SUMMARY

With the advent of recombinant DNA technology, it is now possible to transfer genes across species barrier leading to tremendous growth in utility of proteins as therapeutics agents and other industrial purposes. At present there are more than 120 biotechnology derived therapeutics and vaccines approved by US FDA (Food and drug authority) for medical use and over 800 additional drugs and vaccines are in various phases of clinical trials. These recombinant proteins are made (expressed) in a variety of host systems like bacteria, yeast, insect or mammalian cell lines. However, these host expression systems have a variety of technical or other problems e.g. there are serious limitations in using prokaryotic cells for the production of eukaryotic proteins e.g. many of the eukaryotic proteins undergo a variety of post translational modifications like proper folding, glycosylation, phosphorylation etc.

The soil amoeba *D. discoideum* is an organism that provides an attractive alternative for heterologous expression of human glycoproteins. Though it can be grown and transformed with the same ease as the yeast *saccharomyces*, it has some features that resemble mammalian cells, such as glycosylation (N- and O-type) and chemotaxis. It is capable of expressing functional heterologous proteins which are glycosylated, secreted or inserted into the membrane. Several mammalian glycoproteins have been expressed in *Dictyostelium 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., it still needs to be optimized to maximize the production of heterologous proteins for commercialization. Therefore the overall objective of the present study is to develop *D. discoideum* as an heterologous expression system for production of recombinant proteins. More specifically it involves :

1. Designing of an ideal secretory expression vector for *Dictyostelium discoideum*.
2. Construction of the secretory expression vector.
3. Expression of reporter gene: e.g. Green fluorescent protein (GFP).
4. Expression of test genes: human DNase I and Proteinase K.

During secretion, some important modifications take place (e.g. folding) which are not only required for proper secretion but are also extremely crucial for the

activity of the protein. Secretion is also known to be beneficial during purification of the desired protein since the cells are not required to be lysed-a process during which proteases are activated which might degrade the recombinant protein. Secretion also helps in scaling of the culture since the cells can be continuously grown in the media and proteins can be purified without harvesting them (continuous culture). Considering all these important features and benefits of secretion, our objective was to make a secretory expression vector for *D. discoideum* in all three reading frames having following features:

1. Secretory signal sequence (Prespore antigen leader sequence)
2. Protein purification tag (Histidine tag)
3. Cleavage site to remove purification tag from the protein (thrombin cleavage site)
4. Multiple cloning site (MCS) to clone the gene of interest.

The plasmid backbone from pA15GFP, an integrating vector of *D. discoideum* origin having actin 15 promoter and G418 resistance gene was used for constructing the secretory vector. Single stranded DNA (plus and minus strands) having *D. discoideum* leader sequence, histidine tag, thrombin cleavage site and BssHII site to clone the MCS was synthesized. Both the strands were annealed together and phosphorylated. Annealed DNA was digested and many attempts were made to clone it in pA15GFP (sticky end ligation), but no positive clones were obtained. Since many a times restriction enzyme are inefficient in digesting terminal sites, it was decided to first subclone the annealed DNA without any digestion in pBR322 (blunt end ligation) to make the terminal RE sites internal in the circular plasmid and then digest it with respective enzymes for cloning in pA15GFP. MCS from Bluescript II (KS+/-) plasmid was subcloned in *BssHII* site of the synthesized DNA. The complete synthesized sequence along with MCS from pBR322 was then subcloned in pA15GFP replacing GFP and thus making the secretory expression vector pA15S(1) in first reading frame. Vectors in other two reading frames i.e. pA15S(2) and pA15S(3) were made by modifying the restriction sites in the MCS with exonuclease activity of *Klenow* enzyme resulting into loss of nucleotides and hence shift in reading frame. All three plasmids were sequenced to confirm the synthesized DNA sequence and shift in frame.

To monitor the expression and secretory property of the vector, GFP was used as a reporter gene. GFP is unique among light-emitting proteins in that it does not require the presence of any cofactors or substrates for the generation of its green light. pA15S(2)GFP was constructed by subcloning GFP in the MCS of pA15S(2). Reading frame was confirmed by sequencing. Ax-2 strain of *D. discoideum* was transformed with pA15S(2)GFP by $CaPO_4$ method. Transformants were screened for expression of GFP. No fluorescence was detected either in cells (fluorescence microscopy) or in the medium (spectrofluorimeter). Considering the fact that GC rich sequences (bacterial MCS) might interfere in translation in *D. discoideum* (AT rich genome), GFP was recloned in the starting of the MCS (*sacI*) of pA15S(1) making pA15S(1)GFP. On screening the transformants, no Ssignificant fluorescence was observed in the cells under the microscope but the TCA precipitate of medium showed marginal fluorescence as compared to the nontransformant and negative control confirming the secretion of GFP in the medium. 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 reaffirming the integrative nature of pA15 backbone. RT-PCR amplification of GFP from the total cell RNA further illustrated 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.

