DEVELOPMENT OF OPTIMAL FERMENTATION EXPRESSION SYSTEMS FOR RECOMBINANT PROTEINS

A thesis submitted in partial fulfilment
of the requirements for the degree of

Masters
in
Technology

at Massey University,
Palmerston North,
New Zealand

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2004

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ABSTRACT

DEVELOPMENT OF OPTIMAL FERMENTATION EXPRESSION SYSTEMS FOR RECOMBINANT PROTEINS

by Daniel Manderson

This research set out to maximise the titre of four recombinant protein products (i.e. *Eg*95 vaccine antigen against *Echinococcus granulosus*; a aspartyl protease inhibitor homologue, *Aspin*; a secreted cytokine granulocyte colony stimulating factor (G-CSF); a secreted gonadotropin ovine follicle stimulating factor (*o*FSH)) and develop parameters for the expression of those proteins in a small scale stirred tank bioreactor.

Production of *Eg*95 as inclusion bodies in *E. coli* was influenced by the medium, feeding strategy, induction timing and dissolved oxygen concentration. Expression was greatest using the medium Terrific Broth. Higher *Eg*95 titres were favoured using exponential feeding, a low dissolved oxygen concentration and with cells induced in mid-exponential growth. A maximum titre of 1.73 g/L of *Eg*95 was produced in a fed-batch fermentation controlled at 37°C, pH 7.0 and 30% dissolved oxygen. Induction with 0.1 mM of IPTG added four hours after inoculation, was optimal. The maximum titre attained, was a 360% improvement on fermentations prior to this research.

Aspin was used to investigate the culture conditions for maximizing the production of soluble protein in *E.coli*. Soluble *Aspin* production was favoured at low expression rates. A volumetric titre of 0.220 g/L of soluble *Aspin* was attained in batch fermentation by inducing with 2 g/L of L-arabinose, with the temperature reduced from 37°C to 23°C and by maintaining a low dissolved oxygen (DO) concentration. This yield was relatively high compared to previous reports [1-3].

G-CSF production in the yeast *Pichia pastoris* was influenced by the medium, pH and methanol-to-cell ratio. A maximum titre of 0.028g/L of G-CSF was produced in shaker flasks of enhanced yeast extract Hy-Soy dextrose medium (YEHD), maintained at 200 rpm, 30°C, pH 6.0 and with 1% (v/v) methanol fed per day. Cells were resuspended to an optical density of 8 prior to induction. No improvement in G-CSF was achieved in the fermenter, likely due to an inhibition by toxic materials. The optimised shaker flask yield was consistent with previous reports [4-6].

Production of *o*FSH in insect cells was influenced by the cell density at inoculation and rate of agitation. 0.001g/L of *o*FSH was produced in shaker flasks inoculated at a density of 1 x 10^6 cells/mL, cultured at 27°C and agitated at 140rpm. This represented an improvement over previous yields [7].

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LIST OF ABBREVIATIONS AND SYMBOLS

AOX alcohol oxidase

bla ampicillin resistance gene

bsd blasticidin resistance gene

BCA bicinchoninic acid

BMG buffered minimal glycerol medium

BMM buffered minimal methanol medium

BMGY buffered complex glycerol medium

BMMY buffered complex methanol medium

CARE continuous absorption recycle extraction

CDW cell dry weight

DO dissolved oxygen

DTT dithiothreitol

EDTA ethylene diamine tetra-acetic acid

ELISA enzyme-linked immunosorbent assay

F(t) flow rate of feed at time t (h^{-1})

GFP green fluorescence protein

G-CSF granulocyte colony stimulating factor

GST. glutathione-s-transferase

HCl hydrochloride

HIC hydrophobic interaction chromatography

His_6 polyhistidine epitope tag

HRP horse radish peroxidase

IgG Immunoglobulin G

IMAC immobilised metal chelating resin

INF interferon

IPTG isopropyl β-D-thiogalactopyranoside

LB Luria-Bertani broth

LPM litres per minute

MBP maltose binding protein

MGY minimal glycerol medium

MM minimal methanol medium

MOI multiplicity of infection

NaOH sodium hydroxide

NMW nominal molecular weight

OD optical density

PID proportional integral derivative

PTM *P.pastoris* trace metal solution

RBS ribosome binding site

REC reverse phase chromatography

RO reverse osmosis

SB superbroth

SD Shine-Dalgarno site

SDS-PAGE sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sf *Spodoptera frugiperda*

S_F substrate concentration in feed (g/L)

$Y_{x/s}$ cell titre of substrate (g CDW/g)

μ specific growth rate (h^{-1})

SEC size exclusion chromatography

S(t) substrate concentration at time t (g/L)

TB Terrific Broth

TBS tris buffered solution

TMB 3, 3', 5, 5'-tetramethyl-benzidine

TRX thioredoxin gene

TSB tryptic soy broth

UF ultrafiltration

V(t) reactor volume at time t (L)

WCW wet cell weight (g/L)

X(0) cell concentration at time t

X(t) cell concentration at time t (g CDW/L)

YEPD yeast extract peptone dextrose medium

ACKNOWLEDGMENTS

The author wishes to thank Dr Robert Dempster (Group Leader Fermentation, AgResearch, Wallaceville), Professor Yusuf Chisti (Institute of Technology and Engineering, Massey University) for supervising this work and all the staff at the Wallaceville Animal Research Centre, Upper Hutt, are thanked for their guidance and support.

1 Literature Review

The supply of many eukaryotic proteins which have potential clinical or industrial use is often limited by their low natural availability. The advent of biotechnology and gene cloning has sought to provide an alternative supply for eukaryotic proteins. Agresearch has extensive experience in biotechnology products designed to improve the welfare of animals. Scientists at the Wallaceville facility of Agresearch have developed a number of recombinant protein products which have the potential as animal pharmaceuticals. The objective of this research was firstly to maximise the titre of four recombinant proteins products to improve their profitability and marketability to potential manufactures. The research then looks at developing parameters for the expression of those proteins in a small scale bioreactor to aid the possible transfer of the technology to higher volume production. Finally the research serves to provide a basis of fermentation knowledge to aid the development of other recombinant protein products. The recombinant protein products investigated included a vaccine antigen (*Eg*95) produced as inclusion bodies in *E.coli*, a aspartyl protease inhibitor homologue (*Aspin*) produced in *E.coli*, a secreted cytokine (G-CSF) produced in *Pichia pastoris* and a secreted gonadotropin (*o*FSH) produced in High Five™ insect cells.

1.1 HOST CELL LINE

Three different types of micro-organism hosts are investigated in this research. They are *Escherichia coli*, *Pichia pastoris* and the insect cell line High

Five™. The host cell line appropriateness is dependant on a number of factors including the price and form of the product, protein structure/ size and whether post-translational modifications such as glycosylation are required.

Escherichia coli is one of the most commonly utilised organisms for producing recombinant products. This can be mainly attributed to the wealth of knowledge of the organism's biology, ability to grow to high-cell-densities on inexpensive media and the robustness of its growth. However the use of *E.coli* has many drawbacks including; the production of endotoxins, difficulty in secreting proteins, improper folding, and lack of post-translation modification [1, 8-15]. Eukaryotic proteins expressed in *E.coli* also often form inclusion bodies which add a number of steps to the downstream processing, making recovery more expensive and complicated [10, 16-24].

In contrast, the yeast *Pichia pastoris* can grow to high cell concentrations on inexpensive chemically defined media, can secrete proteins extracellularly simplifying downstream processing and has the ability to perform some eukaryotic post-translational modifications [4, 25-28]. However, proteins produced in *P.pastoris* are not always folded correctly, may be over glycosylated and titres are variable with reports of recombinant protein levels vary from milligrams to grams per litre [4, 26]. *P.pastoris* also grows more slowly than *E.coli* with typical culture time of days instead of hours [29].

Insect cells can perform complex post-translation modifications, produce high active protein concentrations and most proteins are secreted simplifying purification [30]. However, insect cells are extremely delicate, require high

seeding ratios, can be infected by mammalian viruses and are significantly slower to grow than bacteria and yeasts cells [23, 31, 32]. The most common insect cell line used is *Spodoptera frugiperda* (Sf9) which is derived from the pupal ovarian tissue of fall army worms. High Five™ is a cell line which originates from the ovarian cells of the cabbage looper *Trichoplusia ni*. High Five™ cells have a typical doubling time 18-24 hours, can be grown in suspended culture in serum free media and produce 5-28 fold more protein than Sf9 [31-33]. However insect cell media are generally more complex and expensive than prokaryotic media, cells do not grow to high cell densities and protein production is generally in the milligram per litre range [32, 34-37].

1.2 PLASMID EXPRESSION

1.2.1 Expression Vector

The construction of expression vectors requires several elements whose configuration must be carefully considered to ensure high levels of protein synthesis [38]. A typical *Escherichia coli* expression vector is shown in Figure 1. The gene of interest is preceded by a promoter which binds RNA polymerase initialising transcription. A large number of promoters are available for *E.coli* with the most commonly used being the *lac*, *tac*, T7, $p_L(\lambda)$ and *ara*BAD. Desirable traits for a promoter include that it leads to the accumulation of high levels of protein and is tightly regulated to allow the growth of cultures to high densities with minimal protein expression. The *lac* and *tac* promoters

are considered weak promoters with only moderate protein titres and a high basal expression [13, 39]. In contrast the *ara*BAD, T7, and $p_L(\lambda)$ promoters are considered relatively strong promoters with high levels of protein production and low levels basal of expression [22, 40, 41]. The *lac* and *ara*BAD systems are repressed in the presence of glucose [22]. Other desirable traits include the promoter needs to be easily transferable to allow the testing of many strains for protein titres. If the product is destined for large scale production then the simplicity and cost of induction must also be considered. The pET102 plasmid shown in Figure 1 contains the T7 promoter controlled by the *lac* operator (*lac*O) which binds a repressor protein (*lac*I) in the absence of isopropyl β-D-thiogalactopyranoside (IPTG) preventing transcription [13]. IPTG is commonly used in laboratory experiments, however is costly at large scale [1, 42-44]. Less expensive options for large scale production include the $p_L(\lambda)$ promoter which is thermally induced or the *ara*BAD promoter which is induced using L-arabinose [16, 45, 46]. Following on from the promoter is the ribosome binding site (RBS) which consists of a Shine-Dalgarno (SD) site which interacts with the rRNA during translation initiation and a translational spacer before the start codon of the gene of interest [47]. The RBS plays an important role in the efficiency of transcription initiation. Surrounding the gene of interest the pET102 plasmid contains the coded sequences for the fusion partners HP thioredoxin, EK recognition site, V5 epitope and His-6 genes which are discussed in the next section. Downstream of the gene of interest a transcription terminator is located to stop transcription and also to protect the newly created mRNA. A transcription terminator consists of a region of amino acid symmetry which protects the mRNA from exonucleolytic degradation by creating a loop structures in the RNA. Figure 1 shows the plasmid also contains an ampicillin resistance gene (*bla*). A gene that confers antibiotics resistance is used to provide selective pressure on plasmid containing cells. Common antibiotics used in *E.coli* include ampicillin, kanamycin, tetracycline and chloramphenicol. Finally the origin of

replication (ori) gene determines the plasmid copy number. The use of high copy numbers has been shown to in some cases lead to higher titres of recombinant protein [48-50]. However higher plasmid copy numbers have also been observed to lead to lower cell viability [22, 51].

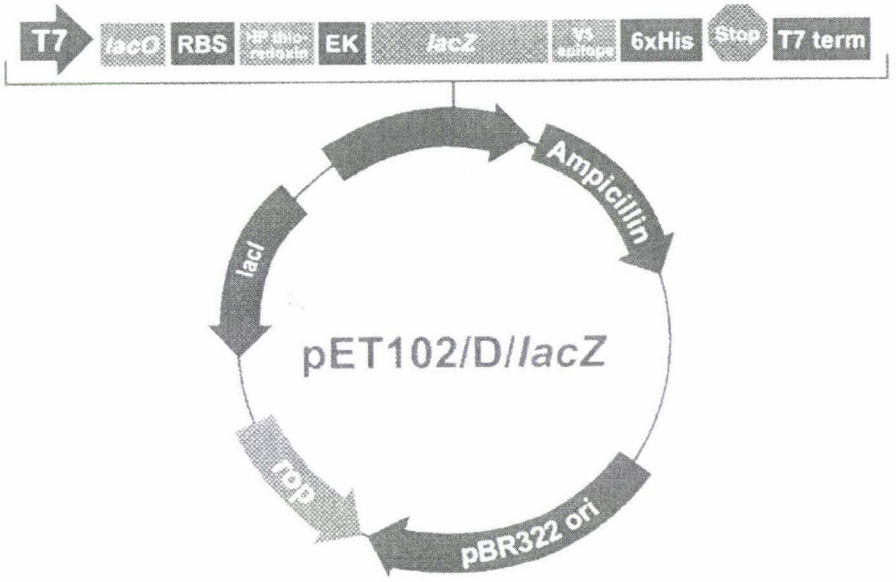

Figure 1: pET102 vector map showing the various genes (source Invitrogen). The *lacZ* gene is shown here as the gene of interest. The ribosome binding site (RBS) proceeds the gene of interest which is expressed with fusion partners HP thioredoxin, EK recognition, V5 epitope and poly-histidine domains. The plasmid contains the T7 promoter regulated by the operator (*lac*O). A T7 translational terminator stabilises the mRNA and ampicillin resistance gene (*bla*) which aids clone selection. An origin of replication (ori) determines the plasmid copy number.

Pichia pastoris is a methylotrophic yeast capable of utilising methanol as its sole source of carbon. The first step in the metabolism of methanol is the oxidation to formaldehyde by the enzyme alcohol oxidase (AOX). The promoter regulating the production of AOX can be used to control recombinant protein expression in *P.pastoris*. The AOX promoter is tightly regulated in the presence of methanol and strongly repressed in the presence

of glucose [5, 26, 52, 53]. Two genes AOX1 and AOX2 code for alcohol oxidase [54, 55]. Isolation of these genes has been utilised to produce two different strains, Mut^+ which contains the AOX1 gene and Mut^S which contains the AOX2 gene [56]. The AOX1 gene is responsible for a majority of the AOX production in *P.pastoris* and produces fast methanol metabolising strains while AOX2 isolates are much slower at metabolising methanol and produce recombinant protein more slowly [54]. A vector map of the pPICZ plasmid which was used in this research for the expression of granulocyte colony stimulating factor (G-CSF) is shown in Figure 2. Transcription is controlled by the AOX1 promoter and the gene of interest cloned into the multiple cloning site (*Stu* 1, *Eco*R I, *Pml* l....). The gene of interest is expressed fused to the c-myc epitope and poly-histidine tag (6xHis). The pPICZ plasmid contains a resistance gene for the antibiotic Zeocin™ to aid the selection of transformed cells. The EM7 and TEF1 promoters constitutively drive the Zeocin™ resistance gene. In *Pichia pastoris* the recombinant plasmid can be incorporated into the genome by homologous recombination. Incorporating the gene of interest into the organism genome has been shown to improve clonal stability [55]. A CYC1 translation terminator is used to aid efficient mRNA processing and the pUC origin of replication allows maintenance and replication of the plasmid when used in *Escherichia coli*. Recombinant protein titres using the pPICZ vector vary between 0.007 g/L to 2 g/L [57-62].

