Chapter 1

Introduction

1.1 Hypercholesterolemia and its risk factors

Cholesterol is a soft, waxy natural substance found in the body. This fat-like substance is produced in the body by liver and also obtained from food that comes from animals (particularly egg yolks, meat, poultry, fish, and dairy products) and is an organic molecule in the class of lipids. It is one of three major classes of lipids that utilize to construct their membranes and is thus manufactured by all animal cells. In addition to cell membrane synthesis, the body needs this substance to make certain hormones, and produce compounds that aid in fat digestion (Ohvo-Rekilä et al. 2002). It plays an important role in maintaining normal physiological body functions. About 20–25% of total daily cholesterol production occurs in the liver; other sites of higher synthesis rates include the intestines, adrenal glands, and reproductive organs. Synthesis within the body starts with one molecule of acetyl CoA and one molecule of acetoacetyl-CoA, which are hydrated to form 3-hydroxy-3-methylglutaryl CoA (HMG-CoA). This molecule is then reduced to mevalonate by the enzyme HMG-CoA reductase, which leads to synthesis of cholesterol. Cholesterol is only slightly soluble in water; it can dissolve and travel in the water-based bloodstream at exceedingly low concentrations. Therefore it is transported in the circulatory system within lipoproteins, complex discoidal particles that have an exterior composed of amphiphilic proteins and lipids whose outward-facing surfaces are water-soluble and inward-facing surfaces are lipid-soluble. For this reason, there are several types of lipoproteins in blood, called, in order of increasing density, chylomicrons, very-low-density lipoprotein (VLDL), intermediate-density lipoprotein

(IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) (Biggerstaff and Wooten 2004). LDL or "bad cholesterol" is responsible for the buildup of cholesterol inside the artery walls. Alternatively, HDL or "good cholesterol" helps in removal of cholesterol from the blood and prevents arterial wall build up. It is also the precursor for steroid hormones, bile acids and vitamin D synthesis.

Hypercholesterolemia is the presence of high levels of cholesterol in the blood which may lead to atherosclerosis. This condition occurs when excess cholesterol present in the bloodstream is deposited in the walls of blood vessels, particularly in the arteries that supply blood to the heart (coronary arteries). The abnormal buildup of cholesterol forms clumps (plaque) that narrow and harden the artery walls. As the clumps get bigger, they can clog the arteries and restrict the flow of blood to the heart. The buildup of plaque in coronary arteries causes a form of chest pain called angina and greatly increases a person's risk of having a heart attack (Berliner et al. 1995; Kratz 2005). Inherited forms of hypercholesterolemia can also cause health problems related to the buildup of excess cholesterol in other tissues. If cholesterol accumulates in tendons, it causes characteristic growths called tendon xanthomas. These growths most often affect the achilles tendons and tendons in the hands and fingers. Yellowish cholesterol deposits under the skin of the eyelids are known as xanthelasmata (van den Bosch and Vos 1998). Cholesterol can also accumulate at the edges of the clear, front surface of the eye (the cornea), leading to a gray-colored ring called an arcus cornealis (Zech and Hoeg 2008).

Hypercholesterolemia is typically due to a combination of environmental and genetic factors. Environmental factors include obesity and dietary choices. Genetic contributions

are usually due to the additive effects of multiple genes, though occasionally may be due to a single gene defect such as in the case of familial hypercholesterolaemia. A number of secondary causes exist including: diabetes mellitus type 2, obesity, alcohol, monoclonal gammopathy, dialysis, nephrotic syndrome, obstructive jaundice, hypothyroidism, Cushing's syndrome, anorexia nervosa, and some medications (Bhatnagar, Soran, and Durrington 2008; Choices 2013).

Major risk factors of hypercholesterolemia are:

- **Heredity**: High levels of cholesterol may be inherited because genes may influence cholesterol metabolism.
- **Foods high in saturated fats:** This is the most common cause of hypercholesterolemia as excess of saturated fats may modestly increase LDL (bad) cholesterol level.
- **Other diseases:** Some people suffering from diseases like diabetes, obesity, hypertension, hyperthyroidism etc may have high levels of cholesterol.
- **Lifestyle Changes:** Changes in lifestyle that lead to decrease in physical activity (sedentary life style), smoking and alcoholism result in increased fat and cholesterol levels.

