Plasmid fermentation for
gene therapy
and vaccination

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Abstract

Plasmid-based gene therapy and vaccination require the production of purified plasmid DNA. There is a current understanding that supercoiled plasmid DNA is the best form for administration and FDA guidelines state that a specification for the minimum level of supercoiled plasmid DNA, in the final product, should be made. Currently, plasmids of 10 kb or smaller are being used in gene therapy trials, but if plasmid-based vaccination is to become a reality then much larger plasmids, with the ability to carry multi-variant genes, will be required, this raises questions of feasibility of production. This study examined two main issues. Firstly a fed-batch strategy to maximise the level of supercoiling of a 6.9 kb plasmid resulting from fermentation of a recombinant *Escherichia coli* strain was developed. Secondly issues relating to the production of larger plasmids, for gene therapy and vaccination, were examined.

A fed-batch fermentation strategy for the production of a 6.9 kb plasmid, pSVβ, in *E. coli* DH5α, on a semi-defined medium, was developed. Starvation of amino acids was shown to induce plasmid amplification and in batch fermentation a maximum biomass of 3.5 g/L dry cell weight (DCW) and maximum plasmid yields of 40 mg/L and 11.3 mg/g DCW were achieved. However, amplified plasmid levels were not maintained. After 31 h 90% of plasmid was in the supercoiled form but only remained so for 1 h. Maximum plasmid yield and maximum plasmid supercoiling did not occur simultaneously. Hence a strategy to delay amplification was investigated. A dual feeding strategy consisting of a linear amino acids feed and an exponential D-glucose feed was employed. In fed-batch culture a mean maximum biomass of 4.9 g/L dry cell weight and mean maximum plasmid yields of 44 mg/L and 9.1 mg/g DCW were achieved. 90% of plasmid was in the supercoiled form after 25 h and remained at this level until harvest at 35 h.

An important consideration in the production of large plasmids is the ability to supply sufficient oxygen to the fermentation. *E. coli* DH5α harbouring either the plasmid pBGS18, a 4.4 kb pUC-based plasmid, or pQR150, a 20 kb derivative of pBGS18, was grown in D-glucose-limited
chemostat culture to investigate the effects of plasmid size and recombinant protein production on oxygen demand. Under conditions where no recombinant protein was expressed the cellular oxygen demand of the two strains was not significantly different. When recombinant protein was expressed cells harbouring pBGS18 demonstrated a statistically insignificant increase in mean specific oxygen uptake rate while those harbouring pQR150 demonstrated a statistically significant increase. It was concluded that plasmid size does not significantly affect oxygen demand. The increase in oxygen demand reported with an increase in plasmid size by other researchers is due to production of recombinant protein.

The production of a 116 kb plasmid, p5176, in \textit{E. coli} DH10\textbeta on complex and semi-defined media was investigated. Fermentations on complex medium were poorly reproducible while those on semi-defined medium were highly reproducible. Maximum plasmid yields were comparable between the two media, while higher biomass yields and lower oxygen requirements were observed with semi-defined medium. Lysis and primary recovery were investigated and indicated that, using current methods, the manufacture of a large plasmid product may be difficult.

A fed-batch fermentation strategy which allows an increased yield of supercoiled plasmid DNA has been developed. It has been established that oxygen supply is not an issue in the production of large plasmids and a base for the production of large plasmids has been established for future work.
In memory of Professor Rob Cumming.

His love of his subject was an inspiration.


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<th>Definition</th>
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<tr>
<td>AAV</td>
<td>Adeno Associated Virus</td>
</tr>
<tr>
<td>ADA</td>
<td>Adenosine Deaminase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BSE</td>
<td>Bovine Spongiform Encephalopathy</td>
</tr>
<tr>
<td>CER</td>
<td>Carbon Dioxide Evolution Rate</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethylammoniumbromide</td>
</tr>
<tr>
<td>DCW</td>
<td>Dry Cell Weight</td>
</tr>
<tr>
<td>DM</td>
<td>Defined Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DNases</td>
<td>Deoxyribonucleases</td>
</tr>
<tr>
<td>DNS</td>
<td>Dinitrosalycilic Acid</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved Oxygen</td>
</tr>
<tr>
<td>DX5</td>
<td>Design Expert 5</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FSU</td>
<td>Fluorescence Spectroscopy Units</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescence Protein</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes Simplex Virus</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-b-D-thiogalactosidase</td>
</tr>
<tr>
<td>LBM agar</td>
<td>Modified Luriana Bertrani agar</td>
</tr>
<tr>
<td>MCB</td>
<td>Master Cell Bank</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
</tbody>
</table>
X-gal ........................................ 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
1 Introduction

1.1 Gene Therapy

The definition of gene therapy has broadened as work in the area has progressed. The term no longer applies simply to the treatment of an existing genetic disorder by replacement of the defective gene with a functional gene but is now more broadly defined as:

'The use of nucleic acid transfer, either RNA or DNA, to treat or prevent a disease'

(Robbins and Ghivizzani, 1998)

'The treatment or prevention of disease by gene transfer”

(Mountain, 2000)

It encompasses the treatment of inherited genetic diseases, such as cystic fibrosis, and acquired diseases, such as cancer (Miller, 1992; Morgan and Anderson, 1993).

The first human gene therapy experiment was conducted in 1990 by W. French Anderson following his outline of such a protocol in 1987 and a pilot gene transfer experiment in 1989 (Morgan and Anderson, 1993). This initial gene therapy experiment involved the introduction of a normal adenosine deaminase (ADA) gene to T-lymphocytes in order to treat the rare genetic disease ADA deficiency. Sufferers of ADA deficiency fail to produce ADA protein which is required for normal immune system function. This initial gene therapy experiment demonstrated that T-cells could be safely harvested from a patient, subjected to genetic change and re-implanted into the patient. Results from this trial were encouraging, T-lymphocytes rose to normal levels and ADA protein was produced at 25% of normal levels.

There are currently 636 completed, ongoing or pending gene therapy clinical trials world wide (Wiley, 2003). Figures 1.1 to 1.3 illustrate the diseases that are currently being treated in clinical trials, the proportion of trials taking place on each continent and the proportion of protocols at each stage of clinical trials respectively. The majority of clinical trials have been or are being
carried out in the USA and, to date, cancer gene therapy has been most prevalent in clinical trials. While the majority of current cancer trials are concerned with gene therapy there are some trials which are investigating the use of genetic vaccination for the treatment of cancers. Vical are evaluating patients treated with their immunotherapeutic vaccine, Allovectin-7®; in Phase II clinical trials (Vical, 2003; National institutes of Health, 2003).

Of the 636 recorded trials only four have fully progressed to phase III and a further five are at phase II/III. Of these nine trials 89% are concerned with the treatment of various cancers and 66% are taking place in the USA (Wiley, 2003). Gene therapy and vaccination show much promise but there is clearly some way to go before gene therapy treatments are readily available in the marketplace.
Chapter 1: Introduction

Figure 1.1. Gene therapy trials split by disease treated.
Figure 1.2. Gene therapy clinical trials split by continent.

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Figure 1.3. Gene therapy clinical trials split by trial stage.
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1.2 DNA Vaccines

In addition to the correction of genetic diseases gene delivery may also be used in vaccination, for disease prevention. Certain diseases such as malaria, Human Immunodeficiency Virus (HIV) and tuberculosis require a cellular immune response in addition to the antibody immune response (Gurunathan et al., 2000a). Currently licensed vaccines induce effective antibody responses but only live vaccines are able to effectively induce cellular immunity. The wide scale use of live vaccines is not attractive due to manufacturing and safety constraints. DNA vaccines mimic live vaccines but do not pose the same safety concerns, additionally they may be produced cost effectively and are easier to store than live vaccines (Gurunathan et al., 2000b).

DNA vaccines have been shown to elicit immunity in animal models (Robinson et al., 1996; Ulmer et al., 1993). In the study by Ulmer et al. both humoral and cellular immunity were demonstrated. DNA vaccines result in expression of foreign proteins which elicit an immune response in the host and hence confer immunity to pathogens displaying these proteins. Unlike currently available vaccines DNA vaccines pose no risk of infection. However, at present the major barrier to using DNA vaccines is their low potency in humans. Clinical trials have required doses of 1 - 5 mg by intra-muscular injection in order to achieve immune responses (Donnelly et al., 2003). The requirement for such doses in general clinical usage could make it difficult to commercially produce DNA vaccines at reasonable cost. Therefore attention is being focused on improving the effectiveness of DNA vaccines in order to reduce the required dose and therefore the cost of the final drug product.

1.3 Gene Delivery

Gene delivery can be categorised as ex vivo or in vivo and the vectors used to deliver the genetic material can be categorised as viral or non-viral.
1.3.1 *Ex vivo*

The *ex vivo* approach to gene delivery has been historically predominant (Gottschalk and Chan, 1998). In this approach target cells are removed from the patient by biopsy. The cells are transfected with the desired genetic material and are cultured *in vitro* until a sufficient number of cells are available for return to the patient. This method of gene delivery has a high gene transfer efficiency, especially when retroviral vectors are used to deliver the genetic material. It also allows for selection of transformed cells when a selectable marker is used. The drawbacks to the method are that it is elaborate, time consuming – in the order of months – and labour intensive. The procedure is inconvenient to the patient and must be tailored for each individual. In addition there are concerns that cellular changes may occur during the passage *in vitro* (Marquet *et al.*, 1995). *Ex vivo* gene delivery is only considered suitable for skin, endothelium, haematopoietic and tumour cells (Gottschalk and Chan, 1998).

1.3.2 *In vivo*

The *in vivo* approach to gene therapy involves direct transfer of genetic material to cells in the body, by injection or inhalation (Gottschalk and Chan, 1998). The *in vivo* approach has several potential advantages over the *ex vivo* approach. The gene therapy can be administered in much the same manner as a traditional pharmaceutical and the same treatment can be used for all patients suffering from the same disorder. The use of non-viral vectors negates the need to use viral components which may cause an immune response or trigger an infection. Current, proven economical methods of manufacturing may be employed. The potential drawbacks to *in vivo* gene delivery are lower gene transfer efficiencies and non-stable integration. Advances in vector optimisation have, however, allowed *in vivo* gene therapy to achieve expression levels comparable to those achieved using viral vectors (Marquet *et al.*, 1995). In some cases the lack of stable integration may be advantageous, for example in vaccination of a healthy population where foreign gene integration is undesirable and in cancer therapy where transient expression may induce tumour destruction (Marquet *et al.*, 1995). Although the *ex vivo* approach to the
administration of genetic material was historically predominant, the majority of clinical trials now use \textit{in vivo} methods of delivery (Wiley, 2003).

1.3.3 Delivery vectors

There are two broad methods of achieving gene delivery, viral and non-viral. Figure 1.4 illustrates the use of different delivery vectors in gene therapy trials. Viral vectors remain predominant but the use of plasmid DNA, either naked or complexed, such as in lipofection, is increasing.
Figure 1.4. Gene therapy clinical trials split by delivery vector

AAV; Adeno-associated virus, HSV; Herpes Simplex Virus. Other includes trials where the vector type was not reported. Reproduced with permission from the Journal of Gene Medicine web site http://www.wiley.co.uk/genmed. © 2003 John Wiley & Sons Ltd.
1.3.3.1 Viral vectors

Viral vectors are generally more efficient in transfecting target cells (Gottschalk and Chan, 1998; Robbins and Ghivizzani, 1998; Wu and Ataai, 2000) and have the ability to target specific cell types. Engineering of viruses to remove their pathogenicity whilst retaining their efficiency of gene transfer and expression renders them suitable vectors for gene delivery. The use of viral vectors does, however, carry safety concerns.

In 1999 the first gene therapy death occurred (Boyce, 1999). Jesse Gelsinger died after receiving gene therapy administered using an adenoviral vector. Adenovirus can provoke an immune response (Gottschalk and Chan, 1998; Robbins and Ghivizzani, 1998; Boyce, 1999), and in the case of Jesse Gelsinger this response was dramatic and lead to his death. The other participants in the trial only exhibited fever upon administration. This incident raised concerns relating to the safety of adenoviral vectors but it was found that the clinical procedures had been poorly handled and the virus itself was not the problem. Adenoviral vectors remain the second most common vectors in gene therapy trials. More recently, three clinical trials for the treatment of Severe Combined Immuno-deficiency (SCID) were suspended in the USA following the development of Leukaemia in two of ten boys treated for the disease in France in 1999 (Sedlak, 2003). The boys were treated in an ex-vivo protocol which used retroviral vectors to deliver the genetic material. Retroviruses integrate into the host chromosome in a random fashion and hence the site of integration can not be controlled (Calos, 1996). It was found that in the two boys who developed Leukaemia the therapeutic gene had been inserted in or close to a cancer promoting gene (Sedlak, 2003). SCID is a fatal disease, whereas Leukaemia although serious is treatable, it may therefore remain preferable to use retroviral gene therapy in such cases, this remains a point for debate.
1.3.3.2 Non-viral Vectors

Initial emphasis for gene delivery was centred on the development of viral vectors but increasingly attention is turning to non-viral methods of delivery. Non-viral delivery methods eliminate concerns relating to the transfer of unwanted viral nucleic acid sequences. In addition they are more amenable to the required manufacturing conditions which must be imposed if gene therapy is to be applied wide scale in medicine (Morgan and Anderson, 1993). Steady improvements in non-viral gene delivery systems are expected to lead to an increase in the number of clinical trials involving non-viral vectors in the coming decade (Templeton and Lasic, 1999).

Genes for non-viral delivery are carried on plasmids most commonly produced in E. coli. Present clinical trials use plasmids of commonly less than 10 kb but as the field moves forward it is expected that larger plasmids will be required (Levy et al., 2000). It has been indicated that the effectiveness of DNA vaccines may be improved by incorporating genes for signalling molecules into the plasmid (Cohen et al., 1998) and gene therapy is expected to progress to the treatment of metabolic and multi-gene diseases (Newgard, 1992; Yarmush and Berthiaume, 1997). These advances will lead to the requirement of plasmids greater than 10 kb for both vaccination and therapeutic applications. Plasmid DNA may be administered ‘naked’ or may be formulated with a carrier to improve delivery.

1.3.3.2.1 Liposomes

Liposomes are the most widely studied and used of the non-viral vector (Robbins and Ghivizzani, 1998, Templeton and Lasic, 1999). They are spherical colloidal particles which can trap or bind nucleic acids protecting them from environmental factors and delivering them to target cells. Both anionic and cationic liposomes exist, with cationic liposomes being the most frequently used in human gene therapy. Cationic liposomes are able to complex plasmid DNA in to small colloidally stable particles and have higher transfection efficiencies than anionic liposomes.
The use of liposomes for gene therapy has several advantages over viral vector delivery systems. They are non-pathogenic and do not provoke an immune response in the host, hence transfected cells are not destroyed by an immune response and liposomes can be used for repeated therapy administration. Liposomes can be complexed with nucleic acids of wide ranging sizes and do not have an upper limit as is the case with viral vectors. In addition the manufacture of liposomes at large scale is relatively simple and low cost. At present the use of liposomes for gene therapy suffers from low efficiency of delivery and gene expression. Plasmid DNA does not integrate to host DNA in the nucleus but remains in the cytoplasm, however advances have recently been made which have improved transfection efficiency (Templeton and Lasic, 1999).

1.3.3.2.2 Targeted liposomes

To improve the efficiency of gene delivery DNA-liposome complexes can be coated with ligands to allow them to be targeted to specific cell types. Templeton and Lasic (1999) reported that delivery to the liver and subsequent gene expression was increased using DNA-liposome complexes coated with succinyalted asialofetuin.

Ligands can be added to complexes using either ionic interactions or covalent attachments. Ionic interactions are of greater interest as repeated administration using covalently attached ligands can lead to a host immune response (Templeton and Lasic, 1999).

1.3.3.2.3 DNA conjugates

DNA conjugates consist of DNA conjugated to a protein ligand, usually via polylysine, the choice of ligand allows certain cell types to be selectively targeted. The majority of research in this area has concentrated on delivery to the liver. The ligands of choice have been asialoglycoproteins and transferrin as hepatocytes display receptors for these proteins, allowing targeted gene delivery (Morgan and Anderson, 1993). An inherent problem of this method is lysosomal DNA degradation following endocytosis (Morgan and Anderson, 1993). In order to avoid DNA degradation conjugation to whole, inactivated adenovirus has been made. This approach takes
advantage of adenovirus' ability to release itself from endosomes and to transport DNA to the host's nucleus (Robbins and Ghivizzani, 1998). Although this method enhances DNA transport to and uptake by the nucleus, in vivo the presence of adenovirus can elicit an immune response against the targeted cell, leading to a loss of the therapeutic gene.

1.3.3.2.4 Gene gun

DNA coated microscopic gold beads can be fired directly into cells using a helium gene gun, to effect gene delivery. This method has the advantages of cheap and easy preparation and can be used to deliver to non-dividing cells, in addition transfer of unwanted DNA does not occur. Using this method DNA does not become integrated to the host chromosome so expression is transient. The applications for gene therapy using this technology appear to be limited to delivery to skin, to promote wound healing, and to ex vivo gene delivery (Robbins and Ghivizzani, 1998). However this delivery method may be useful in vaccine delivery where transient gene expression is desired and it has been shown to elicit a greater immune response than manual vaccination methods. Bennett et al. (2000) found that the immune response of mice, vaccinated with 4 μg of plasmid DNA using a gene gun, was significantly higher than that of mice vaccinated with 50 μg of plasmid DNA using either intra-dermal or intra-muscular injection. The gene gun could provide a method of achieving an immune response without the need for the high doses currently applied in clinical trials using intra-muscular injection.

1.3.3.2.5 Non-viral-viral hybrids

The use of non-viral-viral hybrids is a gene delivery method which is currently being examined in order to overcome the transient gene expression which is observed using non-viral vectors. Sequences have been identified from both human papilloma virus and epstein barr virus which can allow episomal plasmid replication to take place. Inclusion of these sequences in a plasmid carrying a therapeutic gene may allow gene expression to be extended (Robbins and Ghivizzani, 1998).
1.4 Fermentation of plasmid DNA for gene therapy and vaccine applications

1.4.1 Vector construct

A lot of the work in constructing a vector is concerned with the therapeutic gene and the elements which will regulate its expression in the eukaryotic target cells (Durland and Eastman, 1998). From this perspective the safety and potency of the plasmid should be considered, it should be free of viral sequences and open reading frames and it is recommended that several promoters are evaluated for gene expression in the host cell (Marquet et al., 1995). The vector source materials should be thoroughly characterised and the vector should be sequenced (FDA, 1996a, 1996b). The FDA initially advised that at a minimum the therapeutic gene insert should be sequenced but since the revelation of evidence that the plasmid backbone may affect the activity of a vaccine it is now requested that the entire plasmid is sequenced before clinical trials may commence (Smith and Klinman, 2001).

From a manufacturing perspective the behaviour of the plasmid in its bacterial host is of prime importance. Areas of concern are plasmid yield, plasmid stability and the ability to select for plasmid containing cells.

The copy number and therefore yield of plasmid is determined by the origin of replication. In order to maximise the yield the plasmid origin should be carefully chosen. It is generally recommended that the pUC replication origin is used (Marquet et al., 1995; Durland and Eastman, 1998; Prazeres et al., 1999). pUC based plasmids have high copy numbers and are well characterised and understood, as such they present a very good starting point for vector construction. Other plasmid origins derived from naturally occurring plasmids, which have relatively low copy numbers, have also been described. Wade-Martins et al. (1999) described the use of an F-based plasmid which was produced in E. coli and used to shuttle more than 100 kb of genomic DNA into human cells. In human cells the plasmid was stably maintained at approximately 2 copies per cell for 15 months, in the presence of a selective agent and was
recovered unrearranged. In the absence of selection the plasmid could still be recovered from human cells after 198 days. The correction of a human genetic deficiency, *in vitro*, using an F-based plasmid was also demonstrated by Wade-Martins *et al.* (2000). Although F-based plasmids are produced in *E. coli* at low copy numbers and will therefore not be produced at high yields, that they are stably maintained in human cells makes them suitable candidates for gene therapy, although their production and purification in sufficient quantities will present a challenge. Antibiotic selection is the most common method of ensuring that only plasmid containing cells are able to propagate in culture. Ampicillin is commonly used as a selective marker in the laboratory but its use in manufacturing is not recommended (Marquet *et al.*, 1995; FDA, 1996a, 1996b; Durland and Eastman 1998). Ampicillin is unsuitable for use at manufacturing scale for several reasons. Ampicillin is a β-lactam antibiotic that can cause severe allergic reactions in some people, even at very low levels, the use of ampicillin would therefore pose a safety risk to both manufacturing personnel and patients. Ampicillin has clinical uses as an antibiotic and there is concern that in clinical use antibiotic resistance genes could be transferred from gene therapy plasmids to potential pathogens. Additionally, ampicillin is rapidly degraded in culture and is therefore not a strong selective agent. The FDA recommends that aminoglycoside antibiotics such as kanamycin and neomycin are used as selective markers in place of ampicillin (FDA 1996a, 1996b). These antibiotics have low clinical usage profiles and do not pose a significant risk of allergic reaction to patients (Durland and Eastman, 1998).

Cobra hold a patent (Sherratt *et al.*, 1999), for the selection of host cells with high plasmid copy number, which does not require the use of antibiotics. An essential gene on the host chromosome is linked to an operator such that the binding of a repressor to the operator prevents expression of the gene and renders the cell unable to survive. The host cell is transformed with a plasmid which also carries the operator and preferentially binds the repressor. This results in expression of the essential gene on the host chromosome when the cell contains a sufficient number of plasmids. If the plasmid copy number of the host cell declines below a threshold
number then the essential gene is no longer expressed and the cell is killed. Such a strategy allows the maintenance of a high copy number without the need for antibiotic addition to the growth medium.

The Cobra approach to selection is preferable because it does not require the addition of antibiotics to the fermentation medium and hence removes the risk of antibiotic carryover into the final product and the risk associated with the use of antibiotic resistance markers in a drug product. However, if other companies were to use this approach they would be required to pay royalty fees to Cobra which could be prohibitive. In some cases it may be possible to run a fermentation without antibiotic addition. O'Kennedy et al. (2000) reported that E. coli DH5α-pSVβ in batch culture for 96 h without antibiotic selection. If there was sufficient confidence, resulting from development work, that a fermentation could be run without antibiotic selection then such a strategy would be desirable because it would remove the antibiotic-associated cost and the need to remove antibiotic during downstream processing.

1.4.2 Host selection

The most common host for plasmid DNA production is E. coli. There is no common consensus on preferred genotypes/phenotypes but desirable phenotypes may include those which are able to support a high plasmid copy number, those which display high plasmid retention and those which are compatible with subsequent purification procedures (Durland and Eastman, 1998). For example it would be advisable to avoid the use of a host, such as HB101, which produces large amounts of carbohydrates (Prazeres et al., 1999). The host strain should be attenuated, for example to leucine dependence, to prevent growth outside of the manufacturing conditions and hence aid the containment strategy (Marquet et al., 1995). Currently, well characterised strains of E. coli appear to be favoured, but little has been published on host optimisation for plasmid production for gene therapy. Benefits may be obtained through the evaluation of several strains before the final host/vector system is chosen (Marquet et al., 1995; Durland and Eastman, 1998).
When the final host is chosen details of the source of the bacterial cells along with the genotype, phenotype and procedures used to generate the clonal population should be documented. The FDA requires that these details are submitted as part of a new drug application package (FDA, 1996a)

1.4.3 Cell banks

Cell bank preparation is one of the most critical steps in the successful manufacture of plasmid DNA (Durland and Eastman, 1998). The first cell bank to be prepared is the Master Cell Bank (MCB), which is usually derived from a single colony of the recombinant strain. A single colony is used as the inoculum for the MCB culture. A sample of this culture is combined with a cryoprotective agent, such as glycerol, and frozen in several hundred aliquots. In industry cell banks are generally stored in liquid nitrogen in order to prolong their viability and hence useful storage life. The culture is then characterised to determine strain identity, plasmid identity, viability and plasmid retention levels and to ensure that contaminants such as other bacteria and viruses are not present. Such characterisation is an FDA requirement (FDA, 1996a, 1996b) and should be well documented. If the culture meets the required specifications then the frozen aliquots are accepted as the MCB. The production of the MCB must be well documented because it plays a crucial role, being the starting material for every batch of plasmid which is manufactured.

A Manufacturers Working Cell Bank (MWCB) is prepared from the MCB. A single vial of the preserved MCB is used as an inoculum for a fresh culture, this culture is again characterised and, providing specifications are met, is preserved in the same manner as the MCB. This method allows many batches of plasmid to be produced from a single, well documented source.

1.4.4 Media

Media used in the production of plasmid DNA should be capable of supporting a high plasmid yield. Some complex media have been developed for plasmid production and are commercially
available, it may, however, be preferable to develop a medium empirically for a given host/vector system (Marquet et al., 1995). In general, complex media, usually containing yeast extracts and/or hydrolysed proteins, are able to support higher cell densities than defined media and are simpler to prepare but do not result in as high a degree of reproducibility.

An important aspect for consideration in the development of a medium is the source of medium components. In recent years the high incidence of Bovine Spongiform Encephalopathy (BSE) in the UK and other European countries has raised concerns regarding the use of bovine derived materials in the production of drug substances (Federal Register, 1994). The infective agent of BSE is highly resistant to traditional disinfection and sterilisation procedures and has crossed the species barrier and infected humans, causing variant Creutzfeldt-Jakob disease (vCJD, Collinge, 1999). The use of bovine derived material, originating in a BSE positive country, in the production of a drug may therefore represent a threat to human health. BSE is one of a number of Transmissible Spongiform Encephalopathies (TSEs), which are known to occur in other ruminants such as sheep and goats. It is thought that the spread of BSE in some countries was caused by feeding infected sheep and cattle tissue to cattle, therefore it is possible that the use of any ruminant-derived material could present a threat to human health. Based on this the FDA requested that materials derived from ruminants which have resided in or originated from countries where BSE has been diagnosed should not be used in the production of FDA regulated drugs intended for administration to humans (FDA, 1993a, 1993b; Federal Register 1994). This request was widened in 2000 to prevent the use of materials derived from ruminants which were born, raised or slaughtered in countries in which the United States Department of Agriculture could not determine that BSE did not exist (FDA, 2000). These requests do not completely preclude the use bovine-derived materials in the production of drugs but it is preferable to avoid their use whenever possible. Hence, where possible, medium development should centre on non-ruminant derived components.
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1.4.5 Batch culture

An initial examination of growth yield, \( Y_{x/s} \) (kg biomass/kg substrate), may lead to the conclusion that simply increasing the concentration of substrates in the growth medium will lead to an associated increase in biomass. In practice this approach is not used, above threshold concentrations substrates cause inhibition of microbial growth, meaning that the expected growth yield is not obtained. For example it has been reported that a glucose concentration above 50 g/L causes growth inhibition of \( E. coli \) (Riesenberg, 1991). These limitations mean that batch culture is generally not used in manufacturing but some reports of plasmid production in batch culture have been made.

Wan and Goodrick (1996) described a batch process using a complex medium which, after 15 hours, achieved an optical density at 600 nm of 30-60 and a plasmid yield of 20-40 mg/L. Horn et al. (1995) described a batch process, again using a complex medium, which achieved an optical density at 600 nm of 30 and a plasmid yield of 4 mg/L after 10-11 hours. Horn et al. used a low copy number pBR322 derivative whilst Wan and Goodrick did not describe their plasmid. The differences in plasmid yield between the two processes could be due to differences in plasmid type and copy number. Durland and Eastman (1998) reported the use of a batch process using a complex medium which achieved an average optical density of 70 with plasmid yield averaging 120 mg/L, using plasmids with a pUC origin. They noted that plasmid yields were highly plasmid dependent, with certain plasmids giving yields much higher or lower than the average value. Hofmann et al. (1990) investigated amplification of pBR322 in batch and fed-batch culture. They showed that plasmid amplification occurred independently of the culture strategy employed, specific plasmid yield being almost identical in batch and fed-batch culture. However, fed-batch culture resulted in much higher biomass concentration and hence volumetric productivity was significantly enhanced.
1.4.6 Fed-batch culture

Fed-batch cultures are carried out in two distinct phases. In the first phase cells are grown to a maximum density on batched substrates, in the second phase feeding of substrates commences to allow further increases in biomass. Various feeding methods have been investigated in fed-batch culture and are summarised in Table 1.1. The majority of fed-batch regimes have been investigated for *E. coli* harbouring plasmids for recombinant protein production but such techniques are equally applicable to plasmid DNA production for gene therapy.

