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FULL PAPER

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Characterization of the carbohydrase productions of an ectomycorrhizal fungus, *Tricholoma matsutake*

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Abstract To evaluate the potential of the production of the ectomycorrhizal fungus *Tricholoma matsutake* to produce carbohydrases, (1) the distribution of carbohydrase activities among the different strains (18 strains) was investigated and (2) the abilities of *T. matsutake* and saprophytic fungi to produce β-glucosidase were compared. The results showed that the carbohydrase productions patterns of *T. matsutake* still resemble one another. Moreover, this fungus exhibited markedly higher β-glucosidase than did the saprophytic mushrooms. *Tricholoma matsutake* showed weak production of α-amylase and α-glucosidase in a static culture filtrate. On the other hand, glucoamylase activity was not observed. Surprisingly, we discovered that β-glucosidase demonstrated strong activity. This finding suggests that this fungus has saprotrophic abilities. The carbohydrase production systems in *T. matsutake* were characterized from our experimental results. Also, we point out some weak points in the carbohydrase production systems of *T. matsutake*.

Key words β-Glucosidase · Carbohydrase · Ectomycorrhizal fungus · Edible mushroom · *Tricholoma matsutake*

Introduction

Tricholoma matsutake (S. Ito et S. Imai) Singer is one of the most delicious, invaluable, and expensive edible mushrooms in Japan, China, Korea, and North Korea. The annual production of this mushroom in Japan was reportedly 12 000 tons in 1941, 3509 tons in 1960, only 211 tons in 1995, and has decreased substantially to only 34 tons in 2005 (Yamada 2005). The main factors for this decrease are believed to be the accumulation of humus in pine tree forests, death of the trees caused by *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle (the pine wilt nematode) (Futai 2003), acid rain, and the collection of immature fruit bodies from the field.

This fungus has low ability to decompose polysaccharides for use as a growth substrate and grows slowly on artificial media (about 2 cm growth/month) (Hur et al. 2001). Therefore, only glucose and a few other mono- and disaccharides can be used to grow this fungus (Kawai and Abe 1976). This fungus has not previously been known to use any other polysaccharides except starch (Lee et al. 1998).

Ohta (1994) reported that *Lyophyllum shimeji* (Kawamura) Hongo, classified as an ectomycorrhizal fungus, forms mature fruit bodies within a bottle containing a barley grain in a cultivation medium without a host plant. He mentioned that a sufficient quantity of starch used as a carbon source was able to supply the factor that allows successful fruit-body formation without raising the osmotic pressure in the medium (Ohta 1997). The fruit-body formation of this fungus in artificial cultivation was reported by Watanabe et al. (1994) and Yoshida and Fujimoto (1994) at about the same time. Ohta (1997) noted the importance of the amylase systems of this fungus based on their ability to use barley starch as a growth substrate.

We examined the amylase systems used during the fruit-body formation of *L. shimeji* in barley grain medium. We showed for the first time that the amylase is a glucoamylase with a high hydrolyzing ability (Kusuda et al. 2004).

The demand for the artificial cultivation of *T. matsutake* has grown stronger because of the success of the artificial cultivation of *L. shimeji*, but it has not yet been accomplished. Therefore, we investigated the starch-hydrolyzing ability (amylase production systems) of *T. matsutake*.

We found that this fungus exhibited weak α-amylase (Kusuda et al. 2003) and α-glucosidase as a weak activity in a static culture filtrate. On the other hand, no glucoamylase activity was detected in the static culture filtrate. Surprisingly, we discovered that β-glucosidase demonstrated strong

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Table 1. Strains of *Tricholoma matsutake* tested

Strain	Institution, origin
Z-1	Dr. K. Inaba, Kinki University (Inaba et al. 1995)
No. 101, No. 103, No.104, No. 105, No. 114, No. 115	The Institute of Iwaizumi Matsutake Mushroom, Iwate Prefecture, Japan
TM19 and Ichikawa	The Hyogo Forestry Research and Technology Center, Hyogo Prefecture, Japan
KBM06	Kibun Co. Ltd., Tokyo Prefecture, Japan
Hiroshima	Isolated from the fruit body grown under pine tree forest in Higashi, Hiroshima city, Hiroshima Prefecture, Japan
NBRC 6933, NBRC 30604 , NBRC 30605, NBRC 30773	NITE Biological Resource Center, National Institute of Technology and Evaluation, Chiba Prefecture, Japan
ATCC 24461, ATCC 64715	American Type Culture Collection, USA
J-1	Dr. K. Yamanaka, Kyoto Mycological Institute, Kyoto Prefecture, Japan (Yamanaka et al. 2003)

activity in the static culture filtrate. These enzymes had already been purified and characterized. The discovery of β-glucosidase suggests that this fungus has saprotrophic ability, and also that *T. matsutake* is able to utilize the cellooligosaccharides released by cellulose (Kusuda et al. 2003, 2006).

