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LICENTIATE THESIS

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Production and Quantification of Eritadenine, a Cholesterol Reducing Compound in Shiitake *(Lentinus edodes)*

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Abstract

Cardiovascular diseases are among the main causes of death in our society and there is a strong correlation between enhanced blood cholesterol levels and the development of such diseases. The popular edible fungus, shiitake mushroom (*Lentinus edodes*), has been shown to produce a blood cholesterol lowering compound designated eritadenine, and the hypocholesterolemic action of this compound has been quite extensively examined in rats. Eritadenine is suggested to accelerate the removal of blood cholesterol either by stimulating tissue uptake or by inhibiting tissue release; there are no indications of this compound inhibiting the biosynthesis of cholesterol.

If shiitake mushrooms are to be used as a source for a potential cholesterol reducing product, it is of great importance to determine the content of eritadenine in the mushrooms as accurately as possible. Hence, in paper I methanol extraction was used to recover as much as possible of the hypocholesterolemic agent from the fungal cells. In order to analyse the target compound, a reliable and reproducible HPLC method for separation, identification and quantification of eritadenine was developed. The amounts of eritadenine in fruit bodies of four commercially cultivated shiitake mushrooms were determined, and the mushrooms under investigation exhibited up to ten times higher levels of eritadenine (3.17-6.33 mg/g dry mushrooms) than previously reported.

Not only the fruit bodies of shiitake, but also its mycelia contain eritadenine. Growing fruit bodies of shiitake is a fairly demanding and time consuming process. Hence, in search for a source of eritadenine, submerged (liquid) cultivation of shiitake mycelia could be an alternative. The reason why shiitake mushrooms synthesize eritadenine is yet not clarified; i.e. the function of this secondary metabolite and the growth conditions that favour its production are not elucidated. In addition, like other filamentous fungi, shiitake exhibits different hyphal morphologies in submerged cultures depending on cultivation conditions such as medium composition, temperature, pH, inoculum concentration, dissolved oxygen and shear. The fungal metabolism and hence production of secondary metabolites is in turn affected by the

morphology, as have been shown in several studies on filamentous fungi. Submerged cultivation of shiitake mycelia offers a convenient way to change the cultivating conditions in order to improve eritadenine yield and productivity. The study in paper II focused on cultivation of mycelia at different conditions, both in shake flasks and in bioreactors, to investigate the effect of pH and stirring rate on production of eritadenine. The shiitake mycelia were found to produce eritadenine, and the compound of interest was found in both the fungal cells and the growth media. The major part (90-99%) was found in the culture medium, which offers a facilitated downstream processing if large scale production of the compound is to be conducted. The mycelial morphology in the shake flask cultures were macroscopic aggregates, pellets, and the specific productivity of eritadenine was relatively low; 6.56 mg/g dry cell weight (DCW). In the bioreactor cultivations, the mycelia grew as freely dispersed filaments, showing a higher specific productivity than in the shake flasks, ranging between 26.00- 39.58 mg/g DCW. This indicates the influence of morphology on eritadenine production. The biomass yield in shake flasks and bioreactors was in parity; 0.45 g in the shake flasks and 0.25- 0.62 g in the bioreactors. A stirring rate of 50 rpm in the bioreactors was preferable for eritadenine production, whereas for biomass production it was 250 rpm, indicating the influence of agitation on both growth and productivity. The pH did not have any major impact on growth, whereas the specific productivity in the bioreactors was higher when pH was uncontrolled than controlled at 5.7.

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List of papers

I Quantification of the Bioactive Compound Eritadenine in Selected Strains of Shiitake Mushroom (*Lentinus edodes*)

Josefine Enman, Ulrika Rova and Kris A. Berglund

J. Agric. Food Chem. **2007**, 55(4):1177-80

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

Josefine Enman, David Hodge, Kris A. Berglund and Ulrika Rova

Manuscript

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Introduction

Cardiovascular disease is a major health concern in modern Western society and in many countries there is a high frequency of such disease. This type of disease is also the most common cause of death in the world (*1*). Cardiovascular disease is a class of diseases related to the heart and blood which mainly develops from atherosclerosis. Atherosclerosis is in turn caused by a process in which fat substances are attached to the inside of blood-vessels and form plaques. These plaques diminish the size of the vessels, causing a reduced blood flow to central organs such as the heart. The resulting deficit of oxygen and nutrients in turn causes serious heart conditions. There are many risk factors which can be associated with atherosclerosis and cardiovascular disease, such as obesity, diabetes, smoking, stress and genetic factors. One of the most well established risk factor for the development of atherosclerosis, and hence cardiovascular disease, is high levels of blood cholesterol. When circulating in the blood, cholesterol attaches to the walls of blood vessels and promotes atherosclerosis. Thus, there is a strong correlation between enhanced plasma cholesterol levels and the risk of developing cardiovascular disease. Increased mortality in coronary artery disease is also correlated to high cholesterol levels (*2*). Considering the prevalence of cardiovascular disease and its correlation to cholesterol there is a need for substances reducing cholesterol and hence prevent this state of ill-health.

Cholesterol and cardiovascular disease

Although cholesterol (**Fig. 1**) is mainly associated with cardiovascular disease, this lipid is also indispensable to the human body. It is essential as a component of cellular membranes and as a precursor of steroid hormones and bile acids. Like most other lipids, cholesterol is hydrophobic in its nature and thus it is carried in the blood, from its site of synthesis to other tissues, as plasma lipoproteins. These lipoproteins are complexes of carrier proteins, apolipoproteins, with different contents of phospholipids, cholesterol, cholesteryl esters and triacylglycerols.

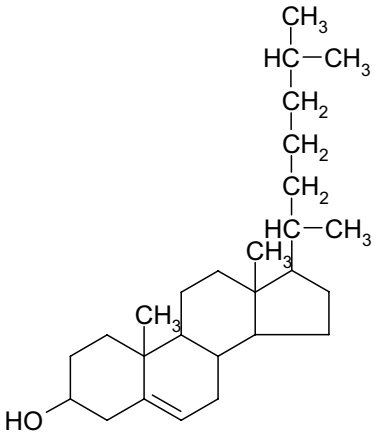

Figure 1. Cholesterol.

The various combinations of proteins and lipids make lipoproteins of different densities. In the blood, cholesterol is principally in the lipoprotein fractions of low density lipoprotein (LDL) and high density lipoprotein (HDL). The main cholesterol transporter, LDL, is very rich in cholesterol and transports it to different extrahepatic tissues, but has the disadvantage of attaching to the walls of the blood vessels and hereby causing atherosclerosis. HDL has a high content of proteins and contains less cholesterol than LDL. The cholesterol content of HDL increases upon the uptake of excess cholesterol in the bloodstream and extrahepatic tissues. In this way cholesterol is transported back to the liver as HDL, for recycling or excretion. Thus, increasing LDL and decreasing HDL cholesterol levels increase the probability of developing atherosclerosis (*2*).

A minor part of cholesterol is obtained from the diet, whereas the major part is produced in the body. The main source of cholesterol in the body is its biosynthesis in the liver (*3*). The rate-limiting step in cholesterol synthesis is the reaction catalysed by the enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (*4*). This is also a major site of regulation of cholesterol biosynthesis. The regulation is mediated at a transcriptional level; the gene encoding HMG-CoA reductase alongside other genes encoding enzymes involved in uptake and synthesis of cholesterol is governed by sterol regulatory element-binding proteins (SREBPs) (*5*). The SREBPs are inactive when cell

cholesterol levels are high, but are activated when the cholesterol levels decrease and hence turn on transcription of their target genes. This regulation of cholesterol synthesis maintains a balance between the supply and demand for cholesterol of the body, and prevents harmful levels of cholesterol circulating in the blood.

Familiar hypercholesterolemia is a genetic disorder in which the individuals suffer from very high blood cholesterol levels. Normally, cellular uptake of LDL is mediated through specific receptor proteins in the cellular membranes, LDL receptors (*6*). The production of these LDL receptors is regulated by intracellular levels of cholesterol and at high intracellular cholesterol levels the transcription of the gene encoding the LDL receptor is decreased. In familiar hypercholesterolemia the LDL receptor is defective and thus cholesterol uptake is prevented in these individuals and the blood cholesterol accumulates in the blood vessels, promoting atherosclerosis. Since cholesterol cannot enter the cell and regulate its own production, the biosynthesis of cholesterol continues despite the already high blood levels.

Treatment of hypercholesterolemia

Hypercholesterolemia is defined as a total cholesterol >5 mM or >3 mM LDL cholesterol. Further, an HDL cholesterol <1 mM is an indication of a higher risk of cardiovascular disease (*7*). Hypercholesterolemia can in some cases be the result of an unhealthy life-style. In these cases the hypercholesterolemia can be treated by changes in the diet. By reducing the intake of fat in general and saturated fats in particular, cholesterol levels can be decreased (*8*). On the other hand, individuals with familial hypercholesterolemia have a disruption in the cholesterol metabolism and need pharmacological treatment. In the latter case drugs that keep the blood cholesterol down is a necessity. There are different drugs on the market for treatment of familiar hypercholesterolemia, of which the statins are frequently prescribed (*8*). The statins are originally a group of secondary metabolites isolated from fungi, such as lovastatin (**Fig. 2**) from *Aspergillus terreus (9)* and mevastatin from *Penicillium citrinium* (*10*). The statins are competitive inhibitors of the enzyme HMG-CoA reductase, which catalyses the

rate limiting step in cholesterol biosynthesis, hence inhibiting cholesterol biosynthesis (*9, 10*). Some of the statins are produced by fungal fermentations, such as lovastatin, which is industrially produced by the filamentous fungus *Aspergillus terreus* (*11*) and is the active substance in the drug Mevacor®. In order to make the statins more efficient, semi-synthetic statins, such as simvastatin have been developed. Simvastatin is a chemically modified derivative of lovastatin with higher inhibitory potency (*12*) and is the active substance in Zocor®. Further, some statins are purely synthetic, like atorvastatin in the hypocholesterolemic drug Lipitor®. Despite being efficient cholesterol reducing compounds, the statins have attracted some attention for their adverse effects, such as liver damage, rhabdomylosis and myotoxicity (*8*). Considering that many of the statin-based drugs are under increased scrutiny due to their negative side effects, there is a demand for new types of cholesterol reducing compounds.

