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# Detection of the Ability of *Tricholoma matsutake* to Utilize Sawdust in Aseptic Culture

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**Abstract**

Four isolates of *Tricholoma matsutake* were investigated for growth on media supplemented with combinations of glucose and sawdust. After 5 weeks of incubation, the transmission electron microscope observations indicated that hyphae of *T. matsutake* (T945) hyphae were able to degrade the xylem cell walls of sawdust in a sawdust-containing medium. After 7 weeks of incubation, the quantity of ergosterol produced by three isolates (T945, T61, and Y1) was significantly greater on the medium containing sawdust than the medium without. The activity of *p*-nitrophenyl-β-D-lactopyranosidase, a cellulolytic enzyme, was monitored in *T. matsutake* cultures as a presumptive assay for extracellular cellulolytic activity. *p*-Nitrophenyl-β-D-lactopyranosidase (*p*-NPL) activity of T945 was significantly

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higher on sawdust containing media containing sawdust and 0.1 g/l glucose than on other media. It is suggested that isolate T945 of *T. matsutake* is capable of utilizing sawdust in aseptic culture. The suitability of beech sawdust as a model substrate in aseptic culture was assessed.

Keywords: Ergosterol, *p*-nitrophenyl-β-D-lactopyranosidase (*p*-NPL) activity, sawdust, TEM, *Tricholoma matsutake*

## 1. Introduction

*Tricholoma matsutake* (S. Ito et Imai) Sing. (Matsutake) is one of the most renowned edible mushrooms in the world. The rapid decrease in natural production since the 1960s, due to the reduction of wood gathering and other human activities in forests (Ogawa, 1978; Iwase, 1997), has raised interest in developing an artificial cultivation system. However, with the exception of the formation of a few primordial (Ogawa and Hamada, 1975) and a single basidioma (Inaba et al., 1985), artificial cultivation of this mushroom has so far proved elusive.

During fruit-body formation, fungi may produce copious amounts of mycelia in which to store or transport nutrients to the developing fruit-bodies. Indeed, the significance of ectomycorrhizal fungi in below-ground carbon cycling has been previously highlighted (Durall et al., 1994) and the ectomycorrhizal fungi's ability to hydrolyze enzymatically various components of the plant cell wall *in vitro* has been reported (Cao and Crawford, 1993; Hutchison, 1990). The utilization of soil organic compounds by the ectomycorrhizal fungi should therefore reduce the carbon drain imposed on the host plant by the mycobiont (Finlay and Söderström, 1992). Norkrans (1950) noted that a number of *Tricholoma* species possessed a range of starch utilization abilities, and Ohta (1997) reported that starch-containing media supported *T. matsutake* growth very well. According to Terashita et al. (1995), *T. matsutake* exhibits high levels of both CM-cellulase and avicelase activities in potato dextrose medium.

We are interested in characterizing *T. matsutake* physiology to understand more about its ability to grow saprophytically as well as in symbiotic mycorrhizal associations. In this present study, the interaction of *T. matsutake* mycelium and sawdust was studied by transmission electron microscopy (TEM) to investigate the utility of beech sawdust as a carbon source to *T. matsutake*. Our aim was then to compare the abilities of different isolates of *T. matsutake* to utilize sawdust under different culture conditions.

## 2. Materials and Methods

*Isolates and culture conditions*

Four isolates of *Tricholoma matsutake* were used in this study. T945 was kindly provided by Dr. K. Iwase (Biological Environment Institute, Kansai Environmental Engineering Center Co. Ltd., Japan), T61 was isolated from a mature fruit-body growing on beneath *Pinus densiflora* Sieb. et Zucc. roots in Niigata, eastern Japan and Y1 and F were kindly provided by Dr. A. Yamada (Shinsyu University, Japan). Stock cultures were maintained on Ohta medium containing 1.4% agar (Ohta, 1990) at 23°C in darkness and were subcultured bimonthly.

