Chapter 13
Microbial Statins

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13.1 Introduction

Statins are a class of antihypercholesterolemic (or cholesterol-lowering) drugs which act on the liver by reducing the biosynthesis of the steroid by inhibiting the activity of HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase, the enzyme responsible for the first step in the synthesis of cholesterol (and other biomolecules). Among statins, there are the molecules that are produced through synthetic means and those produced via fermentation-based processes with their semisynthetic derivatives. The rational production of microbial statins and discovery of new potential statin producers is stimulated by the current market value, with a market share around 60 %. Natural statins have a 2–3 billion dollar market (Bizukojc et al. 2007; Findlay et al. 2007; Morikawa et al. 2002; Murphree 2008; Rozman and Monostory 2010; Seraman et al. 2010; Vilches Ferrón et al. 2005; Weber et al. 2007), while the semisynthetic simvastatin has a 50 % market.

Among the advantages for microbial production of statins, there is the possibility of utilization of agro-industrial by-products for sustainable bioproduction and the possibility of using selected or modified microorganisms to obtain new statins. Statins also have additional biological effects, such as microvascular endothelial protection function (in the presence of hyperglycemia, thus inhibiting the early stage of diabetic microangiopathy), or regression of fatty streak lesions (Arikan et al. 2012; Bizukojc et al. 2007; Nozue et al. 2012; Seraman et al. 2010; Vilches Ferrón et al. 2005).

All bioactive secondary metabolites are common on traditional therapeutic regimens for hyperlipidemia which have been associated with increased risk of coronary

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S.K. Brar et al. (eds.), *Biotransformation of Waste Biomass into High Value Biochemicals*,
DOI 10.1007/978-1-4614-8005-1_13,

disease, stroke, and heart attack. Hyperlipidemia is a leading cause of death in many countries, which is often triggered by hypercholesterolemia (the accumulation of cholesterol in the blood), leading to atherosclerosis (Talayero and Sacks 2011).

This chapter highlights some of the major hypolipidemic properties and aspects relevant to microbial statins, such as the mechanism of action, the market value of these drugs, the potential for new microbial statins, and the production process for the most common microbial statins. It will also present examples of well-characterized non-statin hypolipidemic agents.

13.2 Hyperlipidemia and the Processes Leading to Atherosclerosis

13.2.1 Definitions

Hyperlipidemia or dyslipidemia is a condition characterized by an increased concentration of lipids (triglycerides, cholesterol, or both) and lipoproteins (low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL)) in the blood. Specific terms to increased blood concentrations of triglycerides are referred to as hypertriglyceridemia, while increased blood concentrations of cholesterol are referred to as hypercholesterolemia. The term hyperlipoproteinemia refers to increased blood concentrations of lipoproteins (Talayero and Sacks 2011).

13.2.2 Causes of Hyperlipidemia and Its Association with Atherosclerotic Processes

The mechanisms behind pathologies must be understood in order to develop better drugs. The causes underlying hyperlipidemia and atherosclerosis are discussed in this section.

13.2.2.1 Causes of Hyperlipidemia

Several causes have been reported to cause hyperlipidemia: high-fat diets, obesity, endocrine disorders (such as diabetes mellitus, hypothyroidism, or hyperadrenocorticism), and cholestasis. Among these risk factors, high-fat diets are the main cause of hyperlipidemia. Higher amounts of saturated fat, trans fat, and cholesterol intake in high-fat diet cause increased LDL levels. However, other risk factors and LDL participation have a central role in the atherosclerotic development process.

A vast number of studies confirmed the intimate and causative relationships between dyslipidemias and diabetes (Subramanian and Chait 2012). In diabetes mellitus, there is deficiency of insulin. The chylomicrons and VLDL are released

into blood and should be unloaded by lipases located on the vascular endothelium of tissues. However, these enzymes cannot function to its full extent because of the insulin deficiency, since insulin can regulate lipase gene expression (Bouraoui et al. 2012). In accordance with lipase inhibition, there will be increased triglyceride levels or hypertriglyceridemia. High triglyceride levels are markers for several types of atherogenic lipoproteins, especially apo C-III (Fukui et al. 2011).

Hypercholesterolemia has been stressed as one of the common biochemical findings in primary hypothyroidism. In general, hypothyroidism is associated with hypercholesterolemia mainly due to the elevation of total cholesterol and LDL-C levels, whereas there is an increase in triglyceride levels due to decreased activity of the lipoprotein lipase. There were multiple mechanisms accounting for atherosclerosis in patients with hypothyroidism, including hypercholesterolemia, insulin resistance, and increased oxidation of LDL-C (overview of the atherogenesis below) (Arikan et al. 2012).

Comparative aspects of hyperadrenocorticism and arteriosclerosis have shown that advanced hyperadrenocorticism exhibits widespread arteriosclerosis with calcific complications. This disease shows a higher hypertriglyceridemia and hypercholesterolemia due to the associated metabolic alterations. Cortisol concentrations induce the insulin resistance and lipase activity (Ottosson et al. 1994; Wexler 1971).

Hypercholesterolemia always occurs in cholestasis, a disturbance of the secretion of bile salts by the liver, as bile is the chief pathway for the elimination of cholesterol from the body (Wagner et al. 2009).