In this report, to evaluate the potential of the ectomycorrhizal fungus *T. matsutake* to produce carbohydrases, (1) the distribution of extracellular carbohydrase activities among the different strains (18 strain types) of this mushroom was investigated and (2) the ability of *T. matsutake* and saprotrophic (wood-rotting) fungi to produce β-glucosidase were compared. From these results, we characterized the carbohydrase production systems in the ectomycorrhizal fungus *T. matsutake*. Hereafter, we discuss a few points about the artificial cultivation of this fungus on the basis of our experimental results.

Materials and methods

Microorganism

Eighteen strains of *T. matsutake* isolates were used (Table 1). Moreover, to compare the enzyme activity of the mycorrhizal fungus *T. matsutake* with that of the saprophytic fungi, we used *Flammulina populicola* Redhead and R.H. Peterson NBRC 7777, *Lentinula edodes* (Berkeley) Singer (commercial strain Mori 465; Mori, Kiryu, Gunnma, Japan), *Pleurotus ostreatus* Kitamura (Jacq.: Fr.) Kummer (commercial strain isolated from mature fruit body produced by Kinki Nyugyo Co., 1979), *Grifola frondosa* (Dicks.: Fr.) S.F. Gray (commercial strain isolated from mature fruit body produced by Yukiguni Maitake Co.), and *Polyporus arcularius* Batsch.: Fr. 69B ATCC 24461 as controls. These fungi were stored on a partially modified matsutake agar (PMMA) medium (Terashita et al. 2000) at a low temperature (4°C).

Medium composition and culture condition

A mycelial block (5 × 5 mm) was cut from a plate culture that had grown on a partially modified matsutake agar (PMMA) medium for 40 days at 24°C in a Petri dish (diameter, 90 mm). A partially modified matsutake liquid (PMML) medium was used in these experiments. This medium was composed of 22.7 g glucose, 5.0 g yeast extract, 77.0 g potato extract, and 5.0 g Sunpearl CP (commercial name, Nihon Seishi; prepared from the sulfate pulp waste of softwood) per 1000 ml distilled water, with an initial pH of 5.1. The culture, containing 20 ml medium in a 100-ml Erlenmeyer flask, was autoclaved at 121°C for 5 min before use. Additionally, a synthetic liquid (SL) medium (Kawai and Abe 1976) with soluble starch added to it was used to test the β-glucosidase activities of the mycorrhizal fungus and saprotrophic fungi in the culture filtrate. These fungi were static cultured at 24°C for 7–80 days in a lighted area (about 200 lux), until the surface of the liquid medium was coated with mycelia.

Measurement of mycelial dry weight

Eighteen different strains of *T. matsutake* were cultured in a PMML medium at 24°C for 80 days in a lighted area (about 200 lux). After cultivation, the mycelial mats were washed three times with distilled water, dried in an oven at 80°C to a constant weight, then weighed after cooling in vacuum desiccators. The average value of three different replicates, with six flasks per experiment, is shown in the results.

Preparation of crude enzyme solution and enzyme assay

Separation of the crude culture filtrate after cultivation (60 days after inoculation) was performed using filter paper (No. 2, Advantec, Tokyo, Japan). The crude filtrate was used for an enzyme assay.