*Growth in sawdust pieces-supplemented liquid medium*

Four different media were prepared: modified Ohta liquid medium without glucose (no gluc); modified Ohta liquid medium without glucose but containing 20 g/l beech (*Fagus crenata* Blume) sawdust (no gluc + sd); modified Ohta liquid medium containing 0.1 g/l glucose and 20 g/l sawdust (0.1 gluc + sd) and modified Ohta liquid medium containing 10 g/l glucose and 20 g/l sawdust (10 gluc + sd). The beech sawdust (granulometry: 2 mm, Kakushima-sangyo Nagano, Japan) was dried at 80°C for 24 h before use. Modified Ohta medium consisted of citric acid, 0.1 g; $(NH_4)_2HPO_4$, 250 mg; $KH_2PO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; $CaCl_2 \cdot H_2O$, 50 mg; Ohta mineral solution, 10 ml; Ohta vitamin solution, 10 ml and distilled water to 1,000 ml. The initial pH of all media was adjusted to 5.1 by 1 N KOH before autoclaving at 121°C for 20 min. Sugar concentration of sawdust-containing medium (no gluc + sd) after autoclaving was measured by gas chromatography (Hitachi G-3500) (Vaario et al., 2002).

As inocula, 6-mm-diameter mycelium plugs were cut from the margin of an agar colony growing on Ohta medium. Three plugs of each isolate were cultured in 100 ml Erlenmeyer flasks containing 20 ml of liquid medium. Four replicates were performed for each medium (one replicate was provided for transmission electron microscope observation). The cultures were incubated at 23°C in darkness, in stationary culture.

*Transmission electron microscope observation*

The resulting *T. matsutake* (T945) mycelium-sawdust complexes, cultured in sawdust-containing medium (no gluc + sd) were removed from the culture flask and cut into small pieces approximately 1 $mm^3$. The samples were then fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) containing 1%

acrolein for 2 h under vacuum at room temperature. The samples were washed twice in 0.1 M sodium cacodylate buffer and were then postfixed for 90 min in 2% $OsO_4$ in 0.1 M sodium cacodylate buffer at room temperature. Following three distilled water washes, the samples were taken to 100% propylene oxide in an ascending acetone series. The samples were subsequently infiltrated with Spurr's resin (Spurr, 1969) prior to polymerization at 70°C for 12 h.

*Ergosterol assay and determination of mycelia dry weight*

After 7 weeks of incubation, the mycelium plus undegraded sawdust were collected over a nylon mesh filter (24 × 30 µm) and freeze-dried. Ergosterol, an estimate of mycelial quantity, was then extracted in absolute ethanol (4 ml) and assayed by HPLC (Martin et al., 1990). The control, which was modified Ohta medium containing 20 g/l sawdust, also was done for ergosterol assay. The culture filtrate was collected for enzyme activity assessment.

*Enzyme assays*

*p*-Nitrophenyl-β-D-lactopyranoside (Seikagaku Corp., Tokyo, Japan) was used to assay *p*-nitrophenyl-β-D-lactopyranosidase activity, for presumptive extracellular cellulolytic activity in culture filtrates. Enzyme activity was measured by the modified method of Deshpande et al. (1984). The assay mixture, containing 900 µl culture filtrate and 100 µl of 1 mM substrate in 100 mM sodium acetate buffer, pH 5.0, was incubated at 30°C for 60 min. The enzymatic reaction was stopped by the addition of 100 µl of 2 M sodium carbonate solution and the absorption measured at 400 nm in an Ultrospec 3000 photometer (Pharmacia) at 25°C. Enzyme activity, derived from the amount of *p*-nitrophenol in the culture filtrate, was determined as the concentration of *p*-nitrophenylate ion ($PhNO_2$) calculated using a molecular coefficient ($\varepsilon$ 400=17100 $cm^{-1}$ $M^{-1}$). One unit (U) of *p*-nitrophenyl-β-D-lactopyranosidase was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per minute. Uninoculated culture liquid of four different types of media were also sampled and treated as controls to nullify absorption interference from the culture liquid color.

