THE USE OF ULTRA SCALE-DOWN FOR THE FAST PROCESS CHARACTERISATION OF PLASMID DNA PROCESSES FOR GENE THERAPY

A Thesis submitted for the degree of Doctor of Philosophy

by

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FOR MUM AND DAD
"The mountains melted from before the lord..."

Judges, 5:5
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Abstract

In the production of plasmid DNA for gene therapy the product is separated from chromosomal DNA by an alkaline lysis operation. During this key step a typical rheological profile with respect to time is developed and this has previously been examined using a viscometer as an ultra scale down reactor.

The aim was to elucidate changes in rheological behaviour and plasmid DNA yield of *E.coli* alkaline lysates as a function of fermentation time. This data was used to determine operational windows for fermentation harvesting.

A comparative study of two rheometers showed that the optimum shear rate for the viscosity vs. time profile was 180 s\(^{-1}\). Any rheometer used must achieve this shear rate without breaching the torque limit.

Investigations into *E.coli* cell resistance to physical breakage showed that cells had the least resistance (disruption rate constant (K) 0.9) during exponential growth phase, compared to early exponential and stationary phase values of 0.6 and 0.4 respectively. However, the same effect was not seen for chemical cell lysis, where total cell lysis due to alkaline lysis was maintained at 40 s throughout batch and fed-batch fermentation.

The viscosity vs. time profile for *E.coli* DH1 was seen to change as a function of fermentation time. During exponential growth phase the secondary peak appeared later every hour (from 120 s to 180 s), and the apparent viscosity was higher. This change was due to an increase in chromosomal DNA during exponential growth.

Plasmid DNA yield was seen to peak at stationary growth (69 mg/kg). During early exponential and exponential growth phases the plasmid DNA yield was seen to increase with increasing neutralisation time, except for stationary phase where plasmid yield dropped after 100 s. The reasons for this are undetermined. Yield did not change as a function of fed-batch fermentation time.

In conclusion late exponential / early stationary was determined as the ideal point for fermenter harvest, with the alkaline lysis process requiring efficient mixing to achieve the highest plasmid yield.
1. Introduction and Theory ................................................................. 1
  1.1. Introduction .............................................................................. 1
  1.2. Gene Therapy ........................................................................ 2
    1.2.1. The Theory Behind Gene Therapy ................................ 2
    1.2.2. Vectors Used in Gene Therapy ....................................... 3
    1.2.3. Production of Vectors for Gene Therapy ....................... 4
      1.2.3.1. Fermentation ............................................................. 6
      1.2.3.2. Cell Harvest ............................................................ 7
      1.2.3.3. Purification .............................................................. 7
      1.2.3.4. Finishing ................................................................. 9
    1.2.4. Plasmid DNA Purity ......................................................... 9
    1.2.5. Process Problems ............................................................. 11
      1.2.5.1. Fermentation ............................................................ 11
      1.2.5.2. Downstream Processing ........................................... 12
  1.3. Alkaline Lysis ........................................................................ 13
    1.3.1. Cell Lysis ......................................................................... 14
    1.3.2. Neutralisation ................................................................. 14
1.4. Rheology ................................................................. 15
  1.4.1. Introduction to Rheology ..................................... 15
  1.4.2. Viscosity ......................................................... 17
    1.4.2.1. Measuring Viscosity ..................................... 19
  1.4.3. Elasticity ........................................................ 20
  1.4.4. Viscoelastic Fluids ............................................. 20
    1.4.4.1. Impact of Viscoelastic Behaviour ..................... 22
    1.4.4.2. Normal Stresses and Their Impact on Fluid Behaviour 23
  1.4.5. Non-Newtonian Fluids ........................................ 24
    1.4.5.1. Apparent Viscosity ...................................... 24
    1.4.5.2. Shear-Thinning (Pseudo-Plastic) ...................... 24
    1.4.5.3. Shear-Thickening (Dilatant) ........................ 25
  1.4.6. Measuring Viscoelasticity ................................... 25
    1.4.6.1. The Storage and Loss Modulus ........................ 26
  1.4.7. Rheology in Industry ......................................... 27
  1.4.8. Factors Affecting Rheology .................................. 28
    1.4.8.1. The Effect of Time on Non-Newtonian Fluids .......... 28
    1.4.8.2. The Effect of Temperature on Non-Newtonian Fluids .... 28
  1.5. Summary of Previous Work ..................................... 30
    1.5.1. Rheological Properties of Alkaline Lysates ............. 30
    1.5.2. Classic Alkaline Lysis Viscosity vs. Time Profile ....... 31
      1.5.2.1. Primary Peak ........................................ 32
      1.5.2.2. Secondary Peak ...................................... 33
      1.5.2.3. Pseudo-Steady State ................................ 33
  1.6. Aims .................................................................. 33
2. Materials and Methods ............................................................................... 35

2.1. Introduction ........................................................................................... 35

2.2. Cell Strains and Plasmids .................................................................... 35
   2.2.1. *E.coli* DH5a ................................................................................... 35
   2.2.2. *E.coli* DH1 ..................................................................................... 36
   2.2.3. Strain Maintenance ....................................................................... 36

2.3. Fermentation ........................................................................................ 37
   2.3.1. Growth Medium and Inoculum Preparation ............................... 37
      2.3.1.1. Preparation of Inoculum for 20 L LH Fermenters .............. 37
      2.3.1.2. Preparation of Inoculum for SGI and Applikon Fermenters 37
      2.3.1.3. Preparation of Inoculum for Fed-batch Fermenters ........... 38
   2.3.2. Fermentation Protocol ................................................................. 38
      2.3.2.1. Shake-flask Fermentation .................................................... 38
      2.3.2.2. Batch Fermentation 1 ........................................................... 39
      2.3.2.3. Batch Fermentation 2 ........................................................... 40
      2.3.2.4. Fed-batch Fermentation ....................................................... 41
   2.3.3. Fermenter Sampling .................................................................... 42
      2.3.3.1. Sampling 20 L LH Fermenter .............................................. 42
      2.3.3.2. Sampling Batch and Fed-batch Applikon/SGI Fermenters 43
   2.3.4. Fermenter Harvest ........................................................................ 44

2.4. Alkaline lysis Using Scale-Down Co-axial Rheometer ..................... 44
   2.4.1. Alkaline Lysis Protocol 1: Lysis Step Only .................................. 45
   2.4.2. Alkaline Lysis Protocol 2: Lysis Step Followed By Neutralisation 45
   2.4.3. Reproducibility of Bohlin ............................................................. 45
   2.4.4. Rheological Analysis Using Scale-Down Reactor ..................... 45
      2.4.4.1. Shear Sweep ....................................................................... 46
2.4.4.2. Strain Sweep ................................................................. 46
2.4.4.3. Shear Stress vs. Shear Rate ............................................. 46

2.5. Determining Physical Cell Strength Using An Industrial High Pressure Homogeniser ............................................................... 47
2.5.1. Micron Lab 40 ................................................................... 47
2.5.2. Emulsiflex C5 ................................................................... 48

2.6. Analytical Techniques ................................................................ 49
2.6.1. Measuring Optical Density ................................................... 49
2.6.2. Determination of Protein Concentration (Coomassie Assay) .. 49
2.6.3. Cell Counting ................................................................... 50
2.6.4. Determination of Plasmid DNA (Agarose Gel Electrophoresis) 51
2.6.4.1. Treatment of Alkaline Lysates ........................................... 51
2.6.4.2. Preparation, Loading and Running Gel ............................... 52
2.6.4.3. Staining and Photographing Gel ........................................ 53
2.6.4.4. Analysis of Gel Bands ...................................................... 53

3. The Use of and Reproducibility of Shake-flask, Batch and Fed-batch Fermentations for Plasmid DNA Production ............................................ 57
3.1. Summary ................................................................................ 57
3.2. Introduction ........................................................................... 57
3.2.1. Aims ................................................................................ 58
3.2.2. Scale-up and Scale-down ...................................................... 58
3.2.3. Shake-flask Fermentations .................................................. 58
3.2.4. Batch Fermentations ........................................................... 59
3.2.5. Fed-batch Fermentations ..................................................... 59
3.3. Materials and Methods ............................................................ 60
3.3.1. Shake-flask Fermentation ................................................... 60
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3.2. Batch Fermentation</td>
<td>60</td>
</tr>
<tr>
<td>3.3.3. Fed-batch Fermentation</td>
<td>60</td>
</tr>
<tr>
<td>3.4. Results</td>
<td>61</td>
</tr>
<tr>
<td>3.4.1. Shake-flask Fermentations</td>
<td>61</td>
</tr>
<tr>
<td>3.4.2. Batch Fermentations</td>
<td>62</td>
</tr>
<tr>
<td>3.4.2.1. <em>E.coli</em> DH5a /pQR235 Growth in 20 L LH Fermenter</td>
<td>62</td>
</tr>
<tr>
<td>3.4.2.2. <em>E.coli</em> DH1 /pXY Grown in 3 L Applikon</td>
<td>63</td>
</tr>
<tr>
<td>3.4.2.3. <em>E.coli</em> DH1 /pXY Grown in 7 L Applikon</td>
<td>63</td>
</tr>
<tr>
<td>3.4.2.4. <em>E.coli</em> DH1 Parental Strain Grown in 7 L Applikon</td>
<td>63</td>
</tr>
<tr>
<td>3.4.2.5. <em>E.coli</em> DH1 /pXY Grown in 10 L SGI</td>
<td>64</td>
</tr>
<tr>
<td>3.4.2.6. <em>E.coli</em> DH5a /pXX Grown in 10 L SGI</td>
<td>64</td>
</tr>
<tr>
<td>3.4.3. Fed-batch Fermentations</td>
<td>64</td>
</tr>
<tr>
<td>3.4.4. Maximum Specific Growth Rates</td>
<td>65</td>
</tr>
<tr>
<td>3.4.4.1. Shake-flask Fermentations</td>
<td>65</td>
</tr>
<tr>
<td>3.4.4.2. Batch Fermentations</td>
<td>66</td>
</tr>
<tr>
<td>3.4.4.3. Fed-batch Fermentation</td>
<td>67</td>
</tr>
<tr>
<td>3.5. Discussion</td>
<td>67</td>
</tr>
<tr>
<td>3.5.1. Shake-flask Fermentations</td>
<td>67</td>
</tr>
<tr>
<td>3.5.2. Batch Fermentations</td>
<td>69</td>
</tr>
<tr>
<td>3.5.2.1. Fermenter Configuration</td>
<td>69</td>
</tr>
<tr>
<td>3.5.2.2. Cell Strain</td>
<td>70</td>
</tr>
<tr>
<td>3.5.2.3. Growth Curve</td>
<td>70</td>
</tr>
<tr>
<td>3.5.3. Fed-batch Fermentations</td>
<td>71</td>
</tr>
<tr>
<td>3.6. Conclusion</td>
<td>72</td>
</tr>
</tbody>
</table>
5.2.4. Chemical Cell Strength Measurements ....................................102
5.3. Materials and Methods ..............................................................103
  5.3.1. Physical Cell Strength ..........................................................103
    5.3.1.1. Physical Cell Strength of *E.coli* DH5a /pQR235 ..........103
    5.3.1.2. Physical Cell Strength of *E.coli* DH1 /pXY .............104
  5.3.2. Chemical Cell Strength ..........................................................104
5.4. Results ........................................................................................104
5.5. Discussion ....................................................................................107
  5.5.1. Physical Cell Strength ..........................................................107
  5.5.2. Chemical Cell Strength ..........................................................109
5.6. Conclusion ....................................................................................110

6. Correlating the Changes in the Rheological Behaviour of *Escherichia coli* Alkaline Lysates During Cell Growth ..................................................117
  6.1. Summary ....................................................................................117
  6.2. Introduction ...............................................................................117
    6.2.1. Aims ..................................................................................118
    6.2.2. Alkaline Lysis ......................................................................118
  6.3. Materials and Methods ............................................................119
    6.3.1. Fermentation and Cell Strains ............................................119
    6.3.2. Alkaline Lysis and Scale-Down Reactor .........................119
  6.4. Results ......................................................................................120
    6.4.1. The Impact of Freezing Cell Paste on the Viscosity vs. Time Profil
      e ..........................................................................................120
    6.4.2. Effects of Shake-flask Fermentation Time on Viscosity vs. Time Profile for *E.coli* DH1 /pXY Grown on CIM Media ...................120
    6.4.3. Changes in the Viscosity vs. Time Profile as a Function of Batch Fermentation Growth Phase for *E.coli* DH1 /pXY ...........121
6.4.4. Viscosity vs. Time Profile as a Function of Batch Fermentation Growth Phase for \textit{E.coli} DH5a /pXX ........................................ 122


6.5. Discussion ........................................................................................................ 125

6.5.1. Viscosity vs. Time Profiles for Different Growth Phases of \textit{E.coli} DH1 /pXY Grown on CIM Media in 0.2 v/v Shake-flask .............. 125

6.5.2. Viscosity vs. Time Profile Changes for \textit{E.coli} DH1 /pXY as a Function of Batch fermentation Growth Phase ................... 126

6.5.3. Effect of Strain on Viscosity vs. Time Profiles ........................................ 128

6.5.4. Viscosity vs. Time Profiles for the Alkaline Lysis of \textit{E.coli} DH1 /pXY Samples Grown Using Fed-batch Protocol .............. 128

6.5.5. Apparent Viscosity ...................................................................................... 129

6.6. Conclusions ..................................................................................................... 130

7. Investigating the Causes of the Observed Changes in Rheological Behaviour of \textit{E. coli} Alkaline Lysates during Batch Cell Growth ............. 136

7.1. Summary ........................................................................................................ 136

7.2. Introduction ..................................................................................................... 136

7.2.1. Aims ........................................................................................................... 136

7.2.2. Changes in Viscosity vs. Time Profile for Alkaline Lysates as a Function of Cell Culture Growth Phase ........................................ 137

7.2.3. Selected “Factors” ..................................................................................... 137

7.3. Materials and Methods .................................................................................. 138

7.3.1. Strain and Plasmid Vector ......................................................................... 138

7.3.2. Fermentation .............................................................................................. 138

7.3.3. Alkaline Lysis ............................................................................................ 138

7.3.3.1. Alkaline Lysis Samples For Agarose Gel Electrophoresis 138

7.3.3.2. Treatment with Adjusted Alkaline Lysis Solution ............................... 139
7.3.4. Treatment of Cells to Remove Chosen Factor ........................................... 139
  7.3.4.1. Removal of RNA .................................................................................... 139
  7.3.4.2. Removal of Chromosomal DNA ....................................................... 140
  7.3.4.3. Removal of Intracellular Proteins ...................................................... 140
  7.3.5. Agarose Gel Electrophoresis ........................................................................ 140

7.4. Results ........................................................................................................ 141

7.5. Discussion ................................................................................................... 144

7.6. Conclusions ................................................................................................. 149

8. Changes in Plasmid Quantity and Quality as a Function of Fermentation Age and Neutralisation Time ................................................................. 161
  8.1. Summary .................................................................................................. 161
  8.2. Introduction .............................................................................................. 161
    8.2.1. Aims .................................................................................................. 162
    8.2.2. Plasmid Yield .................................................................................... 162
    8.2.3. Quality Criteria ................................................................................ 162
  8.3. Materials and Methods ............................................................................... 163
    8.3.1. Fermentation and Sampling .............................................................. 163
    8.3.2. Alkaline Lysis .................................................................................... 163
    8.3.3. Agarose Gel Electrophoresis ............................................................. 164
    8.3.4. RNA Removal .................................................................................... 164
  8.4. Results ....................................................................................................... 164
    8.4.1. Analysis of Plasmid DNA Samples .................................................. 164
    8.4.2. Plasmid Yield ..................................................................................... 165
    8.4.3. Plasmid Quality ................................................................................ 166
  8.5. Discussion .................................................................................................. 169

xiv
### Contents

8.5.1. Plasmid Yield ................................................................. 169
8.5.2. Plasmid Quality ............................................................... 171
8.5.3. Correlation Between Viscosity vs. Time Profile and Absolute Mass of Plasmid DNA Present ............................................. 174
8.5.4. Window of Operation for Fermenter Harvest Time and the Time of Neutralisation for Alkaline Lysis Reaction, as Determined by Plasmid Quality and Quantity ...................................................... 175
  
  8.5.4.1. Batch Fermentation .............................................................. 175
  8.5.4.2. Fed-Batch Fermentation ...................................................... 175
8.6. Conclusion ................................................................................. 176

9. Quantitative Study of the Rheology of *E. coli* Alkaline Lysates ....... 184
  
  9.1. Summary ..................................................................................... 184
  9.2. Introduction .................................................................................. 184
  9.2.1. Aims .......................................................................................... 185
  9.3. Materials and Methods ................................................................. 185
  9.3.1. Fermentation and Source of Experimental Samples ............ 185
  9.3.2. Alkaline Lysis and Neutralisation Step .............................. 185
  9.3.3. Rheological Techniques .............................................................. 186
  9.3.3.1. Shear Stress vs. Shear Rate ............................................... 186
  9.3.3.2. Shear Sweeps ................................................................... 186
  9.4. Results .......................................................................................... 187
  9.4.1. Shear Stress vs. Shear Rate Analysis ..................................... 187
  9.4.2. Shear Sweep Analysis ............................................................... 188
  9.4.2.1. Untreated Cell Suspension ............................................... 189
  9.4.2.2. Lysed Samples .................................................................. 190
  9.4.2.3. Neutralised Samples .......................................................... 190
## 9.4.3. Oscillatory Strain Sweep Analysis

9.4.3.1. Untreated Cell Suspension

9.4.3.2. Lysed Samples

9.4.3.3. Neutralised Samples

## 9.5. Discussion

9.5.1. Rheology of Untreated Cell Suspension

9.5.2. Rheology of Alkaline Lysates

9.5.3. Rheology of Neutralised Alkaline Lysates

9.5.4. Significance of Rheological Results on Industrial Process

## 9.6. Conclusion

## 10. Overall Conclusions

10.1. Introduction

10.2. Conclusions

10.2.1. Summary of Conclusions

10.2.2. Characterisation of Alkaline Lysis Process

10.2.3. Operational Window for Cell Harvest

10.3. Industrial Ramifications of PhD

## 11. Further Studies

Appendix A

Appendix B

Appendix C (Glossary)

Appendix D (Nomenclature)

References
# List of Figures

| Figure 1.1 | General process flow diagram for the industrial purification of plasmid DNA | 8 |
| Figure 1.2 | A map of plasmid pQR235 | 11 |
| Figure 1.3 | Photographs of alkaline lysis of *E.coli* cell paste | 16 |
| Figure 1.4 | Sketch of viscosity forces acting on fluid | 19 |
| Figure 1.5 | Logarithmic shear stress plots for Newtonian and non-Newtonian fluids | 21 |
| Figure 1.6 | The result of the application of shear stress to block of Hookean solid | 23 |
| Figure 1.7 | An example of the Weissenberg effect | 24 |
| Figure 1.8 | Application of shear stress and resultant shear strain response of a viscoelastic fluid undergoing oscillatory rheometric analysis | 29 |
| Figure 1.9 | Example of a viscosity vs. time profile for *E.coli* DH5α cells undergoing alkaline lysis | 32 |
| Figure 2.1 | Cross section of Bohlin Rheometer | 54 |
| Figure 2.2 | Detail of co-axial cylinder geometry | 55 |
| Figure 2.3 | Cross section of Micron Lab 40 homogeniser | 56 |
| Figure 3.1 | Classic natural log growth curve | 73 |
| Figure 3.2 | Growth curve for *E.coli* DH1 /pXY grown on CIM media in 0.2 working volume/total volume ratio shake-flask | 74 |
| Figure 3.3 | Growth curves for *E.coli* DH5α /pQR235 grown on LB media in various shake-flasks | 75 |
| Figure 3.4 | Growth curve for *E.coli* DH5α /pQR235 grown on LB media and CFM media in a 20 L LH fermenter | 76 |
Figure 3.5  Growth curve for *E.coli* DH1 /pXY grown on CFM media in a 3 L Applikon fermenter

Figure 3.6  Growth curve for *E.coli* DH1 /pXY grown on CFM media in a 7 L Applikon fermenter

Figure 3.7  Growth curve for *E.coli* DH1 parental strain grown on CFM media in a 7 L Applikon fermenter

Figure 3.8  Growth curve for *E.coli* DH1 /pXY grown on CFM media in a 10 L SGI fermenter

Figure 3.9  Growth curve for *E.coli* DH5α /pXX grown on CFM media in a 10 L SGI fermenter

Figure 3.10 Growth curve for *E.coli* DH1 /pXY grown on FBM media in a 7 L Applikon fed-batch fermenter

Figure 3.11 Relationship between measured optical density, dissolved oxygen tension (DOT) and impeller speed

Figure 4.1  Schematic of the Brookfield viscometer

Figure 4.2  Apparent viscosity vs. time profile for the alkaline lysis of *E.coli* DH1 /pXY cell paste grown on CFM media in a 7 L Applikon batch fermenter

Figure 4.3  Apparent viscosity vs. time profile for the alkaline lysis of *E.coli* DH1 /pXY. Cell paste sampled at 6 h. Three different shear rates: 31 s⁻¹, 49 s⁻¹, 183 s⁻¹

Figure 4.4  Apparent viscosity vs. time profile for the alkaline lysis of *E.coli* DH1 /pXY. Cell paste sampled at: (A) 1 h, (B) 6 h, (C) 10 h. Constant shear rate 31 s⁻¹

Figure 5.1  Coomassie assay calibration curve of optical density (A595nm) vs. protein concentration (mg/ml)

Figure 5.2  Maximum protein release over batch fermentations. 7 L *E.coli* DH1 /pXY in CFM(A); 20 L *E.coli* DH5α /pQR235 in LB(B); 20 L DH5α /pQR235 in CFM(C).
| Figure 5.3 | Protein release at 300 bar as fraction of total protein released at 1200 bar per pass through homogeniser. *E.coli* DH5 /pQR235 20 L ferm on LB | 113 |
| Figure 5.4 | Protein release at 300 bar as fraction of total protein released at 1200 bar per homogeniser pass. *E.coli* DH5 /pQR235 20 L ferm on CFM | 114 |
| Figure 5.5 | Protein release 300bar/protein released 1200bar per homogeniser pass. *E.coli* DH1/pXY 7 L on CFM | 115 |
| Figure 5.6 | Cell lysis time (*E.coli* DH1/pXY) as function of time of neutralisation and fermentation time | 116 |
| Figure 6.1 | Viscosity profile for alkaline lysis of un-frozen DH1/pXY (A) after 1 h fermentation, (B) 6 h, (C) 10 h | 131 |
| Figure 6.2 | Viscosity profile for alkaline lysis of frozen DH1/pXY (A) after 1 h fermentation, (B) 5 h, (C) 9 h | 132 |
| Figure 6.3 | Viscosity profile for alkaline lysis of frozen DH1/pXY (A) after 2 h fermentation, (B) 4 h, (C) 8 h, grown in shake-flask | 133 |
| Figure 6.4 | Viscosity profile for alkaline lysis of frozen DH5α/pXX (A) after 1 h fermentation, (B) 6 h, (C) 10 h | 134 |
| Figure 6.5 | Viscosity profile for alkaline lysis of frozen DH1/pXY grown in fed-batch fermentation a) after 0 h feed regime, b) 2 h, c) 6 h., d) 10 h | 135 |
| Figure 7.1 | Image of plasmid DNA samples run on agarose gels, before (a) and after (b) treatment with RNase | 150 |
| Figure 7.2 | *E.coli* DH1 /pXY cell paste plasmid DNA samples pre-treated with Benzonase and run on agarose gel | 151 |
| Figure 7.3 | Viscosity vs. time profiles for *E.coli* DH1 /pXY from batch fermentation (A) and treated with RNase (B) | 152 |
Figure 7.4  Viscosity vs. time profile for *E. coli* DH 1/pXY batch fermentation: early exponential (A), exponential (B) and stationary (C) phase; all treated with RNase

Figure 7.5  Effect of treatment with Benzonase on *E. coli* DH1/pXY. Panels showing untreated (A) and treated (B) viscosity vs. time profiles

Figure 7.6  Viscosity vs. time profile for alkaline lysis of *E. coli* DH1/pXY pre-treated with Benzonase, sampled during early exponential (A), exponential (B) and stationary (C) growth phases of batch fermentation

Figure 7.7  Effects of Proteinase K treatment on alkaline lysate viscosity vs. time profiles

Figure 7.8  Viscosity vs. time profile for *E. coli* DH1/pXY pre-treated with Proteinase K sampled during early exponential (A), exponential (B) and stationary (C) growth phases of batch fermentation

Figure 7.9  Comparison of viscosity vs. time profile for *E. coli* DH1/pXY cell paste (A) and *E. coli* DH1 parental cell paste (B) from batch fermentation

Figure 7.10  Viscosity vs. time profile for parental *E. coli* DH1 sampled during early exponential (A), exponential (B) and stationary (C) growth of batch fermentation

Figure 7.11  Viscosity vs. time profile for *E. coli* DH1/pXY cell paste sampled at completion of batch fermentation using adjusted alkaline lysis solution

Figure 8.1  Example of agarose gel electrophoresis of plasmid DNA samples removed from batch fermentation

Figure 8.2  Plasmid DNA as function of fermentation growth phase and neutralisation time. *E. coli* DH1/pXY grown in batch fermenter
| Figure 8.3 | Plasmid DNA as function of fermentation growth phase and neutralisation time. *E. coli* grown in batch fermenter. Lysates treated with RNase during lysis | 179 |
| Figure 8.4 | Plasmid DNA from *E. coli* cell culture as a function of fermentation time and neutralisation time, grown in fed-batch fermenter. Treated with RNase | 180 |
| Figure 8.5 | Amount of OC DNA in samples as function of fermentation time and neutralisation time, of batch fermentation | 181 |
| Figure 8.6 | Purity of plasmid for RNase treated (a) batch samples and (b) fed-batch samples | 182 |
| Figure 8.7 | Correlation between plasmid DNA data from the agarose gels and equivalent viscosity vs. time profiles: (a) early exponential; (b) exponential; (c) stationary growth phase | 183 |
| Figure 9.1 | Shear stress vs. rate for cell suspension compared with: early exponential, exponential and stationary cells lysed(A); early exponential, exponential and stationary cells lysed and neutralised after 5 min(B) | 196 |
| Figure 9.2 | Shear sweep (A) and strain sweep (B) analysis of *E. coli* DH1 /pXY cell paste No treatment with alkaline lysis prior to analysis | 197 |
| Figure 9.3 | Example of shear sweep (A) and strain sweep (B) analysis of *E. coli* DH1 /pXY treated with alkaline lysis solution for 5 minutes prior to analysis | 198 |
| Figure 9.4 | Example of shear sweep (A) and strain sweep (B) for cell culture treated with alkaline lysis solution for 5 minutes and then neutralised prior to analysis | 199 |
## List of Tables

| Table 1.1 | Vector systems used for the transfer of therapeutic DNA for gene therapy | 4 |
| Table 1.2 | Final plasmid prep quality criteria | 10 |
| Table 2.1 | Method to make 1 L of Luria Bertani liquid medium | 38 |
| Table 2.2 | The relationship between total and working volume of a fermenter and the inoculum volume used | 41 |
| Table 2.3 | Description of the fed-batch feeding regime | 43 |
| Table 2.4 | TE, PBS and alkaline lysis solution method | 47 |
| Table 2.5 | Volumes of media blank and fermenter cell culture for Optical Density (A\_\text{600nm}) dilutions | 50 |
| Table 3.1 | Fermenter scales used during thesis and working volume/total volume ratio | 61 |
| Table 3.2 | Maximum fermentation optical density achieved | 65 |
| Table 3.3 | Maximum specific growth rates for shake-flasks | 66 |
| Table 3.4 | Maximum specific growth rates from fermentation campaign | 68 |
| Table 3.5 | Inoculum volume to working volume ratios for fermenters | 72 |
| Table 4.1 | The gold standard viscosity vs. time profile for the alkaline lysis of E.coli DH5α at 367 s\(^{-1}\) and 293 K | 86 |
| Table 5.1 | Disruption rate constant (K) for E.coli DH1 and DH5α as a function of fermentation growth phase | 107 |
| Table 6.1 | Variation in timing of primary peak and secondary peak | 123 |
**Table 6.2**  Maximum apparent viscosities for viscosity vs. time profile of cells sampled in early exponential, exponential and stationary growth phases 124

**Table 7.1**  The composition of an average *E. coli* cell 137

**Table 8.1**  Plasmid DNA quality for samples removed from fed-batch fermentation 167

**Table 8.2**  Comparison between batch and fed-batch fermentation plasmid DNA purity and yield 168

**Table 9.1**  Flow behaviour index (\(\varepsilon\)) and consistency index (K) for resuspended cells, lysed and neutralised cells 189

**Table 9.2**  The impact on the phase angle of amplitude applied during strain sweep experiments, for resuspended cells, alkaline lysates and neutralised samples 192

**Table 10.1**  Summary of the operational window for harvest time 204
1. Introduction and Theory

1.1. Introduction

This PhD is concerned with understanding the influence of fermentation conditions on the rheology of the alkaline lysis process - a key stage in the industrial production of plasmid DNA. The experiments will all be conducted at lab scale, and the use of scale-down techniques will aid the characterisation of the alkaline lysis process.

Scale-up and scale-down are tools used in the biotechnology industry to convert lab-scale data to larger scale pilot plant or industrial production. For this reason scale-up is an important step in product development, with its success dependent on the data and process development during lab scale work. If a process needs to be revised, then scale-down techniques can be utilised, by simulating industrial or large-scale conditions in small-scale experiments before subsequent scale-up (Oosterhuis and Kossen, 1983). Scale-down techniques can generate more precise, higher quality scale-up data and hence scale-up design. There are several applications for scale-up. Biological factors that are unimportant at small scale may be at large scale, and so scale-down can be used for screening production strains, or scale-down can be used to collect scale-up data (Jem, 1989 and Humphrey, 1998). Some experiments, for example medium optimisation are only practical to do in shake-flasks (Kennedy et al., 1994), hence the importance of scale-down techniques. The method used in this PhD is ultra scale-down, which comprises volumes measuring only 10 – 20 mL.

This chapter serves as a general introduction to the thesis and is comprised of a number of sections covering the background and theory behind the experimental work. The chapter commences with a review of current literature on gene therapy in general and plasmid DNA production in particular, which is followed by a more detailed analysis of the alkaline lysis process, and an overview of the theory and application of rheology in science.
and industry. This chapter also includes a summary of previous work related to this thesis, conducted by researchers at UCL. A detailed analysis of the aims of the experimental protocols completes this chapter. All references in this thesis are listed in the appropriate section.

1.2. Gene Therapy

Gene therapy is one of the fastest developing areas in modern medical research. In 1992 there were only 37 total protocols, 15 active protocols and 54 patients recorded as being in receipt of gene therapy (Morgan and Anderson, 1993). Currently there are over 532 separate active clinical trials world-wide with roughly 3,400 patients involved, targeting a wide variety of diseases ranging from cancer to HIV, cystic fibrosis to diabetes, immune deficiencies, metabolic disorders and arthritis (J. Gene Med website (www.wiley.co.uk/genmed Clinical trials database) 2001, Blankenstein, 1999 and Mountain, 2000). Most current clinical gene therapy studies involve cancer as the target disease (Martin and Thomas, 1998, Friedmann, 1999). The projected market for gene therapy products could exceed US$45 billion by 2010 (Prazeres et al., 1999).

1.2.1. The Theory Behind Gene Therapy

Gene therapy can be defined as the transfer of new genetic material to the cells of an individual with resulting therapeutic benefit to that individual (Anderson, 1992). The first successful attempt to treat a genetic disease, adenosine deaminase (ADA) deficiency, using gene therapy was performed on 14th September 1990 (Anderson 1992). The patient, a four year old girl, received an infusion of her own T lymphocytes that contained normal copies of the ADA gene: this thirty minute procedure ended five years of laboratory work and three years of government review (Morgan and Anderson, 1993).

The aim of gene therapy varies slightly with different types of diseases: single gene defects may be caused by the lack of a single protein, and therefore only require a single protein to provide treatment. However some
diseases (e.g. cystic fibrosis) require tissue targeting for effective therapeutic treatment. This is especially true for cancer treatment, where the problem is the need to target every single malignant cell. Despite this hurdle, of the 532 active trials, a large proportion are for cancer research (Robinson et al., 1997 and Blankenstein 1999).

1.2.2. Vectors Used in Gene Therapy

There are three main types of vectors that can be used for the delivery of therapeutic DNA: viral, non-viral and physical (Mountain, 2000). Currently the most extensive usage has been with the RNA viruses (the retroviruses). This is mainly because the biology of retroviruses is so well understood, and because long term expression can be achieved. However there is an increasing move towards using naked or plasmid DNA vectors, due to ease of delivery and relatively simple manufacture. By the end of 1996, about 24% of clinical trials under way used plasmids as vectors (Prazeres et al., 1999). This represents a massive shift of focus toward plasmid DNA vectors.

The major vectors that have been developed for the delivery of the therapeutic DNA are:

- Retrovirus vectors
- Adenovirus vectors
- Adeno-associated vectors
- Plasmid DNA

Table 1.1 lists the advantages and disadvantages for each vector in gene therapy, and the frequency of their use in protocols by 1996.

The major problem with gene therapy remains the actual delivery of the gene, both in terms of the risks (e.g. insertional mutagenesis) and the difficulty in delivering genes to the right place (Blankenstein 1999). In September 1990, the ADA deficiency was treated by transduction of bone marrow cells ex vivo. However, this technique has been surpassed by the use of in vivo treatment. The major difference between the two techniques is that
ex vivo requires the removal of cells, whereas in vivo can be achieved by direct injection. This is preferable because it means treatment is easier for both the doctor and patient.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>No. Of Protocols</th>
<th>% Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adeno-Associated Virus</td>
<td>Integration at specific sites.</td>
<td>Requires replicating adenovirus to grow. No helper cell line. Specific integration probably doesn’t occur in absence of viral genes. Very limited insert size.</td>
<td>1</td>
<td>0.9</td>
</tr>
<tr>
<td>Naked DNA</td>
<td>Easy to prepare in quantity. Safe. Unlimited size. No extraneous genes or proteins to produce immune response. No integration.</td>
<td>Very inefficient entry, uptake into nucleus. No mechanism for persistence or stability.</td>
<td>2</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Table 1.1: Vector systems used for the transfer of therapeutic DNA for gene therapy (Orkin and Motulsky, 1995).

1.2.3. Production of Vectors for Gene Therapy

As noted earlier, roughly a quarter of the clinical trials under way use plasmids as vectors. This mode of treatment requires multiple doses, and thus large amounts of plasmid DNA, at a pharmaceutical grade (i.e. suitable for direct injection). For example, a typical dose size for a melanoma patient is 0.3μg, but a full course of treatment could require milligrams (Prazeres et al., 1999). Therefore, it is vital that production methods can meet anticipated demand, and at consistently high levels of purity. This section details how
plasmid DNA is produced, and the problems and bottlenecks that face the biochemical engineer at a large scale.

Plasmid DNA can be manufactured by the fermentation of a recombinant bacterial cell carrying the plasmid vector followed by harvesting of the cell paste and then lysis of the cells. The chromosomal and plasmid DNA can then be separated in a primary recovery step. Cell lysis and DNA separation can be combined in the alkaline lysis procedure (Birnboim and Doly, 1979). This is a crucial step in the production process but suffers from problems ranging from pH control and lysate mixing through to shear-sensitivity of the genomic contaminants and of the product leading to possible yield loss (Levy et al., 2000).

Before it can be produced, a plasmid must first be designed and constructed to perform the required gene therapy. Once a gene therapy plasmid has been designed in this way, it must be established in a suitable production strain of a microorganism, usually a bacteria such as E.coli. Desirable phenotypes include the ability to ferment to a high cell density and high plasmid copy number, minimal generation of non-plasmid containing cells, minimal potential for genetic alterations to the plasmid and compatibility with subsequent purification procedures. Currently, well-characterised strains of E.coli K-12 such as DH5α or DH10B are the industrial standard (Durland and Eastman 1998). The production process can be split into several stages:

- Fermentation
- Harvesting
- Purification
- Finishing

However, before the process begins at an industrial scale, it must be characterised at laboratory and pilot scale, so that should any alterations to the process be attempted, it is easy to estimate the changes to the final yield and purity. Figure 1.1 details a generic process flow chart for industrial-scale manufacture, and the following sections highlight the basic production aims,
followed by the major problems associated with each.

1.2.3.1. Fermentation

Much is already known about the fermentation of *E.coli*, and so this section will only consider the specific requirements relevant for plasmid production in shake flasks and stirred tank reactors (STR). Most *E.coli* strains can be used to propagate plasmid DNA. For the experimental work in this thesis two strains were used: *E.coli* DH5α and DH1. Figure 1.2 shows an example of one of the plasmids used for the experimental work: pQR235. From the diagram it can be seen that it has a pUC origin of replication (and therefore a high copy number), encodes for kanamycin resistance and β-galactosidase selection (other plasmids used are described in Chapter 2, which provides greater detail on the protocols used during experimental work, for each of the steps in the purification process).

*E.coli* fermentations can be either batch or fed-batch (these are preferred over continuous operation since they allow for batch identification of the finished plasmid and for ease of scale-up) and in both cases the fermentation proceeds to a defined end-point, and this is usually the point for maximum plasmid yield, defined empirically (Durland and Eastman 1998).

The main controllable parameters for fermentation are airflow rate (vvm), agitator rate/shaker speed (rpm), pH and temperature. These combine to affect the growth rate and final cell density (and hence plasmid yield). By varying these parameters it is possible to alter the Oxygen Transfer Rate (OTR), and hence cell growth. Large amounts of acetic acid built up while growing *E.coli* in a STR are also detrimental to cell growth. This accumulation is caused by incomplete substrate oxidation in response to oxygen limitation or excess carbon. In high cell concentration fermentations this is often the major task to overcome (Korz *et al.*, 1995). Once the cells have been grown to the required density, they are harvested and the cells lysed, to remove the plasmid. The subsequent sections of this chapter deal with these aspects.
1.2.3.2. Cell Harvest

Bacteria are usually harvested in the late log phase (Horn et al., 1995), and the nature of the product (i.e. extra- or intra-cellular) will determine the process used. Plasmids are intra-cellular, and so cell harvesting involves retaining the cell paste for further treatment. For plasmid DNA production, there are two recognised methods: centrifugation or microfiltration (Prazeres et al., 1999). For the purposes of this report, centrifugation is the method used (see Chapter 2). Following the cell harvesting, the cells are resuspended in TE buffer, for subsequent downstream processing.