Total amylase activity was measured using the Somogyi–Nelson method (Somogyi 1952) after 60 min of reaction at 50°C, with a soluble starch solution (0.4%) as a substrate. One unit of total amylase activity was defined as the activity that forms 1 μmol glucose in 1.0 ml reaction mixture at 50°C over the course of 1 min. α-Amylase activity was assayed at 50°C for 180 min using a soluble starch solution (0.4%) as a substrate. The absorbency was read at 690 nm by a spectrophotometer after the addition of an iodine-potassium iodide solution (Terashita et al. 2000). One unit of α-amylase activity was defined as the activity that decreased the

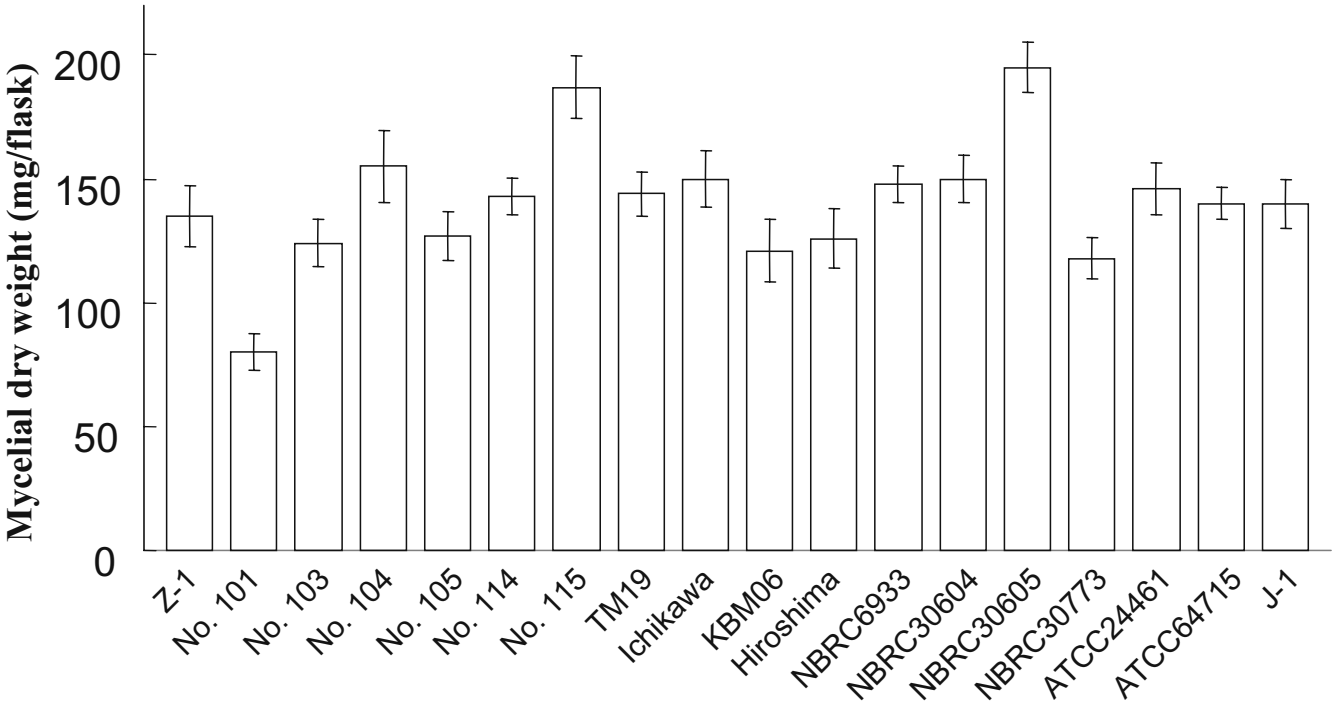

Fig. 1. Mycelial dry weight of *Tricholoma matsutake* in partially modified matsutake liquid (PMML) medium. After inoculation, it was static cultured at 24°C for 80 days in the light (200 lux). Data represent averages of triplicate experiments with six flasks per experiment. *Bar* in each column shows standard division

absorbency of the reaction mixture (1.0 ml) by 0.1 at 50°C over the course of 1 min. Glucoamylase activity was measured at 50°C for 180 min using a soluble starch solution (0.2%) as a substrate. Glucose, which was released from starch by the reaction, was determined using an F-Kit D-glucose (Boehringer Mannheim, Mannheim, Germany) (Terashita et al. 2000). One unit of glucoamylase activity was defined as the activity that forms 1 µmol glucose in 1.0 ml reaction mixture at 50°C over the course of 1 min. The α-glucosidase and β-glucosidase activity were assayed at 50°C for 30–60 min using ρ-nitrophenyl α-D-glucopyranoside (ρ-N-αG) and ρ-nitrophenyl β-D-glucopyranoside (ρ-N-βG) as substrates, respectively. One unit of enzyme activity was defined as the activity that forms 1 µmol ρ-nitrophenol in 1.0 ml reaction mixture at 50°C over the course of 1 min (Kusuda et al. 2006).

