Fungal Production and Solid State Chemistry of Eritadenine

An Integrated Approach to Development of an Active Pharmaceutical Ingredient

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Division of Chemical Engineering
Department of Chemical Engineering and Geosciences
Luleå University of Technology
SE- 971 87 Luleå
Sweden

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Abstract

The present thesis demonstrates an integrated approach to the development of a potential active pharmaceutical ingredient, eritadenine, a cholesterol reducing compound originating from the shiitake mushroom (*Lentinus edodes*). The main areas covered in the thesis are a method for quantification of eritadenine, production of eritadenine by submerged cultivation of fungal mycelia and investigation of the influence of process parameters on mycelial growth and production, and finally solid state characterizations of eritadenine.

The usage of the fungus as a source of eritadenine requires an analytical tool for quantification of the compound. An HPLC method was hence developed for identification and quantification of eritadenine, using chemically synthesized eritadenine as a reference. The amount of eritadenine in fruit bodies of selected strains of shiitake was determined and with the method developed in this study, eritadenine concentrations up to ten times higher than previously reported were detected.

Since both fruit bodies and mycelia of shiitake have been shown to contain eritadenine submerged cultivation of shiitake mycelia was investigated as an alternative source for this compound. The mycelia were cultivated in various submerged conditions, both in shake flasks and in bioreactors. It was found that both the mycelia and the culture media contained eritadenine, of which the major part was detected in the culture media. While the biomass concentrations were higher in shake flasks, the eritadenine concentrations were considerably higher in the bioreactors, which were assigned to morphological variations.

In an attempt to improve the mycelial growth and eritadenine production, a growth promotive substance in the form of a water extract of DDGS, a by-product from dry-grind ethanol facilities, was added to the culture media. It was demonstrated that an amendment of the cultivation media with this extract caused a considerable growth promotive effect on shiitake mycelia in bioreactor cultivations, along with enhanced eritadenine production.

If eritadenine will be used as a pharmaceutical agent, understanding about the solid state chemistry of the compound is required. Raman spectroscopy is a valuable technique for investigation of structural properties; hence, a Raman reference spectrum with line assignments for the solid state of synthetic eritadenine was established.

To further investigate the solid state chemistry of eritadenine, its synthetic analogue was slowly crystallized from water and different ethanol concentrations, at different temperatures. Solids formed from slow cooling of either water or aqueous ethanol showed crystallinity. No polymorphism was detected, irrespective of solvent system or temperature. However, dissimilar thermal behaviours were observed, deducing crystals derived from water as dihydrates and crystals derived from aqueous ethanol as 2.5 hydrates.
Acknowledgements

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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

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

III Growth Promotive Conditions for Enhanced Eritadenine Production during Submerged Cultivation of *Lentinus edodes*
Josefine Enman, David Hodge, Kris A. Berglund and Ulrika Rova
Manuscript to be submitted to Journal of Biomedicine and Biotechnology.

IV Raman Analysis of Synthetic Eritadenine
Josefine Enman, Kerstin Ramser, Ulrika Rova and Kris A. Berglund
*J. Raman Spectrosc.* **2008**, *39*(10):1464-68

V Solid State Characterization of Sodium Eritadenate
Josefine Enman, Anuttam Patra, Kerstin Ramser, Ulrika Rova and Kris A. Berglund.
Submitted to Journal of Pharmaceutical Sciences.
Other Publications by the Author

A New Method for Producing Eritadenine in Liquid Phase Fermentation
Kris A. Berghund, Ulrika Rova and Josefine Enman

Growth Promotive Conditions for Enhanced Eritadenine Production during Submerged Cultivation of *Lentinus edodes*
Kris A. Berghund, Ulrika Rova and Josefine Enman
US provisional patent
Introduction

Cardiovascular disease is a major health concern in modern Western society where there is a high frequency of such disease. This type of disease is also the most common cause of mortality in many affluent countries. Cardiovascular disease is a class of diseases that comprises the heart and blood system and is mainly developed 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 (1). Based on the prevalence of cardiovascular disease and its correlation to cholesterol, there are incentives for development of active pharmaceutical ingredients reducing cholesterol and hence prevent this state of ill-health.

Cholesterol and cardiovascular disease

Although cholesterol (Figure 1) is mainly associated with cardiovascular disease, this lipid is also indispensable to the human body. It is an essential component of cellular membranes and 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.
The various combinations of proteins and lipids give rise to 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. The disadvantage with LDL is its propensity of attaching to the walls of 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 (1).

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 (2). The rate-limiting step in cholesterol synthesis is the reaction catalysed by the enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (3). 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) (4). 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. 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 (5).

**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 (6). 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. In contrast, 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 at a harmless level is a necessity. There are different drugs on the market for treatment of familiar hypercholesterolemia, of which the statins (Figure 2) are most frequently prescribed (7). The statins are originally a group of secondary metabolites isolated from fungi, such as lovastatin from *Aspergillus terreus* (8) and mevastatin from *Penicillium citrinum* (9). The statins are competitive inhibitors of the enzyme HMG-CoA reductase, which catalyses the rate limiting step in cholesterol biosynthesis, hence
inhibiting cholesterol biosynthesis (8, 9). Some of the statins are produced by fungal fermentations such as pravastatin, which is the active substance in Pravachol®. 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 (10) and is the active substance in Zocor®. Further, some statins are purely synthetic, like atorvastatin in the hypocholesterolemic drug Lipitor®, which was the top selling drug worldwide in 2007 (11). Despite being efficient cholesterol reducing compounds, the statins have attracted some attention for their adverse effects, such as liver damage, rhabdomyolysis and myotoxicity (7). 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. Statins used in pharmaceutical applications; lovastatin (A), pravastatin (B) simvastatin (C) and atorvastatin (D).
Eritadenine

A potential candidate as a new active pharmaceutical ingredient against hypercholesterolemia could be the fungal derived compound designated as D-eritadenine, 2(R),3(R)-dihydroxy-4-(9-adenyl)-butanoic acid (Figure 3). This compound is a secondary metabolite mainly occurring in the edible fungus *Lentinus edodes*, shiitake mushroom, wherein it was discovered (12, 13).

![Figure 3. D-Eritadenine.](image)

The cholesterol reducing effect of the shiitake mushroom has been studied on humans. Given 90g of fresh shiitake daily for one week, the serum cholesterol was lowered by 12% in the humans subjected to the experiment (14). This ability of shiitake mushrooms to lower blood cholesterol is mainly ascribed to eritadenine, which was formerly designated as lentinacin (12) and lentysine (13, 15) by the research groups isolating and structurally determined this compound. Upon isolation of eritadenine it was supplied to rats in order to elucidate the effects on serum and liver cholesterol levels, and a diet containing 0.005% eritadenine was found to markedly decrease the serum cholesterol (12, 13). Further studies have shown similar results (16-20), whereas no such studies on humans have been found in the literature.

Although the hypocholesterolemic action of eritadenine has been investigated in several studies on rats, the complete mechanism by which eritadenine causes its hypocholesterolemic effect remains to be clarified. 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 (21) and the hepatic cholesterol levels in rats were not lowered by eritadenine (13, 21). Further, it has been suggested that the hypocholesterolemic action of eritadenine is due to a change in the liver phospholipid metabolism; more exactly a decrease of the phosphatidylcholine (PC)/phosphatidylethanolamine (PE) ratio in rat liver cells (16-19, 22). D-Eritadenine is a very potent reversible inhibitor of the enzyme S-adenosyl-L-homocysteine (SAH) hydrolase (EC 3.3.1.1) (23, 24), which catalyses the hydrolysis of S-adenosyl-L-homocysteine to form adenosine and homocysteine (Figure 4). Hence, the enzyme inhibition by eritadenine causes an increase in the SAH concentration (25).

![Figure 4](image_url)

**Figure 4.** The mechanism of action of S-adenosyl-L-homocysteine hydrolase.

SAH is in turn an inhibitor of different methyltransferases (26) and hereby prevents the PE N-methylation and conversion of PE to PC, catalysed by PE N-methyltransferase (27). In accordance with this mechanism, the eritadenine induced increase in SAH concentration has been shown to inhibit PE N-methylation, with a concomitant increase in the PE content in rat liver microsomes (22) (Figure 5). 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 (19) or decrease the secretion of cholesterol from the liver (22), 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 (16).

\[ \text{Eritadenine} \xrightarrow{\text{SAH hydrolase}} [\text{SAH}] \xrightarrow{\text{PE \text{N-Methyl-}transferase}} [\text{PE}] \xrightarrow{\text{Increased uptake of cholesterol ? Decreased release of cholesterol?}} \text{PC} \]

Figure 5. Proposed mechanism of action of D-eritadenine in rat liver cells.

It should also be emphasized that elevated levels of homocysteine has shown to be involved in pathological states such as cardiovascular disease and stroke, due to an increased risk of developing atherosclerosis (28, 29). Elevated levels of homocysteine have also been associated with the risk of developing Alzheimer’s disease. The mechanism explaining this relation is not established (30), but might be due to the induction of vascular changes by homocysteine (31). It is plausible that eritadenine causes a versatile range of effects on lipid metabolism as commenced by the inhibition of SAH hydrolase, and the resulting increase in SAH concentration and slowdown of homocysteine assembly might in turn have effects on various pathological states (32).

Drug development

The process of developing active pharmaceutical ingredients, from discovery to commercialization, is a multi-disciplinary field which comprises several stages, each subjected to strict regulations. It is estimated that the process from the initial stage of drug development to commercialization typically lasts 10-12 years and that the cost is at least US$ 1 billion. Underlying the development of a new drug is the need of a medication against a certain disease. Thus, generally the first stage in drug development is to find the target, e.g. an enzyme or receptor, which causes or leads to the disease in question. Based on knowledge about this target, chemical or biological compounds are tested against the target in order to find leading drug candidates. Once these lead
compounds are identified they are tested both in vivo and in vitro, to examine the effect on biological systems, in so called preclinical studies. It should also be stressed that many drug substances have traditionally been discovered by empirical observations. In these cases components, mainly of natural origin, that give rise to certain observed biological effects have been isolated and refined. The lead compounds are often modified, e.g. to reduce toxicity and increase performance, in order to optimize the compound properties. When this is accomplished the drug candidate can be applied on human subjects in clinical trials. The criterion for a drug to reach the stage of clinical trials and large-scale production is that it has to be manufactured in such a way that the prevailing regulations for safety, purity, efficacy and consistency are applied (33). A drug substance can be manufactured synthetically, semi-synthetically or be of natural origin. If drug substances, or precursors for drug production, are to be isolated or produced from natural sources, suitable biological systems and production methods are required.

**Fungi as sources for active pharmaceutical ingredients**

Species of the kingdom Fungi constitute a vast reservoir of pharmacologically active substances and are also a useful means of production of several drug products. Some of these are secondary metabolites which 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 have therefore attracted a lot of attention for their commercial significance. 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. These secondary metabolites are also some of the most recognized pharmaceuticals of fungal origin. β-lactam antibiotics include several penicillins and are produced industrially by fermentation of the filamentous microfungus *Penicillium chrysogenum*. Another antibiotic, griseofulvin, was originally isolated from *Penicillium griseofulvum* (34) and is industrially produced from
fermentations of the same fungal species. The cholesterol reducing secondary metabolites, the statins, from different fungi have been developed into drugs and lovastatin is produced industrially by cultivation of the microfungus *Aspergillus terreus* (35). 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 therefore a wide field of therapeutic applications including migraine, parkinsonism and circulatory disturbances (36).

**Medicinal mushrooms**

Medicinal mushrooms are higher Basidiomycetes (macrofungi), which have been used for thousands of years as remedies for diseases, especially in Asian countries, and comprise an immense source of potential new pharmaceuticals, many yet to be discovered. Despite their use in traditional medicine and their pronounced health-promoting effects, few of these medicinal mushrooms have been studied with respect to their commercial and pharmaceutical potential. Conventional drugs derived from medicinal mushrooms are rarely encountered; most commercial products from these organisms are in the form of powdered fruit bodies or fruit body extracts and marketed as nutraceuticals or supplements. Moreover, in Japan, Russia and China, several carcinostatic agents have been purified from mushrooms and mycelia and are available on the market (37). In the last decades the interest in these mushrooms has increased in Western society along with research regarding their pharmacologically active substances (Table 1).
Table 1. Pharmacologically active substances from medicinal mushrooms.

<table>
<thead>
<tr>
<th>Source</th>
<th>Antiviral</th>
<th>Antibacterial</th>
<th>Antitumor</th>
<th>Hypcholesterolemic</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ganoderma pfeifferi</em></td>
<td>Triterpenes against influenza and herpes simplex virus</td>
<td>Ganomycin A and B against <em>Staphylococcus aurea</em></td>
<td>β-D-glucans</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Heteropolysaccharides</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Glykoproteins</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ganoderic acid A and C</td>
<td></td>
</tr>
<tr>
<td><em>Ganoderma lucidum</em></td>
<td>Triterpenes against HIV</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>Flammulina velutipes</em></td>
<td>Ribosome inactivating protein against HIV</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>Grifola frondosa</em></td>
<td></td>
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<tr>
<td><em>Pleurotus ostreatus</em></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>Agaricus bisporus</em></td>
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</tbody>
</table>

For survival in their natural environments mushrooms inherently produce antimicrobial compounds, some of which can be beneficial for humans. As an example, antibacterial farnesyl hydroquinones, ganomycin A and B, produced by *Ganoderma pfeifferi* have been shown to inhibit the growth of methicillin-resistant *Staphylococcus aurea* and other bacteria (38). Antiviral compounds has also been detected in mushrooms, such as several triterpenes from *Ganoderma lucidum* (39) and a ribosome inactivating protein from *Flammulina velutipes* (40), which all act as inhibitors of HIV-1. Further, from *Ganoderma pfeifferi* triterpenes with antiviral activity against influenza virus type A and herpes simplex virus type 1 (HSV-1), have been isolated (41). A majority of all mushrooms contain biologically active polysaccharides, which are known as the most potent antitumor and immunomodulating substances originating from mushrooms. Due to their immunostimulating nature these polysaccharides could have a versatile use in clinical practice. Certain polysaccharides and polysaccharide-protein complexes from mushrooms such as *Ganoderma lucidum* (42) and *Grifola frondosa* (43) among several...
other species have the ability to stimulate the immune system and hereby cause antitumor activity. Further, antitumor activities have also been attributed to lectins, i.e. proteins with the capability of binding to carbohydrate moieties of glycoconjugates, isolated from *Pleurotus ostreatus* and *Agaricus bisporus* (44) and to ganoderic acids A and C derived from *Ganoderma lucidum* (45). Another area where mushrooms can be applied is in the treatment of elevated blood lipid levels; in *Pleurotus ostreatus* the hypocholesterolemic agent lovastatin (46) has been detected and ganoderic acid C from *Ganoderma lucidum* have also been shown able to inhibit cholesterol biosynthesis (47). Moreover, hypoglycemic effects caused by ganoderic acids A, B, G and H isolated from *Ganoderma lucidum* (48) has been observed and a mycelium extract from *Grifola frondosa* (49) has been shown to possess anti-inflammatory effects.

