ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

LOVASTATIN: AN INHIBITOR OF CHOLESTEROL BIOSYNTHSIS FROM PLEUROTUS MUSHROOM

A Thesis submitted to the School of Graduate Studies, Addis Ababa University in partial fulfillment for the requirement of the Degree of Masters of Science in Biology

REDIET SITOTAW

January, 2008

ACKNOWLEGEMENT

Dr. Dawit Abate was supervising the present work. I would like to thank him particularly for giving me the passion of mycology first as an excellent teacher and then by his own enthusiasm in applied mycology. I realize that even if I still have a long way to go in the domain of the research, he has already given me some good keys to arrive safely at the end. In addition, he supplied me with a relevant books, papers and materials.

Ato Tarekegn and Israiel were of great help in using HPLC and I am grateful to them for their all round collaboration during my study as well as to Dr. Negussie Megerssa Director of the Laboratory of Analytical Chemistry in Chemistry Department for allowing me to use the HPLC facility. I must express my sincerest gratitude to Professor Ermias Danye for providing me chemicals and some relevant materials.

.

Financial and material support from Addis Ababa University is gratefully acknowledged. The contribution of Dr. Zewdu Siume, Agere Adisu, and Zenebch Aytenew of Mycology Laboratory are gratefully acknowledged. My thanks also go to Wendwosen for providing his laptop computer while I was writing this manuscript. My thanks also go to all my colleagues and my friends within the Mycology laboratory particularly Zufan, Yemsrach and Kidest.

I am highly grateful to my family especially my dear Dad and also sister Eden, Tedla Eshete, Werku Masho, Berke and Asegdech.

I would deeply thank GOD who keeps me for doing all work according to his plan and will.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATION

BE	Biological Efficiency
CAD	Coronary Artery Disease
EtOAc	Ethyl Acetate
FDA	Food and Drug Administration
HDL	High-Density Lipoproteins
HMG-CoA	Hydroxymethyl Glutaryl-Coenzyme A
HPLC	High-performance liquid chromatography
IUPAC	International Union of Pure and Applied Chemistry
Ki	Inhibition Constant
Km	Michaelis-Menten kinetics
LDL	Low-Density Lipoproteins
MeOH	Methanol
OECD	Organization for Economic Co- operation and Development.
R_f	Retention Factor
SSF	Solid-State Fermentation
TLC	Thin Layer Chromatography
US FDA	United States Food and Drug Administration
UV	Ultra Violet
VLDL	Very Low-Density Lipoproteins
RNA	Ribonucleic Acid
YMG	Yeast extract, Malt extract and Glucose.

ABSTRACT

Lovastatin is a secondary metabolite of certain filamentous fungi. It is one of the members of the drug family, statins. Lovastatin, inhibitor of cholesterol biosynthesis, is widely prescribed for the treatment of hypercholesterolemia. Statins have also recently been shown to inhibit HIV-1 infection by targeting cholesterol in the HIV and host membrane. This research was performed to determine the production of lovastatin from fruit body cultivated on sugarcane bagasse and solid-state fermentation on different substrate (Sorghum, Rice and Wheat bran) of two *Pleurotus* mushrooms species that are edible and commercially cultivated in Ethiopia. The two mushrooms investigated, *P. ostreatus* and *P. sajor-caj,* grown in mushroom growing house on sugarcane bagasse (60% moisture content), and yielded an average bioconversion efficiency of 22.58% and 17.67 % respectively. Ethyl acetate was selected as better solvent for extraction of lovastatin in lactone form from acidified samples that were detected on Thin Layer Chromatography (TLC). The crude extracts from fruit bodies of both species show positive result on TLC with same Retention factor (R_f= 0.57and 0.3 for lactone and acid form respectively) which was identical to the standard lovastatin. The TLC result of Solid-State Fermentation (SSF) on rice and wheat bran showed the presence of lovastatin whereas sorghum gives a negative result. For quantification, extraction of lovastatin (β-hydroxyl acid) from fruit body as well as SSF was performed with mixture of methanol: water (1:1 V/V) that was chosen as the most effective solvent for High-performance liquid chromatography (HPLC) analysis at pH 7.7. Highest amount of lovastatin was found from methanol: water (1:1 V/V) extracts of *P. ostreatus* fruiting body (132µg/g) of dry weight followed by *P. sajor-caju* (129µg/g). Among SSF, methanol: water extracts of fermented wheat bran and rice gave (11.7µg/g) and (3.69µg/g) of dry weight respectively. The HPLC result of samples prepared with ethyl acetate show low amount of lovastatin. The health benefits of consuming the *Pleurotus* mushroom particularly for individuals with high plasma cholesterol as well as HIV infection is promising and it grows on a variety of agricultural residues with high bioconversion efficiency.

1. INTRODUCTION

Cardiovascular diseases, such as hypertension, angina, atherosclerosis and congestive heart failure, have been the main cause of mortality in human (Victoria, 2005). Unlike many other diseases, cases of cardiovascular disorders are growing day by day (Memon and Gilani, 1995). Between 13 and 14 million people in U.S are believed to suffer from this complex and life threatening condition and over 25 million people world wide are expected to die from cardiovascular-related pathologies by the year 2020 (Victoria, 2005). Hypercholesterolemia is considered an important risk factor in coronary artery disease. Thus the possibility of controlling de novo synthesis of endogenous cholesterol, which is nearly two-thirds of total body cholesterol, represents an effective way of lowering plasma cholesterol levels (Manzoni and Rollini, 2004).

Statins, fungal secondary metabolites, selectively inhibit hydroxymethyl glutaryl-coenzyme A (HMG-CoA) reductase, the first enzyme in cholesterol biosynthesis. The mechanism involved in controlling plasma cholesterol levels is the reversible inhibition of HMG-CoA reductase by statins, related to the structural similarity of the acid form of the statins to HMG-CoA, the natural substrate of the enzymatic reaction (Manzoni and Rollini, 2004).

Currently there are seven statins in clinical use. Lovastatin, Pravastatin ,Symvastatin, Pitavastatin, Atorvastatin, Rosuvastatin and Fluvastatin. Lovastatin is the most well studied and the first to be on market among all the statins. The hypocholesterolemic effect of statins lies in the reduction of the Very Low-Density Lipoproteins (VLDL) and Low-Density Lipoproteins (LDL) involved in the translocation of cholesterol, and in the increase in the High-Density Lipoproteins (HDL), with a subsequent reduction of the LDL- to HDL-cholesterol ratio, the best predictor of atherogenic risk (Manzoni and Rollini, 2004).

Several fungal genera including *Aspergillus, Penicillium, Monascus, Paecilomyces, Trichoderma, Scopolariopsis, Doratomyces, Phoma, Pythium, Gymnoascus, Hypomyces* and *Pleurotus* have been reported to be able to produce lovastatin (Gunde-Cimerman *et al.,* 1993; Shindia, 1997).

The general efficiency of processes for lovastatin production is determined by the productivity of fungal strain and the number of steps in the extraction process. For that reason, there is need for methods to produce cholesterol-lowering compounds with a high activity, rather from sources that are not toxic, and by using simple, rapid and inexpensive manufacturing processes. The processes of the production of a cholesterol lowering compounds from edible Basidiomycetes mushrooms have many advantages (Wasser *et al.,* 2002).

Although mushrooms are increasingly being recognized as important food products for their significant role in human health and nutrition their consumption in many developing countries, particularly in Ethiopia, is extremely limited. One reason probably being that the health benefits derived from various edible mushrooms are largely unknown. Different studies show that the addition of dried *P. ostreatus* to a high-cholesterol diet significantly reduced cholesterol accumulation in the serum and liver of tested rats (Bobek*,* 1991).

2. LITRETUR REVIEW

2.1. Fungi as a source of bioactive metabolites

Fungi have proved to be a fertile source of structurally diverse bioactive metabolites, which have yielded some of the most important products of the pharmaceutical industry. These include Penicillin from the filamentous fungus, *Penicillium notatum*, antibacterial agents Cephalosporin (from *Cephalosporium cryptosporium*), immunosuppressive agents [the Cyclosporins, and Rapamycin (from *Streptomyces* species)]; cholesterol-lowering agents (Mevastatin (Compactin) and Pravastatin (from *Penicillium* species). In addition, Lovastatin isolated from *Aspergillus terreus* (Alberts *et al.,* 1980) are further examples supporting today's great interest in new secondary metabolites from fungi (Gordon and David, 2005).

Although the tropical regions of Africa are endowed with a rich variety of fungal flora, fungi are hardly used as traditional medicinal agents or even as foodstuffs. This is mainly because of the lack of familiarity with fungi and the fact that some fungi are toxic (Pegler, 1977; Dawit and Ermias, 1995). In contrast to this, the use of mushrooms for food and medicinal agents goes back over 1000 years in many parts of the Far East (Dawit and Ermias, 1995).

2.2. Nutritional and Medicinal property of mushrooms

2.2.1 Nutritional value of mushrooms

Mushrooms have long been valued as tasty and nutritional foods by many societies through out the world (Chang and Chiu, 1992). Mushrooms are considered a good source of digestible proteins with protein content above most vegetables and somewhat less than most meats and milk. Protein content can vary depending on mushroom type from 10-40% on dry weight bases (Chang, 1999; Breene, 1990).

Fresh mushroom contain 3-21% carbohydrate and 3-35% fiber on dry weight bases. They are also an excellent source of vitamins especially thiamine (B_1), riboflavin (B_2), niacin, biotin and ascorbic acid (Vitamin C). Vitamin A and D are relatively

uncommon although several species contain digestible amount of β-carotin and ergosterol (Smith *et al.,* 2002). While crude fat in mushroom contains all the main classes of lipid compounds including free fatty acids, mono-,di- and triglycerides (Breene, 1990). Mushrooms are effective in concentrating Sodium, Potassium, Phosphorus, Magnesium, Sulpher, Iron, Zink, and Copper in their fruit bodies this makes them to be good sources of minerals (Chang *et al.,* 1981).

2.2.2 Medicinal mushroom

The edible mushroom which demonstrate medicinal property include species of *Lentinula*, *Auricularia, Hericium, Grifola, Flammulina, Pleurotus* and *Tremella* while others are known only for their medicinal properties, such as *Ganoderma* and *Trametes (Cuiolus)* are definitely non-edible due to their coarse and hard texture or bitter tests. Extracts from many medicinal mushrooms have long been used for a wide range of ailment in traditional Chinese medicine. Modern scientific and medical studies are increasingly supporting many of these health claims. The main areas of medical studies include cancer treatment, anti-microbial, blood pressure lowering, liver protective, anti-inflammatory, anti-diabetic and Cholesterol lowering (Smith *et al.,* 2002).

Cholesterol lowering properties

According to traditional Chinese medicine (Breene, 1990; Hobbs, 1995; Zhuang and Mizuno, 1999) mushrooms in general and *Pleurotus spp., Lentinula edodes* and *Grifola frondosa* in particular are almost ideal for diets aiming at preventing cardiovascular disease, due to the high fiber content, sterols, proteins, microelements and low caloric value they possess. However, diet alone is not always successful in controlling hypercholesterolemia; therefore, researchers have tried to identify inhibitors of cholesterol synthesis. One such inhibitor is Mevinolin (Lovastatin), effective in inhibiting the enzyme (HMG-CoA-reductase) which exerts its function in liver microsomes in the initial phases of cholesterol formation.

2.3. Cholesterol and Cardiovascular Diseases

Cholesterol is a precursor to the corticosteroids and sex hormones such as Progestagens, Androgens and Estrogens. It plays a vital role in the maintenance of cell membrane integrity providing greater stability and modulates its fluidity. However, high levels of this lipophilic substance in the body lead to atherosclerosis, a predisposing factor to the development of coronary artery disease (CAD). Total cholesterol level less than 200 mg/dL is desirable where as between 200–239 mg/dL is borderline- high risk and 240 mg/dL and over is high risk. Atherosclerosis involves an accumulation of cholesterol esters and other blood lipids and lipoproteins in macrophage cells found in the intima of arteries. Lipid-engorged macrophage cells become foam cells, and foam cell infiltration progresses to fatty streaks in the arterial wall. Plaque formation, thrombosis, and vessel occlusion can follow, leading to CAD (Victoria, 2005).

Cholesterol is carried in the blood in the form of lipoproteins particles that are low–density lipoprotein (LDL) or bad cholesterol and high density lipoprotein (HDL) or good cholesterol. The proteins serve to solublize the lipids and target them to cells. LDL and VLDL carry cholesterol toward tissues, and elevated levels of these lipoproteins are associated with atheroma formation and lead to cardiovascular disease. HDL, in contrast, carries cholesterol back to the liver and is associated with protection against this disease (Victoria, 2005).

Cholesterol is biosynthesized in a series of more than 25 separate enzymatic reactions that initially involves 3 successive condensations of acetyl-CoA units to form a 6-carbon compound, HMG CoA. This is reduced to mevalonate and then converted in a series of reactions to the isoprenes that are building blocks of squalene, the immediate precursor to sterols, which cyclizes to lanosterol (a methylated sterol) and further metabolized to cholesterol (Victoria, 2005).

