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Production of the Bioactive Compound Eritadenine by Submerged Cultivation of Shiitake (*Lentinus edodes*) Mycelia

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Fruit bodies and mycelia of shiitake mushroom (*Lentinus edodes*) have been shown to contain the cholesterol-reducing compound eritadenine, 2(*R*),3(*R*)-dihydroxy-4-(9-adenyl)butyric acid. In the search for a production method for eritadenine, shiitake mycelia were investigated in the present study. The mycelia were cultivated both in shake flasks and in bioreactors, to investigate the effects of pH, stirring rate, and reactor type on the production and distribution of eritadenine. Both the biomass and the culture broth were examined for their eritadenine content. In the shake flasks, the final concentration of eritadenine was 1.76 mg/L and eritadenine was equally distributed between the mycelia and the growth media. In the bioreactors, the shiitake mycelia were found to contain eritadenine in relatively low levels, whereas the majority, 90.6–98.9%, was detected in the growth media. Applying a stirring rate of 250 rpm during bioreactor cultivation resulted in the highest eritadenine concentrations: 10.23 mg/L when the pH was uncontrolled and 9.59 mg/L when the pH was controlled at 5.7. Reducing the stirring rate to 50 rpm resulted in a decreased eritadenine concentration, both at pH 5.7 (5.25 mg/L) and when pH was not controlled (5.50 mg/L). The mycelia in the shake flask cultures appeared as macroscopic aggregates, whereas mycelia cultivated in bioreactors grew more as freely dispersed filaments. This study demonstrates for the first time the extra- and intracellular distribution of eritadenine produced by shiitake mycelial culture and the influence of reactor conditions on the mycelial morphology and eritadenine concentrations.

KEYWORDS: Eritadenine; *Lentinus edodes*; submerged cultivation; bioactive compounds

INTRODUCTION

The shiitake mushroom (*Lentinus edodes*) is traditionally consumed in East Asia, but in recent decades its consumption has spread worldwide. In addition to being a popular edible fungus, it is well established as a medicinal mushroom because it contains several substances that promote health. Among other attributes, the ability to reduce blood cholesterol in both animals and humans has been ascribed to this mushroom (*1, 2*). The agent responsible for the plasma cholesterol-reducing effect of shiitake is a secondary metabolite designated eritadenine [**Figure 1**, 2(*R*),3(*R*)-dihydroxy-4-(9-adenyl)butyric acid] (*3, 4*). (Eritadenine was designated lentinacin or lentysine by the research groups initially isolating it, before being given its common name.) The hypocholesterolemic action of eritadenine has been investigated in several studies on rats (*3–8*), but the exact mechanism is not fully elucidated. There are no indications of this compound inhibiting the biosynthesis of cholesterol (*8*), and the hepatic cholesterol levels in rats are not lowered by eritadenine (*4, 8*). Rather, eritadenine is suggested to accelerate the removal of blood cholesterol either by stimulated tissue uptake or by inhibited tissue release (*8*).

The eritadenine concentrations in fruit bodies of shiitake, as determined by HPLC analysis, have been found to be in the range of 3.17–6.33 mg/g of dry mushrooms (*9*). The mycelia of shiitake have also been found to contain eritadenine; the amount determined by GC analysis was 0.737 mg/g of dried biomass (*10*). Because the process of growing fruit bodies of shiitake is a fairly demanding and time-consuming process,

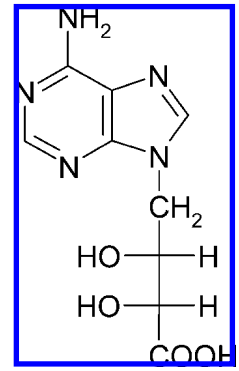

Figure 1. Eritadenine (M_r = 253 g/mol).

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mycelia could be an alternative source of eritadenine. The incentives for shiitake mushrooms to produce eritadenine are not yet clear; that is, the function of this secondary metabolite is not elucidated. However, due to its structural properties, it has been classified as a nucleoside antibiotic, more specifically to the group of inhibitors of RNA synthesis (*11*). Furthermore, the growth conditions that favor the production of eritadenine are not known, and therefore cultivation of shiitake mycelia in controlled submerged conditions offers an approach to identify important factors that can be used for improving eritadenine production.

In recent decades the use of submerged cultivation of filamentous fungi for the production of commercially important products has increased. The cholesterol-reducing compound lovastatin produced by *Aspergillus terreus*, the antibiotic penicillin by *Penicillium chrysogenum*, and citric acid by *Aspergillus niger* represent some examples. It should be emphasized that filamentous fungi are morphologically multifaceted organisms which exhibit different hyphal morphologies in submerged culture, ranging from freely dispersed linear filaments to densely entangled aggregates, pellets (*12*). Several studies have shown the inter-relationship between hyphal morphology, metabolite production, and culture conditions, such as medium composition, pH, inoculum concentration, dissolved oxygen, and agitation (*13–17*).