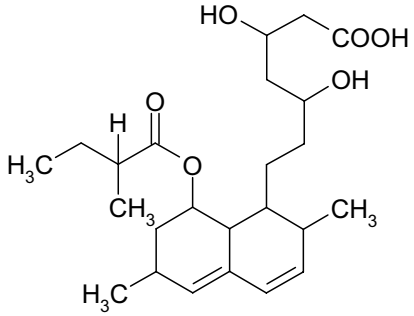

Figure 2. Lovastatin.

The shiitake mushroom

The shiitake mushroom (*Lentinus edodes*) (*13*) is an edible fungus, native to the Far East. In China, Japan and Korea it is a traditional delicacy and its history of outdoor cultivation on hardwood logs dates back to at least a thousand years. In the last decades the techniques for indoor cultivation of this edible mushroom has become more developed and it is now one of the most cultivated edible fungus in the world (*14*). It is denoted as a white-rot fungus since it is a wood decomposer, naturally growing on

dying broadleaf trees such as the shii tree and other Asian oak trees. Shiitake, like many other species of fungi, has a great importance for the ecosystem. It is a saprophyte considering it lives on dead material, thereby enhances the decomposition of these materials. White-rot fungi have one remarkable feature; their ability to completely degrade lignin. Lignin is a complex aromatic polymer in wood, which is considered a by-product in the wood-processing industry and white-rot fungi are the only organisms known to degrade this compound. These white-rot fungi have enzyme systems required for lignin degradation and shiitake mushroom produce two of the major enzymes involved, laccase and manganese peroxidase (15).

The shiitake mushroom belongs to the kingdom Fungi and the phylum Basidiomycota since it produces sexual basidiospores, which are the reproductive units of the fungus. The fruit bodies (basidiocarps) contain the basidiospore-producing structures, the basidia. Each basidium carries four basidiospores, and each basidiospore in turn contains one haploid nucleus. Once basidiospores have been released and the conditions are favourable they germinate to form hypha, a threadlike fungal cell, further divided into hyphal compartments. The hypha is surrounded by a cell wall mainly composed of chitin and glucans (polymers of glucose) having a single nucleus in each compartment (monokaryon). When the hyphae of two different mating compatibility groups fuse with one another a dicaryotic hyphae (**Fig. 3**) are produced, containing two nuclei in each hyphal compartment (one from each compatibility group). The hyphae continue to grow by extension at the hyphal tip and branches repeatedly to form hyphal filaments in a complex network, mycelium, which is the vegetative part of the fungus. The mycelium of the fungus is responsible for the capturing of nutrients for growth and is often hidden underground or in the decaying organic material.

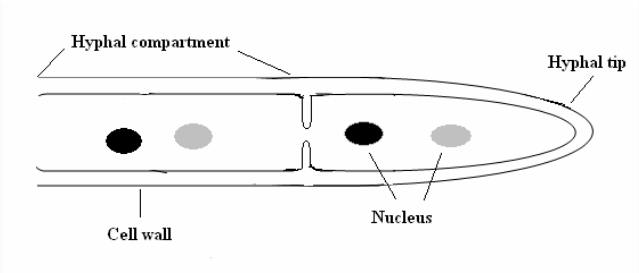

Figure 3. Dikaryotic fungal hypha.

When the surrounding conditions are right, mycelia starts to form a fruit body, completing one life-cycle of the mushroom. All the tissues of the fruit body are composed of dikaryotic hyphae, and within each basidium the two nuclei are fused to produce a diploid nucleus. The subsequent meiosis produces haploid nuclei, which then migrate into the developing basidiospores, one in each of the four basidiospores. The basidiospores reside in the basidium until release and the beginning of a new life cycle of the mushroom.

Shiitake as a medical mushroom

The shiitake mushroom is not only cultivated and consumed as food, it is also used for its medical properties. This mushroom has a long tradition as a medicinal mushroom in the Far East, where it has been used as a remedy for several complaints. In later decades the medical properties of the shiitake mushroom has been under investigation, bringing this mushroom into modern medicine. The most extensive research has been dedicated to the anti-tumour activity of shiitake. One of the agents responsible for the anti-tumour effect, and the most extensively studied, has been identified as a water-soluble polysaccharide denoted as lentinan (*16*). Lentinan exerts its anti-tumour effect by stimulating the immune system, and activating it to counteract tumour growth (*17, 18*). Due to its anti-tumour and immune-modulating properties, lentinan is used in

cancer and human immunodeficiency virus (HIV) therapy, primarily in Japan. Another anti-tumour active polysaccharide, KS-2, has been isolated from shiitake mycelia (LEM) (*19*). Further, extracts from culture media of shiitake mycelia have been found to contain anti-viral substances inhibiting HIV (*20*) and herpes simplex virus type 1 (HSV-1) (*21*) as well as immune-stimulating properties (*22*). Apart from the medical benefits, shiitake is a good source of vitamins and mineral elements (*23*).

Eritadenine

Apart from the previously mentioned medical properties, shiitake mushrooms have been shown to lower the blood cholesterol in both rats (*24-26*) and humans (*27*). Given 90g of fresh shiitake daily for one week, the serum cholesterol was lowered by 12% in the humans subjected to the experiment (*27*). The ability of shiitake mushrooms to lower blood cholesterol is ascribed to the compound designated eritadenine ($_{D}$-eritadenine) (**Fig. 4**). Eritadenine, 2(*R*),3(*R*)-dihydroxy-4-(9-adenyl)-butyric acid, was formerly designated as lentinacin (*28*) and lentysine (*29, 30*) by the research groups individually isolating and structurally determined this compound. Eritadenine is a secondary metabolite produced mainly by shiitake mushrooms.

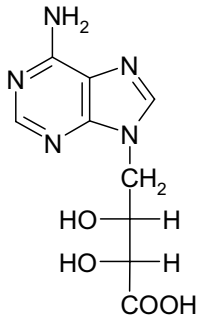

Figure 4. $_{D}$-Eritadenine

Upon isolation of the cholesterol reducing agent, it was supplied to rats in order to elucidate the effect on serum and liver cholesterol levels. A diet containing 0.005% eritadenine markedly decreased the serum cholesterol (*28, 29*) in rats. Further studies

have shown similar results (*31-35*), whereas no such studies on humans have been found in the literature.

The hypocholesterolemic action of eritadenine has been investigated in several studies on rats, however the exact mechanism by which eritadenine causes its hypocholesterolemic effect is not fully elucidated. Eritadenine is suggested to accelerate the removal of blood cholesterol either by stimulated tissue uptake or by inhibited tissue release; there are no indications of this compound inhibiting the biosynthesis of cholesterol (*36*) and the hepatic cholesterol levels in rats are not lowered by eritadenine (*29, 36*). Further, it has been suggested that the hypocholesterolemic action of eritadenine is due to a change in liver phospholipid metabolism; more exactly a decrease of the phosphatidylcholine (PC)/phosphatidylethanolamine (PE) ratio (*31-34, 37*) in rat liver cells. $_{D}$-Eritadenine is a very potent inhibitor of the enzyme *S*-adenosylhomocysteine (SAH) hydrolase (*38, 39*), hereby causing an increase in the SAH concentration (*40*). SAH is an inhibitor of different methyltransferases (*41*) and hence prevents the PE *N*-methylation and conversion of PE to PC, catalysed by PE *N*-methyltransferase (*42*). In accordance with this mechanism, the eritadenine induced increase in SAH concentration has been shown to inhibit PE *N*-methylation, thus increasing the PE content in rat liver microsomes (*37*). Further, studies on rats suggest that a change in the molecular species profile of phospholipids in liver cell membranes, induced by eritadenine, may increase the uptake of plasma lipoprotein cholesterol by the liver (*34*) or decrease the secretion of cholesterol from the liver (*37*), in either way the plasma cholesterol is reduced. There is also a possibility that the change in composition of the membrane phospholipids may activate lipoprotein receptors in liver cell membranes, thus regulating the uptake of plasma lipoprotein lipids (*31*).

The amounts of eritadenine in the fruit bodies of shiitake, as determined by column chromatography fractionation or GC, has been found to be in the range 0.5-0.7 and 0.3-0.4 mg/g dried caps and stems, respectively (*43, 44*). The mycelia of shiitake have also been found to contain eritadenine; the amount determined by GC analysis is 0.737 mg/g dried biomass (*45*).

Fungal biotechnology

Fungal biotechnology is not a new phenomenon as mankind has used fungi for their biochemical activities since the beginning of civilisation. The use of fungi for bread baking and alcohol production has a long history and probably the most well-known industrial use of fungi in modern time is the use of yeasts for brewing and for wine and bread making. In the later decades submerged (liquid) cultivation of fungi for production of commercially important products has increased. These products can be either primary or secondary metabolites produced by fungi. Primary metabolites are referred to as products involved in the growth, development and reproduction, whereas secondary metabolites are not. One of the most well-known groups of secondary metabolites from fungi is antibiotics, which inhibit the growth of microorganisms and function as fungal defence of territory. Many of the secondary metabolites however have no obvious role for the producing organism, yet they are produced in abundance and comprise a wide variety of compounds. Nevertheless, many of the secondary metabolites have been shown to be beneficial to mankind and therefore attracted a lot of attention for their commercial significance. The secondary metabolites are usually produced from common metabolic intermediates, but the production is often species- or strain- specific. The production is accomplished by special enzymatic pathways in the fungi, which usually takes place in the stationary phase when fungi are grown in culture.

Industrial applications

As previously mentioned, fungi produce many compounds which have been shown useful for mankind, and the production of valuable molecules by fungi has enormous potential in industry, medicine, agriculture, and basic science. The cultivation of filamentous fungi for the production of metabolites is diverse and of great economic importance and there is a great variety of industrially important fungal products such as

antibiotics, organic acids, enzymes, foods, and pharmacologically active products (**Table 1**). One of the major fungal biotechnology processes is the production of antibiotics. Penicillin was discovered in 1929 by Alexander Fleming as a metabolite of *Penicillium chrysogenum*, which inhibited growth of *Staphylococcus*. The mass production of antibiotics began during World War II and ever since, industrial-scale processes for production of antibiotics by fungi have been performed. The β-lactam antibiotics include several penicillins and are e.g. produced by fermentation of the filamentous fungus *Penicillium chrysogenum*. Improvements in the fermentation technologies and the productivity of the producer organisms have led to high recovery yields of the penicillins. However, in search for new antibiotics, many of the penicillins produced today are semi-synthetic, i.e. chemically modified natural penicillins (*46*). Another antibiotic, griseofulvin, was originally isolated from *Penicillium griseofulvum* (*47*) and is industrially produced from fermentations of the same fungal species.