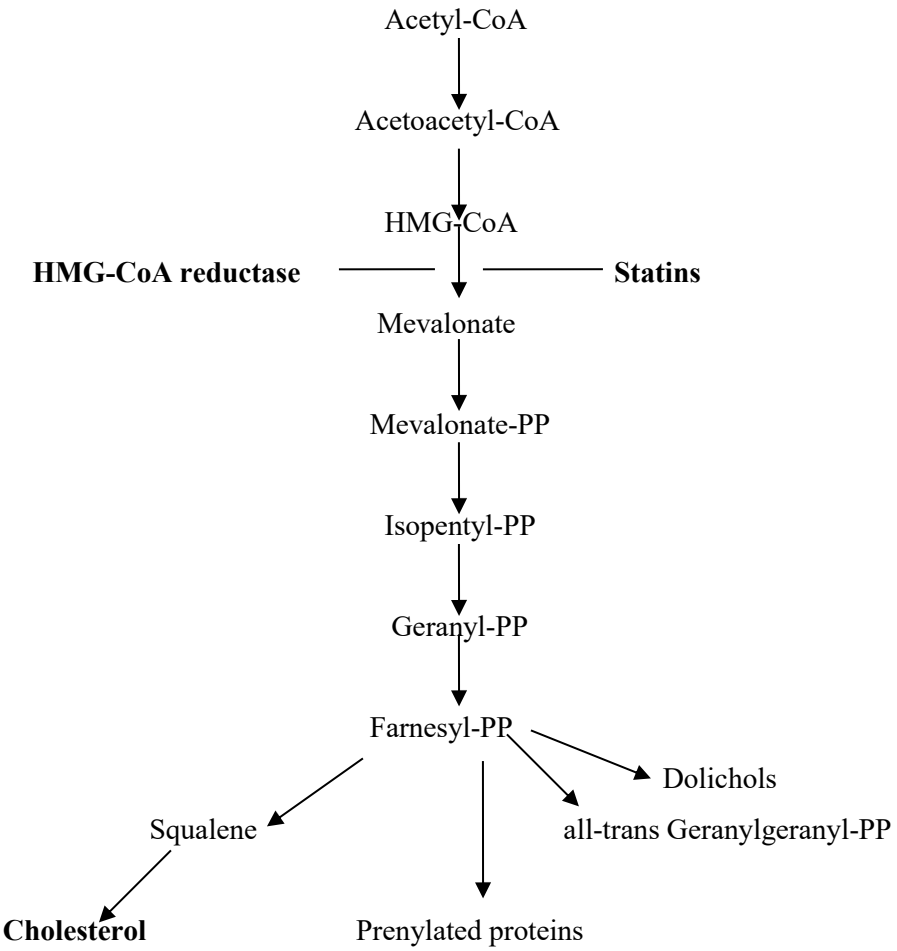


Figure 1.The mammalian Cholesterol biosynthesis.
Adapted from Corsini *et al*. in (Victoria, 2005).

One of the methods to reduce high cholesterol levels is to change lifestyle. This means control body weight, eat a heart-healthy diet, get regular physical activity, avoid tobacco smoke, limit alcohol intake, and limit beverages and foods with added sugars. However in most cases, medication is needed in addition to a healthy diet and lifestyle (Furberg and Curt, 1999).

Several classes of lipid-modifying therapy to decrease elevated cholesterol concentration, particularly LDL-cholesterol are available, including bile acid-binding resins (e.g. cholestyramine, colestipol, colesevalam), nicotinic acid (niacin), the fibrates (e.g. fenofibrate, clofibrate, gemfibrozil, bezafibrate), and more recently the cholesterol-absorption inhibitors (e.g. ezetimibe). Based on clinical trial evidence, the

most commonly prescribed lipid-modifying therapies are the HMG-CoA reductase inhibitors, more commonly known as the statins (Michael, 2004).

2.4. Statins

Statins are the treatment of choice for the management of hypercholesterolaemia because of their proven efficacy and safety profile. They also have an increasing role in managing cardiovascular risk in patients with relatively normal levels of plasma cholesterol. Although all statins share a common mechanism of action, they differ in terms of their chemical structures, pharmacokinetic profiles, and lipid-modifying efficacy. The chemical structures of statins govern their water solubility, which in turn influences their absorption, distribution, metabolism and excretion (Michael, 2004). The statins are classified into two groups based on their source: fermentation-derived (Fungal origin) and synthetic (Table 1).

LDL-lowering potency varies between statins. Cerivastatin is the most potent, followed by (in order of decreasing potency) Rosuvastatin, Atorvastatin, Simvastatin, Lovastatin, Pravastatin, and Fluvastatin (Shepherd *et al.,* 2003) The relative potency of Pitavastatin has not yet been fully established.

The chemical structures of the different statins are shown in figure 3. The structure can be broadly divided into three parts (Alberts, 1998); an analogue of the target enzyme substrate, HMG-CoA (Figure 2); a complex hydrophobic ring structure that is covalently linked to the substrate analogue and is involved in binding of the statin to the reductase enzyme; side groups on the rings that define the solubility of the statin and therefore many of their pharmacokinetic properties.

Table 1. Classification of statin based on source

Statin	Brand name	Derivation
Atorvastatin	Lipitor, Torvast	Synthetic
Cerivastatin	Lipobay,Baycol. (Withdrawn from the market in August, 2001 due to risk of serious adverse effects)	Synthetic
Fluvastatin	Lescol, Lescol XL	Synthetic
Lovastatin	Mevacor, Altocor	Fermentation-derived
Mevastatin	-	Naturally-occurring compound. Found in red yeast rice.
Pitavastatin		Synthetic
Pravastatin	Pravachol, Selektine, Lipostat	Fermentation-derived
Rosuvastatin	Crestor	synthetic
Simvastatin	Zocor, Lipex	synthetic derivate of fermentation product

Source (Manzoni and Rollini, 2004).

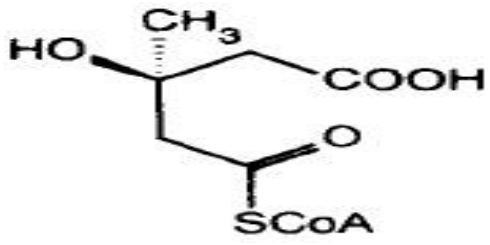


Figure 2. Chemical structures of HMG-CoA
(Michael, 2004).

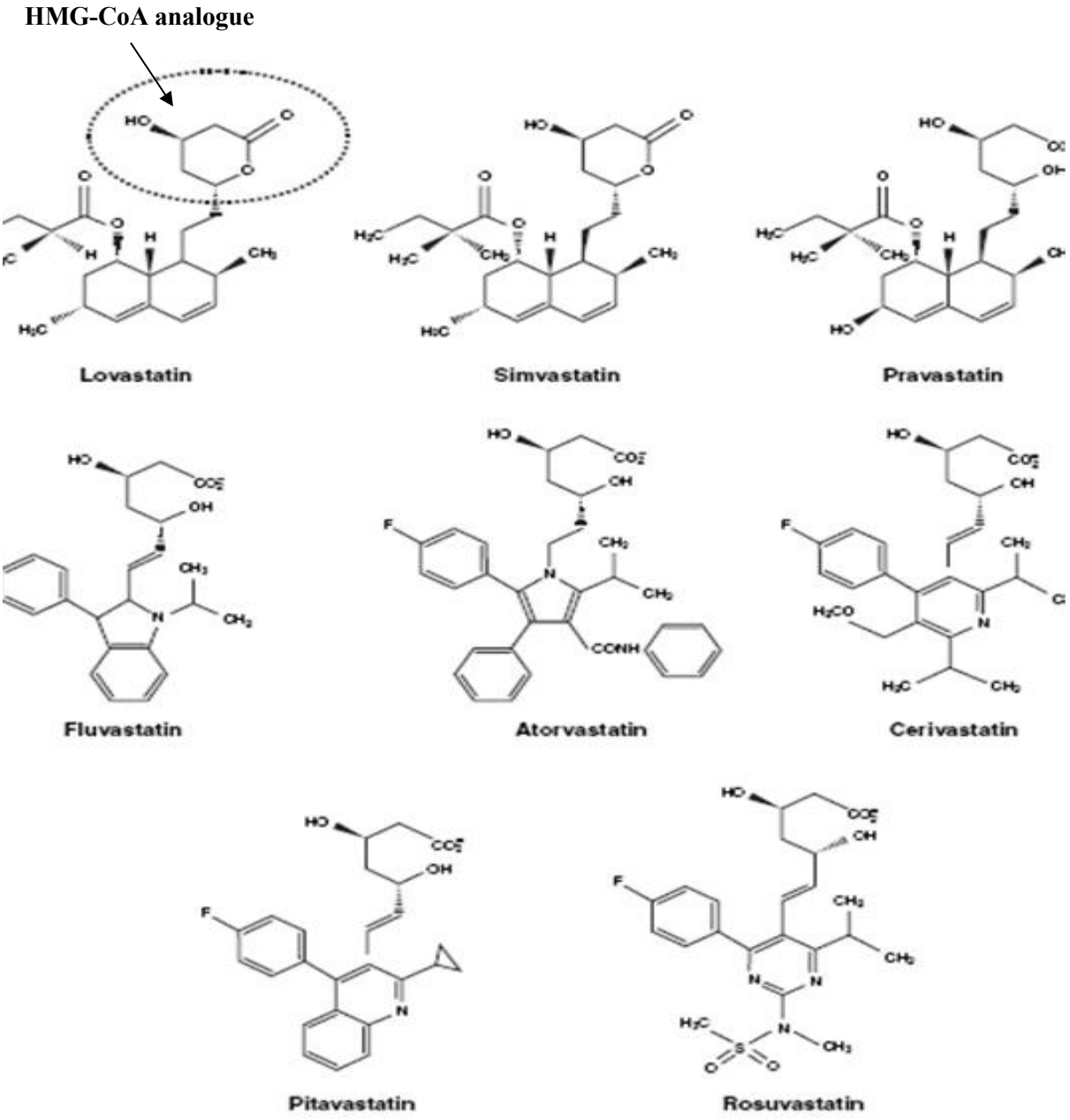


Figure 3. Chemical structures of Lovastatin and related statins
(Michael, 2004).

2.5. Lovastatin

2.5.1. Discovery

A number of early attempts to block the synthesis of cholesterol resulted in agents that inhibited late in the biosynthetic pathway between lanosterol and cholesterol resulting in the accumulation of other steroids (Daniel, 2006). A major rate-limiting step in the pathway is at the level of the microsomal enzyme which catalyzes the conversion of HMG CoA to mevalonic acid in early stage and which has been considered a prime target for pharmacologic intervention (Furberg and Curt, 1999). Inhibition of this enzyme could lead to accumulation of HMG CoA, a water-soluble intermediate that is then capable of being readily metabolized to simpler molecules so it would not lead to accumulation of lipophylic intermediates.

The first breakthrough in efforts to find a potent, specific, competitive inhibitor of HMG CoA reductase occurred in 1976 when Endo *et al.* (1976) reported the discovery of mevastatin, a highly functionalized fungal metabolite, isolated from cultures of *Penicillium citrinum*. Following to the first reports describing mevastatin, efforts were initiated by the pharmaceutical company Merck to search for other naturally occurring inhibitors of HMG CoA reductase. Moreover, lead to the discovery of a novel fungal metabolite Lovastatin isolated from a strain of *Aspergillus terreus* (Alberts *et al.,* 1980)

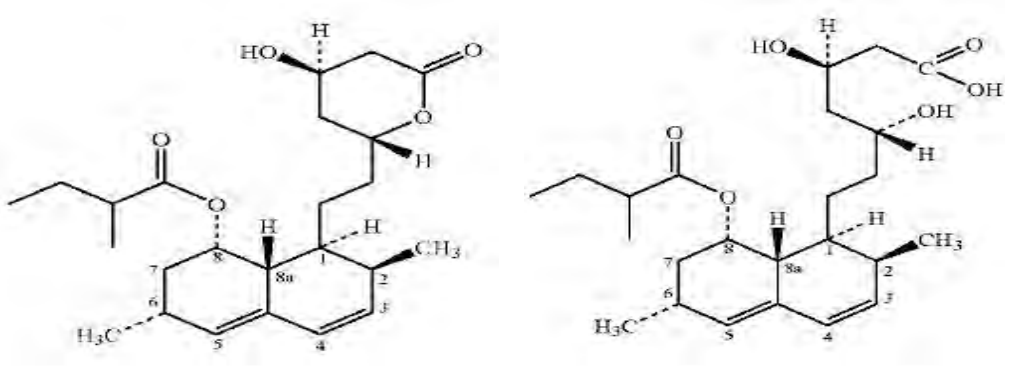
Certain higher fungi such as *Pleurotus ostreatus* (oyster mushroom) and closely related *Pleurotus spp* were also naturally produce lovastatin (Shepherd *et al.,* 2003). The highest amount of lovastatin was found in oyster mushroom fruit-body, especially in the lamellae or gills. *Pleurotus* spp. are promising as medicinal mushrooms, exhibiting hematological, antiviral, antitumor, antibiotic, antibacterial, hypocholesterolic and immunomodulation activities (Cohen *et al.,* 2002).

In 1982 some small-scale clinical investigations of lovastatin, in a very high-risk patients were undertaken, in which dramatic reductions in LDL cholesterol were observed, with very few adverse effects (Daniel, 2006). Large-scale trials confirmed

the effectiveness of lovastatin. Observed tolerability continued to be excellent, and it was the first statin to be approved by the United States Food and Drug Administration (US FDA) in August, 1987.

2.5.2. Chemistry

Lovastatin ($C_{24}H_{36}O_6$, Mw=404.545g/mol), the systematic (IUPAC) name for lovastatin lactone form is 1, 2, 6, 7, 8, 8a-hexahydro-β, δ-di-hydroxy-2, 6-dimethyl-8-(2-methyl-1-oxobutoxy)-1-naphthalene heptanoic acid δ-lacton (Figure 4a). The β-hydroxyl acid is 1, 2, 6, 7, 8, 8a-hexahydro-β, δ-di-hydroxy-2, 6-dimethyl-8-(2-methyl-1-oxobutoxy)-1-naphthalene heptanoic acid (Figure 4b). The percentage composition of the element; C: 71.27%; H: 8.97%; O: 19.77%, and melting point 170.171°C. It is a white, nonhygroscopic crystalline powder that is insoluble in water and soluble in ethanol, methanol, ethyl actate and acetonitrile (Kumar *et al.,* 2003).



(a) (b)

Figure 4. Chemical structure of (a) lactone and (b) β-hydroxyl acid form of lovastatin
(Kumar *et al.,* 2003)

Lovastatin tablets are supplied as 10 mg, 20 mg and 40 mg tablets for oral administration. In addition, each tablet contains the following inactive ingredients: lactose monohydrate, magnesium stearate, microcrystalline cellulose, poloxamer, pregelatinized starch, sodium starch glycolate and Butylated hydroxyanisole (BHA) is added as a preservative (Ucar *et al.,* 2000).

2.5.3. Biosynthesis of lovastatin

In filamentous fungi many secondary metabolites with complex chemical structure are synthesized via the polyketide pathway (Greenspan and Yudkovitz, 1985). Lovastatin is comprised of two polyketide chains derived from acetate that are 8- and 4- carbons long coupled in head to tail fashion. 6-alphamethyl group and the methyl group on the 4-carbon side chain are derived from the methyl group of methionine, and 6 alphamethyl groups is added before closure of the rings (Kumar *et al.,* 2003).

