CHAPTER VII

CONCLUSION

With the advent of our ability to clone and express a foreign gene in the heterologous host, came a remarkable capability to make almost any protein in abundant quantity to be used as therapeutic or diagnostic agent. It quickly led to the realisation that proteins made in different hosts are different in many ways particularly in the post-translation modifications. Eventual objective of producing a desired protein in an economical heterologous host is influenced by a variety of factors. The new found art of maximising production of heterologous proteins for commercial application is still an art. We have begun to understand factors influencing the eventual production of heterologous proteins. These factors are varied and at times poorly understood. Largely the approach remains empirical. However our collective experience will permit us to rationalise our approach in designing heterologous production of commercially important proteins in a variety of expression systems. Subsequent to production, stabilisation and formulation of proteins will pose significant hurdles in utilising the natural biological catalysts and other proteins for therapeutic and industrial purposes.

Recombinant proteins are made in expression systems like bacteria, yeast, CHO cell lines, insect cell lines, etc. Since these expression systems pose various technical or systemic problems, there is a need to develop simple eukaryotic expression system which can be used to make proteins of interest in therapeutics, diagnostics or other applications. Recently, the cellular slime mold *D. discoideum* has been developed as an alternative eukaryotic system for expressing recombinant proteins. The development of a reliable transformation protocol for *D. discoideum* has provided the possibility of expressing heterologous genes in this eukaryotic microbe. Several mammalian glycoproteins have been expressed in *D. discoideum*, including rotavirus VP7, human muscarinic receptors m2 and m3, antithrombin III, a soluble form of mast cell IgE receptor, hCG, hFSH, insulin like growth factors etc., the expression system still needs to be optimized to maximize the production of heterologous proteins for commercialization.

The overall objective of the present study was to develop *D. discoideum* as an expression system for the production of recombinant proteins. More specifically it involved construction of an ideal secretory expression vector for *D. discoideum* and

expressing GFP as reporter molecule and human DNase I and Proteinase K as test proteins to demonstrate the expression capabilities of *D. discoideum*.

We have successfully generated three constructs [pA15S(1), pA15S(2), pA15S(3)] which have leader sequence for secretion, *His* tag for purification, thrombin cleavage site for removal of *His* tag from the protein and multiple cloning site in which any gene of interest can be plugged in and expressed, as desired features. These three constructs represent three reading frames. Therefore insertion of a gene in these three constructs should provide at least one construct in reading frame for proper expression.

Using GFP as a reporter molecule, expression and secretion potential of the secretory vector pA15S has been confirmed. Although no significant fluorescence was detected in the cells, growth medium of transformants showed fluorescence indicating secretion of the expressed protein in the media. PCR amplification of GFP sequence from the genomic DNA of the transformants verified the presence and integration of GFP in the genome of the cells confirming the integrative nature of pA15 backbone. RT-PCR amplification of GFP from the total cell RNA further reaffirmed the presence and transcription of GFP in the transformants. Transformants grew at rates comparable to Ax-2 cells and went through the developmental cycle normally on starvation. Spores as well as glycerol stock of the transformants retained the G418 resistance on revival in the absence of the antibiotic indicating the stability of the integrating plasmid over the generations.

Versatility of *D. discoideum* as an expression system for the production of recombinant proteins of pharmaceutical and industrial importance has been shown by the expression of biologically active human DNase I and proteinase K. Expression of DNase I and Proteinase K shows that *D. discoideum* is capable of making active recombinant proteins. Cellular retention of these proteins could be due to either an inappropriate signal used in mammalian/ fungal cells to control secretion or perhaps to inappropriate glycosylation. Biologically active proteins indicate that they had been processed through part of the secretory pathway.

Although PsA signal has been efficiently used as a secretion signal, and the recombinant proteins have been detected in the stravation buffer indicating that both secretion and cleavage of the expressed protein had occured, in some cases it has failed to do so. Also in some cases higher level of protein production and secretion has been observed when protein's own leader peptide was used as compared to the PsA signal. Therefore choice of the signal sequence remains to be empirical- signal sequence working for one protein is not necessarily going to work for another protein.

Further work is required in optimizing expression of stable proteins which are not deleterious to the host. Optimization process will be at two different stages:

1. Optimization at genetic level by maximizing the genetic expression. One of the parameters that has to be considered for efficient protein expression is the phenomenon called codon bias. *D. discoideum* genome has an AT content of > 75% and codon usage is highly biased towards AT-rich codons. At molecular level, vector as well as the gene needs to be further optimized to suit the codon preference of *D. discoideum.* MCS containing stretches of GC regions (RE sites) needs to be replaced with few RE sites, preferably with AT sequences. Also for expression of toxic proteins inducible promoters needs to be screened so that the toxic protein does not have any effect on growth of cells. Cells can be grown to full density and then protein can be purified on induction of promoter.

2. Optimization at bioprocess level where production, stability, secretion etc. will be examined.

3. Analysis of the post-translation modification status (type and extent of glycosylation, disulfide linkages) of the expressed recombinant proteins and its optimization for maximum activity.

The work presented in this thesis involves use of simple eukaryotic cells of *D. discoideum* for the expression and secretion of recombinant proteins. The success

of the experiments proposed here may lead to an efficient eukaryotic expression system. The expression system can then be used to make biological therapeutics, diagnostics, industrial enzymes or any biomolecule of importance.