Table 1. Industrially important fungal products.

Product	Source
Penicillins G and V	*Penicillium chrysogenum*
Griseofulvin	*Penicillium griseofulvum*
Citric acid	*Aspergillus niger*
Itaconic acid	*Aspergillus terreus*
Microbial protein (Quorn™)	*Fusarium venenatum*
Lovastatin	*Aspergillus terreus*
α-Amylase	*Aspergillus oryzae*
Ergot Alkaloids	*Claviceps purpurea*

The organic acid citric acid is produced by fermentation of *Aspergillus niger* (*48*). Citric acid is used as a constituent of soft drinks and other food products, as a preservative and flavour enhancer. Another organic acid, itaconic acid, is produced on a large scale by

fermenting *Aspergillus terreus*, and can be incorporated into polymers, thus having the potential of substituting petrochemical-based monomers (*49*). The cholesterol reducing agents, the statins, have been isolated from different fungi and developed into drugs. Lovastatin are produced industrially by cultivation of the microfungus *Aspergillus terreus* (*11*).

Quorn™ is a trademark of commercial fungal food products. The main constituent of such foods is mycoprotein produced from *Fusarium venenatum*. These products are protein-rich and serve as an alternative to animal protein sources (*50*). Industrially important starch degrading enzymes like α-amylase are also produced by fungi, e.g. by fermenting *Aspergillus oryzae* (*51*). Ergot alkaloids and their derivatives are secondary metabolites found in fungi of the plant parasitic genus *Claviceps*. This class of compounds is produced on a large scale by e.g. *Claviceps purpurea* and has a high variability of chemical structures. Their pharmacological effects pertain to their structural similarities to neurotransmitters such as dopamine and serotonin. Hence they have effects on neurotransmission and circulation and a wide field of therapeutic applications including migraine, parkinsonism and circulatory disturbances (*52*).

Submerged cultivations of filamentous fungi

Filamentous fungi are frequently used in industrial biotechnology processes, most of them belonging to the phylum Ascomycota. Fungal organisms have a complex metabolism, and the potential of their usage is huge, considering all the beneficial compounds they produce. Thus, on one hand it is possible to benefit from their complexity, on the other hand it has some drawbacks as their morphological complexity causes complications when culturing fungi in submerged condition. The morphology of filamentous fungi differs during different stages in their life-cycle and also with the nature of the growth medium and other chemical and physical factors in their environment. The morphology is also due to genetic factors and thus species or strain specific. Hence, depending on the organism used and the cultivation conditions, the mycelia can exhibit different morphologies in the medium. The morphology in

turn has influences on both the productivity of certain metabolites and on the properties of the growth medium. Influences on the growth medium in turn affect the transfer of e.g. nutrients and oxygen. When cultivating fungi in submerged conditions, there is a complex interrelationship between process parameters, morphology, broth properties and productivity (**Fig. 5**).

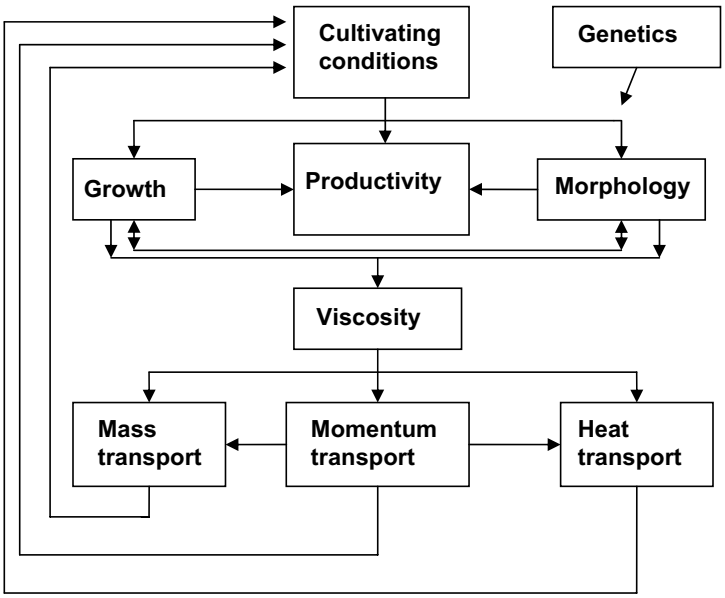

Figure 5. Interrelationships between morphology, growth, productivity, operating conditions, broth and transport properties. Adapted from Kossen (*53*).

Hyphal growth and morphological features

One of the unique features of fungi is their way of growing; hyphal tip (see **Fig. 3**) growth is characteristic for fungi. Filamentous fungi grow by apical extension of hyphae, showing a highly polarized growth which leads to a chemical and structural differentiation between the apical and distal regions of the hyphae. The mechanism and genetics behind hyphal morphogenesis, i.e. the development of a specific morphological form, is not fully elucidated. However, cell wall metabolism is considered central to fungal morphogenesis, but the process of wall synthesis at the

hyphal apex is yet not fully understood. There are different models or theories, trying to explain these events (*54, 55*).

The growth kinetics of filamentous fungi is not as straightforward as for unicellular organisms which reproduce by fission. From a practical point of view it is difficult to study growth kinetics of filamentous fungi in submerged culture. As these kinds of fungi attach to and grow on the walls of the reactor, on agitators and on probes, there is heterogeneity within the biomass. Further, the mechanism of hyphal growth tends to create heterogeneity within the hyphae themselves. For these reasons, growth kinetics of filamentous fungi is mainly based on the more well-known growth kinetics of unicellular organisms, such as some actinobacteria. Despite the differences between filamentous actinobacteria and filamentous fungi, the similar morphologies and growth mechanisms can be useful for studying growth in filamentous fungi (*56*).

As previously mentioned, filamentous fungi consist of hyphae, which are typically branched and form extended structures, mycelia. When grown in submerged culture there are principally two different forms which the fungal hypha can adopt; either dispersed mycelia throughout the medium or as macroscopic aggregates. The dispersed mycelia can range from freely dispersed linear filaments to more complex structures, clumps (**Fig. 6)**. The macroscopic aggregates, pellets, (**Fig. 6**) are more entangled masses of hyphae, which can vary in shape and size; some are loose irregular aggregates and some are more regular and dense spheres. Progress in the development of automatic image analysis systems has provided a valuable tool for a quantitative characterisation of complex mycelial morphologies of filamentous fungi. By examining electronic images, the morphological structures can be evaluated and measured, and their relationship to process parameters and productivity can be studied (*57, 58*). Models using image analysis methods have been developed for hyphal morphology and its relation to penicillin production in *Penicillium chrysogenum* (*59*).

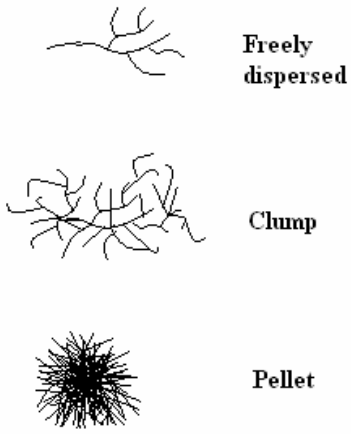

Figure 6. Morphological definitions of filamentous fungi in submerged cultivation. Adapted from Paul and Thomas (*58*).

Morphological influences on the growth medium

The nature of the mycelial morphology affects the physical properties of the broth in submerged cultivations. Freely dispersed mycelia tend to make the broth viscous and to behave in a non-Newtonian manner, i.e. no constant ratio between shearing stress and the shear rate (*60-62*). This non-Newtonian behaviour is due to interactions between the suspended filaments (*61, 62*). Thus, the higher the biomass concentration, the more potential interactions between the filaments and, therefore, increased broth viscosity. Further, the broth behaves differently in the vessel region with high shear as compared to more peripheral regions; in the regions with high shear breakdown of mycelial aggregates occur (*63*). In suspension where the mycelia are in pellet form, the viscosity is less because the discrete pellets exert less influence on the flow properties of the broth (*61*). If a reduction of broth viscosity is aimed at, culturing the fungus in a pellet form is preferable. On the other hand, some productivity can be reduced in this form. Phytase is an enzyme able to break down indigestible phytic acid in grains and thus release digestible phosphorus. It is used as an animal feed supplement, to enhance the nutritional value of plant materials. For the production of phytase from *Aspergillus niger*, filamentous growth of is preferable to large pellets (*64*).

In viscous fermentations different transport systems are affected (**Fig. 5**). Mass transfer at the liquid-solid interphase between cell surface and medium is a concern in cultivation of filamentous fungi; aggregated mycelia can be an obstacle for substrate transport and further enhanced by fluid viscosity (*65*). The main disadvantage with viscous broths is the risk of a heterogeneous system with nutrient gradients established, rather than a homogeneous suspension. In particular, the transfer of oxygen to active cells is crucial in aerobic fermentations and might be a limiting factor in viscous fluids. Normally, oxygen is sparingly soluble in the fermentation fluid and in viscous solutions the oxygen mass transfer is even less efficient (*61*). This can lead to depletion of dissolved oxygen in areas in the slower moving outer areas of the vessel (*66*). The oxygen requirement and its influence on production of lovastatin (*67*) and itaconic acid (*68*) in *Aspergillus terreus* has shown the importance of maintaining a high concentration of dissolved oxygen in the cultivation vessel.

Factors affecting growth, morphology and productivity

The growth and biomass yield of filamentous fungi in submerged culture are influenced by chemical and physical environmental factors, as is the production of metabolites. The nature of the inoculum has been shown important for growth of *Lentinus edodes* mycelia (*69*), and for griseofulvin production in *Penicillium griseofulvum* (*70*). Further, for mycelia of *Lentinus edodes*, a culture pH ranging from 3-7 (*71, 72*) has been reported as suitable for mycelial growth in liquid culture. On the other hand, when the growth optimum pH was found to be 3-3.5, the production of antibacterial substances had an optimum pH of 4.5 (*71*), demonstrating that optimal conditions for growth may not coincide with the optimal conditions for product formation. Similar results have been found in studies on *Aspergillus terreus* for lovastatin production, which demonstrated different temperature optima for growth and productivity (*73*). Agitation conditions have also been shown to affect both growth and productivity in penicillin production by *Penicillium chrysogenum* (*74*) and itaconic acid production in *Aspergillus terreus (75)*.

