Mycoscience (2009) 50:78–81
DOI 10.1007/s10267-008-0447-z

NOTE

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Influence of *Flammulina velutipes* mycelia culture conditions on antimicrobial metabolite production

Received: April 9, 2007 / Accepted: August 27, 2008

Abstract Enokipodins A, B, C, and D are α-cuparene-type sesquiterpenoids antimicrobial metabolites produced in the stationary stage of *Flammulina velutipes* mycelia development in malt extract broth. This study assessed the influence of nutritional and environmental factors on *F. velutipes* mycelia culture for the production of these metabolites. The mycelia growth and antimicrobial activity were assessed by determining dry matter and the diffusion in agar method, respectively. The best *F. velutipes* mycelia growth was observed in dextrose potato broth, and greater antimicrobial metabolite production occurred in complete Pontecorvo's culture medium. Environmental modifications, such as a rise in temperature from 25° to 37°C on the 15th day of *F. velutipes* mycelia culture in malt extract and peptone broth, also optimized antimicrobial metabolite production. The metabolites produced in these treatments were correlated with the enokipodins A and B in thin-layer chromatography (TLC) and the antifungal activity test by TLC bioautography. This study showed that there was no correlation between biomass production and antimicrobial metabolite production, but there may be a correlation between culture medium composition and enokipodins biosynthesis.

Key words Basidiomycetes · Cuparene · Enokipodins A-D · Enokitake · Sesquiterpenoid

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Antimicrobial metabolite production by the basidiomycete mushroom, *Flammulina velutipes* (Curt.: Fr.), against *Bacillus subtilis*, *Escherichia coli*, and *Candida albicans* was reported by Kozová and Rehácek in 1967, and against *Trichoderma harzianum* by Tokimoto in 1985. Sesquiterpenoids of the α-cuparene-type called enokipodins A, B, C, and D were the main compounds responsible for *F. velutipes* antimicrobial activity; these compounds were shown to be active against the Gram-positive bacteria *B. subtilis* and *Staphylococcus aureus* and against the fungus *Cladosporium herbarum* (Ishikawa et al. 2000, 2001, 2005). The antimicrobial action and the uncommon chemical structures of enokipodins A–D triggered interest in the study of their chemical synthesis. Enokipodins A and B were synthesized by Srikrishna and Rao (2004) and recently by Secci et al. (2007). Kuwahara and Saito (2004) were then successful in synthesizing the four enokipodins A–D.

Although complete synthesis has been reported, the study of the viability and yield from the biosynthesis of these compounds is important to assess the advantages and disadvantages of each process for large-scale production. In this study, the influence of culture medium and variations in the conditions in the *F. velutipes* mycelia culture were assessed to investigate possible effects on the biosynthesis of the enokipodins A–D antimicrobial metabolites.

The strain of *F. velutipes* Fv-4 used in this study was a gift from the Laboratory of Forest Resource Biology, Graduate School of Agriculture, Hokkaido University, Sapporo, Hokkaido, Japan. The *F. velutipes* mycelia were kept at 4°C in test tubes containing potato dextrose agar (PDA) medium. To use the fungus as inoculum, part of the stored mycelia was transferred to Petri dishes (9 cm diameter) containing PDA medium. The dishes were incubated at 25°C for 10 ± 1 days; after this time, 9-mm-diameter disks of mycelia were removed from the edge of the culture and used as inocula of the respective treatments. The culture *B. subtilis* LMA0011 is a gift from the Food Microbiology Laboratory, Federal University of Viçosa, Viçosa, MG, Brazil, and it was kept at 4°C in test tubes containing brain heart infusion agar (BHIA) medium. To carry out the antibacterial activity test, a loop was removed from the stock

culture and placed in test tubes containing 10 ml brain heart infusion (BHI) culture medium and incubated at 37°C for approximately 24 h. *Cladosporium herbarum* NRRL2175 culture, from the André Tosello Foundation, Campinas, SP, Brazil, was kept in test tubes containing PDA medium at 4°C. To carry out the antifungal activity, test spores from the stock cultures were placed in tubes containing PDA medium and later incubated at 25°C until the culture presented high quantities of spores.