13.2.2.2 Overview of the Atherogenesis

Atherosclerosis has been reported as a result of hyperlipidemia, and it is regarded as a lipid-induced inflammatory disease where the immune system plays a pivotal role in its initiation and progression. In general, atherogenesis begins at sites of endothelial injury caused by smoking, infection, diabetes mellitus, and hypertension and which results a nidus for monocyte and lipid/lipoprotein accumulation into the underlying arterial intima (Gu et al. 2012). Lipid and lipoprotein accumulation is due to adhesiveness and permeability of the endothelial injury. As LDL accumulates, it may be entrapped extracellularly in arteries, thus being subjected to a milieu conducive to various kinds of enzymatic and chemical modification (oxidation and glycation). Lipoproteins undergo minimal oxidation during circulation but become progressively oxidized within the arterial wall. The monocytes subsequently differentiate into macrophages and ingest modified LDL and other particles (Koga and Aikawa 2012). In a way, scavenger receptors of macrophages bind oxidized LDL but not native LDL, leading to development of cholesterol ester-engorged cells or foam cells, the precursors of atherosclerotic lesions. T cells infiltrating into the endothelial lesion may recognize antigenic signals presented by the activated macrophages and generate an immune response by inflammatory cytokines and growth factors production which stimulate smooth muscle cell migration and proliferation (Kzhyshkowska et al. 2012). The smooth muscle cells, in turn, secrete extracellular matrix components that form a

fibrous cap over the underlying atherosclerotic lesion. Foam cells undergo apoptosis and release their lipid contents to form an extracellular lipid core that is contained under the fibrous cap. All this process will result in thickened intima media and atherosclerosis plaque development in arteries. Moreover, in general, atherosclerotic plaques with thin fibrous cap, large lipid cores, and numerous macrophages are most likely to rupture. Thus, once plaques rupture, the thrombogenic contents are exposed to platelets and coagulation factors in circulating blood, initiating a thrombosis. Thrombosis is a blood clot within a vessel, obstructing or stopping the flow of blood (Steinbrecher 1999; Østerud and Bjørklid 2003).

Atherosclerosis usually does not cause signs and symptoms until it severely narrows or totally blocks a vessel. Therefore, atherosclerosis represents the world's largest problem from modernity by taking 17.1 million lives a year. According to the most recent data available, one in five deaths in developing countries is due to atherosclerosis in its various forms (Ellis et al. 2010; Messner and Bernhard 2010).

Treatments for atherosclerosis may include lifestyle change, surgery, and medicines, such as antihypertensives (angiotensin-converting enzyme inhibitors, calcium channel blockers, thiazide diuretics), antiplatelets, and mainly statins against high cholesterol and LDL levels (Borshch et al. 2012).

The mechanisms discussed about the model of atherogenesis initiation outlined above are mainly based on experimental animal models with high rate of developing lesions, i.e., genetically hyperlipidemic animals and fat fed. However, there is supporting evidence that many molecules, cytokines, growth factors, scavenger receptors, etc. are expressed and produced similarly in humans, validating this whole mechanism and opening several strategies for the development of new treatments.

13.3 The Statin-Based Therapeutic Strategies

13.3.1 General Characteristics of Statins

Statins are oral inhibitors of the 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, which are well-established agents to lower cholesterol levels and prevent cardiovascular morbidity and mortality. HMG-CoA reductase is the rate-limiting enzyme that catalyzes the conversion of HMG-CoA to L-mevalonate, a key pathway for cholesterol biosynthesis. Therefore, statins are the first line of defense in drug treatment of hypercholesterolemia (Morikawa et al. 2002; Rozman and Monostory 2010; Weber et al. 2007).

13.3.2 Chemical Structure and Mode of Action: Statins

Statins have differences in their chemical structure that could translate into different pharmacological properties and pharmacokinetic parameters (bioavailability,

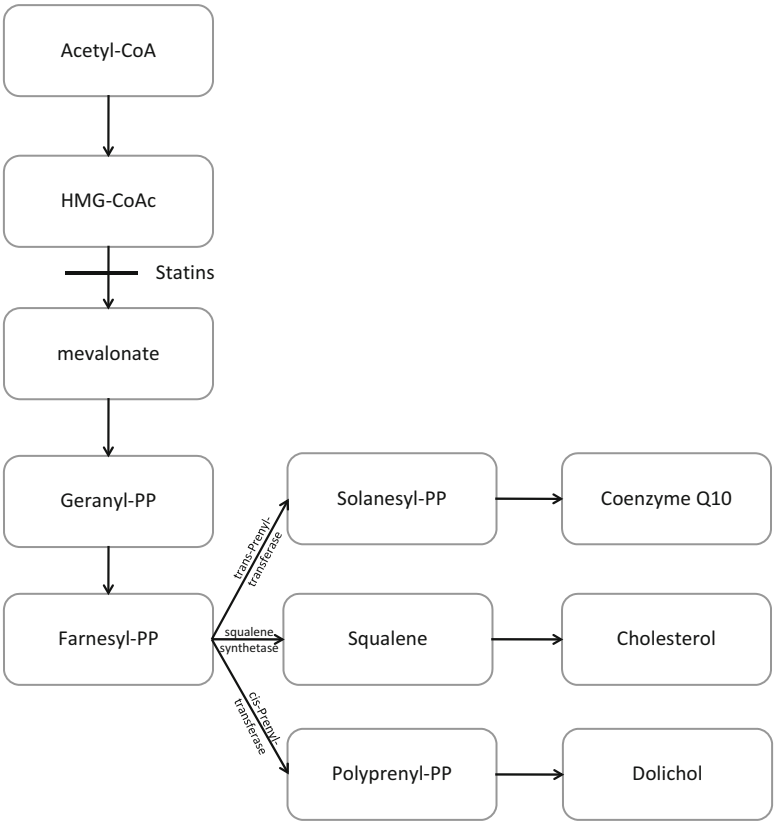

Fig. 13.1 Statins: mechanism of action

half-life, protein binding, metabolism and excretion routes, lipophilicity). All statins have a structural component which is similar to HMG portion of HMG-CoA (Fig. 13.2). Thus, statins act as competitive inhibitors of the HMG-CoA reductase. In general, competitive inhibition of HMG-CoA reductase by statins decreases the conversion of HMG-CoA to mevalonate, the rate-limiting step of cholesterol endogenous synthesis (Fig. 13.1). The reduction of downstream metabolic intermediates leads to increased expression of LDL receptor on the surface of hepatocytes and so increases uptake of LDL-C from the circulation (Campo and Carvalho 2007; Nirogi et al. 2007). Aside from their cholesterol-lowering effects, statins can reduce coenzyme Q10 and dolichol. Coenzyme Q10 is a mitochondrial coenzyme which is essential for the production of ATP and immune system while the role of the dolichol remains elusive (Cantagrel et al. 2010; Kumar et al. 2009).