The composition of the culture medium is another important factor for growth and productivity. Production of α-Amylase from *Aspergillus oryzae* has been shown to depend on the medium glucose concentration, and the specific production was correlated to the specific growth rate of the fungus (*51*). The production of different ligninolytic enzymes in *Lentinus edodes* was shown to be dependent on nitrogen levels in the medium (*15*) and the choice of nitrogen source was important for lovastatin production from *Aspergillus terreus* (*76*). Further, at lovastatin production from *Aspergillus terreus*, higher nitrogen levels generated more biomass, while specific production was less (*67*). Another study demonstrating the influence of medium composition was the addition of a corn fiber extract with growth promotive effects on *Lentinus edodes* mycelia (*77*).

As the mycelial morphology affects the physical properties of the broth, the chemical nature of the broth and physical operating conditions have influences on morphology formation. Also, the physical properties of the reactor itself exert effects on mycelial morphology (*78*). The productivity of certain metabolites, i.e. cell metabolism, is in turn affected by the morphological nature of the mycelia. Agitation is important for proper mixing and mass and heat transfer in submerged fermentations; in aerobic fermentations, oxygen transfer is essential. The agitation is an important physical cultivation parameter influencing morphology and the great diversity of filamentous fungi leads to differences in the response to agitation rate. The influence of mechanical forces on the morphology of filamentous fungi has been the object of investigation in several studies. In penicillin production by *Penicillium chrysogenum* it was shown that both the hyphal length and penicillin production were affected by the agitation intensity and were both decreased at high agitation (*74, 79*). Citric acid production from *Aspergillus niger* was also shown to be dependent on agitation intensity, as was the morphology. Intensive agitation reduced the length of the filaments whereas the thickness increased and the productivity in turn was affected; the shorter the filaments the higher citric acid productivity (*80*). For the production of phytase by *Aspergillus niger*, higher agitation increased the free filamentous form and product formation (*81*).

Another factor influencing fungal morphology and productivity is the nature of the inoculum. Studies have shown that increasing an inoculum of 10^4 spores/mL by five orders of magnitude led to a clear transition from pelleted to dispersed mycelial forms of *Aspergillus niger* (*82*). The pH of the culture can also affect the morphology of the fungal mycelia and productivity, as have been shown in fermentations of *Aspergillus niger* and production of citric acid. Increasing the pH from 2.1 to 4.5 or decreasing it to 1.58 led to markedly decreased production of citric acid and changes in the morphology (*80*). The concentration of dissolved oxygen has also been shown to influence morphology and product formation, as in cultivation of *Aspergillus terreus* for lovastatin production; low levels of dissolved oxygen diminished product formation and pellet formation (*73*).

The composition of the culture medium has also been under investigation in several studies, revealing its effects on morphology and productivity. The growth form of *Aspergillus terreus* in relation to itaconic acid production has been studied, showing that mycelial pellets of different size and forms were obtained depending on the medium composition and the amount itaconic acid produced was correlated to the pellet form (*83*). Similar results have been shown for *Aspergillus terreus* in relation to lovastatin production (*84*). In *Aspergillus niger* the composition of the medium influenced morphology and in turn the production of phytase, for which filamentous mycelia and small pellets were preferable to large pellets (*64*). Further, studies on *Aspergillus niger* morphology and citric acid production showed the effect of the initial glucose concentration in the medium (*85*).

Most certainly are the changes in mycelial morphology due to many interacting factors and productivity in turn is affected. However, most of the work on submerged cultivations of filamentous fungi has been done on species of *Penicillium* and *Aspergillus* due to their economical and commercial significance. As the relationship between fungal metabolism and morphology and the operating conditions shows such versatility and complexity, few statements on the general behaviour of fungi in submerged cultivations can be made.

Present investigation

In light of the prevalence of cardiovascular disease and its correlation to high blood cholesterol levels, the present study was conducted in search for a potential natural medicine against elevated blood cholesterol levels. The shiitake mushroom (*Lentinus edodes*) contains a compound, eritadenine, which has been shown to possess hypocholesterolemic capacities and is the main focus in the present investigation. In order to find a suitable source of eritadenine and a sustainable process for its production the following approaches were applied in the present study:

- Development of a reliable analytical tool to quantify the amount of eritadenine in shiitake mushrooms
- Submerged cultivation of shiitake mycelia at different conditions and investigation of the influence of process parameters on the production of eritadenine

Paper I

The edible fungus, shiitake mushroom (*Lentinus edodes*) produces a cholesterol reducing compound designated eritadenine, 2(*R*),3(*R*)-dihydroxy-4-(9-adenyl)-butyric acid (*28*). Eritadenine does not inhibit cholesterol biosynthesis in the liver, but has the ability to enhance the removal of blood cholesterol, as have been shown in studies on rats (*36*). The mechanism of action of eritadenine is not fully elucidated, but this compound have been suggested to exert its effect by changing the liver phospholipid metabolism, and hereby causing either increased uptake or decreased release of cholesterol (*31-34, 37*). Further, a diet containing 0.005% eritadenine markedly decreased the serum cholesterol in rats (*28*). No studies elucidating the effects of eritadenine on humans have been found in the literature.

In order to establish dose-response effects of eritadenine on human objects and to find a potential source of this compound, the amount has to be accurately quantified. Further, to make the quantification as accurate as possible, the losses in the extraction procedure should be minimised and the amount released from the mushrooms maximised. In search for a potential source of eritadenine, the amounts of eritadenine in the fruit bodies of four different commercial shiitake mushrooms, Le-1, Le-2, Le-A and Le-B were investigated in this study.

To recover as much as possible of eritadenine from the fungal cells, the mushrooms were dried and crushed into fine particles before extraction with hot methanol. Following methanol extraction, the compound of interest was isolated. This was achieved by extraction with diethyl ether, ethanol precipitation and subsequent ion exchange purification. Since eritadenine is a zwitterion, the mushroom extract was in turn applied to a cation-exchange resin and an anion-exchange resin. The completely isolated eritadenine was then confirmed with LC/MS.

In order to analyse the target compound, a high performance liquid chromatography (HPLC) method was developed. To be able to quantify eritadenine with HPLC

analysis, reference samples were needed. Since eritadenine is not commercially available, it was synthesized according to a five-step procedure (*86-88*). To verify the correct product and its purity, NMR analysis was conducted for each step of the synthesis. An LC/MS run further confirmed the final product. A stock solution of the synthesized eritadenine was prepared by dissolving it in distilled water. The stock solution was diluted to obtain reference samples in the range 0.0124-0.198 mg/mL.

For separation, identification and quantification of eritadenine a reversed-phase HPLC method was developed. Since eritadenine absorbs strongly at 260 nm, this wavelength was used for detection of eritadenine. The extraction samples were separated over a C18 column and the application of a gradient elution system showed good resolution of eritadenine. The initial mobile phase was 0.05% TFA in aqueous solution:0.05% TFA in MeCN, in the proportions 98:2 followed by a linear change to 40:60 over 10 min. Triflouro acetic acid (TFA) was added to the mobile phase to improve peak shape and tailing. In order to quantify the amount of eritadenine in shiitake mushrooms a reference curve was constructed from synthesized eritadenine of different concentrations, on the basis of which eritadenine amounts were evaluated. In order to validate the reliability and reproducibility of the proposed method, the reference curve was obtained by triplicate measurements of five different concentrations of the standard. The linear response, r^2, was >0.999 and the relative standard deviation (RSD%) was <2.1%.

The eritadenine content in the shiitake mushrooms under investigation in the present study was in the range 3.2- 6.3 mg/g dried mushrooms, showing the importance of the source for high eritadenine content. The amounts of eritadenine in the fruit bodies of shiitake, as determined by column chromatography fractionation or GC, has been found to be in the range 0.5-0.7 and 0.3-0.4 mg/g dried caps and stems, respectively (*43, 44*). Thus, the amount of eritadenine found in the four different shiitake mushrooms investigated in the present study was up to ten times higher than previously reported for other shiitake strains. This difference can be due to either the extraction or the analytical procedure or to strain specific properties. Further, it was found that methanol extraction was reliable enough for HPLC quantification of eritadenine, i.e.

the peak resolution was acceptable without further isolation. From recovery studies of eritadenine the accuracy values were about 50% and hence, to minimise the losses and achieve an accurate quantification, complete isolation was omitted.

In an attempt to further increase the amount of eritadenine released from fungal cells, enzymes involved in the breakdown of bonds in the major polysaccharides in the fungal cell walls were used in this study. By pretreating the mushrooms with a mixture of hydrolytic enzymes with chitinase and glucanase activity before methanol extraction, it was evaluated if this could enhance the extraction. However, the enzyme pretreatment did not significantly increase the amount of eritadenine released. Most likely the recovery was maximised with methanol extraction, i.e. there was no more eritadenine to be released from the fungal cells.

In summary, this study clearly shows that the HPLC method developed is highly applicable for eritadenine analysis considering identification, separation and quantification of this compound.

Paper II

Both fruit bodies (*28, 29*) and mycelia (*45*) of shiitake (*Lentinus edodes*) have been shown to contain eritadenine. Cultivating shiitake mushroom fruit bodies, however, is fairly demanding and time consuming. Hence, another alternative might be to use shiitake mycelia as a potential source for eritadenine. There is a main advantage of growing mycelia in a controlled environment, as by submerged cultivation in bioreactors or shake flasks; it offers a convenient way of establishing the parameters important for growth and product formation.

