Summary

Japanese encephalitis (JE) virus is a flavivirus causing encephalitis in humans and animals. JE polyprotein consist of structural and non-structural proteins. Among the structural protein, envelope protein comprises of three domains and domain III consists of six anti-parallel β-sheet with one disulphide bond. EIII can generate neutralizing antibodies and thus block the virus entry. A recently identified inhibitory peptide derived from EIII protein can inhibit JEV infection (Li et al., 2012). Considering the increasing potential and immense applicability of EIII protein, there is a need to get high production and higher specific expression of EIII protein per unit volume of fermentation medium. Protein quality, characteristics and yield are the major factors to be considered while selecting the suitable expression host for the recombinant protein expression (Demain & Vaishnav, 2009).

Production of recombinant protein in small quantities is a simple process due to easy availability/manipulation of expression systems and information involved in recombinant protein production but efficiency of the process depends on the protein of interest. Expression of disulphide bonded protein in *E. coli* generally, forms inclusion body which needs to be refolded into its native state while expression in mutant strain of *E. coli* Origami-2, results in its expression in soluble form. The level of expression in both strategies varies as yield depends largely on target protein expression. Therefore, these systems need to be initially tested at shake flask level in order to choose the system further for scale-up.

In the present study, the expression of recombinant EIII in *E. coli* resulted in volumetric expression of 240 mg/l with cell growth of ~3 OD_{600} in LB medium at shake flask level. But the overexpression of EIII in *E. coli* resulted in the formation of IBs, which were soft in nature i.e. solubilized in low concentration of urea. The solubilized inclusion body was refolded by pulse dilution refolding method in refolding buffer containing redox shuffling agent to form the disulphide bond. The refolding yielded 13 % soluble EIII after purification (~31 mg/l of the culture). For the soluble expression of EIII in *E.coli,* Origami-2 strain was used as it had mutations in both the thioredoxin reductase (trxB) and glutathione reductase (gor) genes, which greatly enhanced disulfide bond formation in the cytoplasm. Expression of EIII in Origami-2 resulted in cytoplasmic expression with intact disulphide bond but yielded low amount of protein. The chaperones were coexpressed with EIII in Origami-2 to increase the yield of soluble protein and only GroEL/ES resulted in increased soluble expression of EIII. The final yield of purified EIII after optimization of few parameters which were known

for the increased soluble expression of protein (temperature, inducer concentration and time of induction) was 19.1 mg/l of culture. The coexpression of chaperone with EIII in *E. coli* though, resulted in formation of soluble protein but was unable to form disulphide bond due to reducing cytoplasmic environment. The EIII proteins expressed from *E. coli* and Origami-2 strain after purification to homogeneity were characterized by CD spectroscopy for secondary structure and trypsin digestion (refolded EIII) and Ellman's assay for disulphide bond formation. The comparative yield analysis of EIII production indicated that EIII expression in *E. coli* BL21 followed by refolding was better method to get the protein at shake flask level and same was carried forward for scale-up at fermenter level.

Though the batch fermentation using LB medium yielded higher production than shake flask, there are limitation in cell growths as LB medium was unable to support high cell density. With the objective to increase the cell growth, semi-synthetic medium was used. The volumetric expressions of EIII in semisynthetic medium in shake flask and batch fermentation were 390 and 888 mg/l with cell growth (OD_{600}) of approx. 3.8 and 11 respectively.

Fed batch fermentation has been used to attain high cell densities and this is usually done by controlling the supply of carbon source. Feeding is controlled in such a manner that anaerobic conditions did not develop during fermentation period. Different feeding strategies were used to feed the carbon and nitrogen source during pre-induction phase and post-induction phase as medium requirements were different during these phases. Herein, the two strategies single and dual feed were tried during fermentation period and the difference between these two were mainly in the feeding medium composition. Dual feed system results in ~3 fold higher volumetric expression of EIII as compared to single feed system and this could be due to the presence of increased concentration of precursors for protein synthesis in dual feeding medium. Thus, feeding was divided into two parts, pre-induction feed to increase cell concentration while post induction was standardized to enhance product formation. In subsequent fed batch fermentation only the pre-induction feed rate and composition were studied to increase the volumetric production of EIII. Step changes were used in the feed rate at a regular interval in order to avoid residual glucose accumulation. Series of fed batch fermentations were carried out to further increase the cell concentration. Finally, the cell growth was increased by 27 fold and rEIII production was enhanced 23 fold after 16 h of fed batch fermentation using semi synthetic medium as compared to LB shake flask.

The part of antigen are either isolated and purified from pathogen or produced by recombinant DNA technology. There is an increasing demand for vaccine that provides lifelong and complete immune protection. Generally, the subunit vaccines are often poorly immunogenic and required to be administered with adjuvant and in multiple doses. PLGA based delivery systems provide a feasible alternative as a single dose vaccines for many infectious diseases where neutralizing antibodies provide protective immunity. Development of PLGA based particle faced restriction due to instability of protein during encapsulation, poor loading capacity and encapsulation efficiency. Majority of the instability associated with encapsulation of protein is known to occur during primary emulsification and losses are associated with secondary emulsion while release kinetic depends largely on polymer concentration. Therefore, factors affecting these needs to be considered and for optimization studies lysozyme was used as model protein. During PLGA particles formulation using w/o/w method, process parameters like sonication time (in primary emulsification), homogenization speed (in secondary emulsification), PVA and PLGA concentration were varied to study their effect on properties of polymeric particles. Longer sonication time was found to be detrimental for protein during primary emulsification while high homogenization speed during secondary emulsification helped in controlling the size of the particles. The addition of surface active agent PVA affected the stability of secondary emulsion and thus played a role in controlling the size of particles, encapsulation efficiency and burst release of the entrapped antigen. The BR of encapsulated protein decreased with increase in polymer concentration. These optimized conditions were employed to encapsulate EIII protein, resulted in high LC and EE. EIII loaded particle showed low burst release followed by steady state release with intact structural integrity of EIII protein.

In vivo animal experiments suggested that entrapment of EIII antigen in polymeric particles did not result in significant increase in antibody response after a single administration while formulated EIII polymeric particles along with adjuvant elicited higher antibody response as compared to those without adjuvant. EIII-PLGA particle were able to sustain the antibody response for longer period and showed comparatively same antibody response as obtained with EIII with adjuvant. This indicated that PLGA does behave as a traditional adjuvant. The PLGA based formulation appeared to have sustained release of EIII *in vivo* and thus did not require booster doses.

In the present work, recombinant methods and delivery systems have been employed to enable studies regarding the process development for the production and delivery of Japanese encephalitis virus EIII antigen. Our study describes the development of indigenous process for the production of EIII protein in high cell density fermentation. Also, describes the polymeric particle based delivery of EIII protein that provides prolonged and equivalent antibody response in a single dose as compared to conventional multi-dose regime. This paves the way for development of a Japanese encephalitis subunit vaccine and its delivery in future.