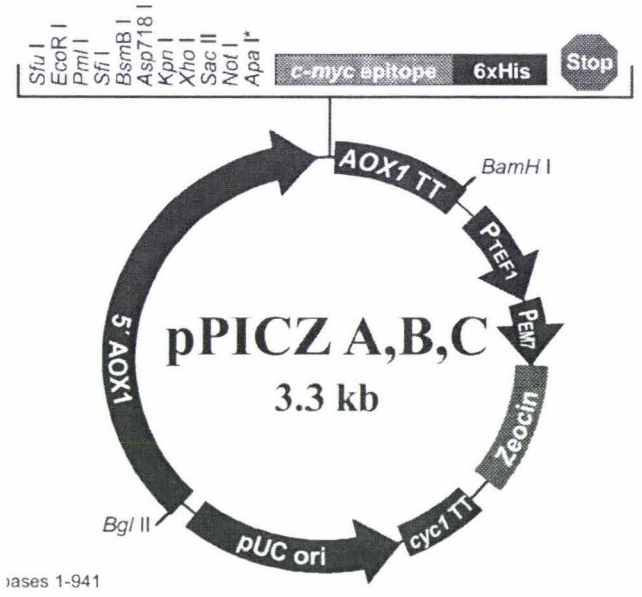

Figure 2: pPICZ vector map showing the various genes (source Invitrogen). The pPICZ vector was used for the expression of colony granulocyte stimulating factor (G-CSF). The gene of interest was cloned into the various cloning sites (*Stu* 1, *Eco*R I, *Pml* I….). Expression is under the control of the alcohol oxidase (AOX1) promoter and the gene expressed with the fusion partners myc-epitope and poly-histidine tag to aid protein detection and purification. The plasmid contains the TEF1 and EM7 promoters which control the expression of the Zeocin™ resistance gene. The AOX1 translational terminator stabilises the mRNA and the pUC origin of replication (ori) aids replication and maintenance of the plasmid in *E.coli*.

Production of recombinant protein in insect cells is initiated by the infection of cells with a baculovirus containing the sequence of DNA for the recombinant protein. This research uses the pMIB vector from the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (OpMNPV) to express ovine follicle stimulating hormone (*o*FSH). Shown in Figure 3 the pMIB vector contains the Op*IE2* promoter which provides constitutive expression of the gene of interest. The gene of interest is expressed with a poly-histidine (6 x histidine) tag and a V5 epitope to simplify purification and detection. A honeybee melitten secretion signal sequence directs the secretion of the protein of interest into the culture medium. The plasmid also contains genes that confer resistance to the antibiotics blasticidin (*bsd)* and ampicillin (*bla).* The antibiotic resistance genes are constitutively

expressed under the control of the EM7 and Op*IE1* promoters. The plasmid is transfected into insect cells using lipid-mediated transfection with stable cloned cells selected using blasticidin.

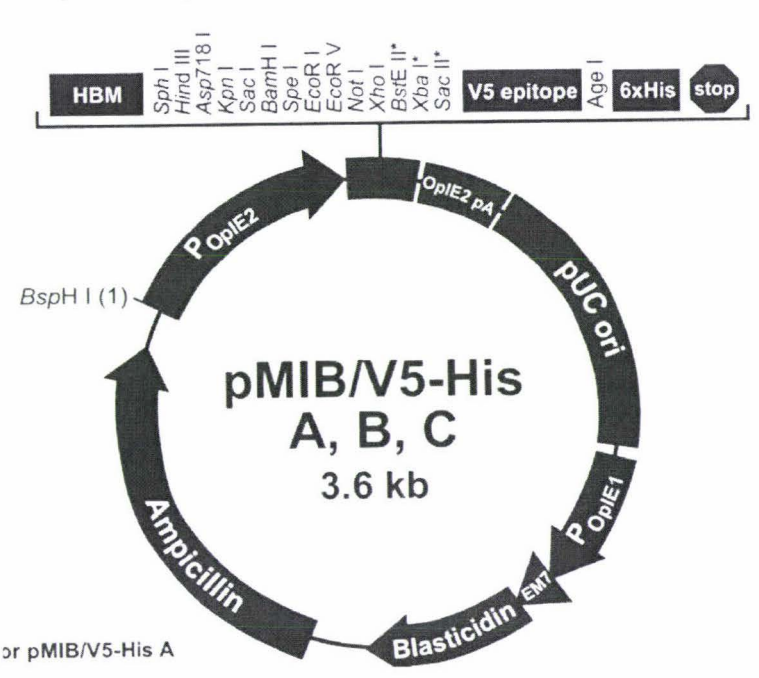

Figure 3: pMIB vector map showing the various genes (source Invitrogen). The pMIB vector was used for the expression of ovine follicle stimulating factor (oFSH). The gene of interest was cloned into the various cloning sites (*Sph* 1, *Hind* III, *Asp*718I….). Expression is under the control of the OpIE2 promoter and the gene expressed with the fusion partners V5 epitope and poly-histidine tag. The plasmid contains the OpIE1 and EM7 promoters which control the expression of the blasticidin and ampicillin resistance gene. The AOX1 translational terminator stabilises the mRNA and the pUC origin of replication (ori) aids replication and maintenance of the plasmid in E.coli

1.2.2 Fusion Partners

Recombinant proteins are often fused to one or more proteins "fusion partner" to aid purification, identification and/or to improve solubility.

One group of fusion proteins consist of small lengths of peptides that create antigenic sites (epitope tags). Commonly used epitope tags include poly-histidine (His_6), green fluorescence protein (GFP), c-Myc and V5. The His_6 epitode tag is probably the most widely used fusion partner in molecular biology [63]. The His_6 tag binds to immobilised metal ion chelating (IMAC) resin under mild solvent conditions providing a quick and relatively inexpensive mode of purification compared to other affinity chromatograph techniques [64]. Its small size (six amino acids) reduces the possibility of steric hindrance and antibodies to the His_6 tag can be used to identify the recombinant protein quickly. The epitope tag GFP produces a fluorescence signal which allows for the expression of recombinant protein to be measured using a spectrometer [65]. V5 and c-Myc are both highly antigenic tags for which commercially available antibodies can be used to identify, immuno-precipitate or immuno-affinity purify the protein.

Another group of fusion partners has been developed to improve the solubility of expressed recombinant proteins. Previously it has been found that fusing recombinant protein to highly soluble proteins improves the overall solubility of the fused protein [66]. The fusion partner glutathione-S-transferase (GST) is a highly soluble protein derived from *Schistosoma japonicum* which has been widely used to improve the solubility of eukaryotic proteins expressed in *E.coli* [9, 13, 66]. However GST large size (15kDa) can hinder proper folding of the recombinant thus effecting their activity [67]. For this reason fusion proteins often contain a sequences such as the *Xa* site which allows the fusion partner to be cleaved once the protein has been purified [67]. Glutathione-sepharose resin can used to purify GST fused protein and commercial antibodies to GST are available. It has been suggested previously that the insolubility of many eukaryotic proteins may be due to the redox state in the *E.coli* cytoplasm, as it tends to be more oxidative than in mammalian

cells [8]. The fusion partner thioredoxin has been used to alter the redox state of bacterial cytoplasm increasing recombinant solubility [8, 11, 66-68]. Maltose-binding protein (MBP) is another common highly soluble protein that has been found to improve translation efficiency and solubility of eukaryotic proteins expressed in *E.coli* [67-69]. Both thioredoxin and MBP have commercially available resin which can be used to purify these fusion proteins [38] however hybrids are now available which contain His_6 sequence allowing less expensive IMAC purification [67].

1.2.3 Induction

Recombinant protein production in *E.coli* fermentations has been reported to be proportional to the specific growth rate at induction [39, 70, 71]. Inducing at the maximum growth rate has the advantage that cell metabolism is at it highest leading to a high cell specific protein production. However, several other studies have found that inducing the cells as late as possible in the exponential growth phase led to the higher expression as higher cell densities could be obtained [72-74]. A balance between cell density and cell specific titre which produces the maximum volumetric titre needs to be empirically determined as the optimum is likely to be a result of a combination of the recombinant characteristics. Care must be taken if the second approach is favoured as induction at high cell densities can shock cultures into stationary phase early [75]. Generally cultures are not induced in stationary phase as culture viability is reduced and a large number of proteases are produced which can degrade the protein of interest [76].

For the production of soluble protein in *E.coli*, cultures are induced early in the exponential phase to slow the culture growth rate. It has been extensively reported that the production of soluble proteins in *E.coli* is inversely related to the specific growth rate of the cultures [4, 22, 73, 77-79]. At high growth rates protein expression can overwhelm chaperones and foldases required for protein folding. This leads to the accumulation of protein intermediates which may form inclusion bodies [10, 66, 80, 81]. However, again a balance must be achieved as inducing the culture earlier reduces the potential to achieve high-cell-densities [9]. Another approach used to increase soluble protein production is to reduce the concentration of inducing agent [81]. Under the control of a strong promoter the concentration of inducing agent can be used to slow the rate of recombinant protein expression. Lim et al. [45] found using 10.7mM of L-arabinose only 10% of interferon-α (INF-α) produced in *E.coli* was soluble but by reducing the concentration of L-arabinose to 2.7mM the fraction of soluble IFN-α increased to above 90%.

In *Pichia pastoris* cultures methanol serves both as a carbon source and an inducer agent. During induction the concentration of methanol must be maintained within a relatively narrow range to avoid growth and expression inhibition. Guarna et al. [52] observed that recombinant protein production decreases above 1.0% (v/v) methanol while other researchers have observed growth inhibition at concentrations above 3% (v/v) [5, 52, 82]. Both these limits are only slightly higher than the 0.5% (v/v) methanol concentration suggested in the Pichia Expression Kit (Invitrogen, San Diego, CA) to initiate induction. Probes that measure the residual methanol concentration are available however in their absence shaker flasks are generally induced with 0.5-1.0% (v/v) methanol per day to avoid inhibition [5, 52, 82-86]. In high-cell-density fermentations the rate of methanol consumption can vary between 0-10 % (v/v) per hour [87] . To avoid the accumulation of methanol

periodic starvation periods are used, in which methanol feeding is halted until methanol depletion occurs as indication by a rapid increase in the dissolved oxygen concentration [5, 52].

Cell density has been shown to have a dramatic effect on the production of recombinant protein in *Trichoplusia ni* cell line with specific recombinant protein production decreasing with increasing cell density [31, 34, 35, 88]. Wickham et al. [31] reported a 6-fold reduction in specific production of β-galactosidase with an 8 fold increase in cellular concentration. For this reason infection of *T.ni* cells is generally performed at low densities. It has been suggested that cellular contact may inhibit the production of viral DNA [31]. Another important aspect in considering when and how to induce is the degradation of recombinant protein. Recombinant protein production triggers stress responses such as the activation of proteases which may damage or destroy the protein of interest [48]. Ramirez and Bentley [75] found that at high rates of induction cells produce more proteases. By reducing the rate of induction either by reducing the amount of inducing agent, inducing at lower cell-densities and over a longer period culture metabolic stress is reduced consequentially reducing the production of proteases.

1.2.4 Plasmid Stability

Instability of recombinant plasmids remains one of the most important problems in the commercial production of recombinant products. The instability is affected by both genetic and environmental factors including the properties of the plasmids themselves, degree of expression, cultivation

temperature, nutrient levels, growth rate and fermentation mode [89]. Culture age increases with fermenter size increasing the chance of reversion to wild-type strains. Unfamiliar conditions of pH, temperature or substrate concentration place added pressures on the organism [90]. In fermentations a distribution of cell copy numbers exist (Gaussian distribution). Cells with lower copy numbers exert less metabolic pressure and therefore have a greater long term stability [51]. Yeast divide by budding therefore are even more likely to form plasmid free cells [91]. To increase plasmid stability often antibiotics are added to a culture or a amino acid dependant strain is used to provide selective pressure on the recombinant population [49]. However, both these methods add expense and some manufacturers do not allow the use of antibiotics. Other methods include the careful selection of appropriate plasmids and their constructs to reduce metabolic stress [50].

1.3 PROCESS CONDITIONS

During fermentation, nutrient concentration, agitation, temperature, dissolved oxygen and pH can be utilised to increase titres and protein expression. Recombinant organisms generally show far more sensitivity to process conditions and require more accurate control than wild-type strains [89].

1.3.1 Media Composition

All micro-organisms require a source of carbon, nitrogen, sulphur/ phosphate and a variety of trace minerals, vitamins and growth factors for optimal growth and protein synthesis.

Glucose has the highest energy potential of all carbohydrates and is the preferred carbon source for most micro-organisms [92]. Other common primary carbon sources include glycerol, lactose, sucrose and in the case of *P.pastoris* methanol. *E.coli* grown on glucose has been observed to grow more quickly and produce higher titres of foreign protein compared to other carbon sources [46, 78, 93, 94]. However the excess supply of glucose causes metabolic overflow and the production of undesirable by-products such as acetic acid [20, 95]. Acetic acid production by *E.coli* has been extensively studied and shown to be a major inhibitor of both growth and recombinant protein production [72, 74, 96-102]. The choice of carbon source is also important in the control of plasmid expression as both the *lac* and *ara*BAD

promoters are repressed in the presence of glucose and the AOX1 promoter by both glycerol and glucose [26, 56, 87, 103]. Prior to induction, the inhibitor substrate must be either removed by replacing the medium with one free of glucose or sufficient time allowed for the culture to consume all the substrate [4, 104]. Extra care must be taken with chemically defined media as induction/repression ratios have been found to be several magnitudes higher than for complex media [22].

Nitrogen can be supplied either from a complex source such as a protein/ vegetable digest or from a synthetic source such as ammonium sulphate, ammonium hydroxide or ammonium chloride. In the production of vaccines a synthetic nitrogen source is often used in preference to a complex source due to concerns over the transmission of disease and variation between batches. Generally growth is slower and recombinant protein titres lower using synthetic nitrogen sources as cells must synthesize their own amino acids [105, 106]. Synthetic media supplements which combine complex mixtures of trace metals and growth factors are available to supplement media; however, generally yeast extract is added to cover all the minor nutrients required for cellular growth [107]. The source of sulphur is usually drawn from the available amino acids in complex media or as part of sulphate salts in synthetic media [108]. Korz et al. [98] found that to increase *E.coli* to high biomass titres phosphate was required to be supplemented. Phosphate is required by cells for the synthesis of DNA, RNA, protein and in cellular respiration [109]. Phosphate is often made available through the use of a phosphate buffer system in the medium. Medium buffering is important to prevent pH fluctuations which may cause culture stress and destabilise secreted proteins [110]. Low concentrations of potassium and sodium are provided by the addition of sodium chloride and potassium chloride [70]. These salts also help balance the osmolarity between the cells and the

medium. Antibiotics such as ampicillin, kanamycin, chloramphenicol and gentamycin are often added to the medium to provide selective pressure on the recombinant to retain its foreign genes and to provide limited protection from contamination by other organisms [38].

Escherichia coli is not fastidious and will grow on almost any medium. For the growth of high-cell-density cultures of recombinant *E.coli* one of the most common medium used is Luria-Bertani (LB) broth [18, 73, 111]. Other common media include M9 minimal medium [45, 75], Terrific Broth (TB) [45] and Super Broth (SB) [112].

Pichia pastoris can also be grown on a wide range of media. Cultures are initially grown in media containing glycerol or dextrose then transferred to an equivalent medium containing methanol. Common media include; MGY/ MM (minimal glycerol or minimal methanol medium) [27, 53, 84, 86], BMG/ BMM (buffered minimal glycerol or methanol medium) [26, 52, 82, 83, 113-115], BMGY/ BMMY (buffered complex glycerol or methanol medium) [5, 26, 82-84, 86, 115] and basal salt media which are commonly used in large scale fermentation [5, 52, 114-117].

Insect cells have much more complex nutritional requirements compared to those of bacteria or yeast cells. This is reflected in the large number of components in the insect culture media. Additional to the basal salt media which provides salts and trace elements, yeastolate, lipid-sterol emulsions and shear protectants are commonly added. Yeastolate is an autolytic digest of yeast which serves as a source of amino acids, vitamins and nucleotides. A lipid-sterol emulsion is added to provide cholesterol which insect cells can not

synthesize, essential fatty acids and α-tocopherol which is an antioxidant. Pluronic F-68 is a common shear protectant used to protect the cell from damage in suspended culture. A number of media optimised for the cultivation of High Five cells are commercially available including; ExCell 405 (JRH Bioscience), Express Five (Life Technologies), IS BAC (Irvine Scientific), and CCM3 (Hyclone).