1.2 Diagnosis of Hypercholesterolemia

Blood test called lipid profile is used to diagnose the cholesterol levels in the blood. Lipid profile gives the information about total cholesterol, LDL, HDL and triglycerides. Having high cholesterol levels, does not itself present any signs or symptoms. Unless routinely screened through regular blood testing, high cholesterol levels will go unnoticed and

could present a silent threat of heart attack or stroke**.** Doctors' guidelines state that everyone over the age of 20 years should have their cholesterol levels checked once every five years. The cholesterol test is done after a period of fasting - no food, drink or pills for 9 to 12 hours - to enable an accurate reading of cholesterol from the blood test. The screening gives information about total cholesterol (TC), HDL cholesterol, LDL cholesterol and triglyceride (TG) levels. The guidelines set cholesterol levels are as follows (Adult Treatment Panel (III) Final Report, 2002)

Table 1.1: ATP III Classification of LDL, Total, and HDL Cholesterol (mg/dL)

Sl. No.	**LDL cholesterol**	
1	Optimal	<100 mg/dL
2	Near-optimal	100 to 129 mg/dL
3	Borderline high	130 to 159 mg/dL
4	High	160 to 189 mg/dL
5	Very high	≥190 mg/dL
	Total cholesterol (TC)	
1	Desirable	<200 mg/dL
2	Borderline high	200-239 mg/dL
3	High	≥240 mg/dL
	HDL cholesterol	
1	Low	<40 mg/dL
2	High	> 60 mg/dL
	Triglycerides (TG)	
1	Normal	<150 mg/dL
2	Borderline High	150-199 mg/dL
3	High	200-499 mg/dL
4	Very High	500 mg/dL

1.3 Consequences of Hypercholesterolemia

High cholesterol levels are an important contributor in the calculation of an individual's risk of having a heart attack. The main effects of high cholesterol are due to a condition called atherosclerosis, which is narrowing and hardening of arteries. If levels of cholesterol are too high, LDLs will leave extra cholesterol in the blood and as HDLs cannot pick up all of this cholesterol, it will begin to build up on artery walls, along with other fats and debris. This buildup of cholesterol is called “plaque formation”. Over time, plaque can cause narrowing of the arteries. This plaque buildup is called atherosclerosis; the main clinical manifestation of atherosclerosis is Coronary Heart Disease (Figure 1.1). The effects of high cholesterol due to atherosclerosis include angina, CHD, heart arrhythmias -- an irregular heart rhythm, transient ischemic attack ("mini" stroke), heart attack, stroke, peripheral artery disease, high blood pressure. The effects of high cholesterol will depend on whether the atherosclerosis partially or completely blocks the artery (Kratz 2005).

1.4 Treatment for Hypercholesterolemia

Lipid levels can be lowered with a combination of diet, weight loss, exercise, and medications. As lipid levels fall, so does the risk of developing cardiovascular disease (CVD), including disease of blood vessels supplying the heart (coronary artery disease), brain (cerebrovascular disease), and limbs (peripheral vascular disease). In certain cases, a healthcare provider will recommend a trial of lifestyle changes before recommending a medication. All patients with high LDL cholesterol should try to make some changes in their day-to-day habits, by reducing total and saturated fat in the diet, losing weight (if

overweight or obese), performing aerobic exercise, and eating a diet rich in fruits and vegetables (Mannu et al. 2013; Varady and Jones 2005). The benefits of such lifestyle modifications usually become evident within 6 to 12 months. However, the success of lipid lowering with lifestyle modification varies widely from individual to individual.

