

# CONTENTS

|                                                                  |          |
|------------------------------------------------------------------|----------|
| <b>1. <u>Chapter I: Introduction</u></b> .....                   | <b>1</b> |
| 1.1 Introduction0.....                                           | 2        |
| 1.1 Table I - Key therapeutic products .....                     | 3        |
| 1.1 Table II - Therapeutic pipeline.....                         | 4        |
| 1.1 Table III - Biotechnology sales in India.....                | 5        |
| 1.2 Heterologous production of proteins.....                     | 2        |
| 1.2 Fig. I(a) - Design of an expression vector.....              | 7        |
| 1.2 Fig. I(b) - Design of an artificial chromosome .....         | 8        |
| 1.3 Available expression system .....                            | 6        |
| 1.3.1 Bacterial system.....                                      | 9        |
| 1.3.2 Yeast.....                                                 | 10       |
| 1.3.3 Insect.....                                                | 11       |
| 1.3.4 Mammalian cells .....                                      | 11       |
| 1.4 Factors affecting intracellular expression .....             | 12       |
| 1.4.1 Initiation of transcription .....                          | 12       |
| 1.4.2 RNA elongation.....                                        | 12       |
| 1.4.3 RNA stability.....                                         | 13       |
| 1.4.4 Gene dosage.....                                           | 13       |
| 1.4.5 Initiation of translation .....                            | 13       |
| 1.4.6 Translational elongation .....                             | 14       |
| 1.4.7 Polypeptide folding.....                                   | 15       |
| 1.4.8 Post translational processing .....                        | 16       |
| 1.4.9 Stability of intracellular proteins.....                   | 17       |
| 1.4.10 Stability of plasmid .....                                | 18       |
| 1.5 <i>Dictyostelium discoideum</i> as an expression system..... | 18       |
| 1.5.1 Classification.....                                        | 19       |
| 1.5.2 Life cycle .....                                           | 19       |
| 1.5.2 Fig. I(c) - Life cycle of <i>D. discoideum</i> .....       | 22       |
| 1.5.3 Genome .....                                               | 21       |
| 1.5.4 Plasmids.....                                              | 21       |

|                                                                     |           |
|---------------------------------------------------------------------|-----------|
| 1.5.5 Model organism.....                                           | 24        |
| 1.5.5.1 Cytokinesis .....                                           | 24        |
| 1.5.5.2 Differentiation.....                                        | 25        |
| 1.5.5.3 Chemotaxis and signal transduction .....                    | 25        |
| 1.5.5.4 Cell motility .....                                         | 26        |
| 1.5.5.5 Expression system.....                                      | 27        |
| 1.6 Objectives.....                                                 | 27        |
| <b>2. Chapter II: Materials and methods .....</b>                   | <b>28</b> |
| 2.1 Materials .....                                                 | 29        |
| 2.1.1 Strains .....                                                 | 29        |
| 2.1.1.1 Bacterial.....                                              | 29        |
| 2.1.1.2 <i>D.discoideum</i> .....                                   | 29        |
| 2.1.2 Sequence of primers .....                                     | 29        |
| 2.1.3 Enzymes .....                                                 | 29        |
| 2.1.4 Fine chemicals .....                                          | 30        |
| 2.1.5 Restriction maps of plasmids .....                            | 30        |
| 2.1.5.1 pBR322.....                                                 | 31        |
| 2.1.5.2 pBluescript II (KS+/-).....                                 | 32        |
| 2.1.5.3 pBluescript II (SK+/-).....                                 | 33        |
| 2.1.5.4 pA15GFP .....                                               | 34        |
| 2.1.5.5 pGEM-T .....                                                | 35        |
| 2.1.5.6 pER III.....                                                | 36        |
| 2.2 General culturing and preservation of <i>D.discoideum</i> ..... | 37        |
| 2.2.1 Axenic growth of <i>Dictyostelium</i> .....                   | 37        |
| 2.2.2 Growth of <i>Dictyostelium</i> on Bacteria .....              | 38        |
| 2.2.3 Development of <i>Dictyostelium</i> .....                     | 39        |
| 2.2.4 Preparation and use of <i>Dictyostelium</i> stocks .....      | 40        |
| 2.2.4.1 Preparing glycerol stocks.....                              | 40        |
| 2.2.4.2 Preparing silica stocks .....                               | 40        |
| 2.3 Construction and confirmation of vectors .....                  | 41        |
| 2.3.1 Transformation in bacteria .....                              | 41        |
| 2.3.1.1 CaCl <sub>2</sub> method .....                              | 41        |