**The shiitake mushroom**

The shiitake mushroom (*Lentinus edodes*) (50) is a popular edible fungus and also a traditional medicinal mushroom, 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, development of the cultivation techniques has enabled the shiitake mushroom to become extensively cultivated throughout the world (51). 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 considered a saprophyte as it lives on dead material and thereby enhances the decomposition of these materials. By secreting hydrolytic and oxidative enzymes, the shiitake mushroom has the ability to convert lignocellulosic materials to extractable carbohydrates, amino acids and other nutrients for growth. The shiitake mushroom is considered to be a weak cellulose degrader and intermediate hemicellulose degrader but a potent starch and lignin degrader (52), the latter due to production of the lignolytic enzymes manganese-dependent peroxidase (MnP), laccase and lignin peroxidase (LiP) (53).
Shiitake growth characteristics

The shiitake mushroom belongs to 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 (Figure 6) is produced, containing two nuclei in each hyphal compartment (one from each compatibility group).

![Figure 6. Dikaryotic fungal hyphae.](image)

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. When the surrounding conditions are right, mycelia start to form primordia, a pre-stage to fruit bodies, and then fruit bodies, completing one life-cycle of the mushroom (Figure 7). The fruit body is formed in response to nutrient, i.e. carbon and nitrogen, limitation.
and also some environmental stimulus (52). 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.

![Diagram of the life cycle of shiitake](image)

**Figure 7.** Life cycle of shiitake.

**Shiitake as a medicinal mushroom**

The shiitake mushroom is not only valued for its culinary uses, but also for its medicinal 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 antitumor activity of shiitake. One of the agents responsible for the antitumor effect, and the most extensively studied, has been identified as a water-soluble
polysaccharide, a β-1,6-branched-β-1,3-glucan designated as lentinan (54). Lentinan exerts its antitumor effect by stimulating the immune system, and activating it to counteract tumour growth (55, 56). Due to its antitumor and immunomodulating properties, lentinan is used in clinical practice for cancer and HIV therapy, primarily in Japan (57). Lentinan has also been suggested to have a prophylactic potential for malaria treatment. Studies on the effects of lentinan in mice infected with malaria parasites have shown that lentinan induced immune responses and hereby managed proliferation of malaria parasites (58). Another antitumor and antiviral polysaccharide, KS-2, has been isolated from shiitake mycelia culture (59) along with other polysaccharides with immunomodulating activities (60). Further, extracts of shiitake mycelia cultures have been found to contain antiviral substances inhibiting HIV (61, 62) and HSV-1 (63) as well as to exert anticarcinogenic (64, 65) and hepatoprotective properties (66). Carbohydrate binding proteins, lectins, have also been found in shiitake. These lectins can have an application in areas such as oncology, cytology, genetics and experimental biology due to their capability of evoking diverse functions in living organisms (67). An antifungal protein, designated as lentin, with an inhibitory activity on HIV-1 reverse transcriptase and on the propagation of leukemia cells, has also been isolated from shiitake mushrooms (68). Further, antibacterial substances against e.g. Streptococcus spp., and Staphylococcus aureus (69-71) have been isolated from shiitake mushrooms and mycelial culture broth. As discussed above, fruit bodies and mycelia of shiitake have been shown to lower blood cholesterol levels in animals (72-74) and humans (14), a feature assigned mainly to the secondary metabolite eritadenine. The amounts of eritadenine in fruit bodies of shiitake mushroom, 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 (75, 76). The mycelia of shiitake have also been found to contain eritadenine (72, 77); the amount determined by GC analysis was 0.737 mg/g dried biomass (77). Apart from the medical benefits, the shiitake mushroom is a nutritionally valuable food. Due to low contents of fat and digestible carbohydrates shiitake mushrooms are low energy-foods, and they also constitute a good source of dietary fibers, amino acids, vitamins and mineral elements (78, 79).
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 filamentous 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. The secondary metabolites are usually produced from common metabolic intermediates, but the production is often species- or strain-specific. The production of secondary metabolites is accomplished by special enzymatic pathways in the fungi and usually takes place in the stationary phase when fungi are grown in culture. However, the repression of enzymes for secondary biosynthesis can be altered by e.g. nutritional and genetic factors and secondary metabolites can be produced during growth (80).

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 2).
One of the major fungal biotechnology processes is the production of antibiotics. Penicillin was discovered in 1928 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. As mentioned in a previous section, 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 (81).

Besides the pharmacologically active metabolites discussed in a previous section, another example of industrially important products from fungi is the organic acid citric acid, produced by fermentation of *Aspergillus niger* (82). 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, produced in large scale by *Aspergillus terreus* fermentations, can be incorporated into polymers and is thus having the potential of substituting petrochemical-based monomers (83). Quorn™ is a trademark of commercial fungal food products. The main constituent of such foods is mycoprotein produced from *Fusarium venenatum*. These products are rich in protein and serve as an alternative to animal protein sources (84). Industrially important starch degrading

<table>
<thead>
<tr>
<th>Product</th>
<th>Source</th>
</tr>
</thead>
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<tr>
<td>Penicillins G and V</td>
<td><em>Penicillium chrysogenum</em></td>
</tr>
<tr>
<td>Griseofulvin</td>
<td><em>Penicillium griseofulvum</em></td>
</tr>
<tr>
<td>Citric acid</td>
<td><em>Aspergillus niger</em></td>
</tr>
<tr>
<td>Itaconic acid</td>
<td><em>Aspergillus terreus</em></td>
</tr>
<tr>
<td>Microbial protein (Quorn™)</td>
<td><em>Fusarium venenatum</em></td>
</tr>
<tr>
<td>Lovastatin</td>
<td><em>Aspergillus terreus</em></td>
</tr>
<tr>
<td>α-Amylase</td>
<td><em>Aspergillus oryzae</em></td>
</tr>
<tr>
<td>Ergot Alkaloids</td>
<td><em>Claviceps purpurea</em></td>
</tr>
</tbody>
</table>

Table 2. Industrially important fungal products.
enzymes like α-amylase are also produced by fungal fermentations, e.g. by *Aspergillus oryzae* (85).

**Submerged cultivations of filamentous fungi**

Filamentous fungi that are used in industrial biotechnology processes are mostly belonging to the phylum Ascomycota. Fungal organisms, like ascomycetes, have a multifaceted metabolism, and the potential of their usage is vast, considering all the beneficial compounds they produce. Thus, on one hand it is possible to benefit from their complexity, on the other hand their usage elicits challenges as their morphological complexity causes complications when culturing fungi in submerged conditions. 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 applied, the mycelia can exhibit different morphologies in the medium. The morphology in turn influences both the productivity of certain metabolites and the properties of the growth medium, which in turn affect the transfer of nutrients and oxygen. Conclusively, when cultivating fungi in submerged conditions, there is a complex interrelationship between process parameters, morphology, broth properties and productivity (*Figure 8*).
Hyphal growth and morphological features

One of the unique features of fungi is their way of growing; hyphal tip (Figure 6) 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 (87, 88).

The growth kinetics of filamentous fungi are 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 are 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 (89).

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 (Figure 9). The macroscopic aggregates, pellets, 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.

![Figure 9](image)

**Figure 9.** Morphological definitions of filamentous fungi in submerged cultivation. Adapted from Paul and Thomas (90).

The nature of the mycelial morphology affects the physical properties of the broth in submerged cultures. Freely dispersed mycelia tend to make the broth viscous and to behave in a non-Newtonian manner, i.e. a fluid without constant viscosity, but for which the apparent viscosity depends on the shear rate and time (91-93). The non-Newtonian rheological properties of the broth are due to interactions between the suspended filaments (91, 93) and 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 (94). 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 (97). If a reduction of broth viscosity is aimed at, culturing the fungus in a pellet form is preferable. On the other hand, the productivity of some metabolites can be reduced by this morphology. For production of antibiotics by *Penicillium Chrysogenum*, dispersed growth has been shown favourable (95), whereas for other metabolites a pellet form is preferred such as for lovastatin production by *Aspergillus terreus* (96).

In viscous fermentations transport phenomena are affected (Figure 8). 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 (97). 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 (97). This can lead to depletion of dissolved oxygen in areas in the slower moving outer areas of the vessel (98).

**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. It should also be emphasized that optimal conditions for growth may not coincide with the optimal conditions for product formation, such as for the cultivation of *Aspergillus terreus* for lovastatin production, which demonstrated different temperature optima for growth and productivity (99). 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. The productivity of certain
metabolites, i.e. cell metabolism, is in turn affected by the morphological nature of the mycelia.

The agitation is an important physical cultivation parameter and the influence of mechanical forces on the morphology and production 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 (100, 101). Citric acid production from *Aspergillus niger* was also shown to depend 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 (102). Further, the agitation speed affected the pellet morphology of *Aspergillus Terreus* and production of lovastatin. It was found that the smaller pellets obtained at highly agitated conditions were unfavourable for lovastatin production and that an upper limit for agitation was essential for high lovastatin titers (103).

Agitation is also important for proper mixing and mass and heat transfer in submerged fermentations; in aerobic fermentations, oxygen transfer is essential. The oxygen requirement and its influence on production of lovastatin (104) and itaconic acid (105) by *Aspergillus terreus* has shown the importance of maintaining a high concentration of dissolved oxygen in the cultivation vessel. Further, for cultivation of *Aspergillus terreus* and lovastatin production the concentration of dissolved oxygen has been shown to influence morphology and product formation; low levels of dissolved oxygen diminished both pellet formation and productivity (99).

The composition of the culture medium is another important factor for growth and productivity and has also been under investigation in several studies, such as for itaconic acid production by *Aspergillus terreus*. In this case it was shown that the amount of itaconic acid produced was correlated to the pellet form and to achieve a growth form resulting in high production rates, the presence of Ca$^{2+}$, Zn$^{2+}$ and Fe$^{2+}$ in the medium was shown to be crucial (106). Production of some secondary metabolites has been
shown to be regulated by catabolic repression such as for the production of penicillin by *Penicillium chrysogenum*, alkaloid production by *Claviceps paspali* (80) and lovastatin production by *Aspergillus terreus* (107), for which glucose has a negative influence on production. In the case of catabolic repression, non-repressive carbon sources are preferentially utilized. Production of α-Amylase from *Aspergillus oryzae* has also been shown to depend on the medium glucose concentration in such a way that high glucose concentrations repressed the production (85). On the other hand, studies on *Aspergillus niger* morphology and citric acid production showed the importance of a high initial glucose concentration in batch cultures for citric acid production and that the morphology was influenced by the glucose concentration in the media (108). The nitrogen source has shown to affect the growth and production of lovastatin by *Aspergillus terreus*; higher nitrogen levels generated more biomass, while the specific production was less (104). Further, the nature of the nitrogen source has been shown to affect both cell morphology and antibiotics production by *Rhizopus chinensis*. The highest antibiotic production was attained by using corn steep liquor as a nitrogen source and pertained to pellet growth (109).

Another factor influencing fungal morphology and productivity is the nature and concentration of the inoculum. Variations in the nature of inocula influenced antibiotic production by *Penicillium chrysogenum*, *Penicillium patulum* (110) and *Penicillium griseofulvum* (111), which could be associated to morphological differences. 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* (112). Further, pH of the culture medium 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 pH from 2.1 to 4.5 or decreasing it to 1.58 led to a markedly decreased production of citric acid and changes in the morphology (102). Lovastatin production in *Monascus purpureus* has also been shown to be influenced by pH of the culture medium (113).

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.

**Submerged cultivations of medicinal mushrooms**

Like the microfungi described above, mycelia of medicinal mushrooms can also be cultivated in submerged conditions and their growth and metabolite production can be altered in response to changes in their environment. Despite their ability to be cultivated in submerged conditions, most commercial products from medicinal mushrooms are derived from the fruit bodies. However, there are some disadvantages when using the fruit bodies as a production source; the cultivation is time consuming and the quality of the final product is difficult to control. Submerged cultivation of medicinal mushrooms is an area of biotechnology which is receiving increased attention as it could serve as an alternative to produce pharmaceutically active substances or other metabolites. Although submerged cultures of medicinal mushrooms could have an industrial potential for metabolite production, the knowledge about their behaviour in these conditions is scant, compared to that on microfungi, and still requires a lot of research. Especially for the biosynthesis of secondary metabolites, which are subjected to complex regulation, much is hitherto unknown. The mushroom industry of today is hence concerned partly with the development of new techniques of fruit body production and partly with the integration of modern biotechnological techniques to produce mushroom derivatives (67). The commercial success of submerged cultivations of mushrooms for production of valuable metabolites relies on the development of efficient production systems which results in an increased product yield.

The most well studied submerged cultivations of medicinal mushrooms with respect to product formation pertain to production of polysaccharides. The maitake mushroom, *Grifola frondosa*, is a well recognized medicinal mushroom, which contains various
pharmacologically active substances. Due to the demand of biomass and active substances, submerged cultivations in bioreactors of this mushroom have been developed (43). It should be emphasized that both the quality and quantity of certain substances derived from this mushroom vary between different strains and also on the locations, culture conditions and extraction procedures applied. As an example, mycelial morphology and exopolysaccharide production have been shown to be significantly altered by culture pH, aeration rate and reactor type (114). Further, at comparatively high oxygen concentrations both cell growth and polysaccharide production were inhibited. On the other hand if 1% of olive oil was added to the medium a relatively high oxygen concentration was beneficial for growth and production (115). Moreover, by doubling the glucose concentration in the medium, production of exopolysaccharides was also doubled and organic nitrogen sources were shown most favourable for mycelial and exopolysaccharide production (116).

Ganoderma lucidum is another medicinal mushroom for which studies on its behaviour in submerged conditions have been conducted. The research comprising the cultivation of this mushroom is mainly focusing on the production of polysaccharides and ganoderic acids, which are considered as highly valuable and attractive metabolites (117). The growth and polysaccharide and ganoderic acid production in Ganoderma lucidum has been improved by controlling pH or by shifting pH during cultivation (118-120). Inoculum density has also been shown important for growth, morphology and production of polysaccharides and ganoderic acid; a large inoculation density caused small pellets and favoured polysaccharide production whereas a low inoculation density gave rise to larger pellet size and high ganoderic acid production (121). The role of oxygen supply has also been investigated with respect to polysaccharide and ganoderic acid production showing that a relatively higher dissolved oxygen tension improved both polysaccharide and ganoderic acid production and that different dissolved oxygen tensions affected growth and morphology (122). Further, by adding 2% of corn oil to the media, growth and polysaccharide production were stimulated (123).

Another basidiomycete of medicinal importance is the oyster mushroom, Pleurotus ostreatus. This mushroom is a white-rot fungus capable of degrading lignin due to its
production of oxidoreductases such as laccase. Laccase and other lignolytic enzymes might have a wide application, especially in the renewable bio-energy industry and has therefore attracted attention. *Pleurotus ostreatus* has been shown to have a considerably higher laccase activity in submerged fermentation than in solid-state fermentation (124). Optimization of the culture conditions for the fermentation of *Pleurotus ostreatus* with respect to laccase production have shown that glucose and Mg$^{2+}$ are the essential medium components for its production (125). However, the usage of lignocellulosic wastes promotes the production of lignolytic enzymes more than when glucose is the carbon source (126). Moreover, the addition of organic nitrogen to the culture medium and keeping the initial oxygen transfer rate comparatively low had a positive influence on biomass production of *Pleurotus ostreatus* and a high glucose concentration and low initial oxygen transfer rate favoured productivity of polysaccharides (127). The cholesterol reducing secondary metabolite lovastatin has been detected both in mycelial cultures (128, 129) and fruit bodies (46) of this mushroom. Further, the amount of lovastatin produced during submerged cultivation of this fungus has been shown to fluctuate highly among different strains (128, 129). For the production of lovastatin by submerged cultures of *Pleurotus ostreatus*, the lovastatin content increased with increasing contributions of carbon and nitrogen, when glucose and an organic nitrogen source were used (128).