Table 2. Extracellular β-glucosidase activities of *Tricholoma matsutake*

Strain	Enzyme activity (mU/flask)	Strain	Enzyme activity (mU/flask)
Z-1	1580	KBM06	1650
No. 101	1905	Hiroshima	1580
No. 103	1140	NBRC 6933	2065
No. 104	1150	NBRC 30604	1220
No. 105	860	NBRC 30605	450
No. 114	1710	NBRC 30773	1390
No. 115	2700	ATCC 24461	1045
TM19	1020	ATCC 64715	1055
Ichikawa	2000	J-1	1075

β-Glucosidase activity was assayed by the ρ-nitrophenol method using ρ-NβG as a substrate; after inoculation, it was static cultured at 24°C for 60 days in the light (200 lux)
Data represent averages of triplicate experiments with 3 flasks per experiment

Results

Mycelial growth of the different strains of *T. matsutake*

The results of the vegetative mycelial growth of *T. matsutake* on a PMML medium at 24°C for 80 days are shown in Fig. 1. The mycelial dry weights varied widely among the 18 different strains of *T. matsutake*. The *T. matsutake* NBRC 30605 (195 ± 10.4 mg/flask) and No. 115 (187 ± 12.6 mg/flask) strains produced considerably higher weights than did the other strains. On the other hand, No. 101 (80.0 ± 7.52 mg/flask), NBRC 30773 (118 ± 8.24 mg/flask), and KBM 06 (121 ± 12.6 mg/flask) produced weights lower than the average value (140.5 mg/flask) among the 18 strains.

Extracellular β-glucosidase activities among the different strains of *T. matsutake*

To compare the β-glucosidase production activities of the different strains of *T. matsutake*, we assayed enzyme activity at 60 and 80 days after inoculation. The results shown in Table 2 are only those from 60 days after inoculation, because the activities measured at 60 days seemed to be