The production and subsequent accumulation of metabolic by-products is the most important growth-limiting factor (Korz *et al.*, 1995) and is well characterised for *E. coli*. The ability to control specific growth rate and hence minimise by-products, such as acetate, is an important aspect of fed-batch fermentation. Chen *et al.* (1997) developed an automated fed-batch fermentation for production of DNA vaccines, using feedback control based on dissolved oxygen (DO) and pH. Their initial studies used a manually controlled feed system and showed that if glucose was allowed to rise above 5 g/L in the medium, growth became suppressed, acetate began to accumulate and inconsistent growth rates were exhibited. Under high growth rate conditions, growth outpaced rate of plasmid production and led to low volumetric plasmid yield. They developed a fed-batch fermentation using DO and pH feedback control of nutrient feeding. The system was designed to control feeding such that the specific growth rate remained at 0.1 1/h, this low growth rate prevented acetate accumulation and allowed maintenance of plasmid replication. The system was successfully scaled up from 7 L to 80 L and was shown to be reproducible at both scales. After 24 hours the system yielded an optical density at 600 nm of approximately 121 and a plasmid yield of approximately 80 mg/L. Such fed-batch fermentations are likely to be used at production scale where high volumetric plasmid yields may be obtained.
### Table 1.1. Feeding control methods for fed-batch culture

Adapted from Lee (1996)

<table>
<thead>
<tr>
<th>Feed control method</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Constant feed</strong></td>
<td>Nutrients are fed at a constant rate and the specific growth rate continuously falls.</td>
</tr>
<tr>
<td><strong>Increased feed</strong></td>
<td>Nutrients are fed at an increasing rate. The specific growth rate decrease can be compensated.</td>
</tr>
<tr>
<td><strong>Exponential feed</strong></td>
<td>Nutrients are fed at an exponential rate. Constant specific growth rate can be achieved.</td>
</tr>
<tr>
<td><strong>DO stat</strong></td>
<td>Nutrients are fed when dissolved oxygen rises indicating substrate depletion.</td>
</tr>
<tr>
<td><strong>pH stat</strong></td>
<td>Nutrients are fed when pH rises indicating carbon source depletion.</td>
</tr>
<tr>
<td><strong>Indirect feedback control</strong></td>
<td></td>
</tr>
<tr>
<td><strong>CER</strong></td>
<td>Carbon dioxide evolution rate (CER) is proportional to the rate of consumption of carbon source and hence is used to control supply. Frequently used to control specific growth rate.</td>
</tr>
<tr>
<td><strong>Cell density</strong></td>
<td>Cell density is measured on-line and substrates are fed in proportion.</td>
</tr>
<tr>
<td><strong>Direct feedback control</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Substrate concentration</strong></td>
<td>Carbon source concentration is measured on-line and fresh substrate is fed accordingly.</td>
</tr>
</tbody>
</table>
1.4.7 Plasmid amplification

Limitation or starvation of amino acids has been shown to induce the amplification of a variety of plasmids leading to increased yields (Hecker et al., 1986; Hofmann et al., 1990; Wegrzyn and Wegrzyn, 1995; Wegrzyn, 1995; Wrobel and Wegrzyn, 1997a, 1997b, 1998; Wegrzyn, 1999; Potrykus et al., 2000). The addition of chloramphenicol to a culture is also known to induce plasmid amplification (Clewell, 1972). The controlled starvation of amino acids presents a simple method of increasing plasmid yields and does not suffer the drawbacks of the chloramphenicol addition method. If chloramphenicol addition was to be used to induce plasmid amplification then there would be a need to demonstrate its removal in the final plasmid product, and as such it presents a regulatory issue. Amino acids are commonly used medium components and therefore do not suffer from the same regulatory issues.

1.4.7.1 Cellular control of plasmid transcription

Initiation of plasmid transcription is regulated by two RNA transcripts of plasmid origin, RNA I and RNA II and by a protein Rom (RNA one modulator) or Rop (Repressor of primer).

RNA II is a 555 nucleotide preprimer transcript which forms a persistent hybrid with the plasmid DNA template close to the replication origin. Cleavage of this RNA II-DNA hybrid at the origin, by RNase H, yields a free 3' OH which acts as a mature primer for elongation of DNA by DNA polymerase I.

RNA I is a 108 nucleotide antisense RNA which negatively regulates transcription initiation. RNA I is exactly complementary to the 5' terminal region of RNA II, and as such is able to form a hybrid with it. RNA I-RNA II hybridisation prevents RNA II-DNA hybridisation and hence RNA I acts to suppress initiation of plasmid transcription.
Rom/Rop is a 63 amino acid protein which has been shown to accelerate RNA I-RNA II binding or to suppress its dissociation, hence Rom/Rop acts as a second negative regulatory element in the initiation of plasmid transcription.

1.4.7.2 The stringent response

The stringent response is exhibited by \textit{relA}^+ strains of \textit{E. coli} and is a complex set of physiological changes which occur in response to amino acid starvation. The hallmark of the stringent response is a dramatic reduction in the rate of RNA production (Jinks-Robertson and Nomura, 1987). When cells become starved of amino acids uncharged tRNAs become present in the cell. Interaction between uncharged tRNAs and codons at the ribosomal A site leads to ribosome idling and activation of the ribosome bound \textit{relA} gene product, guanosine-5'-diphosphate-3'-diphosphate synthetase (ppGpp synthetase I). ppGpp synthetase I catalyses the synthesis of guanosine-5'-diphosphate-3'-diphosphate (ppGpp) which interacts with RNA polymerase, most likely with the $\beta$ subunit. This results in the inhibition of stable rRNA and tRNA synthesis and in the inhibition or activation of synthesis of various mRNAs. This response brings about the necessary changes in the cell to allow it to survive the amino acid starvation.

\textit{E. coli} also exhibits the stringent response in times of carbon source starvation. The response is mediated in a ribosome independent manner, by ppGpp synthetase II, the \textit{spoT} gene product.

1.4.7.3 The relaxed response

The relaxed response is exhibited by \textit{relA}^− mutants which are unable to synthesise ppGpp synthetase I. When \textit{relA}^− mutants are starved of amino acids they are unable to exhibit the stringent response and therefore continue to produce tRNAs, which remain uncharged and accumulate in the cell. In \textit{relA}^− cells subjected to amino acid starvation initiation of plasmid amplification is observed, Wrobel and Wegrzyn (1998) proposed a model for this action. The accumulation of tRNAs to high concentrations in the cell allows them to compete for interactions with RNA I and/or RNA II. These interactions are sufficient to impair RNA I-RNA II hybridisation.
but insufficient to prevent RNA II-DNA hybridisation close to the origin of replication and hence initiation of plasmid replication is able to occur.

1.4.8 Plasmid Instability

There are two forms of plasmid instability, structural and segregational.

Structural instability causes alterations to the plasmid, such as point mutation, deletion, insertion or rearrangement. Such structurally altered plasmids may still be generated and passed to daughter cells, but serve only to lower the productivity of the process, as they are undesired plasmid products. Such plasmids may also complicate downstream processing and hence generation of structurally altered plasmids is undesirable in a manufacturing process.

Plasmids are extra-genomic DNA elements and, as such, are not vital to a cell's viability. When present in a cell a plasmid places extra metabolic burden on the cell, because the cell must produce more DNA, RNA and protein (Kumar et al., 1991; Ryan and Parulekar, 1991).

Defective partitioning of plasmids to daughter cells during cell division is the cause of segregational instability and results in the generation of plasmid-free cells. In the absence of a plasmid maintenance strategy plasmid-free cells are able to replicate at a higher rate than plasmid-bearing cells and therefore quickly become the major constituent of the culture. This leads to a process of poor productivity which is highly undesirable in a manufacturing process.

To prevent instability recombinant fermentation processes must use strategies to maintain the presence of the plasmid in the host cell. The metabolic burden placed on the host has been reported to be dependent on the size of the plasmid, the copy number of the plasmid and whether the plasmid is simply being maintained or is actively expressing protein (Ricci and Hernandez, 2000). This suggests that in fermentations for plasmid rather than recombinant protein production the metabolic load on the host will be lower and plasmid loss may not present such a problem. O'Kennedy et al. (2000) reported that E. coli DH5α carrying the plasmid pSVβ could be
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grown on semi defined medium, without a selective agent, in batch mode for 96 h with insignificant plasmid loss. Therefore in some cases it may be possible to carry out fermentation without employing plasmid selection strategies but this would require investigation for each host/vector combination.

1.4.9 Strategies to maintain plasmid stability

Several strategies to maintain plasmid stability have been investigated and can be broadly classified as selective or non-selective. The majority of strategies are designed to overcome segregational instability because the frequency of structural instability has been reported to be low with many plasmids (Kumar et al., 1991).

1.4.9.1 Selective strategies

The most commonly employed selective strategy is the addition of antibiotic to the growth medium to allow selection for plasmid-bearing cells. Kanamycin is the preferred antibiotic at manufacturing scale, it is not broken down in culture and hence selective pressure is maintained throughout the fermentation process, with high levels of plasmid stability observed.

Other selective strategies which do not require the use of antibiotics, such as that described in a Cobra patent (Sherratt et al., 1999, see section 1.4.1) are available but are less commonly used in manufacturing.

1.4.9.2 Non-selective strategies

Non-selective methods seek to improve plasmid stability rather than specifically select for plasmid-containing cells.

The incorporation of a partitioning locus to the plasmid is one non-selective strategy which has been employed (Kumar et al., 1991; Williams and Thomas, 1992). Low and intermediate copy number plasmids are known to require a partition function in order to ensure even distribution of plasmid to daughter cells at cell division. However, high copy number plasmids, which are
commonly used in recombinant cells, have not been shown to contain such a partition function, and their partitioning mechanism is unclear. Summers and Sherratt (1984) reported that partitioning in high copy number cells is random. The engineering of a high copy number plasmid to contain a partition function may help to improve segregational instability.

Culture conditions have been reported to affect plasmid stability (Kumar et al., 1991, Zabriskie and Arcuri, 1986). In general plasmids are unstable under substrate limiting conditions, glucose, phosphate and magnesium limitation have been shown to result in plasmid loss. The level of instability is very much dependent on the plasmid and on the host, plasmids may be very stable in one host and highly unstable in another. Thus culture conditions must be optimised for a given host/vector system.

1.4.10 Consideration of the impact of increased plasmid size

In order to provide useful products for gene therapy larger plasmids than those currently being used are likely to be required. The insertion of larger fragments of foreign DNA to a cell may be expected to alter the cell’s behaviour with regard to parameters such as growth rate, oxygen demand and plasmid stability.

1.4.10.1 Growth rate

Studies by Cheah et al. (1987), using E. coli JM103 with plasmids of 2.7 kb, 4.4 kb, 5.3 kb and 8.7 kb, showed that increased plasmid size did not significantly alter the growth rate. These authors reported that host and recombinant strains had an approximately equal growth rate, but the maximum cell density obtained was inversely correlated to plasmid size. It was postulated that, as plasmid size increased, general stress on the cell increased and was responsible for the decrease in maximum biomass yield.
Khosravi et al. (1990), using E. coli JM103 with 2.7 kb and 8.7 kb plasmids, also observed an inverse correlation between maximum cell density and plasmid size, in addition they observed a slight decrease in growth rate as plasmid size increased.

Smith and Bidochka (1998) studied the effects of insertion of an isogenic series of plasmids in E. coli XL1 Blue. Using plasmids of 2.96 kb, 3.96 kb and 11.96 kb they reported that there was no significant difference in lag phase or growth rate between the host and the two smaller plasmids. The recombinant strain carrying the 11.96 kb plasmid had a significantly longer lag phase but its growth rate was similar to the other strains.

Warnes and Stephenson (1986) using E. coli HB101 and plasmids of 2 kb, 8 kb and 21 kb reported that the 2 kb plasmid had no effect on the host's growth rate but that the 8 kb and 21 kb plasmids significantly decreased the growth rate. This was attributed to large plasmids placing unacceptable metabolic loads on the host.

These reports indicate that the effect of increased plasmid size is very much dependent on the host/vector system. Thorough evaluation and development work may allow large plasmids to be produced in much the same manner as smaller plasmids.

1.4.10.2 Plasmid stability

Studies on plasmids of increasing size have suggested that as plasmid size increases plasmid stability decreases.

Warnes and Stephenson (1986) investigated the effect of plasmid size on plasmid stability. They reported that a 2 kb plasmid was very stable, an 8 kb plasmid had some transient instability and a 21 kb plasmid was highly unstable. Decreases in copy number for the 21 kb plasmid correlated well with the observed instability and indicated segregational instability. The authors suggested that, as plasmid size increased, deoxynucleotide triphosphates were in greater demand for
plasmid replication. Hence competition between chromosome and plasmid, for deoxynucleotide triphosphates, led to a reduction in plasmid copy number.

Findings by Cheah et al. (1987) and Smith and Bidochka (1998) were in agreement with this. Both groups found that as plasmid size increased plasmid copy number decreased. Additionally Smith and Bidochka (1998) found that in nutrient-limited conditions plasmid loss occurred and the rate of loss was proportional to plasmid size.

1.4.10.3 Oxygen demand
Khosravi et al. (1990) studied the variation in oxygen requirement with plasmid size in *E. coli* JM103. They reported that as plasmid size increased oxygen uptake rate increased while maximum cell density decreased. They suggested that as plasmid size increased, and extra energy was required to maintain the plasmid, cells compensated by increasing their rate of oxidative phosphorylation, and hence oxygen uptake rate increased. Although oxygen uptake rate increased, oxygen may have become a limiting nutrient and hence maximum cell density decreased with increased plasmid size.

1.5 Downstream processing
There are four major classes of impurities which must be separated from plasmid DNA during downstream processing. They are genomic DNA, RNA, proteins and endotoxin. In order to be suitable for use in human gene therapy the purification of plasmid DNA must follow procedures which ensure materials such as toxic compounds, flammables, organics and ruminant derivatives do not contaminate the final product. This somewhat limits the procedures which may be employed. At large scale the process must also yield plasmid DNA of high purity at high yield, in the least number of processing steps, to allow economic feasibility. There is a present understanding that supercoiled plasmid DNA is the most effective form for delivery (Prazeres et al., 1999) and hence the majority of purified plasmid should be in the supercoiled form. The process employed should be scalable to gram or kilogram scale and, most importantly, should be
highly reproducible to yield a consistent product between batches (Durland and Eastman, 1998). Final product should be tested for identity, purity, safety, sterility and potency and should meet pre-determined criteria (FDA, 1996a, 1996b) The use of a highly reproducible manufacturing process is the key to ensuring the final product meets the necessary criteria. Various downstream processing routes have been reported or suggested (Caplen et al., 1994; Horn et al., 1995; Marquet et al., 1995; Lee and Sagar, 2001; Ferreira et al., 1999; Thatcher et al., 1999; Prazeres et al., 1999; Levy et al., 2000; Shamlou, 2003). Any downstream process will consist of cell harvest and lysis to recover plasmid DNA, plasmid purification, polishing, buffer exchange and formulation. A generic plasmid recovery flow sheet is shown in Figure 1.5.
Figure 1.5. A generic process flowsheet for plasmid DNA recovery
Adapted from Shamlou, 2003.
1.5.1 Cell harvest and lysis

The first stages in downstream processing are the separation of the cells from the fermentation medium and lysis of the cells to release the plasmid product. Centrifugation is the commonest method of cell recovery and is scaleable. Once recovered the cells are resuspended, at 10 - 20 times their original concentration, in a buffer designed to facilitate lysis. This buffer usually contains compounds which disrupt ionic or hydrogen bonds between lipids and/or proteins. Removal of ions from the cell wall and cell membrane disrupts their structure, aiding lysis. The activity of magnesium dependent nucleases is reduced and this helps to prevent plasmid degradation. The resuspension buffer also often contains sucrose or glucose to protect plasmid DNA from shear (Prazeres et al., 1999).

At laboratory scale the lysis buffer commonly contains RNase to degrade RNA to small fragments and nucleotides, which are then removed in subsequent purification steps. RNase is commonly used in plasmid manufacture but it has certain disadvantages in that it is commonly bovine derived and at large scale its use becomes expensive. If it is used in a manufacturing process it must be from an FDA approved source. Hanak and Williams (1999) and Cooke et al. (2001) reported the construction of a modified E. coli host strain. A bovine pancreatic RNase expression cassette was integrated to the host chromosome and the cells produced RNase which was transported to the periplasm and hence was only brought into contact with cellular RNA during cell lysis. The host-produced RNase was able to degrade the majority of the RNA present in the cell, thereby facilitating RNA removal downstream without the need to add expensive bovine derived RNase.

Mechanical, heat and chemical methods are available for cell lysis. Carlson et al., (1995) investigated various mechanical lysis methods for plasmid recovery from E. coli. The majority of these methods, while achieving high levels of cell disruption, resulted in low plasmid recovery, generally due to plasmid destruction by shear effects. The bead mill was the only mechanical
lysis which showed any promise, around 90% intact plasmid being recovered. Mechanical devices generally are not considered suitable for cell disruption to recover plasmid because of potential to damage both plasmid and genomic DNA. If genomic DNA is subjected to shear forces it may be degraded to fragments similar in size to the plasmid thereby complicating purification and polishing unit operations. Merck patented a method for large scale isolation and purification of plasmid DNA (Lee and Sagar, 2001) which includes a heat lysis step. Resuspended cells are heated to 70 – 100 °C in a flow-through heat exchanger which results in cell lysis. Merck claim that their method offers a number of advantages over chemical lysis which include higher product recovery, inactivation of DNases, operational simplicity and scalability. However, chemical lysis, based on the method of Birnboim and Doly (1979), is most commonly employed.

Lysis is achieved by mixing the recovered cell suspension with an equal volume of alkaline detergent, normally 1 % w/v SDS in 0.2 M sodium hydroxide. It is very important that the lysis vessel is well mixed, in order to avoid local pH extremes and to gain efficient lysis. A pH greater than 12.5 has been shown to cause irreversible plasmid denaturation (Prazeres et al., 1999). Such denaturation is a significant problem in large scale tanks because pH gradients are likely to occur. Studies by Ciccolini et al. (1998, 1999) showed that a rapid increase in viscosity occurred immediately that the lysis reagent addition was made to the cell suspension. The viscosity increase then stopped for a short period before a further increase was observed, followed by a fall to a constant level. The initial viscosity rise was attributed to cell wall lysis and release of intracellular materials. The second increase and subsequent levelling was attributed to denaturation of genomic DNA. The dramatic increase in viscosity upon lysis reagent addition makes gentle and efficient mixing difficult. Marquet et al. (1995) identified a large barred impeller which was suitable for the purpose and exerted low shear across a wide viscosity range. Levy et al. (1999) demonstrated that shear forces, of the magnitude exerted in bioprocessing equipment, could severely affect the tertiary structure of plasmid DNA, with the level of damage increasing
Chapter 1: Introduction

with increasing plasmid size. It is therefore important that shear is minimised in order to prevent
damage to plasmid DNA to allow maximum supercoiled plasmid yield to be achieved.

The addition of a high salt solution, usually 3 M potassium acetate, results in precipitation of the
solubilised proteins and lipids and the majority of the genomic DNA, whilst plasmid DNA remains
in solution. This stage of lysis requires that minimum shear is exerted in order to avoid damaging
genomic DNA, which could be released back in to solution where it would contaminate the
plasmid DNA. Other precipitation methods have been investigated. A recent report (Lander et
al., 2002) demonstrated the use of cetyltrimethylammoniumbromide (CTAB) to separate
impurities from the plasmid DNA. In this work the authors were able to separate the relaxed
forms of plasmid DNA from the supercoiled form by adjustment of the concentration of CTAB,
thereby eliminating the need for further chromatographic stages.

The precipitated material may be separated from the plasmid-containing liquor by filtration. The
filtration operation should be low shear in order to avoid damaging the genomic DNA which is
contained in the precipitate and releasing it into the plasmid-containing liquor. Filtration is usually
achieved using a filtration medium with a pore size in the range 30 μm – 150 μm (Shamlou,
2003).

1.5.2 Purification

Marquet et al. (1995) used polyethylene glycol (PEG) in a two stage purification scheme. 4 % w/v
PEG 8000 was first used to precipitate the residual RNA and genomic DNA in the clarified lysate.
The precipitate was then removed by filtration. Plasmid DNA was then precipitated using 10 %
w/v PEG 8000. PEG is considered safe and is approved by the FDA for topical and internal use
in humans, it is preferred over short chain alcohols at large scale because it does not require
special facility design or as critical control of process temperature for reproducibility. Precipitated
plasmid DNA was collected by filtration and redissolved before further clarification by the addition
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of ammonium acetate. The addition of ammonium acetate resulted in the precipitation of RNA and lipopolysaccharides which were separated from the plasmid DNA solution by filtration. The plasmid DNA which resulted from this process was considered suitably pure to proceed to high resolution chromatography. Ferreira et al. (1999) also reported the use of PEG 8000 in a clarification procedure.

Ferreira et al. (1999) showed that plasmid polishing may be successfully carried out with the omission of the purification stages, progressing directly from lysis to the chromatographic stages. These authors were able to demonstrate that progressing directly from lysis to polishing resulted in plasmid of equal purity and quality to that obtained via the longer route, and in addition the yield was increased. Endotoxin levels were not measured and hence the suitability of plasmid for gene therapy applications was not determined. The authors demonstrated that plasmid can be purified, at least to molecular biology specifications, in a shorter, less costly process.

1.5.3 Polishing

Impurities which remain in the process stream at the polishing stage are RNA, genomic DNA, endotoxins and plasmid DNA variants. Polishing of plasmid DNA is difficult because the contaminants have similar properties to plasmid DNA and have wide molecular weight ranges.

Prazeres et al. (1998) reported the use of anion exchange chromatography. They achieved separation of supercoiled plasmid from open-circular plasmid and low molecular mass RNA. Their method scaled up well and they obtained consistency at three scales with respect to yield, purity, quality and elution profiles. Endotoxin levels were not measured in this study and it is likely that some endotoxin contamination remained in the final plasmid preparation, it was suggested that a final gel filtration step would render the plasmid suitable for use in gene therapy. Although consistent separation was achieved in this study the authors did note that the anion exchange column had a 3000 fold lower capacity in comparison to the values reported by the manufacturers for protein separation. Plasmid molecules are much larger than protein molecules.
and hence are unlikely to be able to enter the pores of chromatographic matrices hence the
majority of binding will take place on the outer surface of the matrix, severely reducing its
capacity. Although the study by Prazeres et al. (1998) resulted in excellent separation of
supercoiled plasmid from contaminating nucleic acids it is questionable whether such a scheme
could be suitable for larger plasmids given the binding limitations of the chromatographic matrix.

The use of anion exchange membranes in place of chromatographic beads was demonstrated by
Endres et al. (2003) and by Zhang et al. (2003). These studies showed that anion exchange
membranes had up to a 25 fold higher binding capacity for plasmid DNA and could be operated
at flow rates up to 550 times higher than those observed for chromatography beads (Endres et
al., 2003). Although the use of anion exchange membranes did not completely purify the plasmid
significant reductions in contaminants were made meaning that the plasmid was suitable for a
final step such as gel filtration. Anion exchange membranes are supplied ready to use and
therefore avoid the time consuming steps associated with the packing and operation of
chromatography columns. They can also be used in a disposable manner which, in a production
environment would preclude the need for costly cleaning validation. However, as they represent
a significant running cost, a cost analysis of disposable versus fixed technology would be
necessary before a plant committed to the use of disposables.

A final size exclusion or gel chromatography step is required to bring the plasmid to therapeutic
quality. The removal of RNA in earlier process steps effectively increases the capacity of the
polishing step. Genomic DNA, endotoxins and plasmid DNA can be successfully resolved,
genomic DNA runs ahead of plasmid DNA with open-circular plasmid DNA running ahead of
supercoiled plasmid DNA, endotoxins are generally well separated from plasmid DNA. Careful
choice of fractions collected from size exclusion chromatography can allow almost pure
supercoiled plasmid DNA to be collected (Prazeres et al., 1999). The use of size exclusion
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chromatography also allows the plasmid’s buffer to be exchanged for one more suitable for formulation, and allows removal of residual salts.

Once collected the purified plasmid DNA is sterile filtered to give bulk sterile plasmid DNA. Samples are subjected to analytical and quality control testing and, if specifications are met, the plasmid may proceed to formulation and filling (Marquet et al., 1995).

1.5.4 Formulation

Although plasmid DNA may be administered in its ‘naked’ form its large size, short half-life and susceptibility to degradation in the body make this form of delivery very inefficient, resulting in poor transfection efficiencies. Research and development work is being carried out to develop vectors to improve transfection efficiencies.

The majority of formulation strategies aim to condense plasmid DNA to create particles of around 100 nm in diameter although this has proved to be difficult. Condensed particles generally have low interaction energies under physiological conditions which results in physical instability and particle aggregation (Tsai et al., 1999; Lee LK et al., 2001, Maguire et al., 2003). Various condensing agents have been used, including cationic lipids and polymers and anionic liposomes and are usually used in combination with a neutral lipid (Shamlou, 2003). Additional targeting molecules, such as small peptides, are usually conjugated to the complexes in order to target them to specific cells and hence improve their transfection efficiency. In some cases the use of PEG in formulation has been shown to improve the half life of the complexes in the body (Ross and Hui, 1999).

Stable formulations which allow efficient transfection will be vital in the success of plasmid-based gene therapy. In order for a given formulation to be successful it must be produced in a scaleable manner.
1.6 Statistical methods in the design and analysis of experiments

1.6.1 Factorial designs

Factorial designs were first described by Box et al. (1978) and are used in process optimisation experiments and screening experiments.

In traditional 'one at a time' experiments individual factors are varied while the rest are held constant. Such an experimental approach does not yield all of the information which may be of importance in deciding exactly how a process ought to be run.

In factorial design experiments the variables which are thought to influence a process are identified and are then given set values (levels), commonly one high value and one low value. The experiment is then designed in such a manner that all possible combinations of variables (factors) are used in the experiment. For example, a process where three variables are identified and set at two levels is called a 3 factor, 2 level \((2^3)\) design and would have 8 combinations and would require 8 separate runs. As the total number of combinations is equal to the number of levels to the power of the number of variables the number of runs quickly becomes very large. For example a 7 factor, 2 level \((2^7)\) experiment would require 128 runs. It is therefore not recommended that more than 5 factors, which at 2 levels results in 32 combinations, are used in a full factorial design (NIST/SEMATECH, 2003). Once the full experiment has been carried out the results are statistically analysed, using a software package, to determine which factors have a significant effect on the process. The usefulness of full factorial designs is their ability to highlight interactions between factors. For example factor A alone may not have any significant effect on the process but when factor A at a high level is combined with factor B at a high level the process may be significantly improved. Such details would not necessarily be noted in a traditional 'one at a time' experiment.
When the number of factors to be examined exceeds five, running a full factorial experiment can be time consuming and costly. With this number of factors a screening experiment is usually required. Screening experiments can be used to determine the factors which are most significant and if required a smaller factorial experiment may be run with the identified factors. A fractional factorial design may be used in a screening experiment. Such a design uses only a fraction, usually a half or a quarter, of the number of runs required in a full factorial design. A fractional factorial design allows all the main effects and higher order interactions to be examined with a fraction of the experiments required for a full factorial design, presenting a faster, cost effective experiment. Higher order interactions refer to 2 component interactions, for example the interaction of factor A with factor B. Lower order interactions such as the interaction between factors A, B, C, D and E are not identified. However, in the majority of experimental systems lower order interactions are insignificant and can, therefore, be safely left out in initial screening designs with no significant loss of information (NIST/SEMATECH, 2003).

1.6.2 Statistical testing of means

The Student’s t-test is commonly used to carry out statistical comparisons of means but various statistical methods exist for carrying out such comparisons. In order to ensure that the correct method is employed the distributions and variances of the sample populations should first be examined.

Using statistical software a normal distribution may be fitted to the sample population and a Shapiro Wilkes W test for goodness of fit carried out. A probability (P) value of 0.05 or less is sought for statistical significance at the 95 % level. If significance is found then the data is not normally distributed.