**Submerged cultivations of shiitake mushroom**

Although the shiitake mushroom naturally grows on hardwoods it has the ability to grow on a variety of other materials and to be cultivated in submerged conditions. Some agricultural and industrial wastes or by-products have shown to hold the nutritional requirements for fungal growth; shiitake mycelia have successfully been grown in submerged culture on a hot water-soluble fraction from corn fiber, a by-product of the wet corn milling process (130). Further, high mycelial yield and antibacterial metabolite production were obtained in media supplemented with rice bran, vermiculite and molasses (131). The ability to grow on a variety of lignocellulosic and other substrates enables the shiitake mushroom to play an important role in the
managing of organic wastes. By adding agricultural or industrial wastes or by-products the costs might be reduced for production of commercial products and the bioconversion diminish the pollution.

The carbon and nitrogen sources are key factors for growth of all mycelia. Apart from different carbohydrates, shiitake mycelia have been shown to grow well on non-carbohydrate organic substances like ethanol and glycerol (132). Moreover, the growth of this fungus on wood substrate showed that nitrogen availability was the limiting factor for growth and the addition of complex nitrogen sources enhanced growth whereas the addition of extra glucose, xylose or starch did not (133). Further, the carbon and nitrogen sources along with their ratio and pH of the culture medium were shown to influence lectin production. In this case L-arabinose and L-asparagine was shown to be the preferred carbon and nitrogen source, respectively, and a pH of 8-9 in the culture media was beneficial (134). The production of different ligninolytic enzymes by shiitake has been shown to be strain specific and to depend on the lignocellulosic material used for cultivation (135) and also on nitrogen levels in the medium when glucose was used as a carbon source. In the latter case, laccase production was stimulated by high nitrogen content while MnP was suppressed (53).

The nature of the inoculum has been shown important for growth of *Lentinus edodes* mycelia; homogenized inocula resulted in higher biomass production (136) and the size of the mycelial pellets in shake flasks was inversely related to inoculum size (137). Shiitake mycelia have shown able to grow at temperatures between 20-30 ºC and in pH ranging from 3-7 in liquid cultures (131, 138). On the other hand, the optimum medium pH for mycelial growth has been reported to be 3-3.5, whereas production of certain antibacterial substances had an optimum pH of 4.5 (131) and for lentinan production pH greater than 5 was favourable (139), demonstrating that optimal conditions for growth may not coincide with the optimal conditions for product formation.
**Solid state chemistry of drugs**

Since most drugs are delivered as solids, the solid state chemistry of a given active pharmaceutical ingredient is a key concept in the pharmaceutical industry, from drug discovery to successful commercialization. The solid state of a pharmaceutically active substance is decisive for physical properties and performance of the drug, such as solubility, stability and bioavailability. Hence, an understanding of the solid state in relation to its functional properties is fundamental to drug formulation and performance. Solid state technology is a multi-faceted area which covers the characterization of solids and investigation of properties of pharmaceutical importance. The overall aim is to make each drug available in a form which has optimal performance for the application in question (140).

**Pharmaceutical solids**

In the pharmaceutical industry there is a variety of methods applied for production of solids (Table 3), most of which are based on a reduction in solubility of the compound in question.

<table>
<thead>
<tr>
<th>Method</th>
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<tbody>
<tr>
<td>Evaporation</td>
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<tr>
<td>Cooling of a solution</td>
</tr>
<tr>
<td>Seeding a supersaturated solution with crystals of the preferred form</td>
</tr>
<tr>
<td>Freeze drying</td>
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<tr>
<td>Addition of antisolvents</td>
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<tr>
<td>Salting out</td>
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<tr>
<td>Changing pH</td>
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</table>

Since most marketed drugs used are as molecular crystal solids (141), crystallization is the main process for solid formation in the pharmaceutical industry (142). Crystals are defined as molecules arranged in a highly ordered fashion and the arrangement of the molecules in the crystal dictates the physical properties and in turn the performance of the drug. The ordered packing of crystals leaves very little space between the molecules...
and hereby causes a dense structure. Further, the specific local order is defined as a unit cell (Figure 10) and crystals are constructed by the repetition of the unit cell in three dimensions and this three-dimensional array of unit cells is referred to as the crystal lattice. When the unit cell is strictly retained throughout the boundaries of a given particle, that particle is called a single crystal. The number of ways molecules can be packed is governed by certain symmetry operations and their possible combinations, which are defined as space groups. The degree of symmetry of a molecule is another factor which decides how it is packed in the crystal. If molecules have symmetries that lead to a more close packing they are in general more easily crystallized and form better crystals. Organic crystals are held together mainly by non-covalent interactions, which can be either hydrogen-bonding or non-covalent attractive forces. Non-covalent attractive interactions depend on dipole moments, polarizability and electronic distribution of the molecules and hydrogen bonding requires donor and acceptor functional groups.

Figure 10. The most common classes of unit cells for crystals of drugs; triclinic (A), monoclinic (B) and orthorhombic (C). Adapted from Byrn et al. 1999 (140).

In order to form crystals supersaturation is required, in which case the concentration of a solute is higher than the solubility. As mentioned above, most crystallization methods are based on a reduction in solubility of the compound and supersaturation can thus be created by e.g. cooling or evaporation (Figure 11). Supersaturation is not the only prerequisite for crystal growth to occur; once supersaturation is achieved crystals are not necessarily formed. A process referred to as primary nucleation, in which a critical
number of ordered molecules are gathered into nuclei, is required for crystal growth from a supersaturated solution. This nucleation takes place only if the supersaturation is sufficient to surmount the unfavorable energy linked to the creation of new interfaces when the solid phase first forms. Following the primary nucleation is secondary nucleation, when crystallization continues from the initial crystals formed, and the so-called metastable zone is the ideal region for crystal growth once nuclei have formed. It should also be stressed that these nucleation events are decisive for the resulting crystal form, number and size (140, 143).

![Figure 11. Schematic diagram of solubility.](image)

Solid pharmaceuticals can be present in different forms and which form is obtained depends on the crystallization method and conditions applied. The method of crystallization in turn affects the process outcome. As an example, for optimization of the crystallization process of acetylsalicylic acid with respect to product properties and performance a combined cooling/antisolvent system has been shown useful. In this case acetylsalicylic acid was crystallized from solutions with ethanol as solvent and water as antisolvent and it was found that a constant cooling and antisolvent addition was highly applicable to improve productivity and reducing formation of fines (144).

Commonly drugs exist as crystalline polymorphs, i.e. solids which have the same elemental composition but different crystal structures (145). Compounds which crystallize into different crystalline states, i.e. as two or more polymorphs, can exhibit
various physical and chemical properties such as different melting points, solubility and different spectral patterns. The various properties can in turn affect e.g. stability and bioavailability of the drug. If polymorphs have been shown to exist it is necessary to investigate the physical properties for each of these and if they differ, the polymorph with optimal properties for the pharmaceutical application should be chosen. To control the crystallization outcome, i.e. that the desired crystal form is obtained, a method referred to as seeding is commonly used in crystallization processes, in which nuclei of the desired phase are added (145).

The solvent composition has shown to be an important factor for formation of polymorphs. Crystals of the antibiotic agent sulphathiazole have shown different properties when crystallized by cooling of different solvent systems. The size, shape and filtration characteristics of the crystals differed when crystallized from propan-1-ol, water and mixtures of propan-1-ol and water. Further, crystallization from propan-1-ol resulted mainly in one type of polymorph whereas formation of another polymorph was favored when water and mixtures of propan-1-ol and water were used as solvents (146). The anti-inflammatory analgesic agent mefenamic acid as well as the cholesterol reducing agent atorvastatin have also been shown to display polymorphism upon crystallization from different solvents (147, 148). On the other hand, when Nevirapine, a drug used for treatment of HIV infection and AIDS, was recrystallized from different solvent systems with varying polarity the same internal crystal lattice was obtained. However, different habits were obtained (149), i.e. crystals with the same chemical composition and crystal structure, but which display different shapes (140). Moreover, the stirring and degree of supersaturation influenced both the size and shape of the crystals (149).

The temperature has been shown to influence the formation of α- and β-polymorphs of L-glutamic acid. At 25 ºC, growth of the α-form occurred whereas at 45 ºC growth of the β-form took place (150). Carbamazepine is a drug used in the treatment of epilepsy, which has been shown to exist in four polymorphic forms. To improve the mechanical properties of this compound it was recrystallized from aqueous solutions at different pH
values. For all pH values in the study carbamazepine had superior mechanical properties compared to the original samples. Moreover, no changes in crystallinity were observed, irrespective of pH whereas the morphology and size of the crystals differed (151).

Other common types of solid forms are solvates or hydrates. In these cases one or more solvent molecules are incorporated into the crystal structure and act as stabilizers. Pseudopolymorphism is referred to as crystalline forms of a compound that differ in nature or stoichiometry of included solvent molecules (152). Most solvates are hydrates due to the propensity of water molecules to be part of crystal structures and approximately one third of all active pharmaceutical ingredients are able to form crystal hydrates. The small size of the water molecule makes it apt to fill structural voids and the multidirectional hydrogen binding capacity of water further promotes the formation of stable crystal structures. By multidirectional hydrogen binding capacity is meant that water molecules bind both to other water molecules and to other available donor and acceptor functional groups. Depending on the location of water in the crystalline hydrates the latter can be classified into three categories; isolated lattice site hydrates, lattice channel hydrates and metal-ion coordinated hydrates (152). If the lattice integrity is maintained upon desolvation, the crystal forms are referred to as desolvated solvates (145), however, some solvates may convert to amorphous material upon desolvation (153).

Although rarely, some pharmaceuticals exist in amorphous forms (154), which lack the long-range order of crystals. Further, rapid cooling of a compound solution generally gives rise to amorphous materials and freeze drying is a common method for production of amorphous solids in the pharmaceutical industry (155). The advantage of using amorphous forms is that they tend to be more soluble than their crystalline counterparts (149, 156) and hence have a higher bioavailability (157). On the other hand they are generally less stable than their crystalline counterparts (155) and consequently not marketed.
Characterization of pharmaceutical solids

Physical characterization of active pharmaceutical ingredients is essential to successful development of the final drug product and commonly encompasses several complementary techniques. X-ray diffractometry, which comprises two principal applications, is extensively used to characterize pharmaceutical solids (158). X-ray crystallography is a powerful tool to determine the crystal structure and the crystal packing characteristics among the molecules in the solid. The highly ordered arrangement of molecules in crystals enables them to act as a three-dimensional diffraction grating for X-rays that have a wavelength in the atomic size range. Unlike X-ray crystallography, X-ray powder diffraction does not require large single crystals but can be applied to any powdered solid. The information gained from X-ray powder diffractometry measurements can be less detailed, yet it is highly applicable and useful for characterization of pharmaceutical solids.

Thermal analysis methods are also frequently applied for characterization of pharmaceutical solids (159), of which the most important are thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). With these techniques it is possible to record changes of a sample that occur upon temperature changes. In TGA the change in mass of a sample with temperature is measured and with DSC enthalpy changes caused by temperature associated solid state processes can be recorded. Further, the usage of solid state NMR offers valuable information regarding the compound structure. Solid state NMR has the ability to probe the chemical environment of each nucleus and is widely used in the area of polymorphism and pseudopolymorphism. This technique is also suitable for the study molecular dynamics (140).

Vibrational spectroscopy methods, infrared absorption (IR) and Raman scattering, have also attracted attention as valuable tools for characterizing solids. These techniques offer information about motions of functional groups in the solid and are highly sensitive to structure related modifications in molecular compounds. Due to the sensitivity to the crystalline structure of molecular compounds, Raman and IR spectroscopies are
powerful for the identification of polymorphs in drugs (160-162). One advantage with Raman spectroscopy is that it can be used to probe lattice vibrations that are associated with the motion of an entire molecule in a crystalline state (160). For detection of water molecules present in the crystal lattice, IR spectroscopy is applicable and can in some cases be used to make a distinction between anhydrates and hydrates (163).
Present investigation

In light of the prevalence of cardiovascular disease and its correlation to high blood cholesterol levels, the present study encompasses a potential new active pharmaceutical ingredient against elevated blood cholesterol levels. The shiitake mushroom (*Lentinus edodes*) produces a compound designated as eritadenine, which has been shown to possess hypocholesterolemic capacities and is the main focus in the present investigation. Since scarcely any studies so far have been concerned with the development of eritadenine into an active pharmaceutical ingredient this was approached in the present study, by employing the following procedures:

- Development of a reliable analytical tool to identify and quantify eritadenine
- Cultivation of shiitake mycelia at different submerged conditions and investigation of the influence of process parameters on the mycelial growth and production of eritadenine
- Solid state characterizations of the sodium salt of eritadenine
Paper I

The edible fungus shiitake mushroom (*Lentinus edodes*) produces a cholesterol reducing compound designated as eritadenine, 2\((R)\),3\((R)\)-dihydroxy-4-(9-adenyl)-butanoic acid (12). Eritadenine has the ability to enhance the removal of blood cholesterol, but not to inhibit cholesterol biosynthesis in the liver, as shown in studies on rats (21). So far, the mechanism of action of eritadenine is not fully elucidated, but this compound is suggested to exert its hypcholesterolemic effect by amending the liver phospholipid metabolism, and hereby causing either increased uptake or decreased release of cholesterol (16-19, 22). Further, a diet containing 0.005% eritadenine has been shown to markedly decrease the serum cholesterol in rats (12). Hitherto, no studies elucidating the effects of eritadenine on humans have been found in the literature.

To establish dose-response effects of eritadenine in humans and to find a potential source of this compound, it is important to accurately quantify the amount. 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 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 (164-166). 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% trifluoro acetic acid (TFA) in aqueous solution and 0.05% TFA in MeCN, in the proportions 98:2, followed by a linear change to 40:60 over 10 min. TFA was added to the mobile phase to improve peak shape and reduce 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 concentrations 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 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, have been found to be in the range 0.5-0.7 and 0.3-0.4 mg/g dried caps and stems, respectively (75, 76). 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 (12, 13) and mycelia (77) of shiitake 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 of eritadenine. There is a main advantage of growing mycelia in a controlled environment, 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 are industrially used for the production of several important compounds and there is generally a complex relationship between chemical and
physical culturing conditions, such as stirring rate, pH, inoculum, dissolved oxygen and medium composition, and the morphology and production of metabolites by filamentous fungi \( (100, 102, 103, 105, 110) \). Moreover, the response in terms of growth and productivity to environmental factors is as diverse as the different species of filamentous fungi. 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. In light of this, the effects of pH, stirring rate and shear 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 cultivations in shake flasks took place at 150 rpm, 23 °C and pH was not controlled. The bioreactor cultivations took place either in 1 L bioreactors or in a 12 L reactor at 25 °C and a dissolved oxygen flow rate of 1 v/v/min. For bioreactor cultivations in the 1 L reactors, the agitation rate was either 50 or 250 rpm and pH was either controlled at 5.7 or uncontrolled. In the 12 L bioreactor the agitation speed was set to 150 rpm and the pH was 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 ion exchange resins. The mycelia and culture broths were then analysed by HPLC as previously described \( (167) \).