Currently, seven statins are used in medical practice: lovastatin, pravastatin, simvastatin, fluvastatin, atorvastatin, rosuvastatin, and pitavastatin. From these, the last five are synthesized chemically while lovastatin and pravastatin are derived from fungal fermentation. Chemical structures of statins are shown in Fig. 13.2.

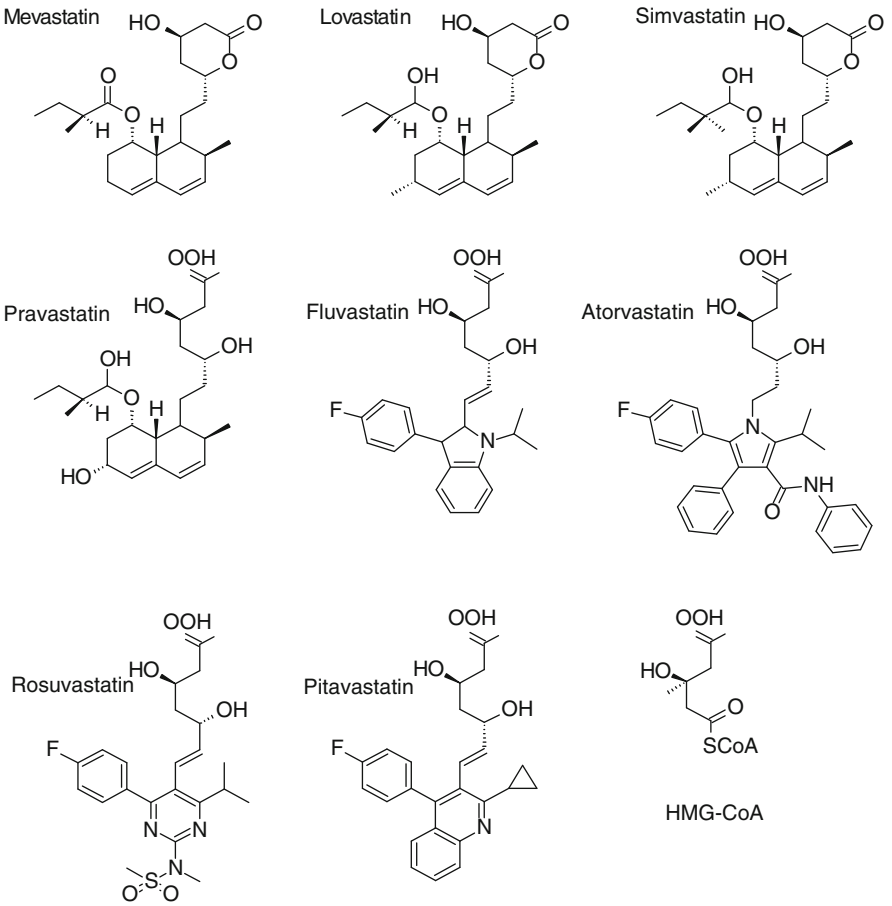

Fig. 13.2 Chemical structure of the statins and HMG-CoA, the molecule they mimic in the cholesterol synthesis inhibition

The recurrent structure in all statins is a hydroxyl carboxylic acid which mimics HMG-CoA. The lactonized forms of statins are converted to the active hydroxy acids in the liver. The efficiency with which statins are absorbed and inhibit the synthesis of cholesterol is affected by their structure, leading to a wide heterogeneity of traditional therapeutic regimens used by statins, as well as their pharmacological properties. Table 13.1 shows the therapeutic doses and half-lives for statins.

13.3.3 Other Relevant Effects of Statins

Aside from their cholesterol-lowering effects, statins are known to have a range of effects, such as vasodilatation, effects on coagulation, inflammatory response modulation, atherosclerotic plaque regression, and immunomodulatory effects.

Table 13.1 Traditional therapeutic regimens used by statins and its pharmacological properties (Betteridge 2010; Catapano 2010; Jones et al. 2003; Knopp 1999)

Statins	Therapeutic dose[a] (mg)	Elimination half-life (h)	Solubility
Fluvastatin	20–80	1–2	Lipophilic
Atorvastatin	10–80	14	Lipophilic
Rosuvastatin	5–40	19	Hydrophilic
Pitavastatin	2–4	11–18	Moderately lipophilic
Simvastatin	5–80	1–2	Lipophilic
Lovastatin	10–80	3	Lipophilic
Pravastatin	10–80	1–2	Hydrophilic

[a]Dose required for inhibition of 50 % HMG-CoA reductase

Endothelium-ameliorating effects of statin therapy were observed in patients with symptomatic heart failure. The beneficial effect of statin therapy on endothelium-dependent vasodilatation in patients was associated with coenzyme Q_{10} reductions. Therefore, statins can increase nitric oxide (NO) bioactivity, which is consistent with enhanced endothelium function, and reduce synthesis of proinflammatory proteins on the endothelial cell surface, which may reduce inflammation (Brull et al. 2001; Gajendragadkar et al. 2009; Koh et al. 2004; Strey et al. 2005).