Flammulina velutipes mycelia were cultured in conditions used by Ishikawa et al. (2005) as the control treatment. Only the size of the Erlenmeyer flask was changed, to 100 ml with 50 ml culture medium inoculated with three mycelia disks. To assess the influence of the quantity of carbon source on the biomass and antimicrobial metabolite production, *F. velutipes* was cultured in malt extract broth (MEB) [3% malt extract (Difco, Detroit) and 0.3% peptone (Biobrás, Minas Gerais, Brazil) in distilled water, w/v] culture medium with quantities of malt extract altered to 1%, 2%, 4%, 5%, and 6% (w/v). In addition, to assess the influence of the quantity of nitrogen source, MEB culture medium with peptone altered to 0.1, 0.2, 0.4, 0.5, and 0.6 was used. The *F. velutipes* mycelia growth and antimicrobial metabolite production were also analyzed in liquid culture media: potato dextrose broth (PDB), Pontecorvo's complete culture medium (MC), and Pontecorvo's minimum culture medium (MM) (Pontecorvo et al. 1953). Mycelia growth was analyzed by determining the dry matter, and the antimicrobial metabolite production was analyzed on the 20th, 25th, and 30th day of incubation by diffusion in agar by the hole plate method. The liquefied BHIA medium (20 ml, about at 40°C) was inoculated with about 10^5 colony-forming units (CFU)/ml of the test bacterium in a Petri dish (9 cm diameter). After the mixture had solidified, 9-mm-diameter holes were punched with a cork borer, and 0.1 ml each of the respective culture filtrate was poured into the hole. These Petri dishes were kept at 4°C overnight and then incubated at 37°C for 12 h. The antibacterial activity was determined by observed of the clear inhibition zone around each hole. Each condition was tested in four replications.

The effect of environmental factors on biomass and antimicrobial metabolites production by *F. velutipes* was analyzed in MEB culture medium. The influence of temperature change during mycelia culture was analyzed by starting the *F. velutipes* culture at 25°C under static conditions and applying one of two treatments on the 15th day of incubation: incubation for 24 h at 4°C (cold treatment) and incubation at 37°C for 24 h (heat treatment). After this period, both cultures were returned to the starting conditions. The influence of exposure to light was tested by culturing *F. velutipes* mycelia at 25°C under constant light throughout the whole incubation period (CTE light) and exposure to light only on the 15th day of incubation for 24 h (24-h light). To analyze the influence of aeration by agitation, mycelia growth was started at 25°C under static conditions, and on the 15th day of incubation the cultures were placed in an orbital shaker at 90 rotations per minute (RPM) (agitation treatment). Differences in mycelia growth between the control and the treatments were analyzed by the Tukey test.

The treatments with *F. velutipes* mycelia culture in MC culture medium and change in temperature to 37°C on the 15th day of mycelia culture in MEB culture medium presented positive results for the optimization of antimicrobial metabolite production. Thus, these treatments and the control (MEB culture medium and constant temperature at 25°C) were repeated to extract the metabolites and confirm the results by the antibacterial paper disk test. In this experiment, the mycelia were cultured in ten Erlenmeyer flasks (250 ml) containing 100 ml culture medium each. Five mycelia disks were inoculated per flask, and the treatments were removed at 15, 17, 20, 25, and 30 days of incubation. For metabolite extraction, culture filtrate was extracted by the methodology reported by Ishikawa et al. (2000, 2001). The test bacterium *B. subtilis* was used in the paper disk method. In this method, incubation conditions and the medium with test bacterium was prepared in the same way that was used in the hole plate method. However, the hole with filtrate was replaced by a paper disk (8 mm) soaked with EtOAc extract equivalent to 1 ml culture filtrates. The experiments were performed in four replicates.