When employing submerged cultivation of shiitake in bioreactors, eritadenine was found in both the mycelium and the culture broth, with the major part, > 90%, in the broth and in concentrations between 3.32 and 10.23 mg/L. Shiitake mycelia have been cultivated and analysed for its eritadenine content in previous investigations, and the intracellular amount as determined by GC was found to be 0.737 mg/g DCW \( (77) \). However, as yet, no data from studies investigating the culture medium for eritadenine has been found in the literature.
A stirring rate of 250 rpm resulted in the highest biomass and eritadenine concentrations in bioreactor cultivations; 0.89 g/L and 10.23 mg/L, respectively, both about twice as large as at 50 rpm, for the same cultivation vessel. The biomass production in shake flasks was 1.77 g/L and thus higher than in any of the bioreactor cultivations whereas eritadenine production was lower; 1.76 mg/L. 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, indicating that the mycelial morphology might influence eritadenine production. Moreover, in the shake flasks, there was a more pronounced lowering of pH, probably due to higher biomass production. In this case the final pH was 3.0, whereas in the bioreactors at 250 and 50 rpm the final pH was 4.2 and 5.0, respectively, when pH was uncontrolled. It is possible that the comparatively low pH in shake flasks also diminish the production and excretion of eritadenine. By studying the dynamics of shiitake mycelial growth and eritadenine production it was deduced that growth and eritadenine production was uncoupled and, finally, carbon was found not to be a limiting factor for growth and production in the present study.

Taken together, 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. Moreover, the observed 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.

**Paper III**

Eritadenine was previously (168) detected both in mycelia and in the culture broth of submerged cultures of shiitake. Based on these results it can be deduced that submerged cultivation of shiitake mycelia might have the potential as a means of production of this hypcholesterolemic compound. In the previous study the growth and eritadenine production was relatively low and the cultivation time fairly long, urging improvements to the process.
The mycelia of shiitake mushrooms have the ability to be cultivated in submerged conditions and alter their growth behavior and production in response to changes in the environment. Previously, shiitake mycelia have been shown able to grow on various wastes or by-products (130, 131, 135) in liquid conditions, due to their capability of extracting nutrients from complex sources. In the present study a water extract of the major by-product from the dry-grind ethanol facilities, distillers dried grains with solubles (DDGS), was added to the culture media with the anticipated increase in biomass and eritadenine production. To reduce the cultivation time, shake flask cultures were used as inocula as an alternative to inoculate directly from mycelial agar plates. Finally, the influence of pH and stirring rate on growth and production of eritadenine was evaluated.

The submerged cultivations took place in 1 L bioreactors in a malt yeast medium, prepared as described previously (168). All bioreactor cultivations took place at a temperature of 25 °C and the dissolved oxygen was controlled at 90%. The initial pH was adjusted to 4.2 or 5.2, and then either uncontrolled or controlled. The stirring rate applied was 125, 250 or 500 rpm and the cultivations lasted between 13– 20 days. In some of the bioreactor cultivations 10% (v/v) of a water extract of DDGS was added, and the inoculum used was either in the form of mycelia from malt yeast agar (MYA) plates or from shake flask cultures. In the latter case submerged cultivation took place in shake flasks for 10 days, at 150 rpm and 23 °C, prior to the inoculation for bioreactor cultivations, in the same malt yeast medium as for the bioreactor cultivations. The mycelial biomasses and culture broths were harvested and prepared prior to eritadenine HPLC analysis as described previously (167, 168).

Addition of a water extract of DDGS to the cultivation media was shown to result in the highest biomass concentrations when comparing all experiments. The biomasses obtained when the culture medium was supplemented with an extract of DDGS were 2-3 times higher than in the control cultivations and the highest biomass concentration achieved was 3.4 g/L. It can thus be deduced that a water extract of DDGS has growth promotive effects on shiitake mycelia in submerged conditions. Further, the observed
growth improvement caused by the water extract of DDGS was attributed to the nitrogen or carbon sources, or to complex effects caused by the various compounds in the extract.

When using a shake flask culture as inoculum, a cultivation time of 13 days resulted in approximately the same biomass concentrations as after 20 days with a mycelial plate as inoculum, if 125 or 250 rpm was applied as the agitation speed and if pH was uncontrolled. However, when the agitation speed was set to 500 or when pH of the media was controlled at either 4.2 or 5.2, the growth was slower, despite the usage of shake flasks as inocula, and hence 20 days was required to obtain a comparable biomass concentration. Further, when an extract of DDGS was added to the media, the bioreactor cultivation time could also be reduced by seven days when shake flask cultures were used as inocula.

All the biomass concentrations in the present study resulting from cultivations devoid of an extract of DDGS were in the same range and slightly higher than in the previous study on bioreactor cultivations of the same shiitake strain. The major differences for all experiments were that no additional glucose was added to the media in the present study and the dissolved oxygen concentration was set to 90% instead of applying a continuous air flow, which could have influenced the resulting biomass concentration.

Like the biomass concentrations, the eritadenine concentrations were in the same range for most experiments devoid of an extract of DDGS in the culture media. When pH was controlled at either 4.2 or 5.2, the eritadenine concentrations were slightly higher compared to other experiments lacking the extract of DDGS, indicating that pH might influence eritadenine production. Compared to the previous study (168), while all biomass concentrations were higher in the present study, not all of the experiments resulted in higher eritadenine concentrations. This can be due to the different aeration method applied or different glucose contents in the media. Moreover, the initial and final pH differed between the two studies, which can also contribute to variations in eritadenine production. The highest eritadenine concentration, obtained when a water extract of DDGS was added to the culture media, was 25.1 mg/L. The higher
eritadenine yield obtained from shiitake mycelia upon the addition of a water extract of DDGS to the culture medium could be due to a higher biomass concentration or that some of the ingredients in the extract promoted production.

**Paper IV**

If eritadenine will be used as a pharmaceutical agent, knowledge about the solid state chemistry of the compound is of great importance. The solid state of the compound is of importance since it dictates the properties and performance of a drug, such as stability and bioavailability. Raman spectroscopy is a valuable technique for investigation of structural properties of organic compounds (160, 169, 170). It is a laser-light based spectroscopic technique which can be coupled to a microscope, and micro-Raman configurations have shown to be a highly valuable tool for the examination of structural properties as well as states of molecules. Hence, in the present study a Raman reference spectrum with line assignments for the solid state of synthetic eritadenine was established.

To establish the Raman spectrum of the cholesterol reducing compound eritadenine its synthetic analogue was used to obtain a high degree of purity of the compound. The Raman reference spectrum with line assignments of the solid state of eritadenine was accomplished by utilizing adenine and D-ribose, two of the main compounds involved in the synthesis of eritadenine, as reference samples, along with a synthesis intermediate. To follow the synthesis by Raman spectroscopy and assigning a reference spectrum to eritadenine, all Raman spectra were compared and analysed with respect to how structural changes affected the Raman shifts.

The intermediate, methyl 5-(6-Aminopurin-9H-9-yl)-2,3-O-isopropylidene-5-deoxy-β-D-ribofuranoside (MAIR), and D-eritadenine were synthesized as previously described (167). Solid state Raman spectra of all compounds were recorded with a micro-Raman spectrometer, using an excitation wavelength of 830 nm. The power
onto the sample was 23 mW and the integration time was 100 s, and for all measurements a 20× long working distance (LWD) microscope objective was used.

In the Raman spectrum of eritadenine, only one vibration detected at 773 cm⁻¹ was unique for this compound. This vibration was ascribed to vibrations in the carbon chain, either to 𝜏(C-C) or 𝜏(C-O). It was deduced that changes at the binding sites of N9 of adenine during synthesis caused the disappearance of Raman lines observed in the spectrum of pure adenine. In addition, since there is no sugar moiety in eritadenine, several of the D-ribose vibrations were not detected in the Raman spectrum of the former.

In summary, by comparing the spectra of the main components, adenine and D-ribose, and a synthesis intermediate of eritadenine, knowledge on the hitherto unassigned Raman spectrum of the solid state of eritadenine was obtained. Based on the information gained, a Raman spectrum with Raman line assignments of eritadenine could be established.

**Paper V**

To further investigate the solid state chemistry of eritadenine, its synthetic analogue in the form of sodium eritadenate was synthesized and its solid state characteristics were analyzed. A given drug substance can be present in several different solid forms; as crystalline polymorphs; as solvates or desolvated solvates, or as amorphous solids, and the solid form in turn affects the properties and performance of the drug (145, 152).

In this study sodium eritadenate was slowly crystallized from water and different aqueous ethanol solutions, at different temperatures, by means of cooling. Sodium eritadenate in water solutions was also rapidly solidified by means of lyophilization and by vacuum drying. The resulting solid forms were analysed by Raman spectroscopy, X-ray diffraction and thermal analysis, to reveal crystallinity, possible polymorphism and solvates or hydrates.
Sodium eritadenate was synthesized as previously described (167). The solubility of this compound in pure water and in 15, 30 and 50% (v/v) aqueous ethanol solutions was studied. Sodium eritadenate was in the concentration range 50-300 mg/mL for pure water, and 5-150 mg/mL for aqueous ethanol solutions. All solubility studies took place in microtiter wells which were placed on an orbital shaker, at 200 rpm. The solubility of sodium eritadenate was observed after 24 hours at 20, 30, 40 and 50 ºC for all solvent systems, and additionally at 60 ºC for pure water.

Based on the results from the solubility studies, different concentrations of sodium eritadenate were added to microtiter wells. For all solvent systems samples were heated and slowly cooled down, and the solid forms obtained at 20, 30, 40 and 50 ºC were collected and dried. Sodium eritadenate in pure water was also subjected to high vacuum drying and to lyophilization.

Solid state Raman spectra of all solids were recorded with a micro-Raman spectrometer. The excitation wavelength was 830 nm, the power onto the sample was 50 mW and the integration time was 10 sec. For the measurements a 20× LWD microscope objective was used. X-ray powder diffraction (XRPD) data were recorded, using CuKα radiation and variable slits. The samples were investigated in the 2-theta range 7-90º, in Bragg-Brentano geometry, with a step size of 0.01, for 15 hours. The thermal behavior of the solid samples was studied by means of differential scanning calorimetry (DSC), by scanning the samples from 20 to 260 ºC, at a heating rate of 10 ºC/min, under nitrogen purge. The changes in sample mass with temperature were measured by thermogravimetric analysis (TGA), by heating the samples from 20 to 260 ºC, at a heating rate of 10ºC/min, under nitrogen purge. Experiments for identification of the evolved gas on heating were conducted in flowing argon while heating the samples from 20 to 260 ºC, at a heating rate of 10 ºC /min.

All solid forms obtained from the crystallization of sodium eritadenate were screened for the occurrence of polymorphism by Raman spectroscopy. However, no
polymorphism with respect to solvent composition or temperature could be observed for solids resulting from slow cooling of solution. The Raman spectra of the solids resulting from fast cooling of solution had a more amorphous pattern. Further, XRPD measurements also indicated that solids resulting from slow cooling were crystalline and their patterns were similar, irrespective of the solvent used, whereas solid forms resulting from rapid cooling gave rise to a more amorphous pattern.

The crystalline solids obtained from slow cooling of solution were further studied by thermal analysis to investigate the thermal behavior and possible occurrence of solvates or hydrates. For sodium eritadenate crystallized from water a mass loss occurred in two steps in the temperature range 70-130 °C, which was identified as a loss of two water equivalents. For crystals forming from aqueous ethanol, a mass loss was detected between 55-125 °C and was again identified as a loss of water, but in this case it was reasonably 2.5 molecules of water per molecule sodium eritadenate. When heating the two different types of crystals to 160 °C, under nitrogen atmosphere, and investigating the anhydrous forms by Raman spectroscopy it could be seen that there was a transition to amorphous phase upon dehydration. Since the water loss resulted in more amorphous materials, water reasonably has a stabilizing effect on both types of hydrates.

In summary, the thermal analysis of crystals derived from water and from aqueous ethanol solutions indicated the former to be dihydrates and the latter 2.5 hydrates. These two types of hydrates showed no difference in crystal structure but clearly displayed different thermal behaviors. However, since the crystal structures appeared the same for both types of hydrates, the difference in water binding is plausibly small and could be attributed to secondary water molecules more outside the lattice. For both types of crystals, water molecules act as stabilizers and are most likely both incorporated into the crystal lattice and more loosely bound to the crystals.
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 shiitake mycelia 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, it was found that the mycelial morphology in shake flasks was as macroscopic aggregates and in bioreactors as more dispersed filaments. The production of eritadenine in shake flasks was significantly lower than in the bioreactors, whereas the biomass concentration was higher. From the results it could be deduced that optimal conditions for mycelial growth and eritadenine production do not coincide and that pH, stirring rate, shear and morphology might influence eritadenine production.

In paper III it was demonstrated that by supplementing the cultivation media with a water extract of DDGS, a considerable growth promotive effect on shiitake mycelia in bioreactor cultivations could be achieved along with enhanced eritadenine production. It was also demonstrated that the bioreactor cultivation time could be reduced by using shake flask cultures instead of mycelial agar plates as inocula, for certain agitation rates and pH settings. Further, by combining the results from this study with the results in paper II there are indications that that agitation speed, pH, glucose concentration and oxygen supply can influence both growth and eritadenine production by shiitake mycelia.

In paper IV a Raman reference spectrum of synthesized eritadenine with Raman line assignments was established by comparing the spectra of the starting materials, adenine
and D-ribose, with the spectrum of a synthesis intermediate and the spectrum of eritadenine. By comparing the structural changes during synthesis in relation to the Raman spectra, information could be gained to construct a Raman reference spectrum for eritadenine.

In paper V solid state characteristics of synthesized sodium salt of eritadenine were investigated by means of Raman spectroscopy, X-ray powder diffraction and thermal analysis. Prior to analysis the compound had been crystallized from water or aqueous ethanol solutions, at various temperatures and cooling methods. Solids forming from slow cooling from either water or aqueous ethanol showed crystallinity and similar Raman and XRPD patterns, irrespective of temperature of formation, whereas rapid cooling resulted in more amorphous solids. However, dissimilar thermal behaviors were observed for crystals derived from water and from aqueous ethanol, and the former were deduced as dihydrates and the latter as 2.5 hydrates. Finally, for both types of crystals water was plausibly both incorporated into the crystal lattice and more outside the lattice and had a stabilizing effect on the structures.

With the present work the initial steps towards the process of developing the cholesterol reducing compound eritadenine into an active pharmaceutical ingredient have been taken. In order to develop a new pharmaceutical, a well established process for its manufacture is required and in the present work submerged cultivation of shiitake mycelia was explored as a potential method for eritadenine production. Moreover, to evaluate dose-response effects of the compound in humans, a purification and quantification method of the compound is needed and these processes were also developed in the present study. Since most pharmaceuticals are supplied as solids, knowledge about the solid state of the compound is essential to obtain a drug with optimal performance. The solid state behavior of the compound, as crystallized from common solvents used in the pharmaceutical industry, was therefore investigated. Taken together, the results from the present study are of significance for subsequent large scale cultivations, downstream processing and clinical trials to establish dose-response effects of eritadenine. However, ultimately, the success of commercialization of a pharmaceutical relies on the process economics. If eritadenine is to be developed
into a drug with the manufacturing process investigated in the present study, significant improvements might be needed to make the process sustainable. If comparatively large quantities of eritadenine are required for cholesterol reducing effects the relatively low yields of eritadenine have to be enhanced and the cultivation time preferably reduced. This could be achieved by selecting high-producing strains of shiitake, or by elucidating the biosynthetic pathway of eritadenine and modify existing strains with respect to growth and productivity. Nevertheless, the approach to the development of eritadenine into an active pharmaceutical ingredient, as commenced by the present study, has opened the path for further exploitation of eritadenine as a new cholesterol reducing product.
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Paper I
Quantification of the Bioactive Compound Eritadenine in Selected Strains of Shiitake Mushroom (Lentinus edodes)

JOSEFINE ENMAN, ULRIKA ROVA,* AND KRIS A. BERGLUND

Division of Biochemical and Chemical Process Engineering, Luleå University of Technology, SE-97187 Luleå, Sweden

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 hypocholes-

* To whom correspondence should be addressed. Tel: +46(0)920-491315. Fax: +46(0)920-491199. E-mail: ulrika.rova@ltu.se.