The goal of the present work was to evaluate if submerged cultivation of shiitake mycelia could be a feasible way of producing eritadenine. The impact of pH, stirring rate, and reactor type on eritadenine production and the resulting extra- and intracellular distribution were studied.

MATERIALS AND METHODS

Fungal Material. The shiitake strain used was *L. edodes* 2 (Le-2). Mycelia of this strain were kindly supplied by Dr. Gary L. Mills, Diversified Natural Products, Inc., Scottville, MI. The mycelia were cultivated on malt yeast agar (MYA) plates composed of, per liter, 20 g of malt extract, 2 g of yeast extract, and 20 g of microbial agar, for 10 days at 23 °C.

Shake Flask Cultivation. Mycelia were cut from half of a MYA plate (90 mm in diameter), homogenized in a 0.05 mM phosphate buffer, pH 5.8, and aseptically transferred to 200 mL of sterilized malt yeast medium composed of, per liter, 20 g of malt extract, 2 g of yeast extract, and 20 g of glucose, with the glucose solution sterilized separately. Triplicate cultivation experiments were performed in shake flasks at 150 rpm for 20 days at 23 °C.

Bioreactor Cultivation. The submerged cultivations took place in 1 L bioreactors (Biobundle 1 L, Applikon Biotechnology, Schiedam, The Netherlands) and in a 12 L bioreactor (BR 12, Belach Bioteknik AB, Solna, Sweden), with total starting volumes of 700 mL and 10 L, respectively. The preparation of the medium and the mycelia for inoculation was done in the same way as described previously, but the number of plates used was 1 and 14 for the 1 and 12 L reactors, respectively. All bioreactor cultivations took place at a temperature of 25 °C and a dissolved oxygen flow rate of 1 v/v/min. In the 1 L reactors a stirring rate of either 50 or 250 rpm was applied, and in the 12 L reactor the stirring rate was set to 150 rpm. In the 1 L reactors the pH was either controlled at 5.7 by automatic addition of 5 M KOH or uncontrolled, and the cultivations lasted for 20 days. In the 12 L reactor the pH was left uncontrolled and the cultivation proceeded for 26 days. The dissolved oxygen concentration (DO) was measured by a pO_2 electrode, and the pH was measured with a pH electrode. From the 12 L reactor samples were taken after 10, 15, 20, and 26 days of cultivation. Duplicate cultivation experiments at 50 rpm and pH controlled at 5.7 were conducted for assessment of the reproducibility.

Biomass Determination. Following cultivation, the mycelia from shake flasks and bioreactors were harvested by filtering the culture broth through Whatman no. 42 filter paper and a subsequent wash with distilled water. The biomass was dried overnight and the dry cell weight (DCW) determined.

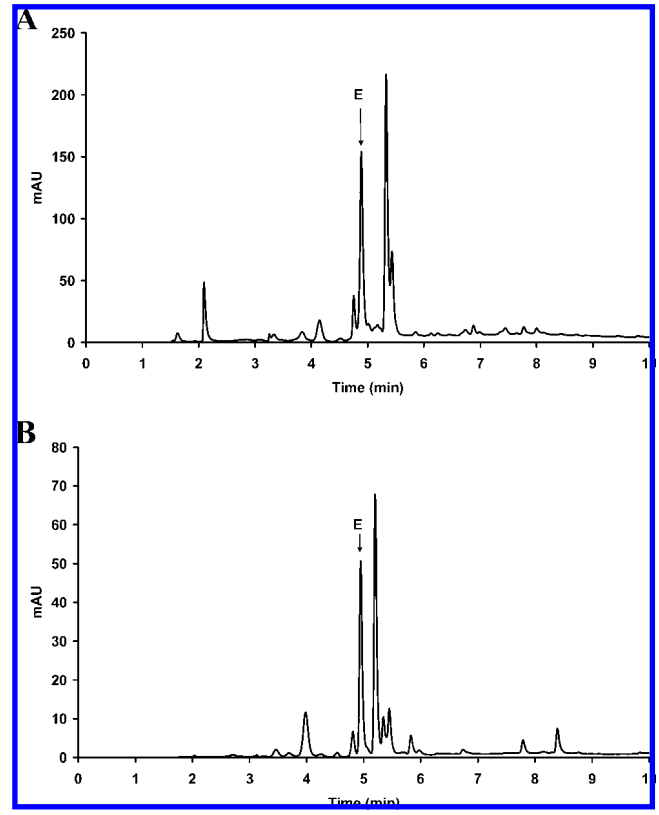

Figure 2. HPLC chromatograms of a methanol extract from shiitake mycelia (**A**) and the purified culture broth (**B**) from submerged cultivation of shiitake mycelia. Eritadenine is eluted at a retention time of 4.9 and is represented by E in the chromatograms.