1.2.3.3. Purification

Figure 1.1 shows the steps involved in the plasmid DNA process. The process has been developed with several factors in mind: the yield and purity must be as high as reasonably possible while the cost, time and number of process steps are minimised; all procedures must be scaleable and all procedures must be reproducible to ensure that all batches of plasmid meet the required specifications.

Purity is measured by the amount of genomic DNA, RNA, protein and endotoxin present in the cell lysate, and after each process unit (Durland and Eastman, 1998). The FDA sets a process standard for human administration that must be adhered to. This is reviewed in section 1.2.4, and is concerned with the amount of impurities and contaminants, as well as the amount of plasmid that is supercoiled. It is preferable to avoid the use of toxic additives, organics, flammables, animal products and expensive enzymes during the process (Durland and Eastman, 1998), and this is reflected in the fact that traditional laboratory scale methods such as organic solvent extraction and the use of ethidium bromide and CsCL centrifugation have been avoided (Horn et al., 1995).
Figure 1.1: General process flow diagram for the industrial purification of plasmid DNA (Horn et al., 1995).
From Figure 1.1 it can be seen that there are five steps in the purification process: alkaline lysis, centrifugation, supernatant filtration, ultra filtration and ion exchange chromatography. Briefly, the filtration steps are performed on the centrifugation supernatant to produce a sterile and homogeneous cleared lysate, which will be used for the chromatography step. The chromatography step intends to remove residual contaminants such as nucleotides, proteins, RNA, single stranded DNA and lipopolysaccharides. The plasmid DNA is then eluted by a high salt buffer and precipitated with isopropanol. The precipitated DNA is then ready for finishing (Rehm and Reed, 1999). This is at pilot plant scale of operation and above, using a STR, and small-scale shake flask experiments generally go no further than alkaline lysis. The most important step in terms of scale up, and the least characterised, is alkaline lysis (see section 1.3). This forms a focal point for this thesis, as described in the thesis aims (section 1.6).

1.2.3.4. Finishing

Following purification, the next step is to concentrate the product, or to exchange unwanted buffers. This is achieved using ultrafiltration or diafiltration. Sterile filtration is often the final step in the production of bulk plasmid, for human usage, and following this polishing step the plasmid is stored at 4°C (Durland and Eastmann, 1998) prior to secondary manufacturing e.g. formulation.

There are requirements for the level of purity and the maximum amount of contaminants allowed in a plasmid DNA batch, and these are detailed in the following section.

1.2.4. Plasmid DNA Purity

When direct gene transfer of plasmid DNA is used for therapeutic purposes, it is vitally important that the plasmid DNA should not produce an immune response. The injected DNA must be free of all contamination, particularly toxic or antigenic substances such as endotoxins (pyrogenic
lipopolysaccharides: common components of the cell wall of gram negative bacteria), and any antibiotics used in growth of production strain (Dertzbaugh, 1998). Therefore, during production a high degree of removal of these impurities is necessary (Schleef et al., 1997).

Table 1.2 provides a summary of the quality criteria demanded by the FDA. If these criteria are not met, then validation of the process and acceptance of the end product will be extremely difficult (Schorr et al., 1995). Good manufacturing practices should be employed at all times (Epstein, 1996). The current understanding is that plasmid vectors should be in the supercoiled, or covalently closed circle form, as it is more effective at transferring gene expression than the open circle, linear, multimeric or partially denatured forms (Prazeres et al., 1999). The current ruling is that plasmid DNA homogeneity should be greater than 90% ccc (Schorr et al., 1995). This is a specification that places significant demands on the biochemical engineer wishing to design and operate a large-scale process.

<table>
<thead>
<tr>
<th>Quality Criteria</th>
<th>FDA Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> genomic DNA</td>
<td>&lt; 10 ng dose$^{-1}$</td>
</tr>
<tr>
<td>Host protein</td>
<td>&lt; 10 ng dose$^{-1}$</td>
</tr>
<tr>
<td>RNA level</td>
<td>None seen on 0.8% agarose gel</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>&lt;0.1 Endotoxin Units (EU) ($\mu$g plasmid)$^{-1}$</td>
</tr>
<tr>
<td>DNA homogeneity</td>
<td>&gt;90% covalently closed circular (ccc)</td>
</tr>
<tr>
<td>Sterility</td>
<td>No colonies after 21 day tryptose broth culture</td>
</tr>
<tr>
<td>Purity</td>
<td>Spectrophotometric scans between $A_{220}$ and $A_{320}$</td>
</tr>
<tr>
<td>Identity</td>
<td>Restriction digest</td>
</tr>
<tr>
<td>Absorbency at $A_{260/280}$</td>
<td>1.75 - 1.85</td>
</tr>
<tr>
<td>Potency</td>
<td>Transfection experiments</td>
</tr>
</tbody>
</table>

Table 1.2: Final plasmid prep quality criteria (Prazeres et al., 1999 and Schorr et al., 1995).
1.2.5. Process Problems

This section looks at the process problems and bottlenecks experienced so far in the two major parts of the production: fermentation and downstream processing.

1.2.5.1. Fermentation

The main fermentation aspect for the biochemical engineer is the question of yield and the problems of scale-up. In a large-scale process, high cell
densities are reached (Optical Density ($A_{600\text{ nm}}$) = 30-100), and therefore to develop an equivalent plasmid yield, a combination of plasmid and host-strain selection with optimisation of media and fermentation are required. It is possible to achieve plasmid yields (expressed per unit volume of cell culture) as high as 220 µg mL$^{-1}$. This is compared to shake flask yields for high copy-number plasmids in E.coli growing in Luria Bertani (LB) broth at 37°C of 3-5 µg mL$^{-1}$ (Prazeres et al., 1999).

1.2.5.2. Downstream Processing

The majority of problems in the production of plasmids are encountered in the downstream processing operations, aimed at removing cellular contaminants (Prazeres et al., 1999). Figure 1.1 shows the unit operations, and the following bullet points consider the important process problems for cell harvesting, alkaline lysis and purification:

A: Cell harvesting - efficient removal of cells from broth with limited loss of cells in the waste stream.

B: Alkaline lysis (see section 1.3) - this is a crucial step in the process, and one with a lot of potential problems (Prazeres et al., 1999). Briefly, the main problems are:

- pH range and control – maintaining pH within the required range for efficient alkaline lysis, as values higher than 12.5 can cause irreversible plasmid denaturation (Prazeres et al., 1998).

- Mixing of lysate – it is important to have good mixing to ensure complete and efficient alkaline lysis throughout the cell sample undergoing lysis (Marquet et al., 1995).

- Shear-sensitivity of lysate – previous work has shown that the resultant gel matrix is shear sensitive (Ciccolini et al., 1998). Mixing should be gentle to avoid shearing gDNA and plasmid DNA, to help with separation downstream (Prazeres et al., 1999).

- Control problems at large scale – including good mixing of lysis
solution and neutralisation buffer.

➢ Lack of reproducibility – the alkaline lysis process is not well characterised, leading to a considerable range in yield.

➢ Loss of plasmid – caused by any or a combination of the above problems. Plasmid DNA can also be lost in the liquid held in the solid phase (Theodossiou et al., 1997).

C: Purification - contaminants remaining include RNA, gDNA fragments, endotoxins and plasmid variants. These are all very similar molecules to the plasmid DNA product and provide a potential purification problem for size exclusion and ion-exchange chromatography: although RNA is easily removed, gDNA fragments and endotoxins may co-purify with the plasmid due to similar binding affinities (Prazeres et al., 1999). The passage of gDNA fragments downstream are of particular concern given the poor resolving capacity of chromatographic methods for this material relative to the plasmid product.

In the next section the role and design of the alkaline lysis process itself is described in detail from the perspective of the process operation.

1.3. Alkaline Lysis

In section 1.2 the use of alkaline lysis for the production and purification of plasmid DNA for gene therapy was introduced. This section provides an introduction to the theory of alkaline lysis. Details of all the protocols used are presented in Chapter 2.

The alkaline lysis reaction was developed as a procedure for extracting plasmid DNA from bacterial cells (Birnboim and Doly, 1979). There are three defined stages to the method, which produces plasmid DNA from the cell paste harvested from the STR. This method is an established industry norm, and the three stages are:

➢ Cell resuspension

➢ Cell lysis
Neutralisation

The cell lysis and neutralisation steps will be considered in detail in the following sub-sections. According to the method of Birnboim and Doly, before the lysis and neutralisation the *E.coli* cell paste is resuspended in TE buffer at a concentration of 12.5 g wet cell weight per 100 ml. The cell lysis stage then follows.

1.3.1. Cell Lysis

The cell lysis step is initiated by the addition of a solution of sodium hydroxide containing sodium dodecyl sulphate (SDS) to the cell paste suspension. The lysis reaction occurs as a result of the interaction of SDS with lipids and proteins in the cell wall, solubilising the cell wall and hence releasing the intracellular contents (Birnboim and Doly, 1979 and Cicollini *et al.*, 1998).

Within a narrow pH range (12 - 12.5) it is known that high molecular weight DNA is irreversibly denatured, and low molecular weight DNA is reversibly denatured. The presence of NaOH in the solution provides the required alkaline pH in the mixture and hence causes the reversible denaturation of the low molecular weight plasmid DNA and the irreversible denaturation of high molecular weight chromosomal DNA.

1.3.2. Neutralisation

After a period of gentle agitation for approximately five minutes, which is believed to be sufficient for complete denaturation (Ciccolini *et al.*, 1998), the mixture can be neutralised by the addition of a chilled acetate salt solution (potassium or sodium). The solubility of SDS decreases at lower temperatures and at high salt concentrations – and a gel matrix aggregate forms containing cell debris, cellular RNA and high molecular weight DNA that becomes trapped in the fluid within the gel matrix. The low molecular weight plasmid DNA renatures and goes into solution. This causes the formation of a two-phase mixture with the SDS-protein floc containing the denatured chromosomal DNA floating on the plasmid bearing lysate (Ciccolini *et al.*, 1998).
The three stages can be clearly seen in figure 1.3(a)–(c). Previous work at UCL (e.g. Ciccolini et al., 1998) investigating the rheological changes that occur during alkaline lysis using a co-axial rheometer is described in section 1.5.

The rheology of the alkaline lysis reaction is important as viscoelastic properties may have a negative impact on the process for manufacturing plasmid DNA when sudden changes in rates of deformation occur, for example during flow start-up and stop. The possible impact is considered in greater depth along with the theory behind rheology in the next section.

1.4. Rheology

This section discusses the theory behind the science of rheology and how it is employed in industry. It also considers how rheology may be used to analyse the alkaline lysis of *E.coli* cell cultures sampled from fermentations run as described in Chapter 3. Finally the rheology section discusses the importance of rheology in the production of plasmid DNA for gene therapy.

1.4.1. Introduction to Rheology

The original definitions of solids and liquids as two sharp and separate physical laws, as established by Hooke and Newton respectively, have been found to be inadequate in their ability to describe the behaviour of a wide class of materials that are now known as viscoelastic solids or elastic liquids (Walters, 1974). The study of these complex materials has come to be known as the study of rheology, and is the topic of this section of the thesis. The viscosity coefficient is traditionally measured in a viscometer. However, as explained in section 1.4.4, the viscometer is inadequate for characterising the behaviour of many of these complex materials, and has to be replaced by a rheometer, defined as an instrument for the measurement of rheological properties (Walters, 1980).

The term “Rheology” was invented by Professor Bingham of Lafayette
Andrew F. Day  

Chapter 1

College, Indiana in the 1920s, and means "the study of the deformation and flow of matter" (Barnes et. al., 1989). Rheology covers the study of the properties and behaviour of a wide range of materials, from asphalt to rubber, and lubricants to plastics.

Figure 1.3: (A) *E. coli* cell paste resuspended in TE buffer at a ratio of 12.5 g wet cell weight per 100 mL of buffer. (B) Photograph of resuspended cells after the addition of alkaline lysis solution. (C) The impact of adding neutralisation buffer to the lysed solution. All photographs appear courtesy of GlaxoSmithKline R&D, Beckenham, UK.
Significant advances have been made in the study of the rheology of many of these industrial and household products; advances in polymer rheology and suspension rheology have lead to a greater understanding of the importance of rheology in the chemical processing industries.

In the field of biology, many body fluids have been shown to have viscoelastic behaviour including blood, synovial fluid and various forms of mucus. This has lead to the development of an international society of biorheology (Walters, 1974), and the increased use of rheological techniques in the biotechnology industry.

Figure 1.4 illustrates how flow is initiated by a shear stress, and this is discussed in greater detail in section 1.4.2. However, fluid flow patterns and the relationship between fluid deformation and stress are more complex generally than the one shown in Figure 1.4. Rheology is the discipline that studies this complex relationship, by obtaining constitutive equations by which stresses may be derived from deformation rates (Perry, 1997).

The following three sections discuss the theory of viscosity, elasticity and viscoelasticity.

1.4.2. Viscosity

Isaac Newton published the "Principia" in 1687, and in it hypothesised that "the resistance which arises from the lack of slipperiness of the parts of the liquid, other things being equal, is proportional to the velocity with which the parts of the liquid are separated from one another." This lack of slipperiness is what we now call viscosity; this term is synonymous with internal friction and is a measure of resistance to flow. The force per unit area (F/A) required to produce motion is denoted by $\tau$ and is proportional to the velocity gradient, or shear rate $U/d$. In other words, if you double the force you double the velocity gradient. This relationship is described by Newton's postulate:

$$\tau = \eta U/d$$  \hspace{1cm} (Equation 1.1)

Where $\eta$ is the coefficient of viscosity (for Newtonian fluids; otherwise known
simply as viscosity) $\tau$ is termed shear stress and $U/d$ is the shear rate (it is usual to write $\gamma'$ for shear rate, and this will be used from now on in the thesis). Viscosity and apparent viscosity are derived using equation 1.1, with units of Pa.s. Kinematic viscosity is the ratio of viscosity to density, measured in m$^2$/s.

$$V = \eta/\rho \quad (\text{Equation 1.2})$$

Where $V$ is kinematic viscosity, $\eta$ is viscosity and $\rho$ is density.

Figure 1.4 illustrates how flow is initiated by a shear stress ($\tau$). In the case of a Newtonian liquid, the flow persists as long as there is a stress applied. Purely viscous fluids can be divided into time-dependent (shear stress depends on past history of shear rates, due to structural build-up or breakdown during deformation) and time-independent (shear stress depends on current shear rate) fluids (Perry, 1997).

Newtonian behaviour in experiments conducted at constant temperature and pressure has the following characteristics:

- The only stress generated in simple shear flow is the shear stress ($\tau$), with both normal stress differences being zero.
- The shear velocity does not vary with shear rate.
- The viscosity is constant with respect to the time of shearing and the stress in the liquid falls to zero immediately the shearing is stopped.

A fluid that deviates from any of the above characteristics has non-Newtonian behaviour (Barnes et al., 1989).

Viscosity is the most important property affecting flow behaviour of a fluid. It has a marked impact on pumping, mixing, mass transfer, heat transfer and aeration of fluids (Doran, 1998).

Hooke's theory of elasticity for solids is discussed next, and section 1.4.4 considers solids and fluids that fit in-between these two extremes.
1.4.2.1. Measuring Viscosity

In order to measure viscosity it is necessary to create a controlled flow environment (rotational motion with laminar flow) where easily measured parameters (torque \( M \) and angular velocity \( \Omega \)) can be related to shear stress \( \tau \) and shear rate \( \gamma \), using approximate formulae depending on the geometry of the apparatus. Figure 1.5 shows the expected shear stress vs. shear rate rheograms for Newtonian, dilatant, pseudoplastic, and Bingham plastic fluids. These values can then be used for evaluation of viscosity for Newtonian fluids, or parameters important in the characterisation of non-Newtonian fluids (Doran, 1998). Experiments for measuring rheological behaviour are always measured at constant temperature and pressure, to avoid changes in measured viscosity/apparent viscosity (see section 1.4.8).

![Figure 1.4: Two parallel planes of area A, at y = 0 and y = d, the intervening space being filled with sheared liquid. The upper plane moves with velocity U and the lengths of the arrows between the planes are proportional to the local velocity Vx in the liquid.](image)

19
1.4.3. Elasticity

Robert Hooke wrote the “True Theory of Elasticity” in 1678, within which he proposed, “the power of any spring is in the same proportion with the tension thereof.” That is, if you double the tension you double the extension. This is the theory of classical elasticity. For a Hookean solid, a shear stress ($\tau$) applied to the surface of a solid results in deformation. Once the deformed state is reached it is maintained as long as the stress is applied.

Figure 1.6 illustrates this principle. The constitutive equation is shown below:

$$\tau = G\gamma$$  \hspace{1cm} (Equation 1.3)

Where the angle $\gamma$ is called the strain, and $G$ is referred to as the rigidity modulus.

1.4.4. Viscoelastic Fluids

For two hundred years the theories for Newtonian fluids and Hookean solids were accepted. However, by 1835 studies performed on silk threads showed the threads to be not completely elastic, and indeed showed some characteristics that were fluid-like. This kind of behaviour was termed viscoelastic. Nowadays it is accepted that fluids usually fall between the classic Newtonian and Hookean extremes; polymeric liquids make up the largest group of viscoelastic fluids (Perry, 1997). Both Newton and Hooke’s laws are linear laws, which assume direct proportionality between stress and strain. However, this is a very restrictive framework for viscoelastic behaviour as the range of stress over which materials behave linearly is limited. Material properties such as rigidity modulus and viscosity can change with applied stress, and any such increase or decrease can occur either instantaneously or over a period of time (Barnes et al., 1989). This non-linearity is discussed in section 1.4.5.
Figure 1.5: Series of rheograms showing logarithmic shear stress plots for Newtonian fluid (A); different types of non-Newtonian fluids (Bingham plastic, shear thickening and shear thinning) (B); and for an arbitrary fluid with $K=10$ pas$^2$ ($\varepsilon = 0.7$) (C) (Source: http://chemeng1.kat.lth.se/staff/ulf-b/mp-rheo.htm).
1.4.4.1. Impact of Viscoelastic Behaviour

Viscoelastic fluids exhibit elastic recovery from deformation when stress is removed. Viscoelastic properties may be important when designing plasmid DNA processes when sudden changes in rates of deformation occur for a number of reasons: during flow start-up and stop; with rapidly oscillating flows; and as fluid passes through sudden expansions and contractions where acceleration occurs. If there is a process with a fully developed flow where none of these factors occur then viscoelastic fluids behave as if they were purely viscous (Perry, 1997). In viscoelastic flows, normal stresses perpendicular to the direction of shear are different from those in the parallel direction, and this impacts the behaviour of the fluid causing the Weissenberg effect where fluid climbs up the rotating shaft in the fluid (Figure 1.7), and die swell where a stream of fluid issuing from a tube may expand to two or three times the tube diameter (Perry, 1997). The Weissenberg effect has a negative impact on mixing, reducing the efficiency of mixing (Walters, 1974).

The Deborah number (De) is an important parameter that can be used to indicate the importance of viscoelastic effects for a particular fluid (see equation 1.4). It measures the ratio of the characteristic relaxation time of the fluid to the characteristic time scale of the flow.

\[ De = \frac{\lambda}{t} \]  
(Equation 1.4)

Where \( \lambda \) is the characteristic relaxation time and \( t \) is the characteristic time scale of the flow.

Materials with a small Deborah number have a fast relaxation time compared to the time scale of the flow and the fluid behaviour is purely viscous. Very large Deborah numbers indicate materials that behave as an elastic solid.
Figure 1.6: The result of the application of a shear stress $\sigma$ to a block of Hookean solid - on the application of the stress the section ABCD is deformed to become A'B'C'D'.

1.4.4.2. Normal Stresses and Their Impact on Fluid Behaviour

Normal stress differences ($N_1$ and $N_2$) are associated with non-linear effects. The generation of unequal normal stress components and hence non-zero values of $N_1$ and $N_2$, arises from the fact that in a flow process the microstructure of the liquid becomes anisotropic. These normal stresses are responsible for a number of effects of laboratory interest and of commercial importance, such as the Weissenberg effect (see Figure 1.7).

Whereas a Newtonian liquid would be forced towards the rim of the vessel by inertia, the elastic liquid produces a free surface that is much nearer the rod. The rise of the surface is independent of the direction of the rotation.
1.4.5. Non-Newtonian Fluids

1.4.5.1. Apparent Viscosity

The logarithmic shear stress rheograms shown in Figure 1.5 show that non-Newtonian fluids do not exhibit the same constant relationship between shear stress and shear rate that Newtonian fluids have: when the shear rate acting upon a non-Newtonian fluid is varied, the shear stress doesn’t vary in the same proportion, or even in the same direction (Brookfield Manual). This means that the viscosity will change as the shear rate is altered, and thus the measured viscosity of a non-Newtonian fluid is known as the apparent viscosity ($\eta_a$). The apparent viscosity is only accurate when experimental parameters and conditions are adhered to and quoted alongside any measured value.

1.4.5.2. Shear-Thinning (Pseudo-Plastic)

In some cases the viscosity of a fluid decreases with an increase in shear
rate. Many polymer melts and solutions, as well as some solid suspensions are shear thinning, and they follow the power law model over a range of shear rates (Perry, 1997). The power law model is shown in equation 1.5.

\[
\tau = \eta_s (\dot{\gamma})^\varepsilon \quad \text{(Equation 1.5)}
\]

Where \(\varepsilon\) is the power law index, which gives a measure of the degree of non-Newtonian behaviour of the material. For shear-thinning fluids, \(0 \leq \varepsilon < 1\).

1.4.5.3. Shear-Thickening (Dilatant)

Some fluids show increasing viscosity with increasing shear rate. They may be described by the power law model (equation 1.5) when \(\varepsilon < 0\).

If the shear stress (\(\tau\)) and the shear rate (\(\dot{\gamma}\)) are plotted on logarithmic axis, the equation obtained is:

\[
\log \tau = (\log \eta) + \varepsilon (\log \dot{\gamma}) \quad \text{(Equation 1.6)}
\]

If \((\log \eta) = K\) (consistency index), then equation 1.6 becomes:

\[
\log \tau = \varepsilon (\log \dot{\gamma}) + K \quad \text{(Equation 1.7)}
\]

This is the equation of the straight line where \(K\) is the intercept with the \(y\)-axis and the degree of Newtonian behaviour (\(\varepsilon\)) is the slope of the line. The consistency index, \(K\), reflects the apparent viscosity of the measured material at a point in time. If it changes as a function of the time during which a strain is applied to the sample the material displays time dependent flow (see section 1.4.8). Other more complex flow equations are described in appendix A.

1.4.6. Measuring Viscoelasticity

The fact that most materials do not conform exactly to either the definition of a Hookean solid or of a Newtonian fluid, but rather a combination of viscous and elastic components was introduced in Section 1.4.4. If a sufficiently large strain is applied then it is possible to break the structure of the material removing any trace of elastic behaviour and force the material to exhibit only
viscous flow – the principle behind viscometry tests. At low strains the elastic component plays a part in the materials behaviour and so it is necessary to define and measure it, using a suitable rheometer (Ciccolini, 1999).

A rheometric measurement normally consists of a strain (deformation) or a stress analysis at a constant frequency (normally 1 Hz) combined with a frequency analysis, for example between 0.1 and 100 Hz. The strain sweep gives information on the elastic or storage modulus (\(G'\)), the viscous or loss modulus (\(G''\)) and the phase angle \(\delta\) (Bolmstedt, 2001). The experimental studies completed for this thesis used only oscillatory techniques based on the protocol used by Ciccolini (1998). This is detailed in Chapter 2. The oscillatory techniques involve the application of a stress or strain whose value changes continuously according to a sine wave equation. The induced response (strain or stress) will also follow a sine wave. The complete cycle of the sine wave is considered as 360°, and the differences between the two phases (applied and response) are termed phase angles (Ciccolini, 1999).

A large value of \(G'\) in comparison to \(G''\) indicates pronounced elastic properties of the fluid being analysed. If the fluid is highly elastic then the phase angle is small, around 20°. A phase angle of 0° means a perfectly elastic material and a phase angle of 90° means a perfectly viscous material (Bolmstedt, 2001). This is because the strain is directly related to the stress in an elastic solid, and so at maximum stress the strain will be at its maximum, and the strain response is totally in phase with the applied stress; with viscous fluids, the strain is completely out of phase with the applied stress (Ciccolini, 1999), as shown in Figure 1.8(a).

The frequency sweep gives information about the elastic strength where a large slope of the \(G'\) curve indicates low strength and a small slope indicates high strength (Bolmstedt, 2001).

1.4.6.1. The Storage and Loss Modulus

Equation 1.3 shows how Hooke's law relates the strain to the stress using a material constant termed the rigidity modulus (\(G\)). In an oscillation test the
strain is constantly changing, and hence so is $G$. The material's complex modulus ($G^*$) is obtained from the ratio of the stress amplitude to the strain amplitude. $G^*$ is the sum of the elastic component and the viscous component, and is defined by equation 1.8:

$$G^* = G' + jG'' \quad \text{(Equation 1.8)}$$

Where $G'$ is the storage modulus, $G''$ is the loss modulus and $j$ is the imaginary number ($j^2 = -1$). The units of $G^*$ are Pa.

The phase angle $\delta$ is the difference between the stress and strain (as shown in Figure 1.8(a)), and is given by:

$$\delta = \tan^{-1} \left( \frac{G''}{G'} \right) \quad \text{(Equation 1.9)}$$

This relationship is shown graphically in figure 1.8(b), as well as the relationship between $G^*$, $G'$ and $G''$. $G'$ and $G''$ can be defined in terms of sine and cosine, as described in equations 1.10 and 1.11:

$$G' = G^* \cos \delta \quad \text{(Equation 1.10)}$$

$$G'' = G^* \sin \delta \quad \text{(Equation 1.11)}$$

### 1.4.7. Rheology in Industry

Rheology has an important role to play in the biotechnology industry: providing much useful behavioural and predictive information for various products (Brookfield manual). Rheological investigations are of potential importance in quality control and process control (Barnes et al., 1989), especially where raw materials must be consistent from batch to batch (Brookfield manual). Flow behaviour studies can also provide a direct assessment of "processibility" e.g. a high viscosity liquid requires more power to pump than a low viscosity one; knowing the rheological behaviour of a fluid is useful when designing pumping and piping systems (Brookfield manual). Rheological measurements, crucially, are also useful for following the course of a chemical reaction, and allow the study of the effects of additives or help to predict and control a large amount of product properties and material behaviour (Brookfield manual).
In the case of this thesis, characterising the rheology of the alkaline lysis process will help to determine both quality control and processibility.

1.4.8. Factors Affecting Rheology

1.4.8.1. The Effect of Time on Non-Newtonian Fluids

The measured shear stress, and hence viscosity (see equation 1.1) can either increase or decrease (reversibly or irreversibly) with the time of shearing. A gradual decrease in viscosity under shear stress followed by a gradual recovery of structure when the stress is removed is called thixotropy. This usually occurs when the liquid is shear thinning.

The opposite type of behaviour, where there is a gradual increase in viscosity under stress followed by recovery is called rheopexy, and this is usually associated with shear-thickening behaviour.

1.4.8.2. The Effect of Temperature on Non-Newtonian Fluids

Viscosity is directly affected by temperature and so this must be carefully controlled during rheological experiments. The temperature dependence of water, for example is 3% per °C at room temperature so that ±1% accuracy requires the sample temperature to be maintained to within ±0.3 °C.

The viscosity of Newtonian fluids decreases with an increase in temperature, according to the Arrhenius relationship:

$$\eta = Ae^{B/T} \quad \text{(Equation 1.12)}$$

Where T is the absolute temperature and A and B are constants of the liquid. The greater the viscosity of Newtonian fluids the larger the dependence on temperature. It is recommended that the accuracy must be greater than ±0.3 °C for fluids with a higher viscosity than water (Barnes et al., 1989).
Figure 1.8: (A) The diagram represents the application of shear stress ($\tau$), and resultant shear strain ($\gamma$) response of a viscoelastic fluid undergoing oscillatory rheometric analysis. The resultant strain is out of phase with the applied stress, and this is represented by the phase angle ($\delta$). (B) Diagrammatical representation of the relationship between complex shear modulus ($G^*$), storage modulus ($G'$), loss modulus ($G''$) and phase angle ($\delta$). (Source: www.sik.se/sik/affomr/star/reointro.html).

In polymeric systems, the solubility of the polymer can increase or decrease with temperature depending on the system – the coiled chain structure may become more open, resulting in an increase in resistance to
flow. It is therefore vital that all rheological experiments are performed using the same methodology and at controlled temperatures.

Section 1.4 has provided a general introduction to rheology to explain the theory behind the protocols listed in Chapter 2, and to describe the importance of rheology to the biochemical engineer, and how it will be used as a tool in this thesis. The next section (section 1.5) explains how rheology has been used in the characterisation of the alkaline lysis process to date at UCL.

1.5. Summary of Previous Work

This section outlines the results of published work investigating the rheology of cells undergoing alkaline lysis. This kind of study forms the major focus of the thesis as detailed in section 1.6. This section will focus primarily on the work by Ciccolini, but there are a number of other published works that investigate how rheological properties can be used to characterise the mixing and heat transfer of fermentation processes (e.g. Zhao et al., 1994).

1.5.1. Rheological Properties of Alkaline Lysates

Previous rheological studies of alkaline lysis have been concerned with investigating the cell lysis reaction time and the rheology of the alkaline lysis reaction. Figure 1.9 shows a typical rheological profile generated during the lysis of a cell suspension and carried out in a co-axial rheometer employed as a scale-down tool. The rheometer provides a defined mixing environment with on-line measurement of the contents' apparent viscosity and allows the study of the kinetics and rheology of cell lysates under laminar flow conditions, at small scale.

Ciccolini (e.g. Ciccolini et al., 1998, 1999) investigated the rheological properties of cell lysates, and determined that a reproducible viscosity vs. time profile resulted from the alkaline lysis process and that the shape of the profile did not alter with changes in the shear rate applied. However, the absolute magnitude of the apparent viscosity did change exhibiting shear rate
dependence, and hence indicative of the development of non-Newtonian flow. They also showed that the data could be modelled with the power law equation (see equation 1.5) with the exponent value of 0.48 indicating significant shear thinning behaviour of the solution containing the freshly denatured chromosomal DNA (Ciccolini et al., 1999).

Investigations into the effect of plasmid on the rheological profile showed no difference between the viscosity vs. time profile of the alkaline lysis of wild type *E. coli* C600 and that of low copy number plasmid containing *E. coli* C600/pR26 (Ciccolini et al., 1999).

The impact of removing NaOH from the alkaline lysis solution was also investigated. In the absence of an alkaline pH the cells lysed but no sharp peaks in the rheological profile were seen, suggesting that it was the denatured chromosomal DNA that gave rise to the second peak seen in the rheological profile (Ciccolini et al., 1999).

The addition of chilled potassium acetate as the neutralisation buffer has an immediate effect on the appearance of the alkaline lysate, as can be seen in Figure 1.3(C). The change in pH causes the renaturation of the low molecular weight plasmid DNA, and the reduction in temperature causes the SDS to come out of solution, forming a floc with proteins and denatured chromosomal DNA (see section 1.3.2). A low shear environment is necessary to maintain the resultant gel matrix that by inspection appears to be viscoelastic (Ciccolini et al., 1999).

This was proven by Ciccolini as the plots of storage and loss moduli showed that the storage modulus had a higher value than the loss modulus, and hence the rheology of the gel matrix is dominated by elasticity (Ciccolini et al., 1999).

### 1.5.2. Classic Alkaline Lysis Viscosity vs. Time Profile

Cells lysed in a co-axial rheometer using the alkaline lysis method show a characteristic viscosity vs. time plot, and there are three distinctive features of the viscosity profile, as shown in Figure 1.9. A change in viscosity occurs as
soon as the alkaline lysis solution is added to the cell suspension, and the apparent viscosity rises for a period of about 30 s to form the primary peak. After a short pause, during which the viscosity remains constant, the apparent viscosity begins to rise again to a pronounced secondary peak after about 100 s. Beyond this the viscosity falls to a steady value the pseudo steady state. The likely events leading to the rheological markers are described in sections 1.5.2.1 – 1.5.2.3.

**Figure 1.9:** Example of a viscosity vs. time profile for *E.coli* DH5α cells undergoing alkaline lysis. The alkaline lysis solution was added at time = 0, and the primary peak, secondary peak and pseudo-steady state can be clearly seen. Shear rate was constant at 367 s⁻¹ and temperature at 293 K.

1.5.2.1. Primary Peak

The primary peak is associated with the time of the cell lysis reaction - the time required to solubilise all the cells in suspension. Stopping the reaction
with the addition of chilled potassium acetate and counting the cells remaining has confirmed this: the primary peak was shown to coincide with the point at which all the cells were lysed (Ciccolini et al., 1999). Different cell strains were shown to have different primary peak timings, with *E.coli* C600 shown to have a primary peak timing of 30 s and DH5α strain primary peak at 40 s (Ciccolini et al., 1998). The mechanism by which alkaline lysis of cells occurs is discussed in detail in section 1.3.

1.5.2.2. Secondary Peak

The secondary peak in the viscosity profile has a higher apparent viscosity than that of the primary peak, and this is believed to be associated with the formation of a viscous network caused by maximum entanglement between denatured DNA and proteins released during cell lysis.

1.5.2.3. Pseudo-Steady State

After the formation of the secondary peak the apparent viscosity drops to a shear-rate dependent pseudo-steady state caused by shear degradation of DNA (Ciccolini et al., 1998).

This characteristic rheological profile will be used in this thesis as a probe to measure any changes that occur to the rheology of alkaline lysates as a function of different fermentation methods. The aims of the thesis, including how the rheological profiles will be utilised are described in detail in the next section.

1.6. Aims

To date only studies of the kinetics and rheology of cells lysed at the end of batch fermentations have been reported. Cells undergo many physiological changes over the time course of a fermentation. This PhD aims to characterise how these changes impact upon the alkaline lysis stage. The specific aims of the thesis are:

- Establish a suitable scale-down reactor for characterisation of the alkaline
lysis stage, using two differing rheometers (Chapter 4).

- Correlate cell resistance to physical breakage with cell resistance to chemical cell lysis, using high-pressure homogeniser studies (Chapter 5).

- Determine how rheology changes as a function of fermentation method and fermentation time, and elucidate the cause of rheological changes observed (Chapters 6 & 7).

- Investigate the impact of fermentation harvest time and the alkaline lysis stage neutralisation time on plasmid yield (Chapter 8).

- Perform an investigation into how the elastic component of alkaline lysates varies as a function of fermentation time (Chapter 9).

- Develop a rheological window of operation for optimum time of cell paste harvest, using the data from all the experimental work.

The significance of this thesis from an industrial perspective is in the increase in knowledge and understanding of scale-down alkaline lysis. The alkaline lysis step is a crucial stage in the production of plasmid DNA, and one that is still not sufficiently characterised. If there are methods that can increase the efficiency of alkaline lysis from a yield and economic perspective, then this will be of benefit to industry. The ultimate aim therefore is to provide insight for industrial application.
2. Materials and Methods

2.1. Introduction

This chapter details all the experimental protocols used in the practical work for this thesis. It is divided into a number of sections that describe the major experimental techniques: fermentation, alkaline lysis, physical cell lysis, rheological analysis and finally a section detailing the analytical techniques that were used throughout the thesis. This chapter lists the generic protocols, and is supported by the specific details listed in the materials and methods section of each particular results chapter.

The protocols were extracted mainly from the literature, and were chosen or designed so as to fulfil the requirements set by the aims in Chapter 1, section 1.6. Aseptic techniques and sterile materials were used throughout all protocols.

The following section describes the plasmids used in the studies.

2.2. Cell Strains and Plasmids

Two *Escherichia coli* strains were used during PhD experimental work: DH5α and DH1. The strains contained one of three recombinant plasmids, or as the plasmid free parental strain.

2.2.1. *E. coli* DH5α

The bacterial strain *Escherichia coli* DH5α (F-, supE44, hsdR17, recA1, gyrA96, endA1, thi-1, relA1, deoR) was transformed with either the plasmid pXX (supplied by GlaxoSmithKline R&D, Beckenham, Kent), a high copy number model plasmid approximately 10 Kb in size, expressing ampicillin resistance; or the plasmid pQR235 (supplied by University College London), a high copy number pUC based plasmid 8.1 Kb in size, expressing kanamycin resistance. An example plasmid map of pQR235 is shown in Figure 1.2. The bacterial hosts were transformed with plasmid DNA using CaCl₂-based transformation, as described by Sambrook (1989). The resultant
transformants were stored in 10 % (v/v) glycerol stocks at −70 °C.

2.2.2. *E. coli* DH1

The bacterial strain *Escherichia coli* DH1 (recA1, endA1, gyrA96, thi-1, relA1, spoT1, mcrA) containing the model plasmid pXY was supplied by GlaxoSmithKline R&D (Beckenham, Kent). This high copy number pUC-based plasmid is approximately 10 Kb and encodes for kanamycin resistance. The bacterial host was transformed with plasmid DNA using CaCl₂-based transformation, as described by Sambrook (1989). The resultant transformants were stored in 10 % (v/v) glycerol stocks at −70 °C. The parental strain of DH1, containing no plasmid was also used. This strain encodes no antibiotic resistance, and stocks were stored as before.

2.2.3. Strain Maintenance

Agar master plates of *E. coli* DH5α pQR235 were provided by Dr J. Ward, Department of Biochemistry and Molecular Biology, UCL. Master plates of *E. coli* DH1 pXY and *E. coli* DH5α pXX were provided by GlaxoSmithKline R&D, Beckenham UK.

Working cell banks (WCB) were constructed for each individual strain and plasmid combination, using the following protocol. Bacterial colonies were scraped from the particular master agar plate on which the required *E. coli* strain was grown and transferred to a conical flask containing 100 mL sterile proprietary complex inoculum medium (GlaxoSmithKline R&D, Beckenham UK). Similar growth can be achieved using an equivalent complex media such as Terrific broth (Sambrook *et al*., 1989). The conical flask was then transferred to an incubator (ISF-I-V, Adolf Kuhner AG, Basel, Switzerland) at 37°C, 230 rpm until the recorded optical density (UV-1201, UV-VIS spectrophotometer; Shimadzu, Milton Keynes, UK) reached 2 OD units. 10 mL of sterile glycerol (BDH Chemicals, Leics, UK) was then added to the cell culture in the conical flask, to make a 10% glycerol cell culture stock. 1.8 mL aliquots were then pipetted into labelled cryo-vials and stored at −70°C.
2.3. Fermentation

This section details the protocols for fermentations using the various makes and scales of batch fermenters available. The different batch fermentation methods are described in section 2.3.2. Each individual results chapter lists the batch fermenter protocols utilised for that particular experimental study. Inoculum preparation is detailed in section 2.3.1. The fed-batch protocol is described in section 2.3.3.