If the sample populations are shown to be normally distributed then the variances of the two sample populations to be compared should be tested. Several tests for unequal variance are available but the Levene test has been shown to be the most robust (Lim and Loh, 1996). Again,
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A P value of 0.05 or less is sought for statistical significance at the 95% level. If significance is found then the variances are unequal.

The Student’s t-tests is only appropriate if the sample populations to be tested are normally distributed and if the variances between the two sample populations are statistically equal. When the sample population is normally distributed, but the variances are statistically unequal, the Welch ANOVA test, which allows for unequal variances, is used. In cases where the sample populations are not normally distributed the Wilcoxon test is used to compare the means. In all cases a P value of 0.05 or less is sought to indicate that there is a statistically significant difference between the two means. This is summarised in Figure 1.6.
Figure 1.6. Appropriate use of statistical tests for comparison of means.
1.7 Electrophoresis methods

Two forms of gel electrophoresis were used in this study, conventional gel electrophoresis and pulsed field gel electrophoresis (PFGE). The differences between the two methods warrants some discussion.

Electrophoresis is used to separate and visualise different plasmid forms. In conventional gel electrophoresis the plasmid sample is loaded onto an agarose gel and is exposed to a constant, uni-directional electric field. Due to the negatively charged nature of DNA the plasmid molecules move through the gel towards the positive pole. The agarose gel is a porous lattice and in order for the DNA to migrate through the gel it must move through the pores of the lattice. The rate at which DNA is able to move through the gel is dictated by its size and conformation. For example a 4 kb plasmid will move at a greater rate than a 20 kb plasmid and a supercoiled plasmid will move at a greater rate than an open circular plasmid of the same size. A supercoiled plasmid is able to move at a greater rate than an open circular plasmid because it is more compact and hence is able to move more easily through the pores of the gel. This phenomenon allows different plasmid conformations to be separated and a plasmid preparation to be quantified in terms of supercoiling. In a conventional electrophoresis gel supercoiled plasmid will always run ahead of open circular plasmid.

Conventional gel electrophoresis can only be used to separate plasmids up to 30 - 50 kb. Above this size plasmids migrate at the same rate regardless of their size and separation can not be achieved. PFGE was first described by Schwartz and Cantor (1984) and has since been developed and improved. Many types of PFGE equipment are now commercially available. In PFGE the direction of the electrical field is periodically changed. Each time it changes the DNA in the gel is forced to reorient itself in order to continue moving towards the positive pole. Smaller plasmid molecules are able to reorient themselves more quickly than larger molecules and hence move through the gel at a greater rate. In PFGE unlike conventional electrophoresis supercoiled
plasmid DNA runs behind open circular plasmid DNA. The theory of PFGE remains a matter of debate but it is thought that open circular DNA is able to reorient itself more rapidly than supercoiled DNA.

1.8 Aims

This study had two main aims. The initial aim was to investigate the use of plasmid amplification, by amino acid starvation, in the production of a small, 6.9 kb, plasmid. From this investigation the aim was to develop a novel fermentation which could produce plasmid DNA predominantly in the supercoiled form and hence was suitable for further processing as a gene therapy product. Although fed-batch fermentations for the production of plasmid DNA have been reported they have not been optimised or developed to produce plasmid of a sufficiently high quality, with respect to supercoiling, to be used as a gene therapy product. Maximising plasmid supercoiling at the fermentation stage allows the maximum possible yield of plasmid to be obtained in downstream processing because these processes are optimised to recover supercoiled plasmid and remove other isoforms.

The second aim of this study was to carry out an investigation to assess the feasibility of producing large plasmids. It is envisaged that large plasmids will be required in the future for both gene therapy and vaccination and hence it is an important challenge to establish that a fermentation for large plasmid production is possible. In order to satisfy this aim a two stage investigation was planned. The first stage was to investigate the effect of increased plasmid size on oxygen demand. Increased plasmid size may be expected to give rise to increased oxygen demand which could make the production of large plasmids difficult, especially at large scale where ability to supply oxygen is limited. If the ability to supply oxygen could be satisfied then the second stage was to examine the fermentation and initial recovery of a large plasmid. Reports of the fermentation of large plasmids for gene therapy and vaccination have not been made and it is not known if their production will be possible. Similarly, although much research into the recovery
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of small plasmids has been carried out reports relating to large plasmids have not been made. It may be expected that the recovery of large plasmids will be complicated by shear related issues.
Chapter 2: Materials and methods

2 Materials and methods

All chemicals were obtained from BDH (Poole, Dorset, UK) unless otherwise stated.

2.1 Plasmids and bacterial strains

The plasmids used in this work were pSVβ (Promega Corp., Massachusetts, USA), pBGS18 (Spratt et al., 1986) and its derivative, pQR150 (Jackson et al., 1995) and p5176 (Wade-Martins et al., 1999).

pSVβ is a 6.9 kb pUC-based plasmid which confers ampicillin resistance and encodes the β-gal gene. pBGS18 is a 4.4 kb pUC-based plasmid which confers kanamycin resistance and encodes the α fragment of the β-gal gene, under control of the lac promoter. The benzoate meta-cleavage pathway of Pseudomonas putida, which encodes catechol 2,3-dioxygenase activity, was cloned into pBGS18 to yield pQR150, a 20 kb plasmid. P5176 is a 116.9 kb F-based plasmid which confers chloramphenicol and hygromycin resistance and encodes the green fluorescence protein (GFP) gene. Plasmid maps are shown in Appendix 1.

pSVβ, pBGS18 and pQR150 were transformed into E. coli DH5α by John Ward (Department of Biochemistry, University College London, London, UK). DH5α is a well characterised strain of E. coli and such strains have been favoured in gene therapy fermentation development work. Competent cells were prepared by the calcium chloride technique and transformed using the standard technique (Sambrook et al., 1989). Both the host and recombinant strains were maintained in 20 % (v/v) glycerol at -85 °C. p5176 was transformed into E. coli DH10β by Richard Wade-Martins (Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, UK) using electroporation and was kindly donated to John Ward. Electroporation results in a higher transformation efficiency than the chemical technique, especially when using larger plasmids, hence this is the method of choice for large plasmid transformations (Wade-Martins, personal communication). Attempts were made to transform p5176 into DH5α using the
Chapter 2: Materials and methods

standard technique but these were unsuccessful, hence all work with p5176 was conducted in DH10β.

2.2 Culture media

All media and stock solutions were prepared in reverse osmosis (RO) water.

Modified Luria Bertani agar (LBM agar) consisted of, per litre, mycological peptone (10.0 g; Oxoid, Basingstoke, Hampshire, UK), yeast extract (5.0 g; Oxoid), sodium chloride (10.0 g) and bacteriological agar (10.0 g; Oxoid).

Superbroth consisted of, per litre, tryptone (32.0 g; Oxoid), yeast extract (20.0 g; Oxoid) and sodium chloride (5.0 g). The pH of Superbroth was adjusted to 7.4 with 4 M sodium hydroxide prior to sterilisation.

Semi-defined medium (SDCAS), consisted of, per litre, D-glucose (10.0 g), magnesium sulphate heptahydrate (1.2 g), ammonium sulphate (4.0 g), potassium dihydrogen orthophosphate (13.3 g), casein hydrolysate (10.0 g; Oxoid), citric acid (1.7 g), EDTA sodium salt (8.4 mg), cobalt chloride hexahydrate (2.5 mg), manganese sulphate (15.0 mg), copper sulphate dihydrate (1.5 mg), boric acid (3.0 mg), sodium molybdate dihydrate (2.5 mg), zinc chloride (13.0 mg), iron (III) citrate (100.0 mg) and thiamine hydrochloride (4.5 mg; Sigma, Poole, Dorset, UK). SDCAS was routinely prepared as follows, to avoid precipitation:

1. A trace elements stock solution consisting of, per litre, EDTA sodium salt (8.4 g), cobalt chloride hexahydrate (2.5 g), manganese sulphate (15.0 g), copper sulphate dihydrate (1.5 g), boric acid (3.0 g), sodium molybdate dihydrate (2.5 g), zinc chloride (13 g) was prepared and stored at room temperature.

2. Stock solutions of Iron (III) Citrate at 100.0 g/L, D-glucose and MgSO₄·7H₂O at 400.0 g/L and 600.0 g/L respectively and casein hydrolysate at 200.0 g/L were prepared, sterilised by autoclaving and stored at room temperature.
3. A stock solution of Thiamine at 4.5 g/L was prepared, sterilised by filtration through a 0.22 μm syringe filter and stored at -20 °C.

4. Base medium consisting of, per litre, ammonium sulphate (4.0 g), potassium dihydrogen orthophosphate (13.3 g), citric acid (1.7 g) and trace elements solution (1.0 mL) was prepared and the pH was adjusted to 6.3 with 4 M sodium hydroxide. Base medium was sterilised by autoclaving or in situ in the case of fermenter cultures.

5. Once the sterilised base medium had cooled to 37 °C, or below, appropriate volumes of each stock solution (from 2 and 3 above) were added to it to obtain final concentrations of each component as stated above.

Where selective conditions were required media were supplemented with ampicillin (100.0 mg/L), kanamycin (25.0 mg/L) or chloramphenicol (25.0 mg/L) according to the strain being cultured. Stock solutions were prepared at 1000 x strength and stored at -20 °C in 1 mL aliquots. Ampicillin and kanamycin were prepared in RO water and sterilised by filtration through a 0.22 μm syringe filter. Chloramphenicol was prepared in absolute ethanol and no sterilisation was required.

2.3 Cell bank preparation

All working cell banks (WCBs) were prepared from streak plates of the master cell bank (MCB). All DH5α master cell banks were provided by Ronan O'Kennedy (Department of Biochemical Engineering, University College London, London, UK). The DH10β p5176 master cell bank was prepared in the same manner as the WCB, described below, from a streak plate provided by John Ward. A single, well separated colony, was picked and inoculated to 100 mL of selective SDCAS in a 500 mL baffled flask. The culture was incubated at 37°C, 200 rpm in a rotary shaking incubator with a 0.05 m throw (G25 incubator shaker, New Brunswick, New Jersey, USA) for 16 h. 20 mL of culture were transferred to a sterile 50 mL tube and placed on ice for 5 minutes before being mixed with 20 mL of sterile 40% (v/v) glycerol. The cell/glycerol suspension
was dispensed as 1 mL aliquots to sterile cryovials (Nalgene Ltd., Hereford, Herefordshire, UK) and stored at -85°C. As previously stated, in an industrial environment cell banks are generally stored in liquid nitrogen but this is both time consuming and expensive. Hence, in an academic research environment the accepted storage method for bacterial cells is freezing at or below -70°C, this method has been standard in the Biochemistry Department at University College London for 20 years (John Ward, personal communication).

2.4 Inoculum preparation

A glycerol stock was defrosted at room temperature and streaked, in triplicate, on to selective LBM agar plates, which were incubated at 37 °C for 24 h. Single, well separated colonies were picked from the plates and inoculated in quadruplicate to 10 mL of the appropriate selective medium in McCartney bottles. The cultures were incubated horizontally, at 37 °C, 200 rpm for 16 h.

For small scale cultures 20 µL of a single overnight culture were used as the inoculum. For shake flask cultures 2 mL of a single overnight culture were used as the inoculum. For 7 L fermenter cultures a single overnight culture was inoculated into 500 mL of the appropriate selective medium in a 2 L inoculation flask which was incubated at 37 °C, 200 rpm for 12 h. This culture was then used to inoculate the fermenter. For 2 L chemostat cultures 3 overnight cultures were bulked and then used to inoculate the fermenter. The various inoculation routes are illustrated in Figure 2.1.
Chapter 2: Materials and methods

Figure 2.1. Inoculation routes used in this work
2.5 Fermentations for the production of pSVβ

2.5.1 Small scale cultures

Casein hydrolysate was removed from SDCAS to give defined medium (DM). Cultures were grown in McCartney bottles, with a 10 mL working volume, which were incubated horizontally at 37 °C, 200 rpm for 21.5 h. For nutrient pool experiments DM was supplemented according to Holiday (1956) (See Table 2.1 and Appendix 2). Design Expert 5 (DX-5, Statease Corporation, Minneapolis, USA) was used to create the quarter fraction duplicated factorial design (see section 1.6.1) and DM was supplemented accordingly (see Table 2.2).
<table>
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Preparation of compounds is detailed in Appendix 2
Table 2.2. Quarter fraction duplicated factorial design

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Abbreviations are standard amino acid and base abbreviations.

*; high concentration; 1.67 mg/L for amino acids, 8.33 mg/L for bases.

- ; low concentration; 0.17 mg/L for amino acids, 0.83 mg/L for bases.
2.5.2 Fermenter cultures

2.5.2.1 Conditions and controls

All fermenter cultures were grown on SDCAS medium. Fermentations were carried out in a 7 L LH210 series bioreactor with a 5.5 L working volume (Adaptive Biosystems Ltd., Luton, Bedfordshire, UK) which was aerated at 5.5 L/min. Oxygen levels were monitored using an Ingold polarographic probe (Mettler-Toledo Ltd., Leicester, Leicestershire, UK) and were maintained at 30 % of saturation by automatic stirrer speed adjustment. Stirrer speed was set to a minimum value of 200 rpm to maintain mixing within the fermenter. pH was monitored using a gel filled pH electrode (Mettler-Toledo) and was maintained at 6.30 ± 0.02 by automatic additions of 4 M sodium hydroxide and 4 M phosphoric acid. Foam was controlled automatically by the addition of polypropylene glycol 2025 (PPG). When the foam probe detected foam the PPG feed was switched on for 5 s and then switched off for 5 s. This cycle continued until foam was no longer detected. Exit gas analysis of oxygen and carbon dioxide was carried out by mass spectrometry (VG gas analysis systems Ltd., Middlewich, Cheshire, UK). Online data was logged by Propack data logging and acquisition software (Acquisition Systems, Fleet, Hampshire, UK).

2.5.2.2 Fed-batch cultures

Fed-batch culture consisted of an exponential 40 % (w/v) D-glucose and a linear amino acids feed. 40% (w/v) D-glucose was fed exponentially to achieve a growth rate of 0.1 (1/h) using a 2 rpm and a 32 rpm Watson Marlow 101U pump (Watson Marlow, Falmouth, Cornwall, UK) controlled through Propack, via a TCS control unit (Eurotherm, Worthing, West Sussex, UK), according to Gregory et al. (1994) (See Figure 2.2).
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The main control algorithm was:

\[ F = \frac{\mu}{Y_{x/glucose}} x(t) V(t) R \]

\[ x(t) = x(t - \Delta t) e^{\mu \Delta t} \]

\[ V(t) = V(t - \Delta t) + \frac{F}{S_f} \Delta t \]

Where:

- \( F \) = D-glucose feed rate (g/h)
- \( \mu \) = required specific growth rate (1/h)
- \( x \) = dry cell weight (g/L)
- \( v \) = Culture volume (L)
- \( Y_{x/glucose} \) = growth yield from D-glucose (g/g)
- \( S_f \) = D-glucose feed concentration (g/L)
- \( R \) = scaling factor to achieve a desired RQ

D-glucose feeding began when a rapid drop in oxygen uptake rate indicated D-glucose exhaustion, which was confirmed by dinitrosalycilic acid (DNS) reducing sugar assay (Miller, 1959). Upon commencement of D-glucose feeding, values for dry cell weight, required growth rate, required RQ, D-glucose feed concentration, growth yield from D-glucose and estimated culture volume were manually input to Propack. Dry cell weight was estimated from OD\(_{625}\) using a previously constructed calibration curve. Growth yield from D-glucose was assumed to be 0.5 g/g from previous work carried out in the department (O’Kennedy et al., 2003). Propack estimated biomass on a 5 min cycle and adjusted the feed rate accordingly. A manual measurement of OD\(_{625}\) was made hourly and dry cell weight was estimated from the calibration curve. Where the manual dry cell weight estimate and Propack’s dry cell weight value were significantly different Propack was adjusted to reflect the manually estimated value. The linear
amino acids feed, at 60 mL/h, was started 5 h after the commencement of D-glucose feeding. Per litre the feed consisted of, leucine (0.64 g), glycine (0.56 g) and histidine (0.17g). The amino acid concentrations of the feed were calculated from amino acid uptake rate data reported by Gschaedler and Boudrant (1994)
Figure 2.2. Set-up for control of exponential D-glucose feeding.
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2.6 Chemostat fermentations

2.6.1 Conditions and controls

Chemostat cultures were grown on SDCAS medium and were carried out in a 2 L (1.2 L working volume) LH 210 series bioreactor (Adaptive Biosystems Ltd.) which was aerated at 1.2 L/min. Monitoring and control was essentially the same as described in section 2.5.2.1 with the exception of foam and temperature control. Foam was controlled by the automatic addition of polypropylene glycol 2025 at 5 s/h. Due to the chemostat configuration there were not enough ports in the top plate to use a heating element and a cooling finger. The temperature was maintained at 37 °C by pumping RO water from a circulating water bath set at 39.5 °C through the cooling finger. The temperature in the water bath was measured using the water bath’s integral thermocouple. The chemostat set-up is shown in Figure 2.3.

Following inoculation, cultures were allowed to grow in batch mode for 22 h before transition to continuous mode was made. Continuous mode was achieved by feeding SDCAS medium, containing D-glucose at 0.5 g/L and kanamycin at 25 mg/L, at a rate of 120 mL/h to achieve a dilution rate of 0.1 (1/h). Fresh medium was pumped into the fermenter, through a medium break (FT Applikon, Ltd., Tewkesbury, Gloucestershire, UK) using a calibrated peristaltic pump (Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). A small volume of sterile air, from the air inlet line, was fed through the medium break to push the fresh medium into the vessel and prevent growth in the medium break. Spent medium was pumped from the vessel, through an overflow tube, via a Watson Marlow 32 rpm 101U pump set to maximum speed. A period of 30 h (3 volume changes) in chemostat mode was allowed before sampling began. 30 mL samples were taken at 3 h intervals. A large sample interval was required to minimise fluctuations in culture volume. Chemostat culture was carried out for 48 h after which isopropyl-β-D-thiogalactoside (IPTG, BioGene, Kimbolton, Cambridgeshire, UK) was added to the vessel and
the medium reservoir to a final concentration of 1 mM. Sampling was continued for a further 48 h.
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Figure 2.3. Chemostat set-up.
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2.7 Fermentations for the production of p5176

2.7.1 Conditions and controls
Monitoring and control was essentially the same as described in section 2.5.2.1. Where Superbroth medium was used pH was controlled to 7.40 ± 0.02 by automatic addition of 4 M sodium hydroxide and 4 M phosphoric acid. A pH setpoint of 7.4 was used for Superbroth as this is what was advised by Wade-Martins (personal communication).

2.8 Analytical techniques
Unless otherwise stated sampling was carried out at 1 h intervals and a range of measurements were made. Optical density was measured at 625 nm (Ultrospec 2000, Pharmacia Biotech or Jenway 6400, Jenway, Dunmow, Essex, UK). Wet cell pellets and culture supernatants were frozen at −20 °C for retrospective analysis.

2.8.1 Wet and dry cell pellets
To obtain wet cell pellets triplicate 5 mL aliquots of culture were centrifuged at 3,500 rpm (GS6R; Beckman, High Wycombe, Buckinghamshire, UK) for 20 min at 4 °C in sterile universals. Supernatant was removed to clean tubes and cell pellets were washed in 5 mL of sterile reverse osmosis (RO) water and recentrifuged. Pellets were resuspended in approximately 1 mL of residual supernatant, transferred to sterile 2.2 mL microtubes and pelleted at 13,000 rpm for 3 min (Biofuge 13, Heraeus, Brentwood, Essex, UK). For DH10β p5176 additional 'large' wet pellets were taken at 2 h intervals. Single 200 mL aliquots of culture were centrifuged at 10,000 rpm (Beckman J2-M1, JA-10 rotor) for 30 min at 4 °C. Supernatant was discarded and cell pellets were washed in 100 mL of sterile RO water and recentrifuged. Supernatant was discarded and the centrifuge bottles were allowed to drain for 1 min. For dry cell weight determination triplicate 5 mL aliquots of culture were centrifuged at 3,500 rpm for 20 min at 4 °C in pre-weighed McCartney bottles. Pellets were washed in 5 mL of sterile RO water and recentrifuged, before being dried at 80 °C for 24 h.
2.8.2 DNS reducing sugar assay

D-glucose concentration in culture supernatants was determined using the dinitrosalicylic acid (DNS) reducing sugar assay (Miller, 1959). DNS reagent was prepared by dissolving 16 g of sodium hydroxide and 10 g of dinitrosalicylic acid in 200 mL of water and dissolving 300 g sodium potassium tartrate in 300 mL of water. Both solutions were brought to the boil and then combined and allowed to cool to room temperature before being used. Culture supernatants were thawed at room temperature. When necessary supernatants were diluted in RO water to bring them in to the range of the calibration curve. 500 µL of supernatant were mixed with 500 µL of DNS reagent in glass test tubes which were covered with foil to prevent evaporative loss. Samples were placed in a boiling water bath for 5 min to allow colour development. Samples were cooled on ice and 4 mL of RO water were added. Absorbance at 540 nm was measured against a water blank and compared to a calibration curve freshly constructed from D-glucose in the range 0 - 4 g/L to determine residual D-glucose concentration.

2.8.3 Amino acid assay

Amino acid concentration in culture supernatants was measured by absorbance at 570 nm after reaction with ninhydrin. 500 µL of culture supernatant was mixed with 500 µL of 60 % perchloric acid in microfuge tubes, to precipitate proteins. Tubes were centrifuged for 3 min at 13,000 rpm and supernatants were removed to clean tubes. Supernatants were diluted 1/100 in RO water and 1 mL was mixed with 1 mL ninhydrin reagent (Sigma) in glass test tubes. Tubes were covered with foil to prevent evaporative loss and transferred to a boiling water bath for 15 min. Samples were placed on ice and 3 mL RO water was added. Absorbance at 570 nm was measured against a blank prepared from RO water and treated in the same manner as the samples.

2.8.4 Percentage plasmid-containing cells

Culture samples were appropriately diluted in sterile 0.9 % (w/v) sodium chloride and plated in triplicate onto agar. For DH5α pSVβ and DH5α pBGS18 LBM-X-gal agar was used to enhance
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X-gal colour development (O'Kennedy and Patching, 1995). X-gal (5-bromo-4-chloro-3-indolyl \( \beta \)-D-galactopyranoside, BioGene) was dissolved in dimethylformylamide (Sigma) and added to autoclaved LBM agar to a final concentration of 40 mg/L. DH5\( \alpha \) pQR150 and DH10\( \beta \) p5176 were plated onto LBM agar. Plates were incubated at 37 °C for 24 h. For DH5\( \alpha \) pSV\( \beta \) and DH5\( \alpha \) pBG518 the percentage plasmid-containing cells was calculated from the number of blue colonies divided by the total number of colonies grown on LBM-X-gal plates multiplied by 100. DH5\( \alpha \) pQR150 plates were sprayed with 0.1 M catechol and incubated at room temperature for 1 min to allow colour development. Plasmid-containing cells were able to metabolise the catechol and colonies turned yellow. Non plasmid-containing cells remained white. The percentage plasmid-containing cells was calculated from the number of yellow colonies divided by the total number of colonies grown on LBM plates multiplied by 100. DH10\( \beta \) P5176 plates were replica plated, using sterile velvet, onto LBM agar containing chloramphenicol at 25 mg/L and both original and replica plates were incubated at 37 °C for 18 h. The percentage plasmid-containing cells was calculated from the number of colonies grown on the replica plate divided by the number of colonies grown on the original plate, multiplied by 100.

2.8.5 Plasmid DNA extraction

2.8.5.1 Standard extraction

The standard extraction was used to extract plasmid from wet cell pellet time course samples, as described by Birnboim and Doly (1979). Wet cell pellets, from 5 mL of culture, were defrosted on ice and resuspended in 300 \( \mu L \) of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8) containing RNase A at 50 \( \mu g/mL \), by vortexing. 300 \( \mu L \) alkaline lysis buffer (1% SDS, 0.2 M sodium hydroxide) was added to the cell suspension and lysis was allowed to proceed for 4 min at room temperature. 300 \( \mu L \) of chilled 3 M potassium acetate (pH 5.5) was added and tubes were incubated on ice for 30 min. The neutralised lysate was clarified by centrifugation at 13,000 rpm for 5 min. The supernatant was transferred to clean tubes and 700 \( \mu L \) of isopropanol was added.
to precipitate DNA. Precipitated DNA was pelleted at 13,000 rpm for 5 min and the supernatant was discarded. After air drying overnight DNA pellets were resuspended in 150 µL, or in the case of p5176, in 50 µL of TE buffer.

2.8.5.2 Qiagen Maxi extraction

Unless otherwise stated all buffers were proprietary and supplied by Qiagen (Qiagen Ltd., Crawley, West Sussex, UK). Qiagen Maxi Columns were used according to the Qiagen Maxi Protocol (Qiagen, 2002) to extract highly purified pSVβ, pBGS18 and pQR150 in order to construct standard curves for PicoGreen plasmid quantification (see section 2.8.6.2). 3 g of cell paste were thawed at room temperature and resuspended, by vortexing, in 10 mL of buffer P1 containing RNase A at 100 µg/mL. The cell suspension was transferred to a 40 mL Beckman tube. 10 mL of buffer P2 were added and the tube was inverted 6 times to ensure efficient mixing. Lysis was allowed to proceed for 4 min at room temperature. 10 mL of chilled buffer P3 were added and the tube was inverted 6 times before being incubated on ice for 20 min. The neutralised lysate was clarified by centrifugation at 17,000 rpm (Beckman J2-M1, JA-17 rotor) for 30 min at 4 °C. The supernatant was removed to a clean 40 mL Beckman tube and the centrifugation step was repeated. During the centrifugation step a Qiagen tip 500 was equilibrated with 10 mL buffer QBT. The supernatant from the centrifugation was applied to the equilibrated tip 500 and the tip was allowed to empty by gravity flow. The tip 500 was washed with 2 x 30 mL of buffer QC, with each 30 mL aliquot being allowed to completely drain from the tip before the second aliquot was applied. DNA was eluted from the tip 500, in to a 40 mL Beckman tube, with 15 mL of buffer QF. DNA was precipitated by the addition of 10.5 mL of room temperature isopropanol, and the tube was centrifuged at 17,000 rpm for 30 min at 4 °C. The supernatant was carefully poured off the DNA pellet and the pellet was washed in 5 mL of 70 % (v/v) ethanol. The tube was centrifuged at 17,000 rpm for 10 min at 4 °C. The supernatant was carefully poured off the pellet and the pellet was allowed to air dry for 10 min before being
dissolved in 1 mL of TE buffer (100 mM Tris HCl pH 8, 10 mM EDTA pH 8). Purified plasmid was stored at –20 °C.