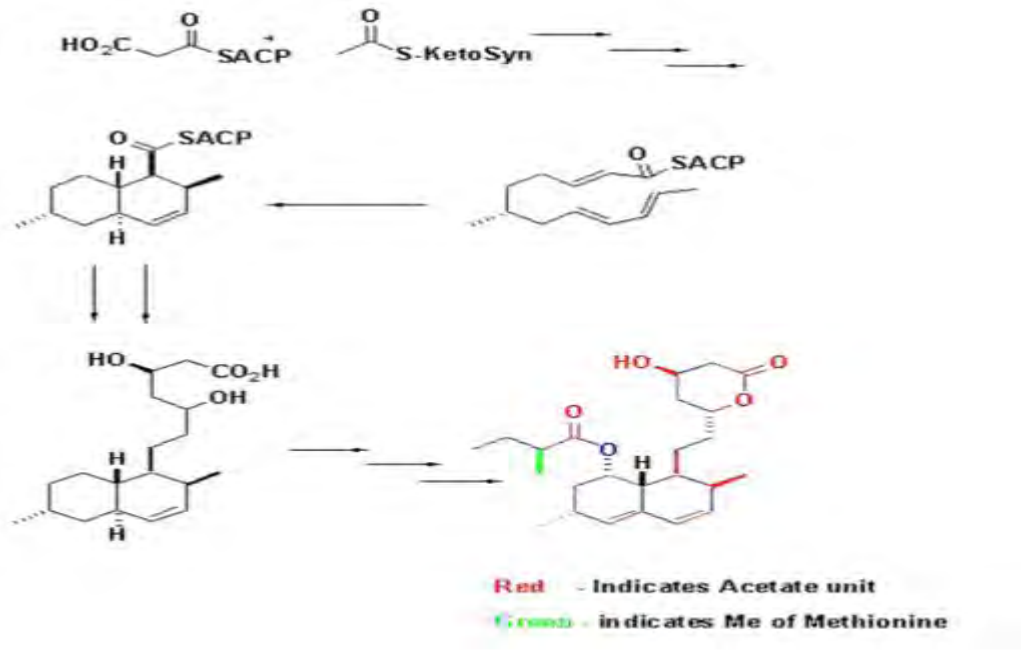


Figure 5. Biosynthesis of lovastatin.
(Kumar *et al.,* 2003).

2.5.4. Mechanism of action

Lovastatin, as all other statins, is a reversible competitive inhibitor of HMG-CoA reductase. Lovastatin, being inactive in the native (lactone) form, the form in which it is administered, is hydrolysed to the active β-hydroxy acid form in the body. The 3, 5-dihydroxy- heptanoic acid portion of these compounds resembles the HMG portion of HMG-CoA. The Michaelis-Menten kinetics (Km) value for mammalian HMG-CoA reductase is ~10 μM, while the Inhibition Constant (Ki) for the ring-

opened acids of lovastatin are in the range of 0.2-1 nM. Thus, the affinity of HMG-CoA reductase for lovastatin analogues is 10000-fold or more than its affinity for the natural substrate, HMG-CoA. The mechanism by which lovastatin inhibits HMG-CoA reductase appeared to be ideal for its development as a drug (Endo, 1992).

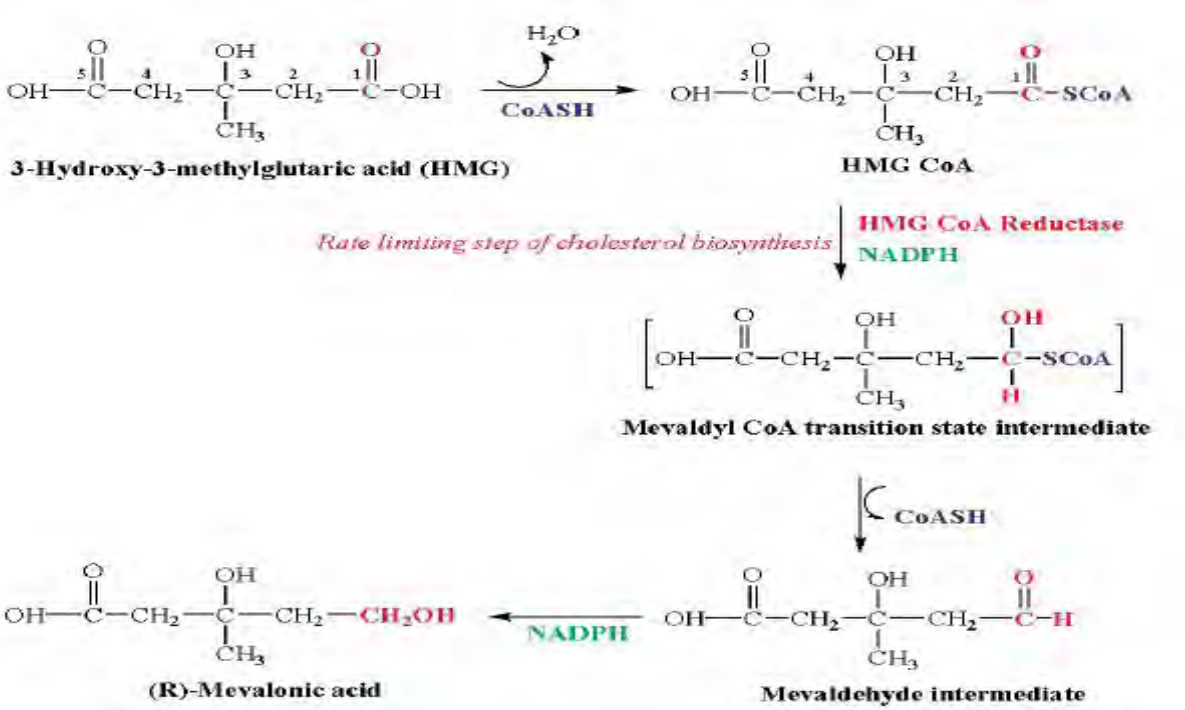


Figure 6. The mammalian mevalonate pathway

Cytochrome P450 (CYP) 3A4 is the main isoenzyme involved in the metabolic transformation of HMG-CoA reductase inhibitors. Individuals with both low hepatic and low gastrointestinal tract levels of CYP3A4 expression may be at in increased risk of myotoxicity due to potentially higher HMG-CoA reductase inhibitor plasma concentrations. The risk of these serious adverse reactions is dose-dependent and may increase when HMG-CoA reductase inhibitors are prescribed concomitantly with drugs that inhibit their metabolism (Ucar *et al.,* 2000).

Another principal action of lovastatin is to increase the number of LDL receptors, but not to inhibit the synthesis of lipoproteins. Rates of synthesis of LDL receptors are inversely correlated with the amount of cholesterol in cells. Studies with experimental

animals revealed that HMG-CoA reductase inhibitors increase messenger RNA for LDL receptors in the liver, enhance the number of LDL receptors expressed on the surface of liver cells, and thus increase the breakdown of LDL (Ma *et al.,* 1986). Lovastatin can also produce slight to moderate increases in HDL, and slight to moderate decreases in triglycerides. Both of these effects are typically beneficial to a patient with a poor lipid profile.

Endo *et al.* (1976), assayed HMG-CoA reductase principally by measuring the incorporation of radioactivity from [^{14}C] HMG-CoA into mevalonate as [^{14}C] HMG-CoA was too expensive to use, they first searched for microbial culture broths that inhibited the incorporation of [^{14}C] acetate into cholesterol. The active broths were then tested for their ability to inhibit lipid synthesis from [^{3}H]mevalonate. Culture broths that were active in the first assay but not active in the second determination were suspected to contain a compound (or compounds) that inhibited the early stages between acetate and mevalonate in the cholesterol synthetic pathway. The principal active component(s) from these culture broths were isolated. Rat liver enzymes were used for these assays (Endo, 1992).

Lovastatins exhibit action beyond lipid-lowering activity in the prevention of atherosclerosis. Researchers hypothesize that statins prevent cardiovascular disease via four proposed mechanisms: Improving endothelial function, modulate inflammatory responses, maintain plaque stability and prevent thrombus formation (Furberg and Curt, 1999).

2.6. Lovastatin producing fungi

Loastatin is produced as secondary metabolite by a varity of filamentous fungi including *Aspergillus, Penicillium, Monascus, Paecilomyces, Trichoderma, Scopolariopsis, Doratomyces, Phoma, Pythium, Gymnoascus, Hypomyces* and *Pleurotus.* Commercial production of lovastatin is based on *Aspergillus terreus* batch fermentation (Siamak *et al.,* 2003).

Aspergillus species

This group of lovastatin producers includes *Aspergillus flavus, A. niger, A. repens*, in a concentration not more than 5 mg/l. *Aspergillus terreus* is the best lovastatin producer (Siamak *et al.,* 2003). An optimal medium for maximizing the production of lovastatin in batch cultures of *A. terreus* should contain 48g/l carbon as lactose, 0.46g/l nitrogen as soyabean meal, and 0.79g/l phosphate. This composition can yield a lovstatin titer of ~230mg/l (Lopez *et al.,* 2004). According to Siamak *et al.* (2003) the production time-course study showed that about 80% of maximum lovastatin production was obtained after 7 days of incubation in an oxygen-rich environment.

Lovastatin production by *Aspergillus terreus* ATCC 20542 in solid-state fermentation (SSF) on various substrates was studied. The results from different studies showed that rice as well as wheat bran was suitable substrate for lovastatin production in SSF. The maximum yield of lovastatin (2.9 mg/g dry substrate) using rice as substrate was achieved after incubating for 11 days at the following optimized process parameters: 50%~60% initial moisture content, at 28°C (Wei Pei-lian *et al.,* 2006).

Monascus species

Monacolin K (Lovastatin or Mevinolin) is a typical secondary metabolite produced by *Monascus* species (Endo, 1979). A serious problem associated with functional *Monascus* products is the mycotoxin-citrinin, another secondary metabolite produced by *Monascus* spp. The only way of avoiding citrinin in the products is to obtain a *Monascus* strain that is genetically citrinin blocked. The functional *Monascus* products containing higher ratio of the open β-hydroxyacid form Monacolin K should be regarded as a higher quality (Ganrong *et al.,* 2002).

Red yeast rice made from rice fermented by red yeast known as *Monascus purpureus* contains significant quantites of the HMG-CoA reductase inhibitor lovastatin a naturally occurring statin. Red yeast rice is sold as dietary supplement for controlling cholesterol. There is strong scientific evidence for its effect in lowering blood levels of total cholesterol, low-density lipoprotein (LDL) and triglyceride levels. Because an

approved drug is identical to the molecule, it has therefore been regulated as a drug by FDA. It has been used for over 1,000 years in China to improve circulation and treat indigestion and diarrhea (Ganrong *et al.,* 2002).

Penicillium species

Endo *et al.* (1976) described a process for the production and purification of mevastatin (compactin) from *Penicillium citrinum*. However, because of multiple side effects compactin was not used as a medicine but it is used as an important source for producing Pravastatin (one of advanced cholesterol-lowering agents). Mevastatin was independently isolated from *P. brevicompactum* as an antibiotic (Shindia, 1997). Lovastatin production from *Penicillium variablehigher* (not more than 4 mg/l), *Penicillium citrinum* (61 mg/l,), *Penicillium chryso-genum* (35 mg/l), *Penicillium funiculosom* (19.3 mg/l) was reported by (Siamak *et al.,* 2003). Along with mevastatin, dihydrocompactin ML-236A and ML-236C have been isolated from *P. citrinum*

Pleurotus species

Pleurotus species, belongs to class Basidiomycetes, subclass Hollobasidiomycetidae, order Agaricales. When fruiting bodies of the edible and commercially available mushroom *Pleurotus* were investigated, it was found that they contained significant amount of the inhibitor (Gunde-cimerman and Cimerman, 1995). The presence of lovastatin was determined in the submerged fermentation broth of *P.sapidus*, in the surface fermentation broth of *P. saca* and also in the sporocarps, lamellae and spores of *P. ostreatus* (Gunde-cimerman *et al.,* 1993). *P. sajor-caju* exhibit hypotensive effect through its active ingredients (Shahdat *et al.,* 2003)

The appearance of the inhibitor during the development of fruiting bodies was followed and amount of lovastatin in the primordial as well as in different parts of sporocarps of different size were determined. Lovastatin at the beginning of mushroom growth is uniformly distributed in small sporocarps, there is no substantial difference between the pileus, and the stipe. During sporocarp growth, the majority of

it is first transferred to the pilleus and later to the lamellae. Part of the lovastatin in the completely mature mushroom is transferred to the basidiospores, which disperse and make lovastatin content smaller than less matured sporocarps (Gunde-cimerman and Cimerman, 1995).

When the determination of lovastatin in sporocarps of two other commercially cultivated species, *P. cornucopiae* and *P. eryngii*, the highest quantities of lovastatin were determined, in the lamellae of not completely matured sporocarps to be from 1.5 to 15 times higher than quantities in the pilei (Gunde-cimerman and Cimerman, 1995).

It was recognized that with the usual extraction method, mixing the fungal biomass with a methanol: water (1:1, v/v) mixture, was not possible to extract lovastatin from the basidiospores, which have two to third times thicker cell walls than hyphal cells. Therefore, for the preparation of extracts from basidiospores, liquid nitrogen was used for cell wall disintegration (Gunde-cimerman and Cimerman, 1995).

The production of lovastatin from *P. ostreatus* in their natural environment and that are grown on a variety of substrate inside greenhouse were compared by Alarcoin *et al.,* (2002). And a result ranging from 0.4 to 2.07% measured in dry weight from cultivated mushroom whereas fruiting bodies collected from their natural environment gave 0.7 to 2.8%, implying that it is possible to increase the yield of lovastatin by modifying the components of the substrate used for cultivation.

2.7. Hypocholesterolemic effect of *Pleurotus mushroom.*

Pleurotus species, and other related mushrooms were used in the traditional oriental medicine as components of natural diets with antisclerotic effect. Oyster mushroom in the diet reduced very profoundly and efficiently cholesterol diet-induced accumulation of cholesterol in blood and several other body organs (including almost 80% decrease of cholesterol content in aorta). Significant reduction of cholesterol levels in blood, aorta and heart muscle by oyster mushroom diet was accompanied by a striking reduction in the incidence and extent of atherosclerotic lesions in aorta and in coronary arteries (Bobek and Galbavy, 1999).

According to Bobek and Galbavy, (1999) the addition of 10% dried fruiting bodies of oyster mushroom (*Pleurotus ostreatus*) to the diet containing 1% of cholesterol reduced serum cholesterol levels by 65% and cholesterol content in liver, heart, long extensor muscle and aorta of male rabbits by 60, 47, 25 and 80%, respectively. The decrease in total serum cholesterol was affected primarily (by 70%) reduced cholesterol content in very low- density lipoproteins (VLDL) while the contribution of high density lipoproteins (HDL) cholesterol increased by a factor of 3.