2.3.1. Growth Medium and Inoculum Preparation

2.3.1.1. Preparation of Inoculum for 20 L LH-Inceltech Fermenters

This fermentation used Luria Bertani (LB) medium for the inoculum medium. Table 2.1 shows the method for production of LB medium. Sterile-filtered kanamycin (Sigma Ltd, Dorset, UK) was added to all growth media to a final concentration of 50 mg/L.

The pre-inoculum was developed by adding 1 mL of a DH5α glycerol stock to each of between 4-6 (depending on the amount of pre-inoculum vessels required) glass screw-top universals containing 5 mL of sterile LB. This was then incubated at 37°C, 230 rpm for 4 hours, after which the pre-inoculum was added to 4 2 L shake-flasks containing 500 mL LB medium and incubated at 37°C, 230 rpm for 16 hours.

2.3.1.2. Preparation of Inoculum for SGI (New Brunswick Scientific Co., Inc., Edison, New Jersey, USA) and Applikon (Applikon, Tewkesbury, UK) Fermenters

Two media were used for batch fermentation using the SGI and Applikon vessels: a proprietary complex inoculum medium (CIM); and a proprietary complex fermentation medium (CFM) formulated at GlaxoSmithKline R&D (Beckenham, Kent), and similar to well characterised complex media such as Terrific broth. Sterile-filtered Kanamycin (Sigma Ltd, Dorset, UK) was added to all growth media to a final concentration of 50 mg/L. The pre-inoculum was
developed by adding 1.8 mL of a DH1 pXY glycerol stock to 100 mL of sterile CIM medium and incubated at 37 °C, 230 rpm. After 6 h growth, pre-inoculum was added to six flasks containing 100 mL of CIM to give an optical density of 0.2 at 600 nm (UV-1201, UV-VIS spectrophotometer; Shimadzu, Milton Keynes, UK). The shake flasks were then incubated at 37 °C, 230 rpm for 16 h.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Supplier</th>
<th>Amount</th>
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<tbody>
<tr>
<td>NaCl</td>
<td>BDH</td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>Oxoid</td>
<td>5 g</td>
</tr>
<tr>
<td>Tryptone</td>
<td>Difco</td>
<td>10 g</td>
</tr>
<tr>
<td>R.O. Water</td>
<td>#N/A</td>
<td>Add 950 ml to vessel; add to make 1 L final volume.</td>
</tr>
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</table>

Table 2.1: List of ingredients, suppliers and measures to add to make 1 L of Luria Bertani liquid medium.

2.3.1.3. Preparation of Inoculum for Fed-batch Applikon Fermenters

The pre-inoculum was developed by adding 1.8 mL of a DH1 pXY glycerol stock to 100 mL of sterile CIM medium and incubated at 37 °C, 230 rpm. After 6 h growth, pre-inoculum was added to six flasks containing 100 mL of CIM to give an optical density of 0.2 at 600 nm (UV-1201, UV-VIS spectrophotometer; Shimadzu, Milton Keynes, UK). The shake flasks were then incubated at 37 °C, 230 rpm for 16 h.

2.3.2. Fermentation Protocol

2.3.2.1. Shake-flask Fermentation

Two shake-flask fermentation protocols were used during the work for this thesis, depending on the aim of the experiment. Both protocols followed the same fundamental process. The materials and methods section for each
individual results chapter details the choice of protocol.

- **Shake-flasks of 1 L total volume or greater:**

  Kanamycin (Sigma Ltd, Dorset, UK) was added to 500 mL working volume of sterile LB medium (see Table 2.1) at a concentration of 50 mg/L in each shake-flask required for the experiment (total working volumes of 1 L, 2 L or 3 L).

  The pre-inoculum universals (see method in section 2.3.1.1.) were then poured into a 100 mL sterile conical flask and mixed well. 5 mL of inoculum was then transferred aseptically, to each shake flask required for fermentation. Each shake-flask was labelled clearly and sealed using a foam bung and foil cover. The shake flasks were then incubated (ISF-I-V, Adolf Kuhner AG, Basel, Switzerland) at 37°C, 230 rpm for 16 hours.

- **Shake-flasks of 500 mL total volume:**

  Kanamycin (Sigma Ltd, Dorset, UK) was added at a concentration of 50 mg/L to 100 mL of sterile CIM medium in each of the 500 mL shake-flasks required for the experiment.

  A controlled volume of inoculum was added to the media to give an optical density of 0.2 measured at 600 nm wavelength (UV-1201, UV-VIS spectrophotometer; Shimadzu, Milton Keynes, UK) within each shake-flask. Each shake-flask was then labelled and sealed with a foam bung and foil cover, before being incubated (ISF-I-V, Adolf Kuhner AG, Basel, Switzerland) at 37 °C, 230 rpm for 16 h.

2.3.2.2. Batch Fermentation 1

The first fermentation method was carried out in a 20 L LH Inceltech series 1075 (LH-Inceltech, Toulouse, France) fermenter, using a 14 L working volume of either Luria-Bertani (LB) media or CFM (GlaxoSmithKline R&D, Beckenham). The fermenter and media were sterilised in situ, using the manufacturers recommended method. Kanamycin was added at a concentration of 50 mg/L, once the medium had returned to room temperature. The fermenter was inoculated using a sterile port attached to a
1 L shake-flask containing 500 mL of the inoculum. The cell strain used was \textit{E. coli} DH5\textalpha /pQR235. The impeller speed and the air mass flow rate at time of inoculation were 250 rpm at 1 vvm respectively. The impeller speed was controlled using feedback to maintain dissolved oxygen tension (DOT\%) at 30\% air saturation and air mass flow rate controlled by operator. Off-gases were analysed using a mass spectrometer (VG Gas Analysis Ltd., Worthing, UK), and pH maintained at 7.00 using controlled addition of 4 M sodium hydroxide and 1 M phosphoric acid. Polypropylene glycol (PPG) (BDH Chemicals, Leics, UK) was used as antifoam agent.

2.3.2.3. Batch Fermentation 2

The second batch fermentation method was used for the SGI 10 L fermenters (New Brunswick Scientific Co., Inc., Edison, New Jersey, USA) and the Applikon 7 L and 3 L total volume fermenters (Applikon, Tewkesbury, UK). All the fermenters used CFM (GlaxoSmithKline R&D, UK) medium with glycerol added at 10 mL/L working volume and 0.1 mL/L PPG also added. Table 2.2 shows the amount of media required for the working volume of each fermenter.

CFM medium was added to the fermenters before the whole unit was sterilised by autoclave. Either \textit{E. coli} DH1 /pXY or DH5\textalpha /pXX was grown as an inoculum (see above) in preparation for fermentation. The fermenters were prepared for inoculation by the addition of antibiotic: kanamycin was added at a concentration of 50 mg/L for DH1 /pXY cell strain and ampicillin at a concentration of 100 mg/L for DH5\textalpha /pXX. Inoculum was added using sterile connection; volumes are listed in Table 2.2.

At point of inoculation, stirrer speed and air mass flow were set at 500 rpm and 1 vvm respectively. Growth conditions were maintained, using feedback control at: 37\degree C by the use of a water jacket; pH 7 by controlled addition of 2 M sulphuric acid and 2 M sodium hydroxide; and DOT at 30\% by control of air mass flow rate and stirrer speed. A controlled feed of 2\% PPG (BDH Chemicals, Leics, UK) solution reduced foaming.
2.3.2.4. Fed-batch Fermentation

The fed-batch fermentation protocol is based on the batch fermentation method. The fed-batch method used a growth-limiting, controlled approach to produce a reduced specific growth rate ($0.14 \ h^{-1}$) by feeding a limited substrate component into the culture.

For this thesis, 7 L total volume Applikon (Applikon, Tewkesbury, UK) fermenters were used, with a working volume of 3 L of a proprietary fed-batch media (GlaxoSmithKline R&D, Beckenham UK). Sterilisation and fermenter station set-up were the same as in the batch fermentation method 2.

Fed-batch medium (FBM) is similar to CFM except with a 20% reduction in carbon source. The feed media (GlaxoSmithKline R&D, Beckenham UK) was made up of the reduced carbon source at a concentration 7.5 times that of CFM.

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<td>0.57</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>SGI</td>
<td>10</td>
<td>5</td>
<td>0.5</td>
<td>0.45</td>
<td>0.1</td>
</tr>
<tr>
<td>Fed-batch Applikon</td>
<td>7</td>
<td>3</td>
<td>0.43</td>
<td>0.1</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**Table 2.2:** The relationship between total and working volume of a fermenter and the inoculum volume used.

The inoculum was prepared as in the method described in section 2.3.1.3, and 100 mL was added to the fermenter at 12.00 am by means of a peristaltic pump (Watson-Marlow Bredel Pumps Ltd., Falmouth, Cornwall,
Andrew F. Day

Chapter 2

UK) on a timer switch. The fed-batch fermenter was run as a simple batch fermenter for 8 h at normal conditions for optimum growth (37°C, pH 7.0, DOT 30%), with feedback control identical to batch fermenters. After 8 h, the feeding regime was started, with sterile feed medium pumped in using the regime described in Table 2.3. The feed regime was run for 11 h before the fermentation was stopped and the cell culture harvested.

2.3.3. Fermenter Sampling

2.3.3.1. Sampling 20 L LH Fermenter

Samples were taken every hour, starting from time of fermenter inoculation. Each time a sample was taken, 20 mL of cell culture was stored on ice for subsequent optical density and dry cell weight assays. At time of sampling, two universals were used. The fermenter outlet was steamed for 5 minutes prior to the sampling, and then one universal was filled and discarded. The next universal, clearly labelled with the time of the fermentation, was filled from the sample point, and then stored on ice. After the sample was taken the port was steamed for 5 minutes.

Samples for homogenisation were taken every 2 h. At sample time sample lines were flushed by filling one universal from the sample port and then discarding the universal. 4 universals were then each filled with 20 mL of broth. These samples were then split equally into a total of 8 universals, each containing 10 ml. The universals were then spun at 3000 g, 4°C for 15 minutes in a bench top centrifuge (Beckman GS-6R Centrifuge, Beckman Instruments Ltd, Bucks, UK). The supernatant was then carefully decanted and the cell pellet resuspended in 10 ml of Phosphate Buffer Solution (PBS: 8.5g NaCl, 1.07g Na₂HPO₄, 0.345g NaH₂PO₄·2H₂O in 1 L water). The universals were then spun again at 3000 g, 4°C for 15 minutes. The supernatant was again decanted and resuspended in 10 ml PBS buffer. The 8 samples were then poured into 4 universals, each containing 20 ml of sample. The samples were then paired to make 2 x 40 ml samples, ready to be used in the homogeniser protocol.
Table 2.3: Description of the fed-batch feeding regime, showing the rate and total volume of feed medium addition.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Feed Rate (mL/h)</th>
<th>Total Feed Added (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.00</td>
<td>00.0</td>
</tr>
<tr>
<td>2</td>
<td>14.40</td>
<td>12.0</td>
</tr>
<tr>
<td>3</td>
<td>17.28</td>
<td>26.4</td>
</tr>
<tr>
<td>4</td>
<td>20.74</td>
<td>43.7</td>
</tr>
<tr>
<td>5</td>
<td>24.88</td>
<td>64.4</td>
</tr>
<tr>
<td>6</td>
<td>29.86</td>
<td>89.3</td>
</tr>
<tr>
<td>7</td>
<td>35.83</td>
<td>119.2</td>
</tr>
<tr>
<td>8</td>
<td>43.00</td>
<td>155.0</td>
</tr>
<tr>
<td>9</td>
<td>51.60</td>
<td>198.0</td>
</tr>
<tr>
<td>10</td>
<td>61.92</td>
<td>249.6</td>
</tr>
<tr>
<td>11</td>
<td>74.30</td>
<td>311.5</td>
</tr>
</tbody>
</table>

2.3.3.2. Sampling Batch and Fed-batch Applikon / SGI Fermenters

Samples for optical density and viable cell count were taken every hour, with first one universal filled and then discarded as described in section 2.3.3.1. A small sample (approximately 5 mL) was then removed and stored on ice in a labelled universal.

Samples for alkaline lysis were removed every hour:

100 mL of culture was removed and pipetted into pre-weighed and labelled 200 mL centrifuge pots. Samples were then centrifuged at 3000 g, 4 °C for 20 min (Heraeus Sepatech Omnifuge 2.0RS, Kendro Laboratory Products Ltd., Herts., UK). The supernatant was then discarded and the weight of the pellet was determined. Cell pellets were then either stored at 4 °C for immediate use or frozen at −20 °C for future analysis.

Samples for homogenisation were taken every 2 h:
Four aliquots of 20 mL were centrifuged in preweighed universals at 3000 g, 4 °C for 20 min (Heraeus Sepatech Minifuge T, Kendro Laboratory Products Ltd). The supernatant was discarded and the pellet washed in 20 mL of phosphate buffered saline (PBS; pH 7.2). The pellet was then weighed and resuspended in 20 mL of PBS. Samples were then pooled to form 2x 40 mL samples.

2.3.4. Fermenter Harvest

Cell culture harvest was carried out by first siphoning the culture into sterile plastic bottles. The fermenter was then sterilised and cleaned according to the required protocol. Cell culture was then transferred to 500 mL centrifuge pots maintaining an even weight distribution, and then spun in a centrifuge (Beckman J2-M1, JA10 rotor, Beckman Instruments Ltd, Bucks., UK) at 3000 g, 4°C for 20 minutes. Cell paste was then stored, frozen at -70°C for future analysis.

2.4. Alkaline lysis Using Scale-Down Co-axial Rheometer

A co-axial cylinder rheometer (Bohlin VOR system C25, Bohlin Instruments Ltd., Glos., UK) was used to monitor apparent viscosity changes during alkaline lysis of cell suspensions derived from the fermentations (see Figure 2.1). The co-axial cylinder arrangement (see Figure 2.2) consists of a fixed bob, (diameter 25 mm) located in an outer rotating cylinder (diameter 27.5 mm). The default torque bar used was 10.15 gcm except where stated otherwise. The rheometer was used as both the lysis mixing vessel and the measuring device, under controlled, laminar conditions as described by Ciccolini et al. (1998). Apparent viscosity was measured every 2 s for 10 min at a fixed shear rate (461 s⁻¹), this having been shown to provide a suitable environment for the study of rheological profiles before (Ciccolini et al, 1998), and the temperature maintained at 20°C.
2.4.1. Alkaline Lysis Protocol 1: Lysis Step Only

Frozen cell paste was defrosted on ice and then re-suspended in TE buffer (see Table 2.4) at a ratio of 100 mL TE buffer per 12.5 g wet cell weight of cell paste. 6 mL of the re-suspended cell paste was then transferred by pipette into the cup of the co-axial cylinder rheometer. The rheometer was then set-up for viscosity measurements as described above, and the bob lowered into the cup. The rheometer was started and alkaline lysis initiated immediately by the addition of alkaline lysis solution (see Table 2.4). Apparent viscosity measurements were recorded every 2 s for 10 minutes, after which the lysed cells were disposed of.

2.4.2. Alkaline Lysis Protocol 2: Lysis Step Followed By Neutralisation

Frozen cell paste was defrosted and re-suspended as in protocol 1. 4 mL of the re-suspended cells were transferred to the cup of the co-axial cylinder rheometer, and the rheometer set up for measurement as described in the manual. The rheometer was started and lysis initiated immediately by the addition of 4 mL of alkaline lysis solution. Apparent viscosity was measured as before for a predetermined period, as required by the experiment, and then the reaction was neutralised by the addition of 4 mL of neutralisation buffer (see Table 2.6). Samples were then transferred to labelled universals and stored on ice for subsequent analysis or purification (see section 2.6.4).

2.4.3. Reproducibility of Bohlin Rheometer Data

Apparent viscosity may differ slightly in absolute value at an error of ± 10%.

2.4.4. Rheological Analysis Using Scale-Down Reactor

Rheological analysis was carried out in the scale-down reactor, using a combination of techniques to characterise the viscoelastic properties of the fluid under analysis. The techniques used were shear sweep, strain sweep and shear strain vs. shear rate analysis. The method for each technique is discussed in the following sub-sections, and is supported by the materials and methods section of Chapter 9, where the samples used are listed. The theory
behind rheological analysis is discussed in Chapter 1, section 1.4. Rheological analysis was only performed on samples from batch fermentations.

Normal practice is to use a cone-and-plate geometry, but initial studies showed this set-up to lack sensitivity, and so the co-axial cylinders geometry was employed for all rheological analysis, with the Bohlin rheometer set at x10 sensitivity.

2.4.4.1. Shear Sweep

12 mL of sample was loaded into the bob, and the rheometer prepared for start, as according to the manufacturer's instructions. 10.15 gcm torque bar was used. The operating shear rate was increased in increments from 1.16 s$^{-1}$ up to 1160 s$^{-1}$ with each shear rate held for 7 s. The apparent viscosity was recorded at each shear rate up to the maximum shear rate, after which it retraced back to 0, again recording apparent viscosity.

2.4.4.2. Strain Sweep

Strain sweeps were carried out on 12 mL samples loaded into the cup. Sensitivity was set at 10 times normal rate, and linear sweeps (see Chapter 1, section 1.4.6 for theory) were conducted in fifteen steps between 40% and 60% amplitude. The frequency of oscillation was maintained at 1 Hz, and the storage and loss moduli, as well as the phase angle were recorded. The torque bar selected was the 0.295 gcm bar.

2.4.4.3. Shear Stress vs. Shear Rate

This technique was used to generate stress diagrams for each of the fluids under analysis. Samples for rheological analysis were removed from the batch fermenter at early exponential, exponential and stationary growth phases as explained in section 2.3.3. 12 mL of the sample undergoing analysis was then loaded into the cup and the rheometer set up to start as explained in the instruction manual. 10.15 gcm torque bar was used. The analysis was started at a very low shear rate (1.16 s$^{-1}$), before increasing in
increments to 1160 s\(^{-1}\) (holding each level for 7 s) and the shear stress generated by that shear rate was recorded. Once the final shear rate was reached, the experiment was terminated.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate Buffered Solution (PBS)</td>
<td>Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na(_2)HPO(_4), and 0.24 g of KH(_2)PO(_4) in 800 mL of distilled H(_2)O. Adjust pH to 7.4 and add water to 1 L. Sterilise by autoclaving.</td>
</tr>
<tr>
<td>TE Buffer</td>
<td>10 mM Tris-HCL (pH 8.0), 1mM EDTA.</td>
</tr>
<tr>
<td>Potassium Acetate (Kac)</td>
<td>Add 11.5 mL glacial acetic acid and 28.5 mL H(_2)O to 60 mL 5 M potassium acetate. The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.</td>
</tr>
<tr>
<td>10% Sodium Dodecyl Sulphate (SDS)</td>
<td>Dissolve 100 g electrophoresis grade SDS in 900 mL H(_2)O, heat to 68(^\circ)C to dissolve. Adjust to pH 7.2 with HCL and adjust volume to 1 L.</td>
</tr>
<tr>
<td>Alkaline Lysis Solution</td>
<td>Add 9 mL of 10% SDS to 90 mL of 2 M Sodium Hydroxide (BDH).</td>
</tr>
</tbody>
</table>

Table 2.4: TE, PBS and alkaline lysis buffer ingredients and their production method (Sambrook et al., 1989).

2.5. Determining Physical Cell Strength Using An Industrial High Pressure Homogeniser

The physical cell strength was determined by the use of industrial high-pressure homogeniser. Two homogenisers were used in this thesis: a Micron Lab 40 and an Emulsiflex C5. The following sub-sections consider the protocol for each homogeniser. For details on the theory behind physical cell strength see Chapter 6.

2.5.1. Micron Lab 40

The physical cell strength of \textit{E. coli} DH5\textalpha{} /pQR235 was measured using a Micron Lab 40 APV Gaulin homogeniser (APV Systems, W.Sussex, UK).
experimental method was similar to that used by Engler and Robinson (1981) and Siddiqi et al. (1995) on yeast. Duplicate 40 mL samples, treated using the protocol described in section 2.3.3.2, were tested. The sample was poured into the feed cylinder and the valve housing secured in place. The required pressure was set manually, and the sample was passed through the valve of the homogeniser (see Figure 2.3). The valve housing was then removed and the homogenate poured into a beaker. This constituted one pass. After each pass, a 0.5 mL sample was transferred to a labelled microfuge tube and stored on ice. Each aliquot was then spun at 6000 g for 5 min in a micro centrifuge (Beckman Microfuge 12, Beckman Instruments Ltd, Bucks., UK), and resuspended in TE buffer, before being tested for soluble protein content. Protein release was measured using the Coomassie protein assay reagent (Pierce and Warriner Ltd., Chester, UK.) as per the protocol described in section 2.6.2, and the protein concentration determined by comparison with the standard curve prepared with BSA.

Maximum protein release was defined by the measured release at a pressure of 1200 bar. The protein release was measured over four passes at a working pressure of 300 bar. Each duplicate sample was subjected to both pressures. Data from the protein assay was used to calculate a rate constant (K) for cell disruption, derived from equation 2.1 (Hetherington et al., 1971), shown below:

\[ KN = \ln \left( \frac{R_m}{R_m - R} \right) \]  

(Equation 2.1)

Where \( R_m \) is the maximum protein release at 1200 bar; \( R \) is the protein released at 300 bar; and \( N \) is the number of passes through the homogeniser. Increasing values of \( K \) indicate increased susceptibility to cell breakage. See Chapter 5 for details of the use of the Hetherington equation.

2.5.2. Emulsiflex C5

Physical cell strength of \( E. \ coli \) DH1 /pXY was measured by disrupting cells in a Emulsiflex C5 homogeniser (Avestin Inc., Ottawa, Canada). Duplicate 40 mL samples from the same fermentation batch stage of cell growth were passed through the homogeniser, using the same pressures and number of
passes as in section 2.5.1. After each pass, 0.5 mL samples were taken and analysed using the Coomassie assay. This data was used to calculate disruption rate constant (K) as described in Chapter 5.

2.6. Analytical Techniques

A variety of techniques were used for determining optical density of fermenter samples, the protein concentration of homogenates, the number of intact cells after alkaline lysis, and the plasmid DNA content of samples. These methods are described in the following sub-sections.

2.6.1. Measuring Optical Density

The growth of a cell culture can be determined off-line by measuring the optical density. Samples are collected every hour as described in section 2.3.3, and stored on ice. 2 mL of culture are added to a cuvette and the absorbance at 600 nm measured in a spectrophotometer (UV-1201, UV-VIS spectrophotometer; Shimadzu, Milton Keynes, UK). The spectrophotometer is zeroed against a cuvette containing pre-inoculum media. This acts as a blank, and a diluent. There is a linear relationship between absorbance and cell biomass up to a reading of 0.8 OD units. If the reading is above this threshold then the sample must be diluted. Dilution is achieved by the addition of the media blank, at the ratios described in Table 2.5.

2.6.2. Determination of Protein Concentration (Coomassie Assay)

The protein concentration was measured using the Coomassie protein assay reagent (Pierce and Warriner Ltd., Chester, UK). A standard curve was constructed using bovine serum albumin (BSA), grade > 96% (Sigma, Poole, Dorset, UK). The BSA standard curve was constructed for known concentrations from 2 mg/mL to 0.025 mg/mL. For each concentration, 950 µL Coomassie reagent and 50 µL of standard was added to a cuvette, and mixed thoroughly before being left to stand for over 5 minutes. Absorbency was measured at 595 nm in a spectrophotometer (UV-1201, UV-VIS spectrophotometer; Shimadzu, Milton Keynes, UK).
All samples were read at 595 nm and 1 mL of Coomassie reagent was used as a blank. To test unknown samples, 50 μL of the experimental sample was added to a cuvette containing 950 μL of the Coomassie reagent. Mixed and stood for 5 minutes. The absorbency was then tested at 595 nm. If the reading was higher than 0.8, the sample was diluted (e.g. 1 in 10) before being measured again. The results were then tested against the standard curve.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Culture Volume (mL)</th>
<th>Media Blank (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neat</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>1/2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1/4</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>1/8</td>
<td>0.25</td>
<td>1.75</td>
</tr>
<tr>
<td>1/10</td>
<td>0.2</td>
<td>1.8</td>
</tr>
<tr>
<td>1/16</td>
<td>0.125</td>
<td>1.875</td>
</tr>
<tr>
<td>1/20</td>
<td>0.1</td>
<td>1.9</td>
</tr>
<tr>
<td>1/32</td>
<td>1 of 1/16 dilution</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.5: Volumes of media blank and fermenter cell culture used to construct Optical Density (A_{600nm}) dilutions.

2.6.3. Cell Counting

Total cell count was estimated using a Thoma haemocytometer (Fisher Scientific UK Ltd., Orme Technologies, Middleton, Manchester, UK). A standard method taken from the literature was employed (Ison and Matthew, 1997) with no cell staining.

A small sample of lysate was pipetted onto the counting slide, after which the cover slip was placed on and the cells were counted under a microscope (Nikon Optiphot, Nikon UK Ltd, Kingston, Surrey, UK) at x400 magnification.
The Thoma haemocytometer has an etched grid of known dimensions. The grid is divided into 25 squares, each of which is further subdivided into 16 small squares. This makes 400 squares, each with a surface area of 1/400 mm$^2$. The number of cells visible in a 16 square portion is counted. The average of 5 different 16 square portions is taken, and from this data the cell concentration is then calculated, using equation 2.2:

$$\frac{(N/4) \times 10^5 \text{ cells/ml}}{\text{(Equation 2.2)}}$$

Where $N$ is the average number of cells counted. If the sample required dilution, the count was multiplied accordingly.

2.6.4. Determination of Plasmid DNA Quality and Quantity (Agarose Gel Electrophoresis)

Agarose gel electrophoresis was used to determine qualitatively and quantitatively (by using the appropriate analysis package) the supercoiled plasmid DNA content of a sample of alkaline lysate.

Alkaline lysate samples were derived as explained in section 2.4.2. The subsequent treatment of the samples is detailed in section 2.6.4.1. The preparation, loading, running and staining of the agarose gels is explained in sections 2.6.4.2 and 2.6.4.3. The analysis of the gels is described in 2.6.4.4.

2.6.4.1. Treatment of Alkaline Lysates

12 mL of neutralised alkaline lysate was used per sample (recovered from the protocol described in section 2.4.2). Each sample was spun for 10 min at 3000 g, 4°C (Heraeus Sepatech Omnifuge 2.0RS, Kendro Laboratory Products Ltd., Herts., UK). The supernatant was then transferred to a universal containing 7.2 mL of Isopropanol (BDH Chemicals, Leics., UK) and stood for 5 min. The mixture was then spun for 10 min at 3000 g, 4°C, after which the supernatant was gently discarded, and the residual supernatant aspirated without disturbing the pellet. The pellet was then washed by the addition of 7.2 mL of ethanol (BDH Chemicals, Leics., UK) followed by centrifugation for 10 min at 3000 g, 4°C. The ethanol was then decanted and
the pellet air-dried for 10 min. The pellet was then resuspended in 0.8 mL TE buffer (1/5<sup>th</sup> of the original cell suspension volume), and transferred to a labelled microfuge tube.

10 μL of 1/10<sup>th</sup> dilution DNA samples to be run were then mixed with the 4 μL of the gel-loading buffer (Sigma, Dorset, UK). The mixture was then loaded by pipette in to the gel wells. The minimum amount of DNA that can be detected is 2 ng in a 0.5 cm wide band and more than 500 ng is overloading.

Supercoiled Lambda DNA ladder (Sigma, Dorset, UK) was used as the marker. The marker was loaded into three wells per gel at volumes of 5 μL, 10 μL and 15 μL with the same concentration of 20 ng/μL. The supercoiled markers contained gel-loading dye.

2.6.4.2. Preparation, Loading and Running Gel

The edges of a clean dry glass plate were sealed with autoclave tape to form a mould. Sufficient 0.5x TBE solution (1 x solution: 0.09 M tris-borate, 0.002 M EDTA) was prepared. A 0.6 % agarose gel mixture was made by adding 0.9 g of agarose (Sigma, Dorset, UK) to 150 mL of TBE buffer in a 250 mL conical flask. The flask was loosely plugged with cotton wool and the slurry heated in microwave to dissolve the agarose. The solution was cooled to 60 °C, before pouring the agarose carefully into the glass tray. The comb was fitted into the appropriate slot so that there was a 1 mm gap between the bottom of the comb and the plate. The gel should be 3-5 mm thick. The gel was left for 30-45 minutes at room temperature to completely set. Then the comb and the autoclave tape were carefully removed and the gel mounted in the electrophoresis tank. The rest of the TBE buffer solution was then added to the tank to cover the gel to a depth of about 1 mm.

The samples and the standards were pipetted into the wells as described in section 2.6.4.1 and then the lid of the tank was fixed on. The electrical leads were attached so the DNA would migrate towards the anode. 20 volts and 400 mAmp current were applied for 16 hours, after which the battery pack (Hybaid PS250, Hybaid Ltd, Ashford, Middlesex, UK) was turned
off and the leads and lid removed from the gel tank.

2.6.4.3. Staining and Photographing Gel

The agarose gel was transferred for staining to a tray of water containing ethidium bromide at a concentration of 0.5 μg/mL. Ethidium bromide solution (10 mg/mL) was prepared by adding 1 g of ethidium bromide (Sigma, Dorset, UK) to 100 mL of RO water and stirring for several hours to dissolve the dye. The solution was then stored in an aluminium wrapped container at ambient temperature. The dye was incorporated into the gel by soaking for 45 minutes.

The gel was transferred carefully to a tray containing water and soaked for 5 min to clear excess ethidium bromide. The gel was then photographed using an UV transilluminator and camera package (Ultra-Violet Products Ltd, Cambridge, UK). The gel photographs were then saved on to disk for later analysis.

2.6.4.4. Analysis of Gel Bands

The analysis was carried out using Scion image analyser (Scion Corporation, Maryland, USA), where the intensity of each band was measured and compared to the calibration generated by the markers (reproducible within ±5%).

The well loaded with 5 μL of marker had a total concentration of 100 ng DNA, the well with 10 μL of marker had 200 ng of DNA and the well loaded with 15 μL of marker had 300 ng DNA. Each band of the supercoiled ladder therefore had a known concentration: each of the 11 bands derived after the supercoiled DNA ladder marker had been run had an equal concentration – the total mass of DNA loaded into the well divided by 11. It is therefore possible to create a calibration curve of the measured band area against the known mass of DNA, from which the unknown bands’ concentration can be derived (see Appendix B).
Figure 2.1: Cross section of Bohlin Rheometer with co-axial cylinder geometry, showing DC motor (1); gear boxes (2); clutch (3); position serve actuator (4); cup and bob (5); temperature sensor (6); bearing (7); output to computer (8); and torque bar (9) (Bohlin Rheometer Handbook).
Figure 2.2: Detail of co-axial cylinder geometry showing the relationship between the cup and bob. **D1** = Bob Diameter (25 mm); **D2** = Cup Internal Diameter (27.5 mm); **H1** = Bob Height (37.5 mm); **H2** = Depth when Closed (12.4 mm); and **α** = Cone Angle (5/6 π).
Figure 2.3: Cross section sketch of Micron Lab 40 APV Gaulin homogeniser showing flow of fluid and position of valve.
3. The Use of and Reproducibility of Shake-flask, Batch and Fed-batch Fermentations for Plasmid DNA Production

3.1. Summary

The inoculum was developed using shake-flasks where the working volume to total volume ratio was maintained at 0.2 v/v to produce a maximum specific growth rate (\(\mu_{\text{max}}\)) of 0.98 h\(^{-1}\).

It was shown that maintaining similar working volume to total volume and inoculum volume to working volume ratios in different fermenters would produce a similar \(\mu_{\text{max}}\) and hence similar growth conditions.

Similar fermenters produced a \(\mu_{\text{max}}\) of 0.38 h\(^{-1}\) for the \textit{E.coli} DH1 /pXY cell strain. The parental strain had a \(\mu_{\text{max}}\) of 0.35 h\(^{-1}\) and DH5\(\alpha\) /pXX cell strain produced 0.46 h\(^{-1}\) proving that different cell strains have different growth characteristics. The impact of growth media was shown in an experiment growing DH5\(\alpha\) /pQR235 in a 20 L fermenter on LB and CFM media. The cells grown on CFM produced a \(\mu_{\text{max}}\) of 0.58 h\(^{-1}\) compared to the cells grown on LB media that had a \(\mu_{\text{max}}\) of 0.32 h\(^{-1}\).

The fed-batch method was shown to maintain the \(\mu_{\text{max}}\) at the desired rate of 0.14 h\(^{-1}\), which would allow some assessment of the effects of the maximum specific growth rate on physical and chemical cell lysis, in subsequent chapters in this thesis.

3.2. Introduction

The first stage in the industrial production of Plasmid DNA is the fermentation of recombinant cells, using a suitable production bacterial strain such as \textit{E.coli} cells (see Chapter 1, section 1.2.3). It is important that the fermentation process is reproducible and well characterised, so as to avoid inconsistencies between batches.
3.2.1. Aims
The aim of this chapter was to create a pool of consistent and standardised samples that could then be either analysed immediately or frozen for later analysis. The fermentation protocol had to be reproducible as described above. All fermentations must be monitored and controlled so that the maximum growth rates ($\mu_{\text{max}}$) could be standardised. Three different fermentation methods were used: shake-flask, batch and fed-batch. This chapter presents the data from all of the fermentation campaigns carried out throughout the PhD.

3.2.2. Scale-up and Scale-down
Scale-up is a vital step in fermentation development. It usually follows the following format: laboratory fermentations are used to screen strains and optimise process conditions; pilot plant fermentations are used to produce enough data for evaluation and further optimisation; finally the process is transferred to a production facility for commercialisation (Jem, 1989). Scale-down can be used to reduce a process to a smaller scale to solve problems, or potential problems at process level, and reduce development times. These small-scale experiments must be, however, representative of the full scale (Jem, 1989; Kossen, 1992; Dahlgren et al., 1993). Scale-up and scale-down uses and problems were considered further in Chapter 1, section 1.1.

3.2.3. Shake-Flask Fermentations
Shake-flask cultures are often used to optimise media and fermentation conditions, or to screen for a desirable strain of bacteria before a production process is developed and scaled up to larger stirred tank reactors (Jem, 1989 and Dahlgren et al., 1993). However, shake-flasks are generally limited by lack of pH control and poor oxygen transfer. In this thesis shake-flask fermentations were used as a way of producing small amounts of cell paste rapidly for initial studies and to generate inoculum for larger stirred tank reactor (STR) fermentations.
3.2.4. Batch Fermentations

A batch fermentation is a closed system where all the materials are present at time t=0, or inoculation; throughout the fermentation nothing is added except air, antifoam and pH controllers, and the cell culture progresses through three classic phases of growth: lag, exponential and stationary phase (Creuger and Creuger, 1989), as shown in Figure 3.1. Batch fermentations are easy to regulate and validate, and provide product in batches that are simple to trace back through the process should there, for example be a purity issue. However, batch fermentations can affect the formation of many secondary metabolites because of catabolite repression by high levels of metabolites. A number of different manufacturer’s models and sizes of batch fermenter have been used throughout the research reported in this thesis, chiefly because of availability of equipment. The fermenters used are listed in the materials and methods section (3.3.2).

3.2.5. Fed-batch Fermentations

Fed-batch systems have been developed to try and overcome the batch fermentation limitation created by the formation of toxic secondary metabolites. An example of this is when E.coli cells are inhibited by the acetate they produce when grown on media containing glucose (Luli and Strohl, 1990). An extra feed stream adding nutrients to the reactor while the fermentation is running is often desired so that inducers can be added for increased product yield, precursors added for the expression of desired products, or in order to maintain low nutrient levels to minimise catabolite repression (Bailey and Ollis, 1986), and help to improve cell biomass and plasmid yield. Fed-batch systems also give some control over the organism’s growth rate, which can lead to an extension of the productive growing conditions (Stanbury et al., 1995). The use of batch and fed-batch fermentations for the production of plasmid DNA is discussed in detail in Chapter 1, section 1.2.3.1.
3.3. Materials and Methods

This section supports the materials and methods in Chapter 2, section 2.3. All protocols are listed in detail within that chapter.

3.3.1. Shake-flask Fermentation

The shake-flask fermentation protocol was as described in Chapter 2, section 2.3.2.1 for all shake-flask scales. Two distinct protocols were employed. The first protocol maintained a 0.2 v/v working volume of CIM media in 0.5 L shake-flask, using *E.coli* DH1 /pXY cell strain. The second protocol for shake-flask fermentations used a variety of different scale shake-flasks (1 L, 2 L and 3 L) with the same 0.5 L working volume of LB media in each of them, hence varying the working volume to total volume ratio (see Table 3.1). The cell strain used for these shake-flask experiments was *E.coli* DH5α /pQR325. 2 L shake-flasks with baffles were also used, to monitor the impact on growth of this mixing enhancer.

3.3.2. Batch Fermentation

There were a number of different models and sizes of fermenters used, as explained in section 3.2.4. The largest scale was 20 L (LH Inceltech series 1075) with a 14 L working volume (0.7 v/v working volume) and using the cell strain *E.coli* DH5α /pQR235. The next scale of fermenter was 10 L SGI (New Brunswick Scientific Co., Inc., Edison, New Jersey, USA) with a 5 L working volume (0.5 v/v working volume). Also used were 7 L Applikon fermenters (Applikon, Tewkesbury, UK) with a 4 L working volume (0.57 v/v working volume) and 3 L Applikon with a 2 L working volume (0.67 v/v working volume). The fermenter total and working volumes are listed in Table 3.1. All fermenters other than the 20 L LH Inceltech used either *E.coli* DH1 /pXY or *E.coli* DH5α /pXX cell strains. All protocols and media selections are described in detail in Chapter 2, section 2.3.2.2.

3.3.3. Fed-batch Fermentation

All fed-batch fermentations were carried out in 7 L (0.43 v/v working volume) Applikons. The *E.coli* DH1 /pXY cell strain was grown on fed-batch media (FBM) for 8 h before the feeding regime (adding feed media) was started
using the method described in Chapter 2, section 2.3.2.4. The aim of this protocol was to maintain maximum specific growth rate ($\mu_{\text{max}}$) at 0.1 h$^{-1}$.