|                                                                                                                                   |        |
|-----------------------------------------------------------------------------------------------------------------------------------|--------|
| 2.3.1.2 Electroporation.....                                                                                                      | 43     |
| 2.3.2 Extraction of plasmid .....                                                                                                 | 43     |
| 2.3.3 Agarose gel electrophoresis.....                                                                                            | 44     |
| 2.3.4 Preparation of double strand oligonucleotides for cloning .....                                                             | 45     |
| 2.3.5 Restriction enzyme (RE) digestion of DNA.....                                                                               | 46     |
| 2.3.6 Phosphorylation of DNA.....                                                                                                 | 47     |
| 2.3.7 Dephosphorylation of DNA.....                                                                                               | 47     |
| 2.3.8 Elution of DNA from gel.....                                                                                                | 48     |
| 2.3.9 Preparation of blunt end DNA fragments.....                                                                                 | 49     |
| 2.3.9.1 End-filling activity .....                                                                                                | 49     |
| 2.3.9.2 Exonuclease activity.....                                                                                                 | 50     |
| 2.3.10 Ligation.....                                                                                                              | 50     |
| 2.3.11 Polymerase chain reaction (PCR) .....                                                                                      | 51     |
| 2.3.12 Ligation of PCR product to pGEM-T vector.....                                                                              | 52     |
| 2.3.13 Screening by colony blot.....                                                                                              | 53     |
| 2.3.14 Sequencing of DNA.....                                                                                                     | 56     |
| 2.4 Transformation and clonal selection in <i>D. discoideum</i> .....                                                             | 56     |
| 2.5 Confirmation of <i>D. discoideum</i> transformants.....                                                                       | 57     |
| 2.5.1 TCA precipitation of growth medium .....                                                                                    | 57     |
| 2.5.2 Extraction of genomic DNA .....                                                                                             | 58     |
| 2.5.3 Genomic DNA PCR for detecting GFP insert.....                                                                               | 59     |
| 2.5.4 Extraction of total cell RNA.....                                                                                           | 59     |
| 2.5.5 RT-PCR for detecting GFP mRNA.....                                                                                          | 61     |
| 2.5.6 Drop test for confirming the effect of expressed recombinant<br>protein on development of <i>D. discoideum</i> .....        | 61     |
| 2.5.7 SDS-PAGE for detecting the expressed recombinant protein.....                                                               | 62     |
| 2.5.8 Western blotting for detecting the expressed recombinant protein.....                                                       | 63     |
| 2.5.9 cAMP induced expression of DNase I.....                                                                                     | 64     |
| <br>3. <b><u>Chapter III: Construction of a secretory expression vector for<br/>    <i>Dictyostelium discoideum</i></u></b> ..... | <br>66 |
| 3.1 Introduction.....                                                                                                             | 67     |
| 3.2 Annealing of complementary strands having signal sequence .....                                                               | 69     |
| 3.2.1 Sequence of HP1 and HP2 .....                                                                                               | 70     |