Filamentous fungi industrially used for the production of several important compounds have been shown to respond in different ways to different chemical and physical culturing conditions, such as stirring rate, pH, temperature, inoculum, temperature and medium composition (*67, 75*). The morphology of filamentous fungi is multifaceted

and affected by the surrounding conditions, as is the production of metabolites (*73, 74, 82-84*). The response in terms of growth and productivity to environmental factor is as diverse as the different species of filamentous fungi, and there is a complex interrelationship between cultivation conditions, morphology and productivity when growing these fungi in submerged cultures. Since the reason for eritadenine production by shiitake mushrooms is unknown, varying the operation conditions during submerged cultivations of the mycelia can be used for establishing which cultivation conditions are favourable for eritadenine production. Two of the operating factors which have been shown to affect morphology and productivity in submerged cultures of filamentous fungi are pH and stirring rate (*74, 79-81, 89*). In light of this, the effects of pH and stirring rate on shiitake mycelial morphology and eritadenine production were elucidated in this study. This was accomplished by growing shiitake mycelia in both shake flasks and bioreactors, at various conditions.

Mycelia of the strain Le-2 were cultivated in malt yeast (MY) medium, composed of (w/v) 2% malt extract, 0.2% yeast extract and 2% glucose, for 20 days. The cultivation in shake flasks took place at 150 rpm and the pH was not controlled. In the bioreactors the cultivation took place at either 50 or 250 rpm and a pH either controlled at 5.7 or uncontrolled. Following cultivation, the mycelia were harvested and the dry cell weight (DCW) determined. The mycelial biomass was then extracted with hot methanol. The culture broths were purified by application to a cation-exchange resin followed by application to an anion-exchange resin. The mycelia and culture broths were then analysed by HPLC as previously described (*90*).

When conducting submerged cultivation of shiitake, eritadenine was found in both the mycelium and the culture broth, with the major part, 90-99%, in the broth. Shiitake mycelia have been cultivated and analysed for its eritadenine content in previous investigations, and the amount as determined by GC was found to be 0.737 mg/g DCW (*45*). However, no data from studies investigating the culture medium has been found in the literature. Excretion of eritadenine into the medium might in turn facilitate downstream processing, making a great advantage if this compound is to be produced on a large scale.

The mycelia in shake flasks were not exposed to shear and grew as macroscopic aggregates, pellets, whereas the mycelia in bioreactors appeared as dispersed filaments. The biomass yield in shake flasks and bioreactors was in parity; 0.45 g in the shake flasks and 0.25-0.62 g in the bioreactors. However, the eritadenine production was significantly higher in the bioreactors as compared to the shake flasks; a specific productivity in the bioreactors ranging between 26.00 and 39.58 mg eritadenine/g DCW as compared to 6.56 mg/g DCW in the shake flasks. This indicates the influence of morphology on eritadenine production.

The optimal stirring rate was 50 rpm for specific productivity (39.58 mg eritadenine/g DCW) and 250 rpm for mycelial biomass yield (0.62 g). The pH did not have any major impact on growth, whereas the specific productivity in the bioreactors was higher when pH was uncontrolled than controlled at 5.7. In the shake flasks, the final pH was 3.0, whereas in the bioreactors at 250 and 50 rpm and uncontrolled pH the final pH was 4.2 and 5.0, respectively. These results demonstrate the differences in fungal cell metabolism as a response to cultivation conditions, and that optimal conditions for growth differ from those favouring eritadenine production.

Conclusions

In paper I the amounts of the cholesterol reducing compound eritadenine in fruit bodies of four different shiitake mushrooms were determined. The HPLC method developed showed to be highly applicable for eritadenine analysis. The amounts found in this study were about 10 times higher than the amounts reported in previous studies for other strains. Further, the use of cell wall degrading enzymes did not significantly increase the eritadenine amount released from fungal cells.

In paper II the mycelia of shiitake mushrooms were cultivated in shake flasks and bioreactors, and the production of eritadenine analysed. It was found that both the mycelia and the culture medium contained eritadenine, and the major part was excreted to the culture medium. Further, the cultivation were conducted at different conditions, in order to investigate the effect of pH and stirring rate on eritadenine production and its relation to mycelial morphology. It was found that the morphology in shake flasks were macroscopic aggregates and in bioreactors as dispersed filaments throughout the medium. Further, the specific productivity in shake flasks was significantly lower than in the bioreactors and both pH and stirring rate affected eritadenine production. It was also shown that optimal conditions for mycelial growth and eritadenine production did not coincide.

The developed method for eritadenine quantification and the results from its production by submerged cultivation of mycelia are all prerequisites for subsequent clinical trials and large scale cultivation. In summary, the obtained results open the path for further exploitation of eritadenine as a new cholesterol reducing product.

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Paper I

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Quantification of the Bioactive Compound Eritadenine in Selected Strains of Shiitake Mushroom (*Lentinus edodes*)

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Cardiovascular disease is one of the most common causes of death in the Western world, and a high level of blood cholesterol is considered a risk factor. The edible fungus, shiitake mushroom (*Lentinus edodes*), contains the hypocholesterolemic agent eritadenine, 2(*R*),3(*R*)-dihydroxy-4-(9-adenyl)-butyric acid. This study was conducted to quantify the amount of the cholesterol reducing agent eritadenine in shiitake mushrooms, in search of a potential natural medicine against blood cholesterol. The amounts of eritadenine in the fruit bodies of four different shiitake mushrooms, Le-1, Le-2, Le-A, and Le-B, were investigated in this study. To achieve this goal, methanol extraction was used to recover as much as possible of the hypocholesterolemic agent from the fungal cells. In addition, enzymes that degrade the fungal cell walls were also used to elucidate if the extraction could be further enhanced. To analyze the target compound, a reliable and reproducible HPLC method for separation, identification, and quantification of eritadenine was developed. The shiitake strains under investigation exhibit up to 10 times higher levels of eritadenine than previously reported for other shiitake strains. Further, pretreating the mushrooms with hydrolytic enzymes before methanol extraction resulted in an insignificant increase in the amount of eritadenine released. These results indicate the potential for delivery of therapeutic amounts of eritadenine from the ingestion of extracts or dried concentrates of shiitake mushroom strains.

KEYWORDS: Eritadenine; *Lentinus edodes*; bioactive compounds; HPLC

INTRODUCTION

The shiitake mushroom is widely cultivated and consumed not only as food but also as a natural medicine because of its medical properties. This mushroom has a high nutritional value and contains several substances with additional positive effects on health, such as the anti-tumor agent lentinan (*1*). One of the health benefits, which this mushroom possesses, is the ability to reduce blood cholesterol as shown in both animal and human studies (*2*, *3*). The cholesterol reducing agent in shiitake mushrooms is a purine alkaloid (**Figure 1**) designated as eritadenine (lentinacin), 2(*R*),3(*R*)-dihydroxy-4-(9-adenyl)-butyric acid (*4*).

Competitive inhibitors of HMG-CoA reductase, the statins, are produced in a large scale as cholesterol reducing pharmaceuticals. Unlike the statins, eritadenine does not inhibit the biosynthesis of cholesterol in the liver but enhances removal of blood cholesterol (*5*). The exact mechanism by which eritadenine elicits its hypocholesterolemic action is not yet fully understood. However, the hypocholesterolemic action of eritadenine has been investigated in several studies on rats. It has been shown that total plasma cholesterol levels are decreased in rats fed eritadenine in their diets and that the hypocholesterolemic action is caused by a decrease of the phosphatidylcholine (PC)/phophatidylethanolamine (PE) ratio (*6*−*10*). Eritadenine is a very potent inhibitor of the enzyme *S*-adenosyl-L-homocysteine hydrolase in rat liver cells (*11*), thereby causing an increase in the *S*-adenosylhomocysteine concentration (*12*). The increase in the *S*-adenosylhomocysteine concentration in turn inhibits the PE N-methylation, thus increasing the PE content in liver microsomes (*9*). Further studies on rats suggest that eritadenine may increase the uptake of plasma lipoprotein cholesterol by the liver and thus reduce the plasma cholesterol

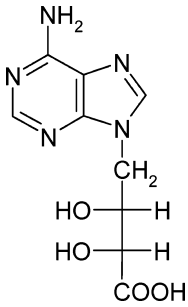

Figure 1. Chemical structure of eritadenine (M_v = 253 g/mol).

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(*10*). There is a possibility that the change in composition of the membrane phospholipids may activate lipoprotein receptors in liver cell membranes, thus regulating the uptake of plasma lipoprotein lipids (*6*).

The amounts of eritadenine in shiitake mushrooms quantified so far are in the range of 50−70 mg/100 g dry weight (dw) in the caps, 30−40 mg/100 g in the stems (*13*, *14*), and 73.7 mg/100 g in shiitake mycelium (*15*). In rats, a diet containing 0.005% eritadenine was shown to lower the serum total cholesterol level with 25% (*4*). Similar studies on humans have not been found in the literature. Previous work indicates the efficacy of shiitake mushrooms in cholesterol reduction; however, the concentrations reported indicate that large quantities may have to be consumed to achieve therapeutic effects (*3*). To establish the dose−response effect of shiitake mushrooms as a cholesterol reducing product, strains producing considerable amounts of eritadenine are favorable, as is a reliable analytical procedure for this substance. To achieve an accurate quantification of eritadenine, losses in the extraction procedure should be minimized and the amount released from the mushrooms maximized.

The goal of the present work was to evaluate the eritadenine content of four commercially cultivated shiitake mushrooms, Le-1, Le-2, Le-A, and Le-B, to determine if they could be used as a viable source of eritadenine, either as a dried product or as an extract. To achieve this objective, a reliable analytical HPLC procedure quantifying eritadenine in shiitake mushrooms was developed.

In an attempt to further increase the amount of eritadenine released, enzymes involved in the breaking of bonds between the polymers in fungal cell walls were used in this study. The cell walls of shiitake mushrooms are mainly composed of the polysaccharides chitin (β-1,4-*N*-acetylglucosamine) and glucans (β-1,3 and β-1,6), which can be degraded by hydrolytic enzymes possessing chitinase or glucanase activity (*16*). Hence, enzymes with these properties were used to enhance the extraction procedure by macerating the fungal cells.

MATERIALS AND METHODS

Fungal Material. Four different commercial shiitake mushrooms were used in this study. The fruit bodies of the *Lentinus edodes*-1 (Le-1) and *Lentinus edodes*-2 (Le-2) strains were kindly supplied by Dr. Gary L. Mills, Diversified Natural Products, Inc., Scottville, MI. The fruit bodies of the two other shiitake mushrooms were bought at local stores and denoted here as *Lentinus edodes*-A (Le-A) and *Lentinus edodes*-B (Le-B). The supplier of Le-A was Limax, Horst, The Netherlands, and the supplier of Le-B was Mykora Oy, Kiukainen, Finland. The fruit bodies were dried in a mushroom dryer. To eliminate individual differences among the mushrooms, dried fruit bodies were subsequently homogenized in a blender, making one batch of 50 g of homogeneous mushroom powder for each strain. The extraction procedures were all repeated 3 times, using 3 g of the homogeneous batches for each extraction.

