Introduction
The Current scenario

The number of deaths occurring from cardio-vascular diseases has increased recent years, becoming the leading cause of motility and morbidity the world over. According the World Health Organization (WHO) estimates, in 1990 cardiovascular diseases killed 14.7 million individuals around the globe and in 2002 and onwards the predicted number will rise to more than 20.7 million each year which accounts to one-third of the total deaths globally (Atlas of Heart Disease and Stroke, WHO, Sept 2004), It has been projected that by 2020, chronic diseases will claim about three-fourth of all deaths (Diet, Nutrition and prevention of chronic diseases, WHO, Geneva, 2003) and out of this 71% of deaths will occur due to ischemic heart diseases. Again 60% of all deaths will occur in developing countries and half of all deaths in women over 50 years of age will be due to heart disease and stroke. Undoubtedly, heart diseases are the major killers world over. Heart diseases comprise a number of ailments; namely Stroke, Congestive Heart Failure, High Blood Pressure, Rheumatic Heart Disease, Coronary Heart Disease, etc.

Coronary heart disease, also known as myocardial infarction, claims the highest share, which is approximately 53% out of the total deaths due to CVD. Current projections suggest that there are more than 45 million estimated patient of coronary artery disease in India. An increasing number of young Indians are falling prey to coronary artery disease due to factors like sedentary lifestyles, unhealthy eating habits, intake of high sugar and fat containing products, higher rate of smoking and hyper-tension (Rissam et al. 2001)

1.1. Coronary heart disease

Coronary (or ischemic) heart or (artery) disease (CHD or CAD) is defined as a condition in which the heart muscles are deteriorated or lose their efficiency because of absence or relative deficiency of oxygen and nutrient deficiency, resulting from reduction in the supply of blood to the specific tissues. The myocardial infarction occurs as a result of the blockage in the coronary artery, due to formation of blood clot. Coronary arteries are the blood vessels that supply oxygen and nutrition to the heart muscles. Over time these arteries develop fatty plaques, a disorder known atherosclerosis (Fig. 1.1). These plaques are mainly made up of cholesterol and other substances like calcium, inflammatory cells, proteins, etc, circulating in the blood
Myocardial infarction occurs due to coronary artery blockage

These arteries supply heart with Oxygen and nutrition

Along with other components, fat and cholesterol are also in circulation

Over the time these arteries deposit these substances on the inner side

Over time the inside of these arteries develop fatty plaques, of different sizes mainly consisting of cholesterol, calcium, protein, etc

With time these plaques increase in size, resulting in the reduction in artery diameter

Due to the pulsing and increasing pressure, sometimes the plaques break. This sometimes ruptures the blood vessels beneath the plaque. Causing blood to ooze out.

Platelets arrive and form clot to stop the bleeding wound known as thrombus

This further reduces the room for the flow of blood and results in occlusion of the coronary artery, known as occlusive thrombosis

This results in reduced blood and oxygen supply to heart. Ultimately resulting in cell necrosis and increased stress on myocardium. The situation commonly known as heart attack

Fig. 1.1. Sequence of events leading to heart attack or myocardial infarction
stream. This reduces the diameter of the artery and reduces blood flow. The plaques formed are hard on the outside and soft on the inside. Sometimes crack develops in these plaques or part of it gets torn out, exposing the tissue beneath. Blood starts oozing out from the ruptured part. Instantly, body’s clotting mechanism comes into play and platelets arrive on the spot. The blood clots which results in further narrowing down the room for the circulation of blood in the artery. Finally the coronary artery gets occluded by the clot, resulting in the death of the surrounding heart muscles. This is known as occlusive thrombosis. Dissolving a clot as a matter of urgency can rapidly improve symptoms and even save the patient’s life. With this in mind, drugs able to dissolve blood clots, called as thrombolytic agents, were developed. These drugs have the property of activating plasminogen into plasmin, the enzyme responsible for dissolving the blood clots.

1.2. The existing therapies

The primary objective of the existing treatments is opening the blockage of the coronary artery, enabling early and efficient reperfusion. This restores the blood and oxygen supply and reduces risk of further complications. However, the benefit of reperfusion is highly dependent on the time it takes to implement the treatment. The choice of treatment available is between the mechanical action carried out primarily by coronary angioplasty or by chemical / enzymatic agents capable of inducing thrombolysis. Angioplasty consists of dilating the coronary artery concerned, which currently done by positioning a stent together with a platelet anti-aggregation treatment (aspirin, clopidogrel, etc). In the case of chemical or enzymatic treatment, the patient is injected with thrombolytic agents that bring the dissolution of the blot clot. The choice is in between chemical or biological agents. The potential biological thrombolytic agents used are urokinase, t-PA, and their derivatives, (e.g. tenecteplase, prourokinase, reteplase, etc) and streptokinase.