|                                                                                              |    |
|----------------------------------------------------------------------------------------------|----|
| 3.2 Fig. III(a) - Schematic summary of annealing of HP1 and HP2.....                         | 71 |
| 3.3 Subcloning of annealed DNA into the designed vector.....                                 | 69 |
| 3.3.1 Confirmation of clone of pMR#1 .....                                                   | 72 |
| 3.3 Fig. III(b) - Schematic summary of construction of pMR#1.....                            | 73 |
| 3.3 Fig. III(c) - Construction and RE digestion pattern of pMR#1 .....                       | 75 |
| 3.4 Modification of MCS in pBSKS .....                                                       | 76 |
| 3.4.1 Confirmation of clone of pMR#2 .....                                                   | 76 |
| 3.4 Fig. III(d) - Schematic summary of construction of pMR#2 .....                           | 77 |
| 3.4 Fig. III(e) - Construction and RE digestion pattern of pMR#2 .....                       | 79 |
| 3.5 Subcloning of modified MCS from pMR#2 into pMR#1 .....                                   | 78 |
| 3.5.1 Confirmation of clone of pMR#3 .....                                                   | 78 |
| 3.5 Fig. III(f) - Schematic summary of construction of pMR#3 .....                           | 80 |
| 3.5 Fig. III(g) - Construction and RE digestion pattern of pMR#3 .....                       | 81 |
| 3.6 Subcloning of desired features including MCS from pMR#3 into<br>pA15GFP .....            | 82 |
| 3.6.1 Confirmation of clone of pA15S(1).....                                                 | 84 |
| 3.6 Fig. III(h) - Schematic summary of construction of pA15S(1) .....                        | 83 |
| 3.6 Fig. III(i) - Construction and RE digestion pattern of pA15S(1).....                     | 85 |
| 3.7 Shift in reading frame of pA15S(1) .....                                                 | 84 |
| 3.7.1 Shift in frame by two nucleotide.....                                                  | 86 |
| 3.7.1.1 Confirmation of clone of pA15S(2).....                                               | 86 |
| 3.7.1 Fig. III(j) - Construction and RE digestion pattern of<br>pA15S(2).....                | 88 |
| 3.7.2 Shift in frame by one nucleotides.....                                                 | 87 |
| 3.7.2.1 Confirmation of clone of pA15S(3).....                                               | 89 |
| 3.7.2 Fig. III(k) - Construction and RE digestion pattern of<br>pA15S(3).....                | 90 |
| 3.8 Sequencing of secretory vector .....                                                     | 89 |
| 3.8 Fig. III(l) - Sequence of pA15S(1).....                                                  | 91 |
| 3.8 Fig. III(m) - Sequence of pA15S(2) .....                                                 | 92 |
| 3.8 Fig. III(n) - Sequence of pA15S(3) .....                                                 | 93 |
| 3.9 Discussion .....                                                                         | 89 |
| 3.9 Fig. III(o) - Schematic summary of construction of pA15S(1),<br>pA15S(2), pA15S(3) ..... | 96 |

|                                                                                                                     |     |
|---------------------------------------------------------------------------------------------------------------------|-----|
| 4. <b><u>Chapter IV: Expression of Green fluorescent protein (GFP) in <i>Dictyostelium discoideum</i></u></b> ..... | 97  |
| 4.1 Introduction: Green fluorescent protein (GFP).....                                                              | 98  |
| 4.1.1 Characteristics .....                                                                                         | 98  |
| 4.1.2 Structure.....                                                                                                | 98  |
| 4.1.3 Gene sequence.....                                                                                            | 99  |
| 4.1.4 Applications.....                                                                                             | 100 |
| 4.2 Construction of pA15S(2)GFP .....                                                                               | 100 |
| 4.2 Fig. IV(a) - Schematic summary of construction of pMR#5 .....                                                   | 102 |
| 4.2 Fig. IV(b) - Construction and RE digestion pattern of pMR#5 .....                                               | 103 |
| 4.2 Fig. IV(c) - Schematic summary of construction of pA15S(2)GFP .....                                             | 104 |
| 4.2 Fig. IV(d) - Construction and RE digestion of pA15S(2)GFP .....                                                 | 106 |
| 4.2 Fig. IV(e) -Sequence of pA15S(2)GFP.....                                                                        | 107 |
| 4.3 Transformation of Ax-2 cells with pA15S(2)GFP .....                                                             | 105 |
| 4.3.1 Fluorescence images of transformed cells.....                                                                 | 108 |
| 4.3.2 GFP fluorescence in the transformed cells.....                                                                | 108 |
| 4.4 Construction of pA15S(1)GFP .....                                                                               | 109 |
| 4.4 Fig. IV(f) - PCR amplified GFP gene.....                                                                        | 110 |
| 4.4 Fig. IV(g) - Schematic summary of construction of pA15S(1)GFP .....                                             | 111 |
| 4.4 Fig. IV(h) - Colony blot of <i>E. coli</i> cells transformed with<br>pA15S(1)GFP .....                          | 112 |
| 4.4 Fig. IV(i) - Construction and RE digestion of pA15S(1)GFP.....                                                  | 114 |
| 4.4 Fig. IV(j) -Sequence of pA15S(1)GFP.....                                                                        | 115 |
| 4.5 Transformation of Ax-2 cells with pA15S(1)GFP .....                                                             | 113 |
| 4.5.1 Fluorescence images and GFP Fluorescence in the<br>transformed cells .....                                    | 113 |
| 4.5.1 Fig. IV(k) - Fluorescence images of transformed cells.....                                                    | 116 |
| 4.5.1 Fig. IV(l) - Relative fluorescence of cells.....                                                              | 118 |
| 4.5.2 PCR amplification of GFP from genomic DNA of transformants .....                                              | 117 |
| 4.5.2 Fig. IV(m) - PCR amplified GFP from genomic DNA of<br>transformants .....                                     | 119 |
| 4.5.3 RT- PCR amplification of GFP from total cell RNA of<br>transformants.....                                     | 117 |