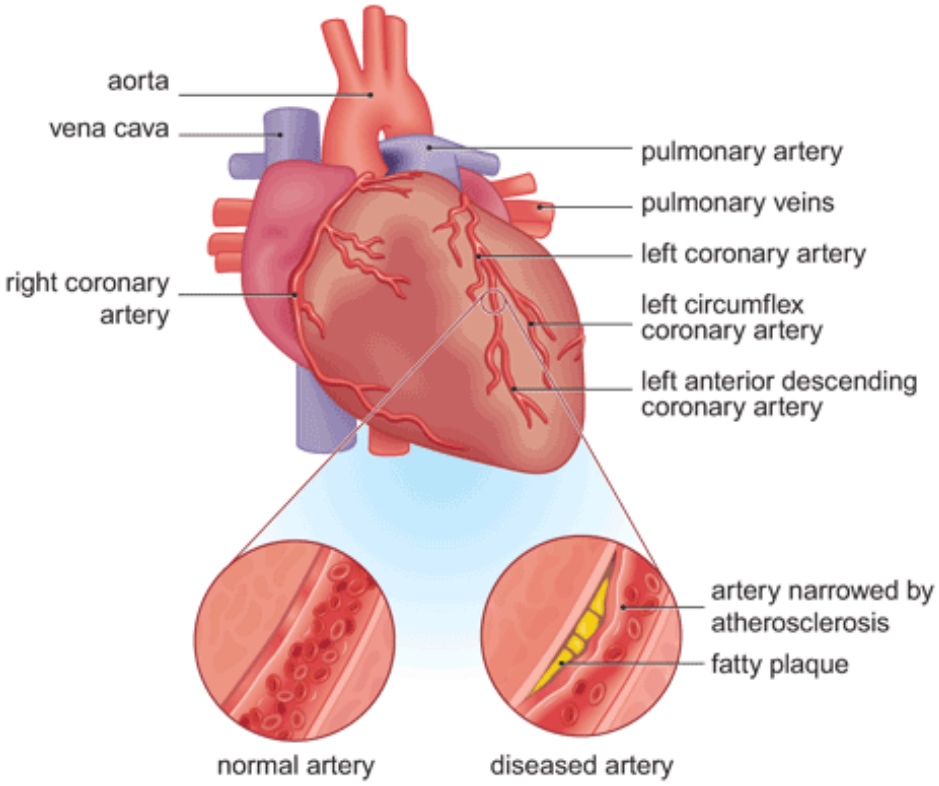

Figure 1.1: Clinical manifestation of atherosclerosis (Task Force Members et al. 2013)

There are many medications available to help lower elevated levels of LDL cholesterol and triglycerides, but only a few for increasing HDL cholesterol. Each category of medication targets a specific lipid and varies in how it works, how effective it is, and how much it costs (Gotto and Moon 2013; Rosenson and Underberg 2013; Lucchi and Vergani 2014). Table 2 describes the various classes of medications used to lower cholesterol levels. Each class of drugs has difference in their mechanism of action and

varying level of cholesterol activity. Out of all these drugs, HMG CoA reductase inhibitors, commonly known as "Statins" are first drug of choice for hypercholestolemia.

Table 1.2: Various classes of drugs used to lower cholesterol levels

S.No	Type of Drug	Mechanism of action	Major Effects	Examples
1	**HMG Co-A reductase inhibitors**	Inhibits cholesterol synthesis in hepatic cells, & up-regulation of hepatic LDL receptors	Lowers LDL-C, TG and raises HDL-C	**Statins (Lovastatin, Simvastatin, Atrovastatin)**
2	Bile acid binding resins	Binds intestinal bile acids interrupting enterohepatic recirculation	Lowers LDL-C, raises TG	Colestipol, Colesevelam
3	Fibric acid derivatives	Inhibits hepatic synthesis of VLDL	Lowers TG with less effect on LDL-C	Fenofibrate
4	Nicotinic acid	Up-regulates hepatic LDL receptors	Lowers LDL-C & TG	Niacin
5	Cholesterol absorption inhibitors	Inhibits intestinal absorption of cholesterol	Lowers LDL-C	Ezetimibe

HMG CoA Reductase Inhibitors

HMG-CoA reductase (or 3-hydroxy-3-methyl-glutaryl-CoA reductase or HMGCR) is the rate-controlling enzyme of the mevalonate pathway, the metabolic pathway that produces cholesterol. This enzyme is thus the target of the widely available cholesterol-lowering drugs. Drugs that inhibit HMG-CoA reductase, known collectively as HMG-CoA reductase inhibitors (or "statins"), are used to lower serum cholesterol levels. They are first-line agents for patients who require drug therapy to reduce cholesterol levels. Statins

work by blocking the enzyme HMGR, the rate-limiting step in the manufacture of cholesterol (Sirtori 2014). Statins reduce low-density lipoprotein cholesterol, total cholesterol, and triglycerides and slightly increase high-density lipoprotein cholesterol. Statins also have anti-inflammatory and other pleiotroptic effects like effect on neurologic diseases, including ischemic and hemorrhagic stroke, Alzheimer disease, Parkinson disease, and multiple sclerosis. These agents have consistently demonstrated a positive safety and tolerability profile, and are recommended by the US National Cholesterol Education Program guidelines and by the European Joint Task Force for Prevention of Coronary Heart Disease to be used after, or in addition to, a first-line approach with diet. Several large-scale clinical trials have shown HMG-CoA reductase inhibitors to be efficacious and well tolerated (Spence 2014; Nilsson 2014).

