The Production of Methyl Trans-cinnamate in the Submerged Cultures of *Tricholoma matsutake* Mycelia

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ABSTRACT

Methyl trans-cinnamate is a significant flavor compound of *Tricholoma matsutake*. Attempts were made to produce this compound by culturing the mycelium using submerged culture. No methyl trans-cinnamate could be detected when the mycelium was cultured in the basal liquid medium. However, the addition of *Pinus densiflora* extracts to the medium, methyl trans-cinnamate was largely produced. To find out compounds or fractions inducing methyl trans-cinnamate, dichloromethane fraction obtained from the wood extracts of *P. densiflora* was subjected to column chromatography. Three sub-fractions were obtained from the CH₂Cl₂ fraction. Submerged cultured mycelium treated with sub-fraction 1 has the highest content of methyl trans-cinnamate. Maximum methyl trans-cinnamate (470.2 μg/g) was obtained when the first sub-fraction of dichloromethane fraction of the *P. densiflora* wood extracts was added to the medium. This indicates that wood extracts of *P. densiflora* contains inducer of the methyl trans-cinnamate production in the *T. matsutake* submerged culture.

Keywords: *Tricholoma matsutake* mycelium, methyl-trans cinnamate, submerged culture, *Pinus densiflora* extracts

1. INTRODUCTION

*Tricholoma matsutake* (called pine mushroom) is mycorrhizal on a number of coniferous tree species (Kranabetter et al., 2002). It has been found to produce useful substances, including anticancer components and thermostable enzymes (Kawagoe et al., 1999). It is one of the most expensive edible mushrooms in Northeast Asia because Korean and Japanese tent to like the mushroom that has a peculiar perfume and chewing taste. The peculiar perfumes of the *T. matsutake* are mainly 1-octene-3-ol, methyl cinnamate, 2-octanol and octyl alcohol, which make up 96.8% of total aroma components (Ahn and Lee, 1986). According to Murahashi (1936), it is also reported that the odor of *T. matsutake* was extractable with ether and consisted of two major components: methyl trans-cinnamate and 1-octene-3-ol. Among these components, methyl trans-cinnamate is a very significant flavor compound in *T. matsutake* (Yaji-
Submerged culture of mushroom has been directed towards the production of fungal mycelium as a source of mushroom biomass or flavor because it is faster and more easily controllable than solid-state culture of mushroom. Although the technique gives good yields in fungal biomass, the biomass contains low level of flavor. Therefore the possibility of producing the flavor by mycelium from submerged culture has been explored. Belinky et al. (1994) reported that the addition of soybean flour and soybean oil to the growth medium enhanced 1-octen-3-ol production from Pleurotus pulmonarius as compared to that obtained on a synthetic medium. Additional study for the production of 1-octen-3-ol was carried out using the submerged culture of Agaricus bisporus 705 (Byun, 1999).

So far, most investigations using the submerged culture of mycelium have been carried out to produce 1-octen-3-ol or other enzymes. No attempts have been made to produce methyl trans-cinnamate using cultivation of the mycelium (Kapich et al., 2004). Compared with T. matsutake obtained from nature, T. matsutake mycelium cultivated in submerged culture has different smell and stiffness, suggesting that cultured mycelium may not be suitable to utilize the processed food. Therefore, it is needed to produce T. matsutake mycelium which has similar degree of smell of T. matsutake mushroom obtained from nature.

As far as our literature survey could ascertain, however, the submerged culture of T. matsutake mycelium for the production of methyl trans-cinnamate has not previously been published. In this study, we demonstrated that addition of P. densiflora extracts to the liquid medium, which on its own does not support the production of methyl trans-cinnamate, led to the production of methyl trans-cinnamate in submerged cultures of T. matsutake. The effects of different parts of ethanolic extracts on the production of methyl trans-cinnamate from T. matsutake mycelium were investigated. We also attempted to isolate the compounds or fractions inducing methyl trans-cinnamate in the submerged culture of T. matsutake mycelium.

2. MATERIALS and METHODS

2.1. Mycelia and Inoculum Preparation

The mycelia of T. matsutake used in this study were obtained from Korea Forest Research Institute. The stock culture was maintained on potato dextrose agar (PDA) slant. The seed cultures were grown in 250 ml flask containing 100 ml SYP medium at 25°C on a shaking incubator for 10 days. The cultured mycelia were homogenized at 13,000 rpm and used as inoculum.