Preparation of Eritadenine Standard. Since eritadenine is not commercially available, it was synthesized. In the first step, methyl 2,3-*O*-isopropylidene-β-D-ribofuranoside was synthesized (*18*). This product was further processed to give the compound methyl 2,3-*O*-isopropylidene-5-*O*-*p*-toluenesulfonyl-β-D-ribofuranoside (*19*). The third step was a reaction of sodium salt of adenine with methyl 2,3-*O*-isopropylidene-5-*O*-*p*-toluenesulfonyl-β-D-ribofuranoside. This reaction gave the product methyl 5-(6-aminopurin-9*H*-9-yl)-2,3-*O*-isopropylidene-5-deoxy-β-D-ribofuranoside. Hydrolysis of this product resulted in 5-(6-aminopurin-9*H*-9-yl)-5-deoxy-D-ribofuranose. The final step was an air oxidation of the previous compound to obtain the product 2(*R*),3(*R*)-dihydroxy-4-(9-adenyl)-butyric acid (i.e., D-eritadenine (*20*)). All chemicals were of analytical grade. To verify the correct product and its purity, NMR analysis was conducted for each step of the synthesis and compared with the literature. An LC/MS run further confirmed the final product. A stock solution (1.98 mg/mL) of the standard was prepared by dissolving synthesized eritadenine in distilled water.

Methanol Extraction and Isolation of Eritadenine. The extraction was a modified version of the method developed by Tokita et al. (*17*). Powder from dried fruit bodies was weighed and extracted with 80% (v/v) methanol for about 3 h under reflux, with a solid−liquid ratio of 1:20. The extract was filtered through Whatman No. 5 filter paper, evaporated to dryness in vacuo at 50−60 °C, and diluted in 50 mL distilled water. The sample was further extracted with 50 mL of diethyl ether 3 times. To the aqueous layer, 4 volumes of 99.5% ethanol was added and incubated at −20 °C overnight. The precipitate was removed by filtration through Whatman OOH filter paper, and the filtrate was evaporated to dryness in vacuo at 50−60 °C. The extract was diluted in 50 mL of distilled water and applied to a column of Amberlite IR-120 (H^+) ion-exchange resin. The substance was eluted with 2% ammonia, showing a high absorbance at 260 nm. The volume collected was evaporated to dryness in vacuo at 50−60 °C, diluted in 50 mL of distilled water, and applied to an Amberlite IRA-67 (OH^-) ion-exchange resin. The substance was eluted with 0.1 M acetic acid, and fractions showing high absorbance at 260 nm were collected. After evaporation to dryness in vacuo at 50−60 °C, the mushroom sample was dissolved in 100 mL of distilled water. The completely isolated eritadenine was confirmed with LC/MS.

Enzymatic Pretreatment. The enzyme preparation, NS 33075, was kindly supplied by Novozymes, Bagsvaerd, Denmark. NS 33075 is a multicomponent carbohydrase preparation originating from *Trichoderma harzianum*. The main components of this enzyme mixture are various α- and β-glucanases, but it also contains some side activities like chitinases and proteases. According to the supplier, this enzyme mixture shows a fairly high activity at pH 4.8 and 50 °C. The enzyme powder was dissolved in water to a stock solution of 2% (w/v). A volume of the stock solution corresponding to 0.2 mg of enzyme per gram of mushrooms was added to either distilled water (pH 6.0) or 0.1 M acetate buffer (pH 4.8). The volume of the water and the buffer used was 10 mL per gram of mushrooms. The enzyme−water or enzyme−buffer mixture was added to the weighed mushroom powder, and the reaction was incubated at 50 °C for 3 h with gentle stirring. Following the enzymatic treatment, a methanol extraction was preformed as previously described.

HPLC Analysis. The eritadenine concentration in shiitake fruit bodies was analyzed by HPLC (Series 200 Quaternary LC pump and UV−vis detector, TotalChrom software, Perkin-Elmer, Wellesley, MA) and separated over a C18 column (RESTEK Ultra Aqueous, 5 μm, 150 mm × 4.6 mm). Prior to analysis, the samples from the extractions were diluted twice with the initial mobile phase and filtered through a 0.2 μm syringe filter. The HPLC analysis was conducted at 23 °C, with a flow rate of 1 mL/min and UV detection at 260 nm. The initial mobile phase was 0.05% TFA in aqueous solution/0.05% TFA in MeCN, in the proportions of 98:2 followed by a linear change to 40:60 over 10 min, and then returned to the initial condition for 15 min. All data were collected and processed using Perkin-Elmer's TotalChrom analytical software. Peak areas from the chromatograms were evaluated on the basis of a reference curve prepared from standard samples of eritadenine diluted in the initial mobile phase to concentrations in the range of 0.0124−0.198 mg/mL.

RESULTS AND DISCUSSION

The usage of shiitake mushroom extracts as food additives against blood cholesterol may have some benefits. Since the active substance eritadenine is water soluble, no excessive fat has to be ingested as is the case with phytosterols used for the same purpose. Eritadenine consumed in combination with cholesterol reducing statins might reduce severe side effects since the mechanism of action for eritadenine differs from the corresponding one for statins. Using shiitake mushroom as a cholesterol reducing natural medicine requires a reliable method

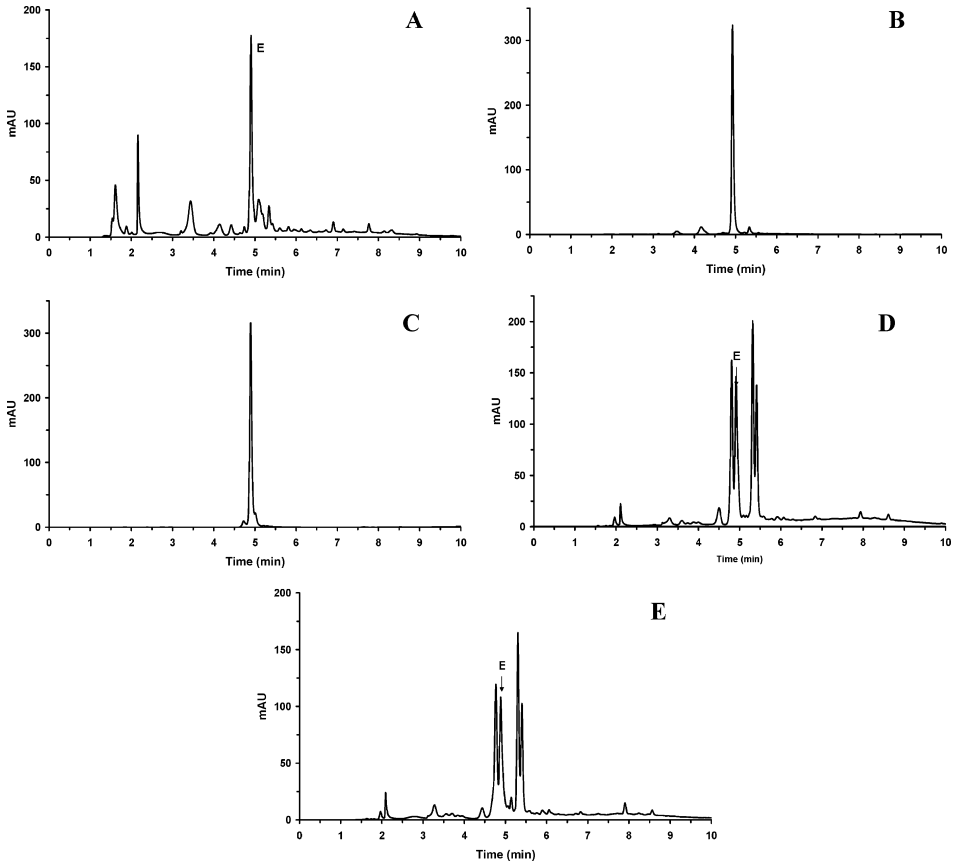

Figure 2. HPLC chromatograms at 260 nm of a methanol extract from shiitake mushrooms (**A**), completely isolated eritadenine from shiitake mushrooms (**B**), synthesized eritadenine (**C**), methanol extract from shiitake mushrooms treated with hydrolytic enzymes in buffer (pH 4.8) (**D**), and methanol extract from shiitake mushrooms treated with hydrolytic enzymes in water (pH 6.0) (**E**). Eritadenine is eluted at a retention time of 4.9 min and is represented by E in the chromatograms of methanol extracts.

of quantifying eritadenine amounts and careful dose−response studies on humans.

To make the quantification of eritadenine as accurate as possible, the losses from the extraction procedure should be minimized. By comparing the HPLC chromatogram from simple methanol extraction (**Figure 2A**) with the HPLC chromatogram of isolated eritadenine (**Figure 2B**), it is clearly shown that methanol extraction, without further purification, is reliable enough for quantification. Further, the chromatogram of the synthesized standard (**Figure 2C**) coincides with the chromatogram of isolated eritadenine (**Figure 2B**). The chromatogram resulting from methanol extraction preceded by enzymatic hydrolysis in either buffer (**Figure 2D**) or water (**Figure 2E**) is not as clean as that from pure methanol extraction but acceptable for quantification. From recovery studies of isolated eritadenine, the accuracy values were about 50%. Since the chromatographic separation was acceptable, methanol extracts were used for quantification, and further isolations were omitted. Samples from the extraction procedures stored in a refrigerator were stable for at least 1 week.

The HPLC analyses of eritadenine content in the fruit bodies of the four different shiitake mushrooms (**Table 1**) used in this study show a statistically significant difference between Le-B and the other shiitake varieties ($p < 0.05$). Each extraction was repeated 3 times, and the spread of the measurements by means of standard deviations was within a reasonable range. There were no statistically significant differences between Le-1, Le-2, and Le-A ($p > 0.05$). These results indicate the importance of the source for high eritadenine content, which can be due to both strain specific properties and cultivation conditions.