<table>
<thead>
<tr>
<th>Shake-flask</th>
<th>Total/Working Vol (v/v)</th>
<th>Fermenter</th>
<th>Total/Working Vol (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 L</td>
<td>0.2</td>
<td>20 L LH</td>
<td>0.7</td>
</tr>
<tr>
<td>1 L</td>
<td>0.5</td>
<td>10 L SGI</td>
<td>0.5</td>
</tr>
<tr>
<td>2 L</td>
<td>0.25</td>
<td>7 L Applikon</td>
<td>0.57</td>
</tr>
<tr>
<td>2 L + baffles</td>
<td>0.25</td>
<td>3 L Applikon</td>
<td>0.67</td>
</tr>
<tr>
<td>3 L</td>
<td>0.17</td>
<td>Fed-batch</td>
<td>0.43</td>
</tr>
</tbody>
</table>

**Table 3.1:** Listing of all shake-flask and fermenter scales used during thesis, and the corresponding working volume/total volume ratio, quoted in text.

### 3.4. Results

This section examines the data generated from the different fermentations carried out over the course of the fermentation campaign. The graphs in this chapter show average fermentation data from at least triplicate data sets, and all error bars are derived from standard deviation analysis of the relevant data sets. The following sub-sections describe the trends observed within the data set for each of the fermenters.

#### 3.4.1. Shake-flask Fermentations

The average growth curve for *E. coli* DH1 /pXY incubated in 0.5 L shake-flasks containing 0.2 v/v complex inoculum medium (CIM) is shown in Figure 3.2. The two curves on the graph show the optical density vs. time (OD growth curve) and the natural log of optical density vs. time (ln(OD) growth curve). The OD growth curve shows the three distinct growth phases: lag phase for the first 2 h of growth; exponential growth up until the peak OD at 9 h and then stationary growth until completion of sampling. The peak OD reached registered at 7.75 units. The ln(OD) growth curve for the data is superimposed on the graph and shows a general smoothing of the data.

The OD growth curves for *E. coli* DH5*Δ* /pQR325 grown in 1 L, 2 L, 2 L-
baffled and 3 L shake-flasks with Luria-Bertani medium at a working volume to total volume ratio of 0.5, 0.25, 0.25 and 0.17 respectively are shown in Figure 3.3. From Figure 3.3(a), by inspection, it is clear that the maximum OD achieved in this experiment is less than half that achieved in the previous shake-flask experiment. The graphs show roughly the same kind of plot as before, with three distinct phases. There are, however a number of differences between each of the curves: the maximum OD reached and the time of fermentation at which it is reached varies as the size of vessel increases. The 1 L shake-flask takes 14 h to reach its maximum OD of 2.215; for the 2 L shake-flask it peaks at 2.76 units after 12 h; the 2 L vessel with baffles reaches 3.12 units after 10 h; and the 3 L vessel reaches a maximum of 3.05 after 10 h. The maximum specific growth rate for each shake-flask is discussed in section 4.4.5. It is clear that the 1 L vessel takes significantly longer to reach its maximum OD than the other shake-flasks, and has an OD a third less than either 2 L baffled or 3 L shake-flasks.

Figure 3.3(b) shows the In(OD) growth curve for the shake-flasks. The profile of the curves is very similar for each of them, with a brief lag phase followed by an exponential growth phase for about 7 h. Stationary growth continues from then on, with any increase in OD not enough to show clearly in the In(OD) growth curve. The maximum specific growth rates derived from these graphs are discussed in section 4.4.5.

### 3.4.2. Batch Fermentations

This section considers the resultant growth curves for the different fermenters used during the fermentation campaign.

3.4.2.1. *E.coli* DH5α/pQR235 Growth in 20 L LH Fermenter

Figure 3.4(a) shows the average growth curves for *E.coli* DH5α/pQR235 grown in Lauria Bertani (LB) medium and Complex fermenter medium (CFM). This allows a direct comparison between the two types of culture medium used during the fermentation campaign. LB medium is a simple culture medium consisting of three components (see Chapter 2) and is therefore cheap and easy to use. CFM is a complex medium designed and supplied by
The differences between the growth of *E. coli* DH5α /pQR235 in the 20 L LH fermenter grown using the two types of culture is highlighted in Figure 3.4(a). Growth on the LB medium reaches its maximum cell density after 5 h. Cells grown in the CFM medium have a lower OD at this point; however, the culture grown in CFM continues to increase for another 3 h, to a maximum OD of 26, before reaching stationary phase and growth slowing. The growth curves for both the LB and CFM media show distinctive lag, exponential and stationary phases. Examination of the ln(OD) growth curves in Figure 3.4(b) show a much steeper initial gradient for cultures grown in LB medium, but stationary phase is reached much earlier while the CFM medium continues to support exponential growth.

Further inspection of Figure 3.4(b) shows no clear lag phase (as seen in Figure 3.1). Instead there is a brief period of accelerating growth and then the exponential growth phase begins.

### 3.4.2.2. *E. coli* DH1 /pXY Grown in 3 L Applikon Fermenter

Figure 3.5 shows the average growth curve for 3 L Applikon fermentations using CFM medium with a working to total volume ratio of 0.67 v/v. The maximum OD reaches 17.3 units after 11 h but there is little growth after 8 h, indicating that stationary growth phase has been reached. The ln(OD) growth curve shows no distinct lag phase, and exponential growth from 1 h to 8 h. Stationary phase proceeds from there.

### 3.4.2.3. *E. coli* DH1 /pXY Grown in 7 L Applikon Applikon

*E. coli* DH1 /pXY were grown in 7 L Applikon fermenters (0.57 v/v working volume) using CFM medium. The average growth curve is shown in Figure 3.6. The OD growth curve shows a similar curve to previous fermentations, but with a final OD of 31. The ln(OD) growth curve again shows no distinctive lag phase. There is a distinct stationary phase after 8 h.

### 3.4.2.4. *E. coli* DH1 Parental Strain Grown in 7 L Applikon Fermenter

The average of the growth curves for the *E. coli* DH1 parental strain is shown
in Figure 3.7. The growth curve is similar to the 7 L pXY plasmid containing cell strain, although the maximum OD reached is higher in the parental strain than with plasmid, reaching 35 units. The error bars are large indicating a large variation in fermentation growth curves. The ln(OD) growth curve again shows a steep lag phase gradient.

3.4.2.5. *E. coli* DH1/pXY Grown in 10 L SGI Fermenter

The working volume in the 10 L SGI fermenters is set at 5 L, and Figure 3.8 shows the average growth curve, with a maximum OD of 27 units. Although the OD growth curve indicates a lag phase, the ln(OD) growth phase shows again that the lag phase has a steeper gradient than the exponential phase. The maximum OD is higher than the 3 L Applikon fermentations but less than the 7 L Applikon fermentations.

3.4.2.6. *E. coli* DH5α/pXX Grown in 10 L SGI Fermenter

The OD growth curve and ln(OD) growth curve shown in Figure 3.9 present similar curves to the previous fermentations, but reach maximum OD of 19 after 7 h.

The maximum optical density reached during the fermentations are listed and compared in Table 3.2.

3.4.3. Fed-batch Fermentations

The growth curve for fed-batch fermentation in a 7 L Applikon using FBM medium is shown in Figure 3.10. The method for fed-batch fermentations is discussed in section 3.3.3, and the aim of the protocol was to limit $\mu_{max}$ to 0.1 h$^{-1}$.

The graph shows a very different plot to the previous OD growth curves and ln(OD) growth curves for batch fermentations. The growth curve shows no indication of any particular growth phases. Instead just a steady controlled growth throughout the fermentation. This highlights the impact of feeding nutrients over time, as opposed to a batch fermentation where all the nutrients are added at the start, and the fermentation is effectively a closed system. The maximum specific growth rates for all the fermentations are considered in
section 3.4.5, with all the growth rates represented in Tables 3.3 and 3.4.

<table>
<thead>
<tr>
<th>Fermenter Type/Size</th>
<th>E. coli Strain /Plasmid</th>
<th>Medium Used</th>
<th>Max OD ($A_{600nm}$)</th>
<th>Ferm Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shake-flask (0.5 L)</td>
<td>DH1 /pXY</td>
<td>Complex Inoculum Medium (CIM)</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Applikon (3 L)</td>
<td>DH1 /pXY</td>
<td>Complex Fermentation Medium (CFM)</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td>Applikon (7 L)</td>
<td>DH1 /pXY</td>
<td>CFM</td>
<td>31</td>
<td>11</td>
</tr>
<tr>
<td>DH1 /parental</td>
<td>CFM</td>
<td>35</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>SGI (10 L)</td>
<td>DH1 /pXY</td>
<td>CFM</td>
<td>27</td>
<td>10</td>
</tr>
<tr>
<td>DH5α /pXX</td>
<td>CFM</td>
<td>19</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>LH (20 L)</td>
<td>DH5α /pQR235</td>
<td>Luria Bertani (LB) Medium</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>DH5α pQR235</td>
<td>CFM</td>
<td>26</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Fed-Batch (7 L)</td>
<td>DH1 /pXY</td>
<td>CFM</td>
<td>26</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 3.2: Comparison between different fermentation protocols and the maximum optical density ($A_{600nm}$) reached.

3.4.4. Maximum Specific Growth Rates

The maximum specific growth rate ($\mu_{\text{max}}$) per hour is the rate at which the cell culture is growing during exponential growth. The following sub-sections discuss the recorded $\mu_{\text{max}}$ of the fermentations carried out during the fermentation campaign (see Tables 3.3 and 3.4).

3.4.4.1. Shake-flask Fermentations

Table 3.3 compares the maximum specific growth rate data for E.coli DH5α /pQR235 grown on LB media in different scale shake-flasks. As explained in section 3.3.1 the working volume was maintained at 0.5 L thus causing the working volume to total volume ratio to decrease as the shake-flask scale increased from 1 L to 3 L.
Table 3.3: Maximum specific growth rates for differing scale shake-flask fermentations.

<table>
<thead>
<tr>
<th>Shake-flask Volume (Total)</th>
<th>Working Volume / Total Volume Ratio</th>
<th>Maximum Specific Growth Rate ($\mu_{max}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 L</td>
<td>0.5</td>
<td>0.62 h^{-1}</td>
</tr>
<tr>
<td>2 L</td>
<td>0.25</td>
<td>0.62 h^{-1}</td>
</tr>
<tr>
<td>2 L + Baffles</td>
<td>0.25</td>
<td>0.71 h^{-1}</td>
</tr>
<tr>
<td>3 L</td>
<td>0.17</td>
<td>0.81 h^{-1}</td>
</tr>
</tbody>
</table>

There is a trend of increasing $\mu_{max}$ as the v/v decreases. The $\mu_{max}$ rises from 0.62 h^{-1} to 0.81 h^{-1} as the v/v decreases from 0.5 to 0.17. The effect on growth rate caused by the presence of mixing enhancing baffles can be seen as the 2 L shake-flask records 0.62 h^{-1} maximum specific growth rate compared to the 2 L with baffles that records a $\mu_{max}$ of 0.71 h^{-1}.

Table 3.4 lists the $\mu_{max}$ for all 0.5 L shake-flask fermentations with a working volume ratio of 0.2 v/v as 0.98 h^{-1} for *E.coli* DH1 /pXY grown in CIM medium. The $\mu_{max}$ recorded in the 0.5 L shake-flask was higher than that recorded in the other shake-flask growth rates detailed in Table 3.3.

3.4.4.2. Batch Fermentations

Table 3.4 lists the maximum specific growth rates for all the batch fermentations. By inspection it is clear that the highest value is for the 20 L fermentation of DH5α /pQR235 grown on CFM media, and the lowest value is for the 3 L fermentation of DH1 /pXY on CFM media. There seem to be a number of trends:

- Fermenter size – the larger the fermenter the higher the $\mu_{max}$ value.
- Cell strain – DH5α has a higher $\mu_{max}$ than DH1.
- Medium – LB media produces a lower $\mu_{max}$ than for growth on CFM media.
The impact each of the above will have on cell paste development will be considered in the discussion.

3.4.4.3. Fed-batch Fermentation

Table 3.4 shows that with the fed-batch fermentation protocol the maximum specific growth rate is maintained at \(0.1 \text{ h}^{-1}\), as intended. The effects of this growth rate on the rheology of the alkaline lysis operation will be investigated in Chapter 6.

3.5. Discussion

The following section discusses the results of the fermentation campaign with particular reference to comparisons between the different fermenter designs and protocols, and whether these differences impact on the aim of producing a consistent source of cell paste samples.

3.5.1. Shake-flask Fermentations

This sub-section will discuss the results from the shake-flask fermentations and the research that will be conducted as an impact of these results.

The shake-flask growth curves show distinctly the three growth phases: lag, exponential and stationary. The experiments show that growth on CIM medium yielded a higher maximum specific growth rate (0.98 h\(^{-1}\)) compared to the best performing shake-flask with growth on LB media (0.8 h\(^{-1}\)). A much higher maximum OD was also reached when grown on CIM media (7.8 OD units) compared to cells grown on LB media (3.1 OD units). This increase in growth could be due the cell strain, the plasmid or the growth media used. From the results in section 3.4.4.2 it is possible to conclude that the choice of CIM media caused the increase in growth rate.

The experiments with a constant 0.5 L of LB medium in different sized shake-flasks show the impact of oxygen transfer limitations on shake-flasks. Each shake-flask experiment will be referred to by its respective working volume to total volume ratio during the following discussion (see Table 3.3). From the results it can be seen that the 0.17 v/v vessel grew the fastest and reached the highest optical density. This can be directly attributed to
increased oxygen transfer through the larger surface area presented in the larger vessel. The impact of improved mixing can be observed from the comparison of the normal 0.25 v/v vessel and the baffled 0.25 v/v vessel. There is a substantial increase in maximum specific growth rate in the vessel with baffles. However, the growth rate is still less than the 0.17 v/v vessel, suggesting that a low v/v ratio is the most influential factor in creating an inoculum.

<table>
<thead>
<tr>
<th>Description of Experiment</th>
<th>Max Specific Growth Rate $(\mu_{\text{max}})$ h$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fermenter Type</strong></td>
<td><strong>E.coli Strain</strong></td>
</tr>
<tr>
<td>Shake-flask (0.5 L)</td>
<td>DH1 /pXY</td>
</tr>
<tr>
<td>Applikon (3 L)</td>
<td>DH1 /pXY</td>
</tr>
<tr>
<td>Applikon (7 L)</td>
<td>DH1 /pXY</td>
</tr>
<tr>
<td></td>
<td>DH1 /parental</td>
</tr>
<tr>
<td>SGI (10 L)</td>
<td>DH1 /pXY</td>
</tr>
<tr>
<td></td>
<td>DH5α /pXX</td>
</tr>
<tr>
<td>LH (20 L)</td>
<td>DH5α /pQR235</td>
</tr>
<tr>
<td></td>
<td>DH5α pQR235</td>
</tr>
<tr>
<td>Fed-Batch (7 L)</td>
<td>DH1 /pXY</td>
</tr>
</tbody>
</table>

**Table 3.4:** Comparison of maximum specific growth rates for the different fermenter designs and protocols used during the fermentation campaign.

Because of the above observations, optimum inoculum development was created using a working volume ratio of 0.2 v/v and growth on CIM media. This resulted in a high maximum specific growth rate (0.98 h$^{-1}$) as shown in Table 3.4.
3.5.2. Batch Fermentations

The reproducibility of the batch fermentations and the fermentation strategy to be employed for the production of cell paste will be discussed in this subsection. There was a distinct difference when using CFM medium compared to LB medium: the former showed no distinctive lag phase, whereas the latter did. The lack of a lag phase may be due to a combination of factors such as the similarity in composition between the CIM and CFM media allowing growth to continue unhampered, the age of the inoculum culture and hence the ability of the culture to begin cell division, and the size of the inoculum (Cartledge, 1992). The area on the growth curves where lag phase would normally be expected appeared to exhibit acceleration phase characteristics (as seen in Figure 3.1), and for the purposes of this thesis will be named the early exponential growth phase from now on. The lag phase will be discussed in detail in section 3.5.2.3.

3.5.2.1. Fermenter Configuration

The different fermenter configurations used were determined primarily by availability, but from the results in Table 3.4 it can be seen that cell cultures grown in the larger fermenters (e.g. 20 L) have a higher \( \mu_{\text{max}} \) than smaller scale (e.g. 3 L). This is in spite of a similar working volume ratio and a higher inoculum to working volume ratio (see Table 3.5) in the 3 L vessel. The inoculum sizes listed in Table 3.5 show that the inoculum ratio was maintained at approximately 0.1 v/v except for the 20 L vessel. This is simply because the \( \mu_{\text{max}} \) for the 20 L fermenter was so high that a greater inoculum volume was not required. It is therefore possible to surmise that there is a threshold for inoculum size required and that beyond 0.5 L absolute inoculum volume there is little advantage to be gained from increasing the inoculum size, even at larger fermenter scales. This could be because the culture is sufficiently large going into a sterile environment to reduce the lag phase and start high growth rates quickly. As expected there is little difference in performance between the 7 L Applikons and the 10 L SGI fermenters, with similar \( \mu_{\text{max}} \) and maximum optical density for the DH1 strain. This is because the various ratios are very similar. From this analysis it is possible to conclude that if inoculum and
working volume to total volume ratios are similar, there is little difference between the choice of fermenters, and therefore overriding factor in fermenter choice will be availability.

The optimum growth media to use for the fermentations is clearly the CFM medium, since this produced a higher maximum specific growth rate and a larger cell density for any given cell strain or fermenter. The next subsection will discuss the impact of cell strain.

3.5.2.2. Cell Strain

From Table 3.4, it can be seen that the DH5α /pXX strain has a higher $\mu_{\text{max}}$ than the DH1 /pXY strain, which in turn has a higher $\mu_{\text{max}}$ than the parental DH1 strain. However, there appears to be a pay-off between maximum specific growth rate and maximum optical density reached. The parental DH1 has the highest final cell density, followed by the DH1 /pXY strain and then the DH5α /pXX strain. The increased final cell density in the parental strain is due to the lack of metabolic load created by the presence of a plasmid (see Chapter 5). It is therefore possible to surmise that the presence of a plasmid can increase maximum specific growth rate, whilst reducing final cell density by a not insignificant amount. The increase in the scale of the fermenter leads to an increase in maximum specific growth rate, as does the use of a complex growth media.

3.5.2.3. Growth Curve

Although the OD growth curves for batch fermentations provide similar shaped plots to the shake-flask results, the In(OD) growth curve shows that there is no distinctive lag phase, as seen in the classic growth curve shown in Figure 3.1. This is probably due to the size of inoculum and the nutrients available in the complex medium encouraging an almost instant acceleration of growth prior to exponential growth phase. It will be important to see how this early exponential phase, in place of where the lag phase would be expected to be in a classic growth curve (1 – 2 h after inoculation) affects the rheology during alkaline lysis of cell culture, and how the physical cell strength alters as an impact of this effect.
An example of the control mechanism to maintain the dissolved oxygen tension (DOT) of a fermentation at 30% is shown in Figure 3.11. The normal growth curve is shown, with the three growth phases clearly visible. The airflow is not shown on the graph as this is controlled by hand, and at the start of a fermentation is pumped through the culture at 1 vvm. The impeller speed is set at a minimum of 500 rpm. During the lag phase the DOT remains high, but once the cell culture moves into exponential phase the DOT drops rapidly. At this point the negative feedback system raises the impeller rate to maintain the DOT at 30%. As the cell density increases, the impeller rate also raises to maintain the DOT. Once the maximum impeller rate is reached the DOT drops steadily until the stationary phase is reached and the DOT starts to rise again as the demands for oxygen reduce. Once the DOT has started to increase and the optical density has stabilised, it is possible to halt the fermentation.

3.5.3. Fed-batch Fermentations

The results from the fed-batch fermentation showed that the maximum specific growth rate was maintained at 0.14 h⁻¹ after the initial limited growth batch fermentation had reached completion, and the feeding regime had begun (see section 3.3.3). This compares with a $\mu_{\text{max}}$ of between 0.3 - 0.4 h⁻¹ for the batch fermentations. The impact upon plasmid yield of achieving a controlled growth rate will be investigated in a subsequent chapter of the thesis (see Chapter 8) as will the fed-batch fermentation’s impact upon the viscosity vs. time profile generated during the alkaline lysis of cell paste (see Chapter 6).

From the perspective of generating an optimum time of cell harvest it would be worth investigating how the physical and chemical cell strength changes over the time course of the fermentation. For example, an investigation into whether cell culture harvested during stationary growth phase has a greater resistance to physical and chemical cell lysis than cell culture harvested during exponential growth phase. This will be the basis for the investigation reported in Chapter 5.

The next chapter continues the theme of establishing consistent results using differing designs of machinery. The aim of the investigation was to
establish whether the viscosity vs. time profiles for resuspended cell paste undergoing alkaline lysis (see Chapter 1, section 1.5) could be reproduced in another design of rheometer to the Bohlin co-axial rheometer used in previous published work (Ciccolin, 1998). This was to determine that the viscosity vs. time profiles were not artefacts of the Bohlin co-axial rheometer.

<table>
<thead>
<tr>
<th>Fermenter Total Volume (L)</th>
<th>Working Volume (L)</th>
<th>Inoculum Size (L)</th>
<th>Inoculum / Working Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>14</td>
<td>0.5</td>
<td>0.04</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>0.45</td>
<td>0.09</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0.2</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 3.5: Inoculum volume to working volume ratio for fermenters used in fermentation campaign.

3.6. Conclusion

Fermentation conditions could be reproduced, and hence a consistent supply of cell paste could be generated, using different designs of fermenter so long as the same growth media was used, and both working volume to total volume and inoculum volume to working volume ratios were the same in the different fermenters. This allowed some flexibility in choice of fermenter design when carrying out the fermentation campaign.

Maximum specific growth rate was affected by the choice of cell strain used and the presence of a plasmid within the strain. The choice of growth media was shown to have a larger impact upon the maximum specific growth rate than the cell strain and plasmid choice. It was also observed that as $\mu_{\text{max}}$ increased the final cell density was reduced.

The optimum inoculum development in shake-flasks was defined as having a working volume to total volume ratio of 0.2 v/v, and as the ratio increased the $\mu_{\text{max}}$ recorded in the shake-flask decreased. The CIM media
was selected as the preferred choice for growth media.

The fed-batch protocol was seen to maintain the $\mu_{\text{max}}$ at the required maximum specific growth rate ($0.1 \text{ h}^{-1}$).

**Figure 3.1:** Classic natural log growth curve showing the three main phases of growth: Lag, Exponential and Stationary growth phases. The graph also shows the lesser-defined growth areas of acceleration and decline (Doran, 1998).
Figure 3.2: Average growth curve for *E.coli* DH1 /pXY grown on CIM media in a 0.2 working volume/total volume ratio shake-flask. Optical density is recorded at an absorbance of 600 nm. Error bars derived from standard deviation from the mean for data set.
Figure 3.3: Growth curves for *E. coli* DH5α /pQR235 grown on LB media in: 1 L shake-flask with 0.5 working vol / total vol; 2 L shake-flask with 0.25 v/v; 2 L shake-flask with 0.25 v/v and baffles; and 3 L shake-flask with 0.17 v/v. (A) Optical Density growth curves (A₆₅₀nm) and (B) ln(Optical Density). Error bars derived from standard deviation from the mean for data set.
Figure 3.4: Average growth curve for *E. coli* DH5α/pQR235 grown on LB media and CFM media in a 20 L LH fermenter with a working volume/total volume ratio of 0.7 v/v. (A) Optical density is recorded at an absorbance of 600 nm. (B) ln(Optical Density). Error bars derived from standard deviation from the mean for data set.
Figure 3.5: Average growth curve for *E.coli DH1* /pXY grown on CFM media in a 3 L Applikon fermenter with a working volume/total volume ratio of 0.67 v/v. Optical density is recorded at an absorbance of 600 nm. Error bars derived from standard deviation from the mean for data set.
Figure 3.6: Average growth curve for *E.coli* DH1/pXY grown on CFM media in a 7 L Applikon fermenter with a working volume/total volume ratio of 0.57 v/v. Optical density is recorded at an absorbance of 600 nm. Error bars derived from standard deviation from the mean for data set.
Figure 3.7: Average growth curve for E.coli DH1 parental strain grown on CFM media in a 7 L Applikon fermenter with a working volume/total volume ratio of 0.57 v/v. Optical density is recorded at an absorbance of 600 nm. Error bars derived from standard deviation from the mean for data set.
Figure 3.8: Average growth curve for *E.coli* DH1/pXY grown on CFM media in a 10 L SGI fermenter with a working volume/total volume ratio of 0.5 v/v. Optical density is recorded at an absorbance of 600 nm. Error bars derived from standard deviation from the mean for data set.
Figure 3.9: Average growth curve for *E. coli* DH5α /pXX grown on CFM media in a 10 L SGI fermenter with a working volume/total volume ratio of 0.5 v/v. Optical density is recorded at an absorbance of 600 nm. Error bars derived from standard deviation from the mean for data set.
Figure 3.10: Average growth curve for *E.coli* DH1 /pXY grown on FBM media in a 7 L Applikon fermenter with a working volume/total volume ratio of 0.43 v/v. Fermentation is carried out using the fed-batch method with nutrient feeding regime. Optical density is recorded at an absorbance of 600 nm. Error bars derived from standard deviation from the mean for data set.
Figure 3.11: The relationship between measured optical density, dissolved oxygen tension (DOT) and impeller speed during a typical fermentation. DOT is measured using an oxygen probe and the impeller speed controlled by negative feedback.
4. Establishing a Suitable Rheometer as a Scale-Down Mimic

4.1. Summary

The maximum shear rate the Brookfield can operate under during alkaline lysis is 180 s⁻¹, because of the low maximum torque level on this design of viscometer. Viscosity vs. time profiles generated at shear rates of 24 s⁻¹ proved to have indistinct secondary peaks and pseudo steady states. Profiles generated at increasingly higher shear rates produced lower maximum torque levels, restricting the clarity of the profile. The primary peak was clear at all shear rates and so the point of total cell lysis could be determined (see Chapter 1, section 1.5).

Due to the limitations caused by the mechanism the Brookfield employs to measure viscosity, it was decided that it was unsuitable for the needs of the experimental work for this PhD, and the Bohlin would be employed as with previous work (Ciccolini, 1998). Initial studies showed that there were differences in the viscosity vs. time profile as a function of the time of the fermentation at which point the cells were sampled. Subsequent experiments in this thesis will investigate this further.

4.2. Introduction

Previous published work (e.g. Ciccolini, 1998) measured the change in apparent viscosity vs. time for the alkaline lysis of E.coli cell paste carried out in a laminar flow environment with a constant shear rate. In the published studies a Bohlin rheometer was employed as the scale-down tool, and a characteristic curve was produced (see Figure 1.9). This chapter details the investigation into whether another rheological instrument could be used as a suitable substitute for the Bohlin.

4.2.1. Aims

The aim of this chapter was to investigate whether the characteristic viscosity vs. time profile previously developed using a particular rheometer as a scale-down tool (Ciccolini et. al., 1998) could be reproduced using a different model
of rheometer. The reasons for this were three-fold: first, to prove the viscosity vs. time profile was not an artefact of the chosen rheometer; second, to see if the benefits of the scale-down tool could be achieved using different equipment available in almost any laboratory; and third, could the same results be achieved using a less sophisticated viscometer design than had been used previously? This would be useful for the wider application of the method used to determine the rheology of alkaline lysates.

4.2.2. The Characteristic Viscosity vs. Time Profile

The characteristic viscosity vs. time profile for the alkaline lysis of *E. coli* C600/pR26 grown on LB media in a 2 L shake-flask with a working volume/total volume ratio of 0.25 v/v is shown in Figure 1.9. There are three distinctive features of the viscosity profile, generated at a constant shear rate of 367 s^{-1} and constant temperature of 293 K (for more information see Chapter 1, section 1.5). The primary peak is associated with the time of the cell lysis reaction - the time required to solubilise all the cells in suspension. The secondary peak in the viscosity profile has a higher apparent viscosity, and this is believed to be associated with the formation of a viscous network caused by maximum entanglement between denatured DNA and proteins. After the secondary peak the apparent viscosity drops to a shear dependent pseudo-steady state caused by shear degradation of DNA (Ciccolini *et al.*, 1998).

4.2.3. The Comparative Scale-down Reactors

The Bohlin co-axial rheometer was used as a scale-down tool for previous work (Ciccolini *et al.*, 1998). By measuring the viscosity over the time of the lysis reaction a characteristic curve can be developed (see Chapter 1, section 1.5).

The equipment chosen for making a comparison with the Bohlin was a Brookfield viscometer. This was chosen, as it is significantly less expensive than the Bohlin and was readily available for analysis. Both models work using the same cup and bob geometry. The temperature could also be controlled in both systems. However, the Bohlin is a rheometer and so can
analyse the elastic component of a substance as well as the viscous component (see Chapter 1, section 1.4); whereas the Brookfield is a viscometer and can measure only the viscosity of a substance, hence it being significantly cheaper.

The following section details the materials and methods particular for this chapter, and compares the protocols for both the Bohlin rheometer and the Brookfield viscometer.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Timing (s)</th>
<th>Apparent Viscosity (mPa.s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary Peak</td>
<td>40</td>
<td>4</td>
</tr>
<tr>
<td>Secondary Peak</td>
<td>100</td>
<td>35</td>
</tr>
<tr>
<td>Pseudo steady state</td>
<td>200</td>
<td>-25</td>
</tr>
</tbody>
</table>

**Table 4.1:** The timing and apparent viscosity of the three main features seen in the classic viscosity vs. time profile for the alkaline lysis of *E.coli* DH5α at 367 s⁻¹ and 293 K.

**4.3. Materials and Methods**

This section supports the general method for alkaline lysis in a scale-down reactor (the Bohlin rheometer), as described in Chapter 2, section 2.4. However, this section also details the precise protocols adapted for this study due to the differences in the configuration and operation of the Bohlin and Brookfield machines.

**4.3.1. Bohlin Rheometer**

The Bohlin rheometer was utilised in exactly the same manner as the protocol described by Ciccolini (1998) and described in Chapter 2, section 2.4.1. In operation a 6 mL sample of cells suspended in TE buffer at a known concentration was mixed with 6 mL of alkaline lysis solution at a constant shear rate. The temperature was maintained at 293 K in all experiments examined in this thesis. Published results produced apparent viscosity vs. time profiles for cell paste at a defined range of shear rates between 46 s⁻¹
and 461 s\(^{-1}\). The sharpest and clearest profile was seen at the higher ranges of shear rate, and so the shear rate was maintained at 461 s\(^{-1}\) during the experimental work for this thesis.

### 4.3.2. Brookfield Viscometer

A Brookfield DV-III viscometer (Brookfield, West Harlow, Essex, UK) was used. The coaxial cylinders (cup and bob) system was used as the measuring geometry on the UL Adapter. Figure 4.1 shows the fixed cup and rotating bob system, with the cooling water from the water bath inlet and outlet.

The following equations were used to calculate viscosity from the geometry and torque measurements:

- **Shear Rate (s\(^{-1}\))**: 
  \[ S' = \left( \frac{2Rc^2}{Rc^2 - Rb^2} \right) \omega \]  
  (Equation 4.1)

- **Shear Stress (dynes cm\(^{-2}\))**: 
  \[ F'' = \frac{M}{2\pi Rb^2 L} \]  
  (Equation 4.2)

- **Viscosity (poise)**: 
  \[ \mu = \frac{F''}{S'} \]  
  (Equation 4.3)

Where: \( \omega \) = angular velocity of spindle (rad/sec)

- \( Rc \) = radius of container (cm)
- \( Rb \) = radius of spindle (cm)
- \( M \) = torque input by instrument (see Brookfield manual)
- \( L \) = effective length of spindle (see Brookfield manual)
- \( S' \) = shear rate at surface of spindle (s\(^{-1}\)).

The protocol used for the determination of a viscosity vs. time profile for the alkaline lysis of *E.coli* cell suspensions was similar to the Bohlin method (see Chapter 2, section 2.5). However, due to the physical size of the cub and bob, 8 mL of cell suspension (defrosted cell paste resuspended in TE buffer at a concentration of 12.5 g per 100 mL) was added to the fixed cup instead of 6 mL in the case of the Bohlin rheometer measurements.

Once the rotating bob was fixed in position, the machine was started at
a fixed predetermined speed (RPM) and 8 ml of alkaline lysis solution was added. Readings were then taken every 2 s for 10 minutes using the viscosity (mPa.s) vs. time program, as in the Bohlin rheometer protocol. The temperature was maintained at 20°C using an external water bath. All experiments were performed in at least triplicate, with an accuracy of 10 %.

4.4. Results

In this chapter, the results from the analysis of the Brookfield data are compared to data from the classic curve created using the Bohlin rheometer. All results for the Brookfield work are presented as an average of at least triplicate runs.

4.4.1. Reproducing the Classic Profile Using the Brookfield Viscometer

The results from the classic curve are shown in Table 4.1. This general shape of curve is reproducible at a range of shear rates between 46 s⁻¹ and 461 s⁻¹ (Ciccolini, 1998), in as much as the timing of the peaks remain the same for a given sample at any shear rate. However, as expected with a non-Newtonian solution, the apparent viscosity reduces as a function of increasing shear-rate. This has an impact on the clarity of the curve, with profiles generated at low shear rates having secondary peaks and pseudo-steady states that are not so easy to distinguish. Published results of apparent viscosity vs. time profiles for the alkaline lysis of *E.coli* cell paste at a range of shear rates (46 s⁻¹, 180 s⁻¹, 231 s⁻¹, 367 s⁻¹ and 461 s⁻¹) showed that the profile at the lowest shear rate was not as clearly defined as the profiles produced at higher shear rates, with the viscosity vs. time profile produced at 461 s⁻¹ having the most distinguishable peaks and pseudo-steady state (Ciccolini, 1999). Therefore, using the Bohlin rheometer the lowest shear rate at which the optimum apparent viscosity vs. time profile is generated is at 180 s⁻¹. The Bohlin rheometer has a motor driven cup and a fixed bob, allowing high shear rates. The Brookfield viscometer has a maximum shear rate of 183 s⁻¹. This is less than the Bohlin rheometer's maximum shear rate (1000 s⁻¹) because a spring system is used to drive the rotating bob of the Brookfield.

From equation 4.2 the recorded apparent viscosity is a function of the
torque input. If the torque reading breaches 110 % during the alkaline lysis of cell paste the computer can no longer calculate the viscosity, and a flat line is produced on the readout. Increasing the RPM of the bob, and hence the shear rate, increases the torque. Thus the maximum viscosity that the Brookfield can record is reduced as the RPM is increased.

The first experiment using the Brookfield viscometer investigated the viscosity vs. time profile for the alkaline lysis of *E.coli* DH1/pXY cell paste at low shear rates, and compared the results with previous published data using the Bohlin rheometer at a low shear rate, of 46 s^{-1} (Ciccolini, 1998). The cell paste was harvested at the completion of the fermentation as with the published data. Figure 4.2 shows the resultant viscosity vs. time profile at a shear rate of 24 s^{-1}. The figure shows a clear primary peak at approximately 40 s after lysis was initiated. The viscosity then rises to greater than 25 mPa.s where the secondary peak can be identified. However, a third peak can be seen 50 s after the secondary peak, and the pseudo-steady state is not clearly defined. Up until the secondary peak, the profile is similar to the classic profile shown in Figure 1.9. However, after the secondary peak the profile does not follow the classic profile.

Figure 4.3 shows the resultant viscosity vs. time profiles for the alkaline lysis of *E.coli* DH1/pXY cell paste sampled from batch fermentation after 6 h i.e. during exponential growth phase. The experiments were carried out as described in section 4.3.2, at three different shear rates (31 s^{-1}, 49 s^{-1} and 183 s^{-1}). The first result on Figure 4.3 represents the viscosity vs. time profile at a shear rate of 31 s^{-1}. The profile is similar to the classic profile initially, with the primary peak clearly visible. The apparent viscosity then rises over the next 100 s, to 28 mPa.s at about 150 s after the initiation of alkaline lysis. However, the apparent viscosity then ceases to rise and flattens out to a completely straight line. This is the point at which the torque reading rises above 110%, and the curve flattens out for the rest of the experiment. The curve produced at a shear rate of 49 s^{-1} is identical to the previous for the first 100 s after initiation of alkaline lysis. The primary peak is visible at around about 40 – 50 s, and the viscosity continues to rise up to about 15 mPa.s before the torque reaches over 110%, about 13 mPa.s lower than the profile...
generated at a shear rate of 31 s\(^{-1}\).

At a shear rate of 183 s\(^{-1}\), there are no conclusions that can be drawn from the curve as to the nature of viscosity changes as a function of time during alkaline lysis, as from the beginning the torque reading is too high to record the apparent viscosity at this shear rate.

4.4.2. Effect of Fermenter Sample Time

From Figures 4.2 and 4.3 two observations determined the next step in this chapter. The first was that a shear rate of 24 s\(^{-1}\) seemed too low to produce a clear viscosity vs. time profile; and the second that samples from the end of a fermentation appeared to have a lower apparent viscosity than cell paste sampled during exponential growth of a fermentation, despite a higher shear rate (31 s\(^{-1}\)).

Figure 4.4 shows the results of a series of experiments to establish how the viscosity vs. time profile changed as a function of fermentation age. The Brookfield viscometer was used as the scale down reactor, and the shear rate was maintained at a constant 31 s\(^{-1}\) for all samples, as lower shear rates produced indistinct viscosity vs. time profiles and higher shear rates resulted in a breach of the torque input maximum at low viscosities. Samples of \(E.\text{coli}\) DH1 /pXY were resuspended, loaded into the cup of the viscometer and treated with an equal volume of alkaline lysis solution, as explained in Chapter 2, section 2.4.

Figure 4.4(A) shows the results for cell culture sampled 1 h after inoculation. The most striking observation is that unlike in Figure 4.3, the maximum torque level of the viscometer is never breached. However, although two peaks within the first 100 s are present, the primary peak shows an unusually high apparent viscosity. It is also unclear whether there is just one peak after the primary peak, or four; as a direct consequence of this it is not possible to identify a pseudo-steady state.

Figure 4.4(B) shows the results of a sample of cell culture removed 6 h after inoculation. This curve is identical to the profile shown in Figure 4.3 at 31 s\(^{-1}\). The primary peak is visible but the apparent viscosity flat lines after 150 s,
as the torque exceeds the machine limits.

The cell paste sampled from the fermenter 10 h after inoculation – at completion of the fermentation is treated with alkaline lysis solution next. Figure 4.4(C) presents a well-developed viscosity vs. time profile that compares closely with the gold standard data obtained from previous studies using the Bohlin rheometer (Ciccolini, 1998). The primary peak and pseudo-steady state are visible, although the latter suffers from almost the same fluctuations as in Figure 4.4(A). However, the actual maximum of the secondary peak is not visible, as the increase in viscosity causes the torque input to temporarily breach the maximum level, making it hard to say precisely at what time the secondary peak occurs.