Statins may affect the expression levels of genes involved in coagulation, such as thrombomodulin and nitric oxide syntase-3. Thrombomodulin is a transmembranous glycoprotein and plays an important role in the anticoagulant system as the receptor of thrombin leading to accelerated protein C activation (Gajendragadkar et al. 2009; Morikawa et al. 2002).

Statins have been demonstrated to inhibit superantigen-induced T cell activation, MHC class II antigen downregulation, and LFA-1 binding; reduce heart and kidney transplant rejection; reduce mortality with staphylococcal bacteremia; and reduce high-sensitive C-reactive protein in patients with coronary artery disease (Fehr et al. 2004; Weber et al. 2007).

13.3.4 Current Market Situation of Statins: The Billion Dollar Drugs

The beginning of the twenty-first century brought an economic growth reduction for most advanced countries, with an average around 2 % per year. Some economies even shrinked, and overall growth in Europe in 2012 was stagnant at 0.2 %. Despite economic losses, statin market was substantially maintained. Accounting for 6.5 % of the total pharmaceutical market share, statin drugs are the most widely sold drugs in history. To date, drug companies are earning $26 billion in annual sale (Fig. 13.3)—a single class of drugs producing more profits than Google−and are expected to continue to increase in the years ahead. The first statin, lovastatin, was launched in America in the 1980s with revenues of $200 million (Findlay et al. 2007; Murphree 2008). The statin market (Fig. 13.3) is still on the rise, although the growth is decelerating after early 2007. While new drugs are developed and the

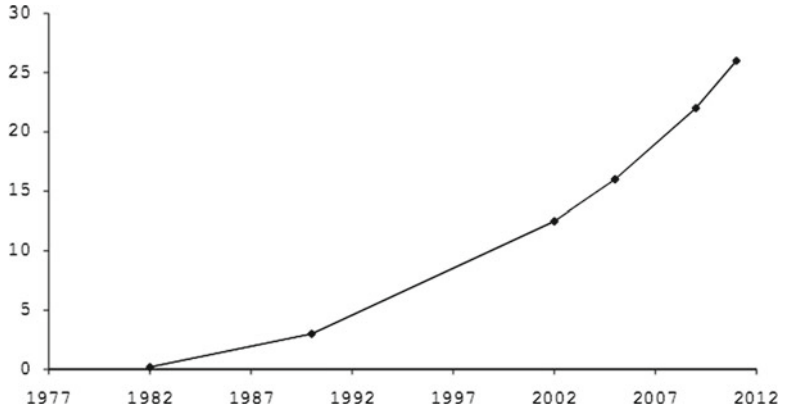

Fig. 13.3 Statin market (in billions). *Source*: Information derived by *Consumer Reports Best Buy Drugs—The statin drugs*. *The Telegraph* and *Forbes magazine*

older patents expire, statin therapies may become even more accessible, and the market may be expected to reach U$35 billion by 2023.

Statin use has increased in recent years as hyperlipidemia is being diagnosed more frequently. There is more evidence supporting the hazard of high levels of lipids in the blood, and consumers have become increasingly aware of beneficial effect of statins. New prescriptions of statins show a market share around 10 % for bio-based compounds (lovastatin and pravastatin) (Findlay et al. 2007) and a 50 % market for the semisynthetic simvastatin. This panorama is probably due to the recent expiration of patent rights for lovastatin (2001) and simvastatin (2006) and the entry of generics. The expiration of atorvastatin patents (2012) will probably bring it back as a generic statin. On the other side, with new drugs in the pipeline and an annual growth of emerging markets (such as Latin America), the microbial statin market is sure to remain huge.

13.4 Microbial Statins: Production Process and Potential for New Substances

Although most of the newer statins are synthetic, microbial statins are of interest because its production skips several synthetic steps, providing a high value-added molecule from low-cost substrates in an eco-friendly process.

ML-236B, known as compactin or mevastatin, was the first breakthrough in efforts to find a hypolipidemic agent by Akira Endo (1976). By 1980, Merck had discovered lovastatin which has been shown to be chemically very similar to mevastatin, differing by one methyl group (absent in mevastatin). Endo also had discovered the same compound, lovastatin (Brown and Goldstein 2004). Soon after the development of lovastatin, several screening efforts led to the development of similar molecules.