Figure 1. Chemical structure of eritadenine ($M_r = 253\,\text{g/mol}$).
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, Kuikainen, 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 homogenous 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 (19). 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-amino-9H-9-yl]-5-deoxy-β-D-ribofuranose. The final step was an air oxidation of the previous compound to obtain the product 2(5R,3R)-1,2,3,4-tetrahydroxy-4-(9-adenyl) butyric acid (i.e., n-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 (5.98 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 of 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 carbohydrate 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 acetic 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 performed 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 (RSTEK 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 95:2 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 analyticalsoftware. 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.
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

<table>
<thead>
<tr>
<th>treatment</th>
<th>Le-1</th>
<th>Le-2</th>
<th>Le-A</th>
<th>Le-B</th>
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<tbody>
<tr>
<td>methanol extraction</td>
<td>3.50 ± 0.26</td>
<td>3.17 ± 0.07</td>
<td>3.24 ± 0.27</td>
<td>6.33 ± 0.03</td>
</tr>
<tr>
<td>enzymatic pretreatment in acetate buffer pH 4.8</td>
<td>3.82 ± 0.30</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>enzymatic pretreatment in water pH 6.0</td>
<td>3.60 ± 0.11</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* All values are mean ± SD from triplicate analyses. *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 slight increase in 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 differed from previous studies (13). Also, in this study, the mushrooms were thoroughly crushed into fine particles to make a homogenate of 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 of the mushroom extract (without any reference sample) (15) 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 0.0124–1.180 mg/mL. This method showed a linear response, r² 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.

ACKNOWLEDGMENT
We acknowledge Diversified Natural Products, Inc. (DNP), Scottville, MI for the contributions of shiitake strains and Novozymes, Bagsvaerd, Denmark for the preparation.

LITERATURE CITED
Paper 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*

Division of Biochemical and Chemical Process Engineering, Luleå University of Technology, SE-971 87 Luleå, Sweden

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,
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, 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 (Biobundle 12 L, 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 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 pH 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.

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 (10) using an HPLC system equipped with a Series 200 refractive index (RI) detector, a guard column, and an ion exchange column (Amnix 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.
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 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, 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 reactors were different, affecting the mass transfer of oxygen and nutrients. The lower biomass and eritadenine concentrations in the 12 L reactor might be attributed to the lower oxygen transfer rate and higher dilution rate.

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

<table>
<thead>
<tr>
<th>cultivation vessel</th>
<th>pH^i/pH^f</th>
<th>rpm (impeller tip speed, cm/s)</th>
<th>total sugar consumption (%)</th>
<th>biomass concn (g/L)</th>
<th>mycelia (mg/g)</th>
<th>broth (mg/L)</th>
<th>total (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>shake flask^cd</td>
<td>5.7/3.0</td>
<td>150 (0)</td>
<td>27</td>
<td>1.77 ± 0.02</td>
<td>0.02</td>
<td>0.50 ± 0.02</td>
<td>0.86 ± 0.05</td>
</tr>
<tr>
<td>1 L reactor</td>
<td>5.7/5.7</td>
<td>250 (58.6)</td>
<td>38</td>
<td>0.89</td>
<td>1.05</td>
<td>8.69</td>
<td>9.59</td>
</tr>
<tr>
<td>1 L reactor</td>
<td>5.7/4.2</td>
<td>250 (58.6)</td>
<td>34</td>
<td>0.84</td>
<td>0.21</td>
<td>10.04</td>
<td>10.23</td>
</tr>
<tr>
<td>1 L reactor^a</td>
<td>5.7/5.7</td>
<td>50 (11.8)</td>
<td>22</td>
<td>0.43 ± 0.06</td>
<td>0.36 ± 0.04</td>
<td>5.07 ± 0.36</td>
<td>5.25 ± 0.34</td>
</tr>
<tr>
<td>1 L reactor</td>
<td>5.7/5.0</td>
<td>50 (11.8)</td>
<td>16</td>
<td>0.36</td>
<td>0.16</td>
<td>5.44</td>
<td>5.50</td>
</tr>
<tr>
<td>12 L reactor</td>
<td>5.7/5.7</td>
<td>150 (78.5)</td>
<td>16</td>
<td>0.34</td>
<td>0.90</td>
<td>3.25</td>
<td>3.32</td>
</tr>
</tbody>
</table>

^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.
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.

LITERATURE CITED


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Paper III
Growth Promotive Conditions for Enhanced Eritadenine Production during Submerged Cultivation of *Lentinus edodes*

Josefine Enman¹, David Hodge¹,**, Kris Arvid Berglund¹²,³ and Ulrika Rova¹*

¹ Department of Chemical Engineering and Geosciences, Luleå University of Technology, SE-971 87 Luleå, Sweden
² Department of Chemical Engineering and Materials Science, Michigan State University, East Lansing, MI 48824, USA
³ Department of Forestry, Michigan State University, East Lansing, MI 48824, USA

* To whom correspondence should be addressed. Tel: +46(0)920-491315. Fax: +46(0)920-491199. E-mail: ulrika.rova@ltu.se

** Currently at Department of Chemical Engineering & Materials Science, Michigan State University, East Lansing, MI 48824, USA
Abstract

Mycelia of the medicinal mushroom shiitake, *Lentinus edodes*, is a potential source for production of the blood cholesterol reducing compound eritadenine, 2(\(R\))-3(\(R\))-dihydroxy-4-(9-adenyl)-butanoic acid. Hence, in the present study the production of eritadenine by shiitake mycelia grown in submerged conditions in bioreactors was investigated. With the objective to increase the biomass and in turn the production of eritadenine, a water extract of distillers dried grains with solubles (DDGS), a by-product from the dry-grind ethanol industry, was added to the culture media. Further, in an effort to reduce the cultivation time, different types of inocula were used for the bioreactor cultivations. The hot water extract of DDGS was shown to considerably increase the growth of shiitake mycelia; the mycelial yield was 2-3 times higher than in the control, containing malt and yeast extract only, and the highest final biomass concentration obtained was 3.4 g/L. Further, by using shake flask cultures as inocula the cultivation time could be reduced by one week for some of the experiments. The highest final titer of eritadenine in the present study was 25.1 mg/L, which was about 2 times higher than in the control, and was also obtained when a water extract of DDGS was added to the culture medium. Finally, the results from the present study indicate that pH and agitation rate might have an impact on mycelial growth and production. When pH was uncontrolled during the submerged cultivations the growth was faster than when pH was controlled at either 4.2 or 5.2, or when the agitation rate was set to 500 rpm. However, when pH was controlled slightly higher eritadenine concentrations were obtained, as compared to other experiments lacking the addition of a water extract of DDGS.
1. Introduction

The shiitake mushroom, *Lentinus edodes*, is widely cultivated and consumed as food and also used as a medicinal mushroom since it is a source of numerous biologically active substances. One of the pharmacologically active compounds derived from this fungus is the blood cholesterol reducing secondary metabolite, eritadenine, \(2(R),3(R)\)-dihydroxy-4-(9-adenyl)-butanoic acid (Figure 1), which has been detected both in fruit bodies [1, 2] and mycelia [3-5] of shiitake.

![Figure 1. D-Eritadenine.](image)

The fruit bodies are usually the source for the manufacture of most commercial products derived from medicinal mushrooms. However, there are some drawbacks when using the former as a production source; the cultivation is time consuming and the quality of the product is difficult to control. On the other hand, submerged cultivation of mycelia is widely applied for production of commercial products from filamentous microfungi. Like other filamentous fungi, cells of medicinal mushrooms have the ability to be cultivated in submerged conditions and their growth and metabolite production can be altered in response to environmental changes [6-10]. Hence, bioactive metabolites from medicinal mushrooms can potentially be produced by submerged cultivation. Since eritadenine is produced by shiitake mycelia [3, 4], submerged cultures of mycelia could be favourable for the production of this compound. In a previous work [3] eritadenine was produced by submerged cultivation of
shiitake mycelia and was detected both in the mycelia and in the culture media. Further, significantly more eritadenine was detected in the culture media than was accumulated in the biomass during cultivation. These findings encourage further studies of the process conditions that can affect growth and eritadenine production.

For submerged cultivation of filamentous fungi there is generally a complex relationship between the process parameters and the growth, morphology and productivity. Factors such as pH, medium composition, agitation and the nature of the inoculum have shown to highly influence the process outcome [11-14]. However, the knowledge about submerged cultivations of mushrooms and the accompanying responses to environmental alterations is rather scarce, compared to submerged cultivations of filamentous microfungi.

One way to promote the growth of mycelia, with an anticipated increase in product formation, is to construct a favourable medium for cultivation. While the preferred substrate is hardwoods in its natural environment, the shiitake mushroom has the ability to grow on a variety of other materials. Hence, shiitake mycelia have successfully been grown in submerged conditions on wastes or by-products such as lignocellulosic wastes in the form of tree leaves and different fruit peels [15], a hot water-soluble fraction from corn fiber [16], rice bran and sugar cane molasses [17]. One industrial by-product which could have the potential to promote growth of shiitake mycelia in submerged conditions is distillers dried grains with solubles, DDGS. This is a major by-product of the dry-grind facilities for ethanol production from grains and is derived from unfermented components of the grains such as protein, fiber, oil and starch and due to the nutritional content in DDGS it is mainly used as a feed ingredient for livestock [18]. It should also be stressed that the shiitake mushroom has the capability to extract nutrients for growth and metabolite production from complex nutrient sources, which could enable its usage of the unfermented components of DDGS.
In this study it was therefore investigated if a water extract of DDGS could be used as a growth promoting substance for the submerged cultivation of shiitake mycelia and if the production of the bioactive compound eritadenine could be enhanced. Moreover, cultivation of shiitake mycelia is quite time consuming [3] and should preferably be shortened to improve process economics. In an attempt to reduce the cultivation time in the bioreactors, two approaches to inoculation were tested; as an alternative to inoculate directly from MYA plates, shake flask cultures were also used as inocula in the present study. Finally, pH and stirring rate might have an impact on growth and eritadenine production of shiitake mycelia and these factors were thus evaluated in the present study.

2. Materials and methods

2.1 Preparation of a hot water extract of DDGS. Agrodrank™ 90 was supplied by Lantmännen Agroetanol (Norrköping, Sweden). A hot water extract of DDGS was prepared mainly according to the procedure of Arai et al. [16]. The extract was obtained by mixing 100 g of Agrodrank™ 90 with 1L of distilled water and then the extraction was carried out for 3 h at 80 °C. The residue was removed by filtration and the filtrate was then concentrated ten times in vacuo, at 40-50 °C.

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

2.3 Inoculum preparation. The mycelia from either MYA plates or shake flask cultures were used as inocula for bioreactor cultivation. In the former case mycelia were cut from a MYA plate (90 mm in diameter) and homogenized in a 0.05 mM phosphate buffer, pH 5.8, prior to inoculation. In the latter case mycelia were cut from a MYA plate (90 mm in diameter),
homogenized in a 0.05 mM phosphate buffer, pH 5.8, and aseptically transferred to 100 mL sterilized malt yeast medium composed of, per L: 20 g malt extract and 2 g yeast extract. Prior to the inoculation for bioreactor cultivations the mycelia were cultivated in shake flasks for 10 days, at 150 rpm and 23 °C.

2.4 Bioreactor cultivation. The submerged cultivations took place in 1 L bioreactors (Biobundle 1 L, Applikon Biotechnology, the Netherlands) with a total starting volume of 700 mL of malt yeast media, prepared as described above. Addition of extra nutrients for growth promotion was supplied to the cultivation media by 10% (v/v) of a water extract of DDGS. All bioreactor cultivations were conducted at a temperature of 25 °C and the dissolved oxygen was controlled at 90 %. The initial pH was adjusted to 4.2 or 5.2 with 2 M H2SO4, and kept either uncontrolled or controlled by automatic addition of 5 M KOH. The stirring rates applied were 125, 250 or 500 rpm, and the cultivations lasted between 13- 20 days.

2.5 Biomass determination. Following cultivation, the mycelia from bioreactors were harvested by filtering the culture broth through Whatman No. 42 filter paper. After a subsequent wash with distilled water the biomass was dried over night and the dry cell weight (DCW) was determined.

2.6 Analysis of eritadenine. The preparation of an eritadenine reference sample and the whole procedure for eritadenine analysis was implemented as described previously [19]. Prior to eritadenine analysis the mycelial biomass was extracted with 80% methanol and the filtered broth was purified by means of ion exchange chromatography. Eritadenine concentrations were then analyzed by HPLC (Series 200 Quaternary LC pump and UV-VIS detector, TotalChrom software, PerkinElmer) and separated over a C18 column (RESTEK ultra aqueous, 5 μm, 4.6 mm x 150 mm). HPLC analysis was conducted at 23 °C, with UV detection at 260 nm and a flow rate of 1 mL/min. A gradient system was applied starting with
0.05% TFA in aqueous solution: 0.05% TFA in MeCN, in the proportions 98:2, and linearly increasing 0.05% TFA in MeCN to 60%, during 10 min.

2.7 Analysis of a hot water extract of DDGS. For analysis of mono- and oligosaccharides and glycerol an HPLC system equipped with a Series 200 refractive index (RI) detector, and either a BioRad Aminex HPX87-P column or a BioRad Aminex HPX87-H column, was used. In the former system the column was kept at 85°C in a column oven and water was used as the mobile phase. In the latter system the column was kept at 65°C and 0.005 M H₂SO₄ was used as the mobile phase. For both systems a flow rate of 0.6 mL/min was applied. For organic acid analysis an HPLC (Series 200 Quaternary LC pump and UV-vis detector, Perkin-Elmer) equipped with a C18 column (Ultra Aqueous, 5 µm, 150 mm × 4.6 mm, Restek) was used. HPLC analysis was conducted at 23 °C, with UV detection at 210 nm and a flow rate of 0.35 mL/min. An isocratic system was applied using 50 mM KH₂PO₄ buffer with 2% MeCN (pH 2.5) as a mobile phase.
3. Results and discussion

In a previous study [3], the hypocholesterolemic metabolite eritadenine was produced by submerged cultures of shiitake mycelia, making the latter a conceivable means of production of this compound. In the present study the objectives was to improve the cultivation process by increasing the mycelial biomass and eritadenine concentrations and also to shorten the bioreactor cultivation time. To achieve this, a potential growth promotive substance, in the form of a water extract of DDGS, was added to the culture media. In feasibility studies (data not shown) of the growth promotive effect of DDGS in shake flask cultures, an addition of 10% (v/v) of the extract was found to be suitable for subsequent bioreactor cultivations. Moreover, the resulting pH in the culture media upon addition of DDGS was found to be 4.2, hence this pH was set as the standard initial pH for the bioreactor experiments. Further, in an attempt to reduce the initial lag phase and in turn the total time required for cultivation in bioreactors, two different types of inocula were evaluated; either mycelia from MYA plates or shake flask cultures.