4.5. Discussion

The results from the viscosity vs. time profile experiments on resuspended cells carried out in the Brookfield viscometer using the coaxial cylinder geometry provide some interesting observations.

4.5.1. Limitations of the Brookfield Viscometer

The first observation is drawn from Figure 4.2. The viscosity vs. time profile at 24 s⁻¹ shear rate shows the entire curve without breaching the maximum torque input level. However, the curve is indistinct implying a degree of shear thinning is required to visualise the secondary peak and the pseudo-steady state. Therefore a higher shear rate should be used for generating the viscosity vs. time profile for the alkaline lysis of E.coli cell paste.

Investigations at a series of increasing shear rates highlighted the primary limitation of the Brookfield viscometer for use in generating viscosity vs. time profiles during the alkaline lysis of E.coli cell paste. Figure 4.3 shows that at a given shear rate there is a maximum apparent viscosity at which point the torque input breaches the maximum level for the machine. From the graph, it can be seen that as the shear rate increases the apparent viscosity above which the torque input breaches the maximum limit actually decreases. This is due to the torque increasing as the shear rate increases. From the graph, shear rates above 49 s⁻¹ produce a torque input limit that restricts the
amount of the viscosity vs. time profile that can be seen.

Clearly having a revolving bob on a spring recording the torque reduces the maximum shear rate the machine can operate under, and this effects the production of accurate viscosity vs. time profiles, as if the apparent viscosity goes above 25 mPa.s at 31 s\(^{-1}\) shear rate the machine cannot register the readings. There is therefore a pay-off between having a shear-rate high enough to produce clear viscosity vs. time profiles and having a high enough torque limit in terms of apparent viscosity to see the entire curve. However, Figures 4.2 and 4.3 show clear primary peaks for all curves and so some value can be taken from viscosity vs. time profiles using the Brookfield viscometer.

There appears to be an anomaly between the profile shown in Figure 4.2 (24 s\(^{-1}\) shear rate) and the curve representing 31 s\(^{-1}\) shear rate in Figure 4.3: the maximum apparent viscosity reached during alkaline lysis is less for the curve at the lower shear rate than at a shear rate of 31 s\(^{-1}\), contrary to the expected pattern, whereby non-Newtonian shear-thinning solutions express lower apparent viscosities at higher shear rates (Ciccolini, 1999). The cell paste used for the experiments in this chapter is sampled from the same design of fermenter and protocol, and using the same cell strain and plasmid. The difference between the two experiments is the time at which the samples were taken from the fermenter. Cell paste for the experiment represented in Figure 4.2 was removed from the fermenter at completion of the fermentation, whereas cell paste for the experiments was sampled after 6 h, during exponential growth. This may well be the reason for the lower maximum apparent viscosity observed in Figure 4.2 despite having a lower shear rate.

This anomaly was investigated further by measuring how viscosity changed as a function of time during the alkaline lysis of *E.coli* DH1/pXY cell paste sampled after 1 h, 6 h and at completion of the fermentation (10 h). The shear rate was kept at a constant 31 s\(^{-1}\) shear rate. The results are shown in Figure 4.4, and the resultant viscosity vs. time profiles indicate that there may be differences in cell rheology over the time course of a fermentation. The apparent viscosity is higher during the alkaline lysis of cell paste sampled after 6 h compared to cell paste sampled at 1 h and 10 h. In Chapter 3, the idea of
testing physical cell strength as a function of fermentation age was introduced. In light of the profiles observed in this chapter this is an experimental study worth conducting as it is possible that there is a connection between chemical and physical cell lysis, and physical lysis may be affected in a similar way to the rheological changes seen here.

From Figures 4.2 to 4.4 it is possible to see the primary peak clearly, and in Figure 4.4(C) the viscosity vs. time profile is similar to the Bohlin rheometer gold standard. Therefore it can be said with confidence that viscosity vs. time profiles seen using the Bohlin coaxial rheometer are, crucially, not an artefact of the Bohlin and are reproducible.

4.5.2. Choice of Scale-Down Reactor

From the results of this study it is clear that the viscosity vs. time profile is reproducible using other viscometers, but the usefulness of the technique is limited by the shear rates available using any given machine. Figure 4.2 shows the effects of a low shear rate. The shear rate for this experiment was set at 24 \( \text{s}^{-1} \), and the cell culture was sampled at the completion of the fermentation. Therefore the closest parallel is with Figure 4.4(C), where the cell paste was sampled at the same time. The viscosity vs. time profile in Figure 4.2 shows that although the torque stays below the maximum range and the primary peak is clear, the secondary peak is indistinguishable from a third peak and the pseudo-steady state is not clear. This is because the shear rate is too low for any shear-thinning effects. Therefore, the shear rate must be maintained above \( 31 \text{s}^{-1} \) for the viscosity vs. time curve to provide the required information.

The Brookfield viscometer can be used for initial studies of the rheology of alkaline lysates, as the primary peak, or point of total cell lysis, is clear, but higher shear rates are required to produce a distinct and clear viscosity vs. time profile for the alkaline lysis of \textit{E.coli} cell paste. It is clear that the Brookfield viscometer has torque limitations, caused by the nature of the rotation mechanism. Therefore a robust design is important when selecting a rheometer for generating viscosity vs. time profiles. For that reason the Bohlin rheometer will be continued to be used for all rheological studies throughout
this thesis.

Chapter 5 details the investigation into how both physical and chemical cell lysis changes as a function of fermentation time course. Chapter 6 looks at the impact of the fermentation time course on the viscosity vs. time profile alone, repeating the experiments started in this chapter using the Brookfield viscometer.

4.6. Conclusion

The classic viscosity vs. time profile reported by Ciccolini is not an artefact of the Bohlin rheometer, and the curve can be reproduced in other similar machines.

Suitable viscometers to be employed, as a scale-down reactor for the alkaline lysis protocol, must have a robust mechanism that can measure apparent viscosity at high shear rates (greater than 180 s\(^{-1}\) ideally).

The viscosity vs. time profiles appear to changes in maximum viscosity as a function of fermentation age, and this will be further investigated using the Bohlin rheometer in subsequent chapters.
Figure 4.1: Schematic of the Brookfield viscometer coaxial cylinder (cup and bob) geometry, highlighting fixed cup and rotating bob.
**Figure 4.2**: Apparent viscosity vs. time profile for the alkaline lysis of *E.coli* DH1 /pXY cell paste grown on CFM media in a 7 L Applikon batch fermenter. Cell paste sampled at completion of fermentation. Alkaline lysis carried out using Brookfield viscometer at 24 s⁻¹ shear rate.
Figure 4.3: Apparent viscosity vs. time profile for the alkaline lysis of *E.coli* DH1/pXY cell paste grown on CFM media in a 7 L Applikon batch fermenter. Cell paste sampled at 6 h after inoculation of fermenter. Alkaline lysis carried out using Brookfield viscometer at three different shear rates: 31 s⁻¹, 49 s⁻¹ and 183 s⁻¹.
Figure 4.4: Apparent viscosity vs. time profile for the alkaline lysis of *E. coli* DH1/pXY cell paste grown on CFM media in a 7 L Applikon batch fermenter. Cell paste sampled at three different fermentation times: (A) 1 h, (B) 6 h, (C) 10 h. Alkaline lysis carried out using the Brookfield viscometer at a constant shear rate of 31 s⁻¹.
5. *Escherichia coli* Resistance to Breakage by Physical and Chemical Methods as a Function of Fermentation Time

5.1. Summary

Physical studies showed that *E.coli* cells had a higher K value, and hence weaker cells at exponential growth. The results also showed that the DH1 strain had a lower K value than DH5α, suggesting the former is more resistant to physical breakage, and hence strain has an impact on cell strength. This correlates with the published data.

However, no correlation was seen between chemical total cell lysis time and fermentation growth phase. This suggests that current alkaline lysis protocols are excessive for the duty required. A change in the protocol may reduce the processing cost at industrial level.

5.2. Introduction

The results presented in this part of the thesis follow on from the initial results seen in Chapter 4, whereby the viscosity vs. time profile for alkaline lysis of *E.coli* cell paste was seen to vary as a function of fermentation time. This chapter intends to see if there are any links between this chemical lysis phenomenon and any possibly linked changes in physical strength of cells.

5.2.1. Aims

Researchers (e.g. Glick, 1995) have reported that cell wall strength may change as a function of growth rate, or fermentation time. This may in turn have an impact on the timing of the primary peak in the viscosity vs. time profile for alkaline lysis, the point of total cell lysis. The first aim of this experimental chapter was to quantify any changes occurring in cell resistance to physical breakage as a function of fermentation time. Cell culture sampled from the fermenter during lag, exponential and stationary phase was passed through a high-pressure homogeniser and the levels of protein released measured. This enabled cell strength to be quantified as described in
Chapter 2.

The second aim of this study was to correlate any changes seen in the physical cell strength with any changes in cell resistance to chemical lysis. Changes in the timing of the primary peak were then compared to those of physical cell strength, to see if the kinetics of this chemical step were influenced by mechanical properties such as cell wall strength.

The primary goal for collecting this data was to try and elucidate any windows of operation for optimum fermenter harvest point as a function of fermentation time, determined by favourable changes in the alkaline lysis reaction (see PhD aims in Chapter 1, section 1.6).

5.2.2. Cell Culture Growth and Associated Metabolic Load

This section introduces the concepts of metabolic load and how *E.coli* cell cultures grow in a batch fermenter.

5.2.2.1. Cell Culture Growth

There are three recognised stages of bacterial cell growth in a batch fermentation: lag, exponential and stationary.

Growth during fermentation is known to have an impact on the physiological composition of the cells (Glick, 1995). Cells in the stationary growth phase differ both chemically and morphologically from cells in the exponential growth phase. Cells in stationary phase are always smaller than cells in exponential growth phase, as cell division continues after the synthesis of most macromolecules has slowed markedly (Ingraham *et al.*, 1983). The following sub-section introduces the concept of metabolic load and how in the particular case of *E.coli* this may be related to the production of plasmid DNA, and determines the best harvest time of the cell culture, as a function of total chemical cell lysis time.

5.2.2.2. Metabolic Load

Metabolic load may be defined as the fraction of a host cells' resources (e.g. ATP, amino acids and metabolites) required to replicate and express foreign DNA as either RNA or proteins in the cell (Glick, 1995). Previous work has
shown some effects of metabolic load relevant to the production of plasmid DNA. Growth rates of plasmid-bearing cultures are generally lower compared to cultures of plasmid-free hosts (Bentley et al., 1990). Plasmid-induced metabolic load is often the consequence of the draining away of cellular energy resources for the transcription and translation of plasmid-borne genes (Hong et al., 1995). Previous evidence suggests that an increase in plasmid size may decrease the growth rate of host cell cultures in exponential growth. Cheah et al. (1987), showed that an increase in plasmid size lowered the maximum cell density achieved by the host strain. To be stably maintained in a cell population, plasmids must propagate in pace with the doubling rate of their hosts (Paulsson and Ehrenberg, 1998), and this metabolic load may place a strain on the plasmid-bearing cell culture (Cheah et al., 1987). Experiments performed with high copy number plasmids showed evidence of the release of a number of heat shock proteins, indicating high levels of strain on the cell (Birnbaum and Bailey, 1990). Previous work by Ciccolini et al. (1998) showed there was no obvious difference between the viscosity profiles of the alkaline lysis stage for plasmid free and plasmid bearing hosts but this analysis was confined to studies of material at the end of batch fermentations where differences in metabolic load might be expected to be smallest.

The following sections describe the theory behind how these physiological changes can be measured as a function of physical and chemical strength.

5.2.3. Physical Cell Strength Measurements

Previous work has shown that the kinetics of protein release from cells disrupted in an industrial homogeniser can be used as an indicator of the physiological changes occurring during fermentation, as explained in the previous section. Measuring the disruption rate constant (K) over a number of passes through the homogeniser can be used to infer a measure of the cell wall strength (Hetherington et al., 1971), and how this varies dependent on fermenter conditions and growth rate (Engler et al., 1981). Equation 5.1 shows the equation for the disruption rate constant (K):
\[ KN = \ln \left( \frac{R_m}{R_m - R} \right) \]  
(Equation 5.1)

Where \( R_m \) is the maximum protein release at 1200 bar; \( R \) is the protein released at the prevailing operating pressure (e.g. 300 bar), and \( N \) is the number of passes through the homogeniser. Increasing values of \( K \) indicate an increased susceptibility to cell breakage, and hence an intrinsically weaker cell wall structure.

Such studies provide an indication of the impact that physiological changes occurring during fermentation may have on mechanical properties such as cell wall strength, which could then be reflected in the performance of other process steps, such as alkaline lysis. This would be pronounced if the kinetics of the chemical release were influenced by cell wall strength in a similar fashion to the changes in mechanical strength.

5.2.4. Chemical Cell Strength Measurements

Chapter 1, section 1.5 details previous work by Ciccolini (1998) whereby a coaxial cylinders rheometer was used as a scale-down reactor for the measurement of viscosity changes as a function of time of cells undergoing alkaline lysis. Figure 1.9 shows an example of the resultant viscosity vs. time profile with its characteristic shape. As explained in Chapter 1 the primary peak relates to the point of total cell lysis. This was confirmed by cell counting using a haemocytometer to determine the point when all the cells were lysed (Ciccolini, 1998). Previous work discovered that the primary peak was cell strain specific: \( E.coli \) C600 cell strain had a primary peak timing of 30 s compared to a primary peak timing of 40 s for the DH5\( \alpha \) strain (Ciccolini, 1998). In the present study cell culture sampled from the fermenter at regular time intervals was lysed in the scale-down reactor using the alkaline lysis technique. These lysates were then neutralised at pre-determined time points and the number of cells remaining counted to determine the point of total cell lysis, and determine whether this is a function of fermentation time.
5.3. Materials and Methods

This section is aimed to support the more complete description for protocols used in this chapter detailed in Chapter 2, section 2.5.

5.3.1. Physical Cell Strength

In this study the disruption rate constant was calculated for *E.coli* DH5\(\alpha\) /pQR235 cells grown in 20 L vessels using LB and CFM medium and *E.coli* DH1 /pXY cells grown in 7 L vessels using CFM medium. This data was derived from protein release results obtained at 300 bar (4.35 Kpsi) pressure expressed as a fraction of the maximum protein release recorded at a pressure of 1200 bar (17.4 Kpsi) per pass through the homogeniser, for all the experimental permutations.

Two designs of homogeniser were used in the practical work depending on whether the work was performed at UCL or GSK. Work at UCL used a Micron Lab 40 homogeniser to test samples of *E.coli* DH5\(\alpha\) /pQR235. At GSK an Emulsiflex C5 homogeniser was used to break *E.coli* DH1 /pXY cells.

5.3.1.1. Physical Cell Strength of *E.coli* DH5\(\alpha\) /pQR235

The physical strength of *E.coli* DH5\(\alpha\) /pQR235 was tested for material grown in a 20 L LH fermenter (see Chapter 2, section 2.3 for fermentation and sampling protocols) using two types of medium: LB and CFM. Samples were taken from time 0 h and subsequently at 2 h intervals for the duration of each fermentation, and treated as described in Chapter 2, section 2.3.3.1.

The samples were passed through the homogeniser a total of 4 times, at a pressure of 300 bar. As described in Chapter 2, section 2.5.1 the protein release was also measured at 1200 bar in order to determine maximum protein release for comparison with that achieved at the working pressure. After each pass a 0.5 ml sample was removed and poured into a labelled microfuge tube, and stored on ice.
Each sample was then analysed for total protein concentration using the Coomassie assay (see Chapter 2, section 2.6.2 for details of Coomassie assay protocol).

5.3.1.2. Physical Cell Strength of E. coli DH1 /pXY

The protocol used to analyse the physical cell strength of the E. coli DH1 /pXY strain is detailed in Chapter 2, section 2.5.2. The cell culture was grown in CFM medium in 7 L Applikon fermenters, and sampled every 2 h throughout the fermentation, as described in Chapter 2. Samples were analysed for total protein concentration using the Coomassie assay.

5.3.2. Chemical Cell Strength

The method for neutralising alkaline lysates is described in Chapter 2, section 2.4.2. The E. coli DH1 /pXY strain grown in CFM medium was tested for chemical cell strength. Cell culture was sampled at 2 h intervals over the course of the fermentation and the samples were treated and resuspended using the standard protocol. Cell samples were lysed in the scale-down co-axial rheometer at a constant shear rate of $461 \text{s}^{-1}$. Each of the samples from particular fermentation times were neutralised by the addition of 4 ml of chilled potassium acetate at a predetermined time after initiation of alkaline lysis (starting at 10 s and then increasing in increments of 10 s up to 40 s), and the intact cells counted in the Thoma haemocytometer (Fisher Scientific UK Ltd., Orme Technologies, Middleton, Manchester, UK).

5.4. Results

Changes in physical cell strength occurring during fermentation were investigated by measuring protein release from cell samples that were fresh, and had not been frozen. This was to prevent any possible structural changes to the cell wall that have been noted by others as a result of freeze and thaw. When cells are freeze/thawed before disruption they become about four times more resistant to homogenisation (Milburn et al, 1994), a feature attributed to the cell wall becoming flaccid in nature.

Figure 5.1 shows the calibration curve created for the Coomassie
assay analysis of the protein samples. The linear fit equation is used as a standard for all the experiments in this chapter.

Figure 5.2 shows the variation in maximum protein release as a function of fermentation time, for both cell strains and types of medium. The maximum protein release in Figure 5.2(A) shows the data for the DH1 strain grown in a 7 L fermenter using CFM medium. By inspection it can be seen that the maximum protein release increased to above 4 mg/ml of cell suspension from an initial concentration of less than 0.5 mg/ml. It should be noted that since all samples in this figure were diluted to have the same initial cell concentration, any increase in protein levels is due to an increase in protein per specific yield.

Figure 5.2(B) presents the maximum protein release results for the DH5α strain grown in a 20 L fermenter using LB medium. The graph shows a much lower maximum protein release (less than 1 mg/mL) over the course of the fermentation, which was complete after only 6 h. In Figure 5.2(C), showing maximum protein release for the DH5α strain grown in a 20 L fermenter using CFM medium, a similar trend is seen to that in Figure 5.2(A). However there are some subtle differences: Figure 5.2(C) had a shorter lag stage, and the protein release reached its maximum after 8 h rather than 10 h.

Data in Figures 5.3 – 5.5 show the change in protein release as a function of batch fermentation time, for a 20 L fermentation of DH5α in LB medium, a 20 L fermentation of DH5α in CFM medium and a 7 L fermentation of DH1 in CFM medium respectively. Each of the figures shows graphs displaying typical release kinetics for batch fermentations at early exponential, exponential and stationary phases of cell culture growth.

Figure 5.3 shows data for a 20 L fermentation of DH5α in LB medium. The early exponential phase (traditionally the lag phase – see Chapter 3) sample is shown in Figure 5.3(A). By inspection, it is not until the fourth pass that the maximum protein release has been achieved. By contrast material obtained under exponential growth is less robust as shown in Figure 5.3(B). Here it requires only 2 passes before the maximum protein release is reached. Figure 5.3(C) for stationary phase material shows a similar curve to
that for the early exponential phase samples, with protein release increasing as a function of pass number to total release at N=4.

The same change in protein release as a function of fermentation growth phase is also seen in Figures 5.4 and 5.5. Hence the data in Figures 5.3 – 5.5 all correlate. This is despite differences in media, cell strain and fermenter scales between the figures.

The release data is supported by the results for the disruption rate constant (K), displayed in Table 5.1. The table contains data for the DH1 /pXY strain grown in CFM media and the DH5α strain grown in CFM and LB media. K values (error of ±0.05 for all values) were calculated by plotting ln(Rm/Rm-R) vs. pass number and calculating the gradient.

The K values for DH1 strain grown in CFM medium were 0.6 for early exponential phase cells, 0.9 for cells growing exponentially, and 0.4 for stationary cells. The higher K value for samples derived from exponential growth phase indicates that cells are more susceptible to physical cell lysis during exponentially growing cultures in E.coli batch fermentations.

The K values for DH1 strain grown in CFM medium were 0.6 for early exponential phase cells, 0.9 for cells growing exponentially, and 0.4 for stationary cells. The higher K value for samples derived from exponential growth phase indicates that cells are more susceptible to physical cell lysis during exponentially growing cultures in E.coli batch fermentations.

The data for the DH5α disruption rate constant values shows a similar general trend to that of the DH1 strain. This is true for DH5α cells grown in LB and CFM media. However, the early exponential K value (at 0.2) for the cells grown in LB media is significantly lower than the early exponential values for the DH5α cells grown in CFM media. The DH5α grown in CFM medium had a result of 0.5, only 0.1 less than the value for DH1 cells grown in CFM media.

The K values for cell culture sampled during exponential phase was the same for all three of the experimental conditions, with DH5α cells grown in LB media only 0.1 less than DH1 and DH5α strains grown in CFM media with a value of 0.8, as opposed to 0.9 for the CFM media.

K values for cells sampled at stationary growth phase were generally lower than the exponential K value for the DH5α fermentations, although the CFM fermentation had a slightly higher K value compared to the DH5α grown in LB media.

Intact cells remaining after ever-increasing periods of alkaline lysis
were counted using a Thoma haemocytometer to estimate the time course of cell lysis. The results are shown in Figure 5.6. It can be seen that within the limits of this method there was no significant difference in the rate of chemical cell lysis during the fermentation. The majority (>90%) of cells lysed in the first 10 s, and essentially all the cells were lysed by 40 s. This was irrespective of the fermentation time at which the cells were sampled, and was a strong function of the alkaline lysis time.

<table>
<thead>
<tr>
<th>Growth Phase</th>
<th>Disruption Rate Constant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. coli</em> DH1 /pXY</td>
</tr>
<tr>
<td>CFM Medium</td>
<td>LB Medium</td>
</tr>
<tr>
<td>Early Exponential</td>
<td>0.6</td>
</tr>
<tr>
<td>Exponential</td>
<td>0.9</td>
</tr>
<tr>
<td>Stationary</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Table 5.1: Disruption rate constant (K) for *E.coli* DH1 and DH5α strains as a function of fermentation growth phase. Both cell strains were grown on CFM media in a STR. K values for *E.coli* DH5α grown on LB media in a STR also shown.

5.5. Discussion

5.5.1. Physical Cell Strength

There are numerous references in the literature about the impact of metabolic load on cells containing plasmid. If exponential cell growth causes an increased strain on cells containing plasmid then it is reasonable to assume that the requirement of cell replication for cell culture growth is such that it is possible that any given bacterium’s cell wall may be weaker during exponential growth than at lag or stationary growth. Results from the physical cell strength studies demonstrate that the physiological state of the cells is crucial with respect to determining susceptibility to cell breakage by high-pressure mechanisms.
Figure 5.2 shows that the increase in protein release as a function of fermentation time follows the cell culture growth rate, and that the cellular protein content increases with cell biomass. The cell strain has little effect on protein increase, whereas the choice of media does. The results suggest that cells growing in the more complex CFM media produce more protein during the fermentation cycle. It could be expected that this increased metabolic load would impact upon the physical cell strength, making the cells grown in CFM media weaker to physical breakage than cells grown in LB media.

The results from the homogeniser studies confirmed that the cell cultures that exhibit early exponential growth (e.g. *E.coli* DH1 /pXY grown on CFM media) instead of traditional lag phase growth (e.g. *E.coli* DH5α /pQR235 grown on LB media in shake flask) displayed the same physical cell breakage characteristics as the lag phase results (see Chapter 3). This confirmed that early exponential cell culture could be treated for the purposes of this thesis as similar to lag growth phase samples.

The data in Table 5.1 show K values for the *E.coli* DH1 pXY of 0.6 for early exponential phase, 0.9 for exponential phase and 0.4 for stationary growth. Previous published data (O’Kennedy *et al.*, 1998) records a disruption rate constant (K) of between 0.8 and 1.4 for *E.coli* DH5α cells. These published experiments used cells grown in complex media. The K values for the DH1 strain achieved during this thesis are significantly lower that published data, which could illustrates that cell strength is probably strain-specific. However, the K values for the DH5α strain achieved for this thesis and recorded in Table 5.1 are also lower than the published DH5α K values, especially the data from cells grown in LB medium. The stationary value for DH5α grown in CFM media is closer to the published data. CFM media is more closely related to the complex media used for the published data. This would therefore suggest that the resistance to cell breakage of the DH5α strain is dependent on the media that is used in the fermentation, as well as the cell strain.

The data obtained for material removed during exponential growth (Figures 5.3(B), 5.4(B) and 5.5(B)), indicate that under exponential growth,
the cells become more susceptible to physical damage, compared to cells in the stationary or early exponential phases of growth. This has been noted before (Engler, 1981) and attributed to a reduction in metabolic effort toward cell wall formation during fast replication, in favour of cell division. Previous data (O'Kennedy et al., 1998) does suggest that strain has some effect on physical cell strength. Media composition data in Table 5.1 suggests that cells grown in complex media are more susceptible to cell damage than those grown in a simple media such as LB. This is probably due to the longer exponential phase and the higher cell density reached, as well as the increased protein production as seen in Figure 5.2, creating an extra strain on cell metabolism. It was noted earlier that there is also evidence of differences in cell strength between strains. However, this was not evident in Figures 5.3 - 5.5. It can be seen that the fraction of protein released per pass as a function of fermentation growth phase for the three different experiments show very similar trends, with any differences between figures within the error of the experiment, indicating that this general phenomenon of weaker cells in exponential growth phase is true for a variety of E.coli strains and media compositions. This suggests that the K value results are not easily observed using other analyses.

5.5.2. Chemical Cell Strength

By contrast the cell strength as measured by the rate of chemical cell lysis remains constant, irrespective of what growth phase the culture is in and therefore does not correlate with the physical cell strength trends. This suggests that for plasmid DNA production using current alkaline lysis protocols, any changes in the physical strength of the cell brought about by changes in metabolic activity will not affect the kinetics of chemical lysis. These results might also suggest therefore that the buffer concentrations are not optimised to reflect changes in cell wall structure, and might be excessive for the duty required. However, as noted in Chapter 1, section 1.5, published work (Ciccolini, 1998) showed a change in the timing of the primary peak in the viscosity vs. time profile for different strains. The results from this chapter prove that fermentation growth phase does not affect the timing of the primary
peak, unlike cell strain.

A useful study might consist of examining, using the scale-down rheometer, the selection of appropriate alkaline conditions so as to achieve adequate cell wall permeability but at more moderate chemical conditions. This would reduce the cost of the process at large scale and reduce the scale of downstream processing units required as the waste and feed stream volumes would be reduced.

In terms of the optimum harvest point as established by chemical and physical cell strength it can be seen that harvesting late in the exponential phase would be ideal if an industrial homogeniser was used as primary extraction. However, from these results there is no ideal point of time for harvesting the fermenter with respect to improving the alkaline lysis process.

The next chapter in this thesis will investigate whether there are any changes to the timing of the secondary peak of the viscosity vs. time profile for alkaline lysates as a function of fermentation time. The next chapter will also endeavour to determine if the viscosity vs. time profile can be used as a probe to measure any alterations to the fermentation process.

5.6. Conclusion

In conclusion it can be seen from the disruption rate constant results that both cell strain and type of media used affect the \( K \) value, with the DH5\( \alpha \) strain having a higher \( K \) value at stationary growth phase than DH1 strain. Exponential growth phase samples had similar cell strength for all parameters tested. CFM media produced weaker cells at early exponential and stationary growth phases than with cells grown in LB media. This is due to the larger amount of protein produced adding to the metabolic load. These results were not easily viewed when simple protein release as a fraction of total protein release was plotted, as both cell strain and media types produced similar results.

There is no apparent optimum point of cell harvest if using alkaline lysis for the extraction of plasmid DNA as fermentation time has no impact on the point of total cell lysis, using the current protocol for the alkaline lysis process.
Despite published data (Ciccolini, 1998) showing that the primary peak of viscosity vs. time profiles for cells undergoing alkaline lysis was dependent on cell strain, and hence suggesting a correlation with physical cell strength, no correlation was seen between chemical cell lysis and fermenter growth phase. This suggests that current alkaline lysis protocols are excessive for the duty required. A change in the protocol may reduce the processing cost at industrial level.

**Figure 5.1:** Coomassie assay calibration curve of optical density (A595nm) vs. protein concentration (mg/ml). This calibration curve is then used to determine the amount of protein in samples that have been passed through the homogeniser.
Figure 5.2: Maximum protein released over the time course of different batch fermentations. The first graph is for 7 L fermentation of *E.coli* DH1 /pXY in CFM medium (A); the second graph is for 20 L fermentation of *E.coli* DH5α /pQR235 in LB medium (B); and the final curve is for 20 L fermentation of *E.coli* DH5α /pQR235 in CFM medium (C).
Figure 5.3: Protein release at 300 bar as a fraction of total protein released at 1200 bar per pass through homogeniser. *E. coli* DH5α/pQR235 cell culture sampled from 20 L fermenter grown on LB media. Cell culture sampled during lag / early exponential phase (A), exponential phase (B) and stationary phase (C) of cell culture growth.
Figure 5.4: Protein release at 300 bar as a fraction of total protein released at 1200 bar per pass through homogeniser. *E. coli* DH5α/pQR235 cell culture sampled from 20 L fermenter grown on CFM media. Cell culture sampled during lag / early exponential phase (A), exponential phase (B) and stationary phase (C) of cell culture growth.
Figure 5.5: Protein release at 300 bar as a fraction of total protein released at 1200 bar per pass through homogeniser. *E.coli* DH1 /pXY cell culture sampled from 7 L fermenter grown on CFM media. Cell culture sampled after 2 h during lag / early exponential phase (A), after 6 h during exponential phase (B) and after 10 h during stationary phase (C) of cell culture growth.
Figure 5.6: Time of complete cell lysis of *E.coli* DH1 /pXY as a function of time of alkaline lysis reaction neutralisation and as a function of fermentation time course. The cells were lysed in the scale-down reactor, and the reaction neutralised after 10 seconds, 20 seconds, 30 seconds and 40 seconds. A sample was removed and examined under microscope in a haemocytometer. Any intact cells were counted and recorded.

Key: (◆) Fermentation hour 2; (■) Fermentation hour 6; (♦) Fermentation hour 8; (x) Fermentation hour 10.
6. Correlating the Changes in the Rheological Behaviour of 
*Escherichia coli* Alkaline Lysates During Cell Growth

6.1. Summary

Frozen cell paste could be used, as there were no observed differences between the alkaline lysis of fresh and defrosted frozen cell paste.

Over the course of shake-flask and batch STR fermentations the timing of the primary peak remained at 40 s. Early exponential samples had no distinct secondary peak and low apparent viscosity (approx 5 mPa.s). Exponential samples had a distinct primary and secondary peaks and a pseudo-steady state with a high apparent viscosity (approx 17 mPa.s for *E.coli DH1 /pXY*). The timing of the secondary peak migrated from 120 s after 2 h of growth to 180 s after 7 h of growth, caused by increasing amounts of intracellular contents such as chromosomal DNA during periods of high cell growth. The stationary growth phase data produced the classic profile, with characteristic primary peak, secondary peak and pseudo-steady state.

Strain specific peak timings and apparent viscosity were observed, but the general trends were followed for all strains.

Viscosity vs. time profiles for the alkaline lysis of cell paste grown using the fed-batch protocol showed consistent patterns throughout the feeding regime, replicating those reproduced by cell paste sampled from batch fermenters early in the exponential growth phase.

6.2. Introduction

The previous chapter detailed the investigation into how cell resistance to physical and chemical lysis changed as a function of fermentation time. The theme of this chapter follows up the results from Chapter 4 where differences in the viscosity vs. time profiles for cell paste sampled from the same fermentation during the three growth phases were observed. This chapter details the experiments using the Bohlin rheometer as a scale-down tool to determine the changes in the viscosity vs. time profiles during cell growth.
6.2.1. Aims

Results from Chapter 4 showed that over the course of a fermentation, the maximum viscosity of the secondary peak and pseudo-steady state of the corresponding viscosity vs. time profiles changes depending on which growth phase the cell culture is in when it is sampled. The aim of this chapter is to investigate these initial findings using the Bohlin rheometer as the scale-down reactor and investigate any changes in the rheology and kinetics of the alkaline lysis of cell paste sampled during the course of shake-flask, batch and fed-batch fermentations.

6.2.2. Alkaline Lysis

Previous work (Ciccolini et al., 1998) was concerned with investigating the cell lysis reaction time and the rheology of the alkaline lysis reaction. Figure 1.9 shows a typical rheological profile (highlighting the two important peaks and the steady state) formed during the lysis of a cell suspension in a co-axial rheometer employed as a scale-down tool.

To date only studies of the kinetics and rheology of cells lysed at the end of batch fermentations have been reported. Cells undergo many physiological changes over the course of a batch fermentation as a result of changes in metabolic load. These changes were identified in Chapter 5 as a measurable change in cell wall strength as determined by the cell's resistance to breakage in an industrial homogeniser. Such changes in cell physiology may be expected also to affect the kinetics and the ensuing rheology of the alkaline lysis viscosity vs. time profile. The maximum viscosity is important when considering the optimum time for cell harvest since rheology defines the flow characteristics and hence the mixing, heat and mass transfer of material in subsequent downstream processes. Viscosity is the most important factor for process considerations (Doran, 1998), and so the relationship between fermentation conditions and viscosity of the alkaline lysates will be investigated in this chapter.
6.3. Materials and Methods

Cell culture from shake-flask fermentations ($\mu_{\text{max}} = 0.98 \, \text{h}^{-1}$) and fed-batch fermentations ($\mu_{\text{max}} = 0.14 \, \text{h}^{-1}$) will be analysed as a function of fermentation time and compared to batch fermentations ($\mu_{\text{max}} = 0.39 \, \text{h}^{-1}$). This investigation will enable the impact of maximum specific growth rate and maximum cell density on the rheology of alkaline lysates to be analysed, and therefore the rheological differences between alkaline lysates from batch and fed-batch fermentation protocols.

6.3.1. Fermentation and Cell Strains

Shake-flask fermentations were performed using the protocol described in Chapter 2, section 2.3.2.1 using 0.5 L shake-flasks containing 0.1 L of CIM medium. For this study the chosen cell strain was *E.coli* DH1 /pXY.

Batch fermentations were carried out in 10 L SGI fermenters with a 5 L working volume as described in Chapter 2, section 2.3.2.3. All fermentations used CFM medium and either *E.coli* DH1 /pXY or *E.coli* DH5a /pXX.

Fed-batch fermentations were carried out as described in Chapter 2, section 2.3.2.4 using 7 L vessels and reduced fermentation medium (RFM) with concentrated feed, developed to maintain the growth rate at 0.1 h$^{-1}$. The cell strain used in all fed-batch fermentations for this study was *E.coli* DH1 /pXY.

All samples were removed and treated as described in Chapter 2, section 2.3.3.

6.3.2. Alkaline Lysis and Scale-Down Reactor

A co-axial cylinder rheometer (Bohlin VOR system C25, Bohlin Instruments Ltd., Glos., UK) was used to measure changes in apparent viscosity during alkaline lysis of cell suspensions derived from the fermentations. The co-axial cylinder arrangement consists of a fixed bob, (diameter 25 mm) located in an outer rotating cylinder (diameter 27.5 mm). The rheometer was used as both the lysis-mixing vessel and the measuring device, under controlled, laminar conditions as described by Ciccolini *et al.* (1998). Apparent viscosity was
measured every 2 s for 10 min at a fixed shear rate (461 s⁻¹), this having been shown to provide a suitable environment for the study of rheological profiles before (Ciccolini et al, 1998).

6.4. Results

Previous results have shown that the viscosity profile for alkaline lysis of several different strains of *E.coli* exhibits three characteristic phases (Figure 1.9). The following data shows that the characteristics of these profiles can change markedly over the time course of a fermentation, reflecting changes in the physiology of the cells.

6.4.1. The Impact of Freezing Cell Paste on the Viscosity vs. Time Profiles

In Chapter 5 fresh samples had to be used when analysing physical cell strength as freezing and subsequently thawing cells affected the cell wall strength (O'Kennedy et al., 1998). So as to establish the impact of using defrosted frozen cell pastes to measure the rheology of alkaline lysates in the Bohlin rheometer a series of profiles were developed for fresh cells harvested at comparable stages for determining rheological data of batch growth. These fresh samples were chemically lysed in the co-axial rheometer. Figures 6.1(a)-(c) display the results of this experiment. A direct comparison between the early exponential, exponential and stationary phase data (Figures 6.1(a-c) respectively) show similar plots to frozen cells (Figures 6.2(a-c), with consistent profiles but slightly higher apparent viscosities for the fresh material. On the basis of the comparable trends it was decided that frozen samples would be used in all subsequent studies.

6.4.2. Effects of Shake-flask Fermentation Time on Viscosity vs. Time Profile for *E.coli* DH1 /pXY Grown on CIM Media

Initial experimental screening was carried out using 0.5 L shake-flasks with working volume to total volume ratio of 0.2 v/v, as described in section 6.3.1. Figure 6.3 shows the viscosity vs. time profiles for alkaline lysis of samples from the three growth phases (early exponential, exponential and stationary)
achieved in a shake-flask fermentation.

Figure 6.3(a) shows an indistinct viscosity vs. time profile for cell paste sampled at early exponential growth phase, without any of the features associated with the classic profile (Figure 1.9). There is a very low apparent viscosity achieved, reaching only 4 mPa.s after 50 s, and then not changing for the rest of the profile.

Figure 6.3(b) represents the data for exponential phase, and shows a similar plot to that seen in batch fermentations (see Figure 6.2(b)), with a primary peak at 40 s and a secondary peak at about 130 s, but generally a lower apparent viscosity than with samples from batch fermentations. The major difference between this profile and that of the classic profile is the pseudo-steady state, which maintains a similar apparent viscosity to the secondary peak, rather than a slight reduction caused by shear thinning.

Figure 6.3(c) shows the expected stationary growth phase curve, very similar to the classic profile in Figure 1.9, with a slightly later timing for the secondary peak and a lower apparent viscosity than in batch fermentations.

6.4.3. Changes in the Viscosity vs. Time Profile as a Function of Batch Fermentation Growth Phase for E.coli DH1 /pXY

The viscosity vs. time profile for the alkaline lysis of E.coli DH1 /pXY was determined throughout a batch fermentation. All of the experiments were performed on samples removed from cell culture and frozen for storage, before defrosting for subsequent lysis. Figure 6.2(a) shows the rheological profile for the alkaline lysis of cell paste suspended in TE buffer, that had been harvested from the cell culture in early exponential phase of growth 1 h after inoculation. A sharp rise to the initial peak at around 40 s is followed by a very gradual increase in apparent viscosity. However, the viscosity remains low at between 4 and 6 mPa.s, and there are no further distinguishable peaks. This is in contrast to previous rheological curves obtained for cells harvested at the end of a batch fermentation (Ciccolini et al., 1998).

