Summary and Conclusion

Cardiovascular diseases (CVD) are a major cause of morbidity and mortality in the world today and will become the leading cause of death and disability worldwide by 2020 (Levenson *et al.*, 2002). The diseases constituting its range of fatal expression include heart attack, myocardial infarction, acute coronary syndrome, congestive heart failure, strokes, kidney disease, and peripheral vascular disease. The world has paid little consideration to the chronic disease and disability profile of labour force and aged populations in the developing world. Even lesser attention has been paid to the economic implications to the disease's increasing trend. There is an urgent need to act and contain the tide of risk factors that lead to CVD, in order to prevent a massive increase in the number of people with end stage illness. In developing countries, where the risk factors are high, a window of opportunity exists to reduce the progression of the disease. If successful, the future costs of death and disability due to CVD will not become an intolerable burden, which is a real problem in the current day. Unfortunately, India is one of the countries where the risk of cardiovascular diseases is increasing at an alarming rate. As a result, the demands for life saving drugs like streptokinase, urokinase, t-PA and its derivatives, etc, have raised manifold. Staphylokinase is one such protein which can be used for treatment of cardiovascular disease, with fewer side effects than its counterparts. The indigenous development of technology for the economical production of staphylokinase will offer a less expensive and better alternative for the sections of society which are not so much privileged with health and hospital facilities or cannot afford expensive surgery. In the present work, detailed studies on production and purification of staphylokinase from recombinant *Escherichia coli* BL21 (λDE3) was undertaken.

The recombinant *Escherichia coli* strain expressed staphylokinase at a concentration of 85 – 90 mg/L, with cell growth (OD_{600nm}) of around 3.2 – 3.4 in LB medium with induction with IPTG. Optimized cell growth (OD_{600nm}) in LB medium reached to 2.74, with SAK expression of 106 mg SAK/L. After optimization at shake flask level, the process was taken to batch and fed batch fermentation in LB medium. Though batch and fed batch with LB medium produced 109 mg SAK/Land 179 mg SAK/L, there was a limit to further increase cell concentration and SAK yield as LB medium does not result in high cell density and therefore is not a suitable medium for high cell density cultures.

In order to increase the cell concentration, synthetic medium was designed and used at shake flask level initially. The cell concentration (OD_{600nm}) reached 3.7, with SAK production of 84.5 mg/L. Batch fermentation resulted in cell concentration (OD_{600nm}) of 10 with SAK production of 235 mg/L. Fed batch fermentation runs produced a cell concentration (OD_{600nm}) of 24.5 and SAK expression of 396 mg/L.

Since SAK expression was intracellular, higher cell density was prerequisite condition for higher volumetric production, without compromising the specific expression of SAK. Therefore, addition of organic nitrogen sources in the synthetic medium was considered, with an insight to improve the staphylokinase production per unit cell mass. In shake flask studies, cell growth (OD_{600nm}) increased to 4.4 in semi-synthetic medium and the volumetric expression was found to increase from 109 mg SAK/L (in LB medium) to 127 mg SAK/L (in semi-synthetic medium).

The medium components play a major role in deciding the rate of cell growth and expression of foreign proteins in microbial cells. Apart from the highly complicated interactions between the microbial cells and medium components, the medium components also interact with each other in a complex manner. Therefore, careful and systematic optimization strategies have to be followed. Addition of various carbon and nitrogen sources (organic and inorganic), in the culture medium was tried to increase SAK production. The highest expression of staphylokinase was found in medium containing glucose at 60.88 mg SAK/gDCW mounting to 133 mg SAK/L, with cell growth OD_{600nm} = 4.6. Hence, glucose was selected for further optimizations on fermentation medium. Cell growth and staphylokinase expression increased with the addition of organic nitrogen sources in the fermentation medium. Higher cell growth (OD_{600nm}) = 4.7 was found to be in tryptone, followed closely by yeast extract and peptone with SAK expression at 135.7 mg SAK/L with tryptone, 123 mg SAK/L with yeast extract and 121 mg SAK/L with peptone. Since cell growth and staphylokinase production levels with all the three organic nitrogen sources were almost at the same level, these three were selected for further optimization studies.

Optimization of medium components by one at a time approach (classical approach) is normally time consuming, labor intensive and tends to give results at suboptimal levels. The results also lack consistency and are still questionable as the optimized conditions largely depend on the skill and expertise in selection of various medium components and their concentration. Response surface methodology was used as a statistical tool for

optimization of medium components for maximizing cell growth and expression of staphylokinase. The components selected were glucose, organic nitrogen sources (tryptone, yeast extract, peptone), potassium di-hydrogen phosphate and di-ammonium hydrogen phosphate and varied at 5 concentration levels. D-optimal design was adopted for optimization of combination of the three organic nitrogen sources at 1 % (w/v). The optimization results for maximizing cell growth and SAK production revealed that the combination of tryptone with yeast extract was the best and it resulted in staphylokinase production of 163 mg SAK/L after D-optimal design as compared to 131 mg SAK/L with tryptone, 142 mg SAK/L with peptone and 137 mg SAK/L with yeast extract. Using this optimized medium composition, obtained by D-optimal design, further experiments were carried out at 5L fermenter scale.