To assess the correlation among the antimicrobial metabolites produced by the *F. velutipes* mycelia in the treatments applied and those already reported, such as enokipodins A and B, crude extract equivalent to 1 ml filtrate was applied to thin-layer chromatography plates (TLC) beside authentic enokipodins A and B eluted in the 40:5:5:2 solvent system (chloroform:acetone:ethyl acetate:methanol). A spore suspension of *C. herbarum* was sprayed over the developed TLC plates, which were incubated at 28°C under humid conditions for 3 days (Homans and Fuchs 1970). The observed inhibitory zones were correlated with the spots seen on the TLC plates under UV (254-nm) light.

The *F. velutipes* mycelia growth profile and the antimicrobial metabolite production when cultivated in the culture medium and at the temperature reported by Ishikawa et al. (2005) were shown to be similar, differing only at the start of antimicrobial metabolite production. Ishikawa and collaborators observed antibacterial activity between the 25th and 30th days, whereas in the present study it was observed between the 20th and 25th day of incubation. This result can be explained by the decrease in the flask size and culture medium volume, a condition that led to the anticipation of the stationary stage of the *F. velutipes* mycelia growth. Concentrations that used altered sources of carbon and nitrogen in MEB medium were not sufficient for significant differences ($P < 0.01$) of mycelia growth (Fig. 1) or antimicrobial metabolite production (Table 1), showing that the increase of these ingredients would increase the expense of culture medium but not the antimicrobial metabolite production. The type of culture medium influenced both the *F. velutipes* mycelia growth and the antimicrobial metabolite production (Figs. 1, 2, Table 1). The PDB culture medium is a simple and cheap medium that resulted in the best mycelia growth (Fig. 1) ($P < 0.01$) but not optimum production of the antimicrobial compound (Table 1). On the other hand, in