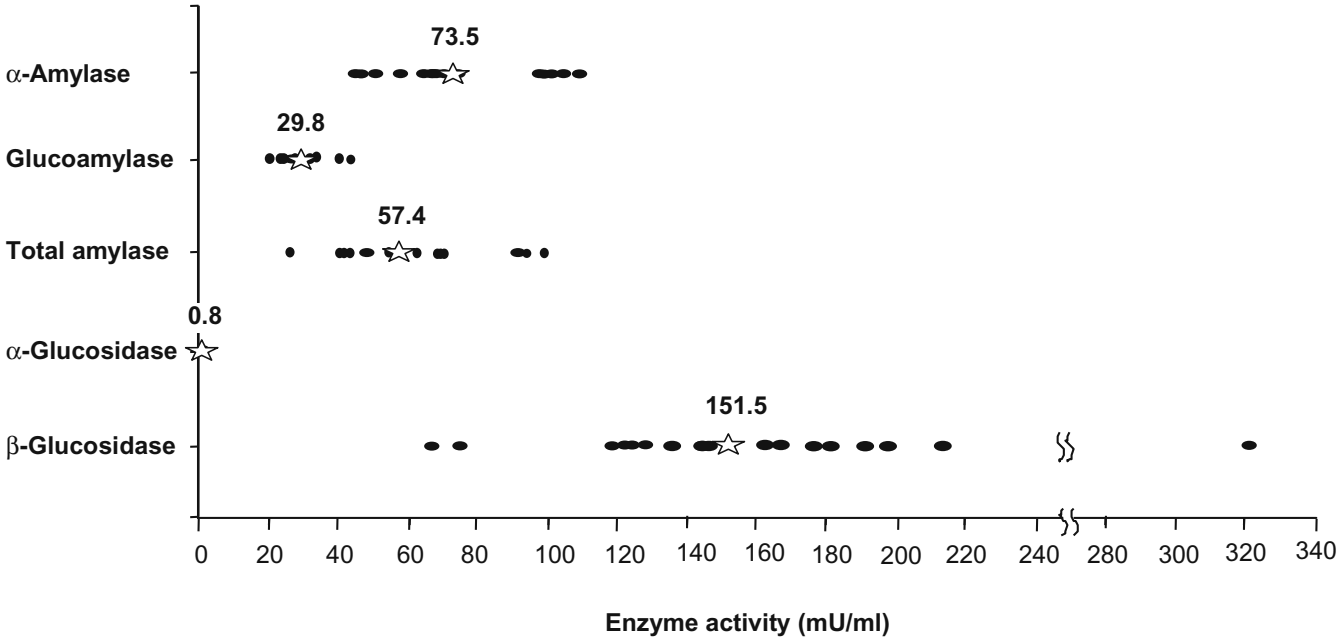

Fig. 2. Production of extracellular carbohydrase activities during the different strains of *Tricholoma matsutake*. Eighteen strains of *T. matsutake* were used for several enzyme assays. After inoculation, it was static cultured at 24°C for 80 days in a light place (about 200 lx). ☆ indicates average value of enzyme activity (mU/ml). Measurement methods for enzyme activity: α-amylase (iodine-potassium iodide method); glucoamylase (F-kit D-glucose method); total amylase (Somogyi-Nelson method); α- and β-glucosidase [p-nitrophenol (synthetic substrate) method]

higher than those measured at 80 days. The No. 115 strain showed the highest total activity (2700 mU/flask) of all the strains of *T. matsutake* tested. On the other hand, the total activity levels of the NBRC 30605 (450 mU/flask) and No. 105 (860 mU/ml) strains were lower than those of the other strains, while the specific activities of No. 115, NBRC 30605, and No. 105 strains showed the values of 14.4, 2.3, and 6.8 mU/mg dry weight of mycelia, respectively. The average amount of total β-glucosidase activity (among the 18 strains) was 1422 mU/flask.

Production of carbohydrase activities by the different strains of *T. matsutake*

To evaluate the potential of the *T. matsutake* strains to produce extracellular carbohydrases (total amylase, α-amylase, glucoamylase, α- and β-glucosidase), we investigated the distribution of enzyme activities among the 18 strains of the fungus stored in our laboratory using a static culture filtrate (Fig. 2). α-Amylase activities, measured using the iodine-potassium iodide method, showed values ranging from 44.5 to 111.7 mU/ml, with an average value of 73.5 mU/ml. Glucoamylase and α-glucosidase activities were very weak, and the average values of these two enzymes were 29.8 mU/ml and 0.8 mU/ml, respectively. On the other hand, β-glucosidase levels ranged from 65.8 to 325.1 mU/ml. β-Glucosidase activity was unexpectedly detected at a dramatically higher level than that of α-amylase, glucoamylase, and α-glucosidase. In particular, *T. matsutake* strain No. 101 showed the highest β-glucosidase value among the 18 strains. The average value was 151.5 mU/ml, and the enzyme production patterns of the 18 different strains of *T. matsutake* resembled one another.

Comparison of the β-glucosidase production of *T. matsutake* and saprotrophic mushrooms

To compare the β-glucosidase production abilities of *T. matsutake* and saprophytic mushrooms, *T. matsutake* J-1 strain, *F. populicola*, and *L. edodes* were static cultured in a synthetic liquid medium (pH 5.2) (Kawai and Abe 1976) with soluble starch added at 24°C for 7–60 days after inoculation. Moreover, 18 strains of *T. matsutake*, *F. populicola*, *L. edodes*, *P. arcularius, G. frondosa*, and *P. ostreatus* were static cultured in a PMML medium (pH 5.1) at 24°C for 7–80 days after inoculation. Each fungus was incubated continuously until it thickened with mycelia on the surface of the liquid medium to synchronize the growth stage of mycelia (Fig. 3A,B).

Moreover, the variations in the productivity of β-glucosidase activities am ong the *T. matsutake* Z-1 strain and several kinds of saprotrophic fungi were examined using a PMML medium. *Tricholoma matsutake* and *F. populicola* showed higher activity than the other mushroom fungi. The β-glucosidase activity of the saprophytic mushrooms was 200–1700 mU/flask, but the activity of *T. matsutake* was 1800–2600 mU/flask.