**2.8.5.3 Modified Qiagen Maxi extraction**

A modified Qiagen Maxi Protocol (Wade-Martins, personal communication) was used to extract highly purified p5176 in order to construct standard curves for PicoGreen plasmid quantification (see section 2.8.6.2). In this method the cell resuspension buffer contained glucose in order to minimise shear damage to the plasmid (Prazeres, 1999). Larger buffer volumes were used and additional filtration, centrifugation and plasmid precipitation steps were included. These additional steps were necessary in order to remove impurities and ensure smooth running of the Qiagen tip 100. This method was also used to extract plasmid DNA from 200 mL samples from 7 L fermentations, as advised by Wade-Martins. All mixing steps were done gently to avoid shearing the plasmid DNA. Pipetting was avoided, with all steps which involved transfer of plasmid-containing liquor from one container to another being achieved by pouring. When pipetting was required, for example in gel loading, 'wide bore' tips were used to avoid subjecting the plasmid DNA to high shear forces. 'Wide bore' tips were made by cutting the ends off standard 200 µL or 1000 µL pipette tips. 3 g of cell paste were thawed at room temperature and resuspended, by vortexing, in 40 mL of GTE buffer (10 g D-glucose, 25 mL 1 M Tris, 20 mL 0.5 M EDTA per litre) containing RNase A at 100 µg/mL. The cell suspension was transferred to a 500 mL Beckman bottle. 80 mL of buffer P2 was added and the bottle was swirled for 5 s to ensure efficient mixing. 60 mL of chilled buffer P3 were added and the bottle was swirled for 5 s before being incubated on ice for 10 min. The bottle was centrifuged at 10,000 rpm (Beckman J2-M1, JA-10 rotor) for 30 min at 4 °C. The supernatant was filtered through a double layer of tissue in to a clean 500 mL Beckman bottle and the centrifugation and filtration steps were repeated. The DNA was precipitated by the addition of 110 mL of room temperature isopropanol and the bottle was centrifuged at 10,000 rpm for 30 min at 18 °C. The supernatant was removed and the pellet was allowed to air dry for 5 min. The pellet was resuspended in 4 mL of TE buffer and
transferred to a 40 mL Beckman tube. The Beckman bottle was rinsed with 8 mL 1.5 x QBT buffer, which was then transferred to the 40 mL tube. The tube was cooled on ice for 5 min and then centrifuged at 17,000 rpm for 10 min at 4 °C, to pellet insoluble impurities. This step was essential to allow the sample to run smoothly through the Qiagen tip 100. During the centrifugation step a Qiagen tip 100 was equilibrated with 4 mL of buffer QBT. The supernatant from the centrifugation was applied to the equilibrated tip 100 and the tip was allowed to empty by gravity flow. The tip 100 was washed with 2 x 10 mL of buffer QC, with each 10 mL aliquot being allowed to completely drain from the tip before the second aliquot was applied. DNA was eluted from the tip 100, in to a 40 mL Beckman tube, with 5 x 1 mL of buffer QF which was pre-heated to 70 °C. DNA was precipitated by the addition of 3.5 mL of room temperature isopropanol, and the tube was centrifuged at 17,000 rpm for 30 min at 4 °C. The supernatant was carefully poured off the DNA pellet and the pellet was washed in 5 mL of 70 % (v/v) ethanol. The tube was centrifuged at 17,000 rpm for 10 min at 4 °C. The supernatant was carefully poured off the pellet and the pellet was allowed to air dry for 10 min before being dissolved in 1 mL of TE buffer. Purified plasmid was stored at 4 °C.

2.8.6 Plasmid analysis

2.8.6.1 A260 analysis

A260 analysis was used to determine the concentration of DNA in Qiagen purified DNA samples. DNA solutions were diluted in TE buffer to allow an A260 reading in the range 0.1 - 0.7 to be obtained. The diluted sample was placed in a quartz cuvette which had been soaked in 0.25 M hydrochloric acid for 20 min and then washed in RO water 5 times, to remove any residual DNA and enzyme activity. The absorbance at 260 nm was read, in triplicate, against TE buffer and the mean reading was used to calculate the DNA concentration in the sample (µg/mL):

\[
[\text{DNA}] = A_{260} \times 50 \times D
\]
A value of 50 was used because a 50 μg/mL solution of double stranded DNA has an absorbance value of 1.0 at 260 nm (Sambrook et al., 1989).

2.8.6.2 PicoGreen total plasmid quantification

A 96 well PicoGreen assay was used to determine total plasmid DNA yields, based on the cuvette method of Noites et al., 1999. PicoGreen reagent and plasmid DNA samples were diluted \( \frac{1}{400} \) in TE buffer to give working solutions. Equal volumes (100 μL) of diluted sample and PicoGreen working solution were mixed in a 96-well plate and incubated, protected from light, at room temperature for 3 min. Fluorescence was read in a 96 well microtitreplate fluorimeter (Fluorocount, Packard Bioscience, Reading, Berkshire, UK) controlled through plate reader software (Packard Bioscience) at excitation and emission wavelengths of 485 nm and 530 nm respectively. Plasmid DNA concentration was determined by comparison of fluorescence with a standard curve constructed using Qiagen purified plasmid DNA in the range 30 – 1000 ng/mL.

Standard plate loading is shown in Figure 2.3
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Figure 2.4. Loading of 96 well plate for PicoGreen assay

TE; TE buffer, S; sample. Numbers in row B refer to DNA concentrations (ng/mL) in the standard curve.
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2.8.6.3 Gel electrophoresis

Gel electrophoresis was used to determine the percentage supercoiled plasmid obtained from DH5α pSVβ fermentations. 0.7% (w/v) agarose gels were made up in 100 mL of 0.5 x TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA). 10 μL of plasmid sample were mixed with 2 μL of loading buffer (Sigma) and 3 μL of TE buffer and the 15 μL solution was loaded on to the gel. Gels were run at 80 V, 40 mA in 1 L of 0.5 x TBE buffer for 2 h. DNA in gels was visualised by staining in ethidium bromide (0.5 μg/mL, Sigma) for 20 min. Gels were scanned using UVP 5000 Gel Documentation System (Ultra Violet Products Ltd., Cambridge, Cambridgeshire, UK). Densitometry plots were obtained using UVP Gelbase (Ultra Violet Products Ltd.). A correction factor of 1.36 was applied to peak areas for open circular plasmid DNA to correct for the increased binding efficiency of ethidium bromide to open circular plasmid DNA (Projan et al., 1983). The percentage of supercoiled plasmid DNA was calculated as the proportion of supercoiled plasmid DNA to total plasmid DNA, multiplied by 100.

2.8.6.4 Pulsed field gel electrophoresis

PFGE was used to determine the percentage supercoiled plasmid obtained from DH10β p5176 fermentations. 1% (w/v) agarose gels were made up in 130 mL of 0.5 x TBE buffer. All plasmid handling was performed with ‘wide bore’ 200 μL tips, made by cutting the ends off standard 200 μL tips, to minimise the effects of shear. 20μL of plasmid sample were mixed with 4 μL of loading buffer and the 24 μL solution was loaded on to the gel. Mid range marker II (New England Biolabs, Hitchin, Hertfordshire, UK) was run alongside plasmid samples to indicate whether the gel had run successfully. The marker is provided embedded in agarose in a 1 mL syringe. Slices of the marker were loaded into the wells once the gel had set. It was found that if marker slices were placed on the gel comb and moulded with the gel, as is sometimes recommended, denaturation occurred and the marker did not run. Mid range marker II is composed of intact lambda DNA annealed to Xba I lambda DNA fragments to form concatamers of various sizes. Xba I produces fragments of 24 kb and 24.5 kb, hence the size range of mid
range marker II is 24 kb – 291 kb. Gels were run at 6 V/cm, 14 °C with an initial switch time of 2 s and a final switch time of 16 s, for 18 h, in 2 L of 0.5 x TBE buffer. The buffer was pre-cooled to 14 °C before the gel was placed in the tank. The pump to the cooling unit was switched off for the first 20 min of the run to prevent samples being washed out of the wells before the DNA had entered the gel. DNA in gels was visualised by staining in ethidium bromide (0.5 μg/mL, Sigma) for 4 h. Gel scanning and densitometric determination of percentage supercoiling was determined as described in section 2.8.6.3.

2.8.6.5 N.BsfNB I digestion of recovered p5176

N.BsfNB I (New England Biolabs) was used to digest a sample of p5176 which was recovered from a 5 L batch fermentation in order to determine what the constituents of the sample were. 160 μL of a p5176 sample were mixed with 56 μL of N.BsfNB I and 24 μL of 10 x NEbuffer (New England Biolabs) in a microfuge tube and the tube was incubated at 55 °C in a water bath. 30 μL samples were withdrawn from the tube after 10, 20, 30, 40, 50, 60, 120 and 240 min and were mixed with 5 μL of loading buffer (Sigma). The samples were loaded on to a 130 mL 1% agarose PFGE gel and the gel was run, stained and photographed as described in section 2.8.6.4, with the exception of the run time which was reduced to 12 h.

2.8.7 Enzyme activity assays

All assays were carried out in triplicate.

2.8.7.1 β-galactosidase assay

β-galactosidase was the marker enzyme used to determine levels of protein production from pBGS18. The assay was carried out according to the method of Miller (1972). 500 μL (non-induced cultures) or 100 μL (IPTG-induced cultures) of culture was added to Z buffer (21.5 g disodium hydrogen orthophosphate dodecahydrate, 6.24 g sodium dihydrogen orthophosphate dihydrate, 0.75 g potassium chloride, 0.246 g magnesium sulphate heptahydrate, 2.7 mL β-mercaptoethanol per litre, adjusted to pH 7 with 4 M sodium hydroxide) to a final volume of 1 mL
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in a 2.2 mL microcentrifuge tube. 2 drops of chloroform and 1 drop of 0.1 % (w/v) SDS from a Pasteur pipette were added and tubes were vortexed for 10 s. Tubes were centrifuged at 13,000 rpm for 5 min to pellet cell debris. Supernatants were decanted to clean cuvettes, 200 μL of o-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma) solution (4 mg/mL in 0.1 M phosphate buffer pH 7.5) were added and tubes were shaken to mix. Colour was allowed to develop for approximately 15 min, before 500 μL of 1 M sodium carbonate were added to quench the reaction, the exact reaction time was recorded. Absorbance at 420 nm was measured against a water blank treated in the same manner as the samples. β-galactosidase activity was calculated as:

\[ \text{Activity(A/min/mL)} = \frac{\text{OD}_{420}}{t \times V} \]

Where:
\( t \) = Reaction time (min)
\( V \) = culture volume assayed (mL)

2.8.7.2 Catechol 2,3-dioxygenase assay

Catechol 2,3-dioxygenase was the marker enzyme used to determine levels of protein production from pQR150. Activity was determined using an adaptation of the method of Huang et al. (1990). 10 mL culture aliquots were centrifuged at 3,500 rpm for 15 min. Supernatants were discarded and cell pellets were resuspended to approximately 0.19 g/mL in 0.1 M phosphate buffer, pH 7.5 supplemented with 10% (v/v) acetone. Exact resuspension volumes were recorded. Cell suspensions were sonicated (Soniprep 150, SanyoGallenkamp, Loughborough, Leicestershire, UK) using 3 x 10 s cycles with a 10 s break at an amplitude of 10 μm. Sonicated samples were centrifuged at 13,000 rpm for 15 min to remove cell debris. Supernatants were decanted to clean microfuge tubes and kept on ice until the activity assay was completed. 40 μL of supernatant were added to 2.7 mL of 83 mM HEPES buffer (Sigma), pH 8 and 300 μL 1 mM catechol in a 4
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mL acetone-compatible cuvette. The cuvettes were inverted to mix and the change in absorbance at 375 nm was measured (Ultrospec 4000, Pharmacia Biotech) against 83 mM HEPES buffer and recorded via Swift II reaction kinetics software (Pharmacia Biotech) for a period of 3 min. When necessary cell extracts were diluted in 0.1 M phosphate buffer, pH 7.5 supplemented with 10% (v/v) acetone to bring the absorbance into the range of the spectrophotometer. Catechol 2,3-dioxygenase activity was calculated as:

\[
\text{Activity (A/min/mL)} = \frac{\Delta A}{0.04} \times V_e \times D
\]

Where:

\(\Delta A\) = Change in absorbance per min, calculated by Swift II reaction kinetics software

\(V_e\) = Total volume of extract (mL)

\(D\) = Dilution factor
3 Development of a rapid 96 well microtitre plate PicoGreen assay

3.1 Introduction

The rapid 96 well PicoGreen assay was developed from the cuvette method of Noites et al. (1999). In this method appropriately diluted plasmid DNA samples, in a 0.5 mL volume, were mixed with 0.5 mL of PicoGreen working solution in a cuvette and were incubated for 3 min. Sample fluorescence was then measured in a laboratory fluorimeter at excitation and emission wavelengths of 480 nm and 520 nm respectively. The cuvette method, although faster than gel electrophoresis, is time consuming when processing large numbers of samples. For example, determination of plasmid DNA concentration over the time course of a fed-batch fermentation involves large numbers of samples, the adaptation of the cuvette method to a 96 well format could significantly decrease the assay time, allowing all samples to be assayed in around 5 minutes.

3.2 Determination of optimum emission filter

Noites et al. (1999) used an excitation wavelength of 480 nm and an emission wavelength of 520 nm. The 96 well plate fluorimeter used in this work (Fluorocount, Packard Bioscience) had fixed wavelength filters. An excitation wavelength of 485 nm was chosen and the available emission wavelengths were tested to determine which was most suitable.

A Qiagen purified pSVβ standard curve, in the range 30 – 1000 ng/mL was loaded in quadruplicate to a 96 well plate, mixed with PicoGreen working solution and incubated at room temperature for 3 minutes, as described in section 2.8.6.2. The plate was then read once using each available emission filter. The results of this test are shown in Figures 3.1 and 3.2.

As Figure 3.1 demonstrates, there was a linear correlation between plasmid DNA concentration and fluorescence for all emission filters. The 530 nm and 570 nm filters both gave ranges of 0 – 70,000 fluorescence spectroscopy units (FSU), the 580 nm filter gave a range of 0 – 13,000 FSU and the 590 nm filter gave a range of 0 – 30,000 FSU. In all cases the assay was highly
repeatable which was demonstrated by the small standard deviations. In order to make the assay as sensitive as possible to small differences in plasmid DNA concentration, the widest FSU range was sought. Both the 530 nm and 570 nm filters fitted this criterion. As Figure 3.2 demonstrates, the absolute background fluorescence was lowest for the 580 nm filter, with the other three filters having similar values. In terms of background fluorescence as a percentage of the filter's fluorescence range the 530 nm and 570 nm were again the most favourable, having values of approximately half those of the 580 nm and 590 nm filters. The 530 nm filter was chosen over the 570 nm filter as it demonstrated a slightly lower background and a smaller standard deviation.

The range of fluorescence values obtained here was not comparable to those reported by Noites et al. (1999). The reason for this is that the fluorescence spectroscopy unit is an arbitrary unit and is dictated by the fluorimeter.
Figure 3.1. Fluorescence Spectroscopy Units vs DNA concentration for four emission filters.

The results shown are the means of quadruplicate determinations made using Qiagen purified pSVβ plasmid DNA. Error bars represent 1 standard deviation. △, 530 nm, ○, 570 nm, ○, 580 nm, □, 590 nm.
Figure 3.2. Background fluorescence for wells containing TE buffer.

■; mean background fluorescence, ■; background fluorescence as a percentage of filter fluorescence range. The results shown are the means of 12 determinations. Error bars represent one standard deviation.
3.3 Determination of optimum sample dilution

Having determined the optimum emission filter for the assay system, the optimum sample dilution was investigated for process samples.

Plasmid DNA was extracted from small scale fermentation samples using the standard extraction method, described in section 2.8.5.1. The plasmid preparations were diluted in the range $\frac{1}{200} - \frac{1}{700}$ and assayed as described in section 2.8.6.2. The results of this investigation are shown in Figure 3.3.

Regardless of sample concentration, a linear correlation was found between sample dilution and fluorescence at dilutions of $\frac{1}{200} - \frac{1}{400}$, at higher dilutions this linearity was lost. The reason for this loss of linearity is unclear and was not reported by Noites et al. (1999), they observed linearity across the dilution range $\frac{1}{90} - \frac{1}{1400}$. A sample dilution of $\frac{1}{400}$ was chosen for the assay to allow the minimum amount of sample to be used without compromising the assay result.
Figure 3.3. Raw FSU vs dilution factor for three samples of pSVβ DNA.
The 3 different symbols represent 3 different samples. Plasmid was purified from small scale cultures using the standard extraction procedure. For clarity only three sets of results are presented, the remaining results can be found in Appendix 6.
3.4 Correlation between PicoGreen and Gel Electrophoresis assays

Plasmid concentrations determined from PicoGreen and gel electrophoresis assays were compared in order to determine the correlation between the two assays. Plasmid DNA was extracted from small scale fermentation samples using the standard extraction technique. The plasmid samples were then assayed by PicoGreen and by gel electrophoresis. Gel electrophoresis samples were run against a pSVβ standard curve to allow their concentrations to be calculated. The concentration of a Qiagen purified sample of pSVβ was determined by A260, as described in section 2.8.6.1 and the sample was diluted to give a 4 point standard curve in the range 25 ng/well – 150 ng/well. The gel was run, stained and scanned as described in section 2.8.6.3 and densitometry plots were obtained. A correction factor of 1.36 was applied to peak areas for open circular plasmid DNA to correct for the increased binding efficiency of ethidium bromide to open circular plasmid DNA (Projan et al., 1983). The total plasmid peak area was determined, by addition of supercoiled and corrected open circular peak areas, and a standard curve of peak area against plasmid DNA/well was plotted. The concentration of gel electrophoresis samples was determined from this standard curve. The correlation between the gel electrophoresis and PicoGreen assays is shown in Figure 3.4.

As Figure 3.4 demonstrates, the linear fit of the data lay very close to the theoretical ideal fit, and an R² value of 0.86 was obtained. It is known that gel electrophoresis has an error of up to 20 % (Projan et al., 1983). The errors in the gel electrophoresis data obtained in this work were in agreement with this. The errors obtained in the PicoGreen assay were typically around 3 % which was in agreement with the errors reported by the manufacturer (Molecular Probes, 2003) and less than the errors reported by Noites et al. (1999). When the errors associated with each assay method were taken into account the data coincided with the theoretical ideal fit, this was in agreement with observations by Noites et al. (1999).
Figure 3.4. Correlation between PicoGreen and Gel Electrophoresis assays.
PicoGreen data are the result of triplicate determinations and error bars represent 1 standard deviation. Gel Electrophoresis data were determined from duplicate samples and error bars represent 1 standard deviation. ·····; theoretical ideal correlation, —; actual correlation.
3.5 Conclusion

A rapid 96 well PicoGreen plasmid DNA assay was developed which compared favourably with both the cuvette assay of Noites et al. (1999) and with conventional gel electrophoresis. The assay allowed up to 24 samples to be assayed in triplicate in around 5 minutes. When sample preparation and plate filling were taken into account the assay took around 45 minutes. This compared very favourably with gel electrophoresis where gel and sample preparation and gel running, staining and scanning may take around 3 hours.
4 Development of a fed-batch fermentation for the production of pSVβ

4.1 Introduction

Small amounts of plasmid DNA may be obtained through standard laboratory techniques, but if gene therapy is to become standard medical treatment much larger quantities of high quality DNA must be produced (Lahijani et al., 1996; Chen et al., 1997), and an efficient fermentation must be established. There is a current understanding that supercoiled plasmid DNA is the best form for administration (Prazeres et al., 1999) and plasmid DNA manufactured for gene therapy should be mainly in the supercoiled form. FDA guidelines require a specification for the minimum level of supercoiled plasmid DNA, in the final product. Ideally a fermentation should therefore produce plasmid DNA in the mainly supercoiled form and contaminants such as genomic DNA and RNA should be minimised in order to reduce the challenge to the downstream process. Expected acceptable contaminant limits in a final plasmid product, per mg of plasmid, are 1 μg of genomic DNA, 10 μg of protein, 5 units of endotoxin and undetectable RNA (Shamlou, 2003).

O'Kennedy et al. (2000) demonstrated that fermentation medium can have a profound effect on plasmid quality and contaminant level. Semi-defined medium (SDCAS) was shown to support a higher cell density and plasmid stability than complex medium. Plasmid extracted from cells grown on semi-defined medium had a greater degree of supercoiling and lower levels of genomic DNA contamination than plasmid produced using complex medium.

Limitation or starvation of amino acids has been shown to induce plasmid amplification, leading to increased plasmid yields. Two areas of research have been reported. The first investigated induction of plasmid amplification in various plasmid types to determine the underlying mechanisms (Hecker et al., 1986; Wegrzyn and Wegrzyn, 1995; Wegrzyn, 1995; Wrobel and Wegrzyn, 1997a, 1997b, 1998; Wegrzyn, 1999; Potrykus et al., 2000)). The second area researched the application of this amplification technique to increase production of plasmid DNA for use in molecular biology or recombinant DNA technology (Hofmann et al., 1990). The use of
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This amplification method for industrial scale production of plasmid DNA has not been reported. This chapter describes an investigation into the applicability of this method to a fermentation for the production of high quality plasmid DNA suitable for use in gene therapy.

4.2 Aim and approach

The aim of the work presented in this chapter was to develop a fermentation strategy which made use of amino acid starvation as a means of increasing the specific yield of plasmid DNA. Based on the earlier findings of O'Kennedy et al. (2000), the semi defined medium SDCAS was used.

Small scale cultures were first used to test the hypothesis that starvation of the complex amino acid source casein hydrolysate was a viable method of increasing specific plasmid yield, for the host-plasmid system E. coli DH5α pSVβ (see section 4.3.1). The results from this experiment confirmed the hypothesis.

Plasmid production was next examined in 7 L batch culture (see section 4.3.2). This work revealed that plasmid amplification did occur, leading to an increase in specific plasmid yield. The highest specific plasmid yield did not occur at the same time as the highest level of plasmid supercoiling. An analysis of productivity, in terms of supercoiled plasmid, showed that the greatest productivity occurred at the point of highest plasmid yield and not highest supercoiling. This was because the amplified plasmid levels were only maintained for a short time and maximum plasmid yield and maximum supercoiling occurred separately.

In order to increase the yield of plasmid a glucose and amino acids fed-batch fermentation was investigated (see section 4.3.5). The idea was to feed glucose exponentially, to relieve glucose exhaustion and increase biomass, and to feed amino acids linearly, to delay amino acid starvation and hence delay plasmid amplification. In this manner it was hoped to increase biomass prior to plasmid amplification in order to improve the volumetric plasmid yield.
Nutrient pool and fractional factorial screening experiments were carried out to identify amino acids which were involved in the mechanism of plasmid amplification (see sections 4.3.3 and 4.3.4). The identified amino acids were then fed, in combination with glucose, in a fed-batch fermentation to delay amino acid starvation and hence delay amplification until the biomass was increased. The rest of this chapter details the results of the experiments described in this section.

4.3 Results and discussion

4.3.1 Effect of casein hydrolysate concentration on final biomass and specific plasmid yield

The semi-defined medium SDCAS was chosen as the medium for the model system based on the report by O'Kennedy et al. (2000), that this medium supported a higher cell density (and plasmid stability than complex medium and that plasmid produced from cells grown on this medium had a greater percentage in the supercoiled form than plasmid produced using complex medium.

DH5α pSVβ was chosen as the host-vector system for the investigation of plasmid amplification by amino acid starvation. As discussed in Introduction section 1.4.7, starvation of individual amino acids has been shown to induce amplification of various plasmid types in relA- hosts, leading to increased plasmid yields. E. coli DH5α is an example of a relA- strain. pSVβ is a ColE1-type plasmid. This class of plasmid has been shown to be amplified, in relA- hosts, by amino acid starvation (Hecker et al., 1986; Reithdorf et al., 1989; Hofmann et al., 1990; Wrobel and Wegrzyn, 1997b).

Small scale (10 mL) cultures, grown in triplicate, were analysed to investigate the effect of casein hydrolysate concentration on final biomass and specific plasmid yields. The cultures were grown in McCartney bottles, on Defined Medium (DM) supplemented with casein hydrolysate to final concentrations of 0, 0.1 or 1 % (w/v) as described in section 2.5.1. The 1 % supplemented
medium was equivalent to SDCAS medium. Final biomass was determined by measuring optical density at 625 nm. Plasmid was extracted using the standard extraction method described in section 2.8.5.1. Plasmid yield was determined using the PicoGreen assay described in section 2.8.6.2. Figure 4.1 shows the results of this experiment.

Over a fixed time, as casein hydrolysate concentration decreased, biomass yields decreased and specific plasmid yields increased. This is consistent with plasmid amplification by amino acid starvation.

In cultures with no casein hydrolysate supplement, cells would have become starved of amino acids rapidly following inoculation and hence demonstrated low biomass and high specific plasmid yields. The small amount of growth that was observed was probably due to a carry over of nutrients from the inoculum. In cultures supplemented with 0.1 % (w/v) casein hydrolysate cells would have become starved of amino acids at a later stage and hence demonstrated higher biomass but lower specific plasmid yields due to the reduced amplification time. The cultures supplemented with 1 % (w/v) casein hydrolysate demonstrated high biomass and low specific plasmid yields, it seems likely that these cultures did not become starved of amino acids during the duration of culture and hence did not show any plasmid amplification.

These results demonstrated, at small scale, that starvation of casein hydrolysate was a viable method of amplifying pSVβ harboured in E. coli DH5α. The model system was deemed suitable for further investigation.
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Figure 4.1. Effect of casein hydrolysate concentration on final biomass and specific plasmid yield for *E. coli* DH5α pSVβ.

Cultures were grown in McCartney bottles containing 10 mL of SDCAS, supplemented as indicated, at 37 °C, 200 rpm for 21.5 h. ■; biomass, ■; specific plasmid yield. Values shown are the means of triplicate determinations, error bars represent 1 standard deviation.
4.3.2 Batch fermentation

It had been demonstrated that starvation of casein hydrolysate led to an increase in specific plasmid yield at small scale. The next step was to determine if this method was applicable at laboratory fermenter scale.

Batch fermentations were carried out in a 7 L fermenter with a 5.5 L working volume and were grown on SDCAS medium, as described in section 2.5.2. Biomass was measured hourly by dry cell weight. Wet cell pellet samples were taken hourly and stored at -20 °C. Plasmid was extracted from thawed wet cell pellet samples using the standard extraction procedure described in section 2.8.5.1. Total plasmid was quantified using the PicoGreen assay described in section 2.8.6.2. The percentage of plasmid in the supercoiled form was determined using gel electrophoresis as described in section 2.8.6.3. Residual D-glucose and total amino acid levels in culture supernatants were measured hourly, as described in sections 2.8.2 and 2.8.3 respectively. Figures 4.2, 4.3 and 4.4 show the oxygen uptake rate profile, the biomass, plasmid yield and residual D-glucose and amino acids profiles and the percentage of plasmid in the supercoiled form, respectively.

Oxygen uptake rate reached a maximum value of 21.1 mmol/L/h at 17.5 h and then began to drop rapidly. It continued to decline steadily, reaching a value of 12.5 mmol/L/h at 21.5 h after which there was a sharp increase for 2 h. A new maximum value of 17.9 mmol/L/h was reached, which was rapidly followed by a very sharp decrease. Oxygen uptake rate fell back to 0.7 mmol/L/h by 25 h and this level was maintained for the remaining 10 h of the fermentation.

Biomass climbed steadily, demonstrating exponential growth, and became level at a mean value of 3.5 g/L dry cell weight by 24 h. Biomass data were not recorded between 16 h and 24 h so it is possible that the culture entered stationary phase between 16 h and 24 h. However, there was a very sharp decrease in OU at 23.5 h, and it seems likely that it was at this point that the culture entered stationary phase. Prior to this decrease the OU, although lower than during the observed
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exponential phase, was well above base level, so although the culture may have been in a
decline phase it seems unlikely that it would have entered stationary phase before 23.5 h. The
maximum growth rate, during exponential growth, was 0.28 1/h (see Appendix 8).

Specific plasmid yield increased from a mean of 6.3 mg/g DCW to 11.3 mg/g DCW by 24 h but
dropped to 7.1 mg/g DCW by 25 h. The percentage of plasmid in the supercoiled form declined
steadily during the fermentation, reaching a mean minimum value of 40 % after 25 h. This level
was maintained until 28 h when it increased sharply and reached a maximum value of 90 % by 31
h, which was maintained for 1 h. A decline to a value of 70 % by 34 h, was then observed.