From the results (Table 1) it could be observed that addition of a water extract of DDGS to the cultivation media resulted in the highest biomass concentrations, when comparing all experiments. The highest biomass concentration, 3.4 g/L, was obtained after 13 days of cultivation when the culture medium was supplemented with a water extract of DDGS and a shake flask culture was used as inoculum.
Table 1. Bioreactor cultivations of shiitake mycelia at various conditions and the resulting biomass and eritadenine concentrations.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Cultivation conditions</th>
<th>Inoculum type</th>
<th>Media</th>
<th>Time (days)</th>
<th>Biomass concn (g/L)</th>
<th>Eritadenine concn (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>250 Rpm 4.2/3.5 Plate</td>
<td>MY</td>
<td>MYd</td>
<td>20</td>
<td>1.1±0.05</td>
<td>10.1±0.80</td>
</tr>
<tr>
<td>2</td>
<td>250 Rpm 4.2/3.5 Plate</td>
<td>10% DDGS</td>
<td>MYd</td>
<td>20</td>
<td>3.2</td>
<td>25.1</td>
</tr>
<tr>
<td>3</td>
<td>250 Rpm 4.2/3.6 Shake flask</td>
<td>MYd</td>
<td>MYd</td>
<td>13</td>
<td>1.5±0.21</td>
<td>10.8±0.38</td>
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<td>4</td>
<td>250 Rpm 4.2/3.6 Shake flask</td>
<td>10% DDGS</td>
<td>MYd</td>
<td>13</td>
<td>3.4±0.28</td>
<td>21±0.66</td>
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<td>MYd</td>
<td>20</td>
<td>1.5±0.14</td>
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</tr>
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<td>MYd</td>
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<td>12.3±0.79</td>
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<tr>
<td>7</td>
<td>500 Rpm 4.2/3.8 Shake flask</td>
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<td>MYd</td>
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<td>1.2±0.04</td>
<td>8.7±0.64</td>
</tr>
<tr>
<td>8</td>
<td>125 Rpm 4.2/3.5 Shake flask</td>
<td>MYd</td>
<td>MYd</td>
<td>13</td>
<td>1.1±0.19</td>
<td>8.4±0.28</td>
</tr>
<tr>
<td>[3]</td>
<td>250 Rpm 5.7/4.2 Plate</td>
<td>MYd</td>
<td>MYd</td>
<td>20</td>
<td>0.84</td>
<td>10.2</td>
</tr>
</tbody>
</table>

* Average values ± SD of two independent cultivation experiments. \(^a\) Initial. \(^b\) Final. \(^d\) Malt yeast medium. \(^e\) Malt yeast medium with 10% (v/v) of a water extract of DDGS. [3] J. Enman et al. 2008.

Experiment 1 and 2 were the same except for the addition of a water extract of DDGS to the cultivation medium in the latter, by which the final biomass concentration increased approximately 3 times. Likewise, experiment 3 and 4 were the same in all respects apart from the addition of a water extract of DDGS in the latter case, which in turn increased the biomass concentration approximately 2 times. From these data it can be deduced that a water extract of DDGS has growth promotive effects on shiitake mycelia in submerged conditions.

The DDGS used in the present study is derived from wheat, barley and triticale and the major components are fibers and protein whereas fat, ash and starch each comprise a smaller fraction (Table 2). However, in the present study a hot water extract of DDGS was used, which is in essence the soluble fraction from the distillation residual that is also known as distillers solubles (DS) or sometimes referred to as syrup. By combining the results from the
analysis of the hot water extract in this study with a previous analysis of a similar extract [18] it was shown to contain mainly mono- and oligosaccharides, organic acids, proteins and fermentation by-products such as glycerol and oil. Shiitake mycelia have been shown to grow well when glycerol was added as a carbon source whereas the growth on succinic acid and some other organic acids was more moderate [20]. Further, nitrogen has been recognized as a limiting factor for growth of shiitake [21] and addition of complex (organic) nitrogen sources in particular have been found to cause a pronounced increase in growth [22].

Table 2. Average percentages of major nutrients of Agrodrank™. Adapted from Lantmännen Agroetanol (Norrköping, Sweden).

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>% per dry substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral detergent fibers</td>
<td>35.5</td>
</tr>
<tr>
<td>Crude protein</td>
<td>34.1</td>
</tr>
<tr>
<td>Crude fat</td>
<td>6.6</td>
</tr>
<tr>
<td>Ash</td>
<td>4.9</td>
</tr>
<tr>
<td>Starch</td>
<td>1.5</td>
</tr>
</tbody>
</table>

While in nature the shiitake mushroom lives on decomposing hardwoods in which polysaccharides are the most abundant components, it is considered to be a strong degrader of starch and lignin, but a weak cellulose degrader [21]. Since the grains used for ethanol production undergo saccharification prior to the yeast fermentations, most of the starch is converted into sugars. However, when shiitake mycelia were cultivated on wood substrate, addition of extra starch did not improve the growth [22]. It is possible that the growth improvement caused by the water extract of DDGS can be attributed to the nature or content of the nitrogen source, or some of the organic carbon sources. Addition of oil for example has been shown to improve biomass production in the medicinal mushroom *Ganoderma lucidum*, by increasing the efficiency of glucose conversion [9]. There is also a possibility that complex effects caused by the various components of the extract enhanced the growth.
To investigate the effect on growth in bioreactor cultivations with respect to the type of inoculum used, in experiment 1 (Table 1) mycelia from a MYA plate were used as inoculum and in experiment 3 a shake flask culture was used as inoculum. When using a shake flask culture as inoculum, a slightly higher biomass concentration was achieved after 13 days of cultivation compared to 20 days of cultivation and using mycelia from a plate as inoculum. In all subsequent experiments shake flask cultures were used as inocula, with the anticipated time reduction for bioreactor cultivations. The faster growth rate as induced by shake flask cultures as inocula could be due to a higher biomass concentration and/or a by-pass of the initial lag phase that can occur upon transferring mycelia from solid state to liquid conditions. However, given that the mycelial growth was not accelerated by using an increased number of plates (data not shown), the latter is more plausible. A cultivation time of 13 days also resulted in approximately the same biomass concentration, yet slightly lower, when 125 rpm was applied as the agitation speed, compared to when 250 rpm was applied. However, when the agitation speed was set to 500 rpm, the growth was rather slow compared to the growth at an agitation rate of 250 or 125 rpm, and hence 20 days was required to obtain a comparable biomass concentration. These results put forward that for these types of cultivation vessels, an agitation speed of less than 500 rpm is beneficial for shiitake mycelial growth. When pH of the media was controlled at either 4.2 or 5.2 the growth rate was also slower as compared to when pH was not controlled, for the same agitation speed, and 20 days of cultivation was needed to achieve a comparable biomass concentration. This suggests that a pH lower than 4.2 in the media might stimulate growth, which was observed previously in shake flask cultures [3]. In other studies [17, 23] it has also been suggested that optimal pH for growth of shiitake mycelia in submerged conditions is in the range 3-3.5. Finally, when a water extract of DDGS was added to the media, approximately the same biomass concentration was
attained after 13 days of cultivation, when a shake flask culture was used as inoculum, as after 20 days of cultivation and using a mycelial plate as inoculum.

All the biomass concentrations in the present study were higher than in the previous study on bioreactor cultivations of the same shiitake strain [3]; at the most approximately 4 times higher (Table 1). The major differences pertaining to all experiments were that no additional glucose was added to the media in the present study and to avoid unnecessary water evaporation the dissolved oxygen concentration was set to 90% instead of applying a continuous air flow. The different aeration methods could in turn affect growth and productivity, which have been demonstrated for submerged cultures of *Ganoderma lucidum* for production of polysaccharides and ganoderic acid [10]. Since the carbon source was found not to be a limiting factor for shiitake mycelial growth and eritadenine production [3], no additional glucose was added to the culture media in the present experiments. In another study glucose was also shown not to be growth limiting for shiitake mycelia and the addition of extra glucose, when the mycelia grew on wood substrate, did not enhance growth [22]. Moreover, in its capacity as wood decomposer, the shiitake mushroom naturally grows by capturing sugars and other nutrients, such as nitrogen, by breaking down complex carbohydrates and it can be speculated that these actions stimulate mycelial growth.

Like the biomass concentrations, the eritadenine concentrations (Table 1) in the present study were in the same range for most experiments devoid of the water extract of DDGS in the culture media. If comparing experiment 1 and 3, for which mycelia from a MYA plate and a shake flask culture were used as inoculum, respectively, there was no major difference in eritadenine concentrations. However, in the former experiment the biomass concentration was less and the growth slower. A comparatively slower growth was also observed when pH was controlled at either 4.2 or 5.2, but the concentrations of eritadenine were slightly higher compared to other experiments lacking a water extract of DDGS in the culture media. Shake
flask cultures of shiitake mycelia have previously [3] been shown to contain lower concentrations of eritadenine compared to bioreactor cultures, which can be due to e.g. low pH of the culture media in the former. The results in the present study also give indications that pH might influence the production of eritadenine. Moreover, the mycelia in shake flask cultures appeared as more dense pellets compared to more dispersed filaments in the bioreactor cultures and the lower eritadenine concentrations in shake flasks could also be due to the mycelial morphology in the shake flasks. However, in the present investigation shake flask cultures were collected after ten days of cultivation and used as inocula. By this time no apparent dense pellet formation was observed, and the induction of shear by the impeller in the bioreactors created more dispersed filaments or loose pellets of mycelia, which might be beneficial for eritadenine production. However, it should be emphasized that the inoculum type might have influenced the production in the present study.

Although all the biomass concentrations in the present study were slightly higher than obtained earlier for bioreactor cultivations of the same shiitake strain [3], not all experiments resulted in a higher eritadenine concentration (Table 1). However, in the present study a different aeration method was applied, as discussed previously, which might influence metabolite production. Different methods for supplying oxygen and the oxygen availability have been shown to have a significant influence on mycelial morphology and lovastatin production by *Aspergillus terreus* [24, 25]. Moreover, the initial and final pH were both lower in the present study compared to the previous [3], which can also contribute to the mode of eritadenine production. The initial and final pH have been shown to effect both growth and product formation in *Ganoderma lucidum* [8]. The extra glucose added to the media in the previous investigation [3] can also influence the production of eritadenine. The carbon source and concentration have e.g. been shown important for lovastatin production by *Aspergillus*
The highest eritadenine concentrations were obtained when a water extract of DDGS was added to the culture media (Table 1); up to 25.1 mg/L when the mycelia were cultured 20 days and mycelial agar plate was used as inoculum. In this case the biomass concentration was also comparatively high. However, the increase in eritadenine concentrations was not directly correlated to the growth increase promoted by the DDGS extract. As carbon and nitrogen sources are important for biomass production, they also influence metabolite production and usually the factors enhancing growth diverge from these optimal for secondary metabolite production. Increased nitrogen has been shown to have a positive effect on biomass production in Aspergillus terreus but the specific production of lovastatin was not improved [24]. As stated above, production of lovastatin by Pleurotus ostreatus has been shown to depend on the carbon and nitrogen source and their ratios. The maximum value of the lovastatin yield in this case was obtained when organic nitrogen was present in the culture medium [7]. Neither the reason for shiitake to produce eritadenine nor the biosynthetic pathway for this compound is yet understood. Secondary metabolites are generally produced from common metabolic intermediates, yet the production is often highly species and strain specific. The higher eritadenine yields obtained from shiitake mycelia upon adding a water extract of DDGS to the culture medium can be due to a higher biomass concentration or there is a possibility that some precursor or otherwise stimulating substance or nutrient for eritadenine production is present in the extract and hereby increase the yield.

4. Conclusions

In the present study it was demonstrated that an amendment of the cultivation medium with a water extract of DDGS caused a considerable growth promotive effect on shiitake mycelia in
bioreactor cultivations, along with enhanced eritadenine production. Hence, the highest final biomass and eritadenine concentrations were obtained when a water extract of DDGS was added to the culture media. It was also demonstrated that the bioreactor cultivation time could be reduced by using shake flask cultures instead of mycelial agar plates as inocula, if pH was uncontrolled and the agitation rate was 125 or 250 rpm, but not at an agitation rate of 500 rpm. Further, when pH was controlled at either 5.2 or 4.2 the mycelial growth rate was not increased despite the usage of shake flask cultures as inocula. On the other hand by comparing all experiments devoid of the water extract of DDGS, the eritadenine concentrations were slightly higher in these two latter experiments.

References


Paper IV
Eritadenine, 2(R),3(R)-dihydroxy-4-(9-adenyl)-butyric acid, is a cholesterol-reducing compound naturally occurring in the shitake mushroom (Lentinus edodes). To identify the unknown Raman spectrum of this compound, pure synthetic eritadenine was examined and the vibrational modes were assigned by following the synthesis pathway. This was accomplished by comparing the known spectra of the starting compounds adenine and D-ribose with the spectra of a synthesis intermediate, methyl 5-(6-Aminopurin-9H-9-y1)-2,3-O-isopropylidene-5-deoxy-β-D-ribofuranoside (MAIR) and eritadenine. In the Raman spectrum of eritadenine, a distinctive vibrational mode at 773 cm$^{-1}$ was detected and ascribed to vibrations in the carbon chain, $\nu$(C–C). A Raman line that arose at 1212 cm$^{-1}$, both in the Raman spectrum of MAIR and eritadenine, was also assigned to $\nu$(C–C). Additional Raman lines detected at 1526 and at 1583 cm$^{-1}$ in the Raman spectrum of MAIR and eritadenine were assigned to $\nu$(N–C) and a deformation of the purine ring structure. In these cases the vibrational modes are due to the linkage between adenine and the ribofuranoside moiety for MAIR, and between adenine and the carbon chain for eritadenine. This link is also the cause for the disappearance of adenine specific Raman lines in the spectrum of both MAIR and eritadenine. Several vibrations observed in the spectrum of D-ribose were not observed in the Raman spectrum of eritadenine due to the absence of the ribose ring structure. In the Raman spectrum of MAIR some of the D-ribose specific Raman lines disappeared due to the introduction of methyl and isopropylidene moieties to the ribose unit. With the approach presented in this study the so far unknown Raman spectrum of eritadenine could be successfully identified and is presented here for the first time.

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KEYWORDS: eritadenine; Raman spectroscopy; Raman line assignment; bioactive compound

INTRODUCTION

Hypercholesterolemia is an accepted risk factor for the development of cardiovascular disease which is one of the most serious health concerns in modern Western society. Frequently prescribed drugs against hypercholesterolemia are the statins which were originally isolated from fungi such as Aspergillus terreus. Although the statins are efficient cholesterol-reducing agents, their side effects are a matter of debate. Another fungal-derived cholesterol-reducing compound is eritadenine, 2(R),3(R)-dihydroxy-4-(9-adenyl)-butyric acid (Fig. 1(D)), naturally occurring in the shitake mushroom, Lentinus edodes. The shitake mushroom has been shown to lower blood cholesterol in both animals and humans and the mechanism of action of eritadenine has been investigated in several studies on rats. Unlike the statins, there are no indications of eritadenine inhibiting the biosynthesis of cholesterol. Rather, eritadenine has been suggested to accelerate the removal of blood cholesterol either by stimulated tissue uptake or by inhibited tissue release. Since eritadenine exerts another mechanism for its hypocholesterolemic effect than the statins, it could potentially be used in combination with the statins to diminish the side effects of the latter such as liver damage, rhabdomyolysis, and myotoxicity.

In the process of developing pharmaceuticals, the solid state of the compound is of great importance since it will affect the properties and performance of a drug, such as stability and bioavailability. One convenient way of determining the solid-state structures of organic compounds is by Raman analysis. Raman spectroscopy is a laser-light based spectroscopic technique that can be coupled to a microscope. Micro-Raman configurations have proven to be most valuable for the examination of structural properties as well as states of molecules.

