Due to the increasing prevalence of thrombo-embolic disorders the demand for life saving clot buster drugs like streptokinase has increased tremendously. The indigenous technology development for the economic production of this drug has become the need of the hour so that this drug becomes easily affordable even to the poorest section of Indian society and other developing countries that are at a much higher risk of these diseases. In the present study, large-scale production and purification of streptokinase from recombinant *E. coli* BL21 (DE3) was studied in detail.

This recombinant *E. coli* strain expressed streptokinase at a concentration of 80 mg/l (74 mg/g dry cells) in LB media at shake flask stage on induction with 1 mM IPTG. The final cell density (OD_{600}) of 2.8 and 3.1 was obtained in induced and un-induced cultures respectively. The batch and fed-batch fermentations in LB media (complex media) resulted in insignificant increase in cell density (maximum OD_{600}=6.0) and was very low compared reported cell density in *E. coli* in high cell density fermentations. A synthetic media was used for further bioreactor experiments. This resulted in cell density (OD_{600}=2.7) and streptokinase expression level (74 mg/l) similar to LB media at shake flask stage. Batch fermentation experiments in the same media with 10 g glucose/l resulted in a maximum cell density (OD_{600}) of 15 and 11 in un-induced and induced runs respectively. Increasing the glucose concentration to 20 g glucose/l did not result in proportional increase in cell density (OD_{600}=17). The expression of streptokinase with 10 g glucose /l, 37 ^{0}C temperature, 6.8 pH (pH control using 2 N NaOH) and 30 % DO concentration was found to be 286 mg/l (i.e. 65 mg/g dry cells). In batch fermentation, increase in DO set point from 30 to 50 % did not improve the expression levels of streptokinase. An IPTG concentration of 0.5 and 1.0 mM was found to be optimal for good expression of streptokinase in these batch fermentations, and a decline in expression level was noticed with 0.1 mM IPTG.

Increase in IPTG concentration to 2 mM, however, did not increase the expression level any further. By changing the pH-controlling agent from 2 N NaOH to 25 % liquid ammonia, little change in cell mass and streptokinase expression was observed.

Fed-batch fermentations with synthetic media as initial batch media and DO concentration and pH controlled at 30 % and 6.8 (using 25 % liquid ammonia) respectively, produced a cell density (OD_{600}) of 44, 72 and 84 at feeding rate of 4.5, 8 and 9.5 g glucose/l/h respectively. The expression of streptokinase after induction at a cell density (OD_{600}) of 32 and 84 (using 0.5 mM IPTG) was found to be 188 and 342 mg/l respectively, corresponding to a specific expression level of 9.4 and 12.4 mg/ g dry cells. The final cell density (OD_{600}) was 38 and 92 respectively. The significant decline in the specific expression level compared to that in shake flask using LB media (74.1 mg/g of dry cells) was due to the decline in the percentage of plasmid bearing cells following induction. In fed-batch fermentation runs, approximately 90 % of the viable cells contained recombinant plasmid at induction compared to only 4 % after 4 h of induction as indicated by the parallel plating technique.

Increase in ampicillin concentration in the feed from 0.1 to 2 g/l did not increase the segregational stability of recombinant plasmid in post-induction phase. On increasing the DO concentration to 50 % a significant improvement in the stability of recombinant plasmid was observed such that ~90 % of the viable cells retained the recombinant plasmid after four hours of induction. The expression of streptokinase also improved significantly and was found to be 722 mg/l corresponding to 47.5 mg/g dry cells. Thus 50 % DO concentration that did not result in any improvement in expression compared to 30 % in batch fermentation, was found to be a major growth parameter affecting the specific productivity in case of fed-batch fermentation. This differential effect might have arisen due to the larger fluctuations in DO value after the onset of feeding phase

(±15 % from the set point) compared to initial batch growth phase as indicated by the data recorded using Advanced fermentation software (AFS) (NBS Co. Inc., NJ) during the course of fermentation. In order to maintain the DO set point at the desired value the supplementation of supplied air with pure oxygen was required in case of fed-batch fermentation as the DO demand of the culture increased with the increase in cell mass. The DO fluctuations arose after the start of addition of feed and supplementation of air with pure oxygen. In the past, fluctuating conditions in DO in PID systems had been reported due to changes in growth phase or growth kinetics, step changes in aeration and agitation rate and increase in viscosity, but the effect of these natural fluctuations on productivity of the protein product was not studied. Only relatively few studies have documented the decrease in plasmid stability and overall yields of the recombinant products with DO fluctuations. The fluctuations in DO in these cases were artificially forced to mimic the large-scale fermentor conditions. In this study, however, the effect of DO fluctuations on plasmid stability and hence streptokinase yield in case of fed-batch fermentation is reported with DO fluctuations naturally resulting due to high oxygen demand at high cell density and different oxygen transfer characteristics under these conditions.