In Figure 6.2(b) the lysis was performed with cells harvested during exponential growth, 5 h after inoculation. There are several important
features. The timing of the first of the alkaline lysis peaks remains the same as before at around 40 s, although the apparent viscosity is higher at about 12 mPa.s. The viscosity then remains constant for about 60 s before increasing to a second peak at about 150 s. The viscosity at this point is 16 mPa.s. The pseudo-steady state of the curve has a very distinctive pattern, with significant fluctuations in apparent viscosity from the mean steady-state value.

In Figure 6.2(c) the viscosity profile for alkaline lysis of cells harvested 9 h after inoculation of the fermenter is shown. The curve has altered yet again. The initial peak remains at 40 s, with a viscosity of 10 mPa.s. However, the secondary peak is no longer clearly discernable from the primary peak. The pseudo-steady state viscosity is lower and also has a noticeable reduction in fluctuations compared to Figure 6.2(b).

The key alkaline lysis parameters for DH1/pXY are presented in Table 6.1. Complete cell lysis time remains consistent at 40 s irrespective of culture age. The most prominent feature is the appearance and movement of the secondary peak in the viscosity vs. time profiles. Samples taken from the fermenter after 1 h (early exponential phase) show no distinguishable secondary peak but after 2 h of growth, there is a discernible peak at 120 s after the initiation of cell lysis. This peak then occurs later as the age of the fermentation progresses. By 7 h it appears 180 s after lysis initiation. It then becomes indistinct from the primary peak after 8 h, returning to 70 s after lysis initiation. This is maintained through to the finish of the fermentation at 10 h.

6.4.4. Viscosity vs. Time Profile as a Function of Batch Fermentation Growth Phase for \textit{E. coli} DH5\textalpha{} /pXX

The viscosity vs. time profiles for \textit{E. coli} DH5\textalpha{} /pXX alkaline lysates sampled from a batch fermentation at early exponential, exponential and stationary phases of growth are shown in Figures 6.4(a)-(c) respectively. The general rheological data is very similar in shape and peak apparent viscosity compared to the DH1 strain data (see Figure 6.2 (a)-(c)), as shown in Table 6.2. Both strains are grown on the same CFM media in the same fermenters under the same conditions, and the secondary peaks for all growth phases
have similar maximum apparent viscosities for both DH1 and DH5α strains.

<table>
<thead>
<tr>
<th>Fermentation Time (hr)</th>
<th>Primary Peak (s)</th>
<th>Secondary Peak (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>40 ±5</td>
<td>150 ±10</td>
</tr>
<tr>
<td>1</td>
<td>40 ±5</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>40 ±5</td>
<td>120 ±10</td>
</tr>
<tr>
<td>3</td>
<td>40 ±5</td>
<td>130 ±10</td>
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<tr>
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<td>40 ±5</td>
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</tr>
<tr>
<td>5</td>
<td>40 ±5</td>
<td>150 ±10</td>
</tr>
<tr>
<td>6</td>
<td>40 ±5</td>
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</tr>
<tr>
<td>7</td>
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</tr>
<tr>
<td>9</td>
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<td>70 ±5</td>
</tr>
<tr>
<td>10</td>
<td>40 ±5</td>
<td>70 ±5</td>
</tr>
</tbody>
</table>

**Table 6.1:** Variation in the time of appearance for the primary peak (time taken for all cells to lyse) and the secondary peak, associated with the formation of a complex network.

However, for exponential phase growth (Figure 6.4(b)), the primary and secondary peaks are not as clearly visible as with DH1 /pXY (Figure 6.2(b)), and the secondary peak timing is some 10 s earlier for the DH5α exponential growth sample with a lower apparent viscosity. For both the DH1 and DH5α strains the primary peak is visible at about 40 s at all growth phases.

The profile corresponding to stationary growth (Figure 6.4(c)) shows a classic viscosity vs. time profile (compare to Figure 1.9) with the primary and secondary peaks even more prominent than in Figure 6.2(c), although at a lower apparent viscosity. Overall, the three curves in Figure 6.4 corresponding to the three growth phases are similar to those shown in Figure 6.2, indicating similar processes are occurring.
6.4.5. Viscosity vs. Time Profile for *E.coli* DH1 /pXY Grown Using Fed-batch Protocol

The fed-batch data in Figure 6.5 differs slightly to all previous rheological data. All samples for fed-batch analysis were removed after the limited growth batch fermentation using FBM media. For fed-batch fermentations 0 h indicates the start of the feeding regime (see Chapter 2, section 2.3.2.4).

Figure 6.5(a) represents the viscosity vs. time profile for 0 h. This curve is identical to that expected early in the *E.coli* DH1 /pXY batch fermentation exponential growth phase, with a secondary peak timing of about 140 s. The primary peak timing is at 40 s, and the apparent viscosity is lower than for batch exponential growth phase.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Fermenter</th>
<th><em>E.coli</em> Strain</th>
<th>$\mu_{\text{max}}$ (h⁻¹)</th>
<th>Max OD (A₆₀₀nm)</th>
<th>Viscosity (mPa.s) of Secondary Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shake-flask</td>
<td>0.5 L /S-F</td>
<td>DH1 /pXY</td>
<td>0.98</td>
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<td>E.Expo: 2</td>
</tr>
<tr>
<td>Batch</td>
<td>10 L /SGI</td>
<td>DH1 /pXY</td>
<td>0.39</td>
<td>27</td>
<td>E.Expo: 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DH5α /pXX</td>
<td>0.46</td>
<td>19</td>
<td>E.Expo: 5</td>
</tr>
<tr>
<td>Fed-batch</td>
<td>7 L /Applikon</td>
<td>DH1 /pXY</td>
<td>0.14</td>
<td>26</td>
<td>E.Expo: 12</td>
</tr>
</tbody>
</table>

**Table 6.2:** Maximum apparent viscosities for viscosity vs. time profile of alkaline lysis of cell suspensions sampled from cell culture in early exponential, exponential and stationary growth phases as a function of fermentation protocol and size, maximum specific growth rate and maximum optical density (A₆₀₀nm) achieved for each experiment.
The profile for 2 h, shown in Figure 6.5(b), shows an identical curve to that for 0 h as shown in Figure 6.5(a). The same can also be said for the sample 6 h into the feeding regime (Figure 6.5(c)), which shows a very similar profile to the previous two fed-batch time points. However, by 10 h into feeding regime (Figure 6.5(d)), the resultant viscosity vs. time profile is similar to that shown previously for cell paste sampled during stationary growth phase of batch fermentations (see Figures 6.2(c) and 6.4(c)).

6.5. Discussion

All of the cell lysates displayed non-Newtonian properties with the viscosity being shear rate dependent. Hence experimental conditions will have an effect on the measured apparent viscosity, and therefore the viscosity is only meaningful when experimental conditions are stated explicitly. In this work all samples were lysed using the same conditions, and measured at the same shear rate (461 s\(^{-1}\)) and temperature (293 K) so enabling comparisons to be made.

The ability to use frozen samples for determining rheological data is confirmed by the results presented in Figures 6.1 (a-c) and Figures 6.2 (a-c). The effects of freezing cells on rheology are negligible. Figure 6.1 (a-c) shows the rheological data from fresh samples removed from the bioreactor. Figure 6.1(a) shows a higher apparent viscosity than its frozen equivalent, but the main features remain the same. Figures 6.1 (b) and (c) show very similar plots to the frozen sample rheological data show in Figures 6.2 (b) and (c), including the primary peak at 40 s. It can therefore be assumed that freeze-thaw effects do not have a major effect on the rheological changes during alkaline lysis.

6.5.1. Viscosity vs. Time Profiles for Different Growth Phases of \textit{E.coli}

\textbf{DH1/pXY Grown on CIM Media in 0.2 v/v Shake-flask}

The shake-flask data presented in Figure 6.3 shows the impact of reduced oxygen transfer on the subsequent rheology of the cell culture alkaline lysates. The presence of a real lag phase (see \textit{ln}(OD) growth curve in Figure 3.1) explains the complete lack of any distinct peaks in Figure 6.3(a), as there
is virtually no cell replication occurring. The exponential growth is clearly indicated in Figure 6.3(b), with the two peaks visible. However, the early timing of the secondary peak suggests that there are not as much cellular contents, and hence the shorter time to form the secondary peak.

Figure 6.3(c) indicates a stationary phase growth curve except for the surprisingly late secondary peak. This appears to be an artefact of shake-flask rheology and may have something to do with the low apparent viscosity recorded in shake-flask derived alkaline lysates, caused by an increase in time to form the matrix of cell debris as there is less internal cell contents due to oxygen transfer restrictions, and hence an extended time for secondary peak formation. This artefact of shake-flasks underlines the need for an investigation into what causes the secondary peak.

6.5.2. Viscosity vs. Time Profile Changes for E.coli DH1 /pXY as a Function of Batch fermentation Growth Phase

The key point of interest in this work is the shift in the timing of the secondary peak as noted in Table 6.1, and its non-appearance during early exponential phase growth of the cell culture. These results may be explained by considering what happens to the chromosomal and plasmid DNA content during cell growth. The plasmids must propagate in pace with the doubling rate of their host in order to be maintained in a cell population (Paulsson and Ehrenberg, 1998). It follows that during late exponential growth there is an increasing amount of plasmid DNA, as well as other associated intracellular material. During exponential growth, cell chromosomes replicate, and there can be as many as 4 genome equivalents per cell at a growth rate of 3 generations per hour within a culture during exponential growth (Kubitschek, 1970), while plasmid DNA is at its lowest. This compares to a culture in early exponential or stationary phase when 1 genome equivalent per cell at a growth rate of 1 generation per hour is more likely. This leads to an increase in chromosomal DNA per average cell for cultures under exponential growth conditions.

If the primary peak corresponding to total cell lysis remains at 40 s no matter what the cell condition (Figure 6.2), then the time between the primary
and secondary peaks is dependent on the length of time required for the internal cell contents to form a matrix by interaction with the SDS. As the cells go through exponential growth, this period increases (Table 6.1), as does the total amount of intracellular DNA and in particular chromosomal DNA. It may therefore be that it is this increase in the concentration of DNA that leads to the extension in the time needed for the secondary peak to form. This does not however, address what the most important factor is in determining the time at which the secondary peak occurs nor the magnitude of it. Both could be functions of the concentrations of RNA, DNA (chromosomal and/or plasmid) or associated proteins, or any combination of these.

In the early stages of the fermentation there is a small DNA content per average cell in the culture as the cell culture is replicating slowly and hence no discernible secondary peak is developed. Although previous data in this thesis suggests that the lag phase does not exist (see growth curves in Chapter 3), this early exponential growth phase that has replaced the lag phase seems to have the same physiological effects on the cell culture as in expected lag phase. Therefore the early exponential data is treated as if it were traditional lag phase data.

As the culture moves through exponential phase into stationary phase so the genomic content of the cells rises and then falls as more of the cells stop replicating exponentially, and more are dying. This may explain both the rise and then reduction in the magnitude of the primary peak and why the secondary peak appears much later under exponential growth (up to 180 s) but during stationary phase returns to 70 s after cell lysis initiation. Since there will be a lower concentration of internal contents within the culture at stationary phase than at exponential, it would take less time for the smaller mass of intracellular contents to interact with the SDS to form the secondary peak in the apparent viscosity profile.

The apparent viscosity measured at the pseudo-steady state is shear-rate dependent. Shear damage to chromosomal DNA above a certain size, determined by the shear conditions, dominates after the formation of the SDS complex, and probably causes the steady state region of the rheological curve, due to shear breakage of the complex into a less viscous material.
NaOH conditions may also impact on the size by alkaline hydrolysis of the DNA. This degradation is an important process parameter to consider because if such chromosomal DNA is reduced to a size comparable to that of the plasmid DNA strands it will remain in the process liquor and proceed downstream where it poses a major challenge for removal by intrinsically low capacity chromatographic operations.

6.5.3. Effect of Strain on Viscosity vs. Time Profiles

Figure 6.4 shows the results for DH5α /pXX experiments for comparison with the DH1 strain. The plots from Figure 6.4 are similar in shape and timing to Figure 6.2 indicating that the same processes are occurring for this strain as well. The timing of the secondary peak is slightly earlier suggesting that there are less internal cell contents in the DH5α cell strain. Table 6.2 lists the maximum optical density reached for the DH5α /pXX fermentations as being lower than the DH1 /pXY batch fermentations. This may be the reason for the observed differences in the viscosity vs. time profiles, and will be discussed further in section 6.5.5. It is clear from these results that there are strain specific viscosity vs. time profiles, as previous work has shown that the timing of the primary peak is dependent on cell strain (Ciccolini, 1998) and apparent viscosities achieved with different strains using identical process parameters are different. The impact of plasmids will be considered in the next chapter. The industrial implications of this observation are that the viscosity of alkaline lysates can be expected to differ with different strains, and hence have differing mixing, pumping and heat transfer characteristics.

6.5.4. Viscosity vs. Time Profiles for the Alkaline Lysis of E.coli DH1 /pXY Samples Grown Using Fed-batch Protocol

Figure 6.5 shows viscosity vs. time profiles for the alkaline lysis of cell paste sampled at regular intervals during the feeding regime (see section 6.3.2). From the panels in Figure 6.5 it can be seen that fed-batch fermentations show a constant pattern for viscosity vs. time profiles throughout the feeding regime. This is an indicator of the effects of a constant reduced maximum specific growth rate, and no distinct growth phases. The cell culture remains in
a state of early exponential growth rate throughout the fermentation up until the cell paste sampled at 10 h. This sample exhibited a stationary growth phase viscosity vs. time profile. By examining the fed-batch growth curve shown in Figure 3.10 it can be seen that the ln(OD) growth rate has levelled off by the 10 h mark, and hence the stationary growth phase curve. It can be seen then that the growth phase of a cell culture within a fermenter can be identified by the characteristic viscosity vs. time profile for the alkaline lysis of the cell paste sample.

6.5.5. Apparent Viscosity

It is important to underline the relevance of the timing of the peaks, and overall shape of the viscosity vs. time curves, compared to the actual apparent viscosity values in this chapter. Although apparent viscosities are relative for samples from each individual fermentation, differences between fermentations can produce slightly different apparent viscosity values.

Therefore, although changes in apparent viscosity are mentioned within this thesis, they are not the most important factor and are used as an indicator rather than an absolute value. However, the results of an investigation into what causes large differences in apparent viscosities are displayed in tabular form in Table 6.2.

At first glance it would appear that maximum apparent viscosities reached are inversely proportional to the maximum specific growth rates, as the highest \( \mu_{\text{max}} \) in shake-flasks produced the lowest apparent viscosities. However, the fed-batch fermentation had the lowest \( \mu_{\text{max}} \) but not the highest viscosity. The same could be said for the maximum optical density reached \( (A_{\text{600nm}})\): the higher the OD reached the higher the maximum apparent viscosity, except for samples from the fed-batch fermentation. This anomaly is probably due to the fact that the fed-batch fermentation protocol uses a reduced media for the initial growth and then a concentrated feed that maintains growth at 0.1 h\(^{-1}\) for the rest of the fermentation. This is probably the cause of the reduction in the resultant apparent viscosity, despite a high maximum OD.
From a process point of view this means that the alkaline lysates will produce certain apparent viscosities depending on the cell strain and method of fermentation used, even if the same wet cell weight is used in the scale-down reactor. This means that downstream equipment must be tailored to meet a broad range of cell strains and mode of fermentation.

The following chapter will outline the investigation carried out to determine the cause of the secondary peak and hence prove why the timing of the secondary peak changes throughout a fermentation. Previous work (Ciccolini et al., 1999) suggested that chromosomal DNA might well be the dominant factor; studies will be performed to support this evidence. Subsequent studies will therefore focus on determining the role of the main cellular components e.g. proteins, RNA, DNA, in defining the suspension rheology (Ingraham et al., 1983 and Levy et al., 2000).

6.6. Conclusions

The viscosity vs. time profile of the alkaline lysis stage changes throughout a batch fermentation, and is dependent on the stage of growth. Whilst cell rupture by chemical lysis is achieved within 40 s the attainment of the peak viscosity caused by the formation of an SDS-genomic DNA complex is related to the physiological state of the cells. For samples taken during exponential growth a longer time is taken for the formation of this maximum viscosity possibly reflecting changes in the culture in terms of increased genomic content relative to other stages of the growth cycle.

Currently practised protocols for alkaline chemical lysis appear to be insensitive to the physiological changes that cells undergo during cell growth in a fermenter. However, Specific cell strain and fermenter choices each produce different apparent viscosities during alkaline lysis, and this has implications on down stream processing.
Figure 6.1: Viscosity profile of alkaline lysis on DH1 /pXY grown in batch fermentation as described in materials and methods. Each panel represents the rheology of alkaline lysis steps carried out using cell biomass isolated over the time course of the fermentation. Cell biomass was not frozen prior to alkaline lysis. (A) 1 h into fermentation, (B) 6 h into fermentation, and (C) 10 h into fermentation.
Figure 6.2: Viscosity profile of alkaline lysis on DH1/pXY grown in batch fermentation as described in materials and methods. Each panel represents the rheology of alternative lysis steps carried out using cell biomass samples isolated over the time course of the fermentation. Cell biomass was frozen prior to alkaline lysis. (A) 1 h into fermentation, (B) 5 h into fermentation, and (C) 9 h into fermentation.
Figure 6.3: Viscosity profile of alkaline lysis on DH1 /pXY grown in shake-flask fermentations as described in materials and methods. Each panel represents the rheology of alternative lysis steps carried out using cell biomass samples isolated over the time course of the fermentation. Cell biomass was frozen prior to alkaline lysis. (A) 2 h into fermentation, (B) 4 h into fermentation, (C) 8 h into fermentation.
Figure 6.4: Viscosity profile of alkaline lysis on DH5α/pXX grown in batch fermentation as described in materials and methods. Each panel represents the rheology of alternative lysis steps carried out using cell biomass samples isolated over the time course of the fermentation. Cell biomass was frozen prior to alkaline lysis. (A) 1 h into fermentation, (B) 6 h into fermentation, and (C) 10 h into fermentation.
Figure 6.5: Viscosity profile of alkaline lysis on DH1/pXY grown in fed-batch fermentation as described in materials and methods. Each panel represents the rheology of alternative lysis steps carried out using cell biomass samples isolated over the time course of the feeding regime after completion of the limited growth medium fermentation. Cell biomass was frozen prior to alkaline lysis. a) 0 h into feed regime (i.e. at completion of limited growth medium fermentation), b) 2 h into feed regime, c) 6 h into feed regime, d) 10 h into feed regime.
7. Investigating the Causes of the Observed Changes in Rheological Behaviour of *Escherichia coli* Alkaline Lysates during Batch Cell Growth

7.1. Summary

The impact of RNase and Proteinase K on the viscosity vs. time profile was negligible, with no significant difference between the treated cell paste and the previous gold standard profile. Viscosity vs. time profiles for the alkaline lysis of parental cell paste had a higher apparent viscosity produced by the reduced metabolic load, but crucially peak timings were identical to the gold standard profile. This result eliminated plasmid DNA from the dominant factor in producing the secondary peak.

The viscosity vs. time profile generated by the alkaline lysis of cell paste pre-treated with DNase produced a flat line with a low apparent viscosity. This absence of any peak highlighted chromosomal DNA as the primary factor behind the generation of a secondary peak. Therefore any changes in secondary peak timing were due to chromosomal DNA, as hypothesised in Chapter 7.

7.2. Introduction

7.2.1. Aims

The aim of this investigation was to identify the likely cause of the observed changes in rheological behaviour that occur over the duration of a batch fermentation and as recorded in Chapter 6.

Ciccolini (1998 and 1999) has proposed that the secondary peak in the rheological profile is due primarily to denatured chromosomal DNA interacting and forming a matrix. Chromosomal DNA is a possible contaminant (Schorr *et al.*, 1995) in the feed stream, and so it is important to understand how it can effect the rheology of the feed stream, and possibly be identified by simple rheological analysis. The impact of plasmid DNA on the formation of
the secondary peak was also investigated by measuring the rheology of the alkaline lysis of the \textit{E.coli} DH1 parental strain.

### 7.2.2. Changes in Viscosity vs. Time Profile for Alkaline Lysates as a Function of Cell Culture Growth Phase

Previous experimental data has shown a characteristic viscosity vs. time curve for the alkaline lysis process, when performed in a scale-down reactor on a sample of \textit{E.coli} cells taken from a fermentation run to completion, and suspended in TE buffer (Ciccolini \textit{et al.}, 1998).

Studies within this thesis have shown a distinct change in the rheological profile occurs throughout a batch fermentation, with different viscosity vs. time profiles for early exponential, exponential and stationary phases (see Chapter 6).

<table>
<thead>
<tr>
<th>Macromolecule</th>
<th>% Total Dry Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>55</td>
</tr>
<tr>
<td>RNA</td>
<td>21</td>
</tr>
<tr>
<td>DNA</td>
<td>3</td>
</tr>
<tr>
<td>Lipid</td>
<td>9</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>3</td>
</tr>
<tr>
<td>Others</td>
<td>9</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>0-3</td>
</tr>
</tbody>
</table>

**Table 7.1:** The macromolecular composition of an average \textit{E.coli} cell (Neidhart, 1987).

### 7.2.3. Selected “Factors”

The most important contaminant species from a process point of view are cellular proteins, chromosomal DNA and RNA (Schorr \textit{et al.}, 1995 and DiPaolo \textit{et al.}, 1999). These are the factors that will be investigated in this study, as well as the effects of the plasmid DNA itself. Their individual effects
on rheology will be analysed by treating the cells with DNase to remove DNA, RNase to remove RNA, Proteinase K to remove proteins and using parental cell lines to test the effects of removing plasmid DNA. The composition of an average \textit{E.coli} cell is shown in Table 7.1, highlighting amount of RNA and protein in an \textit{E.coli} cell.

\textbf{7.3. Materials and Methods}

This section is intended to support and augment the detailed protocols presented in Chapter 2. The experimental work for this chapter involved using the standard protocol devised by Ciccolini (1998) and described in the materials and methods (Chapter 2, section 2.4), and altering the cell samples composition by removing in turn a single “factor” that could influence the rheology of lysed cells, as well as being a major contaminant. The “factors” examined are described in section 8.2.3.

\textbf{7.3.1. Strain and Plasmid Vector}

All experiments for this study were performed using either \textit{E.coli} DH1 /pXY, or the parental strain \textit{E.coli} DH1, as described in Chapter 2, section 2.2.2.

\textbf{7.3.2. Fermentation}

7 L Applikon vessels were employed for batch fermentations, with 4 L working volume using CFM medium. The fermentations were run and sampled using the method described in Chapter 2, section 2.3.2.

\textbf{7.3.3. Alkaline Lysis}

The Bohlin rheometer using co-axial cylinder geometry was used to monitor the apparent viscosity during alkaline lysis, as described in Chapter 2, section 2.4. The shear rate was fixed at 461 s\(^{-1}\) for all experiments, and the temperature maintained at 293 K.

\textbf{7.3.3.1. Alkaline Lysis Samples For Agarose Gel Electrophoresis}

For the production of all samples for agarose gel electrophoresis 4 mL of alkaline lysis solution was added to 4 mL cell suspension in the Bohlin
rheometer. After specified time (10 s, 40 s, 100 s and 250 s after initiation of alkaline lysis) the reaction was neutralised by adding 4 mL chilled potassium acetate buffer as described in Chapter 2, section 2.4.2.

7.3.3.2. Treatment with Adjusted Lysis Solution

The rheology of the alkaline lysis of cell paste was measured as explained in Chapter 2, section 2.4.1. However, normal alkaline lysis solution (see Table 2.4) was replaced with the addition of 6 mL of adjusted lysis solution (9 mL of 10% SDS added to 90 mL of PBS buffer) added to the cell suspension in the Bohlin. This meant that no sodium hydroxide solution was contained within the adjusted lysis solution.

7.3.4. Treatment of Cells to Remove Chosen Factor

Samples were treated for the removal of each of the factors that were described in section 8.2.3.

7.3.4.1. Removal of RNA

Samples were mixed with RNase I Ribonuclease (Sigma, Poole, Dorset, UK) and x10 reaction buffer to remove RNA in advance of treatment with alkaline lysis buffer. The optical density of the cell sample suspended in TE buffer was measured at 600nm using a UV-1201, UV-VIS spectrophotometer (Shimadzu, Milton Keynes, UK), and RNase I added at a concentration of 1 mg RNase per 700 Optical Density (A_{600nm}) units. The cell suspension was then incubated for 30 minutes at 37°C before being added to the cup of the scale-down reactor. The alkaline lysis solution (see Table 2.4) was then added, and the viscosity measured over time, for 10 minutes without KAc neutralisation buffer addition as described in Chapter 2, section 2.4.1. Samples from early exponential, exponential and stationary phase were neutralised at 10 s, 40 s, 100 s and 250 s after the alkaline lysis solution had been added. These samples were then treated as described in the agarose gel electrophoresis method (see Chapter 2, section 2.6.4), and then run on an agarose gel to check that the RNA in the samples had been degraded.
7.3.4.2. Removal of Chromosomal DNA

Chromosomal DNA was removed by treating the cell suspension with Benzonase (Merck KGaA, Darmstadt, Germany), a highly active non-specific endonuclease. The enzyme required the addition of a x10 reaction buffer. Two concentrations of Benzonase were used: 12 µl Benzonase plus 12 µl reaction buffer, or 20 µl Benzonase plus 12 µl reaction buffer. All samples were incubated at 37°C for 30 minutes after addition of endonuclease, before transfer to the scale down reactor and the addition of the alkaline lysis solution. The samples were then measured as before. Samples from early exponential, exponential and stationary phases of cell culture growth were neutralised at 10 s, 40 s, 100 s and 250 s after alkaline lysis had been initiated by the addition of the alkaline lysis. These samples were then treated as described in the agarose gel electrophoresis method in Chapter 2, section 2.6.4, and then run on an agarose gel.

7.3.4.3. Removal of Intracellular Proteins

Proteinase K (Promega UK, Southampton, UK) was added to samples of cell suspension at a concentration of 50 µg/ml plus 12 µl reaction buffer. The enzyme solution was then left for 30 minutes at room temperature. No incubation was needed as Proteinase K is not inhibited by the EDTA in TE buffer, has a wide range of optimum pH and the activity of the enzyme is stimulated by SDS (www.fermentas.com/catalog/modifyingenzymes/proteinasek.htm). Samples were then transferred to the scale down reactor and treated using alkaline lysis protocol 1, as described in Chapter 2, section 2.4.1.

7.3.5. Agarose Gel Electrophoresis

All gels were produced using the method described in Chapter 2, section 2.6.4. All gels were run in triplicate to reduce experimental error when analysing gels qualitatively.
7.4. Results

A number of control studies were performed to check that the enzymes' action was removing the aforementioned factors. These controls were considered important for RNase and Benzonase as they are not particularly active at high pH or in SDS solutions, generated by the alkaline lysis process. Proteinase K however, is active at higher pH and indeed its performance is improved by the presence of SDS, and so no control was considered necessary (see section 7.3.4.3). Figure 7.1 displays captured images of two agarose gels: one untreated (a) and one treated with the RNase protocol (b). By inspection, Figure 7.1(a) clearly shows broad bands of RNA underneath each of the samples. Each sample shows a precise band corresponding to plasmid pXY. Figure 7.1(b) shows no clear indication of RNA despite having been run under the same conditions and length of time as Figure 7.1(a). It also shows pXY bands, as expected, but they appear to be smudged. This is due to the incubation procedure used when treating the samples with RNA. The results of the Benzonase control are shown in Figure 7.2: the bands are extremely faint and there is no sign of any RNA. This suggests that the Benzonase has digested all the RNA and plasmid DNA, as well as chromosomal DNA. The quality of the picture is low as the contrast had to be turned up high so as to view any remaining bands.

The first set of experiments was concerned with the effects of RNA on the viscosity vs. time profiles. The data in Figure 7.3 presents analysis of an average of three runs treated with RNase and three runs untreated, of a cell culture sample removed from the fermenter during early exponential growth phase. Figure 7.3(a) shows the data for the untreated samples. The curve yields the expected behaviour for early exponential growth phase, with a barely distinguishable secondary peak soon after the primary peak, and is almost identical in shape and size to Figure 6.3(a). Figure 7.3(b) shows the data for the rheology of cell culture treated with RNase. There is a reduction in apparent viscosity of about 2 mPa.s compared to Figure 7.3(a), but both the primary peak and, crucially, the secondary peak are visible and there is no alteration in the timing of the peaks.
Figure 7.4 again shows the results of RNase studies, and it can be seen that it is very similar in shape and viscosities relative to the different stages of a fermentation to that expressed in Figure 6.3. The apparent viscosity for exponential and stationary are slightly lower than expected from Figure 6.3, but this is due to the slight viscosity reduction caused by RNA digestion.

The effect of chromosomal DNA on viscosity was investigated by using a non-specific endonuclease, Benzonase, to remove both DNA and RNA. The choice of Benzonase eradicates the difficulties of using a specific DNase that have a notoriously narrow optimum range, especially in the harsh pH environments of the alkaline lysis operation. Figure 7.5 presents two plots of viscosity vs. time for cell culture removed from a fermenter in the stationary phase of growth. Figure 7.5(a) shows data from the sample before Benzonase treatment, while the impact Benzonase has on the cell culture sample is clearly shown in Figure 7.5(b), which shows the same sample treated with 12 μl Benzonase. While the peak timing remains the same there is a marked reduction in apparent viscosity, and the secondary peak is not very well defined. Indeed, it is barely distinguishable from the pseudo-steady state.

Figure 7.6 shows the effects the endonuclease Benzonase had on the viscosity vs. time profiles of samples taken over the time course of a batch fermentation. For the data shown in this figure the concentration of Benzonase was increased to 20 μl per sample of cell paste as opposed to 12 μl used in Figure 7.5. The reasoning for this can be seen in the results. Figures 7.6(a)-(c) show exactly the same trends regardless of the sampling time of the cell paste sample, with no peaks and an apparent viscosity of around about 1.5 – 2 mPa.s, very low for alkaline lysis of cell culture. Increasing the concentration of Benzonase increases the amount of nuclease material that is acted on.

A large proportion of a cell is made up of proteins (see Table 7.1), the chemical composition of E.coli being up to 55% protein by dry weight (Ingraham et al., 1983), and so they could reasonably be expected to have an impact on the viscosity of a solution of lysed cells. Proteinase K and the
required reaction buffer were mixed in with cell culture removed from a fermenter at exponential phase of growth, and the cell suspension lysed in the normal way in the scale down reactor. Figure 7.7 shows a comparative study between cell culture treated and lysed in the normal way, Figure 7.7(a), and cell culture first treated with Proteinase K (Figure 7.7(b)). Figure 7.7(a) shows the expected viscosity vs. time plot for a cell culture removed from a fermenter during exponential growth phase with the primary and secondary peaks easily distinguished from one another and a high apparent viscosity. Figure 7.7(b), following Proteinase K treatment, shows almost an identical plot, with no alteration to the timing of the peaks, which are both clearly defined. The apparent viscosity remains high and essentially comparable with the untreated material.

This behaviour is also seen in Figure 7.8 that presents data from the fermentation time where all cell culture samples had been treated with Proteinase K. The three profiles show the normal differences in peak times and apparent viscosities with respect to each other, as seen in untreated samples (Figure 6.3).

The next experiment was designed to determine the effects that plasmid DNA had on the viscosity vs. time profile. This was achieved by comparing the pXY plasmid bearing strain with the parental *E. coli* DH1 strain. For *E. coli* DH1 pXY, Figure 7.9(a) shows the classic exponential phase viscosity vs. time profile. Figure 7.9(b) presents exponential phase viscosity vs. time profile for the parental strain *E. coli* DH1. The two plots are nearly identical, except the apparent viscosity values are increased 10 mPa.s throughout, compared to the plasmid bearing strain. The secondary peak appears slightly earlier in the parental curve than in Figure 7.9(a), due to sampling an hour earlier (4 hours after inoculation compared to 5 hours). However the major difference is the exceptionally high apparent viscosity achieved for the parental strain. This is a factor of the fermentation, and so could be due to the metabolic differences between plasmid bearing and parental strains.

Figure 7.10 displays the viscosity vs. time profiles for the three growth phases of the parental strain. Figure 7.10(a) shows a classic early exponential
profile with a low apparent viscosity and indistinguishable secondary peak. Figure 7.10(c) shows a similar stationary profile to one expected of a plasmid bearing strain. However, Figure 7.10(b) shows a much higher apparent viscosity than expected for exponential growth, especially as the early exponential and stationary plots have the expected values.

The final investigation carried out for this chapter investigated the impact on the rheology of alkaline lysis of cell paste by removing the sodium hydroxide from the alkaline lysis solution, thus reducing the alkaline conditions to a neutral pH. This adjusted lysis solution is described in section 7.3.3.2. The data from this study is shown in Figure 7.11. The primary peak is visible 40 s after initiation of lysis as when using the normal alkaline lysis solution. The apparent viscosity then climbs by 1.5 mPa.s to a maximum of only 9 mPa.s where the profile levels out and maintains this viscosity before decreasing to 7.5 mPa.s due to shear thinning by 200 s after initiation of lysis.

7.5. Discussion

The factors investigated in this study (RNA, DNA, Proteins and plasmid DNA) are present in a cell culture at high enough concentrations to impact the rheology of any sample of lysed cells. The purpose of this work was to see if there was a single dominant factor that may be causing the observed changes to the secondary peak (see Chapter 6), and therefore determine the most important factor with regards to the viscosity of the feed stream. Chromosomal DNA, RNA and proteins are all contaminants that have to be removed before the plasmid DNA can be ready for therapeutic use.

Figure 7.1(a) shows the image of an agarose gel with untreated samples run through it. The cell culture used was sampled from the same fermentation during the three main phases of growth. The important factor here is the presence of the two dark areas of RNA at the bottom of the image. RNase 1 Ribonuclease is used in this study to remove the RNA from the cell culture samples before alkaline lysis. Figure 7.1(b) shows an image of the same samples, taken from a fermentation during the same phases of growth as in the previous gel. However, in this case the samples are run through a gel after treatment with the RNase enzyme. In this figure it is difficult to make
out any RNA at the bottom of the gel. This suggests that the RNA has been completely digested, and therefore if a viscosity vs. time profile were performed on samples treated in this way within the scale down reactor, RNA would not be affecting the rheological conditions. The pXY plasmid bands in Figure 7.1(b) are not as defined or sharp as in Figure 7.1(a). This is caused by the incubation period that the samples undergo to guarantee that the RNase 1 works (see Chapter 2, section 2.6.4 for agarose gel details).

Figure 7.2 shows the results of a similar control study using the non-specific endonuclease, Benzonase. The samples tested were removed from the fermenter at the same times as with Figures 7.1(a) and 7.1(b), and were treated with 20 µl Benzonase and 12 µl of reaction buffer, with 30 minutes incubation. The agarose gel image shows that the pXY plasmid DNA bands are only barely visible, and from this it can be concluded that the chromosomal DNA has been digested as well. The side effects of using a non-specific endonuclease are apparent from looking at the bottom of the image: there is no evidence of RNA. This could pose a problem in distinguishing if it is RNA or chromosomal DNA having a major impact on the secondary peak of the viscosity vs. time profiles. For this reason, the removal of RNA was investigated first.

The two curves shown in Figure 7.3 were taken from the same cell culture aliquot, sampled from the batch fermentation at stationary growth phase. There is no real difference in the viscosity vs. time profile between the sample treated in the standard way and the sample pre-treated with RNase, allowing for error in the scale down reactor, with perhaps a slight reduction in apparent viscosity in Figure 7.3(b). Figure 7.4 supports this view. The graphs presented in Figure 7.4 are mean values from a number of fermentations. The plots are proportioned as expected for the three growth phases, but as in Figure 7.3 the apparent viscosity is slightly lower than expected.

Therefore, from Figures 7.3 and 7.4 it appears that RNA does not affect the formation of the secondary peak, but it may have a slight impact on the maximum apparent viscosity achieved. However, this change is so slight that it is difficult to prove its significance. Because of the negligible impact of RNA on the viscosity vs. time plots, it is possible to use Benzonase to assess the
independent effects of chromosomal DNA. Benzonase is far more active than most DNase enzymes, and is often used to reduce the viscosity of process feed streams that contain substantial amounts of DNA and RNA (Grabski et al., 2000).

Figure 7.5 displays the results from an analysis of the effects of Benzonase on a sample removed from a fermenter at stationary growth phase, and treated with 12 µl of Benzonase mixed with 12 µl of reaction buffer. Figure 7.5(a) is an average of the viscosity vs. time profiles for alkaline lysis of cell culture sampled at stationary phase, for direct comparison with Figure 7.5(b) that shows the same sample but treated with Benzonase as explained above. The apparent viscosity of the poorly distinguished secondary peak drops to 8 mPa.s and there is a gradual increase of the pseudo steady state viscosity until it reaches close to that of the untreated sample at about 10 mPa.s. Since previous data has shown that RNA has a limited effect on the viscosity vs. time profile the differences seen here must be attributable to chromosomal or plasmid DNA. It is probable that the Benzonase acts to digest the chromosomal DNA to small enough fragments so that no defined matrix causing the secondary peak in viscosity can be formed. Further support for this is given in Figure 7.6 where by increasing the amount of Benzonase to 20 µl per sample no evidence of any peaks or form to the curves is seen, whether sampled at early exponential, exponential or stationary phase of the fermentation. This supports the evidence that the peaks are caused by the binding of long strands of DNA into a matrix. In the 20 µl treated samples there are unlikely to be any long strands left in the treated samples after degradation of DNA by the Benzonase.

The results still leave two questions unanswered: what effect do proteins have on the rheology and how much of the DNA effect is caused by plasmid DNA as opposed to chromosomal DNA. From the evidence seen above, it is reasonable to assume that proteins have a very limited effect on the viscosity observed during alkaline lysis of a sample. Figures 7.7 and 7.8 support this as there is no discernible difference between untreated and treated samples with Proteinase K in Figures 7.7(a) and (b). Both curves show classic viscosity vs. time plots for cell culture sampled during exponential
growth phase. There is a slight decrease in apparent viscosity in Figure 7.7(b) for the treated sample, compared to Figure 7.7(a). This is similar to the results achieved by treating the sample with RNase. Again, however, the significance of the differences is small. Figures 7.8(a)-(c) display exactly the expected plots for the three growth phases of a cell culture in a batch fermentation. The values are very similar to those shown in Figures 6.2(a)-(c), with perhaps a slight reduction in apparent viscosity that could be due to the action of Proteinase K.