Table 1. Eritadenine Content Measured in the Fruit Bodies of Four Different Shiitake Mushrooms from Various Treatments by HPLC Analysis

	eritadenine content (mg/g mushrooms (dw))[a]			
treatment	Le-1	Le-2	Le-A	Le-B
methanol extraction	3.50 ± 0.26	3.17 ± 0.07	3.24 ± 0.27	6.33 ± 0.03
enzymatic pretreatment in acetate buffer pH 4.8	3.82 ± 0.30	NA[b]	NA	NA
enzymatic pretreatment in water pH 6.0	3.60 ± 0.11	NA	NA	NA

[a] All values are mean ± SD from triplicate analyses. [b] NA: not analyzed.

Methanol extraction preceded by enzyme hydrolysis in acetate buffer (pH 4.8) gave the highest amounts of eritadenine for Le-1, followed by enzymatic hydrolysis in water (pH 6.0) with a slightly lower yield of eritadenine, which is reasonable since the pH for the reaction was not optimized in this case. The results indicate that methanol extraction preceded by enzyme hydrolysis may, to some extent, improve the extraction of eritadenine from shiitake mushrooms. However, the difference between methanol extraction preceded by enzymatic hydrolysis in either buffer or water and pure methanol extraction was not statistically significant ($p > 0.05$), and hence, the efficiency of pretreating the mushrooms with cell wall degrading enzymes can be considered unimportant. There is also a possibility that a maximum yield was reached in this case (i.e., there is no more eritadenine to be released from the fungal cells).

In comparison to other studies (*13*, *14*), the amounts of eritadenine in shiitake mushrooms are significantly higher in this study, up to 10 times. There is no information in the previous studies on what specific strains were used, and the eritadenine content might be strain dependent. Another factor that might contribute to the fairly high difference between the amount of eritadenine found in the present study and previous ones is the extraction procedure. In all cases, quantification was preceded by methanol extraction, but there is either no information on how the extraction procedure was performed (*14*) or the temperature, time, and solid−liquid ratio obviously differ from previous studies (*13*). Also, in this study, the mushrooms were thoroughly crushed into fine particles to make a homogeneous fungal material, highly accessible for the subsequent extraction procedure. Finally, the analytical procedures for quantification differ between the studies. The amount of eritadenine has been determined by column chromatographic fractionation without any reference samples (*14*) or by GC (*13*). No data have been found in the literature pertaining to HPLC quantification of eritadenine. Since eritadenine is a nonvolatile compound, it has to undergo derivatization prior to GC analysis; no such modification has to be done to the target compound for HPLC analysis.

To validate the reliability of the proposed HPLC method, a reference curve was obtained by triplicate measurements of five different concentration levels in the range of 0.0124−0.1980 mg/mL. This method showed a linear response, r^2, of >0.999 and a degree of reproducibility expressed as a relative standard deviation (RSD%) of <2.1%. Furthermore, the retention peak obtained for eritadenine in this study indicates a high column efficiency, signifying sufficient resolution for quantification.

In this study, it is clearly shown that HPLC analysis of eritadenine is highly applicable, and it offers a simple and sensitive method for separation, identification, and quantification of this compound.

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Paper II

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

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Abstract

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 search for a source of eritadenine, shiitake mycelia were investigated in the present study. The mycelia were cultivated both in shake flasks and in bioreactors, to investigate the effect of pH and stirring rate on the production of eritadenine. Both the biomass and the culture broth were examined for its eritadenine content. The shiitake mycelia were found to produce eritadenine and the compound of interest was found in relatively low levels in the fungal cells, whereas the major part, 90-99%, was detected in the growth media. In the shake flasks the total specific productivity of eritadenine was 6.56 mg/g dry cell weight (DCW). In the bioreactor, a stirring rate of 50 rpm and an uncontrolled pH resulted in the highest total specific productivity of eritadenine; 39.58 mg/g DCW. When pH was controlled at 5.7, the productivity was slightly less at 50 rpm; 33.83 mg/g DCW. The biomass yield at 50 rpm was not affected by pH; 0.30 g DCW at pH 5.7, and 0.25 g DCW at an uncontrolled pH. Applying a stirring rate of 250 rpm in the bioreactor resulted in increased biomass yield irrespectively of pH; 0.62 g DCW at pH 5.7, and 0.59 g DCW at an uncontrolled pH. A stirring rate of 250 rpm at pH 5.7 gave a specific productivity of 26.00 mg eritadenine/g DCW, which increased to 32.61 when pH was not controlled. The mycelial morphology in the shake flask cultures was macroscopic aggregates, whereas in the bioreactors mycelia grew as freely dispersed filaments. Taking into consideration the differences in eritadenine concentrations, these results point out the relationship between cultivation conditions, morphology and production

of secondary metabolites. The results also indicate that a low stirring rate in the bioreactors promotes eritadenine production, whereas a higher stirring rate favours biomass growth. Further, the results indicate that pH has some influence on eritadenine production, but less effect on mycelial biomass production. The highest specific productivity was achieved at 50 rpm and uncontrolled pH (39.58 mg/g DCW), whereas the highest total amount of eritadenine was achieved when cultivation was conducted at 250 rpm at an uncontrolled pH, resulting in 19.24 mg.

Keywords: Eritadenine, *Lentinus edodes*, submerged cultivation, bioactive compounds

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Introduction

The shiitake mushroom (*Lentinus edodes*) is traditionally used in East Asia, but ever since the last decades it is cultivated and consumed worldwide. In addition to being a popular edible fungus, it is well established as a medicinal mushroom since it contains several substances promoting health. Among other things, the ability to reduce blood cholesterol in both animals and humans has been ascribed to this mushroom (*1-4*). The agent responsible for the plasma cholesterol reducing effect of shiitake is a secondary metabolite designated as eritadenine* (**Fig. 1**), 2(*R*),3(*R*)-dihydroxy-4-(9-adenyl)-butyric acid (*5, 6*).

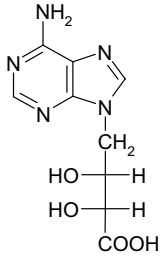

Figure 1. Eritadenine (M_v= 253 g/mol)

* Eritadenine was designated as lentinacin or lentysine by the research groups initially isolating it, before given its trivial name.

The hypocholesterolemic action of eritadenine has been investigated in several studies on rats (*5-14*), but the mechanism by which eritadenine bring about its hypocholesterolemic effect is not fully elucidated. Eritadenine is suggested to accelerate the removal of blood cholesterol either by stimulated tissue uptake or by inhibited tissue release; there are no indications of this compound inhibiting the biosynthesis of cholesterol (*13*) and the hepatic cholesterol levels in rats are not lowered by eritadenine (*6, 13*). Further, it has been shown that plasma cholesterol levels are significantly decreased in rats fed 0.005% eritadenine in their diets (*5-9, 11, 12*) and that eritadenine exerts its hypocholesterolemic action by altering the hepatic phospholipid metabolism (*7-11*).

The amounts of eritadenine in fruit bodies of shiitake, as determined by column chromatography fractionation or GC, have been found to be in the range 0.5-0.7 and 0.3-0.4 mg/g dried caps and stems, respectively (*15, 16*). Later studies pertaining to HPLC analysis of different fruit bodies of shiitake have shown eritadenine amounts in the range 3.17-6.33 mg/g dry mushrooms (*17*). This difference could be due to dissimilarities in the extraction and/or analytical procedure as well as the strains used. The mycelia of shiitake have also been found to contain eritadenine; the amount determined by GC analysis is 0.737 mg/g dried biomass (*18*). Hence, both fruit bodies and mycelia of shiitake contain eritadenine. Growing fruit bodies of shiitake is a fairly demanding and time consuming process and in search for a source of eritadenine, cultivation of mycelia could be an alternative.

The use of fungi for their biochemical activities is not a new event and in the later decades submerged cultivation of filamentous fungi for production of commercially important products has increased. The cholesterol reducing compound lovastatin is industrially produced by *Aspergillus terreus*, the antibiotic penicillin by *Penicillium Chrysogenum* and citric acid by *Aspergillus niger*, to mention a few. Filamentous fungi, like shiitake, are morphologically multifaceted organisms and exhibit different hyphal morphologies in submerged culture; it can range from freely dispersed linear filaments to densely entangled aggregates. The morphology of filamentous fungi in liquid culture depends on the specific organism and the chemical and physical culturing conditions, such as medium composition, temperature, pH, inoculum concentration, dissolved oxygen and shear. Several studies have been conducted showing that formation of fungal metabolites is greatly dependent on the culturing conditions (*19, 20*) as is the hyphal morphology, which in turn affects the production of a certain metabolite (*21-25*). The results from these previous studies imply that

there is no generally preferred mycelial structure; which morphology is desirable for maximal yield depends on the organism and product in question and there is a multifaceted relationship between culture conditions, morphology and productivity.

The reason why shiitake mushrooms synthesize eritadenine is yet not clarified; i.e. the purpose this compound serves for the mushroom as well as the circumstances for its production is not elucidated. Therefore, it is of great interest to investigate shiitake mycelia for eritadenine production; submerged cultivation of mycelia offers a convenient way to change the conditions in order to improve eritadenine yield and productivity. Hence, stirring rate and pH, two factors influencing hyphal morphology (*22, 26-29*) and probably eritadenine production, were investigated in the present study.

The goal of the present work was to evaluate if submerged cultivation of shiitake mycelia could be a conceivable way of producing eritadenine. The mycelia were cultivated under different conditions and both the biomass and culture broth were investigated for its eritadenine content.

Material and methods

Fungal material. The shiitake strain used was *Lentinus edodes*-2 (Le-2). Mycelia of this strain were kindly supplied by Dr. Gary L. Mills, Diversified Natural Products, Inc., Scottville, Michigan, USA. The mycelia were cultivated on malt yeast agar (MYA) plates composed of (w/v) 2% malt extract, 0.2% yeast extract and 2% microbial agar, for 10 days at 23 °C.

Shake flask cultivation. Mycelia were cut from half 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 (w/v) 2% malt extract and 0.2% yeast extract, with a 2% sterilized glucose solution added separately. The submerged cultivation took place in 500 mL shake flasks at 150 rpm for 20 days at 23 °C.