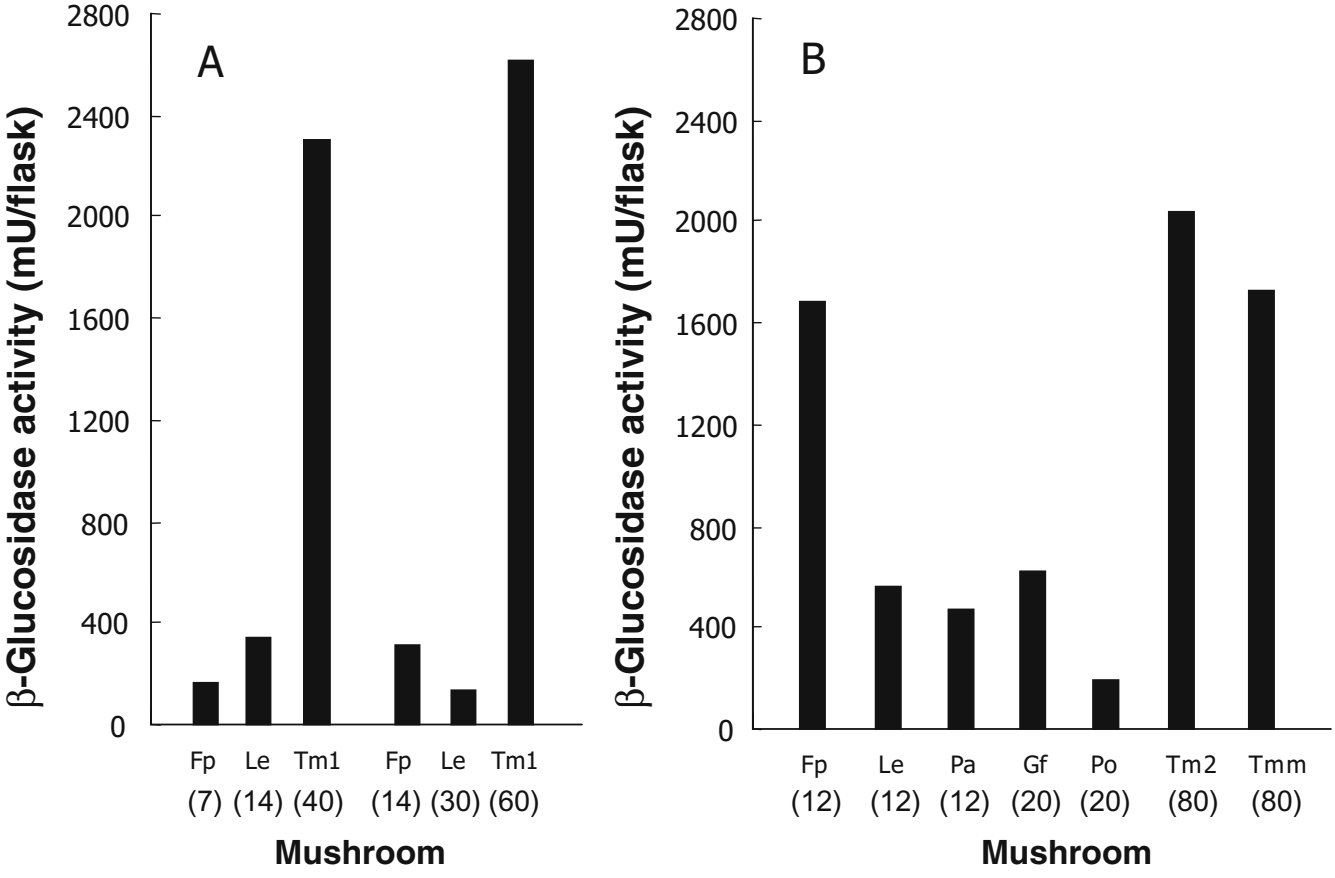

Fig. 3. Comparison of β-glucosidase production between *Tricholoma matsutake* and saprotrophic fungi in the medium. **A** Synthetic liquid medium added starch (Kawai and Abe 1976). **B** Partially modified matsutake liquid medium. Fp, *Flammulina populicola* NBRC 7777; Le, *Lentinula edodes* Mori 465; Tm1, *T. matsutake* J-1; Pa, *Polyporus arcularius* 69B ATCC 24461; Gf, *Grifola frondosa* Yukiguni; Po, *Pleurotus ostreatus* Kitamura; Tm2, *T. matsutake* Z-1; Tmm, Means of 18 strains of *T. matsutake*. Number in parentheses shows incubation days

Discussion

We found that the Z-1 strain produced α-amylase, α-glucosidase, and potent α-glucosidase activity in a static culture filtrate on a PMML medium. The three kinds of enzymes from the Z-1 strain have previously been purified (α-glucosidase is a partially purified enzyme), and their physiological and enzymatic properties have been characterized (Kusuda et al. 2003, 2006). Additionally, the detection of β-glucosidase produced by the Z-1 strain suggests that *T. matsutake* has saprotrophic abilities.