1.3.2 Feeding Regime

The traditional batch fermentation requires the entire nutrient allocation to be added at the beginning of the fermentation. This leads to large concentration gradients which can cause cellular stress and may inhibit growth. Other techniques such as continuous culture, perfusion and fed-batch culture overcome these concentration limits by adding nutrient throughout the fermentation as they are consumed. In continuous fermentation, whole broth is withdrawn from the fermenter throughout the culture and fresh medium added to supplement the volume. Continuous culture uses fermenter time more efficiently as production can be maintained for long periods making it appropriate for products which are required to be produced in large volumes. However, continuous culture is not widely used in pharmaceutical manufacture as production volumes are generally too small to warrant the added expense and also due to concerns over the long term clonal stability of recombinant products [69]. Perfusion is a technique where broth is continuously withdrawn from a fermenter through a filter which retains the cells [34, 79, 118]. The filtered broth is collected and fresh medium added to supplement the culture. Using perfusion culture it is possible to obtain cell densities 10 to 30 times higher than the maximum cell density in batch culture [119]. Perfusion however is not widely used in industry due to difficulties in

maintaining large scale operations. To overcome these problems most small scale fermentations incorporate aspects of both continuous and batch culture by using fed-batch systems [73, 100, 120-123]. In fed-batch cultures, growth limiting nutrients are added as they are consumed to maintain growth for extended periods. The addition of nutrients is controlled either by an empirical formula (constant and exponential feeding being the most common) or in response to process measurements (chemostat, pH-stat, DO-stat) [73].

Chemostats relies on the monitoring of substrate or metabolite concentration to control culture feeding [74, 108, 124, 125]. Common compounds monitored include acetate, glucose, phosphate or the off gas makeup [98]. A vessel mass balance or empirical relationship is then used to relate the amount of feed required to achieve a desired growth profile [9]. On-line measurement can significantly increase the cost and complexity of production and is therefore not extensively utilised for small scale production. A pH-stat feeding system monitors the pH of the fermentations for indications of acetic acid production [69, 71, 74, 126, 127]. The feed is increased until the maximum growth rate is reached as indicated by metabolic overflow causing a decrease in the pH [72, 101]. The pH-stat has two main advantages over chemostat in that it does not require additional monitoring equipment as a pH probe is a standard accessory of most fermenters and in that it ensures that the growth rate is always close to its maximum. One of the most common feeding systems is the DO-stat [39, 44, 74]. The DO-stat regime utilises the fact specific oxygen uptake reaches a maximum at the maximum growth rate [96]. Using pulses of feed the response of the organisms oxygen uptake is used to determine whether the organism is at its maximum growth rate [72]. The popularity of the DO-stat system can be mainly attributed to its simplicity and the quick response time (response observed in less than 30 seconds in *E.coli* cultures [96]). However, DO-stat systems are liable to

control distortions caused by changes in the volumetric oxygen transfer coefficient. Changes can be caused by a number of things including DNA release, antifoam addition, protein production and agitation [94]. Online feeding regimes such as DO-stat and pH-stat are particularly useful during development of fermentation processes as they are easy to implement and adaptive to change however can cause high cellular stress due to fluctuations in culture conditions [89, 121]. Exponential and constant feeding regimes use empirical relationships to regulate the feed rate [25, 26, 74, 78, 93, 126]. In contrast to methods that use online measurement, considerable knowledge of the culture growth kinetics and a robust fermentation process are required to use these regimes. Accumulation or depletion of nutrients may occur if the relationship is a poor fit or if the system undergoes an unanticipated change which affects the utilisation of substrates [72]. An advantage of an exponential feeding regime is that it can be used to manipulate the growth rate of the cells and changes in substrate concentrations are gradual reducing cellular stress [126]

1.3.3 Temperature

Fermentation temperature affects the kinetics of chemical reactions within the cells. At low temperatures organisms are sluggish and the growth rate is reduced as energy production slows. At very low temperatures the micro-organisms may stop growing altogether. In contrast at high temperatures enzymes in the organism may be denatured and lose their facilitative ability.

The optimal growth temperature of wild-type *E.coli* is 37°C [128]. The temperature of recombinant fermentations are often lowered to restrict the

expression rate [9, 12, 13, 48, 112]. Restriction of the expression rate is desirable as it has been extensively demonstrated that the production of active protein is increased at lower expression rates [12-15, 29, 81, 93]. Recombinant strains of *E.coli* have been found to have an optimal protein titre using post-induction temperatures between 20 and 30°C [4, 44, 48, 98, 112]. Maintaining a lower temperature has also been found to increase the amount recombinant protein by reducing the activity proteases [9].

A majority of *P.pastoris* fermentations are controlled at 30°C [4, 5, 26, 27, 83-87, 104, 113-117, 129, 130]. Tight control of temperature is required as above 32°C protein expression is inhibited [5]. During methanol utilisation *P.pastoris* can produce considerable amounts of heat. Coupled with the capability to grow to high cell densities, this means that large cooling systems are often required to control the temperature [117]. Reducing the fermentation temperature below 28°C has been reported to increase the activity of some proteins expressed by *P.pastoris* [5, 82, 117, 129]. Another advantage of lowering the temperature is that at the lower temperatures *P.pastoris* secretes less low molecular weight proteins reducing downstream processing [5].

Insect cell have been successfully grown over a range of temperatures between 25-30°C however the optimal temperature for cell growth and viral infection has been demonstrated to be 27°C [131, 132]. Reuveny et al. [133] found that insect cells produced similar titres of recombinant protein in the range 22 and 27°C, increasing the temperature led to earlier production and an increase in the proportion of recombinant protein secreted in the culture medium. Most recombinant protein productions in *T.ni* are grown at 27°C [37, 79, 118, 134]. Above 30°C protein production and cell viability have been observed to decrease rapidly [133]. No literature was found that used lowered

temperatures to increase recombinant protein solubility or activity in insect cells.

1.3.4 Agitation

Cells require a continuous supply of nutrients and oxygen for growth. The primary role of agitations in fermentation is to improve the transport of these materials in the medium. Agitation helps to create homogeneous conditions throughout the fermenter for consistency and control as well as improves the heat transport in the fluid. For organisms that produce toxic metabolites, agitation also helps to disperse toxins.

During fermentation an operator needs to be aware of the fragility of the organism they are working with. *E.coli* and *P.pastoris* are relatively robust. In shaker flasks cultures of *P.pastoris* and *E.coli* agitation rates of between 150-250rpm are commonly used [4, 5, 85, 115]. *E.coli* fermentation generally use speeds of between 600-1000rpm [74, 93, 97, 98]. High-cell-density cultures of *P.pastoris* require high agitation rates in excess of 1000rpm to attain the oxygen transfer rates required to maintain culture dissolved oxygen levels [5, 113, 116]. Maintaining the dissolved oxygen is especially important during the expression of recombinant protein as *P.pastoris* cannot grow on methanol in the absence of oxygen.

Conversely, insect cells are very shear sensitive as they do not have cell walls, only a thin cell membrane to protect them against rupture [79, 90, 135]. A

balance must be established between the conflicting demands of nutrient transport and shear sensitivity. Wild-type *T.ni* cell are adherent however commercially available strains such as High Five have been selectively passaged to obtain cells that can growth in suspension. Despite this, cells must still be slowly adapted to suspended culture by increasing the agitation rate over a number of passages [37]. Agitation speeds of between 100-160rpm are used for *T.ni* cultures in shaker flasks [30, 37, 88, 134]. In bioreactors marine propellers or fixed matrix supports can be used to reduce the shear on the cells [136]. At low agitations speeds step down gearing may also be required to provide accurate control of propeller movement [30]. Additives are often supplemented to insect cell media to protect cells against shear. Shear protecting additives including Pluronic® F68, poly (ethylene glycol), poly vinyl alcohol and foetal calf serum [137].

1.3.5 pH Conditions

The pH of fermentations affects enzyme mediated reactions within the cell as well as the redox reactions of the cellular transport systems. Expressed protein folding and final conformation are also affected by the pH as different amino acid residues are exposed under various oxidative conditions.

The optimal pH range for growth of *E.coli* is between 6.4 and 7.2 [109, 138]. The pH of *E.coli* fermentations are generally controlled through the addition of 2-3M sulphuric acid and 29% (w/v) ammonium hydroxide (ammonium solution) or 5M sodium hydroxide [139]. In *E.coli* the solubility of expressed proteins has been reported to be effected by pH [140]. Strandberg and Enfors [140] found that amount of recombinant protein expressed as

inclusion bodies increased with decreasing pH. The specific activity of recombinant protein has an optimum pH during expression which maybe outside the range of pH for growth [109]. For this reason new recombinant proteins must be empirically tested over a range of pH with a balance sought between activity and bulk protein production.

P.pastoris is able to grow satisfactory over a wide range of pH (3-7) [117]. This can prove to be useful in optimising expression conditions and protecting secreted proteins from proteolysis. During the accumulation of biomass *P.pastoris* is generally maintain at pH 5.0, however post-induction the pH is often changed to optimise the conditions of protein expression [4, 115]. A number of studies have found that the titre of recombinant protein in *P.pastoris* fermentations can be increased by reducing the pH to between 3-5 post-induction [4, 5, 83, 84, 87, 113, 115, 117, 141]. The increase in titre of recombinant protein at lowered pH has been attributed mainly to the inactivation of neutral proteases [110, 117]. In shaker flasks the same effect is achieved by the use of unbuffered media such MGY/ MM which allows the pH to fall as cell metabolites such as ammonium accumulate in the culture [4-6, 87, 117].

Insect cells are very sensitive to oxidative conditions. For this reason insect cell media is often buffered at 6.2 using a buffer system such as carbonate and bicarbonate. Under the carbonate/ bicarbonate system small changes in pH can be achieved through the sparging of carbon dioxide which applies pressure on the carbonate/ bicarbonate buffer equilibrium. In addition 0.1M sodium hydroxide and hydrochloric acid can be used however the low intensity of agitation in cell cultures can cause localised acid and base pools that may damage the cells [142]. In adjusting the pH of fermentation media,

care must also be taken not to cause precipitation of medium components. Magnesium sulphate or chelating agents such as ethylene diamine tetra-acetic acid (EDTA) are generally added to synthetic media to prevent the precipitation of salts during fermentation [45, 71, 101].

1.3.6 Dissolved Oxygen

The availability of oxygen can affect the nature and rate of metabolic reactions within cells. Due to the low solubility of oxygen in water, oxygen transfer in large scale fermentations is often the main limiting factor in aerobic microbial growth [135].

During culturing, the operator generally has three methods of controlling the dissolved oxygen concentration; by the agitation speed, sparging rate and the oxygen concentration in the sparged gas. Furthermore the dissolved oxygen may be indirectly controlled by regulating the oxygen uptake of the micro-organism by either reducing the culture temperature or rate of feed. Several studies have found that a reduction in oxygen transfer leads to an increase in specific recombinant protein through the reduction of culture growth rate freeing up cellular resources to protein expression [39, 94, 109, 143, 144]. *E.coli* is generally grown in aerobic conditions, as during anaerobic growth it produces a number of metabolic products including; acetate, succinate, formate, lactate, and ethanol. The production of these products reduces the available energy for other process such as growth and protein synthesis [101]. Maintaining a residue oxygen concentration is also important as a number of proteases which may degrade the protein of interest are produced under oxygen starved conditions [111].

P.pastoris is capable of extremely high cell densities (greater than to 150g DCW/L) [4]. Oxygen is required for the first step of methanol catabolism therefore is extremely important to ensure that *P.pastoris* grows on methanol [113]. Generally *P.pastoris* fermentations are controlled at 30-35% of air saturation [5, 26, 87, 114, 117]. At high-cell-densities agitation speeds of up to 2000rpm, 1vvm of air and supplementation with pure oxygen maybe required to maintain the dissolve oxygen concentration [113, 115-117].

Due to a lack of cell wall insect cells are sensitive to shear from agitation and the sparging of gases [79]. As a result the rate of oxygen transfer in cultures is often the limiting factor in expression of recombinant protein [118]. To avoid damage from bubble cavitations, gas is often overlayed on the surface of the culture [145]. This dramatically limits the oxygen transfer rate so pure oxygen supplementation may be required to increase the solubilization driving force. Other methods to reduce the shear while increasing oxygen transfer include the use of tubing which diffuse microscopic bubbles through the culture or fixed matrix supports which cells are adhered to prevent contact.[136]

1.4 DOWNSTREAM PROCESSING

Fermentation products are invariantly found in low concentration in complex and ill defined solutions. The goals of downstream processing include removal of unwanted impurities, bulk-volume reduction with concomitant concentration of the desired protein and the transfer of the product to a stable and active environment [136].

Figure 4 shows the general downstream processing steps for protein production. All recombinant protein purifications begin with the clarification of cells from the fermentation broth. For extracellular secreted protein the pelleted cells are simply disposed and the protein of interest purified and concentrated from the culture supernatant. With recombinant proteins expressed intracellularly the pelleted cells are then be ruptured and the supernatant filtered or centrifuged to remove the cell debris. Denatured proteins must be solubilised before purification and concentration. Depending on the desired form the product may then be lyophilised or packaged in liquid form.

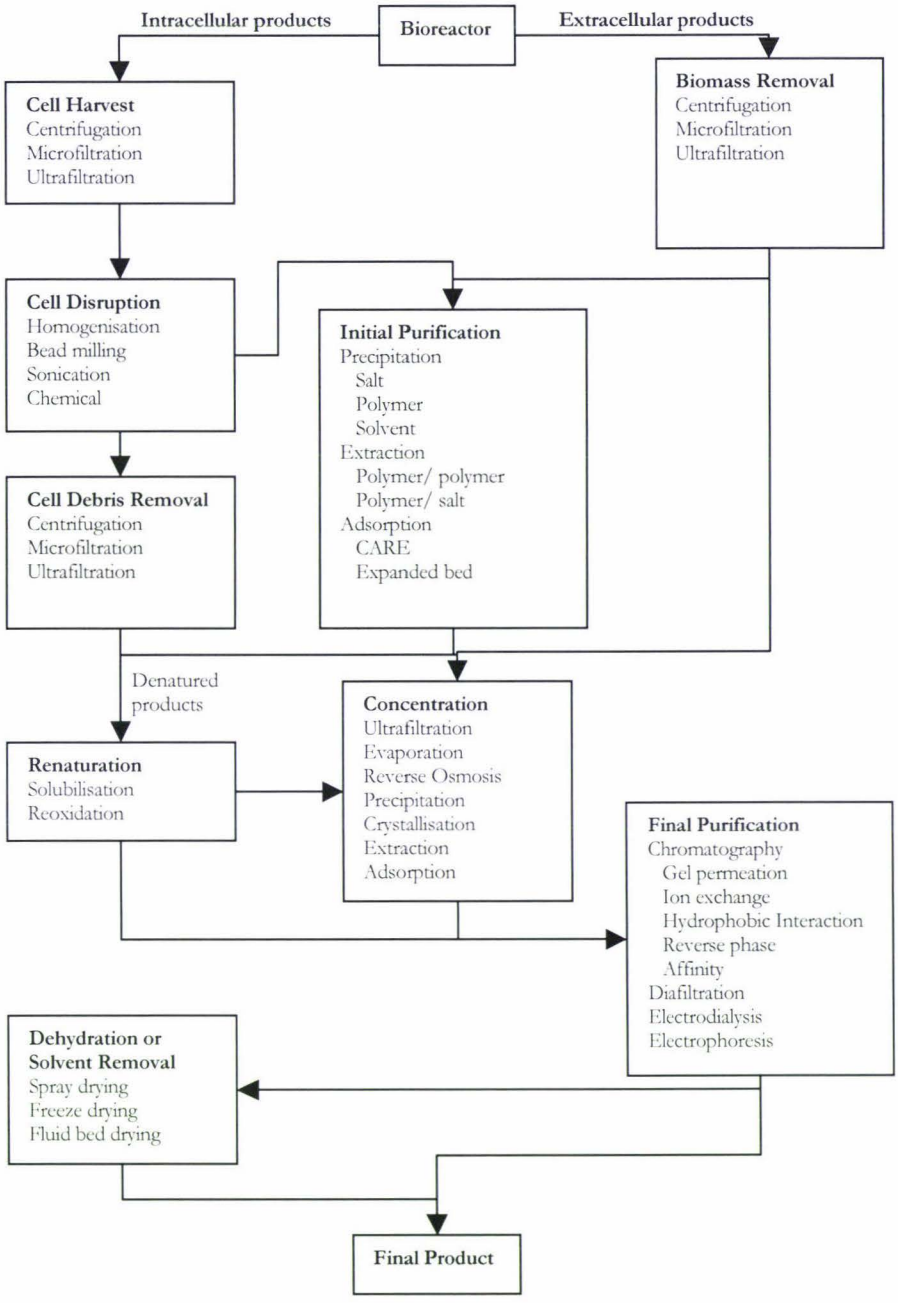

Figure 4: General stages of downstream processing for protein production, adapted from Perry's Engineering Handbook [136].