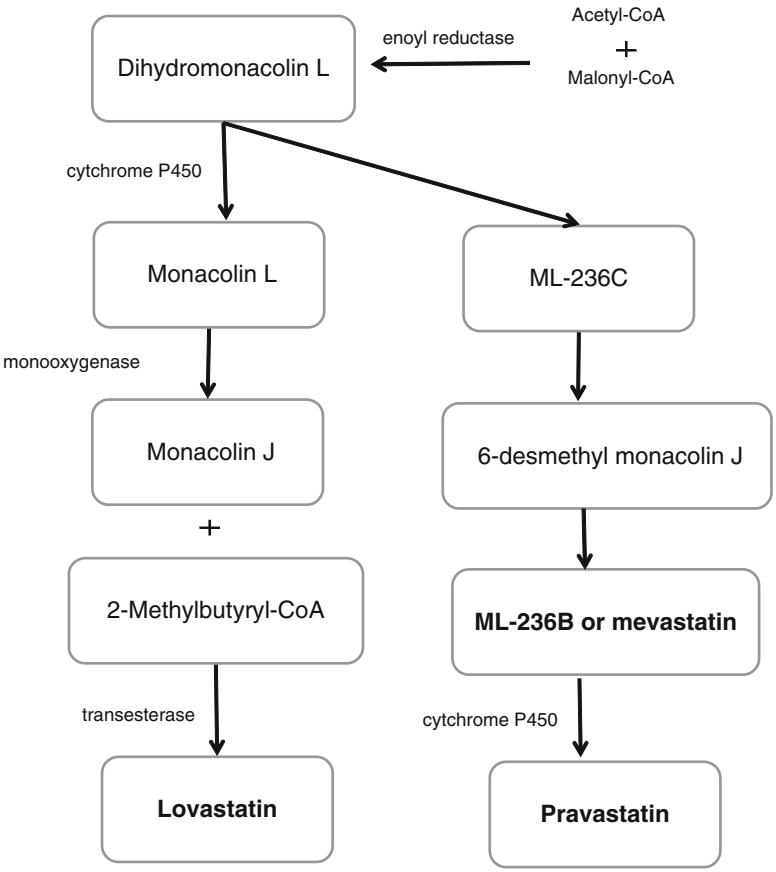

Fig. 13.4 Microbial statins biosynthetic pathway

The biosynthetic pathway for microbial statins starts with the condensation of acetyl and malonyl-CoA (Auclair et al. 2001; Barrios-gonzález and Miranda 2010; Komagata et al. 1989) and proceeds through the mevalonate pathway (Fig. 13.4).

13.4.1 Lovastatin

13.4.1.1 General

Lovastatin is a fungal secondary metabolite discovered in the seventies and introduced in the American market in the 1980s as Mevacor by Merck. It was the first statin to be approved by FDA, and it was formerly called as mevinolin or monacolin K. Lovastatin is administered as β-hydroxy lactone which over the time converts in vivo to the respective hydroxy acid form, partly similar to HMG-CoA. The hydroxyl

acid form is a weak acid ($pK_a = 4.31$) with a molar mass of 422.55 while its lactone form has a higher ($pK_a = 13.5$) (Bizukojc et al. 2007; Brown and Goldstein 2004; Lisec et al. 2012; Seenivas et al. 2008; Seraman et al. 2010).

13.4.1.2 Current and Potential Uses of Lovastatin

Besides its cholesterol-lowering properties, lovastatin has been reported as a potential therapeutic agent for the treatment of various types of cancer. Recent in vitro studies have shown that lovastatin inhibits proliferation of anaplastic thyroid cancer cells through upregulation of p27 by interfering with the Rho/ROCK (serine/threonine kinase Rho kinase)-mediated pathway—this pathway has been suggested to be involved in the regulation of cancer cell motility. Other studies have shown endothelial protection function of lovastatin in the presence of hyperglycemia. Endothelial dysfunction, such as decreased endothelium-dependent vasorelaxation, plays a key role in the pathogenesis of diabetic vascular disease. Lovastatin was able to improve mesenteric responses to acetylcholine (Gajendragadkar et al. 2009; Zhong et al. 2011). Oxidative stress has been linked to the cause of many human diseases, such as heart failure, coronary artery and chronic kidney disease, and neurodegenerative disturbances. It arises from an imbalance between an excessive generation of reactive oxygen species, reactive nitrogen species, and insufficiency of antioxidant agents. Oral administration of lovastatin has been demonstrated to reduce oxidative stress and change the activities of antioxidant enzymes (Kumar et al. 2011).

13.4.1.3 Production Process

Potential Producers

Lovastatin is produced by a variety of filamentous fungi. Some of the important microbial sources are *Monascus* sp., *Penicillium* sp., and *Aspergillus* sp. Species were found to be the most significant producers of lovastatin, such as *Monascus purpureus*, *Monascus ruber*, *Aspergillus terreus*, and *Aspergillus flavipes*. Table 13.2 lists some species evaluated or developed for lovastatin production. Although several species produce low amounts of lovastatin, they are shown in order to illustrate the genera variability.

New rapid screening methods were developed to find new potential producers based on the activity of lovastatin against the yeast *Candida albicans.* In this method, the diameter of the inhibition zones (obtained on plates of *Candida albicans*) correlated linearly with the quantity of lovastatin impregnated in the paper disc (Bizukojc et al. 2007; Seraman et al. 2010; Vilches Ferrón et al. 2005). Other method for detecting lovastatin-producing strain is based on PCR for specific genes related to lovastatin synthesis, detecting suitable strains more quickly and effectively (Kim et al. 2011).

Table 13.2 Lovastatin production (in mg/L of fermented broth) by selected strains

Microorganism	Strain	mg/L	Reference
A. terreus	ATCC 20542	100	Porcel et al. (2008)
Penicillium citrinum	MTCC 1256	589	Ahmad et al. (2010)
Aspergillus terreus	Isolate	400	Szakács et al. (1998)
Aspergillus terreus	DRCC 122 (uv mutant)	2,200	Kumar et al. (2000)
Aspergillus terreus	Isolate	400	Samiee et al. (2003)
Monascus pilosus	MK-1 (a mutant strain)	725	Miyake et al. (2006)
Monascus purpureus	MTCC 369	351	Sayyad et al. (2007)
Biospora sp.	Isolate	13	Osman et al. (2011)
Cylindrocarpon radicicola	Isolate	7.1	Osman et al. (2011)
Penicillium spinulosum	Isolate	15.8	Osman et al. (2011)
Trichoderma viride	Isolate	36	Osman et al. (2011)
Mycelia sterilia	Isolate	15.3	Osman et al. (2011)
A. terreus	DSM 13596	310	Benedetti et al. (2002)

Fermentation

Submerged fermentation and solid-state fermentation (SSF) have been used for lovastatin production. Large-scale processes were developed using *Aspergillus terreus* in submerged fermentation. Enhanced strategies, such as the use of antibiotics, cultivation in fed-batch mode, and medium development, led to higher yields (Jia et al. 2010; Seenivas et al. 2008; Porcel et al. 2008).