The effect of the dose of oyster mushroom in the diet and of the period of application on cholesterol accumulation in blood and body organs was studied by the work of Bobek and Galbavy, (1999) in weanling male Wistar rats. Reduction of cholesterol in serum and body organs was found to be dependent on the amount of dietary oyster mushroom administered. A negative correlation between the mushroom dose and cholesterol level was found. The dose of 1% oyster mushroom did not affect cholesterol levels in serum or body organs. A significant reduction of cholesterol levels was observed in serum (31–46%) and liver (25–30%) at a dose of 5% of oyster mushroom for all periods.

Water-soluble components of fibrous complex from oyster mushroom (β-glucan and pectin, 20% and 6% respectively) are able to bind bile acids thus inhibiting the formation of micelles and cholesterol absorption. Increased excretion of bile acids reduced their re-absorption to liver and accelerated cholesterol catabolism by a feedback mechanism (Bobek, 1991).

It was found that the water-soluble component of β-glucans has the ability to quench reactive oxygen species and that hypocholesterolemic oyster mushroom simultaneously has antioxidative property. The pronounced antiatherogenic effect of oyster mushroom in rabbits could be attributed in the first place to decrease in serum cholesterol and probably also to reduced risk of oxidative modification of low-density lipoproteins (Bobek, 1991).

2.8. Statins and HIV

The human immunodefficiency virus (HIV) has proven to be difficult pathogen to overcome. HIV fusion and infection involves a step- wise process that includes a number of host cell proteins and lipids (Eckert and Kim, 2001). There are only a few currently FDA-approved drugs that target HIV proteins. Although these drugs combination therapies provide effective suppression of HIV virions in individuals, the cost, toxicity and drug resistance remain common concerns.

Recent studies demonstrated that lipids, such as cholesterol and sphingolipids on both the virus and the host cell, can influence the efficiency of virus- cell fusion. As viruses enter and bud from infected cells they do so at cholesterol- rich lipid rafts, resulting in the enrichment of cholesterol in viral membranes as compared to the levels of cholesterol found on host cell. Targeting lipid rafts could possibly provide such a means to inhibiting viral infection. Many studies shows that the disturbance of lipid rafts-specific in the target cell membrane can influence the cell's ability to be infected (Viard *et al.,* 2002).

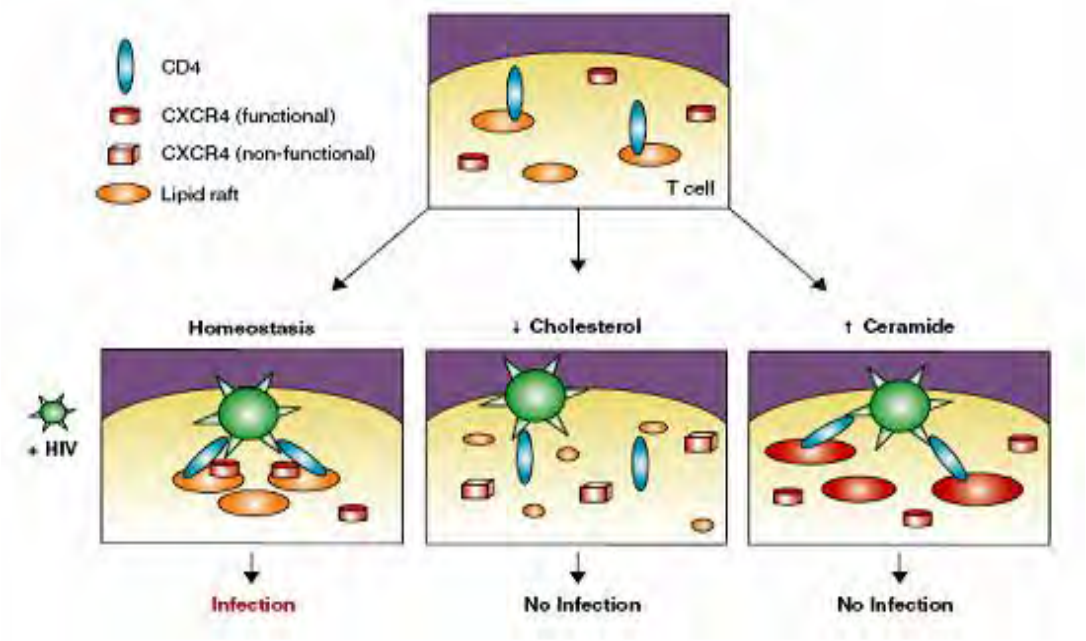


Figure 7. Model for targeting lipid rafts to inhibit HIV infection.
(Viard *et al.,* 2002)

An additional point of interest regarding cholesterol is that statin drugs, inhibit HIV infection both by blocking the interaction of virus and host cell (lowering of cholesterol-rich lipid rafts) and by inhibiting Rho GTPase activity (Giguere and Tremblay, 2004).

2.10. Cultivation of Pleurotus mushroom

In nature, *Pleurotus* species are found distributed in forest through out the Northern Hemisphere (Europe, North America, and Asia) (Shah *et al.,* 2004). *Pleurotus ostreatus* was first cultivated in USA in 1990 whereas *P.sajor-caju*, was initially cultivated in India after the late of 1940`s (OECD, 2005). Although species in this genus are mostly known to be distributed in temperate zones of the world, some species like *Pleurotus lueoalbus*, *P.opuntiae* and *P.flabellatus* have been reported from East Africa by Pegler, (1977) and none of them have been domesticated for commercial cultivation.

According to the report by OECD, (2005), *Pleurotus* is the third most commercially cultivated mushroom in the world, accounting for 25% of total world production of cultivated mushroom. Its production shows an increase of 42% and China was the most important contributor to this growth (Royse and Schisler, 1987). The genus *Pleurotus* has the highest number of species cultivated. This is one of the reasons for the increasing importance world wide of the mushroom group. It is likely that this mushroom type would rank first among the cultivated mushroom in the coming years. (Dawit, 1998).

In Ethiopia, Oyster mushrooms (*Pleurotus ostreatus, P.sajor-caju, P.pulmonarius*) are being cultivated on small scale by a few individual or small scale commercial growers in and around Addis Ababa (Dawit, 1998).

2.10.1. Fruiting body production

All in all, commercial mushroom production is a solid-state-fermentation technology. All agricultural production for plant crops generates enormous waste typically 80–90% of the total biomass of agricultural production is discarded as waste (Chang, 1999). Landfilling with this abundant waste is costly and ineffective in terms of energy flow, and combustion is indeed a waste of resource (Chang and Lai, 1993; Chang and Chiu, 1992).

The fast-growing oyster mushrooms of genus *Pleurotus,* having a complete lignocellulolytic enzyme system, can use a wide spectrum of agricultural and industrial wastes such as wheat straw, leaves, saw dust, waste paper, coffee pulp, sugarcane bagasse for growth and fruiting (Chiu *et al.,* 1998; Hadar *et al.,* 1993; Ortega *et al.,* 1992). Moreover, in the genus *Pleurotus* different species are known to grow in wide range of temperature. Therefore, besides its high biological efficiency and ease in cultivation, oyster mushroom production experienced an increase of more than 200% from 1985 to 1991 (Chang and Lai, 1993).

Among these residues, the use of sugarcane bagasse allows a by product to be utilized in the production of a food of high nutritional value, with a protein content of up to 40% in dry matter. Sugarcane bagasse, which is the major by-product of the sugar cane industry (Rajarathnam and Bano, 1989), contains about 50% cellulose, 25% hemicellulose and 25% lignin. Due to its abundant availability, it can serve as an ideal substrate for microbial processes for the production of value-added products. Attempts have been made to produce protein-enriched animal feed, enzymes, amino acids, organic acids and pharmaceutically important compounds from bagasse substrate (Ashok *et al.,* 2000).

Mushroom can be cultivated through a variety of methods. But the standard mushroom production involves different operations such as selection of mushroom

types or strains, maintenance of mycelial cultures, development of spawn (inoculums), preparation of growing medium, spawn inoculation and colonization of substrates, crop management for mushroom production (Smith *et al.,* 2002). Commercial production techniques for *Pleurotus* mushroom are well developed and are relatively simple and inexpensive when compered to other mushroom groups (OECD, 2005). Dawit (1995) described that traditional brick pots such as`Ensra` could be used to grow Oyster mushroom for small scale production.

Three major treatments can be used for the preparation of substrate to cultivate Oyster mushroom, which are composting, fermentation and pasteurization (Stametes and Chilton, 1983). According to Tilahun, (2006) using pasteurization or fermentation methods of substrate preparation showed no significant difference on mushroom yield in the case of *Pleurotus* (*P.ostreatus* and *P. sajor-caju*) mushroom grown on the three substrate (Teff, Grass and Wheat bran). Therefore, considering the economics of mushroom production, fermenting substrate for small scale cultivation of *Pleurotus* mushroom, especially in low-income communities, becomes interesting since it excludes the pasteurization stage that increase the production cost. Other mushrooms require a sterilized substrate that requires a boiler and an autoclave large enough to sterilize commercial quantities of substrate which is quite expensive.

Oyster mushrooms are so prolific that the mycelium outgrows most contaminants. *Pleurotus* species can also be cultivated in submerged liquid cultures for the production of fungal protein, as a source of spawn or flavoring agents (Hadar and Cohen, 1986).

Oyster mushrooms have several advantages and only a few drawbacks. They are easy to grow using agricultural waste as a substrate. The remaining composted material can be used as a feed for cattle or as a soil amendment after the mushrooms are harvested. On the cautionary side, these mushrooms produce numerous spores, which trigger allergies in many people. Oysters have a relatively short shelf life of approximately 1 week.

One of the drawbacks of using fruiting bodies of wild mushroom as a source of secondary products is the difficulty of getting large enough amounts of fruiting bodies when required particularly from wild. Use of cultures of fungi as a source of secondary products often has advantages. The cultures can be maintained in the laboratory and can be grown on a larger scale when needed. Attempts to improve yield of desired products can also be undertaken by optimizing nutrients, physical conditions of growth or by genetic manipulation and selection (Dawit and Ermias, 1995).

2.10.2. Solid state fermentation (Mycelial production)

Solid-state fermentation (SSF) processes, which involve the growth of microorganisms (typically fungi) on moist solid substrates in the absence of free-flowing water, have considerable economical potential in producing products for the food, feed, pharmaceutical and agricultural industries. SSF was already used for the production of fermented food, enzyme and edible mushrooms in Asia and Africa in the ancient World (Wei Pei-lian *et al.,* 2006). The advantages of SSF when compared to the submerged fermentation includes; effective production of secondary metabolites such as enzymes, aroma substances, coloring substances as well as pharmaceutically active compounds, produce a more stable product, requiring less energy, in smaller fermenters and with easier downstream processing measures. Recent evidence indicates that bacteria and fungi, growing under SSF conditions, are more than capable of supplying the growing global demand for secondary metabolites (Robinson *et al.,* 2004).

2.9. Extraction, Detection and determination

Lovastatin is extracted from different fungi with various procedures. Ethyl Acetate, acetonitrile, toluene, methanol can be used as extraction solvents. Clean-up procedures, column chromatography and solid phase extraction can be used. Thin-layer chromatography was developing for primary screening, which separated the major co-metabolite from lovastatin and hence used for detection. High- performance

liquid chromatography (HPLC) provides a reliable method for quantification of lovastatin (Gunde-cimerman *et al.,* 1993).

Lovastatin is present under two types of structural forms depending on pH of the fungal material to be extracted. In alkaline solution the open β-hydroxyacid form predominates while the lactone and open β-hydroxyacid form being in eqilibrium under acidic condition (Alarcoin *et al.,* 2003). There are also different options for HPLC determination of lovastatin in different fungal extract: 1) determination of the compound in open hydroxy acid form after adjustment of the pH to 7.7 (Shindia, 1997); 2) determination of lovastatin in both open β-hydroxyacid and lactone forms existing simultaneously in an acidic condition (Kysilka and Kren, 1993).

Methanol and ethyl acetate extraction of the compound were compared in the work of Siamak, *et al.,* (2003) and they came up with a result that after methanol extraction, a significant amount of lovastatin remains in open β-hydroxyacid form. Using ethyl acetate for extraction were enable to recover about 90% of existing lovastatin as lactone form, which can be detected easily by the TLC system.

Lactonization can be carried out for conversion of open β-hydroxyacid to lactone form this can be done by after extraction with ethyl acetate; the extract is preconcentrated and followed by lactonization in toluene at 106^{o}C. for 2 hours (Kumar *et al.,* 2003).

3. OBJECTIVE OF THE STUDY

This work was undertaken for the following objective

- General objective
  - ✓ To prove the presence of lovastatin in the Oyster mushroom cultivated in Ethiopia
- Specific objective
  - ✓ Qulitative determination of lovastatin in *P.ostreatus* and *P.sajor-caju* fruiting body.
  - ✓ Selection of substrates for lovastatin production of *P.ostreatus* and *P.sajor-caju* in solid substrate.
  - ✓ To quantify amounts of lovastatin in each extract.

4. MATERIALS AND METHODS

4.1. Cultivation experiments

4.1.1. Organism and culture condition

Fungal culture: *Pleurotus ostreatus* (Jacq.:Fr.) Kumm. and *Pleurotus sajor-caju* (Fr.) Sing. obtained from Mycology Laboratory, Biology Department, Addis Ababa University were used. The two species were grown on MEA (Malt Extract Agar) and plates were incubated at 22°c for 1-2 weeks until mycelial growth had covered almost the entire surface of the plates.

4.1.2. Fruit body production

4.1.2.1. Grain Spawn Production

The spawn used in this study was prepared on whole grains of sorghum. Sorghum was cleaned and socked overnight in water and rinsed three times in distilled water. The excess water was drained off and 10% wheat bran and 2% gypsum (calcium sulfate) were added. The ingredients were thoroughly mixed and distributed equally in to 500ml glass bottles at the rate of 100g seed per jar and autoclaved. After cooling, each bottle was inoculated with eight agar blocks(1cm x 1cm) of 15 days old agar culture and incubated for 20 days at 22°c until the substrate become fully colonized.