1.4.1 Clarification

Clarification helps to reduce process volumes and remove major contaminants which may clog the more sensitive/selective operations. Shown in Figure 4, at several stages in the downstream processing of recombinant protein products clarification is required. Clarification is used to separate cells from the fermentation broth after cell rupture to remove cell fragments and to change buffers solutions between purification steps. Cell separation processes are almost exclusively performed by the mechanical methods centrifugation and filtration [146]. The choice of method depends on the physical properties of the broth (temperature, pH, ionic strength), medium components (cells, polymers, polyvalent cations, presence of other particles) and on the desired final state of the product [147].

Filtration relies on the retention of particles by a membrane based on size, with the driving force for separation created by the pressure across a semi-permeable membrane. Filtration is the best established and most versatile method of removing insoluble material when the particles are dilute, large and rigid however many biological suspensions are difficult and slow to filter as they produce gels [136]. Generally cross-flow operations are used as the tangential flow of materials helps to prevent build-up of gel layers on the membrane [147]. Filter aids can also be used to assist filtration however these often complicate downstream concentration and purification steps [146]. The two main types of filtration membranes used for cell/ debris remove are microfiltration and ultrafiltration [148, 149]. Microfiltration membranes are generally used for particles with average nominal molecular weights (NMW) greater than 500,000 while ultrafiltration is used for proteins below 100,000 NMW [136]. The main advantage of filtration over centrifugation is that it produces a retentate with lower water content reducing process volumes [150].

Centrifugation relies on the enhanced sedimentation of particles of different densities under an externally applied centrifugal force. Centrifugation generally requires more expensive equipment than filtration but is more effective in removing small particles which tend to clog filters [136]. Centrifuges are especially useful for removing cells from fermentation broths, however the retentate produced by centrifugation generally has a higher water content than that produced by filtration [150]. Many different types of centrifuges exist, the most common of which are the tubular and disk centrifuge, scroll conveyor and basket centrifuge [136, 151]. For the removal of *E.coli* and *P.pastoris* cells from fermentation broths generally centrifugal forces of 2,000-5,000g for 5-20 minutes are used [15, 71, 140]. For insect or mammalian cells which are sensitive to shear lower centrifugal speeds of 200-1000g are used to prevent cell rupture [30, 79, 88, 145]. If the protein is intracellular the removal of finely dispersed particle after cell disruption may require forces of up to 30,000g [9, 16, 39, 71, 120]. During centrifugation often a low concentration of denaturant such as Triton X-100, deoxycholate or urea is added to the wash buffer to prevent crude impurities adhering to the surface of the protein aggregates [12, 17, 18, 152].

1.4.2 Cell Disruption

The release of intracellular proteins is achieved through the disruption of the cell walls of the organism using; mechanical, non-mechanical, biological, or chemical lysis.

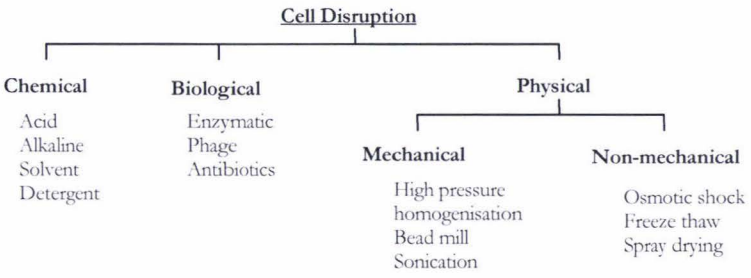

Figure 5: Techniques of cell disruption showning the various chemical, biological and physical methods

Physical disruption methods include high-pressure homogenisation, sonication and bead milling. On the large scale homogenisation is the most common method of cell disruption [153]. High pressure homogenisation is a liquid shear method which utilises rapid changes in pressure and velocity as the cells pass through a small orifice to rupture cells [9, 154, 155]. Operation at high pressures is desirable for effective disruption with units working at pressures of up to 1200 bar [156]. The use of high pressures is limited by the resultant temperature rise in the liquid across the valve and by the avaliability of materials to prevent erosion of the valve [157]. Cooling is required at high pressures to prevent denaturation of the protein of interest. During cell lysis a large amount of DNA and RNA material is released into the solution. This causes an increase in viscosity that can be a problem in downstream processing. Further passes in a homogeniser or the use of DNAase and RNAase help to break down the nucleic acids and reduce the viscosity [94, 153, 156].

Bead mills use agitation of a cell suspension with abrasives such as glass or steel beads to disrupt the cell walls [158]. Disruption of the target cells is due to a combination of collision between the beads, cavitations, and the generation of high shear forces. Bead mills are commonly used in industrial applications due

to ease of scale-up and the ability of the process to achieve high efficiencies with short disruption times [156]. Bead milling like high-pressure homogenisation is not as delicate as enzymatic or chemical lysis and can cause extensive fragmentation of the organism. This causes downstream processing problems with the fragments clogging filtration membranes and adsorption columns [154, 159]. Shear forces during disruption also produce large amounts of heat that can denature the protein of interest therefore cooling of the bead chamber is required.

Sonication is a liquid shear method of cell disruption [43, 160, 161]. The method utilises ultrasonic waves at 15-20 kHz which create micro-bubbles in the medium that on cavitation have the potential to rupture cell walls. Sonication has low operating costs, does not require sophisticated equipment or extensive staff training and the equipment can be easily cleaned [162]. However some proteins are inactivated by shear and heat produced in the process, and fine cell debris is produced which can hamper downstream processing [163]. On the laboratory scale sonication is extensively used however few industrial processes exist due to problems with localised heating [136].

On a laboratory scale a number of other methods are available including the use of detergents, solvents and enzymes to disrupt cells. Few of these have found large scale applicability as they are often too costly and may contaminate the final product, thus increasing the amount of downstream processing [157]. Chemical lysis looks to solubilise the walls of the cells. Common solubilising agents include detergents such as Triton X-100, sodium dodecyl sulphate (SDS) or chaotropes such as urea and guanidine hydrochloride [15, 46, 112]. The choice of solubilising agent is important as many proteins will be denatured in the presence of these compounds and they also may complicate downstream

processing. Enzymatic digestion of the cell wall is also possible using lysozyme or phages [15, 16]. This method is extremely innovative in that it reduces the amount of downstream processing in removing cell walls however this method is currently too expensive for large scale application. Other non-mechanical methods of disrupting cells include osmotic shock in which the cell are exposed to high salt concentrations which causes high osmotic pressures across the cell membrane rupturing them. Similarly cycling of freezing and thawing [39, 68] or pressurising the broth with nitrogen gas and then rapidly releasing it can be used. These methods are not widely used in industry but on the laboratory scale are often used in addition to mechanical methods to improve disruption efficiency [136].

1.4.3 Initial Purification

Purification aims to separate the product from materials with similar properties. This can be achieved using a range of techniques that utilise differences in molecular size, diffusivity, solubility, charge and density (see Figure 6). Methods used in initial purification include; precipitation, extraction and adsorption.

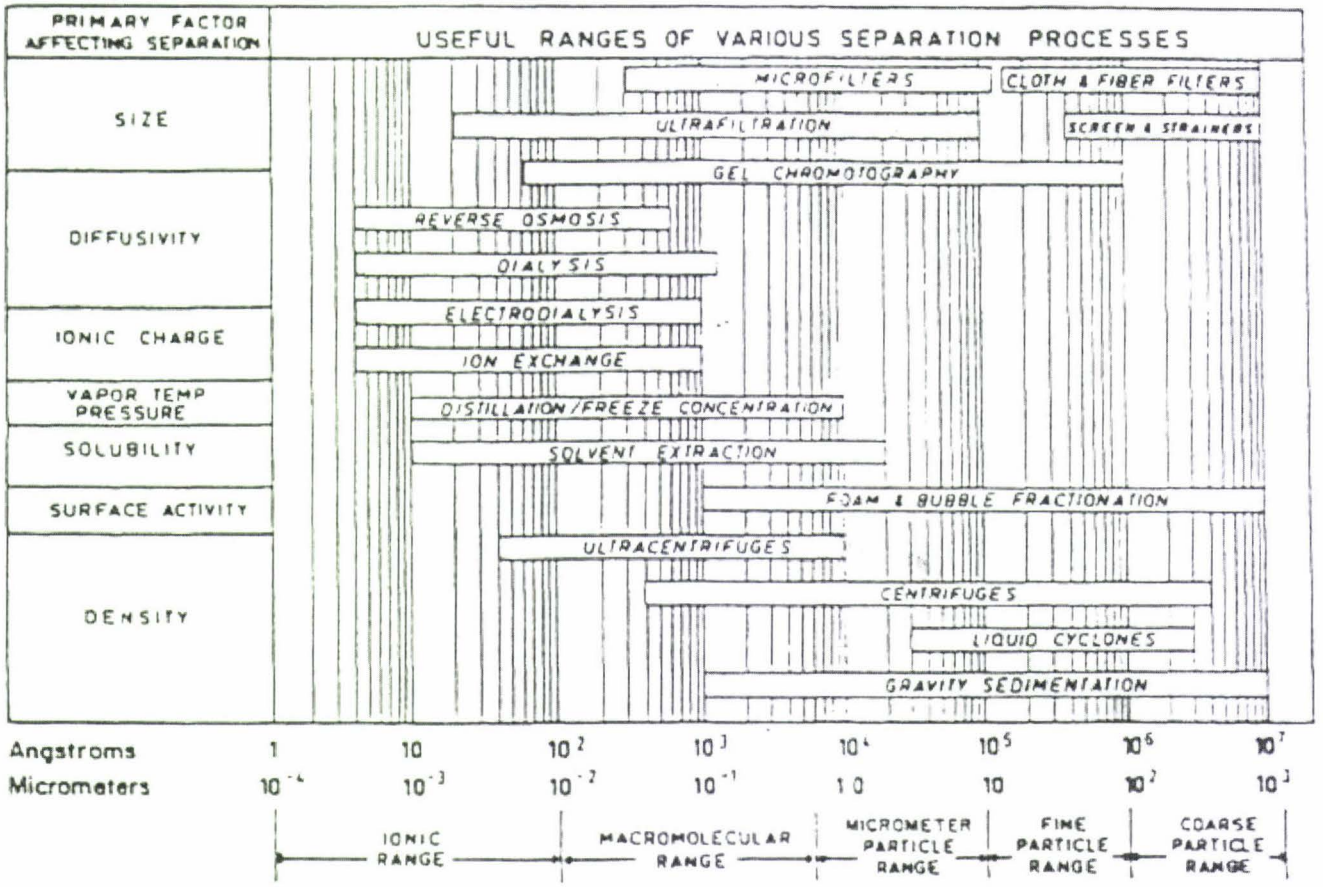

Figure 6: Comparison of various separation processes showing the range of particle and molecular size covered and the primary factor governing the separation process. Source Unit Operations Of Chemical Engineering [164].

Precipitation is a simple well documented method of purifying proteins which is widely used in laboratories [162]. Precipitation can be induced by the addition of solvents, salts, polymers and by adjusting the pH or temperature. The most common precipitate used in biological separation is ammonium sulphate as it is inexpensive and causes little or no denaturation [2, 162]. Ammonium sulphate works by hydrating proteins, causing them to expose their hydrophobic regions which aggregate together causing the protein to precipitate. After precipitation, the pellet is separated by either centrifugation or filtration and washed in ammonium sulphate solution. Other potential precipitants include ethanol, acetone, polyethylene glycol and polyethylene imine polyacrylic acid [136]. Precipitation is generally used in the early stages of separation processes to remove bulk impurities and concentrate the target protein [165]. However precipitation operations are generally not highly selective, are sensitive to process conditions and precipitates can disrupt downstream processing and increase waste disposal costs [162]. The process equipment is the same as for crystallisation (discussed later).

Extraction utilises differences in the solubility of biological materials between two different liquid phases or between a solid and liquid (leaching) [165, 166]. Phase forming reagents include polymers, salts and solvents. A number of modules are available for contacting the two phases however they can be generally divided into two groups; stage wise (mixer settlers) and continuous flow devices (spray towers) [136]. Once sufficient partitioning has occurred the product light phase is either decanted off or removed by centrifugation and the product recovered from the rich phase. The extent of partitioning between the two phases is dependant on the solvents relative molecular weight, concentration and the proteins size, charge and hydrophobicity [167]. Extraction can be used for large volumes and is especially good for unclarified broths as insoluble particles tend to collect at the interface of the phases

allowing easy removal [136]. Solvent based extraction systems have been extensively used in the recovery of antibiotics as they have a low solubility in water but high solubility in organic solvent [147]. Leaching is often used to extract pharmaceuticals from plants [166].

Adsorption is concerned with the concentration of solutes at the surface of particles [168]. Adsorption which uses 'loose' bed operations (stirred tank and expanded bed) are used during the initial purification instead of chromatograph (fixed bed operation) as the crude streams can foul packed columns. Separation is achieved by utilising protein charge, hydrophobicity, or affinity interaction with absorbent particles as for chromatography (discussed later). The most common materials used in adsorption are activated carbon and silica gel. Expanded bed adsorption is a technique incorporating clarification, concentration, and purification capture in a single process [149]. Expanded beds use absorbents with low densities which allow the fluidisation of the bed as the material is pumped up through the column. After the product is absorbed, the loose materials are washed free. The direction of flow is then changed turning the system into a packed bed and the product eluted as in chromatography. Continuous adsorption recycle extraction (CARE) uses a system of two reactors to adsorb and then desorb the protein of interest with the absorbent gel recycled in a continuous operation. This system offers the ability to work with larger volumes and reduces batch to batch variation [149]. The two most common applications of adsorption are in the decolouration and biomass removal from solutions [169, 170].

1.4.4 Concentration

Concentration takes the clarified solution and increases the product concentration by several weight percent. Common methods of concentration include ultrafiltration, reverse osmosis, precipitation, crystallisation, extraction and adsorption.

Reverse osmosis (RO) is a membrane selective process used most commonly for the separation of low molecular weight molecules such as salts or sugars [160]. RO membranes are essentially non-porous, made from materials including cellulose acetate, polyamide and a number of other composite polymers [171]. The molecular separation is based on solubility and diffusivity of the solute through the membrane. In reverse osmosis hydraulic pressure (between 10-60 bar) is applied across a membrane to induce diffusion against the osmotic gradient [172]. Ultrafiltration (UF) is also a membrane selective processes which separate molecules primarily on size [150, 173]. The operation is cross flow, with the solution pumped from a holding tank across the face of the membrane. Major membrane materials include; cellulose, polysulphone, polyethersulphone, polyacrylonitrite with a nominal cut off size of between 1 and 100kDa [150]. Both RO and UF can be performed using a number of modules including plate and frame, tubular and spiral wound formats, with the modules varying in flux rate and the ease of cleaning [172]. Diafiltration is a method commonly used between purification steps to exchange buffer solutions. Diafiltration is the same as normal tangential filtration except that fresh solvent is continuously added to the retentate as the permeate is removed. This aids to wash contaminants from the feed and increases the concentration of the compound of interest in the retentate [136].