Residual D-glucose levels were not measured between 16 h and 24 h, but the sharp decline in
oxygen uptake rate at 17.5 h suggests primary carbon source exhaustion. This is consistent with
the findings of O'Kennedy et al. (2003). Oxygen uptake rate did not drop to base level
immediately following D-glucose exhaustion, but declined steadily for 4 h, this suggests that the
culture was able to utilise the remaining medium components, such as amino acids and other
components of casein hydrolysate. This is supported by the observation that dry cell weight
increased from 3 g/L to 3.5 g/L between 16 h and 24 h. Although this indicates that further
growth occurred following D-glucose exhaustion it is not consistent with the maximum growth rate
of 0.28 1/h during exponential phase. The report by O'Kennedy et al. (2003) does not indicate at
what time stationary phase occurred as no data was reported between 8 h and 23 h. This report
did, however, record a maximum optical density of 7 units at 24 h. Using the standard curve
shown in Appendix A4.1 this equates to a dry cell weight of 3.4 g/L. Therefore the biomass
obtained in this work is comparable to that previously reported (O'Kennedy et al., 2003). The
increase in oxygen uptake rate observed between 21.5 h and 23.5 h is indicative of increased
cellular activity. One possibility is that during this time the culture was utilising acetate as a
carbon source, following the exhaustion of D-glucose. No measurement of acetate levels in
culture supernatants was made so this hypothesis could not be proven or disproven directly.
However, DH5α is not known to utilise acetate. Whilst it is known that *E. coli* produces acetate under conditions of oxygen starvation or limitation (O'Beirne and Hamer, 2000), oxygen was maintained at or above 30% of saturation in order to avoid oxygen limitation. It is therefore unlikely that acetate consumption occurred following D-glucose exhaustion and hence the increase in oxygen uptake rate between 21.5 h and 23.5 h can not be explained by acetate consumption. It may be postulated that the increase in oxygen uptake rate was due to plasmid amplification, induced by amino acid starvation. Increased plasmid production would increase cellular activity leading to an increase in oxygen requirement. Total amino acid levels were not measured between 16 h and 24 h. However, the total amino acid level measured at 24 h was not very different from that measured at 16 h, which may suggest that amino acid levels did not change over this time. This is not supported by the observation of increased biomass during this time and, in the ninhydrin assay different amino acids form different amounts of coloured reaction product, so depletion of one amino acid may not be reflected in the assay result. It is postulated that one amino acid became starved in the medium resulting in plasmid amplification between 21.5 h and 23.5 h. It may also be postulated that plasmid amplification did occur, but was brought about by carbon source limitation, rather than amino acid starvation. As discussed in section 1.4.7.2 *E. coli* ordinarily exhibits the stringent response in times of nutrient starvation which leads to a shutdown of cellular activity. During amino acid starvation this response is mediated by the *relA* gene product, ppGpp synthetase I, and during carbon source starvation by the *spoT* gene product, ppGpp synthetase II. Mutant strains, which do not exhibit the stringent response, can not shut down cellular activity, one result of which is the initiation of plasmid replication (see section 1.4.7.3). *E. coli* DH5α, which was used in this work, is a *relA* mutant but is not a *spoT* mutant, hence plasmid amplification can only be induced in this strain by amino acid starvation. Therefore it is concluded that the increased specific plasmid yield observed at 24 h was as a result of plasmid amplification brought about by amino acid starvation. The plasmid yield increased from a mean of 6.3 mg/g DCW at 16 h to 11.3 mg/g DCW at 24 h and then fell
back to a mean of 7.1 mg/g DCW by 25 h, indicating that amplified plasmid levels could not be maintained for a great length of time. A decline in plasmid yield following amplification was reported by Hofmann et al. (1990), although the rate of decline was much lower than observed here. The work reported by Hofmann et al. (1990) was conducted at 32 °C whereas this work was conducted at 37 °C. Elevated temperatures have been shown to increase the rate of DNA degradation following plasmid amplification in amino acid starved *E. coli* (Neubauer et al., 1996). The difference in temperature between this work and that reported by Hofmann et al. (1990) may explain the increased rate of degradation.

Although plasmid yield was increased via amplification, only 50 % of the resultant plasmid was in the supercoiled form. As previously discussed, final plasmid DNA product for use in gene therapy should be composed of a majority of plasmid in the supercoiled form and non-supercoiled forms of plasmid may be viewed as impurities. Purification techniques which effectively separate supercoiled plasmid from other plasmid species have been reported (Prazeres et al., 1998; Kendall et al., 2001; Lander et al., 2002; Lemmens et al., 2003; Winters et al., 2003). Although it would be preferable to maximise the level of supercoiling at the fermenter stage it may be argued that a purification scheme should contain a unit operation which is capable of separating plasmid species in order to guarantee the supercoiled level of the purified product. The total yield of supercoiled plasmid in the batch fermentation was calculated according to equation 4.1:

\[
\text{Yield (mg)} = \frac{\text{Volumetric Yield} \times \text{Fraction of Plasmid in Supercoiled Form} \times \text{Fermenter Volume}}{\text{Fermenter Volume}}
\]  

(4.1)

The results of this analysis are shown in Figure 4.5. As Figure 4.5 demonstrates, the amount of supercoiled plasmid available for recovery was greater at the point of maximum plasmid supercoiling than at the point of maximum volumetric plasmid yield. Therefore the data available suggests that it would be preferable to harvest the fermentation at the point of maximum plasmid supercoiling. However, it may be argued that it is not worth spending an additional 8 h of
fermenting time for a relatively small increase in yield due to the associated utilities and labour costs.

In this batch fermentation maximum plasmid yield was not coincident with maximum plasmid quality with respect to supercoiling. Due to the relatively small difference in supercoiled plasmid yield between the point of highest volumetric plasmid yield and the point of highest plasmid supercoiling the preferable point of harvest was following amplification at the point of highest volumetric yield if it is assumed that the downstream process would employ a plasmid species separation step.
Figure 4.2. Oxygen uptake rate profile for *E. coli* DH5α pSVβ grown in batch culture.

The culture was grown on SDCAS in a 7 L fermenter with a 5.5 L working volume. The dashed line indicates the onset of D-glucose exhaustion.
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Figure 4.3. Biomass, plasmid and nutrient profiles for *E. coli* DH5α pSVβ grown in batch culture.

A: Δ; biomass, O; specific plasmid yield, ●; volumetric plasmid yield. B; X; D-glucose, ○; amino acids. The culture was grown on SDCAS in a 7 L fermenter with a 5.5 L working volume. The dashed line indicates the onset of D-glucose exhaustion.
Figure 4.4. Percentage of plasmid in the supercoiled form for *E. coli* DH5α pSVβ grown in batch culture. The culture was grown on SDCAS in a 7 L fermenter with a 5.5 L working volume. The dashed line indicates the onset of D-glucose exhaustion.
Figure 4.5. Total supercoiled plasmid profile for supercoiled plasmid obtained from *E. coli* DH5α pSVβ grown in batch culture.

Total supercoiled yield was calculated according to equation 4.1. The dashed line indicates the highest measured volumetric yield and the dotted line indicates the highest measured supercoiled level.
4.3.3 Nutrient pool experiments

The results of the batch fermentation demonstrated that plasmid DNA yield could be increased through amino acid starvation induced amplification. A fed-batch fermentation was chosen as a method of improving plasmid yield. The intent was to use an exponential D-glucose feed and a linear defined amino acids feed in order to increase biomass and delay induction of plasmid amplification. Amino acids would become starved at a later point than in the batch fermentation due to the linear feed. In this manner volumetric plasmid yield would be improved over the batch fermentation, owing to the increased biomass and plasmid amplification would still occur.

The first step in developing such a fed-batch strategy was the identification of the amino acids, which were involved in plasmid amplification, and that could aid biomass increase. Amino acid analysis equipment was not available during this work, previous work in the Department of Biochemical Engineering had attempted to develop an analysis system but met with no success. Hence, other experimental techniques were employed in this work. Nutrient pool experiments were carried out to identify compounds to carry forward into a factorial design.

Triplicate 10 mL cultures of host and recombinant strains were grown on Defined Medium (DM) supplemented according to Holiday (1956) as described in section 2.5.1. Control cultures were grown on DM with no supplements. Final biomass was determined by measuring optical density at 625 nm. Plasmid was extracted using the standard extraction method described in section 2.8.5.1 and plasmid yield was determined using the PicoGreen assay described in section 2.8.6.2. The results of this investigation are shown in Table 4.1.

The investigation was carried out with 12 different nutrient pools, each consisting of 6 compounds, with the exception of pools 1 and 11 which consisted of 5 compounds, as shown in Table 4.1. Increased growth, over the control culture, in a single pool indicated that the strain had a requirement for two or more nutrients in that pool. Which nutrients these were could not be determined solely from that pool. Each of the pools 1-6 (columns) had a nutrient in common with
each of the pools 7-12 (rows). When increased growth was observed in a column pool and a row pool then the nutrient that was common to both pools (crossover nutrient) was the one that was required by the strain.

In order to identify nutrients which contributed to an increase in biomass a significant increase in biomass in comparison to control cultures, was sought. The results of the investigation, for both the host and the recombinant strain, were examined to identify high biomass phenotypes. The analysis identified leucine, glycine, histidine, adenine, guanine and hypoxanthine in the host strain and adenine, hypoxanthine and guanine in the recombinant strain as contributing to increased biomass.

A significant decrease in plasmid yield, in comparison to control cultures, was used to identify amino acids which, when starved, led to plasmid amplification. Control cultures were grown on DM with no supplement. As discussed in section 4.3.1, this had already been shown to induce plasmid amplification. In cultures with a significantly lower plasmid yield than the control, plasmid amplification was assumed not to have occurred and amino acids in those cultures were assumed to contribute to amplification, when starved. Examination of the results from the investigation did not identify any amino acids. It is possible that the concentrations used in this investigation, which were those reported by Holiday (1956), were too low so amino acid starvation did occur and hence crossover nutrients were not identified.

The nutrients identified as contributing to an increase in biomass for both the host and recombinant strains were used in a further screening experiment to attempt to determine their effects on biomass and plasmid amplification. A fractional factorial design was used and is discussed in the next section.
Table 4.1 A. Biomass production of *E. coli* DH5α

<table>
<thead>
<tr>
<th>Pool</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>OD_{625} % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td><strong>Adenine</strong></td>
<td>Biotin</td>
<td>Phenylalanine</td>
<td>Alanine</td>
<td>Arginine</td>
<td>Leucine</td>
<td>230</td>
</tr>
<tr>
<td>8</td>
<td><strong>Hypoxanthine</strong></td>
<td>Folic acid</td>
<td>Serine</td>
<td>Cysteine</td>
<td>Ornithine</td>
<td><strong>Glycine</strong></td>
<td>186</td>
</tr>
<tr>
<td>9</td>
<td>Cytosine</td>
<td>Pantothenic acid</td>
<td>Tryptophan</td>
<td>Threonine</td>
<td>Aspartic acid</td>
<td>Isoleucine</td>
<td>96</td>
</tr>
<tr>
<td>10</td>
<td><strong>Guanine</strong></td>
<td>Pyridoxine</td>
<td>Tyrosine</td>
<td>Sodium thiosulphate</td>
<td>Proline</td>
<td><strong>Histidine</strong></td>
<td>219</td>
</tr>
<tr>
<td>11</td>
<td>/</td>
<td>Thiamine</td>
<td>P-amino benzolic acid</td>
<td>Methionine</td>
<td>Glutamic acid</td>
<td>Lysine</td>
<td>108</td>
</tr>
<tr>
<td>12</td>
<td>Uracil</td>
<td>Riboflavin</td>
<td>Nicotinic acid</td>
<td>Choline</td>
<td>Inositol</td>
<td>Valine</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 4.1 B. Biomass and plasmid production of *E. coli* DH5α pSVb

<table>
<thead>
<tr>
<th>Pool</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>OD_{625} % of control</th>
<th>Plasmid % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td><strong>Adenine</strong></td>
<td>Biotin</td>
<td>Phenylalanine</td>
<td>Alanine</td>
<td>Arginine</td>
<td>Leucine</td>
<td>158</td>
<td>35</td>
</tr>
<tr>
<td>8</td>
<td><strong>Hypoxanthine</strong></td>
<td>Folic acid</td>
<td>Serine</td>
<td>Cysteine</td>
<td>Ornithine</td>
<td><strong>Glycine</strong></td>
<td>130</td>
<td>91</td>
</tr>
<tr>
<td>9</td>
<td>Cytosine</td>
<td>Pantothenic acid</td>
<td>Tryptophan</td>
<td>Threonine</td>
<td>Aspartic acid</td>
<td>Isoleucine</td>
<td>92</td>
<td>113</td>
</tr>
<tr>
<td>10</td>
<td><strong>Guanine</strong></td>
<td>Pyridoxine</td>
<td>Tyrosine</td>
<td>Sodium thiosulphate</td>
<td>Proline</td>
<td><strong>Histidine</strong></td>
<td>150</td>
<td>26</td>
</tr>
<tr>
<td>11</td>
<td>/</td>
<td>Thiamine</td>
<td>P-amino benzolic acid</td>
<td>Methionine</td>
<td>Glutamic acid</td>
<td>Lysine</td>
<td>100</td>
<td>106</td>
</tr>
<tr>
<td>12</td>
<td>Uracil</td>
<td>Riboflavin</td>
<td>Nicotinic acid</td>
<td>Choline</td>
<td>Inositol</td>
<td>Valine</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

Crossover nutrients are shown in bold italics.
4.3.4 Factorial design

Having identified nutrients which contributed to an increase in biomass a screening experiment was required to determine which nutrients had the greatest influence on biomass and if any of the identified nutrients were involved in plasmid amplification. As discussed in section 1.6.1, the fractional factorial design is a much more effective method of screening for best conditions than the traditional ‘one at a time’ experimental design. A fractional factorial design was therefore chosen to screen the nutrients identified in the nutrient pool investigation. In addition to the six nutrients identified in the nutrient pool study threonine was used as a negative control. Threonine was not identified in the nutrient pool study but had previously been implicated in plasmid amplification (Wrobel and Wegrzyn, 1998). A two level, duplicated quarter fraction factorial design, requiring 64 runs, was chosen and created using Design Expert 5 (DX-5) software. The design allowed high order effects and interactions to be identified using a manageable number of cultures.

Triplicate 10 mL cultures of DH5α pSVβ were grown on DM, supplemented according to Table 2.2, as described in section 2.5.1. Final biomass was determined by measuring optical density at 625 nm. Plasmid was extracted using the standard extraction method described in section 2.8.5.1 and plasmid yield was determined using the PicoGreen assay described in section 2.8.6.2. The data was subjected to ANOVA analysis using DX-5 software. As discussed in section 1.6.1, only main effects and high order interactions are realistically likely to have an effect on an experimental system. Interactions of 3 or more factors are unlikely to be significant and can therefore be excluded from the analysis of a fractional factorial experiment. Separate analyses were conducted to determine the effects of the various nutrients on biomass and specific plasmid yield. Results from this analysis are shown in Tables 4.2 A and 4.2 B and 4.3.

The effects identified in the analysis of biomass are shown in Table 4.2 A. Only amino acids were selected as having significant effects on biomass. An effect is the increase in mean
response, in this case biomass, which is observed as a factor (nutrient) increases from a low concentration to a high concentration. A positive effect indicates that the response will be greater at the higher concentration and a negative effect indicates that the response will be greater at the lower concentration. For increased biomass a positive effect is sought. The analysis showed that high concentrations of glycine, leucine and histidine were expected to lead to increases in biomass, while a high concentration of threonine was expected to inhibit biomass increase. This indicated that glycine, leucine and histidine should be considered for use in a fed-batch strategy to increase biomass.

The effects and interactions identified in the analysis of specific plasmid yield are shown in Table 4.2 B. As a negative effect indicates that the response, in this case specific plasmid yield, will be greater at the lower factor (nutrient) concentration, negative effects were sought for specific plasmid yield. Increased plasmid yield with low nutrient concentration indicates that amplification is induced by starvation of that nutrient, and feeding it in a fed-batch strategy could therefore prevent amplification. Individual negative effects were not identified for specific plasmid yield, but a two factor interaction between leucine and histidine was identified. When two compounds interact the outcome of the interaction is dependent on the concentrations of the two compounds. For a two compound interaction there are four possible interactions and hence four possible outcomes. As part of the analysis DX-5 provides an equation which allows the responses for the four possible interactions to be calculated. The calculated values are shown in Table 4.3. The data indicated that the highest specific plasmid yield was expected when leucine was starved in the presence of a high histidine concentration. This indicated that leucine should be used in a fed-batch strategy to prevent plasmid amplification.

When using statistical analysis techniques probability levels are set by the user. In general the level of statistical significance is set at 90 - 95 %, meaning that there is a 5 - 10 % probability that the results could be obtained by chance. In this case, the analysis of biomass gave a probability
> F value of 0.23, indicating significance at the 77 % level, and the analysis of specific plasmid yield gave a probability > F value of 0.13, indicating significance at the 87 % level. As this experiment was conducted for screening purposes these levels of significance were deemed acceptable. Screening experiments reduce the number of variables to be examined and can be revisited and 'fine tuned' if the factors identified in the initial screen prove not to be sufficient. For replicated experiments DX5 reports a probability > F value for lack of fit. As lack of fit is undesirable these values should be greater than 0.05 to indicate that lack of fit is statistically insignificant. Both the biomass and specific plasmid yield analyses gave values much greater than 0.05 indicating that lack of fit was insignificant.
Table 4.2 A. Summary data from ANOVA analysis of biomass in fractional factorial design

<table>
<thead>
<tr>
<th>Compound</th>
<th>Biomass (OD_{625})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td>+ 0.11</td>
</tr>
<tr>
<td>Histidine</td>
<td>- 0.005</td>
</tr>
<tr>
<td>Glycine</td>
<td>+ 0.13</td>
</tr>
<tr>
<td>Threonine</td>
<td>- 0.10</td>
</tr>
</tbody>
</table>

Effect size

<table>
<thead>
<tr>
<th>Probability &gt; F</th>
<th>Analysis</th>
<th>Lack of fit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.23</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Table 4.2 B. Summary data from ANOVA analysis of specific plasmid yield in fractional factorial design

<table>
<thead>
<tr>
<th>Compound</th>
<th>Specific plasmid DNA (mg/L/OD_{625})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td>+ 1.54</td>
</tr>
<tr>
<td>Histidine</td>
<td>+ 2.14</td>
</tr>
<tr>
<td>Leucine/histidine</td>
<td>- 1.60</td>
</tr>
</tbody>
</table>

Effect size

<table>
<thead>
<tr>
<th>Probability &gt; F</th>
<th>Analysis</th>
<th>Lack of fit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.13</td>
<td>0.55</td>
</tr>
</tbody>
</table>
### Table 4.3. Possible specific plasmid yield outcomes for leucine/histidine interactions

<table>
<thead>
<tr>
<th></th>
<th>Histidine</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low concentration (-)</td>
<td>High concentration (+)</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>1.931</td>
<td>3.331</td>
<td></td>
</tr>
<tr>
<td>High concentration (+)</td>
<td>2.929</td>
<td>2.449</td>
<td></td>
</tr>
</tbody>
</table>

High concentration = 1.7 mg/L  
Low concentration = 0.17 mg/L  
Concentrations were the same as those used in the nutrient pool experiment
4.3.5 Fed-batch fermentation

The amino acids leucine, glycine and histidine, identified from the fractional factorial screening experiment were fed in combination with D-glucose in a fed-batch fermentation. The aim was to increase the final level of biomass, in comparison to the batch fermentation, and delay plasmid amplification, allowing a higher yield of plasmid DNA to be obtained than in the batch fermentation.

Fed-batch fermentations were carried out in a 7 L fermenter with a 5.5 L working volume and were grown on SDCAS medium, as described in section 2.5.2. Biomass was measured hourly by dry cell weight. Wet cell pellet samples were taken hourly and stored at -20 °C. Plasmid was extracted from thawed wet cell pellet samples using the standard extraction procedure described in section 2.8.5.1. Total plasmid was quantified using the PicoGreen assay described in section 2.8.6.2. The percentage of plasmid in the supercoiled form was determined using gel electrophoresis as described in section 2.8.6.3. Residual D-glucose and amino acid levels in culture supernatants were measured hourly, as described in sections 2.8.2 and 2.8.3 respectively. Figure 4.5 shows the oxygen uptake rate profile, Figure 4.6 shows biomass, plasmid yields and residual D-glucose and amino acids profiles and Figure 4.7 shows the percentage of plasmid in the supercoiled form.

Oxygen uptake rate reached a maximum value of 24.7 mmol/L/h at 12.2 h and then began to drop steadily, reaching a value of 19.6 mmol/L/h at 15 h. This was followed by a rapid drop, indicating D-glucose exhaustion, which was confirmed by the DNS reducing sugar assay (see Figure 4.6). The exponential D-glucose feed was started immediately after this rapid drop was observed. Oxygen uptake rate rapidly returned to a value of 19.6 mmol/L/h and continued to climb, reaching a new maximum level of 25.6 mmol/L/h at 20.7 h when a linear amino acids feed was started. The start of feeding was based on observations of nutrient limitation during the batch fermentation, reported in section 4.3.2. It continued to climb for a further hour, and reached
a maximum value of 26.6 mmol/L/h. A decline to 7.4 mmol/L/h, at 24.2 h, was then observed.
From 24.2 h to 28.2 h oxygen uptake rate increased to 15 mmol/L/h then dropped to 11 mmol/L/h
and remained steady until harvest at 35 h.

Owing to the length of the fermentation the batch phase was run overnight without sampling,
hence no biomass, plasmid or nutrient profile data was collected during the batch phase.

Biomass climbed steadily and reached a value of 3.7 g/L dry cell weight by 15 h, which was
slightly higher than previous batch fermentations. Following commencement of the exponential
D-glucose feed biomass continued to climb steadily and reached a maximum value of 4.9 g/L dry
cell weight at 21 h which was maintained for the remaining 14 h of the fermentation.

Volumetric plasmid yield reached a mean maximum value of 44 mg/L at 20.7 h which was
maintained for the remainder of the fermentation. Specific plasmid yield remained constant at a
mean value of 9.1 mg/g DCW, no amplification was observed demonstrating that the amino acids
feed was sufficient to prevent amplification. The percentage of plasmid in the supercoiled form
remained at a mean value of 50 % until 22 h when it started to increase. By 25 h 90 % of plasmid
was in the supercoiled form and remained at this level until harvest at 35 h, this is in contrast to
the batch fermentation where high levels of supercoiling were maintained for only 1 h.

The maximum oxygen uptake rate and the biomass in the batch phase were higher than those
observed in the batch fermentation. The casein hydrolysate lot used in the fed-batch
fermentation was different to that used in the batch fermentation, all other medium component
lots were the same. The casein hydrolysate used in the fed-batch fermentation showed very
different physical properties to that used in the batch fermentation. The casein hydrolysate lot
used in the batch fermentation had dissolved easily at room temperature, whereas the lot used in
the fed-batch fermentation only dissolved upon autoclaving. Oxoid were contacted and confirmed
Chapter 4: Development of a fed-batch fermentation for the production of pSVβ

that the two batches were from different countries of origin. Batch to batch variation in casein hydrolysate may explain the difference in these two parameters between the two fermentations.

The decline in oxygen uptake rate observed between 21.7 h and 24.2 h, coupled with the steady biomass suggests that the culture had become starved of a further nutrient necessary for growth. That no plasmid amplification was observed during this time indicates that the amino acid feed was sufficient to prevent amino acid starvation but not sufficient, in combination with the D-glucose feed, to increase biomass. Besides D-glucose, the major components of SDCAS are ammonium sulphate, potassium dihydrogen phosphate and casein hydrolysate. It is likely that the culture had become nitrogen limited. Nitrogen limitation could be alleviated by additional feeding of a nitrogen source or by using ammonia for pH control in place of sodium hydroxide. This requires further investigation. The increase and steadying in oxygen uptake rate observed from 24.2 h is likely to be due to cells entering a steady state of maintenance. Once biomass became steady the D-glucose feed effectively became linear, due to the manual correction of Propack's estimated biomass values (see section 2.5.2.2). All available D-glucose was taken up by the cells, indicated by the very low levels of residual D-glucose (see Figure 4.6), suggesting that it was being used in maintenance activity.

The biomass level was 3.7 g/L dry cell weight at the point at which glucose was exhausted. After 5 h of glucose feeding the biomass had increased to 4.9 g/L dry cell weight. Therefore glucose feeding allowed a 30 % biomass increase over the batch stage of the fermentation. The specific plasmid yield in the fed-batch fermentation was higher than that seen prior to amplification in the batch fermentation. It is unclear why the specific plasmid yield differed between the two fermentation strategies. In the batch fermentation the specific plasmid yield was seen to increase during the first 10 h. This increase was also evident in the report by O'Kennedy et al. (2003). Presumably this also occurred in the batch phase of the fed-batch fermentation, but as no data was recorded this can not be proven. The inocula for all fermentations were grown in shake
flasks under the same conditions, however, as shake flasks are poorly controlled it is possible that the inocula differed between fermentations resulting in the difference in specific plasmid yields. The difference may also be attributed to inherent process variability, further work is required to determine process variability.

The amino acids feed did not increase the biomass yield over that obtained from feeding D-glucose, however within 5 h of initiating the amino acids feed, the percentage of plasmid in the supercoiled form had increased to 90 % and was maintained for the remaining 10 h of the fermentation. This was a great improvement over the batch fermentation where high levels of supercoiling were only maintained for 1 h. Changes in intracellular ATP/ADP ratios, associated with changing environmental conditions, have been reported to lead to increases in plasmid supercoiling (Hsieh et al., 1991a, 1991b; Camacho-Carranza et al., 1995). It is possible that the change in environment, associated with the loss of a nutrient from the medium and a shift in growth phase, led to changes in the intracellular ATP/ADP ratio which led to the increase in supercoiling observed in both the batch and fed-batch fermentations. Why the increase in supercoiling was only transient in the batch culture but was maintained in the fed-batch culture is not known. It seems that feeding amino acids and D-glucose for cellular maintenance allowed the level of supercoiling to be sustained. How and why this occurred is not known and warrants further investigation.

No amplification was observed during the fed-batch fermentation. It is possible that if a nitrogen source had also been fed the culture would have continued to grow and would have become starved of amino acids, inducing plasmid amplification which would have allowed a higher plasmid yield to be achieved. This requires further investigation, to determine if it is the case and how the level of supercoiling is affected. Despite not achieving its intention the fed-batch fermentation resulted in a much greater yield of supercoiled plasmid than the batch fermentation and hence had a higher level of recoverable supercoiled plasmid. The supercoiled plasmid yield
profile is shown in Figure 4.9. At the initial point at which plasmid supercoiling became 90% the supercoiled plasmid yield of the fed-batch fermentation was double that of the batch fermentation. Hence the fed-batch fermentation was the preferable strategy.

The discussion so far has assumed that the downstream process will contain a unit operation for separation of supercoiled plasmid from other plasmid species. In the case of the batch fermentation this step would be vital to ensure the quality of the final recovered product. However, during the fed-batch fermentation plasmid supercoiling was maintained at 90% for the final 10 h of the fermentation. It is not currently known if this is repeatable, but if process variability is such that this maintenance of a high level of supercoiling is repeatable then it may be possible to remove the plasmid species separation operation from the downstream process. This would mean that the downstream process would require less capital investment and would have reduced running costs due to the reduction in requirement of labour, utilities and raw material inputs. The maintenance of a high level of plasmid supercoiling means that the harvest point would not be critical to product quality, although a later harvest would result in greater labour, utilities and materials costs. In a production environment it would be preferable to harvest at the point of maximum productivity but failure to do so would not be detrimental to product quality. This introduces a certain amount of flexibility to the process which is vital in a production environment where unforeseen delays may, on occasion, hold up forward processing of fermenter broth.
Chapter 4: Development of a fed-batch fermentation for the production of pSVβ

Figure 4.6. Oxygen uptake rate profile for *E. coli* DH5α pSVβ grown in fed-batch culture.
The culture was grown on SDCAS in a 7 L fermenter with a 5.5 L working volume. The dashed line represents the start of an exponential 40 % (w/v) D-glucose feed to achieve a growth rate of 0.1 (1/h). The dotted line represents the start of a linear amino acids feed (per litre; leucine 0.64 g, glycine 0.56 g, histidine 0.17 g) at 60 mL/h.
Figure 4.7. Biomass, plasmid and nutrient profiles for *E. coli* DH5α pSVβ grown in fed-batch culture.

A; Δ; biomass, ○; specific plasmid yield; ●; volumetric plasmid yield. B; X; D-glucose, ◊; amino acids. The culture was grown on SDCAS in a 7 L fermenter with a 5.5 L working volume. The dashed line represents the start of an exponential 40% (w/v) D-glucose feed to achieve a growth rate of 0.1 (1/h). The dotted line represents the start of a linear amino acids feed (per litre; leucine 0.64 g; glycine 0.56 g; histidine 0.17g) at 60 mL/h.
Figure 4.8. Percentage plasmid in supercoiled form for *E. coli* DH5α pSVβ grown in batch culture.