1.5 Gap in Existing Research

The microorganisms used for statin production broadly belong to three main groups, two of molds, i.e., *Aspergillus* and *Penicillium* and the yeast *Monascus*. The initial studies were carried out mainly with *Penicillium brevicompactum, P. citrinum,* and *A. terreus*. Recent studies under submerged fermentation conditions have been mostly performed with *A. terreus* and, to some extent, also with *M. ruber*, *M. pilosus,* and *M. purpureus* and *P. citrinum*. The field of industrial biotechnology has utilized only a very small proportion of the natural microbial arsenal in the search for new producers of statins. The fungal strains exploited for statin production are terrestrial, so there is tremendous scope of exploring microorganisms from different ecological niches for statin production. Only single fungal strain, *A. terreus*, has been explored for dedicated acyltransferase based

commercial production of semi synthetic HMGR inhibitor, simvastatin. Presently no bacterium is being used for fermentation derived industrial semi-synthetic production of HMGR inhibitors.

1.6 Objectives of the proposed research

The broad objective of this thesis was to screen microbial population from natural sources for presence of acyltransferase/s and related class of enzymes for enzyme assisted synthesis of statins and their analogs which included isolation and screening of microorganisms followed by strain identification and characterization. The product obtained after downstream extraction of microbial fermentation reaction was characterized and microbes were also screened for the presence of genes for acyltransferase/s and related class of enzymes. The specific objectives achieved are:

- Screening of microbial population from natural sources production of HMGR inhibitors.
- Screening of the potential producers for presence of acyltransferase gene and it's homologues by amplification using specific primers and in silico tools.
- Analytical and biological characterization of the product/s obtained.

1.7 Significance of the study

This work represents an important milestone in biosynthesis of HMGR inhibitors and industrially useful manufacturing tool that can lead to substantial cost reduction and development of potentially novel sources for production of statins, the cholesterol lowering drugs. According to WHO report, "too few people are put on cholesterol lowering drugs". Most people around the globe with high cholesterol are not getting the

treatment they need. So having a new source for fast and economical production of statins would help to manage the global disease burden.

1.8 Outline of the Thesis and Schematics of Work Plan

The present thesis has focused on the microbial production of HMG CoA reductase inhibitors and has been divided into 5 chapters. Chapter 2 summarizes the structure, therapeutic importance, history of discovery and development of statins along with mechanism of action and their biosynthesis reviewing all the studies done in this field. The obtained results are analyzed in chapter 4 following the methodology description in chapter 3. Chapter 5 discusses the results and thesis ends with a succnct conclusion along with specific contribution, limitations and future scope of the study.

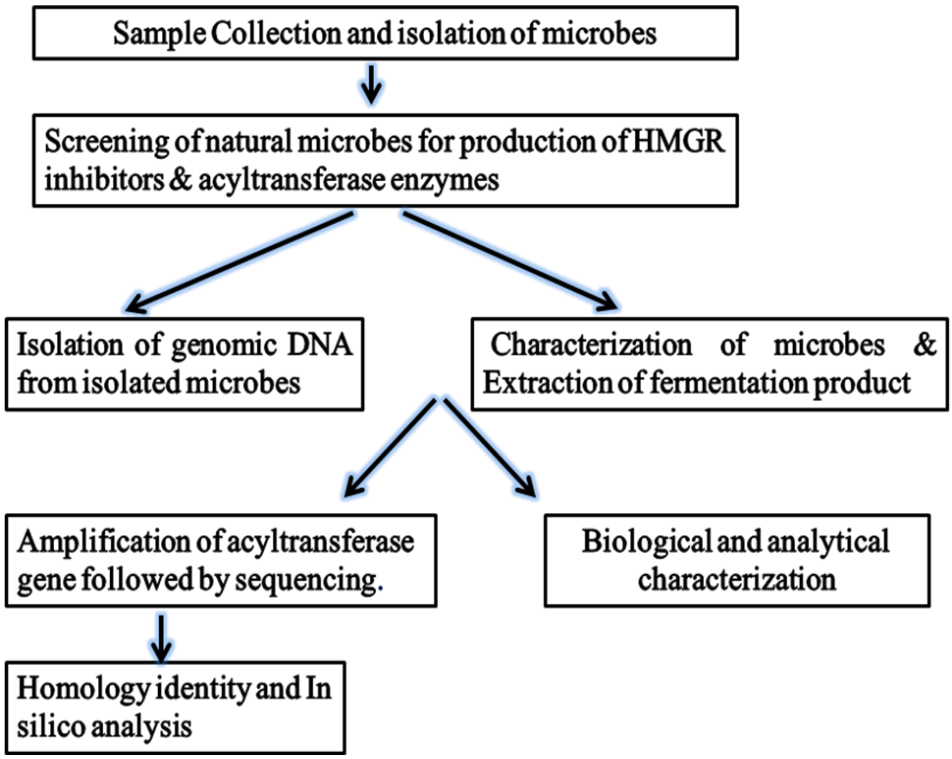

Figure 1.2: Schematics of work plan