*Statistical analyses*

Multiple comparisons of fungal ergosterol content and *p*-nitrophenyl-β-D-lactopyranosidase activity were made using one-way ANOVA, Tukey-Krame test ($P<0.05$). Computations were carried out by SPSS 10.0 (SPSS Inc.) for Windows.

## 3. Results

*Transmission electron microscope observation*

The mycelium-sawdust aggregates (T945) formed after 5 wks of incubation observed by TEM showed the presence of hyphae close to the wood cell wall (Fig. 1a) and the hyphae invading and degrading the plant cell wall (Fig. 1b).

*Determination of mycelia growth (ergosterol assay)*

In the medium lacking carbon (no gluc), all isolates grew slowly and growth was imperceptible to the naked eye. In the medium lacking carbon but containing sawdust (no gluc + sd), slight mycelial growth was noted 2 wks after inoculation, particularly by T945 and T61. When 0.1 g/l glucose was added (0.1 gluc + sd), growth of all four *T. matsutake* isolates was more discernible. In all cultures, several small discrete spherical colonies developed. All isolates grew well in 10 g/l glucose (10 gluc + sd), the fungi forming single large spherical colonies.

The quantities of ergosterol produced by T945 and T61 were significantly higher in medium (no gluc + sd) than in medium (no gluc) (from 33 to 138% increase) (Fig. 2). This growth stimulation could be attributed to hexose release from the sawdust as little free sugar was detected in the nutrient medium during autoclaving (detection limit for glucose from sawdust containing medium was 5 mg/l). However, the range of growth stimulation was still much lower than the recorded in response to glucose concentration. When 0.1 g/l glucose and 10 g/l glucose were added into the media, separately, the stronger growth effects were detected (from 170–235% increase and 245–613% increase, respectively).

*Enzyme assays*

The addition of sawdust did not significantly stimulate the *p*-NPL activity in all tested isolates except that in isolate T945 the *p*-NPL activity was strongly stimulated by addition of sawdust with a small amount of glucose (0.1 g/l glucose). However, addition of a large amount of glucose (10 g/l glucose) reduced the production of *p*-NPL in all isolates (Fig. 3).

## 4. Discussion

*Tricholoma matsutake* is one of the most renowned edible mushrooms in the world and, like *Tuber melansporum* Vitt. (Truffle), *Boletus edulis* Bull. Fr. (cep) and *Cantharellus cibarius* Fr. (chanterelle), is ectomycorrhizal. While there