It was deemed unnecessary to prove the action of Proteinase K by running a PAGE del with Coomassie staining as it has already been quoted that Proteinase K is highly active, and if RNase and Benzonase could be proven to work in these conditions, Proteinase K will also work. The final experiments in this study were to test the effects of plasmid DNA on the secondary peak of the viscosity vs. time profiles, and to see the impact on the secondary peak of reducing the alkalinity of the alkaline lysis solution. Parental DH1 strain was grown in a batch fermenter and sampled as described before. Figure 7.9(a) is the viscosity vs. time plot for the normal plasmid pXY containing DH1 strain. The cell culture was removed at exponential growth phase and exhibits the expected alkaline lysis viscosity changes. Figure 7.9(b) displays the viscosity vs. time plot for a cell culture sample removed an hour earlier, hence the slightly earlier secondary peak, but with the same timing of the primary peak. The point of interest here is the much higher apparent viscosity in the parental cell line curve. By definition these cell culture samples are from different fermentations, and some difference in apparent viscosity could be expected. However, this is a very large increase, outside the normal expected fluctuations. Figure 7.10 shows the time course for the parental cell strain, with samples of cell culture taken at early exponential, exponential and stationary phases of growth. Figure 7.10(a) and 10(c) show the data for early exponential and stationary viscosity vs. time profiles respectively. These profiles are very similar to their respective profiles shown in Figures 6.3(a) and 6.3(c), whereas Figure 7.10(b) displays the high apparent viscosity seen in Figure 7.9(b). This suggests that the increase in apparent viscosity is not a factor of the different fermentations, but
rather of the cell strains themselves. It is possible that freed from the 
metabolic load imposed by the presence of a plasmid, the parental cell strain 
culture grows faster, and hence there is more chromosomal DNA replication, 
and thus a greater apparent viscosity than in the pXY carrying strain. Data in 
Table 3.2 shows that there is no real difference between the maximum 
specific growth rates of the parental and plasmid containing strains, but the 
exponential phase is longer for the parental strain, resulting in a higher 
maximum optical density (Table 3.4). This would explain the greater amount 
of chromosomal DNA in the parental strain exponential sample.

In Figure 7.10(a) and 8.10(c) there is no real difference between these 
plots and their equivalent plasmid-carrying curves (Figures 6.2(a) and (c)). It is 
therefore possible to conclude that it is chromosomal DNA and not plasmid 
DNA that is most likely causing the secondary peaks in the viscosity vs. time 
plots. For this reason it was decided not to further investigate the possible 
effects of lipids and other cellular material as chromosomal DNA was the 
factor creating the secondary peak.

The data from the experiment investigating the rheology of the alkaline 
lysis of E.coli DH1 /pXY cell paste using the adjusted lysis solution is shown in 
Figure 7.11. It can be seen by the presence of the primary peak that cell lysis 
occurs as normal. However, with a neutral pH environment the chromosomal 
DNA responsible for the formation of the secondary peak is not denatured and 
no increase in apparent viscosity due to the formation of a matrix is seen. This 
result adds weight to the fact that it is chromosomal DNA behind the formation 
of the secondary peak.

The next chapter investigates how the quality and the quantity of 
plasmid DNA changes over the course of batch and fed-batch fermentations, 
and also during the alkaline lysis reaction to see if there is an ideal point of 
fermenter cell harvest and an optimum point at which to halt the alkaline lysis 
reaction with respect to the quality and quantity of plasmid DNA.
8.6. Conclusions

In conclusion there are a number of points for consideration. The first is that both RNA and intracellular proteins have a limited impact on the apparent viscosity of the viscosity vs. time profile from the alkaline lysis reaction, and are not the primary factor behind the formation of the secondary peak.

The rheological results from the alkaline lysis of the DH1 parental cell strain indicate that there is evidence of metabolic burden in the viscosity vs. time profiles, but there is no impact on the secondary peak timing caused by the presence of plasmid DNA.

From the results, chromosomal DNA is the major cause of the rheological changes evident in the studies done thus far, and the dominant factor behind the formation of the secondary peak.
Figure 7.1: Image of plasmid DNA samples derived from the alkaline lysis of *E. coli* DH1 /pXY cell paste, run on agarose gels by electrophoresis, before (a) and after (b) treatment with RNase.
Figure 7.2: Image of *E.coli* DH1 /pXY cell paste plasmid DNA samples pretreated with Benzonase and run on agarose gels by electrophoresis.
Figure 7.3: Effects of RNase treatment on alkaline lysate viscosity vs. time profiles. The panels show viscosity vs. time profile for *E.coli* DH1/pXY sampled from batch fermentation 3 h after inoculation (A) and viscosity vs. time profile for *E.coli* DH1/pXY sampled from batch fermentation 3 h after inoculation and treated with RNase prior to alkaline lysis.
Figure 7.4: Viscosity vs. time profile for *E. coli* DH 1/pXY sampled from batch fermentation during early exponential (A), exponential (B) and stationary (C) growth phases. Resuspended cell paste was treated with RNase prior to alkaline lysis.
Figure 7.5: Effect of treatment with Benzonase on resultant viscosity vs. time profile for the alkaline lysis of *E.coli* DH1 /pXY cell paste sampled during stationary phase of fermentation. Panels showing untreated (A) and treated (B) viscosity vs. time profiles.
Figure 7.6: Viscosity vs. time profile for alkaline lysis of *E.coli* DH1 /pXY cell paste pre-treated with Benzonase. Cell paste sampled during early exponential (A), exponential (B) and stationary (C) growth phases of batch fermentation.
Figure 7.7: Effects of Proteinase K treatment on alkaline lysate viscosity vs. time profiles. The panels show viscosity vs. time profile for *E.coli* DH1 /pXY sampled from batch fermentation during exponential growth phase (A) and viscosity vs. time profile for *E.coli* DH1 /pXY sampled from batch fermentation during exponential growth phase and treated with Proteinase K prior to alkaline lysis.
Figure 7.8: Viscosity vs. time profile for alkaline lysis of *E.coli* DH1 /pXY cell paste pre-treated with Proteinase K. Cell paste sampled during early exponential (A), exponential (B) and stationary (C) growth phases of batch fermentation.
Figure 7.9: Comparison of viscosity vs. time profile for *E. coli* DH1 /pXY cell paste sampled during exponential phase of batch fermentation (A) and *E. coli* DH1 parental cell paste sampled during exponential phase of batch fermentation (B).
Figure 7.10: Viscosity vs. time profile for alkaline lysis of parental *E.coli* DH1 cell paste. Cell paste sampled during early exponential (A), exponential (B) and stationary (C) growth phases of batch fermentation.
Figure 7.11: Viscosity vs. time profile for the alkaline lysis, using adjusted alkaline lysis solution, of \textit{E.coli} DH1/pXY cell paste sampled at completion of batch fermentation.
8. Changes in Plasmid Quantity and Quality as a Function of Fermentation Age and Neutralisation Time

8.1. Summary

Results in this chapter showed that for batch fermentations plasmid yield increases as a function of fermentation time and time of neutralisation up until stationary phase, when the plasmid yield drops from 80 mg/kg to 50 mg/kg after 100 s of alkaline lysis reaction. This drop in yield is accompanied by a decrease in the resistance of plasmid DNA to permanent denaturation. Fed-batch data shows a similar decrease in plasmid yield to the batch stationary growth phase data as a function of alkaline lysis time; however, plasmid yield is independent of feed time. The level of open circular (OC) DNA present is an indicator of plasmid purity: the results of this study show an increase in OC DNA as a function of batch fermentation time. As a function of time of neutralisation the OC DNA results show an increase over the first 40 s then the levels stabilise. The OC DNA results for the fed-batch samples shows no change as a function of either fermentation time or time of neutralisation of alkaline lysis reaction.

8.2. Introduction

Previous chapters have investigated the construction of Windows of Operation for the fermentation and alkaline lysis process steps, primarily looking at how physical cell strength changes over the time course of a fermentation and how the rheology of the resultant feed stream is affected by the cell culture growth phase. The likely causes of the observed rheological changes were discussed in the previous chapter, and found to be dependent on the chromosomal DNA content. However, the highest quality and quantity of plasmid DNA yield possible is the ultimate process aim, and so it is important to know how this yield is changed or affected, if at all, during the alkaline lysis process.
8.2.1. Aims

The aims of this experimental chapter are three-fold: to further the characterisation of the alkaline lysis operation; to look at how the quantity and quality of plasmid DNA changes as a function of a number of process factor; and to investigate the possibility of developing Windows of Operation for fermenter harvest time. The process factors to be considered include how the quantity of plasmid DNA changes: during the time course of the fermentation; as a function of the length of time from initiation of alkaline lysis to neutralisation; between batch and fed-batch fermentations.

8.2.2. Plasmid Yield

Processes producing plasmid DNA at large scale should be designed to yield a definitive amount of plasmid DNA within certain specifications of purity, potency, identity, efficacy and safety (Prazeres *et al.*, 1999), whilst remaining cost effective and efficient. Using high copy number plasmids it is possible to achieve a yield of 0.5 g plasmid DNA per 1 kg of wet weight biomass (Schleef, 1997). It is important from a scale-up perspective to understand how the fermentation and the alkaline lysis process units affect the plasmid yield, and to characterise the impact of harvesting and neutralisation decisions on the plasmid DNA quality and quantity.

8.2.3. Quality Criteria

Plasmid DNA produced for therapeutic purposes must be free of any contamination (Schorr *et al.*, 1995), capable of producing patient immune response (Schleef *et al.*, 1997). Table 1.2 (see Chapter 1, section 1.2.4 for greater detail on regulatory requirements) lists the main quality criteria that must be met when producing plasmid DNA. In this chapter there are two important quality criteria to address: the presence of RNA and homogeneity of the plasmid DNA. Chapters 6 and 7 discuss the ramifications of the presence of chromosomal DNA and RNA contamination in a feed stream. In this chapter the concept of plasmid DNA quality is introduced. Table 1.2 lists the requirement of DNA homogeneity as being greater than 90% in supercoiled (SC) form. Supercoiled DNA is considered to be a more effective form of DNA
for transferring gene expression than open-circular, linear, multimeric or partially denatured DNA (Prazeres et al., 1999). This chapter looks at the changes in the quality of plasmid DNA by comparing the amount of open circular DNA present over the course of a fermentation, and between a batch and fed-batch fermentation.

8.3. Materials and Methods

This section supports Chapter 2 by examining some of the protocols used in greater detail.

8.3.1. Fermentation and Sampling

For this study, 7 L total volume Applikon fermenters were used for batch and fed-batch protocols. Batch fermentations had a working volume to total volume ratio of 0.57 v/v and fed-batch fermentations had an initial working volume to total volume ratio of 0.43 v/v. The batch fermentations were sampled at 2 hour intervals, as described in Chapter 2, section 2.3.3, starting at 0 h. The cell culture within the fed-batch fermenters was grown for 8 h before initiation of the feed culture addition. Sampling started at this point, and is referred to as 0 h in this chapter. Samples were taken in the usual way every 2 hours. All samples were spun and frozen for later analysis in the manner described in Chapter 2, section 2.3.3.

8.3.2. Alkaline Lysis

All samples for this study were lysed in the rheometer using the C25 cup and bob geometry at a shear rate of 461 s⁻¹. For samples that were not to be neutralised, 6 ml of resuspended defrosted cell paste was added to the rheometer, and an equivalent volume of lysis buffer added as the shear was applied. The reaction was allowed to run for 10 minutes.

Samples that were to undergo agarose gel electrophoresis were neutralised in the rheometer 10 s, 40 s, 100 s or 250 s after lysis had been initiated. 4 ml of resuspended cell paste was loaded into the rheometer, and lysis initiated by the addition of 4 ml lysis buffer. After the pre-determined time of lysis the reaction mixture was neutralised as described in Chapter 2,
section 2.4.2.

8.3.3. Agarose Gel Electrophoresis

Plasmid DNA yields were analysed as part of this study. The FDA "Points to Consider on Plasmid DNA Vaccines for Preventive Infectious Disease Indications" states that agarose gel electrophoresis may be a useful technique to analyse plasmid DNA. Neutralised samples were prepared for loading on gels as described in Chapter 2, section 2.6.4. Cell paste sampled from early exponential, exponential and stationary phases of fermentation growth were neutralised after 10 s, 40 s, 100 s and 250 s of alkaline lysis and then loaded and run on the agarose gels. Supercoiled DNA ladders (Sigma, Poole, Dorset, UK) were run in triplicate as markers to compare with experimental data. Gels were analysed using Scion Image (Scion Corporation, Maryland, USA), to measure how much supercoiled DNA was present in each sample. All gels were run in triplicate and Scion Imaging equipment produced an accuracy of ±5 % for results, unless otherwise stated.

8.3.4. RNA Removal

RNA was removed from samples by adding RNase I Ribonuclease (Sigma, Poole, Dorset, UK) to cell suspensions at a concentration of 1 mg/700 OD600nm units, and incubating the mixture with x10 reaction buffer for 30 minutes at 37°C.

8.4. Results

The following sub-sections examine the data collected during this study of the effects of fermenter harvest time and neutralisation of the alkaline lysis step on the plasmid DNA quality and quantity.

8.4.1. Analysis of Plasmid DNA Samples

As explained in the introduction, neutralised alkaline lysates were examined using agarose gel electrophoresis. An example of one of these gels highlighting the relevant features is shown in Figure 8.1. All the samples run on this gel show the same size plasmid, with a cloud of RNA at the base of
the image. The markers flanking the eleven samples have two distinct bands: one corresponding to the pXY plasmid and a second dark band halfway between the plasmid band and the well. This is the band corresponding to open circular, or type II DNA (Middaugh et al., 1998 and Levy et al., 2000). Since any sample of plasmid DNA run on agarose gel electrophoresis must be more than 90% supercoiled DNA to satisfy regulatory purity requirements, open circular DNA may be treated as a contaminant and can be used to indicate the extent of purity, as has been done in this study. Other bands correspond to genomic DNA, displaying how this particular contaminant passes through the lysis step and on to subsequent purifying stages that have a low separation capacity for such material (Levy et al., 2000).

8.4.2. Plasmid Yield

Plasmid DNA yield is examined in Figures 8.2 – 8.4. Samples were removed from a batch fermentation at early exponential, exponential and stationary phases of cell culture growth (see Chapters 3 and 6); treated by alkaline lysis in the scale-down reactor and then neutralised at 10 s, 40 s, 100 s and 250 s after lysis was initiated. These samples were then prepared for agarose gel electrophoresis. After being run out gels were stained and photographed and the plasmid yield in mg DNA/kg wet cell biomass calculated from an analysis of the supercoiled markers, as explained in Chapter 2, section 2.6.4. Figure 8.2 displays the results of samples from a batch fermentation without any treatment with RNase. Section 8.2.2 quotes a possible figure from the literature of approximately 500 mg/kg wet cell biomass. From the graph shown in Figure 8.2 it is clear that the plasmid yield achieved by the DH1 cell strain and pXY plasmid was less than the quoted target. Over the course of the fermentation the yield of plasmid DNA increases by about 20 mg/kg biomass, from 60 to 80 mg/kg biomass. The data from the early exponential and exponential phases shows a steady increase in plasmid yield as the neutralisation time from initiation of lysis increases. The graph also shows an increase in plasmid yield over the time of the fermentation, from early exponential through exponential to stationary phase. The data for stationary phase material shows a change in the trend. After a linear increase in plasmid
yield as a function of time of neutralisation from 10 s to 100 s, there is a
distinct drop in plasmid yield between 100 s and 250 s. This is in contrast to
the behaviour of material from the earlier cell culture growth phases.

RNA is a contaminant in the feed stream. Due to its size in relation to
plasmid DNA it is difficult to remove by chromatographic steps (Prazeres et
al., 1999, Varley et al., 1999 and Levy et al., 2000). Figure 8.1 shows the
presence of RNA at the bottom of the image. For the sake of comparison, an
agarose gel of samples treated with RNase is shown in Figure 7.1. Figure 8.3
shows the data from agarose gel images of samples treated with RNase prior
to alkaline lysis. By inspection the overall plasmid yield is slightly higher in this
figure than for non-treated samples. However, the early exponential curve
shows the same trend as in Figure 8.2, with plasmid yield increasing as a
function of neutralisation time. The exponential data has a higher absolute
plasmid yield than the curve for early exponential data, as expected.
However, the increase in plasmid yield as a function of neutralisation time is
not as pronounced as in Figure 8.2. The curve for stationary data shows no
significant increase as a function of fermentation time compared to the
exponential data, whilst the drop in plasmid yield as a function of
neutralisation time shown in Figure 8.2, is more pronounced here.

The previous two figures contained average data from batch
fermentations. Figure 8.4 displays the average data for samples removed
throughout the time course of fed-batch fermentations. The average plasmid
yield is similar to that shown in Figure 8.2, and each curve shows almost
identical characteristics: there is no change as a function of fed-batch feed
time, but as a function of neutralisation time there is a general increase in
yield for the first 100 s, which then falls away between 100 s and 250 s.

8.4.3. Plasmid Quality

As stated earlier a cGMP process must produce plasmid DNA that consists of
at least 90% in supercoiled form. Open circular DNA is therefore a
contaminant, and its presence a good indicator for the effectiveness of scale-
up performance. Section 8.4.2 demonstrated that the removal of RNA helps to
sharpen an agarose gel's image; therefore all batch samples will be treated
with RNase prior to analysis in this section. The graph in Figure 8.5 shows the amount of open circular (OC) DNA present in the samples as a function of batch fermentation time and neutralisation time. All samples had the same cell concentration, were treated with the same lysis buffer and underwent the same shear conditions in the scale-down reactor. The gel bands corresponding to OC DNA were analysed using the image analysis package, and the area of the bands compared to one another.

The band area of OC DNA present increases as a function of the cell culture growth phase. There is also a trend of a gradual increase as a function of increased neutralisation time, although the trend is not as marked as that with fermentation time.

<table>
<thead>
<tr>
<th>Neutralisation</th>
<th>0</th>
<th>1</th>
<th>6</th>
<th>10</th>
<th>AVERAGE</th>
</tr>
</thead>
<tbody>
<tr>
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<td>16</td>
<td>107</td>
<td>88</td>
<td>101</td>
</tr>
<tr>
<td>10</td>
<td>82.5</td>
<td>83</td>
<td>103.5</td>
<td>94.5</td>
<td>91</td>
</tr>
<tr>
<td>40</td>
<td>105</td>
<td>119.5</td>
<td>79</td>
<td>125</td>
<td>107</td>
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<tr>
<td>250</td>
<td>104</td>
<td>89.5</td>
<td>86</td>
<td>86</td>
<td>91</td>
</tr>
</tbody>
</table>

Table 8.1: Plasmid DNA quality for samples removed from fed-batch fermentation. Values correspond to the area of open circular DNA viewed on an agarose gel using Scion imaging package. Samples were removed at different stages of the feeding regime and treated with alkaline lysis solution. Neutralisation times correspond to the amount of time from addition of alkaline lysis solution to the quenching of the reaction using chilled potassium acetate.
Table 8.1 shows similar data to that in Figure 8.5, but now for fed-batch fermentations. The conditions for lysis were exactly the same as with the previous data. This table shows that with fed-batch material there is no evidence of any trend either as a function of neutralisation time or as a function of feeding time.

Figure 8.6 examines the purity of the plasmid DNA as a percentage of all other bands found on the gels (see Figure 8.1), including OC DNA, for RNase treated batch and fed-batch samples. The data for batch fermenter samples is shown in Figure 8.6(a). There is no great change in the % supercoiled as a function of neutralisation time, with only a slight drop off between 100 and 250 s. However, there is a slight drop in purity as a function of fermentation time. The absolute % SC values are lower than the required 90% for a GMP process indicating the need for subsequent recovery and purification operations.

<table>
<thead>
<tr>
<th>Fermentation Time (h)</th>
<th>Yield (mg/Kg)</th>
<th>Purity (% SC DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Batch</td>
<td>Fed-batch</td>
</tr>
<tr>
<td>0</td>
<td>N/A</td>
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</tr>
<tr>
<td>1</td>
<td>57.7</td>
<td>60.3</td>
</tr>
<tr>
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<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>6</td>
<td>68.7</td>
<td>64.6</td>
</tr>
<tr>
<td>10</td>
<td>61.9</td>
<td>63.1</td>
</tr>
</tbody>
</table>

Table 8.2: Comparison between batch and fed-batch fermentation plasmid DNA purity and yield. All fermentations used E.coli DH1 /pXY strain and plasmid. Fed-batch fermentation time refers to feeding regime time. All data is subject to ±5% error.

Fed-batch data shown in Figure 8.6(b) shows little change in SC purity as a function of either time of neutralisation or feed time. Again, as expected, the % SC purity is lower than that required for GMP.
Table 8.2 presents a direct comparison between batch and fed-batch fermentation plasmid yield. The % supercoiled DNA results show that batch fermentation early exponential samples have the highest purity, but by the end of the batch fermentation purity is similar to corresponding fed-batch data (68.7% and 67.5% respectively). The peak plasmid DNA mg/kg wet cell biomass show that fed-batch fermentations have a consistent yield throughout growth, whereas the batch fermentation yield peaks and then reduces slightly. After 10 h of growth, both batch and fed-batch fermentations have similar yields (62 and 63 mg/kg respectively).

8.5. Discussion

There are a number of interesting trends directly related to plasmid yield and plasmid quality that have resulted from this study. The following sub-sections consider these results.

8.5.1. Plasmid Yield

The results for plasmid DNA yield from a batch fermentation show that the yield increases for early exponential and exponential phases as a function of the time of neutralisation and fermentation time. The stationary phase data marks an interesting switch in behaviour. At short neutralisation times a higher plasmid yield than at the start of the exponential phase was determined, and this plasmid yield increased as a function of neutralisation time for the first 100 s. Thus to this point the general trend of plasmid yield increasing as a function of fermentation time and neutralisation time continues. However, between 100 s and 250 s of alkaline lysis before the reaction is halted by neutralisation, there is a significant drop in the plasmid yield, from 80 mg/kg wet cell biomass to 50 mg/kg wet cell biomass. This is an unexpected, but reproducible drop (all data in this study is an average of at least triplicate runs, sampled in duplicate).

The general increase in plasmid yield as a function of neutralisation time is due to a greater extent of irreversible denaturation of chromosomal DNA that accompanies a longer alkaline lysis time, before the neutralising solution was added. Hence when the solution is neutralised after increased
time the separation of plasmid and chromosomal DNA is increased. It may also be due to an incomplete release of plasmid DNA during early lysis.

In a similar way the general trend of an increase in plasmid yield as a function of fermentation time can be attributed to the fact that a plasmid propagates to keep pace with the growth of the host bacterial cell culture (see Chapter 5) to guarantee the presence of the plasmid in the daughter cells. However, generally plasmid growth is fastest at the end of a fermentation, during late exponential and stationary phase. This will explain the higher yield at stationary phase, and the observed increase at exponential phase must be due to the cell culture slowing down growth. When cell replication slows down and the cell culture moves into stationary phase there is a lag before plasmid propagation also slows down, and hence the observation of continued increase in plasmid volume per unit wet cell biomass.

This does not, however, explain the sudden drop in plasmid yield after 100 s time of neutralisation for the stationary phase material. For all these experiments the same volume of the same cell concentration is used, and the same volumes of lysis and neutralising buffer is added. There are a number of theories that could help to explain this phenomenon.

One possible theory could be that reversibly denatured plasmid DNA may interact, to form larger fragments of plasmid DNA that will behave in a similar fashion to the larger proteins and chromosomal DNA, and therefore it may be that by stationary phase the plasmid and chromosomal DNA are not so easily partitioned from each other. The reduction in plasmid DNA yield in stationary phase material may also be due to the amount of cell debris per cell at stationary phase, because of more developed cell walls. Experimental evidence for the occurrence of stronger, more developed cell walls was reported in Chapter 5, and the increase in cell debris within the lysis reaction mixture may cause problems with the partitioning of plasmid and chromosomal DNA.

Another possible theory is that a longer exposure to the alkaline lysis solution irreversibly denatures plasmid DNA, and this has particular impact on plasmid DNA from stationary phase material. This may be due to the fact that
metabolic load acting on cells means that by stationary phase the plasmid DNA is not as resistant to nicking or irreversible denaturation, bringing about a reduction in plasmid DNA yield. This concept is introduced in the literature, where it is suggested that exposure to the lysis solution for too long may irreversibly denature plasmid DNA (e.g. Rush and Warner, 1970).

The observed reduction in plasmid DNA yield is an important process parameter as the mixing time within the lysis vessel at this stage must be long enough to allow good mixing of the alkaline lysis solution with the cell paste before subsequent neutralisation, whilst remaining brief enough so as not to reduce the plasmid DNA yield. Whilst it was not possible - due to time constraints - to determine for definite the reason for the decreases in plasmid yield, it is clearly something that needs to be investigated thoroughly in the future (see Chapter 11), so as to characterise more thoroughly the alkaline lysis process.

The data for fed-batch fermentations is shown in Figure 8.4. Each of the curves shows a similar plot, with no significant changes as a function of fermentation time. This suggests that only the absolute growth rate of the fed-batch fermentation is important, and as the growth rate is maintained by the feeding regime at 0.1 h\(^{-1}\), there is no change in the results as a function of fermentation time. The impact of extending the neutralisation time displays almost the same trend as for the batch fermentation stationary growth phase data.

8.5.2. Plasmid Quality

The data for the presence of open circular DNA (OC DNA) displays trends as a function of both neutralisation time and batch fermentation time. All samples were treated with RNase before they were lysed and subsequently analysed so as to compare the clarity of the resultant gels. The plots (Figure 8.5) show the area of the band corresponding to the OC DNA seen on the gels. The units are arbitrary and comparative only.

The levels of OC DNA increase as a function of batch fermentation time. The data points corresponding to a constant 10 s of lysis time for each of the growth phases show a marked increase from 50 units at early
exponential stage through 63 units at exponential growth to 108 units at stationary phase. This increase links with the plasmid yield which rises by a similar proportion throughout the fermentation. Thus there is more plasmid DNA available to be nicked during the process and hence become open circular in form.

The level of OC DNA as a function of the time of neutralisation has a distinct pattern that is constant throughout all the growth phases. The OC DNA level increases for the first 40 s of lysis, and then levels out with only a slight fluctuation. The results suggest that the harsh conditions of lysis do have an impact on plasmid DNA, with an increasing level of nicked DNA. However, there does appear to be a threshold level where the amount of OC DNA present does not increase as a function of neutralisation time, and therefore the fermentation time is the dominant factor affecting the levels of OC DNA.

From the above it appears that the impact of lysis time on OC DNA from a process perspective is limited. The lysis time has to be long enough to ensure a suitable yield of plasmid DNA, and beyond 40 s, the lysis time has little impact upon the level of OC DNA.

The impact of fermentation growth phase and neutralisation time on samples from fed-batch fermentations is represented in Table 8.1. With the fed-batch data, fermentation time T=0 relates to the point at which the feed regime begins (see section 8.3.1). The average values for amount of OC DNA present in the samples run on agarose gels are shown as a function of both parameters, fermentation time and time of neutralisation. There is no significant difference in the amount of OC DNA present as a function of fed-batch feed time. There is also no change in the band area corresponding to OC DNA on the gel, as a function of neutralisation time for fed-batch fermentations. As the level of OC DNA remains constant in the cells there are therefore no process issues concerning the content of OC DNA to consider as a function of feed time or time of neutralisation when the fed-batch method is employed. This may be due to the controlled growth conditions in fed-batch fermentation.
The FDA indicates that for cGMP the plasmid DNA product for use in gene therapy treatment must be at least 90% supercoiled. Figure 8.6(a) analyses the percentage of supercoiled DNA by measurement of the gel band areas for batch fermentation samples. The first observation is that the yield is slightly lower than that demanded by cGMP. Therefore further purification is required, and maybe the alkaline lysis process employed in this thesis could also be optimised further before scale-up. This optimisation may include changing the shear levels in the scale-down reactor, or the concentrations of the cell suspension and solutions used in the lysis operation. There is a slight drop as a function of fermenter time, possibly corresponding to the increase in OC DNA. Again, this data may be indicative of overly harsh processing conditions that may be causing this reduction in supercoiled DNA. However, it is important to note that ethidium bromide stained gels can over estimate impurities such as OC DNA as less ethidium bromide intercalates into SC DNA. This may mean that purity is a little higher than quoted.

The percentage supercoiled DNA as a function of neutralisation time shown in Figure 8.6(a) shows an initial drop over the first 40 s of the alkaline lysis, prior to neutralisation of the reaction, correlating well with the increase seen in OC DNA. After this initial drop the percentage supercoiled DNA remains stable and there is no change, except in stationary phase, where there is a noticeable drop between 100 s and 250 s. This correlates with the drop in plasmid yield. This also supports the theory that the cause of the decrease in plasmid yield is due to the higher ratio of plasmid to chromosomal DNA present during the stationary phase, and the resultant removal of extra plasmid DNA. There is not a corresponding increase in OC DNA at the same point as seen in Figure 8.5, which suggests that the plasmid DNA is not necessarily being nicked or damaged to form OC DNA, but perhaps actually removed from the system by neutralisation.

Fed-batch data (Figure 8.6(b)) again highlights the need for further purification steps, as the percentage supercoiled DNA is also low for the fed-batch fermentation method. As with the OC DNA results for fed-batch, there is no significant change in percentage supercoiled DNA as a function of either feed time or time of neutralisation, again probably due to the controlled growth
conditions in a fed-batch fermenter.

The comparison between batch and fed-batch fermentation plasmid DNA quantity and quality (Table 8.2) showed that the two fermentation methods have very similar plasmid DNA yield and purity after a 10 h fermentation period. However, the fed-batch method is probably the superior fermentation method for producing plasmid DNA, as the yield was constant throughout the fed-batch feeding regime. This is due to the controlled growth conditions (see Chapter 3) within the fed-batch fermenter, which would appear to encourage constant plasmid DNA expression. Hence, if the fed-batch feeding regime could be extended, leading to higher cell growth, then the mass of plasmid DNA produced would also increase.

8.5.3. Correlation Between Viscosity vs. Time Profile and Absolute Mass of Plasmid DNA Present

Figure 8.7 shows the correlation between plasmid DNA data from the agarose gels and equivalent viscosity vs. time profiles for batch fermentations. Early exponential data is shown in Figure 8.7(a): the actual value for plasmid DNA run on the gel shows a very similar curve profile to the change in apparent viscosity as a function of time, with a sharp rise up to the first peak at 40 s, and then a gradual increase in viscosity and plasmid DNA after that.

Exponential data for plasmid DNA again follows the curve of the viscosity vs. time profile. However, there is only a slight increase in mass of plasmid DNA compared to the mass increase seen in Figure 8.7(a), whereas apparent viscosity is significantly higher. However, the trend shown by the increase in plasmid DNA mass follows that of the apparent viscosity curve, with corresponding peak timings.

The plasmid DNA mass in the stationary phase diagram, Figure 8.7(c), shows a general increase in plasmid DNA mass compared to the previous graphs, but again mirrors much of the apparent viscosity vs. time profile. However, whereas the apparent viscosity reaches a pseudo-steady state, the plasmid DNA mass drops significantly after 100 s of alkaline lysis. The reasons for this have been discussed in section 8.5.1. However, this data
suggests a correlation between the mass of plasmid DNA and the apparent viscosity of the viscosity vs. time profile, except for stationary phase samples where the plasmid DNA mass decreases after 100 s of alkaline lysis, even though the viscosity of the sample does not decrease. This data supports the results from the previous chapter, where chromosomal DNA is shown to be the significant factor behind the observed rheology of alkaline lysis of cell paste.

8.5.4. Window of Operation for Fermenter Harvest Time and the Time of Neutralisation for Alkaline Lysis Reaction, as Determined by Plasmid Quality and Quantity

This section discusses the Windows of Operation determined by the results from this chapter. The optimum point of harvesting the cell culture from the fermenter is discussed, as is the ideal point at which to neutralise the alkaline reaction, for both batch and fed-batch fermentation methods.

8.5.4.1. Batch Fermentation

The highest plasmid DNA quantity (mg/kg wet cell biomass) is derived from stationary phase material. Whilst this material also has the lowest quality of plasmid DNA (% supercoiled DNA), the gain in plasmid quantity from exponential to stationary phase material is greater than the corresponding loss in plasmid quality.

Neutralisation data shows that an optimum alkaline lysis time at the experimental shear rate is 100 s, after which plasmid DNA quantity reduces. There is no significant change in plasmid DNA quality as a function of neutralisation time.

Therefore, for the highest possible quantity and quality of plasmid DNA for these experimental conditions, cell culture should be harvested at stationary phase and neutralised after 100 s of alkaline lysis.

8.5.4.2. Fed-Batch Fermentation

There was no significant impact of fermentation harvest time on plasmid DNA
quantity and quality. The optimum alkaline lysis time for plasmid DNA quantity was seen to be the same as for batch fermentation samples, with neutralisation of the reaction at 100 s for these experimental conditions. After 100 s of alkaline lysis the plasmid quantity reduced. There was no significant impact of alkaline lysis time on plasmid quality (% supercoiled DNA), with the quality remaining constant over throughout alkaline lysis.

Therefore, at the specified experimental conditions, there was no optimum harvest point with regards to plasmid DNA quality and quantity, and only biomass to consider. The optimum neutralisation point of the alkaline lysis reaction was at 100 s after lysis was initiated, as this produced the maximum plasmid DNA quantity.

The fed-batch fermentation method is probably superior to the batch fermentation method for producing plasmid DNA as the yield remains constant throughout the feeding regime, which can ultimately mean a greater mass of plasmid DNA at completion of the fermentation stage.

In the following chapter, the rheology of the alkaline lysis process will be investigated in greater detail, with particular attention to the elastic component of the reaction mixture.

8.6. Conclusion

In conclusion, the experimental data presented in this chapter shows that for batch fermentation, plasmid yield increases as a function of increasing time from initiation of alkaline lysis to addition of neutralising buffer.

However, when stationary phase samples are neutralised more than 100 s after treatment with alkaline lysis solution, the plasmid yield drops. It is unclear as to what is the cause of this important processing issue. It may be due to plasmid DNA being less resistant to irreversible denaturation due to metabolic load, and therefore more plasmid DNA being removed from the liquor when neutralisation occurs.

Supercoiled DNA percentage decreases marginally as the age of fermenter increases, but there is no distinctive decrease at the point when plasmid yield drops as well, suggesting that the impact of OC DNA is limited.
Fed-batch samples show little variation in plasmid DNA quality and quantity as a function of feed time and neutralisation time allowing for a very flexible harvest Window of Operation. This contrasts with the results for batch fermentation samples, where stationary phase is the ideal time to harvest for optimum plasmid DNA quantity. For both batch and fed-batch fermentations, the optimum point for neutralisation of the alkaline reaction was seen to be 100 s, for maximum plasmid DNA production, using the alkaline lysis conditions described in section 8.3.2.

**Figure 8.1:** Example of agarose gel electrophoresis of plasmid DNA samples removed from batch fermentation.
Figure 8.2: Plasmid DNA (mg/kg wet cell biomass) as a function of fermentation growth phase and neutralisation time of alkaline lysates. *E.coli* DH1 /pXY grown in batch fermenter. Lysates not treated with RNase.
Figure 8.3: Plasmid DNA (mg/kg wet cell biomass) as a function of fermentation growth phase and neutralisation time of alkaline lysates. *E.coli* grown in batch fermenter. Lysates treated with RNase during lysis.
Figure 8.4: Plasmid DNA (mg/kg wet cell biomass) extracted from *E.coli* cell culture as a function of fermentation time and neutralisation time of alkaline lysates, grown in fed-batch fermenter. Lysates treated with RNase during lysis.
Figure 8.5: Amount of open circular (OC) DNA present in the agarose gel samples as a function of fermentation time and neutralisation time, of batch fermentation.
Figure 8.6: Purity of plasmid DNA as a percentage of all other bands found on gel, including OC DNA, for RNase treated (a) batch samples and (b) fed-batch samples.
Figure 8.7: Correlation between plasmid DNA (ng) data from the agarose gels and equivalent viscosity vs. time profiles (mPas): (a) early exponential growth phase; (b) exponential growth phase; (c) stationary growth phase.
9. Quantitative Study of the Rheology of *Escherichia coli*

**Alkaline Lysates**

**9.1. Summary**

Shear stress vs. shear rate plots exhibited non-Newtonian behaviour for all samples, and proved that there was no trend in flow behaviour as a function of fermentation growth phase.

The shear and stress sweeps for the cell suspension exhibited a non-Newtonian shear-thinning rheology, with no distinct elastic component. There was no discernible change in the rheology of alkaline lysates as a function of fermentation growth phase, and the shear and stress sweeps showed evidence of an elastic component at lower strains. Neutralised samples were seen to have a dominant elastic component caused by the gel matrix.

The liquid phase containing plasmid DNA has a low apparent viscosity and a simple rheology that will have a limited impact on the choice of process equipment.

**9.2. Introduction**

This chapter contains an analysis of how the rheology of the alkaline lysis process is affected by the changes cells undergo during fermentation (see Chapter 5).

During the alkaline lysis studies reported in previous chapters, rod climbing of the viscous lysate was experienced after the primary peak of total cell lysis had occurred in the scale-down reactor. An example of this phenomenon, known as the Weisenberg effect, is shown in Chapter 1, Figure 1.7. It is associated with the elastic component of non-Newtonian substances, and is caused by the impact of normal stresses (perpendicular to the direction of shear), on viscoelastic fluids. It is indicative of a strong elastic component in the fluid. In Chapter 1, section 1.4.2, viscosity is quoted as being the most important property affecting flow behaviour of a fluid. However, it is also important to characterise the elastic property of *E.coli* alkaline lysates, to
ensure the elastic component will not effect the processing of plasmid DNA, especially pertinent if feed streams are pumped (Chapter 1, section 1.4.4.1).

### 9.2.1. Aims

The above observation led to the decision to conduct a more thorough investigation into the impact of fermentation time on the elastic component of the *E. coli* alkaline lysates and to see whether the elastic component was sufficiently large as to affect the large-scale industrial alkaline lysis process step, at either the lysis stage and/or the neutralisation stage.

### 9.3. Materials and Methods

This section supports the detailed protocols listed in Chapter 2, by providing information on methods particular to parts of this study.

In this study the magnitude of the elastic component of cell culture samples after treatment with alkaline lysis solution and after neutralisation were measured. A number of standard rheological tests were performed to see how the magnitude of the elastic component changed as a function of fermentation growth phase. The tests included up and down shear sweeps, shear stress analysis and oscillatory strain sweeps to derive data on the storage modulus, loss modulus and phase angle of the lysate. Cells resuspended in TE buffer, with no further treatment were tested as a control.