Crystallisation is common used in the later stages of purification for removing closely related materials that filtration and adsorption are unable to distinguish [165]. In contrast to precipitation, crystallisation produces aggregates of protein with very high purity and uniformity. Operations are performed at low temperatures minimising damage to heat sensitive materials and high concentrations are used minimising unit costs and increasing separation factors. Industrial systems are complex, with performance dependant on a number of factors including kinetic processes, hydrodynamics, modes of operation and vessel geometry [174]. The first stage of crystal formation, nucleation, occurs when the solute exceeds saturation and clusters of solute particles are present to provide a surface for crystal growth. Several different types of crystallisers exist differing in the method of supersaturation, mode of operation, and method of handling the crystal suspension [174]. Crystalliser can be designed to operate in both batch or continuous modes with continuous operation more economical for large volumes. Nucleation can be induced by evaporation, cooling or both under a vacuum [164]. Crystallisation has been used in the commercial purification of high quality pharmaceuticals from paracetamol to human vaccines [175].

1.4.5 Renaturation

After isolation of inclusion bodies solubilization is used to break the intramolecular bonds between the proteins to release the monomers into solution. Common solubilizing solutions include urea, sodium thiocyanate, guanidine hydrochloride (HCl) or the detergents Triton X-100 and sodium dodecyl sulphate (SDS) [20, 21, 70]. Most solubilizing solutions use a pH of around 8 with concentration of 4-9M urea or guanidine HCl. A strong reducing agent such as mercaptoethanol and dithiothreitol (DTT) is also often added to

break any disulphide bonds [19, 20, 80]. Once the inclusion bodies are solubilized the solution is filtered or centrifuged again to remove any impurities that may have been caught in the structure of the inclusion bodies and any unsolubilized protein which may clog subsequent purification steps.

Once the protein has been solubilized/ reduced and impurities removed the protein must be refolded into its native active form. This process begins with the gradual removal of the protein from the solubilizing solution into a renaturation buffer [80]. A number of conditions effect the efficiency of renaturation including; temperature, pH, redox potential, ionic conditions, and the concentration of cofactors [66]. Ideally for process efficiency renaturation should be performed using high protein concentrations however a common observation is that the titre of active protein is generally lower for more highly concentrated solution due the formation of unstable intermediates [10, 12]. To slow down the formation of aggregates protein concentration of 0.010-0.100g/L are generally used during renaturation [20, 66]. If the protein contains disulphide bonds then a renaturation buffer requires a reducing potential to allow the reshuffling of disulphide bonds into their correct structure. Disulphide bonds are later reformed in the presence of strong oxidising conditions using thiol reagents such as glutathione and cysteine, or in a copper induced air oxidation system [66].

1.4.6 Final Purification

The final purification steps are responsible for the removal of the last impurities. For pharmaceutical products this step is important to remove traces of materials which may cause adverse immune reaction [153]. A variety of purification

methods are used for the final purification step including; electrodialysis, dia-filtration and chromatography.

Electrodialysis is a membrane separation technique used to separate ionised species from water, macromolecules and other uncharged species through selectively ion permeable membranes. On the large scale electrolysis is used for the production of table salt, removal of salts from polluted water, and the deashing of milk whey [176, 177]. Electrodialysis is commonly used in the biotechnology industry after ammonia sulphate precipitation of proteins to remove the salts from the product and also in the isoelectric separation of proteins from blood serum [178, 179]. Electrodialysis use series of alternating anionic and cationic exchange membranes with an electric field applied across the whole section. Under the electric field ionized species migrate toward their respective electrodes until they reach their opposing selective membrane which creates alternative compartments of desalinisation and concentration. Electrodialysis is highly prone to the formation of precipitates on the membranes. These precipitates as well as reducing the permeability of the membranes to ions, low specific conductivity that substantially increases power consumption and cause localised heating which may denature the protein of interest [180].

Chromatography is the most widely used downstream processing operation in biotechnology mainly due to its versatility, high selectivity and the wide experience in industry dealing with issues of scaling [136]. Separation of proteins using chromatography exploits the range of physical/ chemical properties of proteins and chromatography media. Methods include size exclusion chromatography (SEC), ion exchange chromatography, hydrophobic

interaction chromatograph (HIC), reverse phase chromatography and affinity chromatography.

Size exclusion (SEC) or gel permeation chromatography operates on the principal of separating molecules based on their size. Smaller particles enter the pores of the gel matrix which holds them up whereas the larger particles which are unable to enter the pores pass through the column quickly. The efficiency of SEC is dependant on the gel used, how it is packed, the size and distribution of pores, the volume and concentration of samples and the flow rate of the applied sample [165, 181]. SEC works best when there is a significant difference in the size of the protein of interest and their contaminants [127, 165, 166]. SEC is often the first method of chromatography genrally attempted, as it quick and relatively inexpensive compared to many of the other methods.

Ion exchange chromatography relies on the amphoteric nature of proteins with their net charge dependant on the pH of the solution. Ion exchange units come in two types, cationic-exchangers and anion-exchangers which bind to positive and negatively charged proteins respectively. The capacity of ion exchange columns to bind protein is depend on the size/ distribution of binding sites and the type of electrically charged groups [164]. Ion exchange chromatography is used in biopharmaceutical industry for the purification of insulin produced in *Escherichia coli* [182], α-amylase from *Aspergillus awamori* [183], L-leucine dehydrogenase from *Bacillus cereus* [184] and monoclonal antibodies .

Hydrophobic interaction chromatography (HIC) and reverse phase chromatograph (REC) takes advantage of cluster of hydrophobic residues and ligand groups to discriminate proteins. The difference between the two

methods is in the strength of the interactions. HIC relies on relatively weak interaction primarily from residues on the surface of the proteins. Binding is controlled through the concentrations of salts in the elution buffer with hydrophobic interactions at their maximum at moderate salt concentrations of 1-2M. Elution is achieved by decreasing the concentration of salts and increasing the concentration of polar species. The technique works best at high salt concentrations and is often used after protein precipitation. Various types of proteins have been purified using hydrophobic interaction chromatography including; alkaline phosphatase, estrogen receptors, isolectins, strepavidin, epoxide hydrolase, proteoglycans and hemoglobins [185]. In contrast to HIC, REC uses much stronger interactions which can unfold the protein and expose internal residues. Binding and elution is controlled using a gradient of organic solvent. REC is not widely used for biological extraction as the strong interactions can cause denaturation and irreversible inactivation of delicate proteins [166].

Affinity chromatograph is a powerful separation technique able to achieve very high specificity and purification (up to 1000 times purification in a single pass [186]). Affinity chromatography relies on very specific and well defined interactions between proteins and affinity groups. The term affinity chromatograph is used to encompass a large group of techniques. Examples of affinity interactions include antibody-antigen, hormone-receptor, enzyme-substrate/ analogue/ inhibitor, metal ion-ligand and dye-ligand pairs. Three of the most common affinity groups in recombinant protein production include; glutathione S-transferase (GST), maltose binding protein (MBP) and thioredoxin (TRX) [9, 12, 13, 17, 66, 68, 85]. Chromatography is particularly useful in isolating high purities of protein from crude solutions such as broths and cell lysates. Unfortunately affinity chromatography does have some limitations. Non-specific interactions of the matrixes can result of ion-exchange

and hydrophobic affinity with contaminants and low levels of matrix ligands also have been found to leach into the product [166, 187, 188]. Coupled with the high cost of packing materials this led to their limited use in large-scale application [188]. Immobilised metal ion chromatography (IMAC) is a form of affinity chromatography which is increasingly being used on the large scale [136]. IMAC relies on the interaction between amino acids such as histidine, cysteine, tryptophan on the surface of proteins with metal ions bound to a matrix. Metal ions include Cu^{2+}, Zn^{2+}, Ni^{2+} and Co^{2+} with the adsorption dependant on the chelating group, metal ion, pH and buffer solution. One of the most common IMAC systems uses the affinity between Ni^{2+} and the poly-histidine fusion partner [67, 189-191]. Elution of the bound protein can be achieved using a pH gradient, competitive ligand, organic solvent and chelating agent. The most common eluting agents being ammonium chloride, imidazole or EDTA which cleaves both the protein and metal ion.

1.4.7 Dehydration

Dehydration serves two purposes: it stabilises the bulk product until it can be formulated and it preserves the activity of most biological product [147]. There are many drying processes but as most proteins are heat sensitive many can be discounted as they use relatively high temperatures [136, 147]. Major methods used for to the dehydration of heat sensitive products include vacuum, freeze and spray drying [146].

The most common and versatile method of drying biological materials is vacuum-shelf drying [136]. As low temperatures must be used to prevent denaturation of the protein, a vacuum is required to speed up the drying

process. Heating is provided by way of passing a heated process fluid through the trays or some small dryers are electrically heated evaporating off the solvent. Freeze drying uses similar equipment to vacuum shelf driers however solvent is removed by sublimation as apposed to evaporation. Freeze drying works by quickly cooling and then heating the product subliming the free solvent, which is drawn off in a vacuum. Both vacuum shelf and freeze driers are used extensively in the pharmaceutical industry for dehydrating temperature sensitive, low titre, high value products where the relatively high labour costs are insignificant [165, 192, 193]. Spray drying is another method that can be use to dry sensitive proteins. Spray driers use convection instead of conduction to dry the product. The feed solution is atomised either using a spray nozzle or a rotating disk to increase the drying surface area. The atomised particles then pass through a hot dry gas which dries the material in a matter of seconds. Further drying takes place in the base of the spray dryer by the creation of a fluidised bed. Spray drying is a very effective method of drying on a large scale and is widely used in the production of whey proteins and milk powder, however the capital cost of equipment prevents its use on low titre processes common in the pharmaceutical industry [165, 194].

1.5 ASSAYS

Assays are important in scientific research to allow review between different works. Assays must be robust, reproducible and the degree of accuracy known. It is also important when choosing an assay to ensure the results are specific to project objectives and that a good standard is available for reference throughout your research.

1.5.1 Cell Density

A number of methods are available for the quantification of cell density [195]. Commonly used methods include; optical cell density, dry cell weight, total and viable cell counts. In total cell counts, small samples of cells are counted using a Helber counter chamber which contains a scribed $1mm^2$ grid containing 400 squares. The suspension is diluted by trial and error till 5-10 cells are present per square then 50-100 squares are counted. This method gives a precise indication of cell number, however is difficult to perform when cells are motile or have a tendency to aggregate. Also to obtain an accurate measure, a number of counts are used which is very time consuming. Total cell counts on their own do not give any indication of the number of live cells. For this reason total cell count are often coupled with a viable cell count or a cell exclusion stain such as Trypan blue [196]. In viable cell counts, samples are serially diluted and spread onto agar plates. The plate are incubated and colonies allowed to form. Plates with between 10-20 colonies are counted with each colony assumed to originate from a single cell. Viable cell counts give an indication of the active cell concentration however can take several days for results and use a large amount of consumables therefore are not suitable for large numbers of samples. Viable cell counts are also not highly accurate, even performed with the best possible technique have been found to

still contain errors of ±90%, therefore results need to be replicated and extreme care taken to minimise errors [195]. Optical density is an extremely quick way of assessing the cell density of a culture with the absorbance of a culture measured at 600nm on a spectrometer. However optical density is only accurate over a small range of absorbances (0.01-0.2) requiring the sample to be diluted. On its own the measure also gives no idea of culture viability and measurements can be affected by other particulate matter in the medium. Despite this optical density remains the most common method of measuring cell density [72, 96, 122, 127]. Cell dry weight is a move accurate method of measuring cell density. The cell pellet is collected by centrifugation or filtration and washed several times in saline to remove debris. The pellet is then dried in an oven at 90°C till constant mass and the dry pellet weighted. Optical density is often calibrated to cell dry weight as it is too slow for use in fermentative studies [197].

1.5.2 Protein Assay

Total protein content can be measuring using optical absorbance, through the use of protein stains, or by measuring the amino nitrogen content of samples. The spectrum of proteins in samples can be separated in electrophoresis gels and viewed by staining with Coomassie blue, silver nitrate or transferred on to nitrocellulose for analysis by Western blotting.

Proteins absorb wavelengths in the near ultra-violet range (~280nm) according to the content of the amino acids tyrosine and tryptophan [198]. If a protein specific absorbance is known then its concentration in solution can be measured using a spectrometer, as the absorbance follows the Beer-Lambert Law of absorbance. This method is quick and the sample is not destroyed in the process however it can only be used for clean preparations

with a number of common fermentation compounds including nucleic acid, sodium chloride, sucrose, phosphate buffer and glycerol interfering with the absorbance [198]. Protein dyes methods include the commercially available Bio-Rad and bicinchoninic acid (BCA) test are more accurate however require colour development time and have higher consumable costs. The Bio-Rad test is based on the Bradford method using the stain Coomassie Brilliant Blue G-250 [199]. The BioRad test is the most commonly used protein assay as it is quick (full colour develops in 20-30 minutes), has a high degree of accuracy and has few interfering reagents [199]. However the Bio-Rad test can not be used for samples with high detergent concentrations or high pH. The BCA protein assay is a modified form of the Lowry Method [200] with bicinchoninic acid replacing folin reagent [201]. The BCA assay is useful for testing samples containing high detergent concentrations and/ or with high pH. Unfortunately the BCA assay has a number of interfering substances including; EDTA, DTT and ammonium sulphate. Another less commonly used total protein tests is the Biuret method [202]. The Biuret method has few interfering agents and is more accurate than the Lowry or ultraviolet absorption methods [202] but is not widely used as it consumes much more material than the other methods.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is one of the most popular methods for separating proteins [203]. SDS-PAGE uses the electromotive force of charged species in an electric field to drive molecules through a porous support matrix of polyacrylamide gel. The porous gel acts as a sieve retarding, or in some cases completely obstructing the movement of large molecules while allowing smaller molecules to migrate freely. The gel matrix prevents convective mixing caused by heating and provides a record of the electrophoresis at the end of the run which can be stained and used for scanning, autoradiography or storage. Prior to loading

the gel samples are boiled in buffer containing sodium dodecyl sulphate (SDS) and a reducing agent such as 2-mercaptoethanol or dithiothreitol (DDT). SDS is an anionic detergent which denatures the protein and confers a negative charge to the protein in proportion to its length. It is usually necessary to also add 2-mercaptoethanol or dithiothreitol (DDT) to reduce disulphide bridges in the protein to allow complete infold. Under these denaturing condition SDS-PAGE separations are primary due to molecular weight. A number of reagents are available to stains gels including Coomassie Blue (Coomassie Brilliant Blue G-250), silver stain, zinc stain, copper stain and Ponceau S. Coomassie blue is the most common method of staining SDS-PAGE as it is quick and inexpensive, however is less sensitive than silver staining detecting only bands above 0.3 μg/band [204]. Silver staining is an extremely sensitive method of staining proteins (1ng/band), however the staining procedure takes several hours and stained gels can only be used for scanning densitometry only over a narrow range of protein concentrations [205]. Neither of these two stains are easily reversible, making the protein unable to be recovered intact for other procedures such as Western blotting. Reversible stains such as Ponceau S are used to visualise protein bands prior to Western blotting, however Ponceau S is not widely used in standard SDS-PAGE as it has a low sensitivity, fades over time and has poor photographability [206].

1.5.3 Protein Activity

Biological and immunological activity is often the critical production response for pharmaceuticals. Ultimately *in-situ* tests of final response may be the only reliable test of the activity. However this form of assay is often time

consuming to develop, expensive and impractical to perform large numbers of tests so invitro methods must be used.