Solid-state fermentation is a potential alternative to produce lovastatin which generates less effluent and uses less power. SSF uses various solid substrates, such as besan flour, barley, sago, and long-grain rice (all these substrates yield high lovastatin production, >110 mg/g dry substrate); mixed solids can also be used to formulate economical substrates for commercial production (Subhagar et al. 2009; Valera et al. 2005). The inocula for these processes may be either a liquid culture or a spore suspension; after inoculation, the fermenters are maintained at a temperature, pH, and aeration rate which are characteristics of each strain for several days. Typical values are 28 °C, pH 6.5, 1.5 vvm, and 7 days for *Aspergillus terreus* strains.

Culture Medium Characteristics

As with any fermentation product, the culture medium has a significant effect on the rate of production and yield of lovastatin. The type of carbon source (e.g., fructose, lactose, glycerol), nitrogen source (e.g., soybean meal, corn steep liquor, yeast extract), and the C:N mass ratio used in the medium influenced production of lovastatin and microbial biomass by *A. terreus.* The results have shown that the presence of excess carbon (slowly metabolizable carbon source) under nitrogen limitation greatly enhanced the rate of production of lovastatin. Nitrogen limitation diverts more carbon to lovastatin metabolic pathways (Casas López et al. 2003). Most liquid culture media use glucose as the main carbon source, but some authors suggest

that this sugar strongly represses lovastatin synthesis (Miyake et al. 2006), which explains why continuous or fed-batch processes enhance the yield. In addition to glucose, several culture media also use starches and complex mixed sources, such as oatmeal, soybean meal, peptones, and yeast extract in the culture medium.

A variety of mineral nutrients is also added to the culture media, usually K_2HPO_4, $MgSO_4$, and ammonia, urea, or nitrates. Microelements are seldom added, being provided by the complex nutrients used.

Studies have shown that the supplementation of the culture medium with B-group vitamins enhances lovastatin synthesis by *Aspergillus terreus*. It is probable that the synthesis of lovastatin requires a high throughput of coenzymes, thus the application of its precursors in the form of B-group vitamins would give a positive effect on lovastatin production (Bizukojc et al. 2007). The impact of other supplements, such as linoleic acid, has demonstrated that micromolar concentrations of this fatty acid enhance lovastatin yield. Possibly, early supplementation of linoleic acid anticipates the production of oxylipins, thus mimicking the critical cell mass necessary for the onset of lovastatin production (Sorrentino et al. 2010). Vegetable oils stand out as a promising substrate as an additional carbon source for lovastatin production (Sripalakit et al. 2011).

Downstream Processing of Lovastatin

As lovastatin has a very low polarity, the concentration from the culture broth may be carried out by liquid-liquid extraction. However, there is a substantial portion of intracellular lovastatin (Benedetti et al. 2002), which may not be easily extracted. In addition, the molecule may be oxidized if not properly processed, leading to hard to separate impurities—antioxidants or inert atmospheres should be used. After extraction and concentration, the statin is usually purified by crystallization, although chromatography and ion exchange steps may also be used. The final compound should have a purity of at least 99.5 %. Figure 13.5 illustrates a possible lovastatin downstream process.

13.4.1.4 Simvastatin Production (Derivatization of Lovastatin)

The natural product lovastatin can be derived in its analog semisynthetic simvastatin. Substitution of the α-methylbutyrate side chain with α-dimethylbutyrate is most effective in treating hypercholesterolemia while lowering undesirable side effects. An alternative method for the simvastatin synthesis is a selective enzymatic deacylation of lovastatin. Due to the effectiveness of simvastatin, numerous multistep syntheses from lovastatin to simvastatin have been described in the patent literature. For example, a process for preparing simvastatin from lovastatin or mevinolinic acid in salt form comprises treating either starting material with cyclopropyl or butyl amine. In another process, lovastatin was hydrolyzed to acid form and then isolated in the form of amine salt like cyclopropyl or t-octylpropyl