In this article, to evaluate the potential of *T. matsutake* to produce carbohydrases, we investigated the distribution of extracellular enzyme activities among the 18 different strains of the fungus stocked in our laboratory. β-Glucosidase activity was unexpectedly found to be dramatically greater than other carbohydrase activities. In addition, the enzyme production in *T. matsutake* resemble one another (see Fig. 2). On the other hand, we compared the abilities of *T. matsutake* and popular saprotrophic edible mushrooms to produce β-glucosidase. *Tricholoma matsutake* showed markedly higher β-glucosidase activity (two- to threefold) than did the saprotrophic mushrooms (see Fig. 3).

From the results of our experimental series studying *L. shimeji* (Kusuda et al. 2004) and *T. matsutake* (Terashita et al. 1995, 2000; Kusuda et al. 2003, 2006, 2007), we drew a figure summarizing the carbohydrase production of the ectomycorrhizal mushrooms *L. shimeji* and *T. matsutake*, and we pointed out the weak points of the artificial cultivation of *T. matsutake* (Fig. 4). In our previous article (Kusuda et al. 2004), we examined the production of amylase during the fruit-body formation of *L. shimeji* in a barley grain medium during polypropylene bottle cultivation. We found that glucoamylase activity in these medium increased markedly during fruit-body formation. Next, we isolated the enzymes from *L. shimeji* and revealed its enzymatic properties when using starch as a growth substrate. From these results, we concluded that glucoamylase is the main enzyme used in glucose production during the formation of fruit bodies in *L. shimeji.*

On the other hand, *T. matsutake* produced α-amylase and α-glucosidase in the static culture filtrate. α-Amylase has already been isolated and its substrate specificities have already been described (Kusuda et al. 2003). This enzyme produces oligosaccharides from starch. However, α-glucosidase (partially purified enzyme) activity was very weak. Moreover, glucoamylase activity was not detected in