4.1.2.2. Substrate Preparation and Spawning

Sugar cane bagasse was mixed with gypsum and wheat bran according to the formula (weight basis) shown in (Table 2). Distilled water was used to adjust moisture of the substrate to 40%, 60%, 70% calculated by drying 100 g wet bagasse in an oven at 50°C until constant weight. The substrate is subjected to a heat treatment; according to (Cangy, 1994) the optimal pasteurization treatment for baggase was identified as two hours at 80-90°C. After cooling, the pasteurized substrate aseptically inoculated into polyethylene bags (50x20cm), with 2kg of prepared substrate per bag, together with 100 g spawn (5%: in relation to the wet mass of the substrate).

Table 2. Composition of prepared bagasse substrate

Material	Percentage (%)
Baggase(at-12% humidity) as received	80
wheat bran	10
Gypsum	10

Source: Cangy (1994)

4.1.2.3. Spawn Run and Fruit Body Development

The spawn run and cropping were performed in a room in the Faculity of Science, Addis Ababa University. The mushroom room, made from local plant material, had good ventilation and did not allow direct sunlight. The average temperature during the experimental period was about 20^{o}C. The relative humidity (RH) of the room was 60-80%. The inoculated bags were placed on wooden shelf in growing room. After completion of the spawn run; the bags were opened and unfolded at the upper parts. Pinholes were also made in the bags with needles for air exchange. Water was sprayed, twice a day, to maintain optimal moisture level and induce fruit body formation.

4.1.3. Mushroom Yield and Biological Efficiency

Mature fruit bodies were harvested at appropriate times and the fresh weight was recorded immediately after the harvest. Biological Efficiency (BE), which is defined as the ratio of the weight of fresh fruit bodies to the weight of dry substrate, multiplied by 100 (Fan *et al.,* 2004) was calculated. The total wet mass of mushrooms was obtained by the sum of yields recorded during four flushes.

4.1.4. Condition for Solid State Fermentation

Three different solid substrates sorghum, rice and wheat bran were used for cultivation of the fungus (mycelial stage) for lovastatin production.

4.1.4.1. Moisture content determination

The moisture content of the substrates that was suitable for the growth of the organism was adjusted prior to inoculation. Samples of 100-grams were taken from autoclaved substrate and were dried at 60^{o}C until constant weight was obtained. The moisture content of the three substrates was determined from the weight loss.

$$\text{Moisture level (\%) of substrate} = 100X \frac{(\text{wet weight of the substrate} - \text{dry weight of the substrate})}{\text{Wet weight of the substrate}}$$

4.1.4.2. Preparation of rice grain for solid state cultivation

A method of preparation, described by Dawit, (1999) was employed. A weight of 200g of rice grains was soaked in 200ml of distilled water for three hours in 1000ml Erlenmeyer flasks. The rice was drained and autoclaved at 121^{o}C for 15 minutes. Flasks containing sterile rice grains were inoculated with 150ml of *P. ostreatus* and *P. sajor-caju* inoculm , grown for five days in YMG (yeast extract, 4g; malt extract,10g; glucose, 4g per 1 litter of distilled water). The solid-state fermentation lasted for 2-6 weeks at 22^{0}C samples were taken every five days (15th, 20th, 25thand 30th, 35 th) analyzed for their lovastatin content.

4.1.4.3. Preparation of wheat bran for solid-state fermentation

Two hundred grams of wheat bran was placed in to 500ml glass bottles and sterilized at 121°C for 15 minuites. After cooling, each bottle was inoculated with eight agar blocks of 15 days old agar culture and incubated for 18 days at 22°c until the substrate become fully colonized. Samples were taken every five days after the substrate was completely colonized; (20th, 25th, and 30th, 35 th) and analyzed for their lovastatin content.

4.2. Isolation and Characterization of lovastatin

Chemicals

Organic solvents used in this investigation were Ethyl Acetate (ALDRICH, Germeny), Dichloromethane (Riedel-de Haen, Germeny), n-Hexane (Hexane) (Riedel-de Haen, Germeny), and Methanol (Riedel-de Haen Germeny), Acetonitrile (HPLC grade, ALDRICH, Germeny), Na_2SO_4, NaOH, HCl.

4.2.1 Extraction

4.2.1.1. Preparation of crude extract

Fruit body Extraction for TLC analysis

Fruiting bodies were collected from the mushroom growing room after three months and processed for extraction according to Gunde-cimerman *et al.* (1993). Hundred gram of fresh mushroom fruit body (moisture content 90%) was blended with electrical blander. The blended mushroom biomass was acidified to pH = 3 using 1N HCl. The material was extracted with 200ml of ethyl acetate (EtOAc). Extraction was performed in a shaker at 120 revolutions per minute (rpm) for 2 hours. Then the biomass was filtered with cotton and the biomass was extracted three times. The collected organic phase was separated from the aqueous phase by separatory funnel and dried with Na_2SO_4 and concentrated using rotary evaporator (Buchi Liboratechnic, Switzerland). Crude extract was weighted after completely dried. After dissolving the dried sample with EtOAc then subjected to flash chromatography for clean up. Each fraction collected from flash chromatography was again concentrated and lactonized with toluene at 106^{o}C. for 2 hours then subjected to TLC run.

Fruit body Extraction for HPLC analysis

To hundred gram of blended fresh mushroom, 200ml of a mixture of methanol: water (1:1, v/v) was added. The pH was adjusted to 7.7 with 1N NaOH. Extraction was performed on rotary shaker at 120 rpm at room temperature for 2 h. Then the organic phase was separated by separatory funnel and dried with Na_2SO_4 then concentrated

using rotary evaporator at 50 ºC. Then samples were kept at 4ºC until needed (Gunde-cimerman *et al.,* 1993).

Extraction of sorghum grain culture (SSF) for TLC analysis

Two weeks after inoculation, 100g of completely fermented sorghum was ground with mortar and pestle. After acidification (to pH = 3) of the biomass with 1N HCl, it was extracted with 200ml EtOAc. Extraction was performed on rotary shaker at 120 rpm for 2 hours. Then the biomass was filtered with cotton and the organic phase was separated from the aqueous phase by separatory funnel and dried with Na_2SO_4. The extract was concentrated using rota evaporator near dryness and subjected to flash chromatography for clean up. The collected fraction was again concentrated and lactonized. It was then spotted on TLC plates.

The extraction, concentration and lactonization for rice grain and wheat bran culture were performed in the same manner. All TLC positive samples were further analyzed by HPLC.

Preparation of solid state culture samples for HPLC analysis

Hundred gram solid state culture of grain was ground with mortar and pestle and 200ml of methanol (MeOH): water (1:1, v/v) was added. The pH was adjusted to 7.7 with 1N NaOH. Extraction was carried out in a shaker at 120 rpm at room temperature for 2 h. It was then filtered and the biomass was extracted three times. The collected organic phase was separated from the solution by separatory funnel and dried with Na_2SO_4. Samples were completely evaporated and the dried residue was weighed and dissolved in 5ml acetonitrile that was purifying through a C_{18} solid-phase extraction cartridge. All samples were filtered through Whatman 0.45µm filter (Millipor corp) before injection to get rid of particles that might interfere with the HPLC system.

4.2.1.2. Standard sample preparation

Preparation of Lovastatin standard in lactone form

Pharmaceutical grade lovastatin (lactone form) tablets (MEDOSTATIN® Edochemie LTD, Limassol-CYPRUS) containing 20 mg lovastatin per tablet were used to prepare the lovastatin standards for the TLC analyses. A tablet was dissolved in 5ml EtOAc. After filtration, preparative TLC on 2.5mm thick silica gel G_6 (BDH Chemicals LTD) was undertaken. Pure lovastatin band was scraped from the plate and then dissolved with EtOAc and separated the gel by filtration.

Preparation of standard in open β-hydroxy acid form

The open hydroxy acid form of lovastatin was prepared freshly from lactone form, whenever necessary, Pharmaceutical grade lovastatin (lactone form) tablets containing 20 mg lovastatin per tablet were used to prepare the standards for the HPLC analyses. According to Siamak *et al*. (2003), the lactone form of lovastatin was converted to the β-hydroxy acid form by dissolving the tablets in a mixture of 0.1 N NaOH and ethanol (1:1 by v/v), heating at 50 °C for 20 min, and neutralizing with HCl. The solution was kept at 4°C until needed.

4.2.2. Chromatography

4.2.2.1. Column chromatography

The crude extracts were cleaned up using silica gel column chromatography which separated the major co-metabolite from lovastatin. A column was prepared by adding mixture of silica gel and n-Hexane to a column (3cm x 18cm). The silica gel was allowed to settle while n-Hexane was slowly passed through the column. The dried crude extracts were dissolved in a minimal volume of EtOAc and applied on a silica gel column. The column was eluted starting with n-hexane followed by n-hexane: EtOAc (1:3 v/v) and finally with EtOAc. The collected fractions were concentrated and lactonized befour TLC run (Kumar *et al.,* 2002).

4.2.2.2. Thin layer chromatography (TLC)

Fractions collected from column chromatography were concentrated and applied to 20x 20 cm silica gel $60F_{254}$ TLC plates (Merck Art No. 1.05554). The plates were developed in solvent systems of Dichloromethane: ethylacetate (70:30, v/v). The developed plates, after air-drying, were examined under the Ultra Violate light (UV) at 254nm and afterward stained with iodine vapor. The replicate plates were sprayed with vanillin/sulphuric acid spray reagent then heated until color developed. For each TLC run, lovastatin standards were applied and Rf (ratio to front) value, UV absorption and color reaction were compared.

$$R_f = \frac{\text{Distance traveled by substance}}{\text{Distance traveled by solvent front}}$$

4.2.2.3 HPLC for Lovastatin analysis

HPLC analysis of lovastatin was determined in open hydroxy acid form. It was performed on HPLC (Agilent Technologies 1200 series, chromatography columns ZORBAX Eclipse XDB-C-18, 5µm, 150×4.6mm) diode array detector was used with UV detector at 238 nm. Six-point calibration standard containg lovastatin acid form was prepared in a concentration range of 0.25 to 10mg/L. These standards are used throughout the analysis. The mobile phase was prepared according to Ganrong *et al.* (2002), acetonitrile: water (pH is adjusted to 2.5 with H_3PO_4) at the ratio of 45:55 (v/v). The sample injection volume was 20 µL. The flow rate is 1.0mL/min and the column temperature was 28ºC. Identification was done by comparing the Diod Array UV spectrom of the sample with that of the standard. Quantification was based up on the external standard calibration curve obtained from the plot of peak area verses concentration of standard lovastatin sample runs.

5. RESULT

5.1 Spawn Production

Whole grain sorghum, obtained from local market, which was used as a substrate for spawn production, supported good mycelia invasion by both *Pleurotus* species (*P. ostreatus* and *P.sajor-caju*) (Figure. 8). Both species took 2-3 week to colonize the substrate completely. The moisture content of the sterile- moist sorghum (55-60%) was found to be suitable.

Figure 8 Spawn of *P.ostreatus and P. sajor-caju* on sorghum

5.2. Spawn running and Fruit Body Development

Mycelial development in general took 4-6 weeks on average after inoculation of both species of *Pleurotus* on bagasse substrate. The first pinhead (fruit bodies) appeared on 3-5 days after the bags were opened for both species and mature fruit bodies developed after 3 days. The optimum moisture content of the substrate was found to be 60%.

Figure 9. Mature fruiting body of Oyster mushroom on bagasse substrate

5.3. Harvesting Yield and Bioconversion Efficiency

Fruit body of Oyster mushroom started to appear as early as 35 days on pasturized bagasse substrate colonized by *P. ostreatus* and 38 days by *P.sajor-caju.* The mean mushroom yield during the cultivation period of 72 days from the three replicates is given in Table 3. The table shows the average yield per bag and bioconversion efficiency of the mushroom harvested on supplemented bagasse. About 94% of the total yield was obtained during the first three flushes. Higher yield of around (780.1g /bag) from *P. ostreatus* and (748.57) from *P.sajor-caju* can be obtained form the four harvesting time.

Table 3. Biological efficiency of supplemented bagasse colonized by *P. ostreatus* and *P.sajor-caju*

Mushroom type	Flush*	BE (%)	Production time (weeks)**
P. ostreatus	1st 2nd 3rd 4th	23.7 24.9 25.58 15.66	6 7 8 9
P. sajor-caju	1st 2nd 3rd 4th	13.7 24.5 20.85 11.66	7 8 9 10

*Flush = number of harvests
**Production time = the number of weeks following inoculation

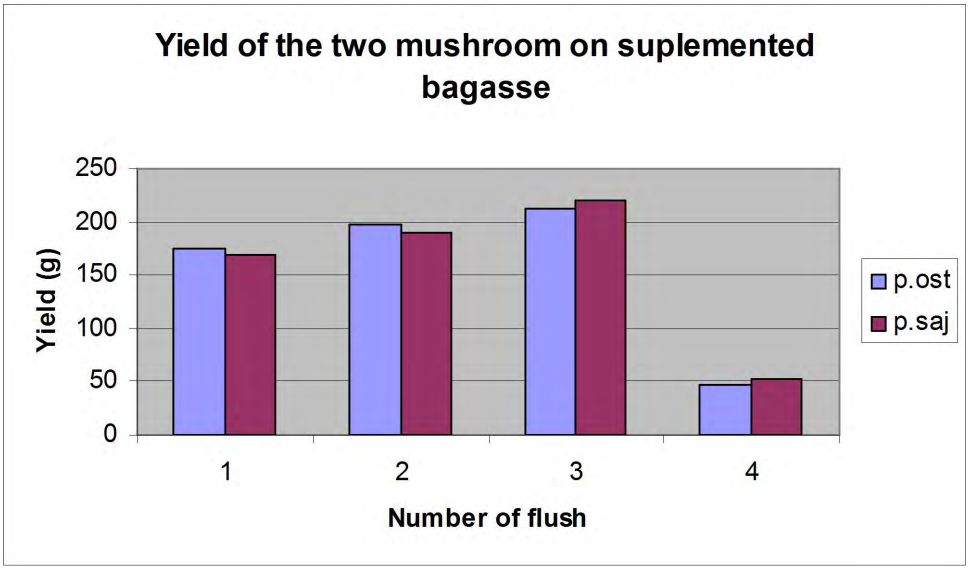


Figure 10. Total yield of supplemented bagasse colonized by *P. ostreatus* and *P.sajor-caju*

5.4. Lovastatin production

Fruiting body of both *P. ostreatus and P.sajor-caju* showed positive result on TLC when compared with the standard. Lovastatin was also detected from culture of *P. ostreatus* and P.sajor-*caj)* that were grown on rice and wheat bran. Lovastatin was not detected from sorghum culture of both mushrooms.