The culture was grown on SDCAS in a 7 L fermenter with a 5.5 L working volume. The dashed line represents the start of an exponential 40 % (w/v) D-glucose feed to achieve a growth rate of 0.1 (1/h). The dotted line represents the start of a linear amino acids feed (per litre; leucine 0.64 g; glycine 0.56 g; histidine 0.17 g) at 60 mL/h. Error bars represent 1 standard deviation.
Figure 4.5. Total supercoiled plasmid yield profile for supercoiled plasmid obtained from *E. coli* DH5α pSVβ grown in batch culture.

Total supercoiled yield was calculated according to equation 4.1. The dashed line indicates the initial point at which plasmid supercoiling became 90%. 
4.4 Conclusions
It was demonstrated that amino acid starvation was a viable method of plasmid amplification at small scale and laboratory fermentation scale. Plasmid amplification allowed a higher plasmid yield to be achieved in batch fermentation. Identification of the amino acids which, when starved, induced plasmid amplification allowed a fed batch strategy to be developed. The strategy allowed plasmid, which was 90% supercoiled to be produced at mean levels of 44 mg/L and 9.1 mg/g DCW. Further work is required to understand the variability of the fermentation processes.
Chapter 5: Impact of plasmid size on oxygen demand

5 Impact of plasmid size on oxygen demand

5.1 Introduction

The size of plasmids currently being used in gene therapy clinical trials is typically less than 10 kb, but there are expectations that plasmid size will increase (Levy et al., 2000). The progression of gene therapy to the treatment of multigene and metabolic diseases (Newgard, 1992; Yarmush and Berthiaume, 1997) will bring about a need for larger vectors. Plasmids of more than 100 kb, have provoked interest as a means of obtaining long term action (Westphal et al., 1998; Willard, 1998; Wade-Martins et al., 1999, 2000). The effectiveness of DNA vaccines may be improved by incorporating genes for signalling molecules into them (Cohen et al., 1998), which will increase their size. In order for such gene therapies and DNA vaccines to become widely available medical treatments large plasmids must be produced at large scale.

An important aspect of large scale plasmid production is the ability to supply sufficient oxygen to the fermentation. Khosravi et al. (1990) reported that oxygen uptake was positively correlated with plasmid size. Increased oxygen uptake with increased plasmid size may present a problem, especially at large scale where oxygen supply is restricted by power input to the impellers and the need to maintain relatively low airflow rates to prevent impeller flooding. Therefore, during the production of large plasmids, oxygen may become a limiting factor and may render large scale, large plasmid production difficult.

It is widely accepted that production of recombinant protein from plasmids places an increased metabolic load on the host (Bentley et al., 1989). The increased load is brought about by the diversion of host resources away from host metabolism towards recombinant protein production (Glick 1995) resulting in a number of physiological changes. Although no direct reports have been made relating oxygen demand to recombinant protein synthesis it seems reasonable that the increased metabolic burden brought about by recombinant protein synthesis would lead to an
increased oxygen demand. The work described in this chapter investigated the relationship between oxygen demand, plasmid size and recombinant protein production.

5.2 Aim and approach

The aim of the work presented in this chapter was to determine if an increase in plasmid size led, in itself, to a significant increase in oxygen demand.

The previous work, reported by Khosravi et al. (1990), investigated the respiration rates of cells harbouring plasmids of 2.7 kb and 8.7 kb during the exponential growth phase, in batch culture. It is difficult to establish a cause and effect relationship in batch culture because the environment is constantly changing. Therefore, in this work, the relationship between plasmid size and oxygen demand was examined during steady state, in chemostat culture.

This chapter describes an investigation into the oxygen uptake rates of two isogenic plasmids of 4.4 kb and 20 kb during plasmid production and recombinant protein production. The work aimed to determine if, for this pair of plasmids, oxygen uptake rate was correlated with plasmid size or whether recombinant protein production made a major contribution to oxygen requirement.

5.3 Results and discussion

Chemostat fermentations were carried out in a 2 L fermenter with a 1.2 L working volume and were fed with SDCAS medium containing D-glucose at 0.5 g/L, as described in section 2.6. The cultures were grown under non-induced, plasmid producing conditions for the initial 48 h. Recombinant protein production was then induced by the addition of IPTG, and the cultures were grown under recombinant protein-producing conditions for a further 48 h. 30 mL samples were taken three hourly to minimise fluctuations in culture volume. Biomass was measured by optical density and dry cell weight. Wet cell pellet samples were taken and stored at -20 °C. Plasmid was extracted from thawed wet cell pellet samples using the standard extraction procedure described in section 2.8.5.1. Total plasmid was quantified using the PicoGreen assay described
in section 2.8.6.2. Residual D-glucose levels in culture supernatants were measured as described in section 2.8.2. The percentage of plasmid-containing cells was measured as described in section 2.8.4. Marker enzyme activity was measured as described in section 2.8.7. Figure 5.1 shows the oxygen uptake rate and respiratory quotient profiles, Figure 5.2 shows the biomass profiles, Figure 5.3 shows the specific plasmid yield and specific protein activity profiles and Figure 5.4 shows the specific oxygen uptake rate profiles for these fermentations.

Respiratory quotient (RQ) was determined by Propack data acquisition software from oxygen and carbon dioxide off gas values and remained at a constant value of 1 for both strains, demonstrating growth on D-glucose. Residual D-glucose concentration remained at the lower limit of detection, of approximately 0.05 g/L, for both strains.

The oxygen uptake rate of DH5α pBGS18 remained in steady state, with a value of 5.2 mmol/L/h for the initial 48 h of culture. Following IPTG induction there was a period of increase for 26.5 h after which oxygen uptake rate settled at a new steady state with a value of 5.7 mmol/L/h. The oxygen uptake rate of DH5α pQR150 underwent an initial period of decline followed by an increase to a steady state of 4 mmol/L/h. Following IPTG induction there was a period of increase after which oxygen uptake rate settled at a new steady state with a value of 5 mmol/L/h.

Biomass remained constant for both strains and indicated that the cultures were under steady-state conditions. Cells harbouring the smaller plasmid pBGS18 exhibited a lower optical density in chemostat culture than cells harbouring the larger plasmid pQR150. However, examination of dry cell weight data for the two strains showed that both strains had a mean dry cell weight of 0.76 g/L in chemostat culture.

The specific plasmid yield of DH5α pBGS18 remained at a constant level throughout the course of the culture, with a mean value of 9.2 mg/g DCW. The specific plasmid yield of DH5α pQR150 declined from a mean of 11 mg/g DCW to 1.5 mg/g DCW over the initial 50 h of culture, after this
time the specific plasmid yield remained constant at 1.5 mg/g DCW. IPTG induction did not appear to affect the specific plasmid yield as no further changes in yield occurred following IPTG addition. The percentage of plasmid-containing cells for both strains remained at 100% throughout the course of the experiments, indicating that all cells contained plasmid.

In order to account for the decline in plasmid yield of DH5α pQR150 enzyme activity was expressed as specific activity (A/min/mg plasmid). Both strains exhibited a low level of 'leaky' expression in the non-induced state. Following IPTG induction specific enzyme activity increased stepwise for both strains indicating that induction was successful. The specific β-galactosidase activity of DH5α pBGS18 increased from 6.5 A/min/mg plasmid before induction to 23.4 A/min/mg plasmid following induction, a 3.6 fold increase. The specific 2,3-catechol dioxygenase activity of DH5α pQR150 increased from 4,100 A/min/mg plasmid before induction to 21,600 A/min/mg plasmid following induction, a 5.3 fold increase.

The fall in plasmid yield observed for DH5α pQR150 must be explained by a decrease in copy number because the percentage of plasmid-containing cells remained at 100% throughout the fermentation. It is possible that during this time plasmid was not replicated but was partitioned upon cell division. This may explain the initial decline in oxygen uptake rate which was followed by an increase to a steady state and seems a more plausible explanation than a scenario where cells were actively breaking down plasmid. This would have required the production of DNases and hence would have been expected to increase the oxygen uptake rate rather than reduce it.

The decline in plasmid yield may be associated with the transition to a growth rate of 0.1 1/h in chemostat culture from a higher growth rate in batch culture. Biomass and plasmid levels were not measured during batch growth in this study, so there is a possibility that plasmid levels began to decline towards the end of the batch phase and continued to fall as the culture made the transition between growth rates. However, several authors have reported that growth rate influences plasmid copy number in chemostat culture. Kim and Shuler (1990) reported that the
copy number of pBR322 in *E. coli* B/r increased from 43.8 copies per cell at a growth rate of 0.08 1/h to 59.8 copies per cell at a growth rate of 0.84 1/h. An increase in copy number of pDM246 in *E. coli* HB101, to a maximum at a growth rate of 0.3 1/h, above which copy number decreased, was reported by Seo and Bailey (1986). Reinikainen and Virkajarvi (1989) observed similar trends in the same strain of *E. coli* harbouring two different plasmids. They reported that highest plasmid copy number occurred at growth rates between 1 and 1.2 1/h depending on the plasmid used. In other reports copy number has been negatively correlated with growth rate. Ryan and Parulekar (1991) reported that the copy number of pUC8 in *E. coli* JM103 decreased from 941 per cell at a growth rate of 0.23 1/h to 50 per cell at a growth rate of 0.4 1/h. A similar negative correlation was reported by Kim and Ryu (1991) in *E. coli* M72 harbouring pPLc23trpA1. All of these studies were conducted with small plasmids and it is difficult to find evidence in the literature relating to larger plasmids. It appears that the correlation between copy number and growth rate is very much dependent on the host/plasmid system and general rules can not be applied.
Figure 5.1. Oxygen uptake rate and RQ (inset) profiles for *E. coli* DH5α, harbouring 2 different plasmids, grown in D-glucose-limited chemostat culture at a dilution rate of 0.1 (1/h). A; pBGS18. B; pQR150. Dashed line represents time of addition of IPTG to a concentration of 1 mM to induce protein production.
Figure 5.2. Biomass profiles for *E. coli* DH5α, harbouring 2 different plasmids, grown in D-glucose-limited chemostat culture at a dilution rate of 0.1 (1/h).

○; pBGS18 OD<sub>625</sub>, ●; pBGS18 DCW, △; pQR150 OD<sub>625</sub>, ▲; pQR150 DCW. Dotted line represents time of addition of IPTG to a concentration of 1 mM to induce protein production.
Figure 5.3. Specific plasmid and specific protein yield profiles for *E. coli* DH5α, harbouring 2 different plasmids, grown in D-glucose-limited chemostat culture at a dilution rate of 0.1 (1/h).

A: pBGS18. B: pQR150. o; specific plasmid yield, Δ; specific marker enzyme yield. Dotted line represents time of addition of IPTG to a concentration of 1 mM to induce protein production.
5.4 Statistical analysis of oxygen uptake rate data

In order to directly compare oxygen uptake rates between the two strains, and to account for the fall in plasmid yield of DH5\(\alpha\) pQR150, mean specific oxygen uptake rates were calculated. Calculations were made using oxygen uptake rate data logged 1 h before and 1 h after each measured plasmid yield data point. The data is presented in Figure 5.4 and Table 5.1.

Both strains demonstrated step increases in mean specific oxygen uptake rate following IPTG induction. DH5\(\alpha\) pBGS18 demonstrated a small increase of 37% while DH5\(\alpha\) pQR150 showed a much larger increase of 415%. The large standard deviations associated with this data were due to the noise inherent in the oxygen uptake rate data. To allow comparison of non-induced and induced mean specific oxygen uptake rates within and between plasmids statistical testing was employed. The data was subjected to normality and unequal variance tests as described in section 1.6.2. The appropriate test for comparison of sample means, as discussed in section 1.6.2 was then used. The normality, unequal variances and comparison of sample means tests are shown in Appendix 9. A summary of the outcome of the tests is presented in Table 5.2.

Comparison of pBGS18 under non-induced and induced conditions demonstrated that mean specific oxygen uptake rate was not statistically significantly different at the 95% level. The small size of the protein expressed under induced conditions did not place a significant metabolic load on the host and hence no significant increase in mean specific oxygen uptake rate was observed.

Comparison of pQR150 under non-induced and induced conditions demonstrated a statistically significant increase in mean specific oxygen uptake rate, following IPTG induction, at the 95% level. 13 proteins are expressed from the meta cleavage pathway in pQR150 and the larger number of proteins expressed placed an increased metabolic load on the host and hence a significant increase in mean specific oxygen uptake rate was observed.
Comparison of pBGS18 and pQR150 under non-induced conditions demonstrated that mean specific oxygen uptake rate was not significantly different between the two strains at the 95% level. This result indicated that increased plasmid size, in itself, did not lead to a significant increase in cellular oxygen demand. Increases in cellular oxygen demand with increased plasmid size may be attributed to increased protein expression from the plasmid. This is supported by the comparison of pBGS18 and pQR150 under induced conditions which demonstrated that mean specific oxygen uptake rate was significantly different between the two strains at the 95% level.

A search of the literature revealed no other reports to have expressed oxygen uptake rate as a function of plasmid content as opposed to a function of cell density. The analysis presented here clearly demonstrates that it is the production of protein from the larger plasmid, and not simply the plasmid’s presence, which leads to an increase in oxygen uptake rate. In this study oxygen uptake rate was measured directly, by mass spectrometry. In the study conducted by Khosravi et al., (1990) growing cells were removed from culture and subjected to centrifugation and washing steps before being added to a non-aerated, air saturated buffer. It is unclear from the report if the temperature during these steps was controlled. Oxygen consumption was then measured for 5 min, using an oxygen electrode, as a function of the decline in the oxygen content of the buffer. This method may well be expected to alter the metabolism and respiration of the cells, therefore not giving a true indication of the oxygen uptake rate in the growing culture.

Although the cell densities, and hence the oxygen uptake rates, achieved in this study were lower than would ordinarily be achieved in a fed-batch fermentation for plasmid production, for gene therapy, it is believed that the conclusions of this study are applicable to fed-batch fermentations. Since the major contributor to an increase in oxygen uptake rate is protein expression rather than plasmid size, it seems reasonable that a fed-batch system that can be used to produce a small plasmid could be just as easily used to produce a larger plasmid. These results indicate that production of large plasmids at large scale will be feasible, as oxygen demand is unlikely to be...
greater than ability to supply provided there is no expression from the inserted DNA in the plasmid during growth of *E. coli*. 
Figure 5.4. Specific oxygen uptake rate profiles for *E. coli* DH5α, harbouring 2 different plasmids, grown in D-glucose-limited chemostat culture at a dilution rate of 0.1 (1/h).

A; pBGS18. B; pQR150. Dotted line represents time of addition of IPTG to a concentration of 1 mM to induce protein production. Specific oxygen uptake rates were calculated by averaging oxygen uptake rate data logged 1 h before and 1 h following manually measured data points.
### Table 5.1. Mean specific oxygen uptake rates during non-induced and IPTG-induced growth.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Mean specific oxygen uptake rate (mmol/L/h/mg plasmid)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-induced</td>
</tr>
<tr>
<td>pBGS18</td>
<td>0.75 ± 0.24</td>
</tr>
<tr>
<td>pQR150</td>
<td>0.78 ± 0.32</td>
</tr>
</tbody>
</table>

Mean specific oxygen uptake rates were determined from the specific oxygen uptake rate data presented in Figure 5.4.

± values represent 1 standard deviation.
### Table 5.2. Results of statistical analysis of mean specific oxygen uptake rates within and between plasmids

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Statistically significant difference?</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBGS18 NI and I</td>
<td>No</td>
</tr>
<tr>
<td>pQR150 NI and I</td>
<td>Yes</td>
</tr>
<tr>
<td>pBGS18 NI and pQR150 NI</td>
<td>No</td>
</tr>
<tr>
<td>pBGS18 I and pQR150 I</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Statistical comparisons were made using the Student’s t-test or the Welch ANOVA test. Details are given in Appendix 9.

NI; non-induced period of culture
I; IPTG-induced period of culture

Yes; statistically significant difference at the 95 % level.
No; no statistically significant difference at the 95 % level.
5.5 Conclusion

Cellular oxygen demand of two recombinant E. coli strains was investigated under both plasmid and protein producing conditions. The results showed that under plasmid producing conditions the oxygen uptake rate was not significantly different between the two strains. Under protein producing conditions, the oxygen uptake rate was significantly different between the two strains. It is concluded that large plasmids themselves do not cause an increase in host cellular oxygen demand but rather the larger amounts of protein produced from such plasmids create an increased metabolic load which leads to an increased oxygen demand.
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6 An examination of the feasibility of large plasmid production

6.1 Introduction

Plasmids of more than 100 kb, have provoked interest as a means of obtaining long term stability and action (Westphal et al., 1998; Willard, 1998; Wade-Martins et al., 1999, 2000) and may have applications in gene therapy because they may be stably maintained and replicated in the host cell. Investigation of their production has not so far been reported. If their use is to become standard in gene therapy and vaccination then they must be produced at large scale.

Chapter 5 concluded that fermentations for the production of large plasmids were unlikely to be limited by cellular oxygen requirement. The next question which presented itself was "Is it feasible to produce large plasmids in fermenters and recover them?"

This chapter describes an investigation into the feasibility of production of large plasmids in a fermenter and a comparison of a batch fermentation to produce large plasmids using two different media.

6.2 Aim and approach

The aim of the work presented in this chapter was to determine if large plasmids could be produced in a fermenter.

A family of plasmids in the size range 33 kb – 242 kb was transformed into E. coli DH10β and kindly donated by Richard Wade-Martins (Wellcome Trust Centre for Human Genetics, University of Oxford, Oxfordshire, UK). Examination of the fermentation and primary recovery, by lysis and clarification, of the mid-sized plasmid p5176, of 116.9 kb was carried out on both Superbroth, a complex medium which was used by Wade-Martins, and SDCAS the semi-defined medium which has been used throughout this study. Both fermentation and primary recovery were possible but the fermentation using the complex medium was not repeatable. The lysis characteristics of the DH10β p5176 system were very different to those observed in DH5α carrying pSVβ, pBGS18
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and pQR150. p5176 was seen to be highly susceptible to shear. The rest of this chapter details the work briefly described here.

6.3 Results and discussion

6.3.1 Batch fermentations of *E. coli* DH10β p5176 at 5 L scale

*E. coli* DH10β p5176 was grown in a 7 L fermenter, with a 5.5 L working volume, on Superbroth and on SDCAS medium, as described in section 2.7. Biomass was measured hourly by dry cell weight. Wet cell pellets from 5 mL culture samples were taken hourly and stored at -20 °C. Plasmid was extracted from thawed wet cell pellet samples using the standard extraction procedure described in section 2.8.5.1. Large wet cell pellets from 200 mL culture samples were taken two hourly and stored at -20°C. Plasmid was extracted from thawed large wet pellet samples using the modified Qiagen extraction technique described in section 2.8.5.3. Total plasmid was quantified using the PicoGreen assay described in section 2.8.6.2 using plasmid extracted using the standard extraction procedure. Plasmid extracted using the modified Qiagen method was run on a pulsed field electrophoresis gel, for visualisation and determination of percentage supercoiling, as described in section 2.8.6.4. Owing to the low copy number of the plasmid (1-2 copies per cell, Wade-Martins, 1999) a large sample had to be extracted for gel analysis in order that sufficient plasmid could be loaded onto the gel to enable visualisation. Figures 6.1, 6.2 and 6.3 show the oxygen uptake rate profiles, the biomass yields and the volumetric plasmid yields respectively for fermentations on Superbroth and SDCAS. Figure 6.4 and 6.5 show pulsed field gel images of plasmid extracted from cells grown on Superbroth and SDCAS respectively. Figure 6.6 shows the percentage plasmid in supercoiled form for fermentations on both media.

Oxygen uptake rate for growth on Superbroth showed the same trend for both fermentations but values were not repeatable. In the first fermentation oxygen uptake rate rose steadily over the initial 5 h of the fermentation then slowed significantly and reached a maximum value of 55
mmol/L/h at 5.9 h. It then dropped rapidly to a value of 9 mmol/L/h/L/h which was maintained to the end of the 7 h fermentation. In the second fermentation the oxygen uptake rate began to slow at 4.5 h and reached a maximum value of 44 mmol/L/h at 6.2 h. It too then dropped rapidly to a value of 9 mmol/L/h/L/h which was maintained until the fermentation was terminated at 7 h.

Oxygen uptake rate for growth on SDCAS was repeatable. In both fermentations oxygen uptake rate rose over the first 6 h of the fermentation and reached a maximum value of 33 mmol/L/h at 6 h. A brief period of decline was followed by a rise, back to 33 mmol/L/h, which was maintained until 7 h. A rapid drop to 20 mmol/L/h was then observed which was followed by a slow increase to a value of 24 mmol/L/h at 11 h. Between 11 h and 13 h the oxygen uptake rate declined steadily and reached a value of 18 mmol/L/h at 13 h. A rapid drop to base level then occurred which was maintained until the fermentation was terminated at 14 h.

For the Superbroth fermentations the maximum growth rate was 0.72 1/h (see Appendix 10). The biomass values were not repeatable between the two fermentations. The maximum biomass was 3.9 g/L dry cell weight in the first fermentation and 3.4 g/L dry cell weight in the second fermentation. In both cases biomass increased between 0 h and 5 h, growth then slowed between 5 h and 6 h and biomass became steady between 6 h and 7 h. Biomass values were repeatable in the SDCAS fermentations. The maximum growth rate was 0.84 1/h and was obtained between 0 h and 6 h, before glucose starvation occurred. A biomass of 2.8 g/L dry cell weight was obtained by 6 h. Following glucose exhaustion further growth was observed, although the growth was linear indicating nutrient limitation (see Appendix 10). Growth had ceased by 13 h and a maximum biomass of 4.8 g/L dry cell weight was obtained.

Volumetric plasmid yield profiles were not repeatable for the fermentations on Superbroth. In the first fermentation a maximum yield of 3.1 mg/L was achieved, at 7 h. In the second fermentation a maximum yield of 2.2 mg/L was achieved at 6 h. For the fermentations on SDCAS the volumetric plasmid yield profiles were very similar. In both cases plasmid yields increased over
the first 5 h of the fermentation reaching values of 1.6 mg/L and 1.9 mg/L respectively. A drop in yield was then observed which reached its lowest value at 7 h. Values of 0.9 mg/L and 1.1 mg/L respectively were observed in the two fermentations at 7 h. Between 7 h and 9 h the yield increased and maximum values of 2.25 mg/L and 2.2 mg/L were obtained. In both fermentations plasmid yield dropped between 9 h and 10 h. Between 10 h and the end of the fermentation, at 14 h, samples were taken at different times and hence did not allow comparisons to be made between the two fermentations. The percentage plasmid in supercoiled form remained above 90 % throughout the first fermentation on Superbroth, unfortunately samples were not available for the second Superbroth fermentation owing to centrifuge failure. The percentage plasmid in supercoiled form for the first SDCAS fermentation remained at 100 % for the first 5 h but dropped to 92 % by 7 h and to 59 % by 9 h, data was not available for the remainder of the fermentation. In the second SDCAS fermentation the percentage plasmid in the supercoiled form was steady at 94 % for the first 5 h of the fermentation, it also dropped to 92 % by 7 h and then demonstrated a slow decline, reaching 70 % by the end of the fermentation at 14 h.

Maximum oxygen uptake values were very different when comparing the two different media, with the SDCAS fermentation having a lower oxygen uptake rate. In both cases the oxygen requirement of the culture was met, dissolved oxygen was maintained at or above 30 % by stirrer speed adjustment. Scaling up based on maximum impeller tip speed shows that at large scale the oxygen requirements of both fermentations could be met (see Appendix 11). The maximum oxygen uptake rate on SDCAS was higher than that previously observed for E. coli DH5α pSVβ on the same medium. This difference in maximum oxygen uptake rate may be due to the difference in host cells or the difference in plasmids or a combination of the two. To examine this further p5176 would have to be transformed into E. coli DH5α pSVβ. To date this has not been achieved. Wade-Martins originally transformed DH10β using electroporation. During this research electroporation equipment was not available for use and attempts to transform E. coli DH5α with p5176 using the standard calcium chloride method met with no success.
The maximum biomass differed between fermentations grown on the two media. Fermentations grown on SDCAS achieved a higher biomass than fermentations on Superbroth. SDCAS fermentations were repeatable with respect to biomass while those grown on Superbroth were not. There was a 13% difference in maximum biomass on Superbroth and only a 4% difference on SDCAS. O'Kennedy *et al.* (2000) noted the same trend when comparing growth of *E. coli* DH5α pSVβ on SDCAS and the complex medium LB.

Maximum volumetric plasmid yields were similar for the fermentations grown on the two different media, with the profiles for the SDCAS fermentation being more repeatable. Wade-Martins (personal communication) routinely recovered approximately 250 µg/L of p5176 from shake flask cultures on Superbroth. The maximum quantities recovered in this work were approximately 10 times this amount. Wade-Martins only ever used shake flask culture and did not optimise his cultures to recover maximum plasmid. Hence comparing the two yields is not very relevant. F-based plasmids, of which p5176 is an example, are stably maintained at 1-2 copies per cell. Using this information in combination with the number of plasmid-containing cells, which was measured during the fermentations as described in section 2.8.4, the theoretical maximum plasmid yields were calculated for the Superbroth and SDCAS fermentations. A worked example is shown in Appendix 12 and a summary of the values is shown in Table 6.1. In both cases the measured plasmid yields at each time point were generally close to the expected yield, assuming the copy number was 1. The agreement between the actual and theoretical yields gives confidence in the plasmid extraction and quantitation techniques. The final plasmid yield for SDCAS was much higher than the calculated value. However, the expected plasmid yields were calculated based on viable cells and the viable cell count dropped towards the end of the fermentation. The dry cell weight did not decrease which suggests that intact (non-lysed) non-viable cells were present in the culture which would increase the plasmid yield obtained through cell lysis over the expected value. A discussion of the lysis characteristics of DH10β p5176 is given in section 6.3.2.
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Plasmid extracted from cells grown on Superbroth was of a suitable quality, with respect to supercoiling, for use as a gene therapy product, as the percentage of plasmid in the supercoiled form remained above 90% throughout the fermentation. The plasmid resulting from the SDCAS fermentations was also of a suitable quality for further processing as a gene therapy product when harvested before glucose starvation occurred. Following the onset of glucose starvation the percentage plasmid in the supercoiled form dropped below the 90% level, making it potentially unsuitable for use as a gene therapy product, as the final yield of supercoiled plasmid, at the end of processing, would be reduced. From the time that glucose starvation occurred to the termination of the fermentation the plasmid yield in the SDCAS fermentations increased significantly. Hence maximum yield was obtained at the end of the fermentation but maximum quality was obtained at 6 h. In an industrial environment it would make most sense to harvest from such a batch fermentation at the 6 h point to obtain maximum quality. The plasmid yield at this point in the fermentation was comparable to that in the Superbroth fermentation, at the same time point. The data collected from the first SDCAS fermentation suggested that the decline in plasmid supercoiling was slowing at the time the fermentation was harvested. Such a trend was observed in the DH5α pSVβ batch fermentation prior to the increase in supercoiling to levels suitable for a gene therapy product. It is possible that if the SDCAS fermentation had been allowed to run on this trend would also have been observed for DH10β p5176. In which case maximum quality and yield could be obtained. Further work is necessary to determine if this will be the case. Additionally, the level of plasmid supercoiling declined once the culture became glucose-starved, it is possible that the high level of supercoiling could be maintained if the fermentation was run in fed-batch mode and was fed with glucose once glucose starvation became evident. Again, further work is necessary to determine if this will be the case. Overall the SDCAS fermentation was preferable due to its high repeatability.
Figure 6.1. Oxygen uptake rate and residual glucose profiles for *E. coli* DH10β p5176 grown in batch culture.

Cultures were grown in a 7 L fermenter with a 5.5 L working volume as described in section 2.7. A; cultures grown on Superbroth. B; cultures grown on SDCAS. ▲; oxygen uptake rate fermentation 1, ▲; oxygen uptake rate fermentation 2, ○; residual glucose fermentation 1, ●; residual glucose fermentation 2. The dashed line indicates the onset of D-glucose starvation.
Figure 6.2. Biomass profiles for *E. coli* DH10β p5176 grown in batch culture.

Cultures were grown in a 7 L fermenter with a 5.5 L working volume as described in section 2.7. A; cultures grown on Superbroth. B; cultures grown on SDCAS. △; fermentation 1, ▲; fermentation 2. The dashed line indicates the onset of D-glucose starvation.
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Figure 6.3. Volumetric plasmid yield profiles for *E. coli* DH10β p5176 grown in batch culture.