Analysis of Eritadenine. Eritadenine concentrations were analyzed by HPLC and separated over a C18 column as previously described (*9*). In brief, HPLC analysis was conducted at 23 °C, with UV detection at 260 nm and a flow rate of 1 mL/min. Initially the mobile phase was 0.05% TFA in aqueous solution/0.05% TFA in MeCN, in the proportion 98:2 followed by a linear change to 40:60 over 10 min. Synthetic eritadenine was used as a reference for identification and as a standard for quantification, as previously described (*9*). Prior to HPLC analysis of eritadenine the mycelial biomass was extracted with 80% methanol, and the filtered broth was purified by application to ion exchange resins, as previously described (*9*). The concentrations of eritadenine were based on the respective starting volumes in the shake flasks and bioreactors.

Sugar Analysis. The concentrations of glucose and maltose were determined as previously described (*18*) using an HPLC system equipped with a Series 200 refractive index (RI) detector, a guard column, and an ion exchange column (Aminex HPX87-P, Bio-Rad). The column was kept at 85 °C in a column oven, and water at a flow rate of 0.6 mL/min was used as the mobile phase. Known concentrations of glucose and maltose were used as standards to determine the total sugar consumption. The concentration of maltose was converted to glucose equivalents for calculation purposes.

RESULTS AND DISCUSSION

In a previous study (*10*) shiitake mycelia from submerged cultivations were investigated for their eritadenine content. However, if eritadenine has some antibiotic activity, its release to the surrounding medium is plausible. Therefore, in the present study not only the mycelia but also the culture broths were analyzed for eritadenine, and the compound of interest was found both in the mycelia (**Figure 2A**) and in the culture broths

Table 1. Cultivation of Shiitake Mycelia at Various Submerged Conditions and the Resulting Total Sugar Consumption, Biomass, and Eritadenine Production

cultivation conditions					eritadenine concn		
cultivation vessel	pH_i[a]/pH_f[b]	rpm (impeller tip speed, cm/s)	total sugar consumption (%)	biomass concn (g/L)	mycelia (mg/g)	broth (mg/L)	total (mg/L)
shake flask[c,d]	5.7/3.0	150 (0)	27	1.77 ± 0.02	0.50 ±0.02	0.86 ± 0.05	1.76 ± 0.08
1 L reactor	5.7/5.7	250 (58.8)	38	0.89	1.05	8.69	9.59
1 L reactor	5.7/4.2	250 (58.8)	34	0.84	0.21	10.04	10.23
1 L reactor[e]	5.7/5.7	50 (11.8)	22	0.43 ± 0.06	0.36 ±0.04	5.07 ± 0.36	5.25 ± 0.34
1 L reactor	5.7/5.0	50 (11.8)	16	0.36	0.16	5.44	5.50
12 L reactor	5.7/5.3	150 (78.5)	16	0.34	0.20	3.25	3.32

[a] Initial. [b] Final. [c] Average values ± SD of three independent cultivation experiments. [d] No impeller-induced stirring. [e] Average values ± SD of two independent cultivation experiments.

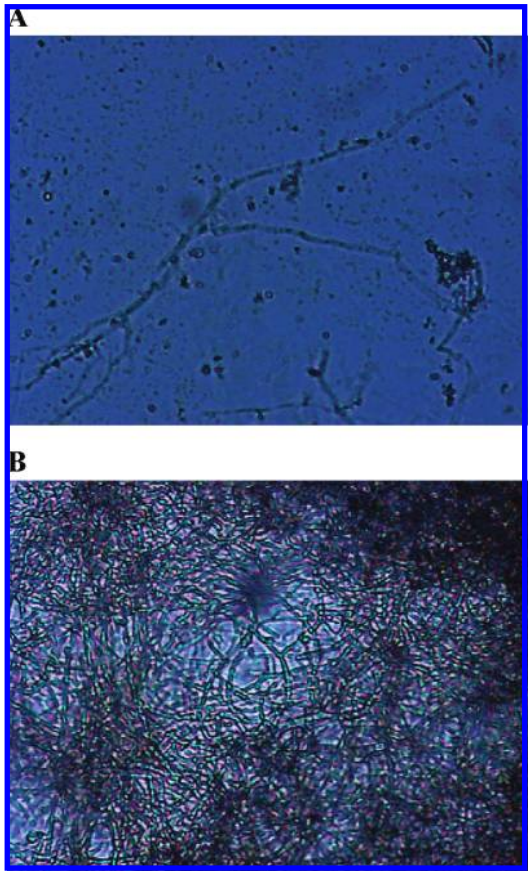

Figure 3. Shiitake mycelia from submerged cultivation in bioreactor (**A**) (20×) and in shake flask (**B**) (10×).

