±0.07

*: $\bar{x}\pm$S.D., **: Okayama Tetsuta.

The strains of *T. matsutake* were stationarily cultured at 24°C for 120 days. Culture filtrates were used as the crude enzyme solution.

Table 3. Productivity of hydrolytic enzymes of mycorrhizal mushrooms on potato-dextrose liquid medium.

Organism	Activity(U)*						
	Proteinase		Chitinase	β-1,3-Glucanase	Cellulase		Xylanase
	Acid	Neutral			CM-cellulase	Avicelase	
Lyophyllum shimeji Kamiakizato-1	3.00±0.56	2.68±0.27	5.22±0.95	2.65±0.18	10.4±0.82	9.87±1.04	9.99±1.13
Lyophyllum shimeji Kamiakizato-3	6.12±1.58	11.2±1.83	3.25±0.57	2.81±0.07	3.86±0.72	6.43±2.01	5.17±1.25
Lyophyllum shimeji Asako	3.64±0.82	3.66±0.93	3.75±1.03	4.32±1.22	9.68±1.71	7.05±0.84	4.47±0.83
Lyophyllum shimeji Yamada-1	3.02±0.74	3.80±0.99	3.97±0.85	5.00±1.06	9.85±1.43	8.32±1.62	4.15±0.84
Lyophyllum shimeji Yamada-2	4.93±1.00	4.95±1.13	1.91±0.71	1.88±0.57	4.32±1.06	10.4±1.02	3.50±1.00
Suillus bovinus Ichikawa	4.20±0.92	9.66±0.81	0.82±0.07	0.86±0.06	3.28±0.69	9.95±1.44	1.84±0.72
Suillus bovinus Kasai	0.72±0.05	0.48±0.03	0.90±0.08	1.40±0.08	4.68±0.74	5.16±0.87	4.91±1.17
Lactarius hatsudake Kasai	2.87±0.37	0.66±0.06	1.08±0.24	0.60±0.07	2.20±0.32	9.89±1.24	0.45±0.07

*: $\bar{x}\pm$S.D.
The strains of *L. shimeji, S. bovinus* and *L. hatsudake* were stationarily cultured at 24°C for 75 days. Culture filtrates were used as the crude enzyme solution.

Table 4. Productivity of hydrolytic enzymes of wood-rotting mushrooms on potato-dextrose liquid medium.

Organism	Activity(U)*						
	Proteinase		Chitinase	β-1,3-Glucanase	Cellulase		Xylanase
	Acid	Neutral			CM-cellulase	Avicelase	
Lentinus edodes Syohatsu	8.15±1.20	1.73±0.09	1.52±0.11	1.43±0.10	0	8.56±0.77	16.8±2.43
Lentinus edodes KB-10	3.43±0.85	0	1.85±0.13	1.74±0.15	2.11±0.64	9.55±0.83	19.6±2.75
Lentinus edodes Niigata	5.82±0.75	0	2.36±0.21	1.64±0.08	3.42±0.28	5.42±0.64	16.0±1.72
Flammulina velutipes Iisui	2.50±0.61	7.24±1.11	3.03±0.48	2.56±0.23	4.81±0.55	39.7±5.72	22.1±3.67
Pleurotus ostreatus Kitamura	2.98±0.34	2.52±0.26	5.02±0.69	4.95±0.53	9.27±1.62	79.4±8.30	11.0±1.04

*: $\bar{x}\pm$S.D.
All fungi were stationarily cultured at 24°C for 25 days. Culture filtrates were used as the crude enzyme solution.

β-1,3-glucanase were lower than that of xylanase in these mushrooms. Of the cellulases, avicelase showed higher activity than CM-cellulase. In addition, the avicelase activity in *P. ostreatus* and *F. velutipes* showed considerably higher values (40–80 U/ml) than other hydrolytic enzymes tested.

Discussion

Little research has been conducted on hydrolytic enzymes of mycorrhizal mushrooms. Nakazawa et al. (1974) reported that *T. matsutake* produced a very weak activity of cellulolytic enzymes, but the detailed profile of the cellulolytic enzymes was unclear. Recently, the diversity of pH spectra of cellulolytic enzymes in Basidiomycetes was reported by Enokibara et al. (1993). They examined the productivity of cellulolytic enzymes in mycorrhizal mushrooms and showed that *Hebeloma vinosophyllum* Hongo and *Laccaria bicolor* (Maire) P. D. Orton produced very weak activities of the sub-neutral cellulases active at pH 5.5–6.8. In contrast, *Lepista nuda* (Bull.: Fr.) Cooke produced strong activities of the acid cellulase active at pH 3.0–5.5. They suggested that the enzymatic properties of cellulases, such as optimum pH, were different among the mushrooms depending on the pH habitat. In our experiments, the cellulase activities from mycorrhizal mushrooms were assayed at pH 5.0 although the active pH ranges of the enzymes were not determined.

Acid proteinase was found to be produced at relatively high levels in *T. matsutake* strains, although their values varied among the strains tested. In our recent article on the proteolytic activity and the enzymatic properties of *T. matsutake* and related species (4 species, 17 strains), we reported that carboxyl proteinases (those inhibited by S-PI (Pepstatin Ac) and diazoacetyl-DL-norleucine methylester (DAN)) were present in all test strains (Terashita and Kono, 1989). The carboxyl proteinases in the culture filtrate and fruit-bodies were highly purified (Terashita and Kono, 1987). These enzymes showed higher S-PI sensitivity (high-affinity) and higher optimum temperature (about 70°C) than those produced by wood-rotting and saprophytic mushrooms. Zhu et al. (1990) have reported the production, purification and characterization of a carboxyl type of acid proteinase from the ectomycorrhizal mushroom *H. crustuliniforme*. The acid proteinases of mycorrhizal mushrooms tested in the present study can be classified as carboxyl proteinases.

However, their S-PI sensitivity and optimum temperature have not yet been investigated.

Comparison of productivity of hydrolytic enzymes between the group of wood-rotting mushrooms and that of mycorrhizal mushrooms showed considerably higher xylanase activity in the former than the latter. Ohga (1992) reported that xylanase activity was the highest among all of the test enzymes from different strains of *L. edodes*. Similar results have also been reported by Ishikawa et al. (1983), who found high xylanase activity (11–21 U/ml) in *P. ostreatus* and *F. velutipes*. Kawai (1973) has reported that almost all strains of wood-rotting mushrooms had high productivity of xylanase.

The strains of mycorrhizal mushrooms used in this study seemed to have relatively strong saprophytic activity, because they grew on the artificial medium, potato-dextrose medium. Thus the potential for production of hydrolytic enzymes by mycorrhizal mushrooms appeared to be relatively high. It was also shown that *T. matsutake* and *L. shimeji* produce many kinds of hydrolytic enzymes in their culture broth. However, more detailed infomation will be needed to reveal the function of these enzymes in the growth of mycorrhizal mushrooms in nature.

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