Enzyme-Linked Immunosorbent Assay (ELISA) is a commonly used invitro test of immunological activity. ELISA uses series of antibodies raised against the material of interest such as a specific proteins to bind a colour substrate which can be measured using a spectrometer [207]. The samples are firstly bound to the specially coated wells of an ELISA plate. A blocking solution is then added to all the wells to fill the empty binding sites, thus preventing non-specific binding. After washing, the primary antibody is then added to the wells binding to the molecule of interest. An ELISA has been previously developed for *Aspin* [208]. The primary antibody used is purified IgE raised in mice against the *Aspin* molecule. A secondary antibody goat anti-mouse horse radish peroxidase conjugate (HRP) then binds to the primary antibody. Finally the colourless substrate 3,3',5,5'-tetramethyl-benzidine (TMB) is added. TMB binds to the HRP forming a yellow complex. The absorbance of the complex can then be measured on a plate reader and compared to dilution curves of samples of known activity. Western blotting is another immunological test which allows the visualisation of the protein of interest in a protein mixture. Separated proteins from a SDS-PAGE gel are transferred to a membrane such as nitrocellulose. The membrane is firstly blocked with a concentrated protein solution (100g/L foetal calf serum, 50g/L non-fat milk powder) to prevent further non-specific binding of proteins. This is followed by incubation of the membrane in antibody solution raised to the protein of interest. After washing of the membrane it was then incubated in diluted conjugated probe antibody or other detecting substrate. Depending on the substrate the protein can be colorimetric/ autoradiographic or chemiluminescent identified. Chemiluminescent substrates are commonly used for low concentration samples because of their greater detection

sensitivity. ELISA and Western blotting are powerful methods which can detect low concentration of material (μg/L to ng/L in solution) and large amounts of samples can be run at once. Both methods are highly dependant on the quality of the raised antibodies and require considerable optimisation to ensure quantitative analysis.

In vitro biological activity can be measured using specialised cell lines. The proliferation/ inhibition or production of signal compounds by cells exposed to the material of interest can be used as an indicator of activity. The biological activity of the immunostimulate Granulocyte Colony Stimulating Factor (G-CSF) is measured by monitor the production of interleukin-3 by murine myeloblastic cell line [209]. Biological tests are generally the closest representation to *in-situ* testing however is not always possible, as specific tests and cell lines may not be available. Biological tests are also generally costly as they require the purchase and maintenance of specialised cell lines

1.6 CONCLUSION

The objective of this research was to maximise the titre of four recombinant proteins products and develop parameters for the expression of those proteins in a small scale bioreactor. The research also aims to provide a basis of fermentation knowledge to aid the development of other recombinant protein products.

The optimal growth conditions of *E.coli* is an aerobic environment at 37°C and pH between 6.4-7.2. Common media used to grow *E.coli* include Luria broth, Terrific Broth, Super Broth and M9 medium. Due to substrate concentration limits feeding of a carbon source and trace nutrients is required to achieve high-cell-densities. DO-stat, pH-stat are online feeding strategies and exponential feed an off-line strategy which have been extensively used to achieve high-cell-densities. A large number of promoters are available for *E.coli* with the most commonly used being the *lac*, *tac*, T7, $p_L(\lambda)$ and *ara*BAD. A good promoter should be strong, have a low level of basal expression, be easy to switch between strains and be inexpensive to induce. Fusion partners such as GST, MBP or $(His)_6$ are often added to recombinant protein to improve their solubility and aid purification. The production of recombinant protein is effected by; the choice of fusion partner, regulatory system, medium composition, concentration of inducer, time of induction, dissolved oxygen concentration and rate of feeding. Eukaryote proteins produced in *Escherichia coli* commonly aggregate to form inclusion bodies made up of inactive protein. If soluble recombinant protein is required then important factors include; the regulatory system, fusion partner, induction time, inducer concentration, medium composition, post–induction temperature.

The optimal temperature for *Pichia pastoris* culture is 30°C above this temperature protein expression is inhibited. *P.pastoris* can grow over a wide range of pH which can be utilised to reduce the action of proteases. The AOX1 promoter is repressed in the presence of glycerol or glucose and derepressed in the presence of methanol. Cultures are grown up first on glycerol/ dextrose media and then transferred to equivalent media with methanol in place of glycerol/ dextrose. Common media include MGY/ MM, BGY/ BMM, BMGY/ BMMY and basal salt medium. *P.pastoris* is capable of achieving very high cell density (>150g/L dry cell weight) and is often only limited by the capacity to supply oxygen and remove excess heat. During expression the dissolved oxygen concentration is extremely important as oxygen is required for the first step of methanol catabolism. The level of recombinant protein expression in *P.pastoris* is dependant on whether the protein is intracellular or secreted, the culture medium used, final cell density, length of expression and the action of proteases.

Insect cell lines are extremely sensitive to environmental conditions and shear. Cultures are grown at 27°C and buffered at pH 6.2 using a carbonate/ bicarbonate system. Insect cells lack a cell wall therefore are susceptible to damage by shear. Agitation should be just sufficient to maintain the cells in suspension and gas overlay or tubing containing micro holes can be used to reduce shear from aeration. High Five™ grow much quicker than tradition insect cell line and can be grown in suspension. Insect cells have much more complex nutritional requirement compared to bacterial or yeast cells and this is reflected in the large number of components in the insect culture media. On top of a basal salt medium which provides salts and trace elements, yeastolate, a lipid-sterol emulsion and shear protectant are commonly added. Important factors in the production of insect cells include: regulatory system, production medium, agitation, seeding ratio, multiplicity of infection and period of expression.

Extracellular secretion of recombinant protein reduces much of the downstream processing. The culture broth is simply centrifuged or filtered to remove the cells and the supernatant passed directly onto purification. Downstream processing of intracellular protein begins with removal of the cells from the medium by centrifugation or filtration. Cells are then disrupted to release the intracellular contents and the lysate centrifuged to remove cell fragments. The most common methods for large scale cell disruption are high pressure homogenisation and bead milling. Other methods on the laboratory scale include sonication, chemical and enzymic disruption. If the protein is soluble then the supernatant proceeds to purification and concentration. Purification aims to separate the product from materials with similar properties. Methods include; precipitation, extraction and adsorption. Ultrafiltration and reverse osmosis, precipitation, crystallisation, extraction and adsorption are then used to concentrate the process stream. For inclusion bodies proteins, the pellet is solubilisation in a reducing solution such as 8-9M urea or guanidine hydrochloride. Denatured protein is then refolded in to its natural form by dialysising the protein in reducing concentration of denaturant. A wide range of methods are available to purify soluble proteins including; gel filtration, ion exchange, hydrophobic interaction chromatography or affinity chromatography. The method most appropriate is dependent on a number of factors including the structure of the protein, market price of the final product, required purity and volume. The protein may finally be dehydrated to stabilise and preserve the biological activity of the product

2.1 INTRODUCTION

Escherichia coli is one of the most commonly utilised organisms for producing recombinant products due to the wealth of knowledge of the organism's biology, ability to grow on inexpensive media and the robustness of its growth. Unfortunately eukaryotic proteins expressed in *E.coli* often form insoluble aggregates or inclusion bodies [10, 16, 17, 71, 126, 140, 210]. Recombinant protein aggregated in inclusion bodies are usually inactive. To recover protein activity inclusion bodies are solubilised and then refolding into their native form which adds additional steps and expensive to a production process [66, 81, 152]. Despite this, many products are still produced as inclusion bodies as alternative systems that produce soluble active protein are either not available or produce insufficient product quantities to be economical [10, 16-24].

AgResearch and Melbourne University scientists have previously developed a recombinant vaccine that protects livestock from hydatids disease which is caused by the tapeworm *Echinococcus granulosus* [211]. Adult *E. granulosus* tapeworms inhabit the small intestine of dogs. The eggs from the tapeworms are excreted by dogs and are ingested by herbivore intermediate hosts such as cattle and sheep where they develop into cysts. Humans can also become accidentally infected with the tapeworm eggs and suffer from hydatids disease. The cysts of *E. granulosus* can grow to contain several liters of fluid. These cysts can interfere with the function of internal organs such as the liver and if burst can cause severe reaction. *Eg95* is a sequence of DNA derived from the *E.granulosus* oncospheres. *Eg*95 has been cloned into *E.coli* cells which expresses the protein in inclusion bodies [212]. Vaccination with

solubilised inclusion bodies has been shown previously to provide immunity to natural challenge by *E.granulosus* eggs in sheep and cattle [212]. The objective of this chapter is to investigate methods to increase the titre of *Eg*95 and develop parameters for production in a small scale bioreactor to improve the profitability of the product. This section also aims to provide a basis of fermentation knowledge for the development of future recombinant protein products produced in *E.coli* as inclusion bodies.

2.2 MATERIALS AND METHODS

2.2.1 Strain and Plasmid

The clone for this study which expresses the recombinant protein *Eg*95 was kindly supplied by David Heath (Agresearch, Upper Hutt New Zealand). The host strain used in this study was *Escherichia coli* BB4 (Strategene, La Jolla, CA, USA). The gene sequence for *Eg*95 was cloned into the pGEX plasmid [LE392.23, F'*lacI*^{q}ZΔM15, proAB, Tn10 (tet^r)] (Strategene) which contains the glutathione S-transferase gene of *Schistosoma japonicum* [213] and the ampicillin resistance gene (*bla*) [211]. Expression is under the control of the *lacI*^{q} allele which is induced by the inclusion of IPTG or lactose. Production seeds were stored in SOB medium with 200g/L glycerol at -80°C in the presence of 0.12g/L ampicillin.

2.2.2 Media and Chemicals

Reagent and chemicals were purchased from Sigma (St Louis, MO, USA), BioRad (Hercules, CA, USA), Merck (Darmstadt, Germany) and Difco (Sparks, MD, USA). All fermentations unless otherwise stated used Terrific Broth which contained per litre 24g yeast extract (Merck), 12g soy-peptone (Merck), 4.8g potassium di-hydrogen orthophosphate, 2.2g di-potassium hydrogen orthophosphate and 5g glycerol. Fed-batch fermentations used a feeding solution of 315g/L of glycerol and 315g/L of yeast extract (Merck).

2.2.3 Inoculum Development

Frozen production seed (1mL) was thawed at room temperature and added to 50mL of Terrific Broth with 0.12g/L of ampicillin in a 250mL baffled shaker flask to ensure adaptive cell populations. The cultures were incubated for 16 hours on a rotary shaker at 180rpm and 37°C. For fermentation studies 1mL of production seed was added to a 200mL baffled shaker flask containing 150mL of Terrific Broth with 0.12g/L ampicillin which was incubated at 180rpm for 24 hours.

2.2.4 Medium Trials

Medium trials aimed to quantify recombinant protein production on a number of commonly used media found in publications. Duplicate culture of five different media; Luria Broth (LB), Terrific Broth (TB), Super Broth (SB), S.O.B, and M9 Minimal Medium were assessed for *Eg*95 production. All the medium compositions were made according to recipes outlined in Handbook of Microbiological Media [214]. 1mL of inoculum was added to 50mL of each of the five media in separate 250mL shaker flasks. The cultures were then incubated at 180rpm and 37°C. Samples were taken every hour to measure cell density and pH. After 4 hours cultures were induced with 0.1mM IPTG. Total protein, recombinant protein content and final cell density were measured 8 hours after inoculation.

2.2.5 Inducer Concentration Trial

Shaker flask trials were used to test the effect of various concentration of IPTG on the production of recombinant protein. 1mL of overnight seed was added to twelve 250mL shaker flasks containing 50mL of Terrific Broth and 0.12g/L ampicillin. Duplicate shaker cultures were induced four hours after inoculation with; 0.0001, 0.001, 0.01, 0.1, 0.5 and 1mM of IPTG. All cultures were incubated at 180rpm and 37°C. Total protein, recombinant protein content and final cell density were measured after 8 hours.

2.2.6 Induction Time Trial

Shaker flask trials were used to test the effect of induction time on the production of *Eg95*. 1mL of overnight seed was added to eight 250mL shaker flasks containing 50mL of Terrific Broth and 0.12g/L ampicillin. Duplicate cultures were induced at 0, 2, 4 and 6 hours after inoculation with 0.1mM of IPTG. All cultures were incubated at 180rpm and 37°C. Total protein, recombinant protein content and final cell density were measured after 8 hours.

2.2.7 Fermenter Trials

All fermenter trials where performed using a 3300mL glass jar fermenter (BioFlo 3000, New Brunswick Scientific, Edison, NJ, USA) as shown in Figure 8. The fermenter was fitted with pH and dissolved oxygen probes (Mettler Toledo, OH, USA). Temperature control was achieved through a

base mounted water filled jacket. Agitation was provided by two centrally mounted six bladed Ruston turbines and aeration through a sparging ring at the base of the fermenter. Dissolved oxygen (DO) was controlled at 30% of air saturation using a sequential cascade of agitation between 50-800rpm, aeration between 2-10 litres per minute with pure oxygen blended into the sparging gas at high-cell-densities. The pH was controlled at 7.0 using 10% (v/v) phosphoric acid and 10M sodium hydroxide. Antifoam 289 (Sigma) was added automatically to control the foaming according to a conductivity probe located 5cm above the culture. All fermentation cultures used a feed solution of 315g/L of glycerol and 315g/L yeast extract adjusted to pH 7.0 with 2M HCl. Three different feeding strategies were used: A DO-stat, pH stat and exponential feeding regime. The DO-stat and pH-stat were controlled by the automated program BioCommand (New Brunswick Scientific). Feed was supplied initially at a rate of 15mL/h, increasing by 0.15mL/h for every minute above their respective set point. For the exponential feeding regime the rate of feeding was calculated according to the equation outlined in Figure 7, with a desired specific growth rate of $0.15h^{-1}$.

$$F(t) = \frac{\mu_i V(t) X(t)}{(S_F - S(t)) Y_{x/s}} \exp(\mu t)$$

Figure 7: Equation for exponential feeding regime [93]. F(t) is the flow rate of feed at time t, μ is the desired specific growth rate (h^{-1}), V(t) the reactor volume at time t (L), S_F the substrate concentration in feed (g/L) and S(t) is the substrate concentration in culture at time t (g/L), X(t) cell concentration at time t (g cell dry weight/L), $Y_{x/s}$ cell titre on glucose (g dry cell weight/g), t is time (h).

All experiments unless otherwise stated had an initial medium volume of 1400mL and were controlled at 37°C. Fermentations were inoculated with 100mL of culture grown for 16 hours in a 2000mL shaker flask at 37°C and 180rpm to an optical density of approximately 1.5. Fermentations were induced with 0.1mM IPTG after 4 hours and maintained until two sequential

reductions in cell density were measured as indication the culture was going into stationary phase.

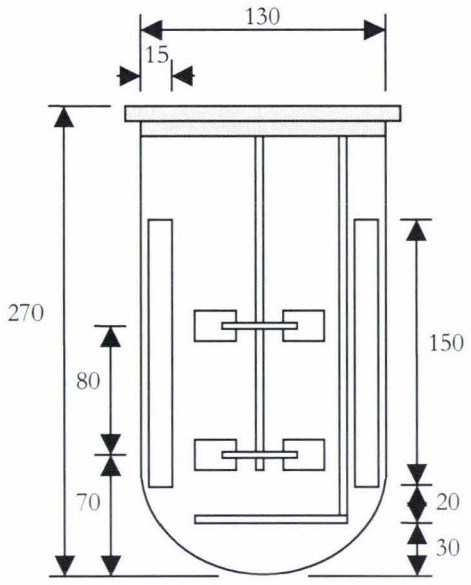

Figure 8: Vessel specification for the 3300mL BioFlo 3000 bioreactor (New Brunswick Scientific., Edison, NJ, USA). All dimensions are in mm.