Batch fermentation experiments showed a final cell concentration (OD_{600nm}) of 12 after 8 h of incubation, with controlled DO and pH at 50 % air saturation and 6.8 respectively. Specific staphylokinase expression went to 69.5 mg SAK/gDCW, with volumetric SAK expression level at 415.3 mg SAK/L.

Optimization of feeding was carried out to further increase cell concentration. In set of uninduced fed batch fermentations, feed medium containing glucose along with yeast extract and tryptone (as optimized from earlier experiments) was fed in fermenter at a constant rate at 2.5, 5 and 7.5 g/h/L of fermentation broth. Cell growth increased with increase in glucose concentration in feed medium, but the cell concentration achieved with feeding of 7.5 g glucose/h/L of fermentation broth didn't increased in proportion to that obtained with 5.0 g glucose/h/L of fermentation broth. Therefore, in further experiments the feed rate was kept at 5 g glucose/L/h. In the first phase of nutrient feeding, the aim was to increase the cell density, so that the cell concentration at the time of induction is high, while the expression of foreign protein requires more nitrogen, precursors, trace elements, etc. As feeding in fed-batch fermentations was done by addition of mainly glucose and organic nitrogen, the ratio of carbon to organic nitrogen was reduced in post-induction feed medium as compared to pre-induction feed medium. The C/N ratio of post-induction feed medium was varied and it was found to have no significant affect on cell density. However, the staphylokinase production increased to 1290 mg SAK/L at C/N ratio of 1, from 1080 mg SAK/L at C/N ratio of 2.

With semi-synthetic medium, the specific expression of staphylokinase was less in fed batch fermentation, 51.5 mg SAK/gDCW as compared to 83 mg SAK/gDCW in shake

flask. The lowering down of specific expression was known to be a common phenomenon in fermenters as the plasmid stability is affected by effective IPTG concentration per unit cell mass, specific growth rate and also accumulation of by-products such as acetate. Addition of IPTG from 0.5 mM to 2 mM didn't affected cell growth, but increased SAK production to 1308 mg SAK/L. With two stage IPTG addition, staphylokinase production increased by 8 – 9 % and reached to 1399 mg SAK/L without significantly affecting cell growth. In order to further increase SAK production, liquid ammonia was used for pH control in combination with constant and stepwise increase in nutrient feeding. Cell growth (OD_{600nm}) increased to 72.4 with constant feeding, with staphylokinase production reaching 2566 mg SAK/L and with step increase in feeding at 7.5g glucose/h, it reached 3500 mg SAK/L. Finally, we were able to increase cell growth by 28 folds and staphylokinase production 40 folds as compared to shake flask studies with LB medium.

The effect of DO was studied from 5 – 35 % saturation. It was observed that with low oxygen concentration, the protein expression reduced. In order to overcome oxygen limitation, *Vitreoscilla* haemoglobin (VHb) was expressed along with SAK. VHb has a pleotropic effect on the host physiology. In addition to binding to oxygen and releasing it when the cell is under oxygen deprivation, VHb can affect more than one cellular biochemical process, e.g. energy metabolism, oxygen uptake, electron transport, etc. The beneficial effect of VHb may be due to the combined effect of VHb. Keeping this in mind; a new plasmid construct was designed by ligation of pUCVHB containing *Vitreoscilla* haemoglobin along with its oxy promoter, with pSAK. Increase in cell growth and SAK expression were observed in shake flask experiments. Cell concentration increased to 12 – 16 % and SAK expression increased to 15 – 25 % batch fermentation experiments, with maintained at different DO concentrations.

Cell lysis is one of the major and critical steps in the recovery of any product that is located intracellularly and its directly affects further purification processes and overall economy of the process. A maximum of 2.4 mg/ml total protein was recovered after sonication from a cell suspension of OD_{600nm} of 20. Large scale cell lysis was carried out by bead-milling. The efficiency of bead-milling process is largely dependent on the variables such as bead loading percentage, bead size, bead-milling time, agitation speed, cell loading and flow rate. Optimization studies showed that the beads of smaller size (0.2 – 0.45 mm) were most effective in cell lysis. The protein release increased with

bead-milling time and reached a plateau after 30 minutes of grinding showing no further increase in the amount of protein released. Bead loading of 80 – 90 % was found to be more effective with 6.1 mg of protein released from cell slurry of OD_{600nm} of 50. Statistical optimization of lysis process was done by using central composite design with 4 factors, varied at 5 levels coded as +2, +1, 0, -1 and -2, *i.e.* cell slurry feed rate, bead loading, cell loading and run time. The release of proteins followed a near linear relation with bead loading and run time, but the effect of cell loading was not much pronounced. The release of intracellular proteins reduced with increase in the feed rate. At the optimum conditions the amount of protein release and staphylokinase were found to be 7.67 g/L and 1.62 g/L respectively.