2.2. Medium Compositions and Cultures

The medium contained the following components (g/l): glucose (40); KH₂PO₄ (1); MgSO₄ (0.5); glutamic acid (3); and yeast extract (1). Medium pH was adjusted to 5.5 with 5 M NaOH before sterilization. The first culture was grown in 1000 ml flasks containing 400 ml of basal medium and 5% (w/v) of the mycelium of T. matsutake at 25°C on a rotary shaker incubator at 150 rpm. After 10 days, 4 ml of ethanol or solvent fraction containing 40 mg extract were added to the pre-cultured flask. The cultivation was performed for 15 days.
2.3. Preparation of the ethanolic extract and solvent fraction

Dried parts (wood, bark, leaf and bark) of *Pinus densiflora* were finely ground, extracted twice with ethanol (EtOH) and then evaporated to give the crude extract. The crude extract of wood was successively partitioned with organic solvents, including n-hexane, dichloromethane (CH$_2$Cl$_2$) and ethyl acetate (EtOAc) (Fig. 1). Among these fractions, CH$_2$Cl$_2$ fraction was subjected to column chromatography on silica gel eluted with CH$_2$Cl$_2$-methanol (30:1, v/v) to yield 3 sets of fraction Fr. 1, Fr. 2 and Fr. 3.

2.4. Determination of Methyl Trans-cinnamate by HPLC

After cultivation, the mycelia were extracted with methanol and were subjected to sonication for 1 h followed by filtration with 0.2 μm pore membrane. The water and solvent were then evaporated in vacuo to give solid extracts. The extracts were then mixed with 2 ml of methanol and analyzed by HPLC (Thermo Separation Products) using 5 μm LiChrospher 100 RP-18 (26 × 8.0 mm) column. The UV spectra were recorded from 200 to 600 nm and the chromatograms were monitored at 300 nm by UV detector (TSP, spectrum system UV 3000 HR). The elution consisted of 65% acetonitrile and 35% water for 15 min and the flow rate was 0.8 ml/min.

3. RESULTS and DISCUSSION

3.1. Culture Growth in Shake-flask

The changes in mycelial growth and pH in the flask during cultivation of *T. matsutake* are shown in Fig. 2. The mycelial dry weight reached 1.15 g at 29th day and the pH remained within the range from 5.5 to 4.5 throughout the culture (Fig. 2). The overall growth rate, Rx, defined as the quotient of the cell concentration at the end of the culture divided by the total cultivation time was 0.09. Although the Rx value reported by Kawagoe (1999) was 0.20, this method was not suitable for the production of mycelium biomass. However, as the purpose of this study is not to produce biomass but to produce methyl trans-cinnamate, we used this culture condition.

3.2. Analysis of Methyl Trans-cinnamate

Since the analysis of methyl trans-cinnamate in the fruit-body of *T. matsutake* was already performed by Lee et al. (2005), we also
analyzed the methyl trans-cinnamate in the mycelium using this method. Using HPLC, methyl trans-cinnamate extracted from the cultured mycelium was analyzed. The retention time of this compound is 6.4 min. at 300 nm. Fig. 3 shows the chemical structure of methyl trans-cinnamate and its HPLC chromatogram.

3.3. Effect of the Extracts from Different Parts of *P. densiflora* Additions on Methyl-trans Cinnamate Production

Although the use of agricultural residues in microbial cultivation is being encouraged due to their ease of availability and inexpensive (Pan-
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![Graph showing mycelial weight of biomass (g) for different parts of extracts](image)

(a)

![Graph showing MTC content (ug/g) for different parts of extracts](image)

(b)

Fig. 4. Effect of extracts from the different parts of *Pinus densiflora* addition on biomass production (a) and methyl trans-cinnamate production (b) in the submerged cultures of *Tricholoma matsutake*.

dey et al., 2001), it is rare to use the extracts from wood in microbial cultivation. To produce methyl trans-cinnamate from mycelium in the submerged cultures of *T. matsutake*, ethanol extracts from the wood, bark, leaf and root of *P. densiflora* were added in the medium after 10 days cultivation. In the 1000 ml flasks containing 400 ml of basal medium and 5% (w/v) of the mycelium of *T. matsutake*, 4 ml of ethanol extracts containing 40 mg extract were added to the pre-cultured flask. The cultivation was performed at 25°C on a rotary shaker incubator at 150 rpm for 15 days.