|                                                                                                  |     |
|--------------------------------------------------------------------------------------------------|-----|
| 4.5.3 Fig. IV(n) - RT- PCR amplified GFP from total cell RNA of transformants .....              | 120 |
| 4.6 Discussion .....                                                                             | 121 |
| <b><u>5. Chapter V: Expression of human DNase I in <i>Dictyostelium discoideum</i></u></b> ..... | 124 |
| 5.1 Introduction: human DNase I .....                                                            | 125 |
| 5.1.1 Characteristics .....                                                                      | 125 |
| 5.1.2 Structure .....                                                                            | 125 |
| 5.1.3 Gene sequence .....                                                                        | 126 |
| 5.1.4 Therapeutic applications .....                                                             | 127 |
| 5.2 Construction of pA15S(1)DNase I .....                                                        | 128 |
| 5.2 Fig. V(a) - PCR amplified DNase I gene .....                                                 | 129 |
| 5.2 Fig. V(b) - Schematic summary of construction of pA15S(1)DNase I .....                       | 131 |
| 5.2 Fig. V(c) - Colony blot of <i>E. coli</i> cells transformed with pA15S(1)DNase I .....       | 132 |
| 5.2 Fig. V(d) - Construction and RE digestion pattern of pA15S(1)DNase I .....                   | 133 |
| 5.2 Fig. V(e) - Sequence of pA15S(1)DNase I .....                                                | 134 |
| 5.3 Transformation of Ax-2 cells with pA15S(1)DNase I .....                                      | 130 |
| 5.3.1 Morphology of cells transformed with pA15S(1)DNase I .....                                 | 135 |
| 5.3.1 Fig. V(f) - Morphology of cells transformed with pA15S(1)DNase I .....                     | 136 |
| 5.3.2 Drop test for viability and impaired development .....                                     | 135 |
| 5.3.2 Fig. V(g) - Drop test for viability and impaired development .....                         | 137 |
| 5.3.3 Trypan blue staining for viability .....                                                   | 135 |
| 5.3.3 Fig. V(h) - Trypan blue staining for viability .....                                       | 139 |
| 5.3.4 SDS-PAGE of cells transformed with pA15S(1)DNase I .....                                   | 138 |
| 5.3.4 Fig. V(i) - SDS-PAGE of cells transformed with pA15S(1)DNase I .....                       | 140 |
| 5.3.5 Detection of DNase I by Western blotting .....                                             | 138 |
| 5.3.5 Fig. V(j) - Western blotting of DNase I expressed in <i>E. coli</i> .....                  | 141 |
| 5.3.5 Fig. V(k) - Western blotting of DNase I expressed in <i>D. discoideum</i> .....            | 142 |



|                                                                                |     |
|--------------------------------------------------------------------------------|-----|
| 6.3.5 Fig. VI(j) - Western blotting of cells transformed with pA15S(1)PK ..... | 168 |
| 6.4 Discussion .....                                                           | 165 |
| <b><u>7. Chapter VII: Conclusion</u></b> .....                                 | 171 |
| <b><u>8. Bibliography</u></b> .....                                            | 176 |
| <b><u>9. Summary</u></b> .....                                                 | 185 |
| <b><u>10. Publications</u></b> .....                                           | 191 |