The addition of yeast extract alone and in combination with tryptone, peptone and casein acid hydrolysate in initial fermentation media and feed media (at a glucose to organic source ratio of 4:2) resulted in a streptokinase expression level of 933, 810, 760 and 860 mg/l respectively. The final cell density (OD_{600}) was ~38 in all cases at harvest (11 h of fermentation). Since, no further improvement in plasmid segregational stability resulted, hence the increase in expression of streptokinase might have been due to the presence of biosynthetic precursors in these complex nutrients that helped in reducing the cellular burden for the production of heterologus proteins. Upon substitution of

25 % liquid ammonia with 2 N NaOH for the control of pH, the streptokinase expression increased and was found to be 1120 mg/l corresponding to a specific expression level of 77.7 mg/g dry cells. The effect of ammonia on expression in case of fed batch fermentation might be due to increase in the amount of ammonia (155 ml) as compared to batch fermentation (50 ml). Glucose to organic nitrogen source (yeast extract and tryptone) ratio of 4:1 in the pre-induction feed and 4:2 in post-induction feed and batch media was found to be optimum for the high level expression of streptokinase (1120 mg/l) and a specific expression level of 77.7 mg/g of dry cells.

Overall, compared to shake flask stage, streptokinase production increased 14-fold from 80 mg/l in LB media to 1120 mg/l in optimized media in fed-batch fermentation. This increase in expression was proportional to similar increase in cell density (OD_{600}) from 2.7 in case of shake flask to 38 in fed-batch. The specific expression level of streptokinase was similar in both cases (74.1 and 77.7 mg SK/ g dry cells in shake flask and fed-batch respectively). The main parameters that were found to be affecting the expression of streptokinase in fed-batch fermentation include dissolved oxygen concentration (50 %), incorporation of yeast extract and tryptone and the use of 2 N NaOH to control pH. Previously a streptokinase expression of 425 X 10^5 IU was obtained from 5 l fermentation broth (Estrada *et al.*, 1992) corresponding to 85 X 10^5 IU streptokinase/l. Hernandez *et al.* (1992) had reported a yield of 300 mg streptokinase/l in 5 l fermentation. Although Zhang *et al.* (1999) had not indicated the exact expression level of streptokinase in fermentation but from the published experimental data the expression level was found to be ~720 mg/l by back calculations.

Increase in the cultivation temperature to 40 ^{0}C following induction and maintaining at that temperature for post-induction period of 20 h resulted in the expression of 27.6 % of the total expressed streptokinase as inclusion bodies. In

keeping with the known effect of temperature to induce inclusion body formation, with a further increase in the temperature to 42 ^{0}C following induction (or after 4 h of induction), ~90 % of the total expressed streptokinase was found to be present in the insoluble fraction of the cytosol after 20 h of induction. The increase in temperature to 42 ^{0}C only for 6 h following induction and then again bringing back to 37 ^{0}C did not result in the expression of streptokinase as inclusion bodies with 90 % of the expressed streptokinase being present in the soluble fraction after 20 h of induction. Although the literature contains reports on the production of different recombinant protein in either soluble or insoluble form by the manipulation of cultivation conditions but till date there is no report on the expression of streptokinase in both soluble and insoluble form by the same recombinant strain. The flexibility of such a system offers obvious advantages in downstream process optimization for this protein.

Disruption of wet cells by bead mill (KDL-dyno mill) resulted in a lysis efficiency of ~100 % (w.r.t. amount of soluble protein released in sonication) after 25 min with 0.5-0.75 mm diameter glass beads at a cell concentration of 1:4. The lysis efficiency in terms of streptokinase activity was found to be 96.3 %. The total amount of soluble protein released (3.6 g/l of fermentation broth) and present in insoluble fraction (2.3 g/l fermentation broth) was similar in both sonication and bead mill. Cell lysis using 8 M urea (in buffer of pH=7.2) resulted in a lysis efficiency of 96.6 in terms of amount of total soluble and insoluble protein (5.7 g/l of fermentation broth compared to 5.9 g/l in sonication) and ~96.3 % recovery of streptokinase activity. The use of urea resulted in the cell lysis as well as solubilization of inclusion bodies being accomplished in a single step. Only a single previous report has the use of 6 M urea for the simultaneous lysis of *E. coli* cells and solubilization of inclusion bodies of a recombinant protein been reported but therein the process was not demonstrated at a cell density more than

12 while in this study 8 M urea was used at a cell density of more than 80 with a lysis efficiency of 96 %.