1.3. Drawbacks of existing therapies

The drugs produced by genetic engineering have been a subject of modification time to time, for better efficacy and fewer side-effects. The existing therapies though offer good results but suffer certain drawbacks. Apart from being pricey (therefore, remain out of reach of the poor or financially weaker section of the society), these agents are immunogenic and cannot be administered repeatedly. A major side effect of these
agents is the bleeding complications they induce, by converting circulating plasminogen to plasmin, leading to systemic plasmin generation leading to intra-cranial bleeding and hemorrhage.

Owing to these shortcomings, there is a need for a better thrombolytic agent with no hemorrhagic complications. A number of thrombolytic agents have been developed and are available with promising potential but none of them are capable of encircling major drawbacks presented by earlier thrombolytic agents. Thrombolytic agents despite having a good potential induce hemorrhagic complications, which is a serious problem.

1.4. Production of recombinant proteins

Although a fair amount of literature is available on the production of heterologous proteins in *Escherichia coli*, it should be comprehended that each expression system is unique in terms of its promoter system, host vector interactions, sequence and characteristics of recombinant product and the effect of expressed foreign protein on the host cell physiology. Hence the requirements of culture for optimum growth and protein expression changes with change in host cell and cloning vectors. Therefore, delicate variations can have a profound effect on efficiency of specific expression of the given protein. The importance of growth parameters can be deduced from the fact that although the parameters like maintenance of recombinant plasmid, its copy number, the degradation of the recombinant protein are a function of the genetic makeup of the host vector system, these are also known to be largely affected by the culture conditions, media components and their concentrations (Zabriskie and Aricuri, 1986; Yee and Blanch, 1992; Lee S Y, 1996). The product yield of any recombinant protein, which is being produced intra-cellularly, is directly proportional to the concentration of the cells in the fermentation broth and its specific yield (amount of protein produced per unit cell mass). Hence, the production of any recombinant protein from *Escherichia coli* requires its cultivation at a higher cell density with a detailed study of the effect of different culture conditions and medium constituents, so that the effects of key parameters affecting the production of recombinant are investigated.

1.5. Purification of recombinant proteins

The proteins having therapeutic potential are subjected to strict purification protocols and stringent quality control, with huge impetus on the purity of the final product. Keeping in view the stringency and the economy of the process, it becomes very
necessary to design and optimize purification protocols that give the highest yield, in combination with maximum purity. It’s well known that each unit operation adds to the cost of the process significantly and decreases the overall yield. Hence, reduction of the overall number of steps along with the simplification of the process by proper designing and meticulous planning is highly desirable. The application of expanded bed chromatography in the last decade (Mc Cormick. 1993) has resulted in the realization of the goal of reduction of unit operation without necessarily compromising the quality of the finished products (Chase H A. 1994; Hjorth R. 1997). By careful and strategic selection of chromatographic conditions one can exploit this technique to obtain maximum yield and purity of the finished product in a cost effective manner.

1.6. Staphylokinase - A potent thrombolytic agent

Staphylokinase, a small molecule with molecular weight in the range of 14-16 kDa, has immense thrombolytic potential. It is produced by certain strains of *Staphylococcus*, namely *S. aureus*. The gene expressing staphylokinase, from *Staphylococcus* can be cloned and easily expressed in *Escherichia coli*, producing staphylokinase in gram quantities. This will noticeably bring down the cost of its production. Hence, the formulation is expected to be cheaper and available to a larger spectrum of people.
Objectives

With this background in mind, the current assignment was undertaken, with the prime objective of developing an indigenous, economical and efficient process for the production and purification of recombinant staphylokinase using *Escherichia coli* BL21 (λDE3), which was genetically engineered at our institute, with the following objectives:

1. Formulation of media, optimization of media and process parameters for laboratory-scale process.

2. Formulation of feeding methods for high cell density culture.

3. Strategies such as cloning of bacterial hemoglobin gene will be employed to increase the availability of oxygen so that cell growth and product yield is not significantly affected by oxygen supply.

4. Formulation of downstream processing protocols for efficient and cost effective recovery and purification of the desired protein.

5. Enzymology studies on staphylokinase will be carried out.