Bioreactor cultivation. Mycelia from two MYA plates (90 mm in diameter) were cut out and homogenized in a 0.05 mM phosphate buffer, pH 5.8, and aseptically transferred to 700 mL sterilized malt yeast medium composed of (w/v) 2% malt extract, 0.2% yeast extract, with a

2% sterilized glucose solution added separately. The submerged cultivation took place in 1 L bioreactors (Biobundle 1 L, Applikon Biotechnology, the Netherlands) with a stirring rate of either 50 or 250 rpm, a temperature of 25 °C, a dissolved oxygen flow rate of 1vvm, and a pH either controlled at 5.7 by automatic addition of 5M KOH or uncontrolled, for 20 days. The dissolved oxygen concentration (%DO), was measured by a pO_2 electrode and the pH was measured with a pH electrode.

Extraction of eritadenine from mycelia. Following cultivation, the mycelia from shake flasks and bioreactors were harvested by filtering the culture through Whatman OOH filter paper and a subsequent wash with distilled water. The biomass was dried over night and the DCW determined. The filtrated broth was collected for further analysis. The mycelial biomass was extracted with 80% (v/v) methanol for about 3 hours under reflux, with a solid-liquid ratio of 1:20. The methanol extract was then filtered through Whatman No. 5 filter paper and washed with distilled water. The resulting filtrate was concentrated in vacuo at 50-60 °C and analysed.

Ion exchange purification of culture medium. The broth was concentrated in vacuo at 50-60 °C and the pH adjusted to 5.8 and applied to a column of Amberlite IR-120 (H^+) ion exchange resin. The substance was eluted with 2% ammonia, and fractions showing high absorbance at 260 nm were collected. The accumulated volume was evaporated to dryness in vacuo at 50-60 °C, diluted in 50 mL distilled water and applied to an Amberlite IRA-67 (OH^-) ion exchange resin. The substance was eluted with 0.1 M acetic acid and fractions showing high absorbance at 260 nm were collected. After evaporation to dryness in vacuo at 50-60 °C the resin was dissolved in distilled water and analysed.

HPLC analysis. The eritadenine concentrations in shiitake mycelia and culture broth were analysed by HPLC as previously described (*17*), using synthesized eritadenine as a reference.

Results and discussion

In search for a potential source of the blood cholesterol lowering compound eritadenine, shiitake mycelia were investigated in this study. The incentives for these mushrooms to produce eritadenine are not yet clear; i.e. the function of this secondary metabolite and the

growth conditions that favour its production are not known. Submerged cultivation of mycelia offers a convenient way to control the operating conditions in order to identify important factors affecting eritadenine yield and productivity. Filamentous fungi, like shiitake, exhibit different hyphal morphologies in submerged cultures, depending on the cultivation conditions. Previous studies have shown that culturing conditions, such as pH (*27, 28*) and stirring rate (*22, 26, 29*) affects the morphology of filamentous fungi and in turn the production of fungal metabolites. With reference to these previous findings, the mycelia in the present study were cultivated at different conditions to investigate the effect of pH and stirring rate on production of eritadenine.

Previous studies (*18*) have investigated the mycelia from submerged cultivation for their eritadenine content. In the present study not only the mycelia, but also the culture broths were analysed for eritadenine and the compound of interest was found both in the mycelia (**Fig. 2A**) and the culture broths (**Fig. 2B**), in various amounts (**Table 1**). The main part of eritadenine was found in the culture medium (**Table 1**). Until now, no data from studies investing the broth from liquid cultures of shiitake mycelia for eritadenine content have been found in the literature. The observation that certain growth conditions favour excretion of eritadenine to the growth medium might have a profound effect on large scale production of the cholesterol reducing compound, where an increased availability of eritadenine in the liquid phase might facilitate subsequent downstream processing.

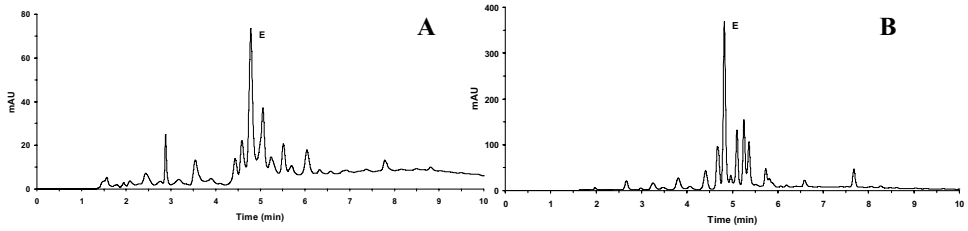

Figure 2. HPLC chromatograms of a methanol extract from shiitake mycelia (**A**) and the culture broth (**B**) from submerged cultivation of shiitake mycelia. Eritadenine is eluted at a retention time of 4.8 and is represented by E in the chromatograms.

The lowest specific productivity of eritadenine was detected in the shake flask cultures; 6.56 mg/g DCW, of which 90% was found in the broth. In this case there was no shear and the

mycelia formed macroscopic aggregates (**Fig. 3A**). The mycelial morphology in the bioreactor cultivations was as freely dispersed filaments (**Fig. 3B**), and the eritadenine content higher than in the shake flasks, showing that the mycelial morphology has an important influence on the production of eritadenine. When investigating the complex morphology of filamentous fungi and the effects on productivity, image analysis methods are a very useful tool (*30*). However, in this study, the morphological distinction was based on the pellet structures found in the shake flasks and the dispersed filaments found in the bioreactors; a more detailed study on the mycelial morphology was not conducted. Applying a stirring rate of 50 rpm and no pH control resulted in the highest specific productivity of eritadenine; 39.58 mg/g DCW, of which 99% was found in the broth. At the same stirring rate (50 rpm), but maintaining a pH of 5.7, 33.83 mg/g DCW eritadenine was detected, of which 99% was in the broth. A stirring rate of 250 rpm and either no pH control or a pH controlled at 5.7, resulted in 32.61 and 26.00 mg eritadenine/g DCW, respectively. In these cases, 99 and 95%, respectively, of the eritadenine detected was in the broth. These results indicate that a relatively low stirring rate is favourable for eritadenine production.

Table1. Eritadenine content measured in the shiitake mycelia and culture broth from various submerged cultivations by HPLC analysis.

	Cultivation conditions		Biomass (g DCW)	Eritadenine content (mg)			Specific productivity (mg/g DCW)
	pH	rpm		Mycelia	Broth	Total	
Shake flask	nc[a]	150	0.45	0.29	2.66	2.95	6.56
Bioreactor	5.7	250	0.62	0.87	15.25	16.12	26
Bioreactor	nc	250	0.59	0.12	19.12	19.24	32.61
Bioreactor	5.7	50	0.30	0.11	10.04	10.15	33.83
Bioreactor	nc	50	0.25	0.04	9.86	9.90	39.58

[a] Not controlled

In all cases the initial pH was 5.8, but during growth pH dropped to a final value of 3.0 in the shake flasks. Further, the final pH in the bioreactors with uncontrolled pH was 4.2 and 5.0 at 250 and 50 rpm, respectively. Clearly, the mycelia change their metabolism and acid production, depending on the culture conditions. Applying a stirring rate of 250 rpm in the bioreactor resulted in a mycelial biomass of 0.62 g DCW at pH 5.7, and 0.59 DCW g when pH was uncontrolled. A stirring rate of 50 rpm resulted in lower biomass production; 0.30 g

DCW at pH 5.7 and 0.25 g DCW when pH was uncontrolled. These results indicate that the stirring rate in bioreactors is more important for growth than is pH, which might be explained by a more efficient mass transfer of oxygen and nutrients. Further, the specific productivity in combination with the biomass yield will affect the total product yield. In this case the highest specific productivity of eritadenine was obtained at uncontrolled pH and 50 rpm, but the higher biomass yield obtained during cultivation at 250 rpm resulted in the highest total product yield, 19.24 mg.

The low final pH in shake flasks combined with the relatively low amount of eritadenine indicate that for eritadenine production, a pH higher than 3.0 is preferable. On the other hand, mycelial growth in the shake flasks was in parity with the mycelial growth in the bioreactors (0.45 g). Further, the results from the cultivations in bioreactors indicate that a pH lower than 5.7 favour eritadenine production, at the same stirring rate. According to previous studies (*31*) optimum pH for growth of shiitake mycelia is 3.0-3.5, while for production of antibacterial substances the optimum pH is 4.5, showing that growth and metabolite production does not have the same pH optimum. Other studies have shown similar results; i.e. the optimal cultivating parameters for cell growth differ from those for product formation, a general case for secondary metabolites (*19, 24*).

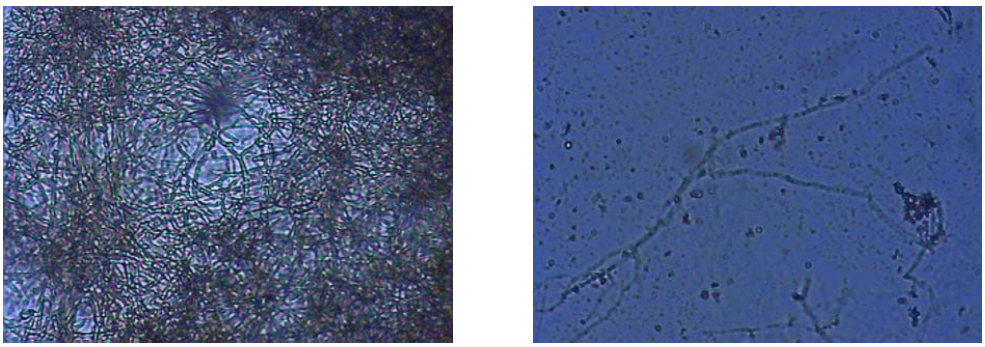

Figure 3. Shiitake mycelia from submerged cultivation in shake flask (**A**) and bioreactor (**B**) (20 x).

Taken together, the results from this study show that eritadenine is produced by shiitake mycelia and the major part of it is excreted to the surrounding medium. Further, the results from the present study indicate that agitation rate and shear influence shiitake mycelial

morphology and in turn eritadenine production; the dispersed filament favouring a higher excretion of eritadenine than the macroscopic aggregates. The pH had less influence on growth than did stirring rate, but a noticeable effect on eritadenine production. On the other hand, the mycelial morphology itself affects pH and probably in turn product formation. This clearly shows the complex interrelationship between culture conditions, morphology and productivity in filamentous fungi. The results from this study also indicate that the optimal conditions for biomass production and eritadenine formation not necessarily coincide.

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