After cell lysis, recovery of protein was done in normally three steps. The first step was clarification of the lysate to clear it off from cell debris and the other two were chromatography steps to purify the protein to homogeneity.

The process of purification was designed initially as a two step chromatography procedure from the clarified cell lysate obtained after purification. The first step was ion-exchange chromatography followed by hydrophobic interaction chromatography, as the second step. SP – Sepharose was used for ion-exchange process followed was by HIC with phenyl sepharose. The overall yield of the combination of two step chromatographic purification process was close to 48 %. The first step yielded 76 % and the recovery in the second step was 63.7 %. As we used cation exchange chromatography process, we speculated on pH based elution of staphylokinase adsorbed on the matrix. This would save process cost as there is no addition of NaCl, it would not require steps of salt removal from the eluted protein molecule and consequent ease off the effluent treatment required for environment protection. The binding of staphylokinase was done in 10 mM Tris-HCl buffer at normal room temperature and after washing of unbound protein, staphylokinase was eluted with the same buffer with a pH of 6.9, at very slow flow rates. This kept the eluted fraction to be less in volume and the concentration of eluted protein to be high. Staphylokinase was found to elute in the form of a single band and there was no presence of any contaminating bands seen on SDS-PAGE profile, even after silver staining the gel. The overall staphylokinase yield was found to be close to 82 % with homogeneity in one single step, proving the process to be better in cost effective.

The cell lysate was centrifuged to remove the cell debris and the clear lysate was further used for purification. As the volume of the fermentation increases, the volume to be handled for cell lysis and purification also increases. Centrifugation is a tedious and cumbersome step that requires considerable labor, need for CIP and SIP procedures, maintenance and time affecting the economy of the process. The problem of centrifugation of cell lysate obtained after bead-milling process was circumvented by expanded bed chromatography. It has been reported by many research groups, that expanded bed chromatography offers the flexibility of loading total cell lysate along with cell debris directly into the chromatography column. Expanded bed chromatography was performed with Streamline SP sepharose, a cation exchanger, the binding and elution conditions were maintained constant as in case of packed bed chromatography. Total cell lysate without clarification was loaded to the column in expanded mode and the bound staphylokinase was eluted in packed bed mode by reversing the direction of flow. The yield of the process was found to be close to 75 % with pure staphylokinase present in the form of a single band on SDS-PAGE after silver staining method. Optimizations of a number of parameters affecting EBC such as number of sample recirculations, protein loading, loading buffer concentration and flow rate of elution were carried out. It was evident from the results that after 8 recirculations of the sample the column saturated and no further increase in binding occurred. A maximum of 1.8 mg staphylokinase was found to bind per ml of the chromatography matrix. Though loading buffer concentration didn't play a crucial role, the concentration of staphylokinase in the eluted fractions decreased with increase in the flow rate of elution. The purity of staphylokinase was also confirmed by Matrix Assisted Laser Ionization and Desorption (MALDI - TOF).

Further studies on enzymatic properties of staphylokinase suggested that the enzyme was more active in phosphate buffer. The temperature and pH stability profile showed the robustness of the enzyme towards temperature up to 45 ^{0}C. Staphylokinase was stable at 37 ^{0}C for 12 h with 12 % loss in activity and lost its activity rapidly at 65 ^{0}C. It was found to be stable at pH 6.0 – 8.1 and slight loss in activity was observed after 8 h of incubation at 37 ^{0}C. The K_m and V_{max} for staphylokinase was calculated by chromogenic assay. Optimization of enzyme activity was done by statistical methods, using central composite design. The variables chosen were buffer concentration, pH and

temperature. The optimum values observed for SAK activity were pH 7.4, 60 mM phosphate buffer and temperature of 37.4 ^{0}C.

PEG-ylation was attempted to derivatize staphylokinase in order to reduce its immunogenicity, Polyethylene glycol was chosen and the purified molecule was reacted with activated PEG molecule. The reaction proceeds quickly and staphylokinase becomes heavily PEG-ylated as the number of lysine residues are high in number that was strongly indicated by the large change in molecular weight of PEG-ylated SAK molecule. The derivatized staphylokinase loses its activity suggesting that random PEGylation is not a preferred means to derive the molecule.

The study resulted in the development of an indigenous know-how technology on large scale production and purification of a potential thrombolytic drug. To the best of our knowledge the production of intracellular soluble staphylokinase at a concentration more than 3.5 g/L of fermentation medium has not been reported anywhere till date. Part of this work has already been packaged as a know-how technology and has been transferred successfully to an industry. It is been strongly felt that these type of application oriented work in the field of biotechnology will bridge the gap and help building strong coordination between academic and industry in between academics and industry and help in building nation.