To check the versatility of the secretory vector and *D. discoideum* as an expression system, human DNase I and proteinase K were expressed in the newly designed vector [pA15S(1)] by replacing GFP with cDNA of test proteins. DNase I is a Ca^{2+}/Mg^{2+}- dependent endonuclease, ubiquitously expressed in mammalian tissues and phylogenitically highly conserved. It is used to reduce the viscosity of Cystic fibrosis sputum by hydrolysing the extracellular DNA released by degenerating leukocytes that accumulate in response to the infection. PCR amplified human DNase I cDNA sequence without its authentic leader sequence was cloned in pA15S(1) and reading frame was confirmed by sequencing. *D. discoideum* was transformed and transformants were screened for DNase I

expression. Transformants showed different phenotype as compared to the untransformed cells i.e. cell death. The cells were able to grow for a certain period of time and then getting detached from the plate and becoming round (characteristic of dying cells) indicating the lethal effect of DNase I expression. Cell death was further confirmed by trypan blue exclusion. Tranformants at the earlier stage of growth also showed delayed development as confirmed by drop test. Proteomics of the transformants showed few alterations (appearance of new bands with disappearance of others) as compared to the untransformed cells. A band at 31.4 kD corresponding to DNase I was expected on SDS-PAGE and Western blot but because of the uncertainty of the extent of glycosylation and the possible degradation of the precursor, it was difficult to ascertain whether DNase I was being expressed or not. Since cells were not able to grow for a longer time, media could not be tested for DNase I expression. To confirm that cell death was because of DNase I activity, DNase I was subcloned in pER III with *ras* promoter. *ras* is a promoter inducible with cAMP. On induction with cAMP, cells showed impaired development due to death of a fraction of cells as compared to untransformed cells confirming the lethality due to DNase I. DNase I antigen by Western blotting was neither detectable in the cells nor in the 50 fold concentrated starvation buffer. Further confirmation of DNase I activity in the transformants is in process.

Proteinase K is an extracellular endopeptidase which is synthesized by the mold *Tritirachium album* Limber. This fungal Proteinase belongs to the class of the serine endopeptidase. Proteinase K is used as a reagent to digest cellular proteins from genomic DNA preparations. Proteinase K cDNA sequence which was codon optimised for *D. discoideum* was PCR amplified and subcloned in pA15S(1) leading to construction of pA15S(1)PK. Reading frame and sequence was confirmed by sequencing. *D. discoideum* cells were transformed and transformants were screened for Proteinase K expression. Transformants showed endogenous cellular protein degradation (confirmed by coomassie staining) suggesting Proteinase K expression and its activity. Cellular protein degradation also resulted into cell death (confirmed by trypan blue staining and drop test). Proteinase K antigen was not detected on Western blot. This might be due to its own proteolytic activity. The amount of Proteinase K in the cells was insufficient for detection on Western blot but was sufficient for its catalytic activity inside the cells. Proteinase K in the media

could not be tested since the cells were not able to grow much due to endogenous cellular protein degradation. No further confirmation of expression was possible due to lethality of the expressed protein.

We have successfully constructed the expression vector for *D. discoideum* in all three reading frames having leader sequence for secretion, histidine tag for purification, thrombin cleavage sequence for removal of His tag from the protein and multiple cloning site in which any gene of interest can be plugged in and expressed. Using GFP as a reporter molecule, expression and secretion potential of the vector has been illustrated. Versatility of the vector and *D. discoideum* as an expression system for production of recombinant proteins of pharmaceutical and industrial importance has been shown by the expression of 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. Further work is required in optimising expression of stable proteins which are not deleterious to *Dictyostelium discoideum*. At molecular level, vector as well as the gene to be inserted needs to be codon optimized in preference to *D. discoideum* AT codon bias to achieve optimum expression level. The successful operation of the experiments proposed 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.