2.2.8 Protein Extraction

Extraction of intracellular proteins from shaker flasks were performed in accordance with the B-PER™ reagent instructions for midi-scale bacteria protein extraction (Pierce Chemical Company, Rockford, IL, USA). 40mL of each sample was centrifuged at 3000g for 10 minutes. The supernatant was decanted off and the pellet suspended in 2.5mL B-PER by repeatedly drawing through a 1mL pipette. The sample was then incubated on a rotating wheel at

room temperature for 15 minutes, before centrifugation at 27,000g for 15 minutes. The supernatant was decanted and pelleted inclusion bodies resuspended in 2.5mL B-PER with 0.010mL of 10g/L lysozyme before incubated at room temperature on a rotating tray for 5 minutes. 15mL of 1:20 B-PER in distilled water was added and the samples centrifuged at 27,000g. The supernatant was decanted, pellet resuspended in 20mL of 1:20 B-PER and centrifuged at 27,000g. The pellet was washed twice more before the extracted inclusion bodies were resuspended in 2mL of solubilising buffer (484.8g/L urea, 11.25g/L glycine buffer, 0.37g/L of EDTA adjusted to pH 9.0 with 10M NaOH and 0.77g/L of DTT added just prior to use).

Fermenter culture broth was centrifuged at 3500g for 30 minutes. The supernatant discarded and pellet reconstituted in 1000mL of wash buffer (181.8g/L urea, 11.25g/L glycine 0.37g/L of EDTA and 0.77g/L DTT). Suspended cells were disrupted in a high pressure homogeniser (APV-1000 single stage, APV Co, London, UK) at 1000 bar. Disrupted cells were centrifuged three times at 10,000g for 30 minutes. Each time the supernatant was discarded and pellet suspended in 1000mL of washing buffer using an ultra-trurrex (Drive T25, IKA). The pelleted inclusion bodies were then resuspended in solublising buffer and placed on a shaking tray for 1 hour before centrifuging at 10,000g for 1 hour to remove any insoluble material. Solubilised material was finally filtered through a 0.22μm Durapore tangential flow unit (Millipore, Billerica, MA, USA).

2.2.9 Analytical Methods

Culture growth was monitored by measuring optical density at 595nm using a Genesis 2C spectrophotometer (Thermo Electron Corp, New York, NY, USA). The dry cell weight (DCW, g/L) was calculated from a calibration curve prepared using eighteen culture samples. 20mL of culture was centrifuged at 5000g for 10 minutes. The supernatant was decanted into a crucible and the wet cell pellet weighed. 20mL of culture was placed in separate crucibles and both were dried over 16 hours at 90°C. Cell dry weight was calculated according to the formula in Figure 10. The culture specific growth rate was calculated according to the equation in Figure 9 which has been described previously [215]. A line of best fit was determined using a linear regression line of best fit function in the program Excel (Microsoft Corporation).

$$\mu=(\log_e X_t-\log_e X_0)/t$$

Figure 9: Equation for the calculation of the specific growth rate (μ). X_t is the optical density at time t, X_0 is the optical density at time 0. t is the time period between 0 and t.

$$\text{DCW [g/L]} = \frac{\text{Dried sample}}{20} - \frac{\text{Dried supernatant}}{20}\left(\frac{100}{100 + \text{WCW}}\right)$$

Figure 10: Formula for the calculation of the dry cell weight. All measurements are in grams. Wet cell weight (WCW), dry cell weight (DCW).

Protein measurements were perforomed using the BIO-RAD protein assay which is based on the Bradford Method [199]. Protein standards were made using bovine albumin serum (Sigma) in distilled water. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed according to the method outlined previously [203]. Protein

separate was carried out on a 0.75mm thick 15% (w/v) acrylamide separating gel with 4% (w/v) slacking gel in a Mini-Protean II electrophoresis unit (Bio-Rad). Coomassie brilliant blue stain was used to visualise the protein and low molecular weight markers (Amersham Biosciences, Uppsala, Sweden) used as standards. The amount of Coomassie stained recombinant protein was evaluated densitometrically from SDS-PAGE gels using an Imaging Densitometer GS-800 (Bio-Rad) and Quantity One (BioRad) analytical imaging software. For an immunological confirmation of *Eg*95, proteins were then transferred electrophoretically to a mixed ester nitrocellulose membrane (Hybond-C; Amersham Biosciences, Uppsala, Sweden) in alkaline transfer buffer (2% (v/v) methanol, 3g/L Tris, 14g/L glycine) using the Mini trans-blot (BioRad). The filter was then incubated in blocking solution (3g/L Tris-HCl, pH 7.5, 29g/L NaOH, 0.1% (v/v) Tween-20, 50g/L non-fat milk powder) for 1 hour at 37°C. The filters were washed in TBS buffer (3g/L Tris-HCl, pH 7.5, 38g/L NaCl, 0.1% (v/v) Tween 20) and then incubated overnight at room temperature in sheep antibody raised to *Eg95* (provided by David Heath, AgResearch, Upper Hutt, New Zealand) diluted 1/200 in blocking solution. Unbound primary antibody was removed by washing in TBS buffer and the filter incubated for 6 hours at room temperature in donkey anti-sheep IgG (Cappel) conjugated to horseradish peroxidase (HRP) diluted 1/1000 in blocking solution. Unbound secondary antibody was removed by washing in TBS buffer. The Western blot was finally developed by incubating in developing solution (50g/L o-phenylenediamine in methanol).

2.3 RESULTS AND DISCUSSION

2.3.1 Media Trials

Shown in Table 1 are the production characteristics of the *Eg*95 clone grown on various *Escherichia coli* media. The purity of *Eg*95 in the inclusion body fraction of all the cultures varied little, with all the samples containing between 29% and 32% recombinant protein (data not shown). Super Broth (SB) achieved the highest final cell density however Terrific Broth (TB) produced 20% more inclusion body and 9% more volumetric *Eg*95 than all the other cultures. SOB and SB produced similar inclusion body and *Eg*95 titre but due to a much higher final cell density SB had a lower specific *Eg*95 titre. Luria Broth (LB) produced the lowest final cell density and recombinant protein titre out the four complex media tested with 50% less *Eg*95 produced than the culture grown on TB. The only chemically defined medium in the trial M9 Minimal Medium performed poorly, achieving a cell density of only 0.371g DCW/L after 10 hours and the subsequent *Eg95* production was not measurable as no inclusion body protein was recovered after extraction. Cultures grown in chemically defined media generally grow more slowly and produce lower levels of recombinant proteins as the cells are required to synthesise their own amino acids [3, 106, 216]. Many researchers will use defined media despite this as they commonly produce more consistent titre, allow better control and monitoring, can simplify downstream processing and make product registration easier [1, 70, 105, 217, 218]. Complex media are generally used during product development as trace metals and amino acids are provided as part of bulk components making them less problematic to optimise.

Table 1: Production characteristic of *E.coli* expressing *Eg*95 grown on various media. Media included; Terrific Broth (TB), Super Broth (SB), S.O.B, Luria Broth (LB) and M9 Minimal Medium (M9). 1mL of inoculum was added to 50mL of each of the five media in separate 250mL shaker flasks. The cultures were then incubated at 180rpm and 37°C. After 4 hours cultures were induced with 0.1mM IPTG. Production characteristics were measured 8h after inoculation.

Media	Production characteristics			
	Final cell density [DCW g/L]	Inclusion body protein [g/L]	Volumetric *Eg95* [g/L]	Specific *Eg*95 [g/g DCW]
TB	1.123	0.218	0.059	0.053
SB	1.184	0.170	0.053	0.045
S.O.B	1.078	0.164	0.054	0.050
LB	1.014	0.094	0.031	0.030
M9	0.371	0.007	#	#

No inclusion body pellet was recovered

Shown in Table 1 the cultures grown on Super Broth (SB) and Terrific Broth (TB) achieved the highest final cell densities. This is likely due to TB and SB both having high contents of yeast extract and containing the phosphate buffer salts potassium di-hydrogen orthophosphate and di-phosphate hydrogen orthophosphate. Yeast extract is often added to prevent trace substrate limitations and has been shown to ease cellular stress responses such as protease production during recombinant protein synthesis [45, 77]. Phosphate has been shown to be important for high-cell-densities cultures of *E.coli* as it can become a limiting substrate [98]. In addition to providing a source of phosphate, the phosphate salts in the medium provide a buffering

capacity to prevent pH fluctuations which may disrupt normal metabolic activity. Shown in Figure 11 is the pH profile of the cultures grown on various media. The pH of unbuffered media such as SOB fluctuated between pH 6.4 and 7.6 during a standard culture which extends out of the optimal pH range recommended for *E.coli* growth [138]. The four complex media SB, LB, SOB and TB demonstrated a characteristic fall then rise in pH (Figure 11) suggested previously to be caused by the utilisation of nitrogen by cells [219]. During the first 4-6 hours the pH decreases as cells consume the free ammonium in the culture media. Once most of the free ammonium in solution is consumed further nitrogen requirements for growth are meet by the decomposition of proteins in the media as indicated by the increase in pH.

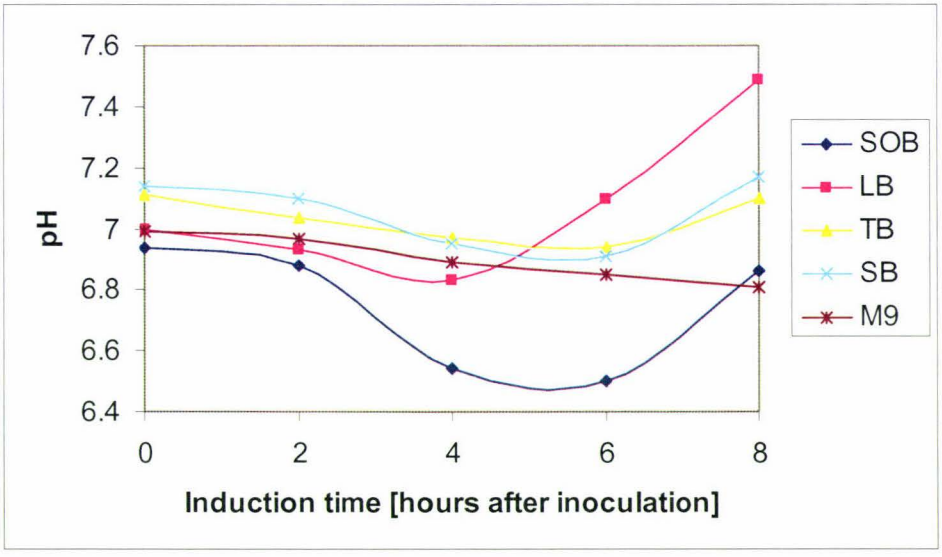

Figure 11: Broth pH variation in shake flask cultures of various media: Terrific Broth (TB), Super Broth (SB), S.O.B, Luria Broth (LB) and M9 Minimal Medium (M9). 1mL of inoculum was added to 50mL of each of the five media in separate 250mL shaker flasks. The cultures were then incubated at 180rpm and 37°C. After 4 hours cultures were induced with 0.1mM IPTG with the pH measured every two hours thereafter.

Shown in Figure 12 the production of *Eg*95 increased with increasing final cell density up to 1.12g DCW/L which was achieved using TB. SB achieved a higher cell density than Terrific Broth (1.18g DCW/L) however had a lower than expected *Eg*95 titre. This reduction in titre is likely a result of competing cellular requirements for growth and expression. At induction SB also had the highest cell density (data not shown). This in turn may have reduced the available energy for recombinant protein synthesis. Surprisingly LB which is one of the most commonly used media for *E.coli* expression [2, 3, 12, 48, 71, 73, 111, 112] produced the lowest final cell density and recombination protein titre. This result is consistent with previous medium studies which also observed that LB produces lower growth and recombinant protein expression than more complex media such as TB and SB [3, 112]. This result has been suggested to be due to the presence of lower amounts of readily accessible carbon and nitrogen in LB [3]. Terrific Broth produced the highest titre of *Eg95* with 0.059g/L of broth and subsequently was selected for fermentation trials.

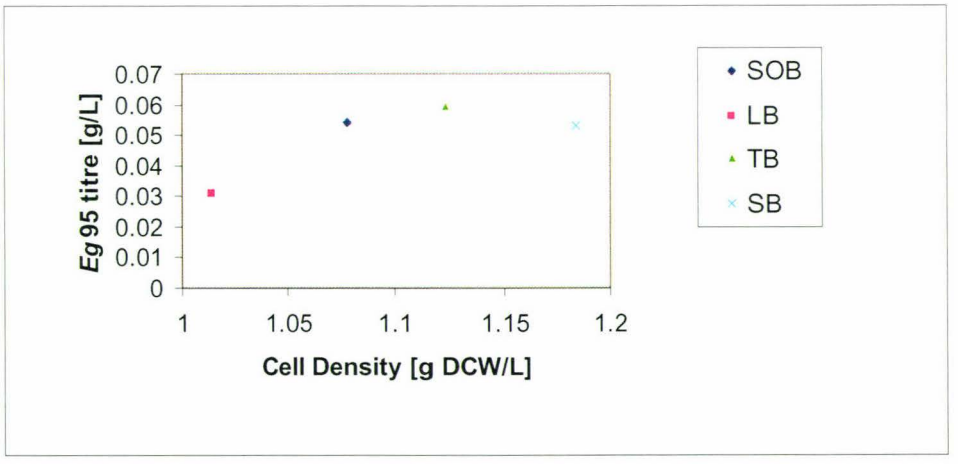

Figure 12: Comparison of the final cell density and volumetric titre of *Eg*95. Media used included: Terrific Broth (TB), Super Broth (SB), S.O.B, Luria Broth (LB) and M9 Minimal Medium (M9). 1mL of inoculum was added to 50mL of each of the five media in separate 250mL shaker flasks. The cultures were then incubated at 180rpm and 37°C. After 4 hours cultures were induced with 0.1mM IPTG. Total protein, recombinant protein content and final cell density were measured after 8 hours.

2.3.2 Inducer Concentration Trial

Due to high cost of isopropyl β-D-thiogalactopyranoside (IPTG) the addition of excess to ensure complete depression of the *lac* promoter is undesirable. The minimum concentration of IPTG required to fully induce the *lac* promoter was therefore tested in shaker flasks. Shown in Figure 13, recombinant protein expression increased with increasing IPTG with the specific titre of *Eg*95 doubling between 0.001 to 0.1mM. Above 0.1mM the *lac* promoter appears to be fully induced with the specific titre of *Eg*95 remaining constant. A range of concentrations of IPTG between 0.1mM and 2mM have been used previously in studies to induce the *lac* promoter [12, 13, 39, 71, 74, 111, 112]. The maximum volumetric titre of 0.048g/L of *Eg*95 was produced using 0.1mM of IPTG. Comparison of this result is difficult as the concentration of IPTG used to induce cultures and the corresponding titre of recombinant protein varies widely between clones. This is well demonstrated by Madurawe et al. [112] who produced 0.010g/L of decorin-binding lipoprotein in *E.coli* using 0.1mM of IPTG, however Robbens et al. [2] used 2mM IPTG to produce 0.016g/L of recombinant murine interleukin-2 in *E.coli*. Interestingly the volumetric titre of *Eg*95 decreased at IPTG concentrations above 0.1mM. This was likely due to a decrease in the final cell density of cultures as a result of growth inhibition at the high IPTG concentration (data not shown). Yee and Blanch [74] found that IPTG at concentration above 1mM can cause a reduction in the growth rate of *E.coli* cells by up to 20% due the toxic effect of recombinant protein expression.

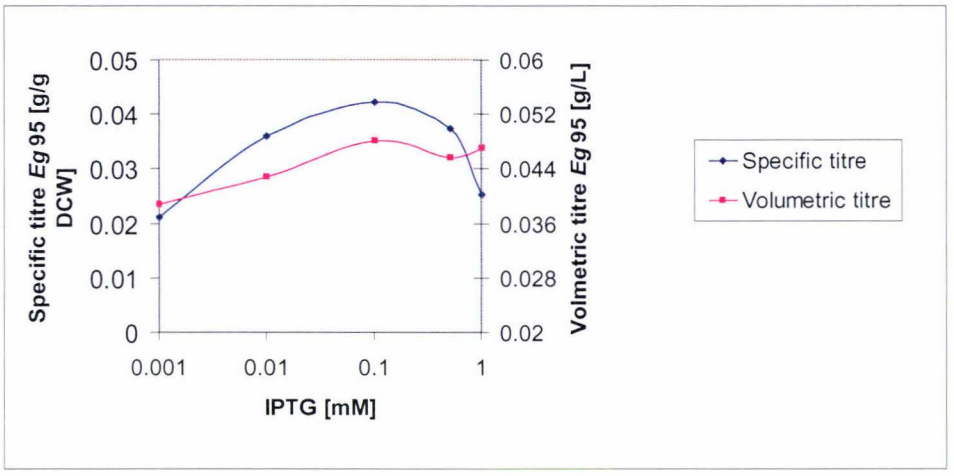

Figure 13: Effect of IPTG concentration on the specific and volumetric titres of *Eg*95. 1mL of overnight seed was added to twelve 250mL shaker flasks containing 50mL of Terrific Broth. Duplicate shaker cultures were induced four hours after inoculation with; 0.0001, 0.001, 0.01, 0.1, 0.5 and 1mM of IPTG. All cultures were incubated at 180rpm and 37°C. Total protein, recombinant protein content and final cell density were measured after 8 hours.