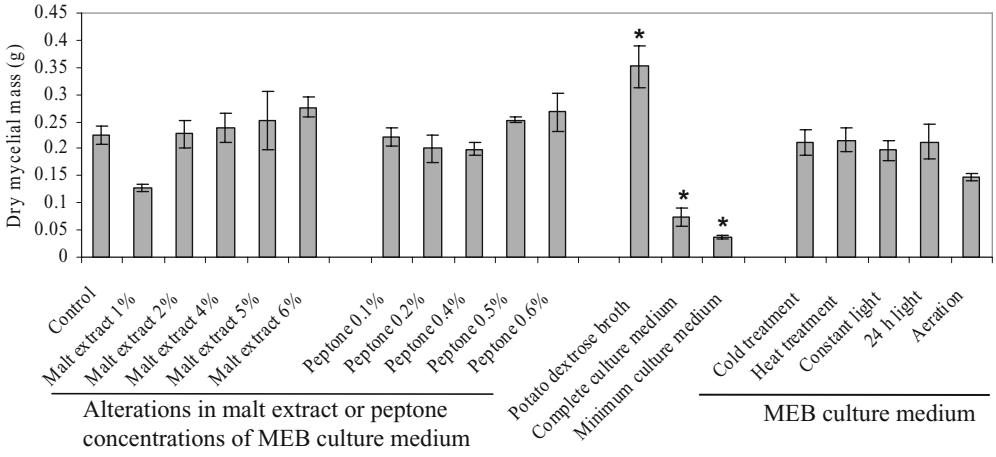

Fig. 1. Effect of culture medium and environmental modification on *Flammulina velutipes* (Fv-4) mycelial growth. Control: malt extract broth (*MEB*) culture medium, at 25°C for 30 days. Alterations were made in malt extract or peptone concentrations of MEB; other growth conditions are same as the control. In potato dextrose broth and Pontecorvo's complete or minimum culture medium, other growth conditions are the same. For cold and heat treatment, the tested strain was cultured in MEB starting at 25°C; on the 15th day, incubated for 24 h at 4°C and 37°C, respectively; after this period, returned to 25°C. For constant light, the strain was cultured in MEB at 25°C under constant light. For 24 h light, the flasks with mycelia were incubated under light for 24 h only on the 15th day. For the aeration treatment, the flasks were cultured in MEB at 25°C starting under static conditions and after the 15th day incubated in an orbital shaker at 90 rotations per minute (RPM). *Showing significant difference with the control at the level $P < 0.01$ by the Tukey test (average of four replications)

Table 1. Antibacterial activity of culture filtrate of *Flammulina velutipes* (Fv-4) mycelia against *Bacillus subtilis* (LMA0011) in different culture media and conditions by the hole plate diffusion method

Treatments[a]	Period of cultivation (days)		
	20	25	30
Control	–	++	+++
Malt extract 1%	–	+	+
Malt extract 2%	–	+	+
Malt extract 4%	–	+++	+++
Malt extract 5%	–	++	+++
Malt extract 6%	–	++	+++
Peptone 0.1%	–	–	+
Peptone 0.2%	–	+	+
Peptone 0.4%	–	+	+++
Peptone 0.5%	–	+	+
Peptone 0.6%	–	+	+
Potato dextrose broth	+	+	–
Complete culture medium	+++	+++	+++
Minimum culture medium	+	++	++
Cold treatment	–	++	+++
Heat treatment	++	+++	+++
Constant light	+	+	+++
24-h light	–	+	+++
Aeration	–	+	+++

Experiments were done in four replicates
–, no inhibition; +, inhibitory zone <2 mm; ++, inhibitory zone = 2–4 mm; +++, inhibitory zone >4 mm
[a] Control: Malt extract broth (MEB) culture medium, at 25°C. Alterations were made in malt extract or peptone concentrations of MEB, at 25°C; in potato dextrose broth, or Pontecorvo's complete or minimum culture medium, at 25°C. For cold and heat treatment, the tested strain was cultured in MEB starting at 25°C; on the 15th day, culture was incubated for 24 h at 4°C and 37°C, respectively, and after this period returned to 25°C. For constant light, the strain was cultured in MEB at 25°C under constant light. For 24-h light, the flasks with mycelia were incubated under light for 24 h only on the 15th day. For the aeration treatment, the flasks were cultured in MEB at 25°C starting under static conditions; and after the 15th day incubated in an orbital shaker at 90 rotations per minute (RPM)

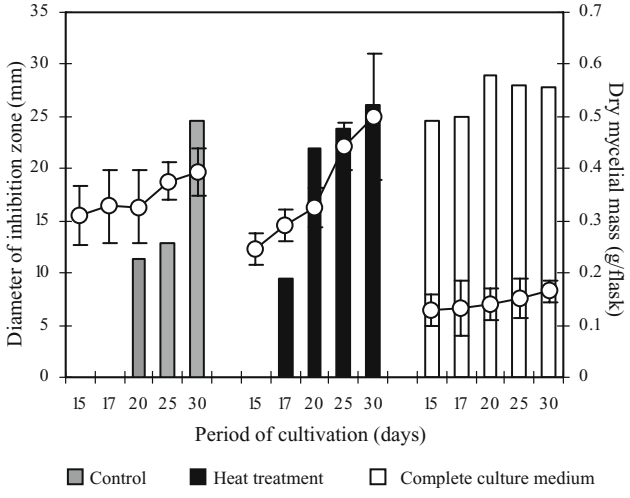

Fig. 2. Effect of culture medium and temperature modification on *Flammulina velutipes* (Fv-4) mycelial growth (*lines*) and antimicrobial compound production (*columns*). *Control:* Cultured in MEB culture medium at 25°C. *Heat treatment*: The tested strain was cultured in MEB starting at 25°C; on the 15th day, incubated at 37°C for 24 h; after this period, returned to 25°C. *Complete culture medium*: Cultured in Pontecorvo's complete culture medium at 25°C. Antibacterial activity of culture filtrate against *Bacillus subtilis* (LMA0011) was measured as diameter of the inhibitory zone around a paper disk soaked with EtOAc extract equal to 1.0 ml culture filtrate, by the paper disk method