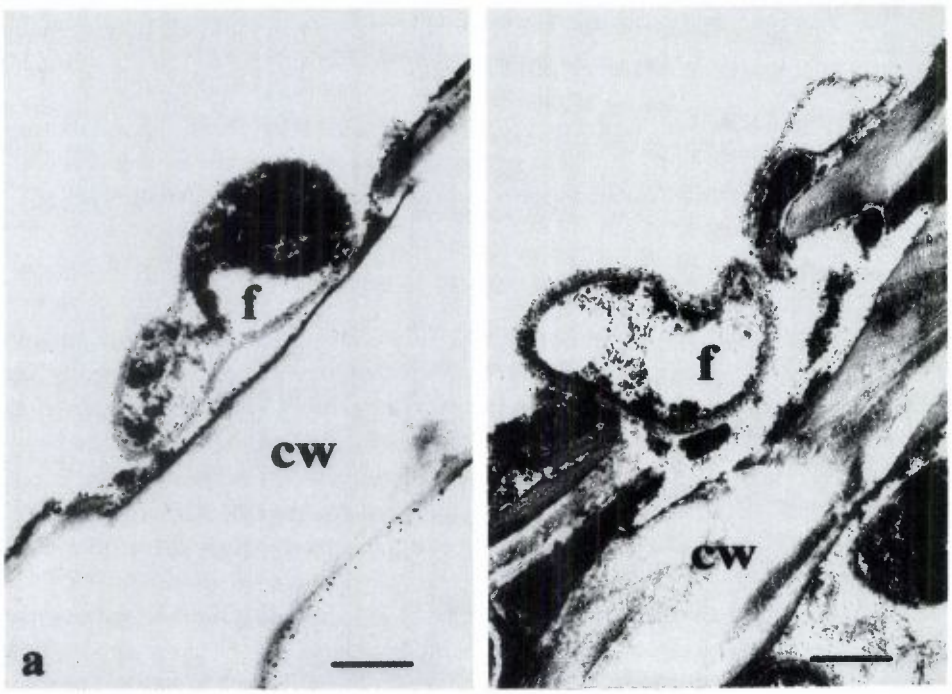


Figure 1. Ultrastructural features of *T. matsutake* (T945) growing on medium without glucose (no gluc) in pure culture after 5 weeks of incubation. (a) A fungal cell (F) adjacent to the sawdust cell wall (CW). (b) the fungal cell partly degrading the sawdust cell wall. Bar = 300 nm.

has been conflicting debate over many years regarding the trophic status of *T. matsutake* (Wang et al., 1997), recent work has demonstrated that *T. matsutake* is capable of forming a typical ectomycorrhizal association (Yamada et al., 1999; Gill et al., 1999, 2000).

Recently, a number of researchers have described the aseptic *in vitro* synthesis of Matsutake mycorrhizas (Yamada et al., 1999; Guerin-Laguette et al., 2000; Vaario et al., 2000), however, the maintenance and further development of these artificial associations presents difficulties. The carbon supply is regarded as being an influential factor in the establishment of ectomycorrhizas (Duddridge, 1986). To address this problem, we have considered those carbon sources, which are most appropriate for *T. matsutake* and whether *T. matsutake* is able to partially utilize a complex carbon source.

In this present study, the partial degradation by T945 of the sawdust cell wall in aseptic culture was initially indicated by TEM observation. We

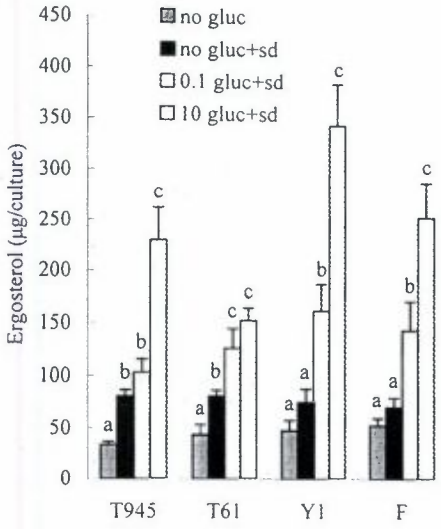


Fig. 2.

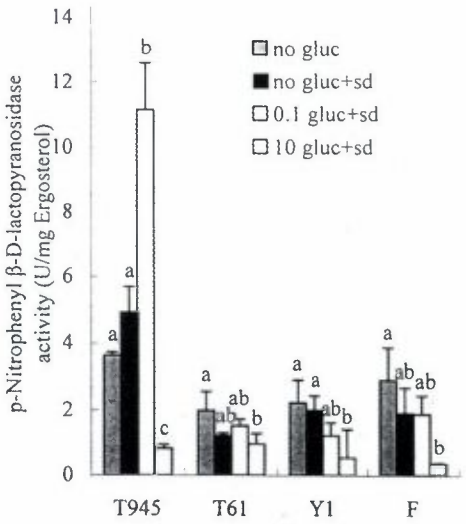


Fig. 3.

Figure 2. The quantity of ergosterol of four *T. matsutake* isolates in different media after 7 weeks of incubation. Values are the means of three replicates ± SD. For each isolate, common letters indicates no significant differences (one-way ANOVA, Tukey-Kramer, $P<0.05$).