Cultures were grown in a 7 L fermenter with a 5.5 L working volume as described in section 2.7. A; cultures grown on Superbroth. B; cultures grown on SDCAS. Δ; fermentation 1, ▲; fermentation 2. The dashed line indicates the onset of D-glucose starvation.
Figure 6.4. A 1% pulsed field gel of p5176 extracted from *E. coli* DH10β p5176 grown in batch culture on Superbroth.

Plasmid was extracted using the modified Qiagen tip 100 protocol as described in section 2.8.5.3. MRII; mid range marker II (24 kb – 291 kb), SC pDNA; supercoiled plasmid DNA, OC pDNA; open circular plasmid DNA, the numbers above the lanes indicate the sampling time in h. The numbers to the right of the gel indicate the marker fragment sizes in kb, where two numbers are given two fragments are present (see section 2.8.6.4). Results refer to samples from fermentation 1.
Figure 6.5. A 1 % pulsed field gel of p5176 extracted from *E. coli* DH10β p5176 grown in batch culture on SDCAS.

Plasmid was extracted using the modified Qiagen tip 100 protocol as described in section 2.8.5.3. MRII; mid range marker II (24 kb – 291 kb) marker fragment sizes are as indicted in Figure 6.4, SC pDNA; supercoiled plasmid DNA, OC pDNA; open circular plasmid DNA, the numbers above the lanes indicate the sampling time in h.
Figure 6.6. percentage plasmid in supercoiled form profiles for *E. coli* DH10β p5176 grown in batch culture.

Cultures were grown in a 7 L fermenter with a 5.5 L working volume as described in section 2.7. A; cultures grown on Superbroth. B; cultures grown on SDCAS. Δ; fermentation 1, ▲; fermentation 2. The dashed line indicates the onset of D-glucose starvation. Values were calculated by densitometric scanning of gels shown in Figures 6.4 and 6.5 as described in section 2.8.6.3. Plasmid was only extracted from Superbroth fermentation 1.
Table 6.1. Theoretical plasmid yields for DH10β p5176 grown on Superbroth and SDCAS.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Theoretical plasmid yield (µg/L)</th>
<th>Actual yield</th>
<th>% of theoretical yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 copy per cell</td>
<td>2 copies per cell</td>
<td>µg/L</td>
</tr>
<tr>
<td>SDCAS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>29</td>
<td>58</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>353</td>
<td>706</td>
<td>1147</td>
</tr>
<tr>
<td>6</td>
<td>1273</td>
<td>2546</td>
<td>1799</td>
</tr>
<tr>
<td>8</td>
<td>1389</td>
<td>2778</td>
<td>1261</td>
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<tr>
<td>12</td>
<td>1119</td>
<td>2238</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>887</td>
<td>1774</td>
<td>3137</td>
</tr>
<tr>
<td>Superbroth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>45</td>
<td>90</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>504</td>
<td>1008</td>
<td>450</td>
</tr>
<tr>
<td>7</td>
<td>1952</td>
<td>3904</td>
<td>1991</td>
</tr>
</tbody>
</table>

For a worked example see Appendix 12.
6.3.2 Lysis characteristics of DH10β p5176

Lysis and plasmid recovery was carried out at two scales for both fermentations. Small scale extractions were carried out on cell pellets obtained from 5 mL of culture and large scale extractions were carried out on cell pellets obtained from 200 mL of culture. The cell pellets were stored at –20 °C before plasmid extraction was carried out.

For the small scale extractions the cell pellets from the Superbroth fermentations resuspended easily in TE buffer whereas the pellets from the SDCAS fermentations were difficult to resuspend and appeared viscous possibly indicating that some cell lysis had taken place during thawing of the cell pellets. Separation of the potassium acetate precipitate from the plasmid-containing liquor was difficult to achieve for samples from both fermentations. The ‘pellets’ resulting from centrifugation had a very open gel-like structure and held a lot of liquor. Re-centrifuging did not aid in the separation of the pellet and the plasmid-containing liquor. The liquor could not be poured off the pellet as the pellet was too loose, instead the pellet was picked out of the liquor with a toothpick. During this process loss of liquor, which was entrained in the pellet, occurred. Recovered liquor volumes were not quantitatively measured but from observation were generally approximately half of the expected volume. In work with DH5α pSVβ, pBGS18 and pQR150 the pellet was very dry and was easily separated from the plasmid-containing liquor by centrifugation. This suggests that the different lysis characteristics observed here may be host related rather than plasmid related, however further work will be required to determine if this was the case.

The lysis characteristics observed at small scale were also observed at large scale. In order to separate the potassium acetate precipitate pellet from the plasmid-containing liquor two centrifugations and two filtrations were required. The liquors were noticeably clearer following the second centrifugation and filtration. However, further processing and a further centrifugation step were still necessary to pellet insoluble debris before the sample could be loaded onto a Qiagen column. In instances where these extra steps were not carried out the Qiagen columns became
blocked, indicated by very low flowrates through the columns. During such occurrences the columns took hours rather than minutes to process.

The loss of plasmid-containing liquor during plasmid recovery suggests that the measured plasmid yields may have been as much as half of the actual amount of plasmid contained in the cells. The loss of as much as 50% of the potential yield in the primary recovery stage is very high and warrants further investigation to reduce the losses. A starting point would be to examine the use of a different host, as discussed above.

6.3.3 Identification of plasmid and impurities

When p5176 was analysed by pulsed field gel electrophoresis, in order to determine the percentage of plasmid in the supercoiled form, a significant band was observed very close to the wells (see Figures 6.4 and 6.5). This band was more pronounced in p5176 obtained from fermentations on SDCAS and was thought to be either a nucleic acid impurity or a plasmid concatamer. A plasmid concatamer, being much larger than a single plasmid might not have been able to enter the gel. If the band was a plasmid concatamer then the analysis of the percentage of plasmid in the supercoiled form was flawed as this band was not included in the analysis. A restriction digest of one recovered plasmid sample was carried out in order to determine if the band consisted of plasmid. The 5 h sample from the first SDCAS fermentation was digested with N.BstNB I as described in section 2.8.6.5. The PFGE gel of the time course of this digestion is shown in Figure 6.7.

The band close to the well was unaffected by the restriction enzyme over the 4 h digestion period, demonstrated by the fact that it remained at the same position in the gel. The lower band was digested by the restriction enzyme, demonstrated by the movement of the band down the gel and the appearance of other bands below it indicating that the plasmid had been cut into smaller fragments. This result showed that the analysis of the percentage of plasmid in the supercoiled form was not flawed as the band close to the well was not composed of plasmid concatamers but
Chapter 6: An examination of the feasibility of large plasmid production

was a nucleic acid impurity carried through lysis and primary recovery. The amount of nucleic acid in the plasmid and impurity bands was not quantitatively assessed, however, it appears from the gel that the levels of recovered plasmid and recovered impurity are approximately equal. This may present a challenge in downstream processing and the ability to separate the plasmid and impurity warrants further work.
Figure 6.7. A 1% pulsed field gel of *N. Bsf*NB 1 digested p5176. DNA was taken from SDCAS fermentation 1, 5 h sample. MRII; mid range marker II (24 kb - 291 kb) marker fragment sizes are as indicted in Figure 6.4, the numbers above the lanes refer to the digestion time in min.
6.3.4 Susceptibility to shear

All mixing operations during lysis and plasmid recovery were carried out using very gentle manual shaking as advised by Wade-Martins (personal communication). Loading of final plasmid samples onto gels or into 96 well microtitre plates was carried out using 'wide bore' pipette tips made as described in section 2.8.5.3. These steps were taken to minimise shear and leave the plasmid intact. When plasmid samples were pipetted with normal rather than 'wide-bore' pipette tips and then run on a PFGE gel no plasmid was seen on the gel. Running the same samples on a conventional electrophoresis gel also resulted in no plasmid being visualised. These observations suggest that the plasmid is highly susceptible to shear forces and is broken down into very small fragments which ran off the end of a conventional electrophoresis gel. This presents another problem in large scale plasmid recovery. At large scale lysis is ordinarily carried out in a stirred tank in order to obtain a uniform environment in which lysis may occur (Prazeres et al., 1999; Marquet et al., 1995). In a stirred tank shear forces are present and this may mean that in such an operation p5176 would be destroyed. For a plasmid as large as p5176 alternative methods of achieving sufficient mixing will be required. Ciccolini et al. (1999) reported the use of a twin jet mixing device. In this device the cell suspension and the lysis solution were pumped simultaneously through two separate pipes whose outlets directly faced each other. The two components mixed effectively within a few milliseconds in the jet impingement zone and hence plasmid was only subjected to shear forces for a very short time. Such a method may be useful in the large scale extraction of p5176. Once lysis has occurred the lysate must be neutralised, Clemson and Kelly (2003) reported that neutralised alkaline lysis solution is shear sensitive. A 20 kb plasmid, in a neutralised alkaline lysis solution, stirred with a Rushton turbine for 10 min suffered a 20% decline in supercoiling. It may therefore be expected that p5176 would be destroyed if subjected to neutralisation in a stirred tank. Again alternative methods may need to be sought for plasmids such as p5176. Further unit operations such as pumping, centrifugation, filtration and dispensing may also exert significant stresses on a flow stream (Levy et al., 1999).
Chapter 6: An examination of the feasibility of large plasmid production

It was demonstrated by Levy et al. (1999) that plasmids greater than 20 kb were sensitive to the shear forces which may be found in bioprocessing equipment. These authors developed a model which allowed the prediction of the shear sensitivity of larger plasmids. A plasmid of 89 kb would be reduced from 100 % to 0 % supercoiling within 0.5 s of exposure to the level of shear found in large scale bioprocessing equipment. This has significant implications for the downstream processing of p5176 because it suggests that p5176 would be destroyed if processed using current accepted methods. Much further work on the downstream processing of p5176 needs to be carried out to determine if and how it may be recovered in a suitable form for use as a gene therapy product.

6.4 Conclusion

The ability to produce a large 116.9 kb plasmid, at the 5 L fermentation scale, was investigated using a complex and a semi-defined medium. Both fermentations produced plasmid which was recoverable. The fermentation using the semi-defined medium, SDCAS, was repeatable whereas that using the complex medium, Superbroth, was not, making the semi-defined fermentation preferable. In both cases the oxygen demand of the organism was easily met and a scale up based on tip speed indicated that this would also be the case at 8,000 L scale. Maximum plasmid yields and maximum supercoiling were not coincident in the fermentation on semi-defined medium. Further investigation will be required to determine if plasmid supercoiling would increase if the fermentation was run for a longer period of time.

Large losses during lysis and primary plasmid recovery and the high susceptibility of the plasmid to shear indicate that large scale downstream processing of p5176 may be difficult. Further work is required to determine what the limitations in downstream processing are, and how they may be overcome.
7 Discussion

Discussion of the results has been covered within the corresponding results chapters. This chapter will summarise the findings of the thesis and discuss their relevance to an industrial process.

Chapter 4 demonstrated that amino acid starvation was a viable method of inducing plasmid amplification and thereby increasing specific plasmid yield. A fed-batch fermentation, to increase plasmid yield was investigated. This fermentation resulted in a high yield of plasmid with a high quality, 90% being in the supercoiled form. The yield and level of supercoiling were stably maintained for 10 h. This fed-batch fermentation would be relatively easy to implement at an industrial scale.

With regard to the semi-defined medium, SDCAS, the requirement for the separate sterilisation of a number of the medium components could be met by the use of a continuous sterilisation system. This would be preferable to a scenario where all the medium components were sterilised together, in the fermentation vessel. If all the nutrients were sterilised together precipitation of some medium components may occur, leading to a reduction in the nutrients available to the organism. Additionally, batch sterilisation procedures require longer heating times in order to bring the medium to the minimum sterilisation temperature, due to the large volumes when compared to continuous sterilisation procedures. The major advantage of a continuous sterilisation system would be the maintenance of medium quality due to the minimum degree of nutrient degradation. In addition, continuous sterilisation systems offer ease of automation leading to reduced variability, a reduced sterilisation cycle time and the reduction of fermenter corrosion (Stanbury et al., 1995).

SDCAS medium contains casein hydrolysate, which is a derivative of bovine milk. In recent years the high incidence of BSE in the UK and other European countries has raised concerns regarding the use of bovine derived materials in the production of drug substances (Federal Register,
Chapter 7: Discussion

The infective agent of BSE, which is highly resistant to traditional disinfection and sterilisation procedures, has crossed the species barrier and infected humans, causing variant Creutzfeldt-Jakob disease (Collinge, 1999). The use of bovine derived material, originating in a BSE positive country, in the production of a drug may therefore represent a threat to human health. Based on this the FDA requested that materials derived from cattle which had resided in or originated from countries where BSE has been diagnosed should not be used in the production of FDA regulated drugs intended for administration to humans (FDA, 1993a, 1993b; Federal Register 1994). This request was widened in 2000 to prevent the use of materials derived from ruminants which were born, raised or slaughtered in countries in which the United States Department of Agriculture could not determine that BSE did not exist (FDA, 2000). These requests do not completely preclude the use bovine-derived materials in the production of drugs but it is preferable to avoid their use whenever possible. If this fed-batch fermentation was to be used in the production of a gene therapy drug, SDCAS could be used as the medium provided all casein hydrolysate was from a certified BSE-free source, and all documentation was in place to provide an audit trail (FDA, 1993b, 2000). Alternatively other complex amino acid sources could be used to replace casein hydrolysate. O'Kennedy et al. (2000) replaced the casein hydrolysate in SDCAS with soya amino acids to give a medium designated SDSOY. Cultures grown on SDSOY gave lower final biomass yields and had lower plasmid retention levels when compared to cultures grown on SDCAS. This indicates that further development work would be necessary if this fed-batch fermentation was to be used with a bovine-free medium.

As industrial scale fermentation plants are staffed by operators who are likely to be running several fermentations at once, it is important that each fermentation is relatively simple and straightforward to operate with minimum observation required. The two feeds employed in this fed-batch fermentation would be easy to implement at industrial scale. The amino acid feed could be started at a set fermentation age, and as it is a linear feed could then be left to run without further operator intervention. The glucose feed could also be started at a set fermentation
Chapter 7: Discussion

age and would require a simple program to run it as an exponential feed (Gregory et al., 1994). Running the glucose feed exponentially would require an operator to measure biomass on an hourly basis and correct discrepancies in the predicted and actual biomass values. This may be too great a requirement in a busy plant. The glucose feed only ran in exponential mode for 6 h before it effectively became linear, owing to limitation of some other nutrient. Therefore, at large scale, the glucose feed could be incrementally increased to pre-set values before becoming linear, rather than running in a true exponential manner. This would ensure that each fermentation was reproducible with respect to glucose feeding and would minimise operator intervention or error.

The stable maintenance of both plasmid yield and supercoiling for a 10 h period in this fed-batch fermentation means that the harvest time is not critical to ensuring product quality. Hence this fed-batch fermentation would be amenable to large scale production and would not suffer yield or quality issues if a delay in harvest occurred. This fermentation could be scaled up for industrial production of a gene therapy drug, however some further development of the medium and feed regimes may be necessary.

Chapter 5 explored the relationship between plasmid size and cellular oxygen demand and concluded that increased plasmid size does not lead to a significantly higher oxygen demand. This has important implications for the large scale production of plasmid DNA. It is envisaged, that as the field of gene therapy progresses, larger plasmids will be required for vaccinations and in the treatment of multigene and metabolic disorders (Levy et al., 2000). At large scale the ability to supply oxygen may be limited by impeller power and by the need to maintain a relatively low air flowrate in order to prevent impeller flooding. That larger plasmids do not induce a significantly higher oxygen demand means that at large scale the oxygen demand of cells harbouring larger plasmids will be met. This infers that fermentations producing larger plasmids may achieve the same productivity as those producing smaller plasmids.
Chapter 6 examined the feasibility of the production of a large, 116.9 kb, plasmid, p5176. The work showed that the plasmid could be produced in fermentation, the cellular oxygen demand could be met and scale up calculations indicated that this would also be the case at large scale. Initial plasmid recovery proved much more difficult than was the case with the 6.9 kb plasmid, pSVβ, which was used in the work reported in Chapter 4, and p5176 was seen to be highly shear sensitive. It was not determined whether the difficulty in plasmid recovery was due to the plasmid itself or due to the host. If it is postulated that the differences in plasmid recovery observed in Chapters 4 and 6 were due to the differences in host strain then a solution to facilitate ease of plasmid recovery would be to transfer p5176 into a different host.

The shear sensitivity of p5176 is a much greater problem from a downstream processing perspective. Current downstream processing methods for plasmids present significant shear forces and while plasmids of less than 20 kb are not significantly damaged by these forces (Levy et al., 1999, 2000) there is evidence that larger plasmids are (Levy et al., 1999; Clemson and Kelly, 2003) A method for achieving efficient alkaline lysis while exposing the plasmid to shear for only a very short period of time has been reported (Ciccolini, 1999) and may be a suitable method for use with p5176. However, alkaline lysis is only the first of several downstream processing operations and much further work on the downstream processing of p5176 needs to be carried out to determine if and how it may be recovered in a suitable form for use as a gene therapy product. However, the ability to produce the plasmid in fermentation is a very positive start to the process.
8 Conclusions

This thesis examined three areas of plasmid production, the development of a suitable fermentation strategy to produce a 6.9 kb plasmid, the ability to meet the oxygen requirements of fermentations for the production of larger plasmids and the feasibility of fermentation and primary recovery of a large, 116.9 kb, plasmid.

It was demonstrated, at the 10 mL scale, that amino acid starvation was a viable method of plasmid amplification. In batch fermentation, at the 5 L scale, starvation of amino acids induced plasmid amplification which allowed a higher maximum plasmid yield to be achieved. Following amplification the maximum yield was not maintained and the plasmid fell back to the pre-amplified level of 7.1 mg/g DCW. This lower yield of plasmid was 90 % in the supercoiled form. Comparison of total supercoiled plasmid yield between the point of maximum volumetric yield (24 h) and maximum plasmid supercoiling (32 h) showed that the yield of supercoiled plasmid was higher at 32 h. However, the difference in yield was small and it was unlikely to be worth the additional 8 h fermentation time to obtain this difference. A fed-batch fermentation strategy was investigated in order to improve the yield of plasmid. It was thought that feeding an exponential glucose feed in combination with a linear amino acids feed would allow the biomass to be increased prior to amino acid starvation, hence the volumetric plasmid yield would be increased. Nutrient pool and factorial design experiments were carried out to allow the identification of the amino acids which, when starved, induced plasmid amplification. Leucine, glycine and histidine were identified as such and allowed a fed-batch fermentation strategy to be developed. The strategy aimed to delay plasmid amplification by employing a linear amino acids feed and improve biomass by using an exponential glucose feed. In this fermentation mode the biomass was increased and plasmid amplification was prevented rather than delayed. However, the fermentation resulted in plasmid which was 90 % supercoiled. The high level of plasmid supercoiling was maintained for an extended 10 h period which meant that harvest time was not
The fermentation was considered suitable for application in an industrial process although it was acknowledged that some further development work may be necessary.

The cellular oxygen demand of two *E. coli* strains, DH5α pBGS18 and DH5α pQR150, carrying a 4.4 kb plasmid and a 20 kb plasmid respectively, was investigated in continuous culture under both plasmid and protein producing conditions. Under plasmid producing conditions the oxygen uptake rate between the two strains was not significantly different whereas, under protein producing conditions, the oxygen uptake rate was significantly different. The results demonstrated that large plasmids do not, in themselves, cause an increase in host cellular oxygen demand but rather the larger amounts of protein produced from such plasmids create an increased metabolic load which leads to an increased oxygen demand. The results indicated that a fermentation for the production of a larger plasmid would not be limited by the ability to supply oxygen.

The feasibility of producing a 116.9 kb plasmid, at the 5 L fermentation scale, was investigated using a complex and a semi-defined medium. Both fermentations produced plasmid which was recoverable although losses due to shear were observed. The fermentation using the semi-defined medium was repeatable whereas that using the complex medium was not, making the semi-defined fermentation preferable. In both cases the oxygen demand of the organism was easily met and a scale up based on tip speed indicated that this would also be the case at the 8,000 L scale. Maximum plasmid yields and maximum supercoiling were not coincident in the fermentation using semi-defined medium. Further investigation is required to determine if plasmid supercoiling would increase if the fermentation was run for a longer period of time as was seen in the fermentations to produce the 6.9 kb plasmid.

The susceptibility of the 116.9 kb plasmid to shear during small scale lysis and recovery indicated its large scale downstream processing was likely to be difficult. Further work is required to determine what the limitations in downstream processing are, and how they may be overcome.
9 Future work

A fed-batch fermentation for the production of a 6.9 kb plasmid, of which 90% was in the supercoiled form at harvest, was investigated at the 5 L scale.

The variability of fed-batch fermentation is not known and requires further investigation to determine if it is repeatable and therefore suitable for further development and scale up as outlined below. It was thought that the fed-batch fermentation became nitrogen limited which prevented further biomass increase and meant that the cells did not use amino acids to the point of starvation, hence amplification was not induced. It would be worthwhile to investigate feeding of a nitrogen source to determine the effect on biomass, plasmid yield and plasmid supercoiling. It may be the case that the yield of supercoiled plasmid could be further increased by employing a nitrogen source.

In its current format the fermentation uses a bovine-derived medium component as a complex source of amino acids. As discussed in chapter 8, although the use of bovine derived materials is not forbidden in the production of FDA regulated products intended for administration to humans, it is preferable to avoid them where possible. It is therefore anticipated that manufacturers of gene therapy products will be keen to avoid the use of animal derived components. One possible direction for future work is the development of a medium with a non-animal amino acid source. O’Kennedy et al. (2000) reported that the replacement of casein hydrolysate with soya amino acids resulted in a lower biomass yield and a lower plasmid retention. These authors used the same mass of soya amino acids as casein hydrolysate. The amino acid composition of soya is not the same as that of casein and hence the replacement of casein requires further investigation and development.

The fed-batch fermentation was developed at the 5 L scale and although problems are not foreseen in scale up it is not proven at larger scales. Research into the effects of scale up is therefore a clear path for future work.
Chapter 9: Future Work

The fed-batch fermentation was developed using pSVβ as a model plasmid. A further area for future research would be the investigation of the production of different plasmids using the fed-batch strategy presented in this thesis, especially larger plasmids. This ties in with future work using p5176, the 116.9 kb plasmid. The production of this plasmid was investigated using both Superbroth and SDCAS medium, the fermentation using SDCAS was viewed as preferable because it had a higher degree of repeatability. This thesis only examined the production of p5176 in batch fermentation. Examining its production in fed-batch fermentation would be a valuable route for further work because a fed-batch fermentation would allow a higher biomass level to be achieved and hence would improve volumetric plasmid yield. In the batch SDCAS fermentation, the level of supercoiling of p5176 at harvest was not sufficient to allow it to be used as a gene therapy product. Further examination and development is required to determine if fermentation conditions may be manipulated in order to obtain the necessary plasmid quality with respect to supercoiling.

Further work is required to investigate and overcome the problems encountered in the downstream processing of p5176.

Large losses were made in the initial plasmid recovery stages owing to the formation of a very loose pellet, following potassium acetate precipitation, which was very difficult to separate from the plasmid containing liquor. Such losses were not encountered in the recovery of pSVβ, pBGS18 or pQR150. One possible reason for the very different recovery characteristics is the difference in host strain. p5176 was harboured in DH10β while all of the other plasmids used in the work reported in this thesis were harboured in DH5α. Further work should focus on transforming p5176 into DH5α to determine if the recovery characteristics are improved.

p5176 was seen to be highly susceptible to shear but work to quantify the effect of shear was not performed. A thorough analysis of the effect of shear on the plasmid should be carried out in order to provide information about the likely damage to the plasmid in industrial downstream
Chapter 9: Future Work

recovery operations. Certain equipment in current plasmid recovery processes generates shear. The shear generated in such processes does not damage the small plasmids which are currently being used in gene therapy trials but it is likely that much larger plasmids will be damaged. Hence it is highly likely that alternative processing routes will need to be sought for large plasmids. The determination of new routes for processing highly shear sensitive plasmids should be a direction for future work.
Chapter 10: Potential for commercialisation of research

10 Potential for commercialisation of research

10.1 Introduction

During the course of my PhD I was given the opportunity to take a module from the Master Of Business Administration course at London Business School. This period of study was funded by the Centre for Scientific Enterprise, London. This chapter provides an appraisal of potential commercialisation routes for the fed-batch fermentation reported in this thesis.

10.2 The current commercialisation of gene therapy

It is expected that the first gene therapy products will enter the market in 2006 and that genetic vaccines will follow (Technical Insights, 2003). There are currently 159 companies active in the areas of gene therapy and genetic vaccination (Wiley, 2003). Figure 10.1 shows the technologies that these companies are exploiting. Some companies are involved in more than one technology, these tend to be the larger companies who have been able to buy out smaller start up companies or have partnered with them. Many of the smaller companies possess a core technology, such as a delivery vector or a therapeutic gene. Those companies that possess a single core technology are likely to license it to other companies or partner with larger companies in order to progress development. Although the possession of intellectual property relating to a core technology is an asset it will not enable a product to be brought to market. In order to bring a product to market a company must have access to all of the necessary technologies (Bossart and Pearson, 1995). These include a therapeutic gene, production, processing and formulation methods and a delivery device. These technologies may be wholly owned by the company, but some of them are likely to be licensed from a third party or be owned by a partner company.
Chapter 10: Potential for commercialisation of research

Figure 10.1. The technologies being exploited by current gene therapy and genetic vaccine companies.

- Worldwide, ■; UK, ■. Some companies are using more than one technology. Reproduced with permission from the Journal of Gene Medicine web site http://www.wiley.co.uk/genmed. © 2003 John Wiley & Sons Ltd.
Chapter 10: Potential for commercialisation of research

10.2 Potential commercialisation routes

Possible routes of commercialisation for the fed-batch fermentation described in this thesis are licensing, licensing and consultation and contract manufacture. These routes are discussed below. Some research is also amenable to development into a product. The fed-batch fermentation described in this thesis is a technology rather than a product hence this route is not applicable.

10.2.1 Licensing

Licensing is a method of creating income from a patent without setting up a company around the technology. Universities often license their technologies. Licensing arrangements do not require a great deal of capital in order to be put in place because the patent owner does not require a great deal of infrastructure. The onus is on the licensee to have the correct facilities and equipment in order to use the technology to its best advantage.

There are two major methods of licensing. Third parties may be licensed to use the technology simply by payment of a one-off licensing fee. However, such schemes severely limit the amount of income that can be generated from the patent and in order to be successful a significant marketing effort is required in order to license many companies and hence increase the income from the patent. A second licensing method involves licensing a third party to use the technology and if a product is successfully brought to market as a result of this then further royalty payments, as a percentage of product sales, are made. This type of arrangement allows higher earnings to be made from the patent without any significant extra input from the patent holder.

10.2.2 Licensing and consultation

As for licensing arrangements, licensing and consultation arrangements do not require a great deal of capital to put in place because the license owner does not require a great deal of infrastructure. The onus is again on the licensee to have the correct facilities and equipment in order to use the technology to its best advantage. However, unlike a simple licensing
arrangement a licensing and consultation arrangement requires input from the patent owner in terms of time and sound technical advice. Some of the success of the product becomes the responsibility of the patent owner.

As with a simple licensing arrangement a fee would be charged to license the third party to use the technology. The patent owner would offer technical advice on the implementation of the technology, in return for regular consultation payments. Again a royalty scheme would allow the most income to be made from the patent.

10.2.3 Contract manufacture
A third option for commercialisation is contract manufacture. Contract manufacture requires a much greater level of investment that either of the previous scenarios. Contract manufacture requires a great deal of investment in equipment, facilities and infrastructure. Production should be carried out according to good manufacturing practice. Suitable production and analytical testing facilities need to be in place along with appropriate quality and documentation systems. A skilled workforce is a necessity of such a venture.

10.3 Discussion
As a result of the research reported in this thesis we have a fed-batch fermentation for the production of small plasmids which is proven at 5 L scale. The fermentation is novel in that it is designed to maximise the yield of supercoiled plasmid DNA, rather than the overall yield of plasmid DNA. This facilitates the recovery of high quality plasmid at maximum yields in downstream processing.