In the present investigation a Raman spectrum of the cholesterol-reducing compound eritadenine, was collected...
Raman analysis of synthetic eritadenine

and analyzed. To obtain a high degree of purity, synthetic eritadenine was used. The goal of this study was to obtain a Raman reference spectrum with line assignments of the solid state of eritadenine. In order to accomplish this, adenine and D-ribose, two main compounds involved in the synthesis of eritadenine were used as reference samples. A spectrum of a synthesis intermediate was also investigated to follow the course of synthesis.

EXPERIMENTAL

Adenine (Fig. 1(A)) (>99% purity) and D-ribose (Fig. 1(B)) (>99% purity) were obtained from Sigma, and methyl 5-(6-Aminopurin-9H-9-yl)-2,3-O-isopropylidene-5-deoxy-β-D-ribofuranoside (MAIR) (Fig. 1(C)) and D-eritadenine (Fig. 1(D)) were synthesized as previously described.

In brief, the synthesis starts with the attachment of a methyl group to O1, and an isopropylidene moiety to O2 and O3 of D-ribose. Then an adenine moiety is bound to the ribofuranoside moiety at C5 (Fig. 1(C)). In the last step, the ribofuranoside ring is opened and the final product eritadenine, 2(R),3(R)-dihydroxy-4-(9-adenyl)butyric acid (Fig. 1(D)) is obtained. The synthesis intermediate, methyl 5-(6-Aminopurin-9H-9-yl)-2,3-O-isopropylidene-5-deoxy-β-D-ribofuranoside was here designated as MAIR due to the lack of a trivial name.

The solid-state Raman spectra of all compounds were recorded with a Renishaw 2000 micro-Raman spectrometer. The excitation wavelength was 830 nm; the power onto the sample was 23 mW; and the integration time was 100 s. For all measurements a 20× LWD microscope objective was used.

RESULTS AND DISCUSSION

The Raman spectrum of eritadenine was established by following the synthesis pathway. Spectra of the two starting components, adenine (Fig. 2(A)) and D-ribose (Fig. 2(B)), as well as of MAIR (Fig. 2(C)) and the final product eritadenine (Fig. 2(D)) were recorded, and Raman lines that significantly changed throughout the synthesis process are listed in Table 1. The assignments of the Raman lines of adenine are according to the study of Santamaria et al. and of D-ribose, according to Carmona and Molina. The Raman spectrum of eritadenine was established by comparing the spectra of all compounds involved and by investigating how new bonds and breaking of existing bonds, i.e. structural changes, affected the Raman shifts.

Polarization measurements performed for eritadenine verified a random orientation for the solid state. However, by changing the polarization direction by 90°, the lines at 1066 and 1085 cm\(^{-1}\) were slightly increased. These lines are due to the v(C–O) and v(C–C) of the carbon chain moiety whose structural properties enable certain orientations. The Raman lines observed in the spectrum of eritadenine are summarized in Table 2.
Figure 2. Raman spectra of the solid states of (A) Adenine, (B) D-Ribose, (C) methyl 5-[8-Aminopurin-9H-9-yl]-2,3-O-isopropylidene-5-deoxy-β-D-ribofuranoside (MAIR), and (D) 2(R,3(R)-dihydroxy-4-[8-adenyl]-butyric acid (eritadenine).

Several D-ribose Raman lines were not observed in the spectra of MAIR and eritadenine. For instance, the Raman shift at 1123 cm$^{-1}$ for D-ribose is caused by $\nu$(C2–O). The shift was absent for MAIR due to the isopropylidene moiety at O2'. In eritadenine, on the other hand, the absence of this Raman line can again be explained by the lack of a ribose moiety.

Raman lines at 1148 and 1160 cm$^{-1}$ assigned to $\nu$(C–O) and $\nu$(C–C) and to $\nu$(C1–O1) and $\nu$(C1–C2), respectively, could not be detected in the spectra of MAIR and eritadenine. In MAIR the ribofuranoside unit is more rigid than D-ribose due to the introduction of a methyl group at O1'. In eritadenine, the opening of the ring structure is the reason for the disappearance of these Raman lines.

The Raman bands at 1280 and 1290 cm$^{-1}$ are assigned to $\nu$(C5–H2). These lines were missing in the spectra of MAIR and eritadenine, since C5 is bound to adenine in both cases. This implies an increased rigidity, which in turn affects the torsion.

At 1330 cm$^{-1}$, the $\delta$(C2–H) and $\omega$(C5–H2) bands were observed for D-ribose. In MAIR C2–H is linked to the isopropylidene group, and hence this vibration was not detected. C5–H2, on the other hand, is linked to the adenine part both in MAIR and eritadenine, hence explaining this missing vibration.

Finally, the Raman line at 1460 cm$^{-1}$ in the spectrum of D-ribose is due to $\delta$(C5–H2) and was neither observed in the Raman spectrum of MAIR nor in the spectrum of eritadenine due to the introduction of the adenine moiety.

Some D-ribose Raman lines observed in the spectrum of MAIR could not be observed in the spectrum of eritadenine. The line at 400 cm$^{-1}$ in D-ribose and in MAIR is due to $\delta$(C–C–C) and could not be detected for eritadenine. In MAIR the ribose moiety is still intact, but during the last step of the synthesis the ribose ring structure is opened; thus this bending mode is not possible in eritadenine. The symmetric ring bend of D-ribose at 551 cm$^{-1}$ was present in the Raman spectrum of MAIR but was not detected in the Raman spectrum of eritadenine due to the opening of the ring structure. Certain D-ribose Raman lines, on the other hand, were observed in the spectrum of eritadenine, but not in the spectrum of MAIR. For instance, the line observed in the Raman spectrum of D-ribose at 880 cm$^{-1}$ was assigned to a mixture of $\nu$(C–C) and $\nu$(C–O). These vibrations were not observed in the Raman spectrum of MAIR but arose again in the Raman spectrum of eritadenine. In eritadenine the stretch is possible at (C2 – O) and (C3 – O), since C2 and C3 are bound to hydroxyl groups. In MAIR there are no hydroxyl groups bound to the carbon sites and hence this vibration does not take place.

Some of the adenine Raman lines could not be observed in the spectra of MAIR or eritadenine. More precisely, the deformation of the five-membered ring (R5) and the squeeze of N7–C8–N9 of adenine at 910 and at 1038 cm$^{-1}$ disappear. This can be explained by the fact that the N9 site is either

was not observed in the spectrum of eritadenine because of the absence of a ribose ring structure.

The Raman shift at 1123 cm$^{-1}$ for D-ribose is caused by $\nu$(C2–O). The shift was absent for MAIR due to the isopropylidene moiety at O2'. In eritadenine, on the other hand, the absence of this Raman line can again be explained by the lack of a ribose moiety.
Table 1. Raman lines and their assignments for the solid states of adenine (Santamaria et al.\textsuperscript{15}), ribose (Carmona and Molina\textsuperscript{16}), MAIR, and eritadenine (from this study)

<table>
<thead>
<tr>
<th>Adenine [cm\textsuperscript{-1}]</th>
<th>Ribose [cm\textsuperscript{-1}]</th>
<th>MAIR [cm\textsuperscript{-1}]</th>
<th>Eritadenine [cm\textsuperscript{-1}]</th>
<th>Assignment adenine</th>
<th>Assignment ribose</th>
<th>New lines</th>
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<td>-</td>
<td>-</td>
<td>400</td>
<td>-</td>
<td>$\delta$(C–C–C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>417</td>
<td>500</td>
<td>$\delta$(C–C–C) + $\delta$(C–C–O)</td>
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<tr>
<td>536</td>
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<td>-</td>
<td>538</td>
<td>Def R6 sqz group (N1–C6–C5–C2–N3–C4)</td>
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<td></td>
</tr>
<tr>
<td>550</td>
<td>551</td>
<td>548</td>
<td>-</td>
<td>Symmetric ring bend</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
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<td>$\delta$(C–C–C) + $\delta$(C–C–O)</td>
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<td>794/830</td>
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<td>-</td>
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<tr>
<td>880</td>
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<td>$\nu$(C–C) + $\nu$(C–O)</td>
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<td>910</td>
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<td>-</td>
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<tr>
<td>935</td>
<td>-</td>
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<td>$\nu$(C1–C) + $\nu$(C1–O) + $\nu$(C1–H)</td>
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<tr>
<td>1038</td>
<td>-</td>
<td>-</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1123</td>
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<td>-</td>
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<td></td>
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<td>-</td>
<td>-</td>
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<td>1148</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>$\nu$(C–O) + $\nu$(C–C)</td>
<td>$\nu$(C–H\textsubscript{2})</td>
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<tr>
<td>1160</td>
<td>-</td>
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<td>$\nu$(C1–O) + $\nu$(C1–C2)</td>
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<td>-</td>
<td>Def R5, R6($\nu$(C5–N7)), $\delta$(N10–H), $\delta$(N10–H)</td>
<td></td>
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<tr>
<td>1212</td>
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<td>$\nu$(C4–C5) + $\nu$(C3–C4)</td>
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<td>1460</td>
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<td>$\delta$(C2–H) + $\omega$(C–H\textsubscript{2})</td>
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<td>1526/1583</td>
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<td>Def R5, R6 v(N9–C5) + v(N9–C4)</td>
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bound to the ribofuranoside moiety (for MAIR) or to the carbon chain (for eritadenine); hence these structures are more rigid. In addition, the Raman lines at 1138 and at 1175 cm\textsuperscript{-1} in the adenine spectrum are due to the deformation of the five-membered ring, R5, and the six-membered ring, R6, as well as $\delta$(C2–H, C8–H, N9–H). The absence of the bands in the spectrum of MAIR and of eritadenine implies that N9 binds to either C5\textsubscript{0} or C4\textsubscript{0} rather than H15.

One Raman line, the rocking NH\textsubscript{2}, as well as the wagging C2–H and C8–H at 550 cm\textsuperscript{-1}, was observed in the spectra of adenine and MAIR but not in the spectrum of eritadenine. Since the adenine moiety of eritadenine is bound to a straight and rigid carbon chain at N9–C4, the vibration of the neighboring atom, C8, is affected. In MAIR this binding site (N9–C5\textsubscript{0}) is less rigid due to a bend at the C5\textsubscript{0} position between the adenine and the ribofuranoside part of the molecule.

There were some Raman lines that arose both in the spectrum of MAIR and in the spectrum of eritadenine. At 1212 cm\textsuperscript{-1} there was a band in the Raman spectrum of MAIR that could be ascribed to the C4–C5 vibration. In the Raman spectrum of eritadenine also this band was observed, but in this case due to the stretching of C3–C4. Moreover, at 1526 and 1583 cm\textsuperscript{-1} lines appeared both in the Raman
The Raman spectrum of synthesized eritadenine was established by comparing the spectra of the starting materials adenine (Fig. 2(A)) and D-ribose (Fig. 2(B)) with the spectrum of a synthesis intermediate (Fig. 2(C)), here denoted as MAIR, and the spectrum of eritadenine (Fig. 2(D)). In the Raman spectrum of eritadenine, there was one vibration at 773 cm\(^{-1}\) exclusively detected for this compound. This vibration is ascribed to vibrations in the carbon chain, i.e. \(\nu(C–C)\). In both the Raman spectrum of MAIR and eritadenine a Raman line arising at 1212 cm\(^{-1}\) was observed, which is also due to \(\nu(C–C)\). Further, Raman lines seen in eritadenine and MAIR at 1526 and at 1583 cm\(^{-1}\) are assigned to vibrations in the carbon chain, i.e. \(\nu(C–C)\). The N9–C bindings also caused Raman lines observed in the spectrum of adenine to disappear. In addition, several of the D-ribose vibrations were not detected in the Raman spectrum of eritadenine due to the lack of a sugar moiety in the latter.

To summarize, by comparing the spectral development of the main components, adenine and D-ribose, and a synthesis intermediate of eritadenine, a good knowledge on the so far unassigned Raman spectrum of the solid state of eritadenine was gained. On the basis of this information, a Raman spectrum with Raman line assignments of eritadenine could be established.

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Paper V
Solid State Characterization of Sodium Eritadenate

Josefine Enman¹, Anuttam Patra¹, Kerstin Ramser², Ulrika Rova¹* and Kris Arvid Berglund¹,³

¹ Department of Chemical Engineering and Geosciences, Luleå University of Technology, SE-971 87 Luleå, Sweden

² Department of Computer Science and Electrical Engineering, Luleå University of Technology, SE-971 87 Luleå, Sweden

³ Departments of Forestry and Chemical Engineering & Materials Science, Michigan State University, East Lansing, MI 48824 USA

* To whom correspondence should be addressed. Tel: +46(0)920-491315. Fax: +46(0)920-491199. E-mail: ulrika.rova@ltu.se
Abstract

Knowledge of the solid state is of great importance in the development of a new active pharmaceutical ingredient, since the solid form often dictates the properties and performance of the drug. In the present study, solid state characteristics of the sodium salt of the candidate cholesterol reducing compound eritadenine, 2(\(R\)),3(\(R\))-dihydroxy-4-(9-adenyl)-butanoic acid, were investigated. The compound was crystallized by slow cooling from water and various aqueous ethanol solutions, at different temperatures. Further, the compound solution was subjected to lyophilization and to high vacuum drying. The resulting solids were screened for polymorphism by Raman spectroscopy and the crystallinity was investigated by X-ray powder diffraction. Further, thermal analysis was applied to study possible occurrence of solvates or hydrates. Solids obtained from slow cooling showed crystallinity, whereas rapid cooling gave rise to amorphous solids. No polymorphism was detected when slow cooling was applied, regardless of the solvent system or temperature used. Thermal analysis revealed that crystals obtained either from water or from aqueous ethanol solutions were hydrates, but that the water derived crystals were stoichiometrically dihydrates, whereas the crystals derived from aqueous ethanol were 2.5 hydrates.
Introduction

Cardiovascular disease is one of the most serious health concerns in Western society and hypercholesterolemia is a well established risk factor for the development of such disease. To counteract hypercholesterolemia, various statins, some of which were originally isolated from fungi such as *Aspergillus terreus*\(^1\), are frequently used. Another hypocholesterolemic compound of fungal origin is D-eritadenine, \(2(R)\)\(3(R)\)-dihydroxy-4-(9-adenyl)-butanoic acid (Figure 1), found in the shiitake mushroom, *Lentinus edodes*\(^2,3\).

![Figure 1. Chemical structure of D-Eritadenine.](image)

The shiitake mushroom has been shown to lower the blood cholesterol in both animals and humans\(^4-6\), and the cholesterol reducing mechanism of eritadenine has been investigated in several studies on rats\(^2,3,7-10\). The complete hypocholesterolemic mechanism of eritadenine remains to be clarified, but it has been shown that eritadenine accelerates the removal of blood cholesterol, with no indications of the compound inhibiting the biosynthesis of cholesterol\(^10\), unlike the statins\(^1\). In search for new cholesterol reducing drugs, eritadenine might have a potential as an active pharmaceutical ingredient, conceivably as a complement to the statins.