Lovastatin production by SSF on Rice

Complete colonization of both *Pleurotus* on rice substrate lasted for about 15 days. Lovastatin production was slow at the first 20 days after inoculation, the productivity increased during the period from 25th day to 35th day. The highest yield of Lovastatin, 3.69µg/g dry weight was obtained at 30thday of cultivation after where the Lovastatin content remained as the same level.

Figure 11. Solid state fermentation of *P.ostreatus and P. sajor-caju on rice grain*

Time course of lovastatin production from mycelial culture of the two fungi grown on rice.

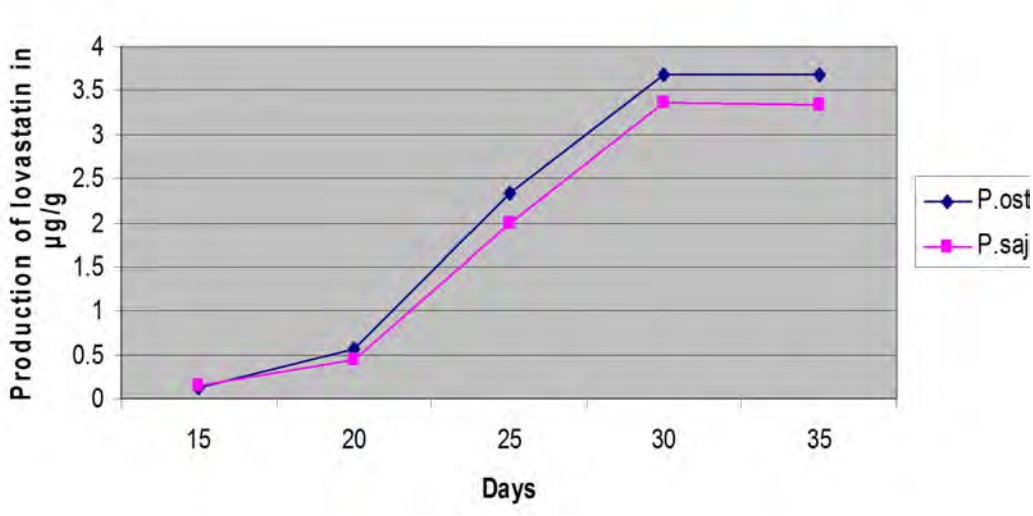


Figure 12. Lovastatin production of the two fungi culture on rice.

Lovastatin production by SSF on wheat bran

A complete colonization of both *Pleurotus* on wheat bran lasted for about 20 days. A relatively high yield of lovastatin was obtained at the level of 11.7µg/g at 30th day of cultivation.

Figure 13. Solid state culture of *P.ostreatus and P. sajor-caju on wheat bran*

Time course of lovastatin production from mycelial culture of the two fungi grown on wheat bran

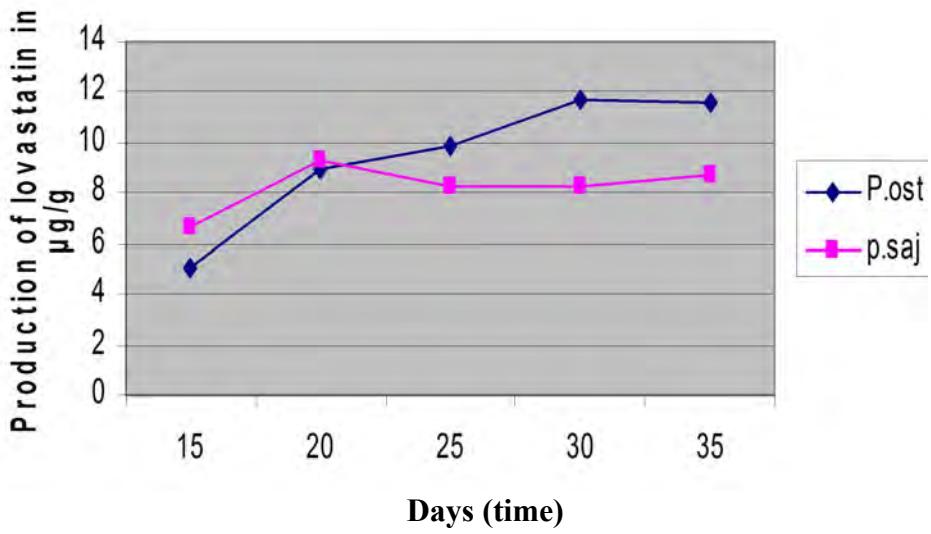


Figure 14. lovastatin production on culture of Oyster mushroom on wheat bran

5.5. TLC analysis

A TLC separation was done for primary screening. The extracts gave spot that have the same R_f value when compared with the standard sample of lovastatin. Lovastatin

in the lactone form was easily detected with UV absorption at 254 nm and after staining with iodine vapor. Where as the β-hydroxyacid form was easily detected by the developed color reaction after sprayed with vanillin/sulphuric acid. The R_f value of the lactone and the β-hydroxyacid form on silica gel TLC were 0.3 and 0.57, respectively. Both mushrooms are capable of producing lovastatin in notable amount.

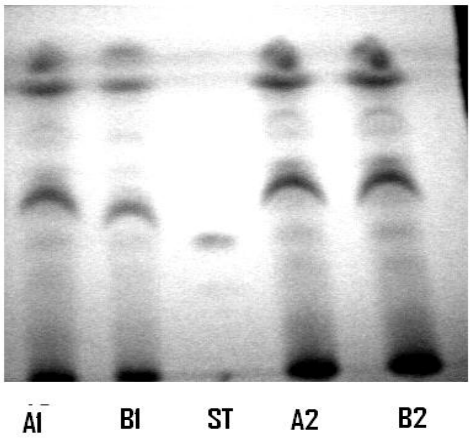


Figure 15. TLC of crude extract of culture of the two fungi on Rice and Wheat bran and lovastatin standard

ST = Lovastatin Standard
A1 = Rice culture of *P.ostreatus*
A2 = Wheat bran culture of *P.ostreatus*
B1 = Rice culture of *P. sajor-caju*
B2 = Wheat bran culture of P.*sajor-caju*

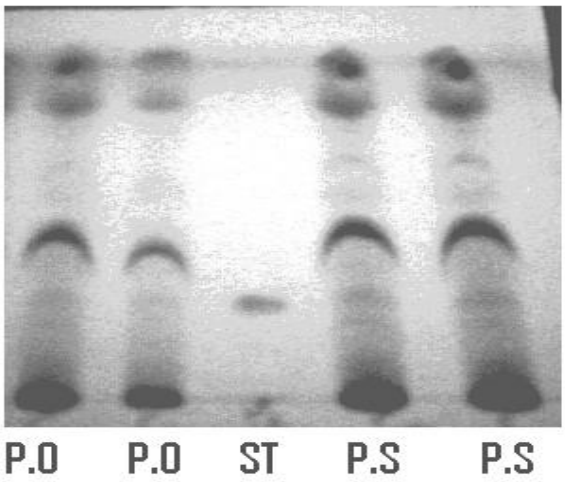


Figure 16. TLC of crude extract of fruit bodies of the two fungi

ST= Lovastatin Standard
P.O= Fruiting body of *P.ostreatus* and
P.S= Fruiting body of *P. sajor-caju*

5.6. HPLC analysis

For HPLC analysis, the extraction was carried out with methanol without any acidification so that lovastatin is preserved at the original status. HPLC spectrums of some of the samples from both fungi show the same spectrum that is in Figure 17 and 18. The retention time for the acid form was compared with the standard sample and it was 16.8min. The amount of Lovastatin produced from *P.ostreatus* and *P. sajor-caju* fruiting body, mycelial culture grown on rice and wheat bran are given in Table 4. The maximum lovastatin production (132µg/g) and (129µg/g) were obtained from fresh fruiting bodies of *P.ostreatus* and *P. sajor-caju* respectively.

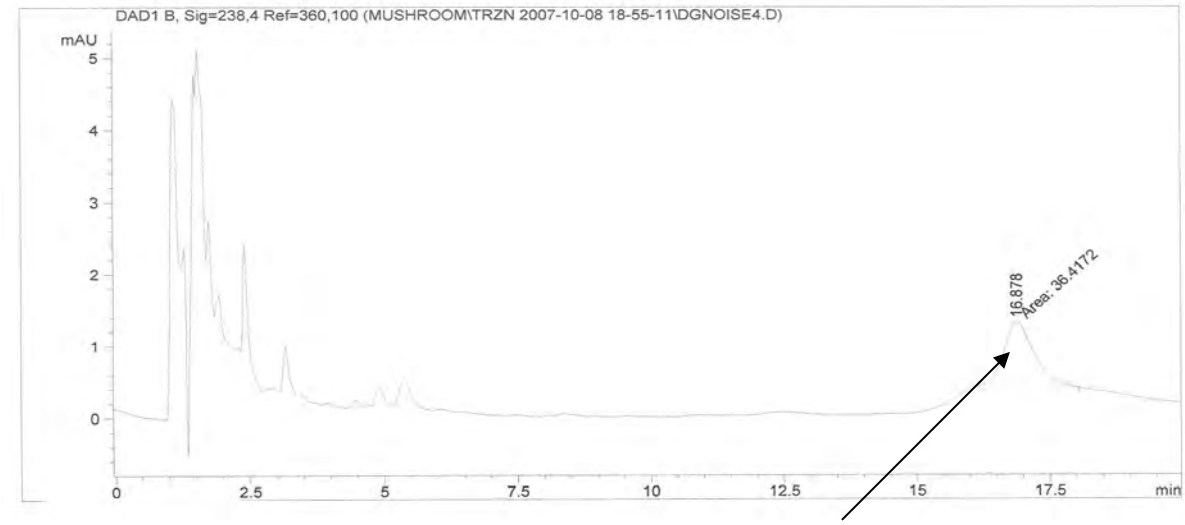


Figure 17. HPLC spectrum of lovastatin from extracts of mycelial culture of P.sajor-caju on wheat bran.

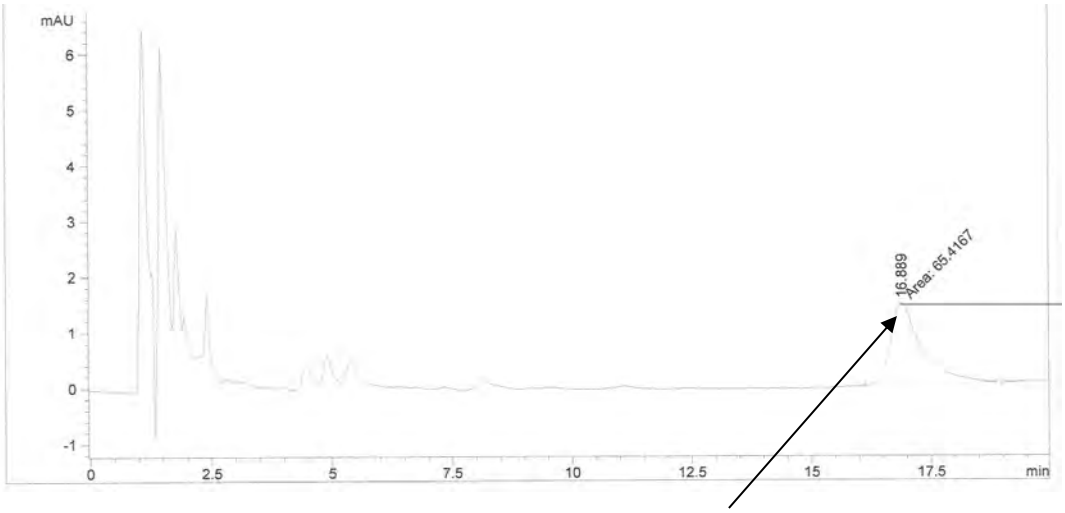


Figure 18. HPLC spectrum of lovastatin from extracts of fruiting bodies of *P.sajor-caju* .

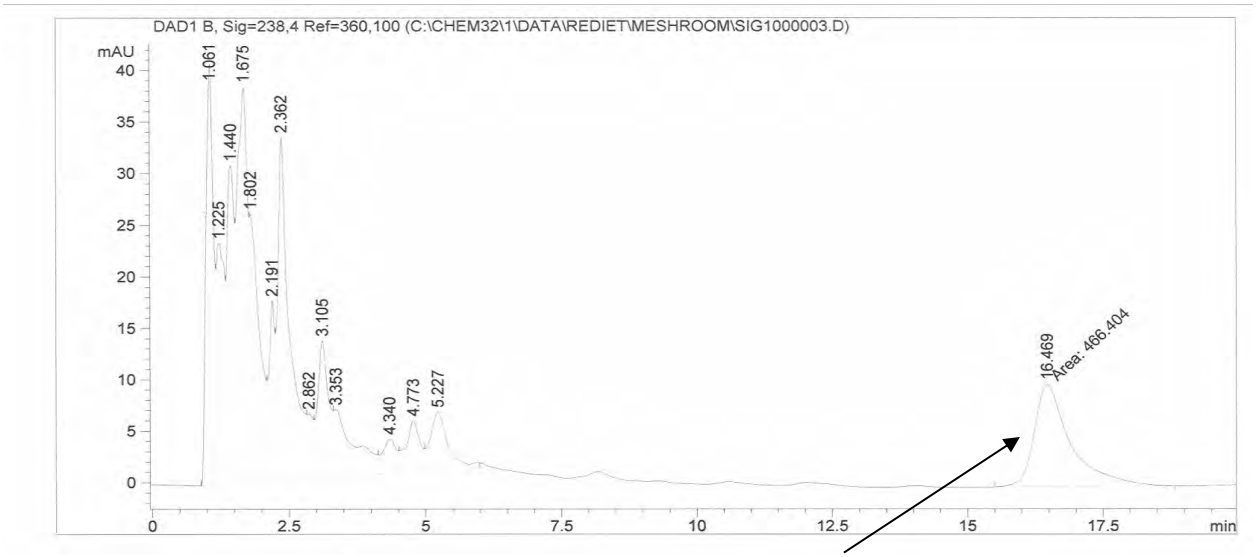


Figure 19 . HPLC spectrum of lovastatin from extracts of *Pleurotus ostreatus* fruiting bodies.