This discussion assumes that a patent will be sought, and granted, in relation to the fed-batch fermentation for the production of high quality plasmid. While patents for the production and recovery of pharmaceutical grade plasmid DNA do exist (Marquet et al., 1994; Thatcher et al., 1999) a search of the US patent and trademark office database (www.uspto.gov) did not reveal
any patents relating to the fermentation of highly supercoiled plasmid DNA. Hence it seems likely that a patent would be granted.

The licensing of the fed-batch fermentation to third party companies would be one method of realising income from a patent. Large, established, pharmaceutical and biotechnology companies may be willing to enter into a simple licensing arrangement because they will have sufficient expertise and infrastructure within the company to use the technology and develop processes around it. However, larger companies may attempt to negotiate an exclusive agreement which could severely limit the earnings potential of the patent. Additionally larger companies may be less willing to enter into royalty agreements, hence reducing potential patent income. Smaller, less established companies may not find a simple licensing arrangement attractive. Smaller companies tend to be less diverse, research-based and focus on a single or a small number of technologies. Hence a small company which may be interested in licensing our technology would be likely to have a single technology, such as a plasmid carrying a therapeutic gene, and would be seeking a method of production. In such a situation the company would be likely to require technical support and consultation as a minimum and may also require aid in full plasmid production.

An alternative to licensing to a gene therapy company is to license to a contract manufacturer. Contract manufacturing companies need to make high quality plasmid in a manner which is cost effective for both them and their customers. If they were to license our technology they would not need to use downstream processing steps to separate different plasmid forms because the plasmid resulting from fermentation would be of a sufficiently high quality for use as a gene therapy product. Hence the scale or number of fermentations required to produce sufficient high quality bulk material would be reduced. This would be attractive to a contract manufacturing company because it would lower their production costs for a given mass of purified plasmid DNA. This would either allow them to make a greater profit on each contract or to pass on their savings
Chapter 10: Potential for commercialisation of research

to their customers and hence offer a lower cost service than their competitors and make them more attractive than the competition. A contract manufacturing company may be more amenable to a royalty agreement because they are able to factor in the royalty cost in the cost to their customers.

As discussed above a licensing and consultation arrangement may be more attractive to smaller companies than a simple licensing arrangement. If we were to enter into a licensing and consultation arrangement we would need to be very sure that we could balance our academic responsibilities with the consultation work or, that the earnings from the consultation work would be sufficient to support it as a full-time venture. If a small company was to enter into a licensing and consultation arrangement they would still need access to the relevant facilities and equipment to produce plasmid. This could be achieved by employing a contract manufacturing company to produce the plasmid using the licensed fermentation method. This would ensure the production of high quality plasmid in a GMP environment. However, it may be easier for us to license directly with a contract manufacturer and agree royalty terms than to become the middle man between a small company and a contract manufacturer. A licensing and consultation arrangement with a contract manufacturer is not a viable option. Contract manufacturers are continually developing new processes for their clients so a licensing and consultation agreement with a contract manufacturer would require technical input for each client. Under such an agreement the patent holder would essentially become a process development employee of the contract manufacturer.

For some companies licensing and consultation alone would not meet their needs, they would require full manufacture. Although contract manufacture would realise the largest turnover it also represents the largest investment input and largest risk in terms of failure. As shown in Table 10.1, there are four established contract manufacturing companies in the UK alone. These companies are internationally respected names in the contract manufacture of materials for
Chapter 10: Potential for commercialisation of research

clinical trials and as such represent severe competition. It would be difficult to establish ourselves as a contract manufacturing company in the UK at the present time given the competition and the large investment which would be required.
### Table 10.1. Details of companies that provide contract manufacturing services for plasmid-based gene therapy and genetic vaccine products

<table>
<thead>
<tr>
<th>Company</th>
<th>Exclusive</th>
<th>Services</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Althea Technologies</td>
<td>Yes</td>
<td>Process development and testing</td>
<td>US</td>
</tr>
<tr>
<td>Incorporated</td>
<td></td>
<td>Process optimisation and scale up</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bulk drug production and packaging</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Regulatory consultation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vector and strain development</td>
<td></td>
</tr>
<tr>
<td>Cobra Biomanufacturing</td>
<td>No</td>
<td>Process optimisation and scale up</td>
<td>UK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Analytical testing</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Regulatory consultation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Consultation from vector design to clinical trials</td>
<td></td>
</tr>
<tr>
<td>PlasmidFactory</td>
<td>Yes</td>
<td>Manufacture of pre-clinical and clinical trials</td>
<td>Germany</td>
</tr>
<tr>
<td></td>
<td></td>
<td>material</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biosafety testing</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Virus clearance validation</td>
<td></td>
</tr>
<tr>
<td>Q-one Biotech Limited</td>
<td>Yes</td>
<td>Contract manufacturing – viral and non-viral vectors</td>
<td>UK/US</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Regulatory affairs consultancy</td>
<td></td>
</tr>
</tbody>
</table>

Exclusive relates to whether the company is working exclusively in the area of gene therapy and/or genetic vaccination. The information in this table was obtained from Wiley (2003) and Cobra (2003).
Chapter 10: Potential for commercialisation of research

10.4 Conclusions

The fed-batch fermentation which was developed as part of the research conducted in this study does have potential for commercialisation if it is assumed that a patent would be granted. The commercialisation route which holds most promise is a licensing and royalty arrangement with a contract manufacturer.
Chapter 11: References

11 References


Chapter 11: References


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Chapter 11: References


Chapter 11: References


Warnes A and Stephenson JR. The Insertion of Large Pieces of Foreign Genetic Material Reduces the Stability of Bacterial Plasmids. Plasmid. 1986. 16, 116 - 123.


Chapter 11: References


Appendices

A1 Plasmid maps

A1.1 pSVβ

Figure A1.1. pSVβ plasmid map.

This map was kindly provided by John Ward, Department of Biochemistry, University College London, London, UK.
Figure A1.2. pBGS18 plasmid map.
This map was kindly provided by John Ward, Department of Biochemistry, University College London, London, UK.
Figure A1.3. pQR150 plasmid map.
This map was kindly provided by John Ward, Department of Biochemistry, University College London, London, UK.
Figure A1.4. p5176 plasmid map.

This map was kindly provided by Richard Wade-Martins, Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, UK.
## A2 Preparation of stock solutions for nutrient pool experiments

### Table A2.1 Preparation of stock solutions for nutrient pool experiments

<table>
<thead>
<tr>
<th>Compound</th>
<th>Stock concentration (g/L)</th>
<th>Solvent</th>
<th>Sterilisation treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>5.0</td>
<td>0.1 M Hydrochloric Acid</td>
<td>Filtration</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.0</td>
<td>RO Water</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.0</td>
<td>RO Water</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>1.0</td>
<td>0.2 M Sodium Hydroxide</td>
<td>Filtration</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.001</td>
<td>RO Water</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Choline</td>
<td>1.0</td>
<td>RO Water</td>
<td>Filtration</td>
</tr>
<tr>
<td>Cysteine</td>
<td>1.0</td>
<td>RO Water</td>
<td>Filtration</td>
</tr>
<tr>
<td>Cytosine</td>
<td>5.0</td>
<td>0.1 M Hydrochloric Acid</td>
<td>Filtration</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.05</td>
<td>0.01 M Sodium Hydroxide</td>
<td>Filtration</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>1.0</td>
<td>0.4 M Sodium Hydroxide</td>
<td>Filtration</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.0</td>
<td>RO Water</td>
<td>Filtration</td>
</tr>
<tr>
<td>Guanine</td>
<td>5.0</td>
<td>0.1 M Hydrochloric Acid</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.0</td>
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<td>Filtration</td>
</tr>
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<td>Hypoxanthine</td>
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<td>0.1 M Ammonium Hydroxide</td>
<td>Filtration</td>
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<td>Inostitol</td>
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<td>RO Water</td>
<td>Filtration</td>
</tr>
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<td>RO Water</td>
<td>Filtration</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.0</td>
<td>RO Water</td>
<td>Filtration</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.0</td>
<td>RO Water</td>
<td>Filtration</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.0</td>
<td>RO Water</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.5</td>
<td>RO Water</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Ornithine</td>
<td>1.0</td>
<td>RO Water</td>
<td>Filtration</td>
</tr>
<tr>
<td>p-Amino Benzoic Acid</td>
<td>0.5</td>
<td>RO Water</td>
<td>Filtration</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>0.05</td>
<td>RO Water</td>
<td>Filtration</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.0</td>
<td>0.01 M Hydrochloric Acid</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Proline</td>
<td>1.0</td>
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<td>Autoclave</td>
</tr>
<tr>
<td>Pyridoxine</td>
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<td>RO Water</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Riboflavin</td>
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<td>0.02 M Acetic Acid</td>
<td>Filtration</td>
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<tr>
<td>Serine</td>
<td>1.0</td>
<td>RO Water</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Sodium Thiosulphate</td>
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<td>RO Water</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.001</td>
<td>RO Water</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.0</td>
<td>RO Water</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.0</td>
<td>0.2 M Hydrochloric Acid</td>
<td>Filtration</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.0</td>
<td>0.4 M Sodium Hydroxide</td>
<td>Filtration</td>
</tr>
<tr>
<td>Uracil</td>
<td>5.0</td>
<td>0.1 M Ammonium Hydroxide</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Valine</td>
<td>1.0</td>
<td>RO Water</td>
<td>Filtration</td>
</tr>
</tbody>
</table>

Autoclave; autoclaved at 121 °C for 20 min
Filtration; filter sterilised through a 0.22 μm syringe filter

16.7 μL of each stock were combined for each pool to give the correct working concentrations. Pools were added to 9.9 mL of defined medium (DM) in McCartney bottles.
A3 2,3 catechol dioxygenase assay development data

The 2,3 catechol dioxygenase assay was tested on whole broth to determine if the assay could be used without the centrifugation and sonication steps in order to simplify the assay and reduce the assay time from approximately 40 minutes to approximately 5 minutes. Whole broth was used in place of the extract supernatant in the assay described in section 2.8.7.2. The results of this investigation are shown in Figure A3.1.

The assay using whole broth was linear across a range of culture volumes and the specific activity was independent of culture volume. However, the standard deviations were very large with values up to 89 %, and hence the assay was not suitable for use on whole broth.

Figure A3.2 shows the results of the assay when performed on cell extracts. Again the assay was linear across a range of extract volumes and was independent of extract volume. The standard deviations were very small, with the largest standard deviation being 8 %. An assay volume of 40 μL was chosen as this was the volume reported by Huang et al. (1990).
Figure A3.1. 2,3 catechol dioxygenase activity assay using whole cells
A; enzyme activity as a function of culture volume.  B; specific enzyme activity as a function of culture volume.  Error bars represent 1 standard deviation.
Figure A3.2. 2,3 catechol dioxygenase activity assay using cell extracts.
A; enzyme activity as a function of culture volume. B; specific enzyme activity as a function of culture volume. Error bars represent 1 standard deviation.
A4 Typical calibration curves

A4.1 Dry cell weight

\[ y = 0.47x + 0.07 \]

\[ R^2 = 0.98 \]

Figure A4.1. Dry cell weight calibration curve for *E. coli* DH5α·pSVβ.

Dry cell weight pellets were prepared as described in section 2.8.1 and calibrated against \( \text{OD}_{625} \) to allow \( \text{OD}_{625} \) values to be converted to dry cell weight for input to Propack's exponential D-glucose feeding programme.
A4.2 DNS reducing sugar assay

\[ y = 0.55x + 0.05 \]
\[ R^2 = 0.99 \]

Figure A4.2. D-glucose calibration curve for DNS reducing sugar assay.
Standard solutions were assayed, in triplicate, as described in section 2.8.2.
A4.3 PicoGreen total plasmid assay

Figure A4.3. pSVβ plasmid DNA calibration curve for PicoGreen total plasmid assay. Standard solutions of Qiagen purified plasmid DNA were assayed, in duplicate, as described in section 2.8.6.2.
Figure A4.4. pBGS18 plasmid DNA calibration curve for PicoGreen total plasmid assay. Standard solutions of Qiagen purified plasmid DNA were assayed, in duplicate, as described in section 2.8.6.2.
Figure A4.5. pQR150 plasmid DNA calibration curve for PicoGreen total plasmid assay. Standard solutions of Qiagen purified plasmid DNA were assayed, in duplicate, as described in section 2.8.6.2.
Figure A4.6. p5176 plasmid DNA calibration curve for PicoGreen total plasmid assay. Standard solutions of Qiagen purified plasmid DNA were assayed, in duplicate, as described in section 2.8.6.2.
Figure A5.1. A typical 0.7% (w/v) agarose gel for determination of percentage pSVβ in supercoiled form.

The samples are a time course from a batch fermentation. The gel was run and stained as described in section 2.8.6.3. The numbers above the lanes indicate sampling times in h.
Figure A6.1 Raw FSU vs dilution factor for 5 pSVβ samples.
The 5 different symbols represent 5 different samples. Plasmid was purified from small scale cultures using the standard extraction procedure described in section 2.8.5.1.
Appendices

A7 Standard operating procedure for exponential fed-batch fermentations via Propack

1 Introduction

This document describes the steps necessary to set up and run an exponential fed-batch fermentation in vessel LH7L04, using Propack to control feeding.

2 Equipment

- 2 rpm Watson Marlow 101U pump
- 32 rpm Watson Marlow 101U pump
- Top pan balance
- TCS unit
- 2 feed bottles connected to a single needle

3 Pump calibration

The density of feed is required to successfully complete the calibration

1. Place the feed bottles on the top pan balance
2. Connect the feed lines to the pumps
3. Connect the needle to the fermenter
4. Prime the feed lines for both pumps
5. Set the fermenter to operating conditions
6. Connect the labelled TCS communication lines to the pumps. Ensure the TCS outputs for B 2 rpm and B 32 rpm are at 0 (display will be 0.4 this is normal). DO NOT connect the TCS communication line to the wall socket
7. Set the pumps to automatic operation and the TCS unit to manual operation
8. Set the TCS output for the 2 rpm pump to 70 %
9. Switch the pumps off
10. Tare the top pan balance
11. Set a stop watch to 5 min. Start the stopwatch and switch on the 2 rpm pump simultaneously
12. After 5 minutes have elapsed record the reading on the top pan balance (Use Table 1 to record the data)

13. Repeat steps 9-12 at TCS outputs of 50% and 20%

14. Repeat steps 8-13 for the 32 rpm pump, but use a time of 1 min

15. Prime the feed lines to within 1 cm of the needle, clamp the lines and switch the pumps off

Table 1. Pump calibration data

<table>
<thead>
<tr>
<th>2 rpm</th>
<th>Mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCS output (%)</td>
<td>Pump speed (rpm)</td>
</tr>
<tr>
<td>20</td>
<td>0.4</td>
</tr>
<tr>
<td>50</td>
<td>1.0</td>
</tr>
<tr>
<td>70</td>
<td>1.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>32 rpm</th>
<th>Mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCS output (%)</td>
<td>Pump speed (rpm)</td>
</tr>
<tr>
<td>20</td>
<td>6.4</td>
</tr>
<tr>
<td>50</td>
<td>16</td>
</tr>
<tr>
<td>70</td>
<td>22.4</td>
</tr>
</tbody>
</table>

16. Calculate the flowrate (mL/min) for each % output for each pump:

\[ F = \left[ \frac{(M_{\text{end}} - M_{0})}{(\rho_{\text{feed}} \times t)} \right] \]

Where:

\( F \) = flowrate (mL/min)

\( M_{\text{end}} \) = mass at time 5 or 1 min (g)

\( M_{0} \) = mass at time 0 (g)

\( \rho_{\text{feed}} \) = density of feed (g/mL)

\( t \) = time (min)
17. Plot graphs of flowrate vs rpm and fit a linear trendline to the data. Typical graphs are shown in Figure 1

Figure 1. Typical calibration curves for Watson-Marlow pumps
A; 2 rpm pump. B; 32 rpm pump.
4 Propack fed-batch set-up

1. Click on the fed-batch menu button in the 7 L 04 menu

2. Click on the growth rate in the value column at the left hand side of the screen

3. Enter the required values:
   - Growth rate 1 – enter desired value (1/h)
   - Feed concentration – enter feed concentration (g/L)
   - Cell concentration – leave blank until feed is required, see section 5
   - Scaling factor = 1
   - Sample volume – enter sampling volume (L)
   - Sampling interval – enter interval between samples (h)
   - Tube bore = 1.6 mm
   - Growth rate 2 = n/a
   - Time 1 = n/a
   - Time 2 = n/a
   - Delay = n/a

4. Press escape to return to the fed-batch menu

5. Click the pump set-up button at the right hand side of the screen

6. Click the view pump status button

7. Enter the values for slope and offset, obtained from the pump calibration curves, for both pumps in BOTH boxes.

   NOTE: Text is reversed, enter slope value as offset and offset value as slope.

8. Hit escape twice to return to the fed-batch menu

5 Starting feeding

   A DCW/OD calibration curve is required to complete this step

1. Attach the TCS communication cable to the communication port in the wall

2. Set the TCS unit to automatic operation
Appendices

3. Unclamp the feed lines

4. Switch the pumps on

5. Take a sample from the fermenter and measure its optical density

6. Convert the optical density reading to DCW and enter this DCW value into Propack.

7. Click the activate fed-batch button to start feeding

8. The values above the pump status bar graphs are the values that Propack is sending to the TCS unit, check that the TCS unit is displaying the correct values

9. Each time optical density is measured convert the reading to DCW and check the value against Propack, if the values are significantly different update Propack with the new value
A8 E. coli DH5α pSVβ growth rate analysis

![Line graph showing growth curve for E. coli DH5α pSVβ in 7 L batch culture on SDCAS.](image)

**Figure A8.1.** Growth curve for *E. coli* DH5α pSVβ in 7 L batch culture on SDCAS. Dry cell weight was measured in triplicate, values shown are the means of three independent fermentations. Growth rate was determined from a linear fit carried out using Origin data analysis software (Microcal Software Inc., Northampton, Massachusetts, USA).
Appendices

A9 Statistical analysis of chemostat oxygen uptake rate data

In order to determine if there were any significant differences in oxygen uptake rate, within and between plasmids, statistical tests were required to allow comparison of mean oxygen uptake rates. The data was subjected to statistical testing, as discussed in section 1.6.2. The results of these tests are shown in Tables A9.1 and A9.2.

JMP statistical software (SAS Institute Inc., Cary, USA) was used to analyse the mean specific oxygen uptake rate data from the chemostat work presented in Chapter 5.
Table A9.1. Outcomes of the Shapiro Wilkes test for goodness of fit for a fitted normal distribution for the mean specific oxygen uptake rate data.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Non-Induced</th>
<th>IPTG-induced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P value</td>
<td>Normal?</td>
</tr>
<tr>
<td>pBGS18</td>
<td>0.223</td>
<td>Yes</td>
</tr>
<tr>
<td>pQR150</td>
<td>0.580</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Table A9.2. Outcomes of the Levene test for unequal variances in mean specific oxygen uptake rate within and between plasmids

<table>
<thead>
<tr>
<th>Comparison</th>
<th>P value</th>
<th>Unequal variances?</th>
<th>Test required</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBGS18 NI and I</td>
<td>0.441</td>
<td>No</td>
<td>Student's t test</td>
</tr>
<tr>
<td>pQR150 NI and I</td>
<td>0.010</td>
<td>Yes</td>
<td>Welch ANOVA</td>
</tr>
<tr>
<td>pBGS18 NI and pQR150 NI</td>
<td>0.433</td>
<td>No</td>
<td>Student's t test</td>
</tr>
<tr>
<td>pBGS18 I and pQR150 I</td>
<td>0.010</td>
<td>Yes</td>
<td>Welch ANOVA</td>
</tr>
</tbody>
</table>

NI: non-induced period of culture
I: IPTG-induced period of culture
Figure A9.1 Student’s t test comparison of mean specific oxygen uptake rate for non-induced and IPTG-induced *E. coli* DH5α pBGS18 in chemostat culture

18 OUR; mean specific oxygen uptake rate of DH5α pBGS18 (mmol/L/h/mg plasmid), NI; non-induced period of culture, I; IPTG-induced period of culture. *P* value = 0.128; there is no statistically significant difference in mean specific oxygen uptake rate between non-induced and IPTG-induced *E. coli* DH5α pBGS18 in chemostat culture at the 95% level.

JMP provides a graphical output for the comparison being made and for the Student’s t-test.

In the graph window the black markers are the individual data points that the sample population is composed from, the line across the middle of the green diamond represents the mean of the sample population and the top and bottom of the green diamond represent plus and minus 1 standard deviation from the mean.

In the Test window the circles give a visual indication of the results of the Student’s t test. Overlapping circles of the same colour indicate that there is no significant difference in the means of the two sample populations. Separated circles of different colours indicate that there is a significant difference in the means of the two sample populations. The greater the separation of the circles, the greater the significance.
Figure A9.2 Comparison of mean specific oxygen uptake rate for non-induced and IPTG-induced *E. coli* DH5α pQR150 in chemostat culture

150 OUR; mean specific oxygen uptake rate of DH5α pQR150 (mmol/L/h/mg plasmid), NI; non-induced period of culture, I; IPTG-induced period of culture. P value = < 0.0001; there is a statistically significant difference in mean specific oxygen uptake rate between non-induced and IPTG-induced *E. coli* DH5α pQR150 in chemostat culture at the 95% level.

Where a Welch ANOVA test is applied no visual indication of the outcome of the test is provided by JMP.
Figure A9.3 Student's t test comparison of mean specific oxygen uptake rate for non-induced *E. coli* DH5α pBGS18 and pQR150 in chemostat culture

Nl; mean specific oxygen uptake rate during non-induced period of culture (mmol/L/h/L/h/mg plasmid), 150; DH5α pQR150, 18; DH5α pBGS18. P value = 0.790; there is no statistically significant difference in mean specific oxygen uptake rate between non-induced *E. coli* DH5α pBGS18 and pQR150 in chemostat culture.
Figure A9.4 Comparison of oxygen uptake rate for IPTG-induced *E. coli* DH5α pBGS18 and pQR150 in chemostat culture

I; mean specific oxygen uptake rate during IPTG-induced period of culture (mmol/L/h/mg plasmid), 150; DH5α pQR150, 18; DH5α pBGS18. P value = 0.0001; there is a statistically significant difference in mean specific oxygen uptake rate between IPTG-induced *E. coli* DH5α pBGS18 and pQR150 in chemostat culture.
A10 E. coli DH10β p5176 growth rate analysis

Figure A10.1. Growth curve for E. coli DH10β p5176 in 7 L batch culture on Superbroth. Dry cell weight was measured in triplicate, values shown are the means of two fermentations. Growth rate was determined from a linear fit of the natural log of the biomass data carried out using Origin data analysis software (Microcal Software Inc.)
Figure A10.2. Growth curve for *E. coli* DH10β p5176 in 7 L batch culture on SDCAS. Data is for the initial part of the fermentation before glucose exhaustion had occurred. Dry cell weight was measured in triplicate, values shown are the means of two fermentations. Growth rate was determined from a linear fit of the natural log of the biomass data carried out using Origin data analysis software (Microcal Software Inc.)
Figure A10.3. Growth curve for *E. coli* DH10β p5176 in 7 L batch culture on SDCAS. Data is for the latter part of the fermentation after glucose exhaustion had occurred. Dry cell weight was measured in triplicate, values shown are the means of two fermentations and error bars represent 1 standard deviation. Growth rate was not determined due to the linearity of the data. The linearity of the data indicates that the culture was nutrient (glucose) limited and hence could no longer attain its maximum growth rate.
Appendices

A11 Assessment of the ability to meet the oxygen demand of DH10β p5176 at large scale

_E. coli_ DH10β p5176 exhibited its maximum oxygen uptake rate during the first fermentation on Superbroth. The maximum stirrer speed required to maintain dissolved oxygen at 30% of saturation during this fermentation was 800 rpm (see Figure A11.1). Details of the impeller configuration for the 7 L tank used in this work and an 8,000 L industrial tank are given in Table A11.1. Impeller tip speed may be calculated using:

\[
\text{St} = \frac{D_i \times S_i \times \pi}{2} \quad (11.1)
\]

Where:

- \( \text{St} \) = tip speed (m/min)
- \( D_i \) = impeller diameter (m)
- \( S_i \) = impeller speed (rpm)

During the time of maximum oxygen requirement, and hence maximum agitator speed, the tip speed of the agitators in the 7 L tank was:

\[
\text{St} = 0.0627 \times 800 \times 3.142 = 157.6 \text{ m/min}
\]

Rearranging equation 11.1 to determine the impeller speed required in the 8,000 L industrial tank to match the tip speed of the 7 L tank gives:

\[
S_i = \frac{S_t \times D_i}{D_i} \quad (11.2)
\]

\[
S_i = \frac{157.6 \times (3.142 \times 0.71)}{0.71} = 70.7 \text{ rpm}
\]

The industrial vessel on which this calculation was based has a maximum airflow rate of 1 vvm, which is the same rate that was used in the 7 L vessel, and a maximum agitator speed of 188 rpm. Hence, using a scale up analysis based on tip speed the oxygen requirement of DH10β p5176 could be met at large scale.
Figure A11.1. Agitator speed profile for DH10β p5176 grown on Superbroth in a 7 L fermenter with a 5 L working volume.
Table A11.1. Impeller configuration details for the 7 L and 8,000 L vessels

<table>
<thead>
<tr>
<th>Vessel volume (L)</th>
<th>Number of impellers</th>
<th>Impeller diameter (m)</th>
<th>Impeller spacing from base of vessel (% of vessel diameter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>2</td>
<td>0.0627</td>
<td>39</td>
</tr>
<tr>
<td>8,000</td>
<td>2</td>
<td>0.71</td>
<td>42</td>
</tr>
</tbody>
</table>

The values for the 8,000 L vessel relate to a vessel which is currently used in the pharmaceutical industry for antibiotic production.
A12 A Worked example of the theoretical plasmid yield from an *E. coli* DH10β p5176 fermentation

The molecular weights of the four nucleotides from which DNA is composed are shown in Table A12.1. The average molecular weight of a DNA nucleotide is 309 g/mol, this assumes that the four nucleotides are present in the molecule in equal quantities. From this value the average mass of a nucleotide may be calculated:

$$M_n = \frac{M_r}{C}$$

Where:

- $M_n$ = average mass of nucleotide (g)
- $M_r$ = average molecular weight of nucleotide (g/mol)
- $C$ = Avogadro's constant = $6.022 \times 10^{23}$

So:

$$M_n = \frac{309}{6.022 \times 10^{23}}$$

$$M_n = 5.13 \times 10^{-22} \text{ g}$$

The mass of a plasmid may be calculated using:

$$M_p = M_n \times \text{BP} \times 2$$

Where:

- $M_p$ = mass of plasmid (g)
- BP = number of base pairs

p5176 has 116 kilobase pairs so the mass of the plasmid is:

$$M_p = 5.13 \times 10^{-16} \times 116 \times 10^3 \times 2$$

$$M_p = 1.19 \times 10^{-16} \text{ g}$$

$$M_p = 1.19 \times 10^{-10} \mu g$$
The theoretical plasmid yield may be calculated using:

\[ \text{Yield} = M_p \times N_c \times VCC \times P_f \]

Where:

- \( N_c \) = plasmid copy number
- \( VCC \) = viable cell count (cells/L)
- \( P_f \) = fraction of the population which is plasmid containing

For example, the second SDCAS fermentation had a viable cell count of \( 1.8 \times 10^{13} \) cells/mL at 10 h, and 100% of cells were plasmid containing. Assuming 1 plasmid copy per cell, the expected plasmid yield was:

\[ \text{Yield} = 1.19 \times 10^{-10} \times 1 \times 1.8 \times 10^{13} \times 1 = 2142 \, \mu g/L \]
### Table A2.1. Molecular weights of DNA nucleotides

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxyadenosine monophosphate</td>
<td>313</td>
</tr>
<tr>
<td>Deoxycytidine monophosphate</td>
<td>304</td>
</tr>
<tr>
<td>Deoxyguanosine monophosphate</td>
<td>328</td>
</tr>
<tr>
<td>Deoxythymidine monophosphate</td>
<td>289</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>309</strong></td>
</tr>
</tbody>
</table>

Molecular weights were calculated from chemical structures presented by Voet and Voet (1995)