the MC culture medium, although the mycelia growth was significantly smaller (Fig. 1) ($P < 0.01$), antimicrobial metabolite production was anticipated and greater than in the control MEB culture medium (Table 1). This result showed that there was no correlation between biomass production and antimicrobial metabolite production, but there may be a correlation between culture medium composition and

enokipodin production. Secondary metabolites are formed only after the requirements of cell growth have been satisfied. When growth stops, it seems that some biochemical pathways are not shut off; some compounds accumulate, and then these are used like raw material to manufacture new end products (Griffin 1994; Kendrick 2000). The MC medium presents a smaller amount of carbon source and energy in relation to PDB and MEB medium, as well as including a solution rich in minerals and vitamins. It is suggested that exhaustion of the carbon nutrition of the MC medium, and/or the excess of some mineral and/or vitamin component, has activated the enokipodins biosynthetic route. Among the modifications in the culture conditions applied using MEB culture medium, treatment with heating of the culture environment from 25° to 37°C for 24 hours on the 15th day did not significantly alter mycelia mass production compared to the control (Fig. 1), but it was shown to improve antimicrobial metabolite production (see Fig. 2, Table 1). The treatment with cooling to 4°C, constant light, light for 24 h on the 15th day, and aeration after the 15th day delayed antimicrobial metabolite production compared to the control (see Fig. 1, Table 1).

The antifungal activity of an ethyl acetate extract from the culture of mycelial *F. velutipes* grown in control condition, culture in MC culture medium, and in the heating treatment were examined by TLC bioautography with the authentic enokipodins A and B samples, using *C. herbarum* as the test fungus. The two main antifungal spots observed at *Rf* 0.73 and 0.88 were correlated with enokipodins A and B, respectively. A discrete spot was observed at *Rf* 0.35, which indicated it was enokipodin C. Because enokipodins C and D are formed mainly after the 30th day of culture (Ishikawa et al. 2005), they were not shown in this study.

To date, only the antimicrobial activity of enokipodins A–D has been assessed. Thus, obtaining these compounds in greater quantity, either by the biosynthetic path or by their synthesis, will contribute to analysis of the efficacy of these compounds for their development as new antibiotics, along with assessment of other biological activities of pharmacological interest, including antitumoral, antimalarial, antioxidant, and antiinflammatory activity as previously reported for other natural sesquiterpenoid compounds (Fraga 2001, 2002).

This study showed that antimicrobial metabolite production by *F. velutipes* mycelia is optimized in the MC medium and by temperature change from 25° to 37°C for 24 h (see Fig. 2). From the biotechnology aspect, the result with use of MC medium was interesting, but for the viability of large-scale production, MC can be a complex and expensive medium. In contrast, the alternative of the use of MEB culture medium with changes in temperature is simple and cheap for enokipodin A–D biosynthesis optimization. From the physiological aspect, it is known that as food runs out, or staling factors build up, or reserves reach an appropriate level, or specific environmental signals are received, the fungus switches into the reproductive mode (Kendrick 2000). Changes in temperature are also one of the main factors that unchain the cellular differentiations leading to mushroom fructification (Chang and Hayes 1978; Kendrick 2000). More experiments are needed, but we think that the conditions for start of the antimicrobial secondary metabolites, enokipodins A–D, and the environmental factors for fructification induction can be correlated.

Acknowledgments The authors are grateful for financial support from the Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq, and to MSc. Vargas-Isla R. for reading the manuscript and giving useful suggestions.

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