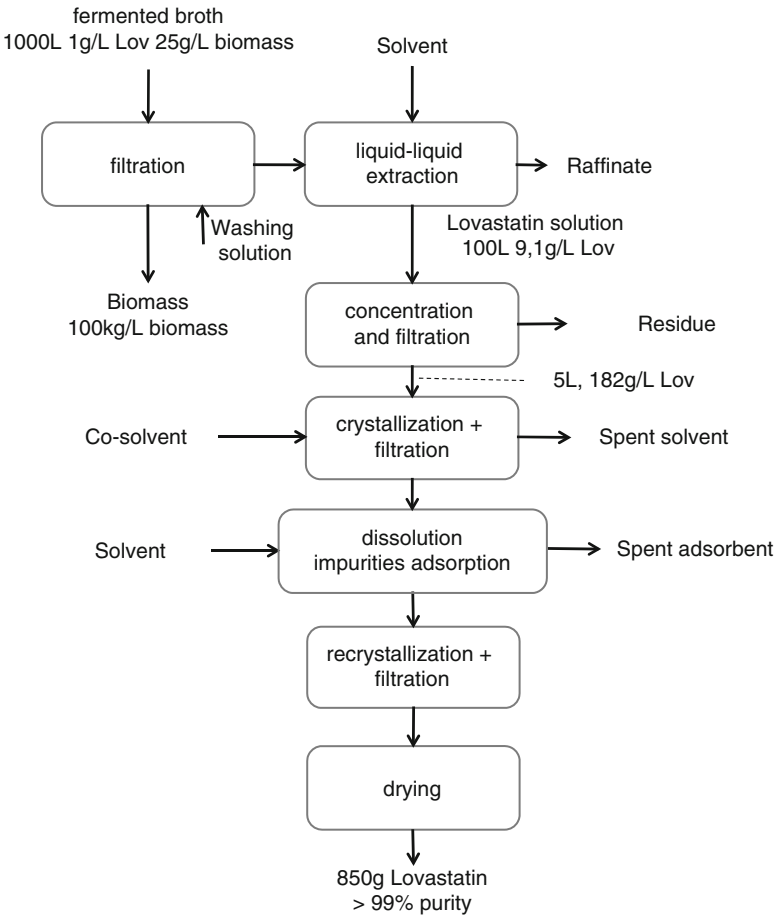

Fig. 13.5 Lovastatin downstream processing relying on crystallization operations

amines. The salts isolated were directly methylated without any protection or deprotection of hydroxyl groups. Then, simvastatin ammonium salts were converted to simvastatin by conventional methods of lactonization (Kumar et al. 1998; Vaid and Narula 2006).

13.4.2 Pravastatin

13.4.2.1 General

Pravastatin was discovered as a bioactive metabolite of mevastatin, in efforts to develop new statins. It was launched on the market in 1991 (Li 2009). Pravastatin as

well as the other statins exists in two forms, lactone and open-ring hydroxy acid form (active form); besides the open ring, pravastatin has an extra hydroxyl group in comparison with lovastatin, being hydrosoluble. Pravastatin is more effective than lovastatin in moderate doses. In human plasma, pravastatin can be determined by enzyme immunoassay or in human urine by HPLC and UV detection (Darwish et al. 2009; Whigan et al. 1989).

13.4.2.2 Current and Potential Uses of Pravastatin

Neuroprotection by pravastatin in acute ischemic stroke has been demonstrated in rats even when given after stroke onset. Among the mechanisms responsible for improved neurological outcome is the inhibition the release of potentially damaging cytokines such as interleukin-6 in the early phase of cerebral ischemia (Berger et al. 2008). Other potential uses of pravastatin are in diabetic nephropathy. Diabetic nephropathy (DN) is the principal cause of end-stage renal failure in the Western world and leads to major mortality. DN is characterized by endothelial dysfunction following a variety of proinflammatory insults. Studies have provided evidence of the protective properties of pravastatin through an upregulation in endothelial constitutive nitric oxide synthase expression in diabetic group (Casey et al. 2005).

13.4.2.3 Production Process

Overview

This compound is obtained by two-step fermentation; firstly mevastatin is produced by *Penicillium citrinum* or other potential producer, such as *Streptomyces carbophilus,* and converted by hydroxylation of mevastatin to form pravastatin. The hydroxylation of mevastatin in *S. carbophilus* is catalyzed by a $CytP450_{sca}$ monooxygenase system (Sakaki 2012; Serizawa 1996). Recent advances in the molecular characterization of the $CytP450_{sca}$ and their responsiveness to mevastatin have been achieved. For example, molecular approaches for transcriptional regulation of the cytochrome $P450_{sca}$ from *S. carbophilus* by mevastatin sodium salt or cloning, characterization, and expression of the gene encoding $CytP450_{sca}$ from *S. carbophilus* involved in production of pravastatin. $CytP450_{sca}$ DNA sequences have been annotated in GenBank® (Sakaki 2012; Watanabe et al. 1995; Watanabe and Serizawa 1998). A new strain of *Streptomyces flavovirens* has been used to produce pravastatin (Gururaja et al. 2003). A *Monascus ruber* strain is capable to produce 3000 mg/L of pravastatin in a short fermentation, according to Benedetti et al. (2002). In relation to bioconversion, it has been established that actinomycetes could hydroxylate mevastatin to pravastatin. The degree of conversion by cells was 65–78 % of mevastatin added and 65–88 % of mevastatin taken up (Peng and Demain 2000).

Downstream Aspects

Pravastatin fermentation is followed by isolation and purification. A method of isolating and purifying pravastatin or its pharmaceutically salt involves a step of extracting using an organic solvent, such as ethyl acetate (Nobunari et al. 2003). Other studies have reported purification methods which use high-performance liquid chromatography (HPLC) (Haytko and Wildman 1992). Some polymorphs of pravastatin sodium have been described as obtainable from a process wherein aprotic and protic solvents are used (Pater and Wnukowski 2012). The fact that pravastatin is hydrophilic has the benefit of limiting the contamination by lipophilic compounds in the initial step of the process and the control of solvent extraction by broth acidification but may lead to partial dimerization in the concentration steps of the solvent extract.

Traces of mevastatin can still be present in the end product after lyophilization to remove solvent. Mevastatin and pravastatin are structurally closely related. Thus, purification of pravastatin is tedious but important for the production of a safe and efficient drug. Methods have been proposed for the extraction of pravastatin and the concomitant removal of impurities. It has been found that the ratio pravastatin/mevastatin has increased when the solution is added to water-immiscible solvent (e.g., isopropyl acetate, methyl isobutyl ketone) with water or an aqueous solution at a pH value ranging from 5.0 to 6.5 (Johannes et al. 2009). Mevastatin can also be highly toxic to microorganisms responsible for biotransformation, especially to mould fungi; thus mevastatin concentration must only be maintained at a low level during industrial production (Minquan et al. 2006).