#### 9.3.1. Fermentation and Source of Experimental Samples

Both 3 L and 7 L Applikon vessels were employed for batch fermentations, with 2 L and 4 L working volume respectively, using CFM medium. The fermentations were run and sampled using the method described in Chapter 2, section 2.3.

#### 9.3.2. Alkaline Lysis and Neutralisation Step

The Bohlin rheometer was employed as the scale-down reactor for this study. Samples for rheological analysis were either resuspended cell paste, cell paste that had undergone alkaline lysis, and cell paste that had been lysed and then neutralise. The protocols for alkaline lysis and subsequent
neutralisation were as described in Chapter 2, section 2.4. All experiments were carried out at a constant temperature of 20°C, with a x10 sensitivity setting.

9.3.3. Rheological Techniques

9.3.3.1. Shear Stress vs. Shear Rate

The method for developing stress diagrams by measuring how shear stress changes as a function of shear rate is described in Chapter 2, section 2.4.3.3. Samples from the fermenter that were analysed included resuspended cells that had no prior treatment, cell samples that had been lysed for 300 s, and samples that had been lysed and subsequently neutralised. Samples that were treated prior to rheological analysis were sampled from the fermenter at early exponential, exponential and stationary phases.

Resuspended cell samples that were analysed without any prior treatment were prepared by resuspending cell paste in TE buffer at a concentration of 12.5 g in 100 mL buffer. 12 mL were then loaded into the cup of the rheometer and the experiment begun.

In order to examine the rheological characteristics of cell samples that had undergone treatment with the alkaline lysis solution, early exponential, exponential and stationary samples were lysed for 300 s at 461 s\(^{-1}\) shear rate, using the protocol described in Chapter 2, section 2.4.1. The rheometer was then stopped and rheological analysis started.

The rheological analysis of cells that were lysed for 300 s at 461 s\(^{-1}\) shear rate and then neutralised as described in Chapter 2, section 2.4.2 was also performed on all the cell sample growth phases as explained above.

9.3.3.2. Shear Sweeps

Shear sweeps and oscillatory strain sweeps were performed on cell samples that had been treated with alkaline lysis solution, treated and then neutralised, and resuspended cells that had no treatment. All conditions were as described in the previous sub-sections. The methods for shear sweeps and strain sweeps are described in Chapter 2, section 2.4.3.1 and 2.4.3.2
respectively.

9.4. Results

This section reviews the data collected during the practical work on the changing rheology of alkaline lysates. The co-axial rheometer was used as a reactor for preparing alkaline lysates and neutralised samples instead of a low shear rate vessel such as a universal as there was no pronounced difference in elastic component observed either in lysates produced in the scale-down reactor or in universals.

9.4.1. Shear Stress vs. Shear Rate Analysis

The results of these studies are shown in the stress diagrams in Figure 9.1 and the data presented in Table 9.1. The data presented in Figure 9.1(a) is for cell culture material lysed in the scale down reactor before undergoing shear sweep analysis. As a control and for comparison between lysed and neutralised stress diagrams, cell culture suspended in TE buffer with no further treatment was also analysed. The curves show that the alkaline lysis samples exhibit very little change in rheology as a function of fermentation growth phase, with any differences fitting within the expected error of the scale-down reactor. The curve representing cells suspended in TE buffer, and hence not treated with alkaline lysis solution prior to the shear stress analysis appears below those for the lysis samples. All of the samples display non-Newtonian behaviour and would appear to exhibit shear-thinning characteristics, as seen in the stress diagrams in Chapter 1, Figure 1.5.

By fitting the power law model to the data represented in Figure 9.1(a) it is possible to quantify the consistency index \( K \) and the flow behaviour index \( \varepsilon \) for the variables (see Chapter 1, section 1.4.5). The results of this analysis are listed in Table 9.1. The consistency index reflects the apparent viscosity, and the \( K \) values reflect the apparent viscosity of the samples. The results for the flow behaviour index confirm the evidence of shear-thinning behaviour seen in the stress diagrams in Figure 9.1(a). The untreated cell suspension had the lowest \( K \) value, suggesting the lowest apparent viscosity. The results for the lysed cells showed that culture sampled at exponential
growth phase has the highest value for K, followed by stationary and then early exponential growth phase material. Neutralised samples exhibited a higher apparent viscosity overall than that seen in lysed cells. The results support the apparent viscosity changes observed in Chapter 6.

The flow behaviour index confirms that all the samples tested exhibit non-Newtonian, shear-thinning properties. The cell suspension samples proved to exhibit the most shear-thinning attributes, and the exponential sample the least shear-thinning, followed by stationary and early exponential samples. However, the difference between the untreated and lysed samples is greater than the difference between the individual lysed samples.

Figure 9.1(b) presents the stress diagrams for cell culture samples lysed in the scale-down reactor, subsequently neutralised and then analysed. It also repeats the plot for untreated cell suspension to allow comparison between Figures 9.1(a) and 9.1(b). The plots for neutralised samples have a shallower gradient than for cell culture samples that have only been lysed. There is also a greater spread between the individual curves than in the former graph. Table 9.1 lists the power law model indices for the neutralised samples. The K values are larger than with samples that were just lysed, indicating a higher apparent viscosity for neutralised samples. The cell culture sampled during stationary phase has the highest K value, followed by early exponential and then exponential phase samples. This trend is different from that seen in the lysed samples.

The flow behaviour index data shows all the neutralised samples to be very non-Newtonian and shear-thinning, more so than either the untreated cell suspension or the lysed samples. Again the flow index for the stationary growth sample is far more extreme than either of the other samples, both of which have similar values.

9.4.2. Shear Sweep Analysis

This sub-section considers the data for the shear sweep studies conducted on the untreated cell suspension (used as the control), the lysed samples and the neutralised samples.
Table 9.1: Table shows the flow behaviour index ($\varepsilon$) and consistency index ($K$) for resuspended cells, lysed cells and neutralised cells. The gradients are taken from the shear stress vs. shear rate graphs shown in Figure 9.1. The formula for the gradient is: $\log \tau = (M \times \log \gamma^0) + C$. When fitted with the Power Law model ($\tau = \eta_0 (\gamma)^n$) the equation becomes: $\log \tau = \varepsilon (\log \gamma) + K$.

9.4.2.1. Untreated Cell Suspension

Figure 9.2(a) represents the data for the shear sweeps performed on samples of untreated cell suspension. The graph shows how apparent viscosity changes as a function of an increase in shear rate up to 1160 s$^{-1}$ (the up sweep) and then back to stand-still (the down sweep).

From the graph it can be seen that the cell suspension exhibits classic shear-thinning behaviour at low shear rates during the up sweep until a particular shear rate is reached (in this case above 50 s$^{-1}$) and the sample then displays Newtonian characteristics, with no change in viscosity, with shear rate.
The down sweep produces Newtonian flow at high shear rates; at low shear rates there is a slight hysteresis of the plots, as the apparent viscosity of the down sweep is slightly above that of the up sweep. This indicates the presence of mild time dependent rheopectic behaviour, as the viscosity rises over time.

9.4.2.2. Lysed Samples

There is no discernable change in the shear sweep rheology over the time course of a fermentation. Any differences are within the expected accuracy of the machine. Figure 9.3(a) therefore is representative of the shear sweep curve for all the cell culture growth phases.

The up sweep produces shear-thinning behaviour for a brief shear rate range (from the start until 100 s\(^{-1}\)), followed by a period of mild shear-thickening between 200 s\(^{-1}\) and 800 s\(^{-1}\), before returning to shear-thinning behaviour at high shear rates above 800 s\(^{-1}\).

The down sweep shows shear-thinning behaviour, but there is some hysteresis as the down sweep has a higher apparent viscosity at lower shear rates. This indicates some increased time dependent rheopectic behaviour.

9.4.2.3. Neutralised Samples

Figure 9.4(a) shows the typical shear sweep behaviour for all the neutralised samples, regardless of the cell culture growth phase at which the sample was removed. The up and down sweeps exhibit shear-thinning behaviour with no hysteresis. At very high shear rates (above 900 s\(^{-1}\)) the flow behaviour becomes Newtonian.

9.4.3. Oscillatory Strain Sweep Analysis

9.4.3.1. Untreated Cell Suspension

The data presented in Figure 9.2(b) shows the results of the strain sweep analysis of the untreated cell suspension. The phase angle data shows that the viscous component dominates within this sample, and although the elastic (storage) modulus (G') appears above the shear (loss) modulus (G'') at the
end of the cycle, they are so close together as to have little impact on the elastic component. Table 9.2 shows the results of Tan (Phase angle), and it is clear that for this sample the viscous component dominates. The variation seen in the phase angle curve is probably due to increased noise as the rheometer was run at x10 sensitivity (see section 9.3.3.), and hence any background disturbances are amplified.

9.4.3.2. Lysed Samples

Figure 9.3(b) represents the strain sweep analysis of the lysis samples. It is again presented as one figure as there were no distinct differences as a function of fermentation time. The graph clearly shows that the storage modulus (G') remains above the loss modulus (G'\") throughout the amplitude range, although there is a reduction in the difference between the two moduli (in Pa). The phase angle is very low at low amplitudes, but after 52 % it increases rapidly to a value of over 80 \(^\circ\). Table 9.2 supports this evidence, showing the Tan (Phase angle) to be below 1 at low amplitude and above 1 at higher amplitudes. The exception is for stationary samples. Unfortunately this anomaly could not be tested further due to equipment failure, but judging by data in Figure 9.1(a) and Table 9.1, it is expected that the stationary sample data would not differ much from the early exponential and exponential data, and so it is reasonable to reject the stationary data.

9.4.3.3. Neutralised Samples

The data for the neutralised samples is presented in Figure 9.4(b) and shows a very clear picture: the elastic component dominates even at high amplitudes, although there is a slight reduction in storage (G') modulus as a function of increasing amplitude. The phase angle remains at 20\(^\circ\) throughout the strain sweep and Table 9.2 shows that all neutralised samples have a Tan (Phase angle) value remaining below 1, regardless of the amplitude.
Table 9.2: The impact on the phase angle (Tan (δ)) of strain/amplitude applied during the strain sweep experiments, for resuspended cells, alkaline lysates and neutralised samples.

9.5. Discussion

9.5.1. Rheology of Untreated Cell Suspension

The untreated cell suspension was used as the experimental control. The data collected from the shear sweep analysis proved, that the cell suspension was non-Newtonian and shear-thinning, with a low overall apparent viscosity. This behaviour is very similar to fermentation media such as *E. coli* grown in LB medium. The plot of apparent viscosity against shear rate (see Figure 9.3) showed shear-thinning behaviour followed by Newtonian flow above a shear rate of 50 s⁻¹. There was some indication of hysteresis on the down sweep, showing time dependent rheopectic flow. This increase in apparent viscosity over time may be due to coagulation of cells perhaps aided by the fact that the cells were suspended in a buffer that reduces osmotic stress and not fermentation medium.

9.5.2. Rheology of Alkaline Lysates

The results of the rheological study show that there is no significant change in the shear stress vs. shear rate relationship as a function of fermentation growth phase, with the flow behaviour and consistency indices each being
slightly higher for exponential phase, almost certainly due to the increase in chromosomal DNA content observed and proven in Chapters 6 and 7 respectively.

The shear sweeps were seen to be almost identical for all the lysis samples, irrespective of fermentation growth phase. Figure 9.3(a) shows the up sweep expressing shear-thinning behaviour initially followed by shear-thickening. This shear-thickening behaviour is due to the aggregation of the chromosomal DNA to form the secondary peak. The behaviour becomes shear-thinning again past 600 s⁻¹, as the chromosomal DNA is sheared. The down sweep shows the same shear-thinning behaviour initially. At low shear rates there is a hysteresis, showing time dependent rheopectic behaviour. This is due to chromosomal DNA aggregating again at the lower shear rates, but it can be said that overall there is shear-thinning behaviour.

The impact of the elastic modulus is shown in Figure 9.3(b). Again, there is no real change in elastic component as a function of fermentation time, with any differences well within the accuracy of the machine (see Chapter 2, section 2.4.3). However, the elastic component is visible, and this would cause the rod climbing observed. The phase angle data shows that as the strain is increased above 52% the viscous component dominates. This is probably due to the breakdown of the cell wall and chromosomal DNA interactions that most likely cause the elastic response. The tan (phase angle) data supports this evidence, showing the viscous component dominates at higher amplitude/strain. However, the data in Table 9.2 is somewhat difficult to interpret due to the mechanical failure of the rheometer. However, from the other data it is possible to be confident that this data is most likely an artefact of the rheometer upon imminent failure, and not a true reflection of viscous or elastic components.

### 9.5.3. Rheology of Neutralised Alkaline Lysates

In order to study the rheology of neutralised alkaline lysates, samples were lysed for 5 minutes before being neutralised and then analysed using shear and strain sweep analysis.

The results (Figures 9.4 (a) and (b)) show shear thinning behaviour in
both the shear stress and apparent viscosity against shear rate analysis. There was no evidence of any hysteresis in the down sweep, defending the hypothesis that the hysteresis seen for samples prior to neutralisation was due to chromosomal DNA interactions. The shear-thinning behaviour seen in Figure 9.4(a) is due to the shear rate overcoming the elastic component of the neutralised solutions.

Figure 9.4(b) identifies that the elastic component dominates in the neutralised alkaline lysates. The stationary phase has both higher consistency and flow behaviour indices, suggesting that the apparent viscosity and non-Newtonian behaviour is greater in the stationary phase than with early exponential and exponential growth phase materials. This may be due to a larger matrix mass forming from stationary phase samples because this contains a larger amount of cell wall material compared to exponential phase samples. This line of reasoning is supported in Chapter 5 where cell walls were shown to be physically weaker during exponential phase.

9.5.4. Significance of Rheological Results on Industrial Process

The results show that there is a significant difference between the rheology of untreated cell suspension, cell culture treated with alkaline lysis solution and cell culture that has been lysed and then neutralised. Previous work has considered the effect of the rheology of the gel matrix only (Ciccolini et. al., 1999), and this is essentially the waste stream containing chromosomal DNA in the matrix. From the analysis of the neutralised sample it can be seen that the gel matrix dominates the rheology of the two-phase mixture.

The implications of these rheological results on the industrial manufacturing process are varied. With respect to process design these include:

- Mixing should be achieved at low shear rates for the addition of alkaline lysis solution to the cell suspension. During the lysis mixing period the viscous element of the alkaline lysate will dominate, with limited impact from the elastic element at typical levels of shear rate, aiding mixing. At higher shear rates Newtonian flow behaviour will occur.
• After neutralisation the rheology will be dominated by the gel matrix, whose properties will be a dominant factor when pumping this two-phase system to subsequent separation stages.

• The liquid phase containing plasmid DNA will have a low apparent viscosity and therefore have a simple rheology with a limited impact on the selection of process equipment.

• The gel matrix, as it is highly elastic, will require the development of high shear rates (high ΔP) in order to flow easily.

• The viscoelastic properties are not a strong function of fermentation growth phase. And as such, there is no apparent window of operation with regards to an optimum fermentation harvest time. However, it was seen that stationary phase samples that were neutralised had an increased elastic component compared to early exponential and exponential phase neutralised samples, caused by the increased density of the gel matrix due to the presence of more developed cell walls. This suggests that there may be a case for optimum cell harvest time at late exponential growth phase.

9.6. Conclusion

The untreated cell suspension exhibited the expected non-Newtonian shear-thinning behaviour. The rheological analysis of alkaline lysates showed that there was little difference between sample results as a function of fermentation growth phase. There was evidence of an elastic component until a strain greater than 52% amplitude after which the viscous component dominated. Neutralised samples showed a shear thinning rheology with a dominant, elastic component. There was a significant difference between the untreated cell suspension, cell culture treated with alkaline lysis and subsequently neutralised cell sample. The gel matrix dominates the rheology of the two-phase mixture. The liquid phase contains the plasmid DNA and has a simple rheology with a low apparent viscosity, meaning that the rheology will have a low impact on the selection of downstream process equipment.
Figure 9.1: Shear stress vs. shear rate for untreated cell suspension compared with: early exponential, exponential and stationary cells lysed in scale down reactor (A); and early exponential, exponential and stationary cells lysed and then neutralised after 5 minutes alkaline lysis (B).
Figure 9.2: Shear sweep (A) and strain sweep (B) analysis of *E.coli* DH1 /pXY cell paste re-suspended in TE buffer. No treatment with alkaline lysis was performed on the cell paste prior to analysis.
Figure 9.3: Example of shear sweep (A) and strain sweep (B) analysis of *E. coli* DH1 /pXY cell culture that has been treated with alkaline lysis solution for 5 minutes prior to rheological analysis.
Figure 9.4: Example of shear sweep (a) and oscillatory sweep (b) for cell culture that has been treated with alkaline lysis solution for 5 minutes and then neutralised prior to rheological analysis.
10. Overall Conclusions

10.1. Introduction

This part of the thesis summarises the discussions from the previous results chapters and links the different sections of the research back to the original aims – to characterise the scale-down alkaline lysis and to investigate possible windows of operation for fermentation harvest. The chapter also discusses the impact the conclusions from the thesis have on industry.

This chapter is divided into two sections. The first section (10.2.) considers the studies of the changes in physical and chemical cell strength over the time course of a fermentation, the causes of the observed changes in rheology during alkaline lysis, changes in the plasmid DNA quantity and quality over the course of the fermentation and during alkaline lysis, and the elasticity of cell lysates as a function of fermentation time. This section links all the conclusions together to consider the overall aim of characterising the alkaline lysis process and formulating windows of operation for fermentation harvest.

The second section of this chapter (10.3.) considers the possible implications of the thesis results on industrial practice.

10.2. Conclusions

The investigation into characterising the alkaline lysis stage progressed from the previous studies conducted by Ciccolini (see Chapter 1, section 1.5). This scale-down probe technique was utilised in many of the studies for this thesis.

10.2.1. Summary of Conclusions

The first study conducted using scale-down techniques investigated the choice of scale-down reactor on the ability to measure consistent viscosity vs. time profiles during the alkaline lysis process. It was concluded that a rheometer that can achieve a minimum shear rate of approximately 180 s⁻¹, but at moderate torque is required. Despite torque limitations, both the rheometer and the viscometer tested were still capable of showing the primary
peak clearly, although the secondary peak was indistinct. The Bohlin rheometer was therefore used as the scale-down reactor for all subsequent studies characterising the alkaline lysis reaction, as the mechanism for driving the rotating parts could achieve high shear rates at low torque levels.

Previous publications had suggested that cell wall strength changes as a function of fermentation growth phase. To confirm if this was true for the E. coli cell culture propagating plasmid DNA, an investigation into cell wall resistance to physical disruption was performed. The results showed that cell culture sampled from exponential growth phase was less resistant to physical disruption than either early exponential or stationary growth phase.

The next study was aimed at determining if the effect of growth phase on physical cell strength would be reflected by changes in the efficiency of chemical lysis. A distinctive change in the profile was seen over the course of batch fermentations. The primary peak remained stationary throughout the fermentation, implying that cell lysis was unaffected by cell culture growth phase. However the secondary peak and the overall shape of the viscosity vs. time profile altered as a function of batch fermentation time. The time interval between the primary and secondary peaks was seen to increase during exponential growth, along with an increased apparent viscosity of the pseudo-steady state. This change was due to increased levels of contaminant genomic DNA, and not plasmid DNA. The fact that the primary peak remains stationary implies that the currently practised method for alkaline lysis is insensitive to the physiological changes that cell cultures undergo during growth. These results imply that the currently applied protocol for alkaline lysis may be excessive in the levels of reagents used to achieve cell lysis.

The results from the investigation into plasmid yield proved that the yield of plasmid increased both as a function of increasing batch fermentation time and as a function of the time interval between initiation of alkaline lysis and neutralisation. Stationary phase material appears to be the exception to this. The reason for this is not clear, but may be due to plasmid DNA becoming less resistant to irreversible denaturation at the end of a fermentation. Fed batch fermentations proved that maintaining a constant
growth rate resulted in an invariant plasmid yield with increasing fermentation time.

Rheological studies of the neutralised matrix showed that there was little change in the elasticity of cell samples as a function of batch fermentation time. Neutralised samples showed a dominant elastic component. The impact of these results means that the liquid phase containing plasmid DNA will have a low apparent viscosity and simple rheology, whereas the waste stream will have a very strong elastic component, which may make the waste stream difficult to process.

The results have shown that the Bohlin rheometer protocol can also be used as an off-line measurement of chromosomal DNA contamination of alkaline lysates, and accurately measure when the cell-culture is moving from exponential to stationary growth phase.

10.2.2. Characterisation of Alkaline Lysis Process

In this section the impact the conclusions have on the aim to characterise further the alkaline lysis process will be discussed.

Physical cell strength experiments showed that cells were less resistant to physical cell breakage in exponential phase compared to early exponential and stationary phase. When the physical cell strength data was compared to the timing of the primary peak, which corresponds to the point of total cell lysis in the viscosity vs. time profiles, for the three growth phases there was seen to be no correlation between the physical cell lysis results and the chemical lysis data as the primary peak remained at 40 s after initiation of lysis for early exponential, exponential and stationary phase samples. This suggests that the volumes of lysis buffers used may be in excess of that required for the lysis of \textit{E.coli} cells. It has been shown in previous work (Ciccolini, 1998) that timing of the primary peak is strain specific, and therefore for any given cell strain complete cell lysis will occur at the strain specific time for chemical lysis.

The secondary peak magnitude and timing is dependent mainly on the chromosomal DNA content in the cell samples, with plasmid DNA and cellular RNA levels being less significant. During exponential growth of batch
fermentations, the secondary peak is delayed in its formation, as the larger amount of chromosomal DNA takes longer to interact and increase the apparent viscosity. It can therefore be said that the secondary peak of the viscosity vs. time profile is a function of growth phase of the cell culture.

The rod climbing observed during lysis is due to the elastic component within the alkaline lysates. There was no apparent correlation between fermentation time and elastic component, implying that the increase in genomic DNA during exponential growth caused an increase in viscosity alone. At high strains the viscous component of the lysates dominates. Neutralised cell samples have a very strong elastic component, attributed to the gel matrix, and again there is no change in elastic component as a function of fermentation time.

10.2.3. Operational Window for Cell Harvest

A major component of the aim of the thesis was to see if there was a window of operation for optimal cell harvest time, that could be derived from the experimental research investigating the plasmid yield, rheology of the lysates and cell strength as a function of fermentation time. The conclusions from this section are summarised in Table 10.1.

The data generated from the physical cell strength experiments raised the question of whether it would be advisable to harvest late in the exponential phase, as opposed to in the stationary phase. The physical cell strength studies showed that cell culture growth phase has a direct impact on cell strength of *E.coli* cells, with exponential cells requiring less pressure to lyse. If using a physical cell disrupter such as a high-pressure homogeniser, then there would be a strong argument for harvesting at late exponential phase instead of stationary phase, as seen in Table 10.1. However, when similar studies were executed using chemical lysis, total cell lysis took the same amount of time, irrespective of fermentation age. It is reasonable to conclude from this that there is no specific harvest time required with respect to optimising the output of the alkaline lysis process using the current protocols.
The experiments investigating the rheology of alkaline lysis were intended to characterise further the alkaline lysis process. The results for viscosity vs. time profiles showed that alkaline lysate viscosity exhibited a dependence on fermentation time, with an increased viscosity during exponential phase. This suggested that, as a fluid with a lower viscosity is easier to process, it would be preferable to harvest late exponential and stationary phase lysates.

<table>
<thead>
<tr>
<th>Experimental Parameter</th>
<th>Optimum Harvest Point</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exponential</td>
</tr>
<tr>
<td>Physical Cell Lysis</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>Chemical Cell Lysis</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>Lysate Rheology</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>—</td>
</tr>
<tr>
<td>Plasmid Quantity</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>—</td>
</tr>
</tbody>
</table>

**Table 10.1:** Table summarising the operational window for harvest time conclusions. The parameters investigated are listed alongside the resultant optimum harvest time. The strength of the parameter is indicated by the symbols. Key: strong positive (+++); positive (++); weak positive (+); negative (—).

The investigation into the elasticity of alkaline lysates proved that after neutralising the alkaline lysis reaction, the elastic component of the gel matrix dominated rheology and this elasticity did not alter as a function of fermentation time. Therefore the elastic component is not a process issue.
when considering fermenter harvest time. As the liquid phase of the neutralised solutions had a very simple rheology it can be assumed that the gel matrix contributes the elastic strength of the neutralised lysates.

Perhaps the two most pertinent conclusions that impact upon a window for optimum cell harvest are derived from the investigation into plasmid DNA quantity and quality. The increase in plasmid yield up to stationary phase inclines the process towards as late a harvest time as possible. The conclusion that fed-batch fermentations showed no great change in rheology or plasmid yield as a function of fermentation age implies that fed-batch fermentations have a very wide window for cell harvest time, and therefore plasmid DNA yield will not be a factor in determining the harvest point of a fed-batch culture.

10.3. Industrial Ramifications of PhD

The aims of this thesis were established with a mind to bring value to the biotechnology industry. This section highlights the areas of the thesis that relate to process issues often found in industry.

A common problem in industry is the need to analyse processes at a small scale, and a small cost. On a large scale economics is an important factor in the development of a process strategy (Rosen, 1985) and so it is important to optimise the process at a small scale. The results from the various rheometer studies (Chapters 4 and 6) showed that more than one rheometer can be used as a scale-down reactor. The scale-down reactor is a cheap and useful scale down tool, especially as a probe for measuring the impact of varying fermentation parameters on the rheology of alkaline lysates.

It is important for industrial processes to be well characterised and economical due to the large scale at which these processes are often run. Existing lysis protocols may be excessive and thus not as economical as they should be. From the evidence in this thesis, the alkaline lysis solution and neutralisation buffer are too concentrated. Confirmation of this could mean a reduction in running costs.
The dominant rheology of the neutralised alkaline lysates was caused by the high elastic component of the gel matrix. The implications of this fact from an industrial perspective are that the waste stream will have a very elastic rheology, whereas the feed stream to downstream processing will have a simple rheology. Materials with elastic rheology will be difficult to process because of large normal stresses.

There is always a need and desire to enhance the yield of a process. Plasmid quality and quantity studies (Chapter 8) provided two conclusions of industrial significance: firstly the need for a low shear environment (to provide an increased plasmid yield), and secondly the need for a quick mixing time, due to loss in plasmid yield experienced after 100 s of alkaline lysis time for stationary phase samples. This requires a typical engineering solution to achieve a compromise between low shear rates and good mixing.

Two methods of fermentation were utilised in the thesis: batch and fed-batch. It is important to know which method would be the best one to use for the industrial production of plasmid DNA. The strength of batch fermentation is the ability to produce validated batches of product. The data from the experimental studies suggests that the fed-batch fermentation method would be the best to use, as it produced constant plasmid yield per kg wet cell biomass throughout the fermentation. The implication of this result is that with optimisation the fed-batch fermentation will be able to produce more plasmid DNA after a longer feeding regime.

With the fed-batch protocol, it is clear that the point of harvest of a fed-batch fermentation is not as important as the need for good mixing in the alkaline lysis tank, so as to reduce the alkaline lysis time and hence maximise plasmid DNA yield.

The next chapter considers the experiments that would naturally follow on from the thesis, if further studies were to be carried out.
11. Further Studies

It is the nature of research that there are often more questions as a result of any experimental investigation than at the outset. This chapter considers some of the key issues raised by the thesis work that may warrant further investigation.

If the research were to be continued, the next stage in the practical work would be to investigate and characterise the pH conditions and control of the alkaline lysis reaction, as pH is a vital parameter in the alkaline lysis process, and its impact on plasmid yield needs to be well characterised and understood. Another issue regarding the alkaline lysis was raised during the investigation into the complete cell lysis time, where there was no correlation between physical and chemical cell strength, and this raised the question of whether the alkaline lysis solutions used are too extreme. It would be useful to devise experiments investigating the impact of both differing solution concentrations, and the pH of the solution on the primary peak timing of the viscosity vs. time profile and plasmid DNA yield.

There are also a number of other studies that could enhance the results and conclusions from this thesis. The first regards the plasmid itself. All of the work conducted for this thesis used relatively small (~10 Kb), high copy number plasmids. With the future interest in plasmid DNA likely to focus on larger plasmids (Levy et al., 2000), it would be of significant interest to study the impact of larger plasmids on the rheology of alkaline lysis and the plasmid quality and yield. This would help to determine the exact impact that plasmid size has on the alkaline lysis process.

Another issue worth investigating further would be the shear sensitivity of the gel matrix formed on neutralisation of the alkaline lysis reaction, and the effect of high shear rates on plasmid yield and subsequent genomic DNA contamination, as shearing the gel may allow DNA back into solution. It may be beneficial to conduct further studies into the optimum shear environment for highest plasmid yield. These studies could be instrumental in developing the fastest mixing time possible at the lowest shear rate, so as to maximise
the plasmid DNA yield from the alkaline lysis process. It would also be worth investigating the plasmid yield experiments using differing shear rates in the scale-down reactor, to quantify any correlation between shear rate and plasmid DNA yield.

This leads on to the next piece of work that would complement this thesis, and that would be to understand why the plasmid yield decreases after 100 s neutralisation time for cell culture sampled at stationary phase. This becomes more poignant when the fed-batch fermenter results are considered – cell culture sampled from fed-batch fermentations exhibited a significant decrease in plasmid yield after 100 s neutralisation time throughout the time of the fermentation. It is not known for certain why this occurred, and is an important process consideration.

These additional investigations would help to answer many of the questions raised by the present practical work, and augment our understanding of interactions between fermentation and primary processing conditions for the reliable and consistent realisation of high quality plasmid DNA produced at scale.
Appendix A

Additional Models For Defining Rheology Of Fluids

This appendix complements the power law model for shear thickening and shear thinning fluids described in Chapter 1, section 1.4.5 by listing a number of other models describing non-Newtonian fluids.

Bingham Plastic

Bingham plastic fluids require a finite stress before continuous deformation occurs. This is displayed clearly in the rheogram shown in figure 1.5, where an "infinite" viscosity is exhibited until a sufficiently high stress is applied, at which point flow begins. In the rheogram of a Bingham plastic material, once flow is initiated, a constant slope called the infinite shear viscosity is described. The simplest yield-stress material as shown in equation A1:

\[ \tau = \tau_y + \eta_s \gamma \]  
(Equation A1)

Where \( \tau_y \) is the Bingham yield stress, or limiting shear stress. Highly concentrated suspensions of fine solid particles frequently exhibit this behaviour.

Herschel-Bulkley

This model incorporates the elements of the three previous models, and is used to define many industrial fluids when designing process plants (www.bohlinusa.com). The model is described in equation A2:

\[ \tau = \tau_y + \eta_s (\gamma^*)^c \]  
(Equation A2)

Special cases of the model:

A pure Newtonian material has limiting stress = 0 and power law index = 1.

Power law fluid has limiting stress = 0 and power law index \( \neq 0 \).

A Bingham fluid has limiting stress \( \neq 0 \) and power law index = 0.
Models For Complex Flow Behaviour

The following models have been developed to enhance the fundamental relationships described above – they can give a more realistic prediction of flow over a wide range of conditions.

- **Ellis**: this model describes materials with power law behaviour at high shear rates but Newtonian behaviour at low shear rates; this model is often used to describe polymeric systems, and can give a better representation than the power law model (www.bohlinusa.com).

  \[ \gamma^* = K_1 \tau + K_2 (\tau)^\varepsilon \]  
  \hspace{1cm} (Equation A3)

  Where \( K_1 \) and \( K_2 \) are sample constants and \( \varepsilon \) is the index of the material.

- **Casson**: this model is used for materials that have Newtonian flow at stresses much higher than the material’s yield stress, and is often used for suspensions, and molten chocolate (www.bohlinusa.com). The Casson model relates the shear stress produced to the shear rate applied through the equation A4.

  \[ \tau^{1/2} = (\tau_y)^{1/2} + K(\gamma^*)^{1/2} \]  
  \hspace{1cm} (Equation A4)

  Where \( K \) is the materials constant.

- **Moore**: flow properties over a wide range of shear rates can be predicted using this model as it incorporates terms for both limiting low shear rate and high shear rate viscosities (www.bohlinusa.com).

- **Cross**: acts as an extension to the Moore model and is often used to describe accurately the shear thinning behaviour of disperse systems (www.bohlinusa.com).

  \[ \eta = \eta_0 + (\eta_0 - \eta_\infty) / \left[ \left( 1 + K(\gamma^*)^\delta \right) \right] \]  
  \hspace{1cm} (Equation A5)

  Where \( \eta_\infty \) is a constant viscosity attained at very high shear rates, and \( \eta_0 \) is a finite value or zero rate viscosity; \( K \) is the material’s constant.

- **Sisko**: can be used to describe a material with a limiting high shear rate viscosity, although it does not generally describe a material with yield (www.bohlinusa.com).
Appendix B

Calibration of Agarose Gels Using Markers

This appendix demonstrates how the agarose gels used in Chapters 7 and 8 were calibrated in order to calculate the plasmid DNA yields. The data was obtained from running supercoiled DNA ladders on agarose gels and measuring the band area using Scion Imaging (see Chapter 2, section 2.6.4 for detailed protocol).

Mass of DNA per Band

<table>
<thead>
<tr>
<th>Vol of Marker Added (μL)</th>
<th>Total DNA Mass in Well (ng)</th>
<th>DNA Mass per Band</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>100</td>
<td>9.1</td>
</tr>
<tr>
<td>10</td>
<td>200</td>
<td>18.2</td>
</tr>
<tr>
<td>15</td>
<td>300</td>
<td>27.3</td>
</tr>
</tbody>
</table>

Average Peak Areas Around Target Plasmid Size

<table>
<thead>
<tr>
<th>Vol of Marker Added (μL)</th>
<th>Approx DNA Size for Band (bp)</th>
<th>Average Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6000</td>
<td>7500</td>
</tr>
<tr>
<td>5</td>
<td>118</td>
<td>156</td>
</tr>
<tr>
<td>10</td>
<td>185</td>
<td>228</td>
</tr>
<tr>
<td>15</td>
<td>237</td>
<td>247</td>
</tr>
</tbody>
</table>
Agarose Gel Calibration Curve

\[ y = 5.4449x + 98.633 \]
Appendix C

Glossary

Alkaline lysate: Upon alkaline lysis of the cell paste and release of intracellular material, a crude alkaline lysate is formed.

Alkaline lysis process: Involves three steps: cells are suspended in TE buffer, lysed using a solution of SDS-NaOH and then neutralised with chilled potassium acetate.

Early exponential growth phase: Replaces lag phase when large inoculum used. However, cell culture has similar characteristics to lag phase.

Fermentation time course: Time over which *E. coli* cell culture achieves maximum cell biomass from small inoculum in fermenter.

Lag growth phase: Time taken for cells in inoculum to adjust to new conditions and begin growing.

Primary Peak: Point on viscosity vs. time profile at which total cell lysis occurs.

Pseudo steady state: Period of stable apparent viscosity on viscosity vs. time profile caused by shear thinning of cellular contents.

Secondary peak: Point on viscosity vs. time profile at which released intracellular contents interact to form matrix.

Stationary growth phase: Point of fermentation where death of cells equals growth of cells, usually caused by exhaustion of substrate or production of toxic by products.

Time of neutralisation: Amount of time after initiation of alkaline lysis that chilled potassium acetate is added to alkaline lysate.

Viscosity vs. time profile: Characteristic profile generated by measuring the apparent viscosity of the alkaline lysis reaction in a scale-down reactor.
## Appendix D

### Nomenclature

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>Concentration of unlysed \textit{E.coli} cells at time $t$</td>
<td>\text{cells/mL}</td>
</tr>
<tr>
<td>$C_0$</td>
<td>Concentration of unlysed \textit{E.coli} cells at time 0</td>
<td>\text{cells/mL}</td>
</tr>
<tr>
<td>$D_e$</td>
<td>Deborah number</td>
<td>[-]</td>
</tr>
<tr>
<td>$G$</td>
<td>Rigidity modulus</td>
<td>\text{Pa}</td>
</tr>
<tr>
<td>$G'$</td>
<td>Elastic / storage modulus</td>
<td>\text{Pa}</td>
</tr>
<tr>
<td>$G''$</td>
<td>Viscous / loss modulus</td>
<td>\text{Pa}</td>
</tr>
<tr>
<td>$G^*$</td>
<td>Complex modulus</td>
<td>\text{Pa}</td>
</tr>
<tr>
<td>$K$</td>
<td>Consistency index</td>
<td>\text{Pa.s}</td>
</tr>
<tr>
<td>$M$</td>
<td>Torque</td>
<td>\text{N m}</td>
</tr>
<tr>
<td>$N$</td>
<td>Number of cells counted / homogeniser passes</td>
<td>[-]</td>
</tr>
<tr>
<td>$N_1$</td>
<td>Normal stress difference 1</td>
<td>\text{Pa}</td>
</tr>
<tr>
<td>$N_2$</td>
<td>Normal stress difference 2</td>
<td>\text{Pa}</td>
</tr>
<tr>
<td>$OD$</td>
<td>Optical density absorbance at $x$ nm</td>
<td>[-]</td>
</tr>
<tr>
<td>$R$</td>
<td>Protein release at 300 bar</td>
<td>\text{mg/mL}</td>
</tr>
<tr>
<td>$R_m$</td>
<td>Maximum protein release at 1200 bar</td>
<td>\text{mg/mL}</td>
</tr>
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</table>

### Greek Letters

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
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</thead>
<tbody>
<tr>
<td>$\delta$</td>
<td>Phase angle</td>
<td>[$^\circ$]</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>Flow behaviour index</td>
<td>[-]</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>Strain</td>
<td>[-]</td>
</tr>
<tr>
<td>$\gamma^*$</td>
<td>Shear rate</td>
<td>[s$^{-1}$]</td>
</tr>
<tr>
<td>$\eta$</td>
<td>Viscosity</td>
<td>\text{mPa.s}</td>
</tr>
<tr>
<td>$\eta_a$</td>
<td>Apparent viscosity</td>
<td>\text{mPa.s}</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>Characteristic relaxation time</td>
<td>[s]</td>
</tr>
<tr>
<td>$\mu_{\text{max}}$</td>
<td>Maximum specific growth rate</td>
<td>[s$^{-1}$]</td>
</tr>
<tr>
<td>$\rho$</td>
<td>Density</td>
<td>\text{Kgm}^{-3}</td>
</tr>
<tr>
<td>$\tau$</td>
<td>Shear stress</td>
<td>\text{Pa}</td>
</tr>
<tr>
<td>$\Omega$</td>
<td>Angular velocity</td>
<td>[rad s$^{-1}$]</td>
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</tbody>
</table>
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Andrew F. Day

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