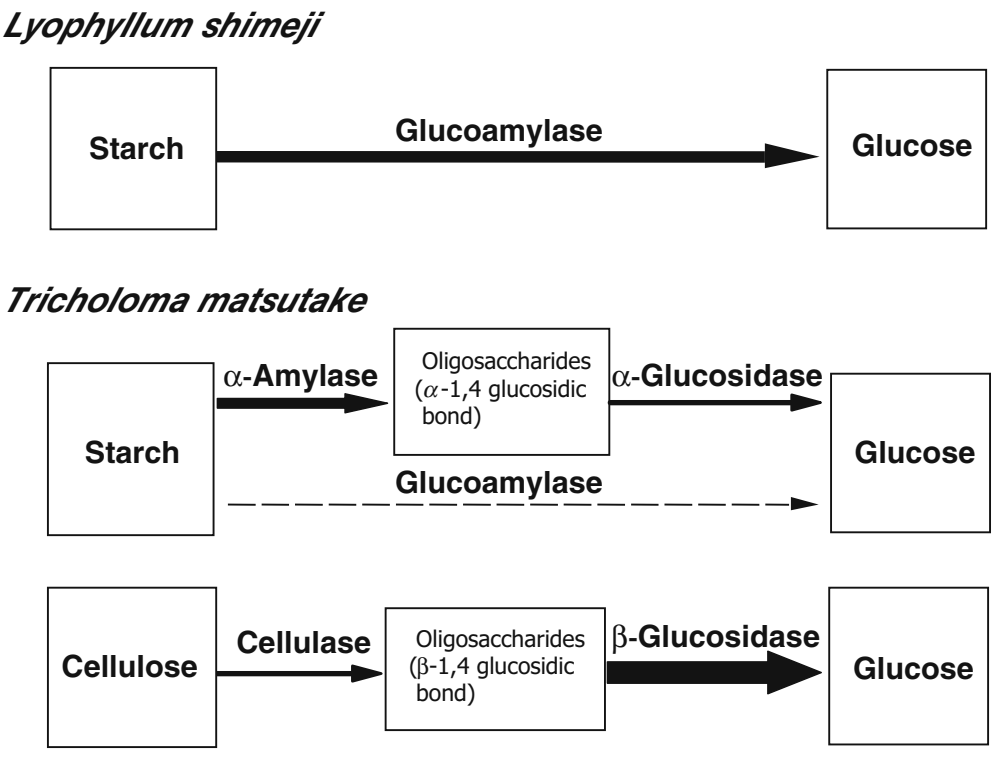

Fig. 4. Characterization of carbohydrase production systems in ectomycorrhizal mushroom, *Tricholoma matsutake*. Enzyme activity: ⟶ Strong (>100 mU/ml); ⟶ Moderate (50–100 mU/ml); ⟶ Weak (<50 mU/ml); ----► Slight or lacking (<5 mU/ml)

the culture broth of *T. matsutake*. We recently reported (Kusuda et al. 2006) that *T. matsutake* produces a β-glucosidase with high activity in a static culture filtrate. This finding suggests that this fungus has saprotrophic abilities. In addition, we reported effective purification procedures and described the enzymatic properties of highly purified β-glucosidase for the first time. The purified enzyme was remarkably more active in the presence of Ca^{2+} (about 2.9 times as active as the control) and Mn^{2+} ions (about 2.6 times as active). Using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) analysis with hydrolysates obtained from enzyme reactions, we showed that β-glucosidase hydrolyzes cellobiose and cellotriose with a β-1,4-glucosidic bond. This enzyme did not act upon polymer cellulose at a β-1,4-glucosidic bond or disaccharides and polysaccharides with α-1, 4 or α-1, 6 bonds. Terashita et al. (1995) have reported the production of endo-type cellulolytic enzymes by *T. matsutake*. CM-cellulase and avicelase activity was very weak, according to their report. On the other hand, the β-glucosidase from *T. matsutake* seems to be similar to the CMCase IIIa from a saprophytic mushroom, *Polyporus arcularius*, whose substrate specificities were described by Ishihara et al. (2005). The CMCase IIIa from *P. arcularius* acts on oligosaccharides ranging from di- to hepta-. Recently, Ohnishi et al. (2007) identified a possible mechanism for the cognition and degradation of insoluble crystalline cellulose by fungal cells. They also pointed out that the CMC IIIa from *P. arcularius* was induced by cellopentaose obtained from cellulose. These findings represent a very interesting phenomenon concerning the β-glucosidase of *T. matsutake*.

When a mushroom forms fruit bodies, large amounts of mycelia may be needed either to store the nutrients for the growth substrates of the fruit bodies or to transport the nutrients to the fruit bodies (Hirato and Kitamoto 1995). However, it is very difficult to cultivate large amounts of mycelia using monosaccharides in a pure culture because of the osmotic pressure in the medium. For example, the osmotic pressures of a 2% glucose solution, a cellobiose solution, and an amylose (MW 2900) solution are 274, 144, and 17 osm/l, respectively. Our experimental results showed that the mycelial growth of *T. matsutake* is inhibited at concentrations above about 300–500 osm/l.

Moreover, it is very difficult to cultivate large amounts of mycelia with *T. matsutake* without a host plant, as its growth substrates are obtained from the host plant through ectomycorrhizae. In general, ectomycorrhizal fungi are believed to obtain much of the carbon necessary for their growth from the host plants through ectomycorrhizae. Conversely, Vaario et al. (2002) found that the carbon transfer of *T. matsutake* via mycorrhizae may be lower than in their experimental results. Quantification of the source of the carbon flow from the host plant to the mycobiont *T. matsutake* is also important. More detailed information is needed about these points to understand the growth mechanism of *T. matsutake*.

Finally, we discuss a few points about the artificial cultivation of this fungus on the basis of our experimental results. Inserting several enzyme genes seems to be a very suitable method for achieving the artificial cultivation of *T. matsutake*, but using this technique is in itself a problem because consensus among researchers is difficult to obtain at the present time in Japan. For this reason, cultures using the cellooligosaccharides as growth substrates seem to be the most probable choice for the potent production of β-glucosidase from *T. matsutake.*

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