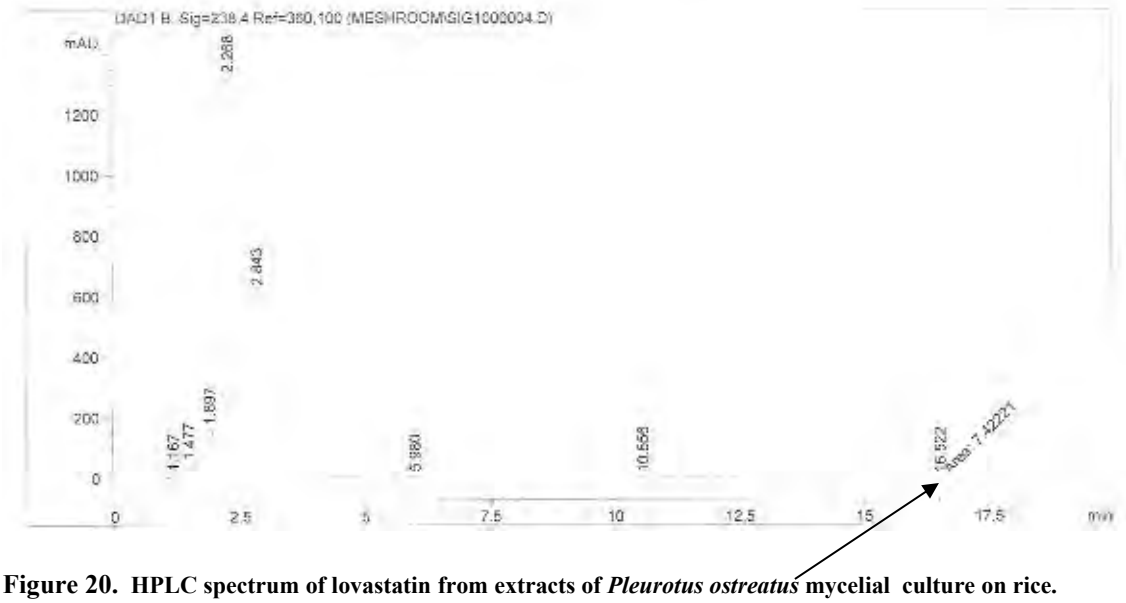


Figure 20. HPLC spectrum of lovastatin from extracts of *Pleurotus ostreatus* mycelial culture on rice.

Table 4. Lovastatin production in (µg/g) dry weight of the two species of *Pleurotus*

Test portion	Lovastatin production (µg/g)dry weight	
	P.ostreatus	***P. sajor-caju***
Fruiting bodies(MeoH)	132	129
Fruiting bodies(EtOAc)	0.08	0.12
mycelial culture on sorghum(EtOAc)	-	-
mycelial culture on rice(MeoH)	3.69	3.36
mycelial culture on rice(EtOAc)	0.18	0.29
mycelial culture on wheat bran (MeoH)	11.7	9.97
mycelial culture on wheat bran (EtOAc)	0.93	1.36

6. DISCUSSION

The oyster mushroom is one of nearly 40 edible species of the genus *Pleurotus*. It is highly admired for its tender flesh, velvety texture, and mild flavor coupled with its distinctive flavor aroma and excellent drying and preservation qualities. The genus *Pleurotus* species are efficient utilizers of lignocellulosic agricultural residues with biotechnological and environmental application (Cohen *et al.,* 2002). Various strategies have been developed to utilize the vast quantities of lignocelluloses waste generated annually through the activities of agricultural, forestry, and food processing industries. One of the most significant, in terms of producing a higher value product from the waste, is the cultivation of edible mushrooms by solid-state fermentation (Hadar *et al.,* 1993). Thus, mushroom cultivation can usually be viewed as an effective means to extract resources left behind in agricultural solid wastes

These mushrooms use a wide spectrum of agricultural and industrial wastes such as wheat straw, teff straw, leaves, saw dust, waste paper, coffee pulp, grass, sugarcane bagasse. In countries where sugar cane is grown for sugar production, bagasse is extensively and increasingly being utilized for mushroom cultivation. It should be noted that bagasse is a material that does not necessitate any chopping or any treatment to break down its fibers to allow mycelial penetration when it is used in mushroom production. In Mauritius, Mexico and Cuba bagasse has been identified as a better raw material for cultivation of Oyster mushroom than other locally available substrate such as straw or corncobs, (Soto-Velazoc *et al.,* 1991; Cangy, 1994). The three sugar factories in Ethiopia (Wonji, Shewa, Metahara) produces about 476 000 tones (235,000 tones of dry matter) of bagasse each year (FAO, 1988) and it is attractive to use the fungus to digest this waste and by doing so produce a cash crop of mushroom.

In this study both species of Pleurotus (*P.ostreatus* and *P. sajor-caju)* showed dense and heavy filamentous mycelial growth on the entire surface of the spawn substrate (i.e. sorghum grain) indicating that the grain provided good food base for the fungal species to grow throughout the substrate. Dawit (1998) also described that sorghum

support good mycelial growth and it is better available substrate in some areas in Africa including Ethiopia.

In this study pasteurized and supplemented sugarcane bagasse was used as substrate for cultivation of *P.ostreatus* and *P. sajor-caju*. Mycelial colonization of the two species was visually observed. At 40% moisture content the mycelial growth was slow and the mycelium was thin implying that this amount of water is much less than that mushroom mycelium requires for growth. Since mushrooms are aerobic organism, they require free oxygen in the substrate, in 70% moisture condition, growth of mycilum was limited to the surface where there is enough oxygen. From the result of these study 40 and 70% moisture content was not good condition for cultivatin of Pleurotus mushroom (Dawit, 1998). Moisture content of 60% was found to be good for mycelial growth on bagasse substrate (Cangy, 1994). In fruit body formation period, *P.ostreatus* was faster than *P. sajor-caju*, fruit body starting to appear 35 days and 38 days on average respectively.

About 94% of the total yield was obtained during the first three flushes that are in line with Dawit (1998) the highest crop of the mushroom is obtained during the second and the third flush. In view of total yield from 72 days of harvest period the bagasses colonized by *P.ostreatus* give higher bioconversion efficiency than *P. sajor-caju* (Table 3). Similarly the average bioconversion efficincy of *P.ostreatus* and *P.sajor-caju* was 22.9% and 17.67% respectively. In related study, (Shah *et al.,* 2004). reported a biological efficiency of 13-19% when *P.sajor-caju* was cultivated on pasteurized sugarcane bagasse.

Mold contamination was observed on some bags, susceptibility of substrate to mold invasion could be due to the residual sugar. After removal of the juice by mills, bagasses contains about 50% water, 45% cellwall material 5% water soluble substances mainly sugar (Dawit, 1998).

Recently, more attention has focused on a second area of exploitation following the discovery that many of these mushrooms produce a range of metabolites of intense interest to the pharmaceutical/nutriceutical (antitumor, immunomoodulating and hypocholesterolemic agents) and food industries (Cangy, 1994).

The result of this findings indicated that extracts prepared from fruit bodies and mycelial culture of two species of the genus *Pleurotus* contain Lovastatin , which have been shown to produce this inhibitor in notable amount as reported by Gunde-cimerman *et al.* (1993).

In this work, EtOAc extracts of fruit body as well as the mycelial culture grown on rice, sorghum and wheat bran were evaluated. Our results in terms of lovastatin production of the cultivated Oyster mushrooms of Ethiopia are in line with the work of Gunde-cimerman *et al.* (1993) from Oyster mushroom in Slovenia. The extracts from fruit bodies show positive result using TLC. The mycelial culture grown on rice, and wheat bran also show positive result where as that of sorghum did not show any spot on TLC. This may be due to good support for mycelial growth of sorghum that implies sorghum does not favor secondary metabolite production in good amount so it can not be detected on TLC or MeOH: water extract of the sorghum culture may give a better result.

The MeOH: water extract of fruiting bodies of both mushrooms (*P. osteratus*, and *P. sajor- caju*) contained 132 µg/g and 129µg/g of lovastatin respectively in dry weight. This figure is lower than reports by Alarcoin *et al.* (2003) who reported 400µg/g after cultivation of the mushroom on wheat straw from Chile and Gunde-Cimerman and Cimerman (1995) reported 875.6µg/g from Slovenia. This may be due to strain difference and the type and quality of the substrate used to cultivate the mushroom. Moreover, Gunde-Cimerman and Cimerman (1995) has shown that the amount of lovastatin in Oyster mushroom fruit body is variable depending on the age of (maturity) the fruiting body.

We were able to determine lovastatin only in β-hydroxyacid form during HPLC analysis and the lactone form was not determined. Using EtOAc for extraction of acidified sample were allow to recover about 90% of existing lovastatin as lacton form but after MeOH:water (1:1v/v) extraction, (pH =7.7) a significant amount of lovastatin remains in open β-hydroxyacid form (Siamak *et al.,* 2003). In this experiment the extraction was performed in two different ways EtOAc and MeoH. As indicated in Table 4 the MeOH extracts gave higher yield than the EtOAc. This result showed that most of the lovastatin in MeOH extract exist in β-hydroxyacid form and using EtOAc for extraction were enable to recover about 90% of existing lovastatin as lactone form.

In this study the R_f value of the lactone and the β-hydroxyacid form on silica gel TLC were 0.3 and 0.57, respectively which is in agreement with the result that was obtained by Gunde-cimerman et al. (1993).

According to Ganrong, (2002) and Vilches *et al.* (2005) most lovastatin producing fungi produce lovastatin in the β-hydroxyacid form (~ 65-80%), so this method (preparing extracts with MeOH: water without acidification) eliminated the conversion step to lactone form (lactonization) that save time and solvent. Using the β-hydroxyacid form allowed rapid analysis because this form is eluted earlier from a chromatography column than does the lactone form of lovastatin. In addition, the β-hydroxyacid is quite stable in solution.

At present 80-85% of all medicinal mushroom products are derived from the fruit bodies while only ~15% are based on extracts from mycelial and only a small percentage are from culture filtrate (Cangy, 1994; Lindequist *et al.,* 2005).

However one of the shortcoming of using fruiting bodies as a source of secondary products is the difficulty of receiving adequate amount of fruiting bodies when required particularly from wild mushroom. Use of cultures of fungi as a supply of secondary products often has advantages. The cultures can be maintained in the

laboratory and can be grown on a larger amount when needed. Efforts to get better yield of preferred products can also be carried out by optimizing growth condition (Dawit and Ermias, 1995).

In this study the mycelial culture of Oyster mushroom grown on rice, and wheat bran show the presence of lovastatin in the fungal biomass. MeoH: water extract of the culture of *P osteratus*, grown on rice, and wheat bran gave (in dry weight) 3.69 µg/g and 11.7µg/g respectively whereas *P. sajor-caju* gave 3.36µg/g and 9.97µg/g on rice and wheat bran respectively. However, the yield of EtOAc extract was low, indicated in Table 4. Wheat bran was found to be a good substrate for lovastatin production when compared to the other tasted substrate. However large amount of lovastatin was obtained in fruiting body (Alarcoin *et al.,* 2003).

In general, the effiency of lovastatin production determined by the amount of lovastatin produced by the various fungi strains together with the efficiency of the extraction procedure. *Aspergillus* strains are more productive than other fungi, but they produce a wide range of toxic substances including terrain, patulin, citrinin and citreoviridin besides lovastatin, and it requires complex and additional extraction and purification procedures to obtain pure lovastatin (Wasser, 2002). Not only are these procedures more expensive but require use of large number of solvents, which in turn are toxic such as benzene, toluene, acetonitrile or ethyl acetate. Hence working with these solvents endangers the health of a persons involved and requires multi-step purification procedures.

Accordingly, there is a need for methods to produce cholesterol-lowering compounds with high activity, preferably from source that are not toxic, and by using simple, rapid and inexpensive manufacturing processes. Many recent studies, suggested the production of this compound from edible Basidiomycetes mushroom (*Pleurotus* species) has many advantages (Wasser, 2002). Nutritionally, it has unique flavor and aromatic properties; and it is considered to be rich in protein (21.3% in *P. ostreatus* and 18.03 in *P. sajor-caju),* fiber, carbohydrates (32.5% in *P. ostreatus* and 23.5 in *P.*

sajor-caju), vitamins and minerals (Shahdat, 2003)**.** Unlike other mushroom species, Oyster mushroom are the easiest, fastest and cheapest to grow, require less substrate preparation time, production technology, and with high biological efficiency.

Pleurotus fungi have been known that have a positive effect on human health and nutrition for centuries (Cohen *et al.,* 2002). Oyster mushroom in the diet reduces very profoundly and effectively cholesterol diet-induced accumulation of cholesterol in blood and several other body organs. This effect of Oyster mushroom was investigated by Bobek and Galbavy (1999). They use male rabbit as experimental animal with an average body weight of 2.25kg, 1% cholesterol was added to normal diet. The animal also receive 10% dried Oyster mushroom fruiting bodies instead of cellulose after 3 months they observed 30% reduction in total cholesterol(TC) and LDL and 20% reduction in VLDL. From this result in order to have the same effect on human with body weight 50kg one has to consume 10% dried oyster mushroom that contain (1.32mg of lovastatin) for about 13 months. Otherwise, one can increase the dose to shorten the time.

The minimum doses of lovastatin prescribe to early stage hypercholestrolaemic individual is 20mg per day (Tobert, 1987). Depending on the result obtained in this study, consumption of 1515.1g fresh oyster mushroom daily may have the same effect as the drug in the tablet form. It is also possible to use the mushroom and the mycelial culture as food supplement for life long with out side effect there is little scope for toxicity and overdose. *Pleurotus* spp also exhibit hematological, antiviral, antitumor, antibiotic, and immunomodulation activities (Cohen *et al.,* 2002) in addition to its high nutritive value. A regular intake may enhance the immune response of the human body thereby increasing resistance to disease and in some cases cause regression of a disease state.

7. CONCLUSION

Different filamentous fungi produce Lovastatin, the cholesterol-lowering agent. The presence of lovastatin in the fruiting body and mycelial culture of edible Pleurotus mushroom makes the fungus regarded as a functional food or/and pharmaceuticals.

Further studies should be conducted in areas of improving the yield of lovastatin by selecting best strain and by introducing various supplement to substrate used for cultivation.

A mushroom-related industry based on utilization of the lignocellulosic waste material that are abundantly available in rural and urban areas can have positive global impact on long-term food nutrition, health, environmental conservation, regeneration, economic and social change especially in developing countries.

Mushroom cultivation, in particular Oyster mushroom, should be practice in Ethiopia and thus can be integrated as an extra-agricultural activity. Awareness of the people to wards the beneficial effect of oyster mushroom should be increased by develop simple cultivation methods which could be practiced by small groups of farmers particularly women to supplement their cash income and protein needs of the family.