Figure 3. *p*-Nitrophenyl-β-D-lactopyranosidase activity of isolates in different media after 7 weeks of incubation. Values are the means of three replicates ± SD. For each isolate, common letters indicates no significant differences (one-way ANOVA, Tukey-Kramer, $P<0.05$). *no gluc, modified Ohta medium without glucose; no gluc + sd, modified Ohta medium without glucose supplemented with 20 g/l sawdust; 0.1 gluc + sd, modified Ohta medium containing 20 g/l sawdust and 0.1 g/l glucose; 10 gluc + sd, modified Ohta medium containing 20 g/l sawdust and 10 g/l glucose.

subsequently demonstrated the capability of *T. matsutake* to utilize natural organic matter (sawdust) in two ways. *T. matsutake* isolates were grown, albeit slowly, in sawdust-containing medium under the culture conditions employed. Among the four isolates tested, the growth of T945 was significantly greater in the sawdust-containing media than in carbonless medium. While other researchers have described the ability of *T. matsutake* to utilize complex carbon sources as limited (Kawai and Abe, 1976; Ogawa, 1978), our findings suggest that some isolates of *T. matsutake* are able to utilize sawdust and lend support to the observation that *T. matsutake* grows well on starch medium (Ohta, 1997).

Secondly, cellulase activities were quantified by *p*-NPL assay. In our previous experiment, we have seen that *T. matsutake* had the ability to utilize cellobiose as a carbon source (data not shown). This assay is a useful tool for screening because *p*-NPL mainly releases cellobiose from cellulose. Here, the *p*-NPL activities were not regulated by supplement of sawdust or glucose in three isolates of *T. matsutake* (T61, Y1 and F), however, enzyme activity was stimulated in sawdust-containing medium augmented with 0.1 g/l glucose and increased glucose concentrations reduced *p*-NPL activity in the T945 isolate. The reduction in *p*-NPL activity at 10 g/l glucose is probably due to some sort of catabolism inhibition. Terashita et al. (1995) reported that *T. matsutake* produced relatively high levels of CM-cellulase and avicelase although they were lower than those of *Lyophyllum shimeji*.

Norkrans (1950) also found that a mycorrhiza former, *Tricholoma vaccinum*, was able to decompose cellulose following the addition of glucose as a "starter" carbon source. In these reports, varying results were achieved under different culture conditions. Our results showed that the organic matter (sawdust) with starter glucose content stimulated *p*-NPL production in the case of T945. It may be presumed that Matsutake produces relatively little cellulolytic activity, and that the medium is a key factor in regulating *T. matsutake* enzyme activity. Terashita et al. (1995) concluded that there was insufficient information to reveal the function of these enzymes in *T. matsutake* mycelial growth. In fact, observed cellulolytic activity could be explained in a number of ways. One could consider that the cellulase degrading abilities might be related not to the use of plant litter as a source of organic carbon, but rather to the penetration of root tissues (Smith and Read, 1997). Others consider that many ectomycorrhizal Basidiomycetes are facultative mycorrhizal fungi and can survive as saprophytes and form basidiomata in the absence of host trees by degrading complex carbon compounds such as cellulose, lignin and pectin in the organic layer of the soil (Smith and Read, 1997). Our findings are in agreement with the latter suggestion. TEM observations indicate that mycelium of isolate T945 is capable of degrading the plant cell wall in media lacking an exogenous carbon source thus primarily satisfying its carbon requirement from plant material. In recent studies, we also found saprotrophic growth of *T. matsutake* in soil and over pine bark (Guerin-Laguette et al., 2001; Vaario et al., 2002).

We previously developed a technique to rapidly synthesize *in vitro* ectomycorrhizas between *T. matsutake* and *Pinus densiflora* on soilless (artificial) media, including sawdust but excluding sugar (Vaario et al., 2000). It appears that media containing a low concentration of monosaccharide supplemented with organic matter may be the key to mastering artificial mycorrhization and fruiting of Matsutake in non-sterile conditions.

In the future, understanding the mechanism(s) by which ectomycorrhizal fungi interact with a complex carbon source at the physiological and molecular

level, and investigation of expression and control of the enzyme systems, will provide new insights into the ecological functioning of ectomycorrhizal associations.

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