(**Figure 2B**). The lowest overall concentration of eritadenine was detected in the shake flask cultures (**Table 1**), of which 48.9% was found in the broth. Cultivations in bioreactors, at a stirring rate of 250 rpm, resulted in the highest eritadenine concentration in the broth, whereas reduction of the stirring rate resulted in a marked decrease in eritadenine concentration. Irrespective of pH and stirring rates in the bioreactors, the majority, 90.6–98.9%, of eritadenine was detected in the broth. During bioreactor cultivation shear was present as a result of impeller-induced stirring, and the mycelial morphology was more as freely dispersed filaments (**Figure 3A**), as compared to the macroscopic aggregates (**Figure 3B**) formed during shake flask cultivation. It is possible that shear affects mycelial morphology, which in turn seems to have a profound effect on eritadenine production and excretion.

The stirring rate in the bioreactors had also a considerable effect on mycelial biomass production, irrespective of pH (**Table 1**). A higher stirring rate promoted growth in the bioreactors, which might be explained by a more efficient mass transfer of

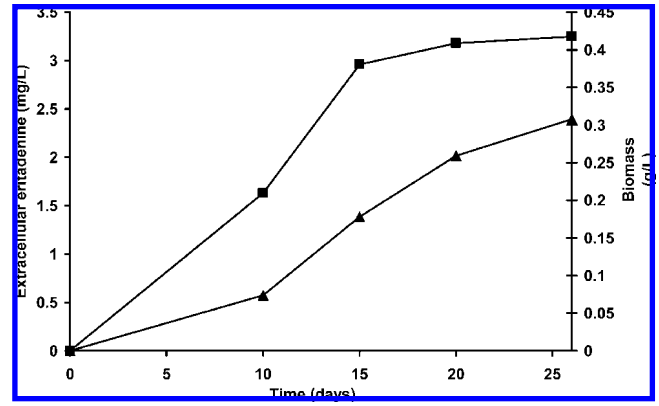

Figure 4. Extracellular eritadenine concentration (■) (mg/L) and biomass concentration (▲) (g/L) at various times of cultivation.

oxygen and nutrients. On the other hand, the biomass production in the shake flasks was higher than for any of the reactor conditions. However, in the shake flask cultures the mycelia were predominantly in the liquid phase, whereas in the bioreactors the mycelia had grown on probes and walls, thus complicating the quantification. For all cultivations the initial pH was 5.7, but during growth the pH decreased in the shake flasks and in the bioreactors with uncontrolled pH, with the most pronounced change in the shake flasks. The relatively high biomass production and/or the mycelial pellet structure in the shake flasks might contribute to a considerable lowering of pH. If eritadenine has some antibiotic effect, a low pH might diminish its release because a low pH is protective in itself. According to previous studies (*19*) the optimum pH for the growth of shiitake mycelia is 3.0–3.5, whereas for the production of certain antibacterial substances the optimum pH is 4.5, showing that growth and metabolite production do not have the same pH optimum.

To investigate the dynamics of mycelial growth and eritadenine production, the mycelia were grown in a 12 L reactor and samples were taken after 10, 15, and 20 days and at the end of cultivation (26 days). There was a marked increase in both extracellular eritadenine and biomass concentration (**Figure 4**) until day 15, when the eritadenine concentration began to level out. The growth, however, continued after day 15, suggesting that growth and eritadenine production are uncoupled as would be expected for a secondary metabolite. At the end of cultivation, <50% of the sugars were consumed (**Table 1**); hence, carbon source deprivation was not a limiting factor for biomass and eritadenine production. The same pertained to all cultivations because in all cases enough carbon for anabolic and catabolic reactions was available.

Both eritadenine and biomass concentration were lower than in the 1 L reactors, and 97.9% of the eritadenine was excreted to the medium. The reactor geometries in the 1 and 12 L

bioreactors differ, resulting in a variation of the oxygen transfer, mixing, and shear, which might affect biomass and eritadenine production. Moreover, the incoming light in the 12 L reactor is significantly less than in the 1 L reactors. Whether or not the light exerts any considerable effect on shiitake mycelial growth or eritadenine production is uncertain, but light has been shown to be an influential factor of growth and biochemical processes in many fungi (*20*).

The results from this study show that impeller-induced agitation during cultivation results in the formation of dispersed mycelial filaments, which favor extracellular excretion of eritadenine. Until now, no studies of eritadenine content in liquid cultures of shiitake mycelia have been found in the literature. This study demonstrates the possibility of influencing the production and distribution of eritadenine by altering the culture conditions. Increased excretion of eritadenine might have a profound effect on large-scale production of this cholesterol-reducing compound, and an increased availability of eritadenine in the liquid phase might facilitate subsequent downstream processing.

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