Further purification was done with either packed bed or expanded bed chromatography as the first adsorption (capture) step. In the former case, cell lysis was followed by centrifugation, washing, solubilization and refolding of inclusion bodies and then further purification was done using hydrophobic interaction and ion exchange matrix chromatography matrices both in packed mode. While in the latter case the cell lysate was directly loaded on to the hydrophobic interaction chromatography matrix (in expanded mode) to capture either inclusion bodies or the active streptokinase protein.

Using the first approach, streptokinase inclusion bodies obtained after the centrifugation of cell lysate (from bead-mill) were twice washed in buffer, solubilized using 8 M urea and refolded by dilution. Further purification of streptokinase using hydrophobic interaction and ion exchange chromatographic steps using Phenyl agarose and DEAE-Sepharose fast flow in packed bed chromatography columns, an overall purification yield of 60.7 % and a specific activity of ~0.9 X 10^5 IU/mg was obtained.

Our attempts at purifying streptokinase by direct capture of inclusion bodies present in the cell lysate (obtained from Dyno-mill) on hydrophobic interaction chromatography in expanded mode were not very successful and a streptokinase yield of only 10 % could be obtained.

On the other hand, the purification of streptokinase from the cell lysate (from chemical lysis) containing solubilized inclusion bodies was done using hydrophobic interaction chromatography in expanded bed mode without intermediate centrifugation, washing and solubilization step. For this cell lysate (diluted 10 times) was purified on a Streamline–50 column containing Streamline phenyl (Hydrophobic Interaction chromatography matrix) resulting in a yield of ~80 % with specific activity of

0.53 X 10^5 IU/mg. Further purification on packed bed column containing DEAE–Sepharose fast flow resulted in an overall yield of 60 % and specific activity of ~0.9 X 10^5 IU/mg. A purification yield of 61.5 % and specific activity of 0.88 X 10^5 IU/mg was obtained with the use of same approach for the purification of streptokinase expressed in soluble, active form after cell lysis using 8 M urea. Thus purification using expanded bed chromatography resulted in similar streptokinase recovery irrespective of whether streptokinase was expressed in soluble form or insoluble form. More than 20 purification runs through the same matrix did not cause any observable change in the purification efficiency.

Previously, streptokinase expressed in soluble form in *E. coli* had been purified using Plasminogen-Sepharose affinity column as the first chromatographic step followed by ion exchange chromatography on DEAE-Sephacel column and a final yield of around 50 % was reported with a specific activity of 0.52 X 10^5 IU/mg (Estrada *et al.*, 1992). A similar yield of 51 % had been reported in case of purification of streptokinase inclusion bodies from recombinant *E. coli* with a specific activity of 1X 10^5 IU/mg (Zhang *et al.*, 1999). The steps involved washing, solubilization and refolding of inclusion bodies after cell disruption followed by purification using ion-exchange chromatography on Q-Sepharose and Gel filtration Sepharose G-10 column. Thus in terms of overall purification yield (~60 %) and number of unit steps required for purification the present work scores significantly over the previous reports.

Previously, wherever expanded-bed adsorption has been used to recover protein of interest from *E. coli* homogenates without prior centrifugation or filtration, yields greater than 70 % have been reported in most cases (Frej *et al.*, 1994; Johansson *et al.*, 1996). The step yield in the hydrophobic interaction chromatography in expanded mode in our study was ~82 %. There are very few reports on the use of expanded bed

adsorption to capture inclusion bodies from cell lysates of recombinant *E. coli*. Frez (1996) has reported 100 % yields during the purification of recombinant human interleukin 8 (IL-8) from *E. coli* inclusion bodies.

Thus, the present study describes the development of an indigenous process, which is efficient and economical in terms of the highest production of streptokinase in high cell density fermentation obtained so far and use of less number of unit operations during purification. Also, the purification yield of streptokinase was similar for both soluble and insoluble streptokinase. Process development work of this kind can help in the speedy realization of the goal of biotechnological (BT) revolution in India and open ways for the fruitful dissemination of knowledge, intellectual expertise, skills and facilities between the R & D sector and industry.