8. References:

Alarcoin, J., Agula, S., Aranicibia-Avil, P., Fuentes, D., Zamorano-ponce, E. and Hernandez, M. (2003). Production and Purification of Statins from Pleirotus ostreatus (Basidiomycetes) strains. *Exp. Mycology*. **58:** 62-64.

Alberts, A. W. (1998). "Discovery, Biochemistry and Biology of Lovastatin". *The American Journal of Cardiolo y* . **62:** 10-15.

Alberts, A. W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffmann, C., Rothcock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapley, E., Albers-Schonberg, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Liesch, J. and Springer, K. (1980). Mevinolin: A highly potent competitive inhibitor of 3 hydroxy 3 methylglutaryl Coenzyme A reductase and a cholesterol lowering agent. *Proc Natl. Acad. Sci.* **77:** 3957-3961.

Ashok, P., Carlos, R., Soccol, P. N. and Vanete, T. S.(2000). Biotechnological potential of Agroindustrial residues sugar cane bagasse. *Bioresource Technology*. **74:**1-6.

Bobek, P. (1991). Cholesterol-lowering effect of the mushroom *Pleurotus ostreatus* in hereditary hypocholesterolemic rats. *Annals of Nutr and Metabol*. **35:**191-195.

Bobek, P., and Galbavý, S. (1999). Dose-and time-dependent hypocholesterolemic effect of oyster mushroom (*Pleurotus ostreatus*) in rats. *Nutrition* **14** (3)**:** 282-286.

Breene, W. M. (1990). Nutritional and medicinal value of speciality mushrooms. *Journal of Food Protection* **53:** 883-894.

Cangy, C. L. (1994). The cultivation of Pleurotus in Mauritious. Proc. 1st Conference on African Mycology, 10-15 June, Mouritius.

Chang, S. T. (1999). Global impact of edible and medicinal mushrooms on human welfare in the 21st century: Nongreen revolution. *Int. J. Med. Mushrooms*. **1:** 1–7.

Chang, S. C. and Lai, M. M. (1993). The Culture and Cultivation History of the Chinese Shiitake, *Shanghai Science and Technology*, pp. 202. Shanghai, China.

Chang, S. T. and Chiu, S. W. (1992). Mushroom production— An economic measure in maintenance of food security. **In:** *Microbial Technology: Economic and Social Aspects*, pp. 110–141. Cambridge University Press, Cambridge.

Chang, S. T., Lau, O.W. and Cho, K.Y. (1981). The cultivation and nutritional value of *Pleurotus sajor- caju*. *Euro. J. Appl. Microbiol. and Biotechnol.* **12**:.58-62.

Chiu, S. W., Chan, Y. H., Law, S. C., Cheung, K. T. and Moore, D. (1998). Cadmium and manganese in contrast to calcium reduce yield and nutritional value of the edible mushroom *Pleurotus pulmonarius. Mycol. Res*. **102:** 449–457.

Cohen, R., Persky, L. and Hadar Y. (2002). Biotechnological applications and potential of wood-degrading mushrooms of the genus Pleurotus. *Appl. Microbiol. Biotechnol*. **58:** 582-594.

Daniel, S. (2006). An interpretive history of the cholesterol controversy: The discovery of the statins and the end of the controversy. *J Lipid Research*. **47:** 1339-1351.

Dawit, A. (1995). Cultivation of the Oyster mushroom in traditional brick pot. *Mycologist.* **9**(4): 179-181.

Dawit, A. (1998) Mushroom cultivation: *A practical approach*. Berhanema Selam PrintingPress, Addis Ababa.

Dawit, A. (1999). Secondary products by solid state cultivation of Fungi. **In:** *Chemistry, Biological and Pharmaeceutical properties of African Medicinal Plants*. International Organisation for Chemical Sciences in Development. (IOCD)-2nd International Symposium. BL 10. February 28th – March 3rd. Addis Ababa, Ethiopia.

Dawit, A. and Ermias, D. (1995). Bioactive compound from plants and higher fungi of Ethiopia. **In:** *Phytochemistry of plants used in traditional medicine* eds. Hostettmann, K., Marston, A., Millard, M. and Hamburger, M., pp. 295-312. Oxford University Press.

Eckert, D. M. and Kim, P.S. (2001). Mecanism of viral membrane fusion and its inhibition. *Ann, Rev. Biochem*.**70:** 777-810.

Endo, A., Kuroda, M. and Tanzawa, K. (1976). Competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by ML-236A and ML-236B, fungal metabolites having hypocholecterolemic activity. *FEBS Lett.* **72:** 323-326.

Endo, A. (1979). Monacolin K, A new hypocholesterolemic agent produced by a *Monascus* spesies. *J. Antibiotics*. **32:** 852-854.

Endo, A. (1992). The discovery and development of HMG-CoA reductase inhibitors. *J Lipid Res*. **33:**1569-82.

Fan, L., Padez, A., Mohan, and Soccol, C.R. (2004). Use of various coffee industry residues for the cultivation of pleurotus ostreatus in solid-state fermentation. *Act.Biot*. **20**(1)**:** 41-52.

FAO (1988). Animal Production and Health papers: Sugarcane as feed. (72)

Furberg, S. and Curt D. (1999). "Natural Statins and Stroke Risk". *Circulation.* **99**: 185-188.

Ganrong, X., Chen Y., Ling, X. (2002). Production of Monacolin K in Solid-state Fermentation of *Monascus sp.* 9901. *J. Chromatogr.* **28**(10)**:** 16-23.

Giguere, J.F. and Tremblay, M.J (2004). Statin compounds reduce human immunodefficiency virus type 1 replication by preventing the interaction between viron- associated host intrecellular adhesion molecules and its natural surface ligand LFA-1. *J. Virol*. **78:**12062-12065.

Gordon, M. C. and David, J. N. (2005). International collaboration in drug discovery and development from natural sources. *Pure Appl. Chem*. **77**(11)**:** 1923–1942.

Greenspan, M. D. and Yudkovitz, J. B. (1985). Mevinolinic acid biosynthesis by *Aspergillus terreus* and its relationship to fatty acid biosynthesis. *J. Bacteriol*. **162:** 704-707.

Gunde-Cimerman, N., Friedrich, J., Cimerman, A. and Benicki, N. (1993). Screening fungi for the production of an inhibitor of HMG CoA reductase: Production of mevinolin by the fungi of the genus Pleurotus. *FEMS Microbiol. Lett.* **111:** 203-206.

Gunde-Cimerman, N., Plemenitas, A. and Cimerman, A. (1993). Pleurotus fungi produse mevinolin an inhibitor of HMG-CoA reductase. *FEMS microbiology letters*. **113:** 333-338.

Gunde-Cimerman, N. and Cimerman, A. (1995). Pleurotas Fruiting Bodies Contain the Inhibitor of Hydroxy-3-Methylglutaryl-Coenzyme A Reductase- Lovastatin *Exp. Mycology*. **19:** 1-6.

Hadar, Y. and Cohen-Arazi, E. (1986). Chemical composition of edible mushroom *Pleurotus Ostreatus* produced by fermentation. *Appl. Env. Microbiol*. **56**(6)**:** 1352-1354.

Hadar, Y., Kerem, Z. and Gorodecki, B. (1993). Biodegradation of lignocellulosic agricultural wastes by *Pleurotus ostreatus*. *J. Biotechnol*. **30:** 133–139.

Hobbs, C. (1995). Medicinal Mushrooms**:** *An exploration of Traditional, Healing and Culture.* Santa Cruz, CA., Botanica Press.

Kumar, H., Srinivasan, R. and Pardeep, D. (2003). Prosses for the isolation of lovastatin. United States Patent 7052886.

Kysilka, R. and Kren, V. (1993). Determination of lovastatin (mevinoln) and mevinolinic acid in fermentation liquids. *J. Chromatogr*. **630:** 415-417.

Lindequist, U., Niedermeyer, T. H. and Julich, W. (2005). The Pharmacological Potential of Mushroom. CAM. **2**(3)**:** 285-299.

Lopez, C.JL., Perez, S. JA., Sevilla, F. JM., Fernandez, A. FG., Grima, M.E. and Chisti, Y.(2004). Fermentation optimization for the production of lovastatin by Aspergillus terreus: use of response surface methodology. *J. Chem. Technol. Biotechnol*.**79:**1110-1126.

Ma, P.T., Gil, G., Sudhof, T.C., Bilheimer, D. W., Goldstein, J. L., Brown, M. S. (1986). Mevinolin, an inhibitor of cholesterol synthesis, induces mRNA for low density lipoprotein receptor in livers of hamsters and rabbits. *Proc Natl Acad Sci.* **83:**8370-4.

Manzoni, M. and Rollini, M. (2004). Biosynthesis and biotechnological production of statins by filamentous fungi and application of these cholesterol-lowering drugs. *Appl. Microbiol. and Biotechnol*. **58:**555-564.

Memon, R. A., and Gilani, A. H. (1995). An update on hyperlipidemia and its management. *J. Pak. Med. Assoc*. **45:** 275-282.

Michael, S. (2004). Chemical, pharmacokinetic and pharmacodynamic properties of statins: an update .*Fundamental & Clinical Pharmacology*.**19 :**117–125.

OECD Envirnoment, health and safety publication. (2005). Consensus document on the biology of *Pleurotus* species (Oyster mushroom). ENV/JM/MONO, Paris.

Ortega, G. M., Martinez, E. O., Betancourt, D., Gonzalez, A. E., and Otero, M. A. (1992). Bioconversion of sugarcane crop residues with white-rot fungi *Pleurotus* spp. *World J. Microbiol. Biotechnol*. **8:** 402–405.mentation broths.

Pegler, D. N. (1977). A preliminaryAgaric flora of East Afriva. London Her Majesty's Stationary Office, UK.

Rajarathnam, S., and Bano, Z. (1989) *Pleurotus* mushrooms: Part III. Biotransformations of natural lignocellulosic wastes commercial applications and implications. *Critl. Rev. Food Sci. Nutr.* **28:** 31-113.

Robinson, T., Singh, D. and Nigam P. (2004). Solid-state fermentation: a promising microbial technology for secondary metabolite production. *Appl. Microbiol. Biotechnol*. **55**(3)**:** 284-289.

Royse, D. J. and Schisler, L.C. (1987). Yield and size of *Pleurotus ostreatus* and *Pleurotus sajor-caju as* effected by delayed-release nutrient. *Appl*. *Microbiol*. *Biotechnol*. **26**:191-194.

Shah, Z. A., Ashraf, M. and Ishtiaqch. M. (2004). Comparative study on cultivation and yield performance of oyster mushroom (*Pleurotus ostreatus*) on different substrates (wheat straw, leaves, sawdust). *Pakistan J. Nutrition*. **3**(3): 158-160.

Shahdat, H., Michio, H., Emran, K., Choudhury, N. A., Shahjalal, H., Moynul H., Shahabuddin, K. C. and Ishtiaq M. (2003). dietarymushroom (*pleurotus ostreatus* ameliorates atherogenic lipid in hypercholesterolaemic rats. *Clinical and Experimental Pharmacology and Physiology.* **30:** 470–475.

Shepherd, J., Hunninghake, D.B., Barter, P., McKenney, J.M., Hutchinson, H.G. (2003). Guidelines for lowering lipids to reduce coronary artery disease risk: a comparison of rosuvastatin with atorvastatin, pravastatin, and simvastatin for achieving lipid-lowering goals. *Am. J. Cardiol.* **91** (5)**:** 11-17.

Shindia, A.A. (1997). Mevinolin production by some fungi. *Folia Microbio*. ***42:*** *477-480.*

Siamak, M., Samiee, N., Moazami, S., H., Farzaneh, A. M., Saeid, M. and Mohammad, R. B. (2003)**.** Screening of Lovastatin Production by Filamentous Fungi. *Iran*. *Biomed*. *J.* **7** (1)**:** 29-33.

Smith, J. E., Rowan, N. J. and Sulivan, R. (2002). Medicinal Mushroom: Their Therapeutic Properties and Current medical usage With Special emphasis on Cancer Treatement. University of Strachclyde, Cancer research, UK.

Soto-Velazoc, C., Guzman-Davalos, L. and Tellez, C. (1991). Substrate for cultivation of Pleurotus in Mexico II: Sugarcane bagasse and corn stover. *Mushroom Journal of Tropics*. **11:** 34-37.

Stametes, S. and Chilton, J.S. (1983). The mushroom cultivator: *A practical guide to growing mushrooms at home*. Agrickon Press, Olympia, WA.

Tobert, J.A. (1987). New development in lipid- lowering therapy: the role of inhibition of hydroxymethylglutaryl-coenzyme A reductase. *J American Heart Association* . **76:** 534-538.

Tilahun, Z. (2006). Bioconversion effiency of Oyster mushroom (*Pleurotus ostreatus and Pleurotus sajor-caju*) on fermented and non fermented straw. M.sc. Thesis, Addis Ababa University, Ethiopia.

Ucar, M., Mjorndal, T., Dahiquist,R. (2000). HMG-CoA Reductase Inhibitors and Myotoxicity. *Drug Safty*. **22**(6)**:** 441-457.

Viard, M., Parolini, I., Sargiocomo, M., Fecchi, K., Ramoni, C., Ablan, S.and Blumenthal, R. (2002). Role of cholesterol in human immunodefficiency virus type 1 envelope protein-mediated fusion with host cell. *J Virol*. **76:**11584-11595.

Victoria, F. R. (2005). Antihyperlipidemic Statins: A Self-Contained, Clinically Relevant Medicinal Chemistry Lesson. *American Journal of Pharmaceutical Education*. **69** (4)**:** 77-85.

Vilches, M.A., Ferro, J.L., Lo´ pez, J.A., Pe´ rez, J.M., Ferna, N. S. and Chisti´ Y. (2005). Rapid screening of Aspergillus terreus mutants for overproduction of lovastatin. *World J. Microbiol. Biotechnol.* **21:**123-125.

Wasser, B., Solomon, P., Sergey, V. (2002). Process for producing, methods and Compositions of cholesterol lowering agents from higher Basidiomycetes mushroom. Free patents online.Com pp.1-15.

Wei Pei-lian , Xu Zhi-nan and Cen Pei-lin. (2006). Lovastatin production by *Aspergillus terreus* in solid-state fermentation, pp. 1521-1526, Zhejiang University Press.

Zhuang, C. and Mizuno, T. (1999). Biological responses from Grifola frondosa. *International Journal Medicinal Mushrooms,* **1:** pp. 317-324.