Despite known pravastatin production process, still there are problems that need to be solved. During fermentation steps, there is a common problem characterized by degradation of pravastatin (e.g., hydrolysis of pravastatin) resulting in loss of product. This phenomenon also can occur with lovastatin. Therefore, use of specific nitrogen or carbon sources to avoid statin hydrolysis during fermentation, as well as deletion of genes encoding enzymes that hydrolyze statins, is necessary (Klaassen et al. 2009). During work-up procedures, unwanted loss of product occurs as result of lactonization leading to pravastatin lactone. Any breakthrough approach suppressing lactonization is therefore of great relevance in productive process. Elevated temperatures, certain pH regimes, and traces of other molecules can promote unwanted lactonization. Aprotic solvents have been appointed to suppress lactonization in pravastatin sodium downstream process (Pater and Wnukowski 2012).

13.5 Perspectives of the Non-statin Hypolipidemic Agents

High statin doses are often associated with the increased frequency of adverse effects. In addition, all statins have been observed to cause myopathy, and the risk of adverse effects on muscle increases with the use of high doses (Riphagen et al. 2012). Therefore, non-statin hypolipidemic drugs can be an alternative treatment option.

There are several promising novel therapeutic approaches for the treatment of hyperlipidemia and atherosclerosis based on non-statin hypolipidemic agents which are expected to be of great benefit for patients with adverse effects. Some of these agents may be produced using bioprocesses.

13.5.1 Niacin

Niacin, also known as vitamin B_3 or nicotinic acid, at levels higher than the required vitamin dose functions as a vitamin, favorably affecting atherogenic lipoprotein release, as well as decreases cholesterol, triglycerides, and LDL and VLDL levels and increases HDL cholesterol levels. Niacin acts on raising apoAI and lowering apoB. These apoliproteins are the main protein components of circulating HLD (apoAI) and of LDL and VLDL (apoB). However, the absolute magnitude of these lipid-modifying effects is highly dependent not only on the daily dose but also on the lipid phenotype at baseline (Chapman et al. 2010; Rozman and Monostory 2010).

13.5.2 Ezetimibe

Ezetimibe, a cholesterol-absorption inhibitor, acts on cholesterol from diet and does not affect the absorption of fat-soluble vitamins, triglycerides, or bile acids. The effect of ezetimibe on the progression of atherosclerosis remains unknown. It selectively inhibits cholesterol absorption by binding to the Niemann-Pick C1-like 1 (NPN1L1) protein. Combined therapy with ezetimibe and a statin provides an incremental reduction in LDL-C levels of 12–19 % (Rozman and Monostory 2010; Teramoto et al. 2012).

13.5.3 Cholesteryl Ester Transfer Protein (CETP) Inhibitors

CETP promotes the transfers of cholesteryl esters from antiatherogenic HDLs to proatherogenic apoB–principle protein components of circulating LDL and VLDL. Moreover, it facilitates the transport of triglycerides between the lipoproteins. Thus, CETP transfers lipids from one lipoprotein to another that results in equilibration of lipids between lipoprotein fractions. A deficiency of CETP is associated with increased HDL levels and decreased LDL levels, supporting the therapeutic potential of CETP inhibition as an approach to retarding atherogenesis (Rozman and Monostory 2010; Tzotzas et al. 2011).

13.5.4 Fibrates

Fibrates are generally effective in lowering elevated plasma trycerides and cholesterol. It is a class of amphipathic carboxylic acids. Fibrates can implicate five major mechanisms underlying the modulation of lipids: (a) induction of lipoprotein lipolysis, (b) induction of hepatic fatty acid uptake and reduction of hepatic triglyceride production, (c) increased removal of LDL particles, (d) reduction in neutral lipid exchange between VLDL and HDL, and (e) increase in HDL production and stimulation of reverse cholesterol transport. In general, fibrates are considered to be well tolerated, with an excellent safety profile (Rozman and Monostory 2010; Saha and Arora 2011).

13.6 Perspectives

The fermentation process for the production of statins has been generally well studied and optimized process w.r.t. process parameters and cost. It is well known that downstream processes can be considered one of the main factors involved in the final cost of the product as it can include multiple steps such as filtration, extraction, chromatography, crystallization, adsorption flow through, and drying (Straathof 2011; Winkelnkemper et al. 2011). Thus, the use of fungal biomass can be an interesting alternative in controlling lipid profile and avoid a complex downstream process (Table 13.3). There are already reports investigating the hypolipidemic effect of fungal biomasses (Santos et al. 2012). The support of this idea comes from herbal medicines which basically use plants in raw state and produce therapeutic action.

The use of statins has radically improved the therapy of coronary disease since 1990. The huge market for these molecules means that constant effort is being pursued by pharmaceutical companies and research institutes for developing new and more efficient molecules. Although semisynthetic derivatives will likely dominate the market in the near future, microorganism screening may lead to the development of new microbial

Table 13.3 Hypocholesterolemic effect of fungal biomass or its fractions on several animal models

Microorganism	Animal model	Amount/fraction	Reference
Auricularia auricula	ICR mice	Ethanol extract 150 mg/kg/day b.w.	Chen et al. (2011)
Cordyceps sinensis	ICR mice	Hot-water extract from mycelia 150 and 300 mg/kg/day	Koh et al. (2003)
Coriolus versicolor	Rats	Water extract	Hor et al. (2011)
Pleurotus ostreatus	Wistar rats	20 % biom/feed	Santos et al. (2012)
Pleurotus ostreatus	Rabbits	10 % dried fruit/feed	Bobek and Galbavy (1999)
Pleurotus ostreatus	Humans	30 g dried fruit/soup	Schneider et al. (2011)

statins. On the other side, traditional foods are being thoroughly studied in order to elucidate other cholesterol-lowering or synergistic mechanisms. This opens the path for the development of novel nutraceutical foods and dietary supplements.

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