Since active pharmaceutical ingredients are commonly delivered as solids, one key aspect in their development is research comprising the solid state chemistry of the compound. The solid form of a pharmaceutical substance greatly influences its properties and
performance, such as stability and bioavailability, and an understanding of the solid state in relation to its functional properties is fundamental when developing a new drug\textsuperscript{11,12}. A given drug substance can be present in several different solid forms, commonly as crystalline polymorphs, i.e. solids with the same elemental composition but different crystal structures; as solvates, in which solvent molecules are present in the crystal structure; as desolvated solvates for which the crystal structure is principally retained upon solvent loss, or as amorphous solids, which show no or only partial crystallinity\textsuperscript{11}. In the pharmaceutical industry a variety of methods are applied for production of solids, such as cooling of a solution and freeze drying\textsuperscript{12}. Crystallization is the main process for solid formation in the pharmaceutical industry\textsuperscript{13} and which crystal form is obtained depends on the crystallization conditions applied, e.g. solvent composition\textsuperscript{14} and temperature\textsuperscript{15}. Characterization of pharmaceutical solids commonly encompass complementary techniques such as Raman spectroscopy\textsuperscript{16}, X-ray diffractometry\textsuperscript{17}, IR spectroscopy\textsuperscript{18}, solid state NMR\textsuperscript{19} and thermal analysis\textsuperscript{20}.

In the present investigation the sodium salt of the cholesterol reducing compound eritadenine, sodium eritadenate, was slowly crystallized from water and different aqueous ethanol solutions, at different temperatures, by means of cooling. Further, rapid cooling was induced by either lyophilization or drying under high vacuum. The resulting solids were collected and analyzed by Raman spectroscopy, X-ray powder diffraction and thermal analysis, to investigate for crystallinity and for the occurrence of possible polymorphs, solvates or hydrates.
Materials and methods

Synthesis of sodium eritadenate

Sodium eritadenate was synthesized as previously described\(^2\). In summary, methyl 2,3-\(\text{O}\)-isopropylidene-\(\beta\)-D-ribofuranoside was first synthesized\(^2\) and processed to methyl 2,3-\(\text{O}\)-isopropylidene-5-\(\text{O}\)-\(p\)-toluenesulfonyl-\(\beta\)-D-ribofuranoside\(^3\). The latter reacted with the sodium salt of adenine to give the compound methyl 5-(6-aminopurin-9\(H-9\)-yl)-2,3-\(\text{O}\)-isopropylidene-5-deoxy-\(\beta\)-D-ribofuranoside, which underwent hydrolysis to 5-(6-aminopurin-9\(H-9\)-yl)-5-deoxy-D-ribofuranose. In the final step, oxidation of the latter in alkaline media resulted in sodium eritadenate\(^4\).

Solubility studies

The solubility of sodium eritadenate in pure water and in 15, 30 and 50\% (v/v) aqueous ethanol solutions was studied. The concentrations of sodium eritadenate used were in the ranges 50-300 mg/mL and 5-150 mg/mL, for pure water and aqueous ethanol solutions, respectively. All solubility studies took place in microtiter wells, which were tightly covered and placed in a Thermo forma orbital shaker, at 200 rpm. The solubility was observed after 24 hours at 20, 30, 40 and 50 °C for all solvent systems, and additionally at 60 °C for pure water.

Crystallization procedure

Based on the solubility studies, different concentrations of sodium eritadenate for all solvent systems were added to microtiter wells, which were tightly covered. The samples were heated and slowly cooled down, using a Thermo forma orbital shaker. Solid forms obtained at 20, 30, 40 and 50 °C were collected and dried. Eritadenine in pure water was also subjected to high vacuum drying and to lyophilization.
**Characterization of solids**

**Raman measurements**

The solid state Raman spectra of all solids were recorded with a Renishaw 2000 micro-Raman spectrometer. The excitation wavelength was 830 nm; the power onto the sample was 50 mW and the integration time was 10 sec. For all measurements a 20× long working distance (LWD) microscope objective was used. Correction for the energy sensitivity of the spectrometer was performed by measuring the spectrum of a calibrated light source and calculating the intensity wavenumber response curve. Each Raman spectrum was filtered by the noise-reduction algorithm according to Eilers\textsuperscript{25}. The background was automatically subtracted using the algorithm by Cao et al.\textsuperscript{26}, which fits a piecewise modified polynomial to the spectrum. The spectra were vector normalized to get equal integrated areas. The preprocessing algorithms, except the one by Eilers, were written in-house and implemented in Matlab (version R2007b including Statistics Toolbox version 6.1).

**X-ray powder diffraction measurements**

X-ray powder diffraction (XRPD) data of selected solid samples were recorded with a Siemens D5000 diffractometer, using CuKα radiation and variable slits. The samples were investigated in the 2-theta range 7-90º, in Bragg-Brentano geometry, with a step size of 0.01, for 15 hours.

**Thermal analysis**

**Differential scanning calorimetry (DSC).** The thermal behavior of selected solid samples was studied using a Thermal Advantage DSC Q1000 (TA instrument). The samples were scanned from 20 to 260 ºC, at a heating rate of 10 ºC/min, under nitrogen purge.
Thermogravimetric analysis (TGA). The changes in sample mass with temperature were measured by thermogravimetric analysis (TGA) using the Thermal Advantage TGA Q5000 (TA instrument-Waters, LLC) module. The samples were heated from 20 to 260 °C, at a heating rate of 10°C/min, under nitrogen purge.

Evolved gas analysis- mass spectrometry (EGA-MS). For identification of the evolved gas on heating, a Netzsch STA 409 instrument equipped with simultaneous thermo-gravimetric (TG), differential scanning calorimetric (DSC) and quadropole mass spectrometric (QMS) analysis was used. The experiments were conducted in flowing argon while heating from 20 to 260 ºC, at a heating rate of 10 ºC/min.

Results and discussion

For acidic drug products the sodium salts are commonly the chosen derivatives, because of higher solubility and thus increased biocompatibility\(^2\); therefore the sodium salt of the cholesterol reducing compound eritadenine, sodium eritadenate, was investigated. The solubility of sodium eritadenate was comparatively high in pure water and virtually insoluble in absolute ethanol (Figure 2); hence the highest ethanol concentration used for crystallization in this study was 50% (v/v). Sodium eritadenate was crystallized from water and aqueous ethanol solutions, by slow cooling, and additionally subjected to rapid cooling from water solutions. The resulting solids were then investigated by means of Raman spectroscopy, X-ray powder diffraction and thermal analysis.
Figure 2. Solubility of sodium eritadenate in pure water (●), 15% aqueous ethanol (Δ), 30% aqueous ethanol (♦) and 50% aqueous ethanol (□).

Raman spectroscopy

Raman spectroscopy is a highly valuable technique for investigating structural properties of molecules, such as polymorphism. All solid forms of sodium eritadenate obtained from the crystallization procedure were screened for the occurrence of polymorphism, by Raman spectroscopy. Three different Raman spectra were recorded for each solid form and the spectra were background corrected and normalized in order to optimize comparison. The Raman spectra of all solids resulting from slow cooling of solution had the same appearance, irrespective of temperature and solvent composition (Figure 3A-B). They were highly reproducible and correlated exactly with the earlier assignments of the eritadenine Raman spectrum. Hence, no polymorphism with respect to solvent composition or temperature could be observed. The absence of polymorphism irrespective of the temperature used might facilitate a possible crystallization process for drug production. Given that the same solid forms are obtained, the temperature needs not to be meticulously controlled. Moreover, neither comparatively high nor low temperatures have to be applied for crystallization. The Raman spectra of the solids resulting from fast cooling of solution, induced by either high vacuum drying or lyophilization, had a more amorphous pattern (Figure 3C-D). The latter is
plausible since amorphous forms are readily obtained by lyophilization or other means of rapid cooling\(^{30-32}\).

Figure 3. Raman spectra of sodium eritadenate solids obtained by slow cooling from water (A), by slow cooling from aqueous ethanol (B), by lyophilization (C), by high vacuum drying (D), by dehydration of A (E) and by dehydration of B (F). The inset shows the region between 1000 and 1150 cm\(^{-1}\), where the Raman lines arise from vibrations in the carbon chain moiety. The upper graph in the inset is from (C), while the lower graph is from (B).

**X-ray powder diffraction**

X-ray powder diffraction is a powerful technique for identifying crystalline phases\(^{17}\) and was employed to establish if the solids of sodium eritadenate were crystalline or amorphous. The selection of solids for XRPD was based on the results from the Raman analysis, i.e. identical
Raman spectra were obtained for all solids resulting from slow cooling (Figure 3A-B), and for both solid forms obtained from fast cooling (Figure 3C-D). Hence, one solid obtained from slow cooling of water and one from 50% aqueous ethanol were chosen for XRPD measurements, along with the lyophilized sample. The XRPD data presented are in the 2-theta range 10-50º, since all distinctive peaks were found in this range (Figure 4A-C).

The XRPD data also indicated that solids resulting from slow cooling were crystalline and their patterns were similar, irrespective of the solvent used (Figure 4A-B). In accordance with the Raman results (Figure 3C-D) the lyophilized solid gave rise to a more amorphous pattern (Figure 4C). However, some crystalline pattern could be observed for the lyophilized solid and this could be attributed to small crystals in the amorphous phase. By optical microscopy studies (data not shown) it was verified that there were small crystals within the amorphous sample. In some cases, the amorphous forms of pharmaceuticals are used as products because of enhanced solubility13 and hence bioavailability14, but generally this form is not marketed due to lower chemical stability than the crystalline counterpart12. In the present study, the amorphous materials were not subjected to any further investigations.
**Thermal analysis**

The same crystalline solids used for XRPD were further studied by thermal analysis to investigate the thermal behavior and possible occurrence of solvates or hydrates. The TGA curve (Figure 5) of sodium eritadenate crystallized from water showed a mass loss in the temperature range 70-130 °C, which was estimated to 12%. The corresponding temperature range in the DTG curve showed that the mass loss occurred in two sequential steps, and the EGA-MS further identified it as water loss.

![Figure 5. Thermal analysis for sodium eritadenate crystallized from water.](image)

The total mass loss calculated amid the two peaks shown in the DTG curve, suggest that two water equivalents departed the crystal structure, one after another. Hence, the water molecules are differently bound in this type of hydrate, which could be supported by the
crystal structure of sodium eritadenate determined previously. Approximately one third of all active pharmaceutical ingredients are able to form crystal hydrates and sodium salts of all different types of drugs are particularly apt to form crystal hydrates. In the DSC curve, the water loss was represented by an endothermic peak, which was followed by a sharp endothermic peak starting at about 135 °C. It is plausible that the latter peak corresponds either to melting of the compound or to a phase transition, since no mass change occurred.

In a separate experiment, upon heating the crystals to 160 °C under nitrogen atmosphere, no melting could be observed. However, by investigating this anhydrous form by Raman spectroscopy (Figure 3E), it could be seen that there was a transition to amorphous phase upon dehydration. Solvent molecules commonly stabilize the crystal structure and desolvation can thus cause amorphous materials. In this case the water molecules act as stabilizers, due to their incorporation in the crystal structure, and hence dehydration would reasonably disrupt the crystal structure. Since water is a small molecule it is apt to fill vacancies and its exceptional hydrogen bonding capacity, to other water molecules or functional groups, enables the formation of stable crystal structures. Starting at about 225 °C, an endothermic peak was observed in the DSC curve (Figure 5); however, at the same temperature a mass change was seen in the TGA curve. This, combined with carbon dioxide formation as shown by EGA-MS, indicated that decomposition of the compound took place.

For the crystals forming from aqueous ethanol, a mass loss was detected between 55-125 °C in the TGA curve (Figure 6) and estimated to 14%. The mass loss was further observed by one broad peak in the DTG curve and a broad endothermic peak in the DSC curve. The EGA-MS showed that water was departing the crystals, whereas no ethanol could be detected. Hence, in these types of crystals there are reasonably 2.5 molecules of water per molecule sodium eritadenate, according to the mass loss.
As stated above, sodium salts of drug molecules are prone to form hydrates. It should also be emphasized that, due to a low ability of forming multi-point hydrogen bonding, ethanol is distinguished by a low likelihood to be incorporated into solvates. Accompanying the water loss, a diffuse endothermic peak could be observed in the DSC curve. Again, upon heating the crystals to 160 °C, under nitrogen atmosphere, and investigating the anhydrous form by Raman spectroscopy (Figure 3F) it could be seen that there was a transition to amorphous phase upon dehydration. Starting around 235 °C, a sharp endothermic peak was detected in the DSC curve (Figure 6). Since there was no mass loss at this temperature, the compound was plausibly melting.
The thermal analysis of crystals derived from water and from aqueous ethanol solutions indicated the former as dihydrates and the latter as 2.5 hydrates. These two types of hydrates showed no difference in crystal structure, as indicated by Raman spectroscopy and XRPD, but clearly displayed different thermal behavior. A higher temperature was required for loss of water from the dihydrates, and the loss was more stepwise. Based on these observations it is plausible that one or more water equivalents are attached differently and/or more loosely to the crystal lattice of the 2.5 hydrates, as compared to the dihydrates. However, since the crystal structures appear to be the same for both types of hydrates, the difference in water binding is plausibly small and tentatively pertains to secondary water molecules more outside the lattice. Further, for both types of crystals the water loss resulted in more amorphous materials, verifying that water has a stabilizing effect on both types of hydrates.

The decrease in order for the amorphous materials was especially apparent in the region between 1000 and 1150 cm\(^{-1}\), where the Raman lines are due to vibrations of the carbon chain moiety (Inset of Figure 3). Previously carried out polarization measurements showed that a change of the polarization direction by 90\(^\circ\) resulted in an increase of the lines at 1066 and 1085 cm\(^{-1}\) ascribed to the \(\nu(C\text{–}O)\) and \(\nu(C\text{–}C)\)\(^{29}\). The carbon chain moiety has structural properties that enable certain orientations, which are stabilized by adjacent water molecules. For solid states of sodium eritadenate achieved through lyophilization, high vacuum drying or dehydration by heating above 120\(^\circ\)C, these bands more or less disappeared (Figure 3C-F). Hence, by removing the stabilizing water molecules, the order of the solid pertaining to the carbon chain moiety is lost and a more amorphous structure is achieved.

Conclusively, for both types of crystals, water molecules are presumably both highly incorporated into the crystal lattice and more loosely bound to the crystals. Further, the additional water molecule for every two molecules of sodium eritadenate in crystals derived from aqueous ethanol solutions, as compared to water derived crystals, is plausibly attached to
the crystal lattice, but not as a part of the crystal structure. The thermal behavior as described above was shown to be highly reproducible for both the dihydrates and the 2.5 hydrates.

**Conclusions**

The solid forms of sodium eritadenate, as crystallized from water or aqueous ethanol solutions, at various temperatures and cooling methods, were investigated. Solids forming from slow cooling from either water or aqueous ethanol showed crystallinity and similar Raman and XRPD patterns, irrespective of temperature of formation, whereas rapid cooling resulted in more amorphous solids. Thermal analysis of crystals derived from water and from aqueous ethanol showed that the crystals were stoichiometrically dihydrates and 2.5 hydrates, respectively. However, the difference in water equivalents had no significant effect on the crystal structures, as observed by Raman spectroscopy and XRPD, but gave rise to dissimilar thermal behaviors. Finally, both anhydrous forms were more amorphous than their hydrated counterparts, verifying the stabilizing effect of water on the crystal structures.

Future studies have to reveal whether there are any differences between the two hydrates with respect to stability and bioactivity and hence which solvent system to use for crystallization. Further, if the amorphous material of sodium eritadenate will be shown to exhibit equal biocompatibility and fulfil stability requirements, this form might also be considered as an active pharmaceutical ingredient. In this case solidification can be achieved significantly faster.

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