The biomass production and the contents of methyl trans-cinnamate in the cultured mycelium treated with the extracts derived from different parts (wood, bark, leaf and bark) of *P. densiflora* were shown in Fig. 4. From the results, it can be known that the addition of wood extracts increased methyl trans-cinnamate contents in the submerged cultures. Although the maximum mycelial growth (1.15 g/400 ml) was achieved in control medium, no methyl trans-cinnamate could be detected.

Since the addition of wood extracts was effective on the production of methyl trans-cinnamate, different concentrations (50, 100 and 200 ug/ml) of wood extracts from *P. densiflora* were added to the culture media. The changes in the yield of mycelium and methyl trans-cinnamate content in mycelia were evaluated (Fig. 5). The mycelial production is decreased by added wood
extracts concentration in the culture media. The highest yield of methyl trans-cinnamate (100.21 ug/g) was obtained when 200 ppm of wood extracts from *P. densiflora* were added. These results can be explained that when exposure to pathogen attack or stresses, cell cultures create various responses, such as accumulation of phytoalexin or other secondary metabolites (Mehdy 1994).

3.4. Effect of the Dichloromethane Fraction from the Wood of *P. densiflora* Addition on Methyl-trans Cinnamate Production

Based on the results of Figs. 3 and 4, wood extracts from *P. densiflora* was successively partitioned with organic solvents, such as hexane, dichloromethane, ethyl acetate. Each fraction obtained from the wood extracts were added in the medium after 10 days cultivation. After added each fraction was added, the cultivation was performed at 25°C on a rotary shaker incubator at 150 rpm for 15 days.

The biomass production and the contents of methyl trans-cinnamate in the cultured mycelium treated with fraction partitioned from wood extracts of *P. densiflora* were shown in Fig. 6. From the results, we can conclude that higher methyl trans-cinnamate content in the mycelia did not lead to the higher production of the mycelial biomass. Among the four fractions (hexane, CH₂Cl₂, EtOAc and residue fraction), dichloromethane fraction was most effective in the
production of methyl trans-cinnamate (100.39 μg/g). However, ethyl acetate fraction and residue fraction did not induce the production of methyl trans-cinnamate in submerged culture. These results suggest that the production of methyl trans-cinnamate by adding ethanolic extract of *P. densiflora* in the submerged cultures of *T. matsutake* mycelia is partially attributable to dichloromethane fraction.

To find out compounds or fractions inducing methyl trans-cinnamate, dichloromethane fraction obtained from the wood extracts of *P. densiflora* was subjected to column chromatography. Three sub-fractions were obtained from the CH$_2$Cl$_2$ fraction.

Fig. 7 showed the TLC chromatogram of sub-fractions, and the mycelial growth and methyl trans-cinnamate production in the submerged cultures of *T. matsutake* added each sub-fraction. After adding sub-fractions in the medium, the cultivation was performed for 15 days. From the results, it can be known that mycelium treated with sub-fraction 1 has the highest content of methyl trans-cinnamate. From the results of Fig. 7, it can be known that maximum methyl trans-cinnamate (470.2 μg/g) was obtained when the first sub-fraction of dichloromethane fraction from the *P. densiflora* wood extracts was added to the medium. Similar research was performed to increase biological activity by addition of some compounds or extract in the submerged culture. It is reported that
in the culture of *Pleurotus eryngii*, the addition of natural products including brown rice and *Acanthopanax* to media can enhance its Angiotensin converting enzyme (ACE) inhibitory activity (Kang et al., 2003).

### 4. CONCLUSIONS

This is the first report to produce methyl trans-cinnamate by culturing the *T. matsutake* mycelium using submerged culture. Methyl trans-cinnamate is a significant flavor compound of *T. matsutake*. However, no methyl trans-cinnamate could be detected when the *T. matsutake* mycelium was cultured in the basal liquid medium. By adding the extracts from *P. densiflora* to the medium, methyl trans-cinnamate can be produced.

Some compounds or fractions derived from the extracts of *P. densiflora* induce the production of methyl trans-cinnamate in culture. To find out compounds or fractions inducing meth-
yl trans-cinnamate, CH$_2$Cl$_2$ fraction obtained from the wood extracts of *P. densiflora* was subjected to column chromatography. Three sub-fractions were obtained from the CH$_2$Cl$_2$ fraction. Submerged cultured mycelium treated with sub-fraction 1 has the highest content (470.2 µg/g) of methyl trans-cinnamate. This indicates that wood extracts of *P. densiflora* contains inducer of the methyl trans-cinnamate production in the *T. matsutake* submerged culture. Further, it is need to find single compound which can induce the methyl trans-cinnamate from sub-fraction 1.

REFERENCES