The *lac* promoter has been previously reported to be a weak promoter with moderate levels of basal expression under non-induced conditions [13, 111]. Basal expression of *Eg*95 here was found to 40% of the maximum expression obtained under purposeful induction (data not shown). Basal expression is generally undesirable as it reduces the energy available for growth and may lead to growth inhibition if the recombinant protein is toxic to the cells. Basal expression can be reduced by replacing the glycerol in the media with glucose [13, 22, 220], however emphasis was placed in this research on reducing any repression of the *lac* operator.

2.3.3 *Induction Time*

Shaker flask trials were used to test the effect of induction time on the production of *Eg95*. Table 2 shows that induction at seeding severely limited culture growth and subsequent recombinant protein production, likely as a result of metabolic stress. The expression of foreign genes in *E.coli* places considerable pressure on the cells metabolic systems and limits the available energy for growth [161]. Shown in Figure 14, the specific titre of *Eg*95 increased as the pre-induced period of growth was increased for up to four hours. The volumetric titre of *Eg*95 was also greatest when induced four hours after inoculation at which time the culture was at its highest specific growth rate (Table 2). This result is consistent with Lim and Jung [70] who found that recombinant protein production in *E.coli* fermentations was proportional to the specific growth rate at induction as cell metabolic capacity is at its greatest.

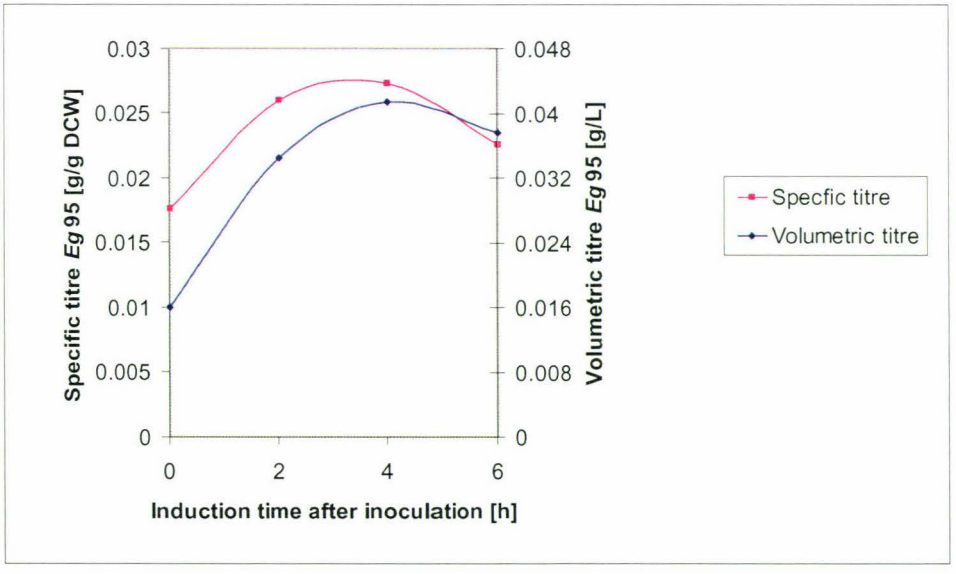

Figure 14: Effect of induction time on the volumetric and specific titres of *Eg*95. 1mL of overnight seed was added to eight 250mL shaker flasks containing 50mL of Terrific Broth. Duplicates cultures were induced at 0, 2, 4 and 6 hours after inoculation with 0.1mM of IPTG. All cultures were incubated at 180rpm and 37°C. Total protein, recombinant protein content and final cell density were measured after 8 hours.

Shown in Table 2 induction late in the exponential growth phase allowed greater levels of cells to accumulate however the specific titre of *Eg*95 decreased. Several studies report that induction late in exponential growth leads to higher volumetric recombinant protein production by increasing the final cell density [3, 25, 74]. Yee and Blanch [74] found that induction late in exponential growth led to 50% more recombinant trypsin being produced however the specific cellular titre decreased. Inducing at higher cell densities causes greater culture stress which reduces individual cells metabolic capacity and subsequent specific protein production [25]. Cellular responses to induction are dependant on a number of interacting factors including the host/ vector system and properties of the expressed protein. The timing of induction of new recombinants therefore needs to be empirically determined [217].

Table 2: Effect of induction time on the final cell density, specific growth rate of cells, specific and volumetric titres of *Eg*95. 1mL of overnight seed was added to eight 250mL shaker flasks containing 50mL of Terrific Broth. Duplicates cultures were induced at 0, 2, 4 and 6 hours after inoculation with 0.1mM of IPTG. All cultures were incubated at 180rpm and 37°C. Total protein, recombinant protein content and final cell density were measured after 8 hours.

Induction time [hours after inoculation]	Properties			
	Final cell density [g DCW/L]	Specific growth rate at induction [hr^{-1}]	Specific titre *Eg*95 [g/g DCW]	Volumetric titre *Eg95* [g/L]
0	0.908	0.08#	0.018	0.015
2	1.326	0.55	0.026	0.034
4	1.516	0.93	0.027	0.041
6	1.662	0.27	0.023	0.038

growth rate measured over the hour after inoculation

2.3.4 DO concentration

Previously, *E.coli* fermentations have been controlled at DO concentrations between 20-70% of air saturation [44, 46, 74, 93, 97-99, 217, 221]. The effect of dissolved oxygen concentration on *Eg*95 production was tested in three DO-stat fermentations with relative set-points of 30, 50 and 70% of saturation with air. As shown in Figure 15, the final cell density of the *E.coli* cultures was inversely related to the DO concentration. At a DO set-point of

30% the culture grew for 7 hours longer before going into stationary growth and produced two-thirds more biomass than when the dissolved oxygen concentration was controlled at 70%. No previous references to increased biomass at lower DO could be found, however the reduction in cell density is likely a result of cellular damage. Elevated DO can cause oxidative stress to cells, leading to the damage of nucleotides and oxidation of proteins [222].

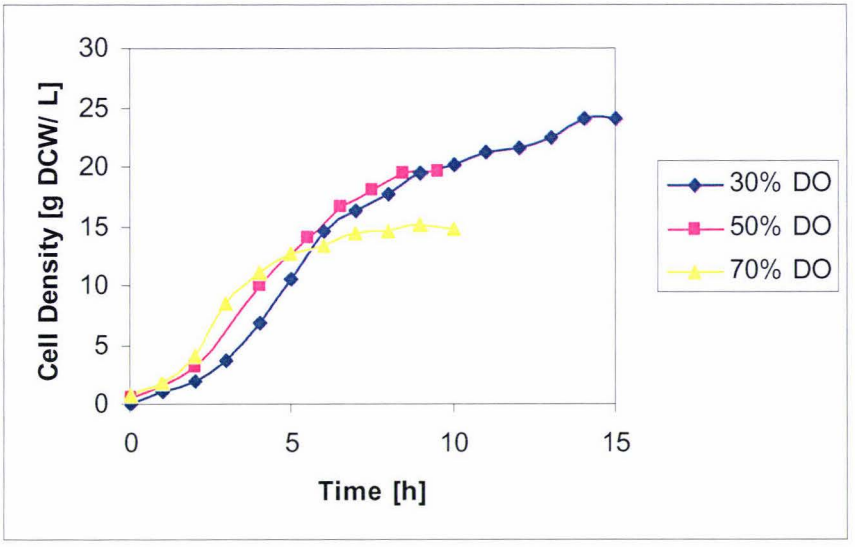

Figure 15: Effect of dissolved oxygen concentration on biomass growth in fed-batch fermentations. Fermentations were inoculated with 100mL of seed OD~1.5 in 1.4L of Terrific Broth. The dissolved oxygen (DO) was controlled at 30%, pH at 7.0 and temperature at 37°C. All fermentation cultures used a feed solution of 315g/L of glycerol and 315g/L of yeast extract. Feeding was controlled by the automated program BioCommand (New Brunswick Scientific). Feed was supplied initially at a rate of 15mL/h, increasing by 0.15mL/h for every minute above their respective set point. Fermentations were induced with 0.1mM IPTG after 4 hours and maintained until two sequential reductions in cell density were measured as indication the culture was going into stationary phase.

Table 3 shows that the specific titre of *Eg95* per cell dry weight increased three-fold when the DO set point was reduced from 70 and 50% but decreased slightly when reduced further to 30%. Overall, the volumetric titre of *Eg*95 increased four and half fold when the DO concentration was reduced from 70% and 30%. The increase in volumetric titre is likely due to a combination of increased specific titre and higher final yield of biomass at the lower DO set-point. Several studies have also found that recombinant proteins expressed in *E.coli* are lower at higher DO [144, 223, 224]. Doig et al. [223] reported that high dissolved oxygen concentrations were deleterious to the production of cyclohexanone monooxygenase (CHMO) due to shear damage. Maintaining a high dissolved oxygen level in high-cell-density cultures requires high rates of agitation and aeration. This can damage cells

and sensitive proteins, and also increases fixed capital and process costs as larger motors and compressors are required.

Table 3: Effect of dissolved oxygen concentration on biomass and protein production of *Eg95* in fermentation cultures. Fermentations were inoculated with 100mL of seed OD~1.5 in 1.4L of Terrific Broth. The dissolved oxygen (DO) was controlled at 30%, pH at 7.0 and temperature at 37°C. All fermentation cultures used a feed solution of 315g/L of glycerol and 315g/L of yeast extract. Feeding was controlled by the automated program BioCommand (New Brunswick Scientific). Feed was supplied initially at a rate of 15mL/h, increasing by 0.15mL/h for every minute above their respective set point. Fermentations were induced with 0.1mM IPTG after 4 hours and maintained until two sequential reductions in cell density were measured as indication the culture was going into stationary phase.

Dissolved oxygen concentration	Fermentation characteristics			
	Final cell density [g DCW/L]	Inclusion body total protein [g/L]	Volumetric titre *Eg95* [g/L]	Specific titre *Eg95* [g/ g DCW]
30%	24.0	3.07	0.72	0.030
50%	19.6	2.70	0.67	0.034
70%	14.8	0.95	0.16	0.011

2.3.5 Feeding Strategy

The use of fed-batch cultures has been shown to increase the cell-density and specific protein production by overcoming inhibitory substrate concentrations encountered in batch culture [46, 94, 122, 127, 220, 225]. In this study three different feeding strategies were investigated: A DO-stat, pH-stat and exponential feeding regime. All the cultures actively grew for approximately

16 hours. However, using the exponential feeding regime the culture achieved a final cell density of 46.3g DCW/L (Table 4). The DO-stat and pH-stat both produced similar final cell densities but only half the biomass obtained using the exponential feeding regime. Shown in Table 4 all three feed regimes had similar specific titres of *Eg*95 between 0.030 and 0.037g/g DCW. This led to a close correlation between the volumetric titres with the final cell densities with the exponentially fed culture producing 130% more *Eg*95 than both the DO-stat and pH-stat. This result is consistent with Yee et al. [74] who reviewed a number of feeding regimes and found that exponentially fed cultures achieved superior titres to online feeding systems such as DO-stat and pH-stats. Online feeding systems are widely used in process development as they are adaptive to changes in culture growth rate; requiring less process experience and control. However online systems place large amounts of stress on cultures due to fluctuations in the culture nutrient levels [112]. Not surprisingly the pH and DO-stat produced similar final cell densities and titres of *Eg*95, as both methods rely on similar environmental indicators of carbon depletion to maintain the culture on the brink of starvation.

Table 4: Relative biomass, total protein and specific *Eg*95 production using various feeding regimes. Fermentations were inoculated with 100mL of seed, OD~1.5 in 1.4L of Terrific Broth. Dissolved oxygen (DO) was controlled at 30%, pH at 6.8 and temperature at 37°C. All fermentation cultures used a feeding solution of 315g/L of glycerol and 315g/L yeast extract. The DO-stat and pH-stat were controlled by the automated program BioCommand (New Brunswick Scientific). Feed was supplied initially at a rate of 15mL/h, increasing by 0.15mL/h for every minute above their respective set point. For the exponential feeding regime the rate of feeding was calculated according to the equation described previously [93], with a desired specific growth rate of $0.15h^{-1}$. Fermentations were induced with 0.1mM IPTG after 4 hours and maintained until two sequential reductions in cell density were measured as indication the culture was going into stationary phase.

Feeding strategy	Production characteristics		
	Final cell density [g DCW/L]	Volumetric titre *Eg95* [g/L]	Specific titre *Eg*95 [g/g DCW]
DO-stat	24.0	0.72	0.030
pH-stat	22.9	0.74	0.032
Exponential feeding	46.3	1.73	0.037

A maximum titre of 1.73g/L of *Eg*95 was produced in a fed-batch fermentation controlled by an exponential feeding regime, maintained at 37°C, pH 7.0 and DO 30% of air saturation. This final titre of solubilised *Eg*95 represents a 363% increase over the previous highest titre achieved at Agresearch prior to this research (Table 5). Consistency was confirmed with two fermentations performed under these new conditions with the final *Eg*95 titre within 0.01g/L (data not shown).

Table 5: Comparison of the previous best production characteristics prior to this study with the production characteristic achieved in this study.

Criteria	Previous best[a]	Best in this study
Final OD	30.3	48.3
Washed weight of inclusion bodies [g/L]	15.03	39.0
Total inclusion body protein [g/L]	0.750	2.78
Eg95 [g/L]	0.215[#]	0.995[b]

[a] Data from previous fermentations provided by David Heath (Agresearch, Upper Hutt, New Zealand)

[b] Soluble *Eg*95 titre after filtration through a 0.22μm filter.

2.4 CONCLUSION

The objective of this chapter was to investigate methods to increase the titre of *Eg*95, develop parameters for the production in a small scale bioreactor and provide a basis of fermentation knowledge for the development of future recombinant protein products produced in *E.coli* as inclusion bodies. The production of *Eg*95 was influenced by the medium composition, feeding strategy, induction timing and dissolved oxygen concentration. *E.coli* grown on complex media rich in yeast extract and containing the phosphate buffer system was found to support higher growth and produce more recombinant protein than other media. 0.1mM was found to be sufficient to fully induce the *lac* promoter. Induction four hours after inoculation when cell were at their maximum growth rate produced the highest volumetric titre of *Eg*95. Decreasing the DO concentration from 70 to 30% lead to a 60% increase the final biomass and a four fold increase in volumetric *Eg*95 titre. Fed-batch as apposed to tradition batch fermentation was found to significantly increase in cell-density and specific protein production. An exponential feeding regime produced higher volumetric titres than online feeding regimes such as DO-stat and pH-stat. A maximum titre of 1.73g/L of *Eg*95 was produced in an exponentially fed fermentation induced with 0.1mM of IPTG and controlled at 37°C, pH 7.0 and 30% DO. This study led to a 360% increase in volumetric titres of *Eg*95 compared to that achieved previous to this research.

3 Soluble Protein Production in E.coli

3.1 INTRODUCTION

Many eukaryote proteins expressed in *Escherichia coli* commonly aggregate to form inclusion bodies made up of inactive protein [80]. Solubilisation and renaturation of native protein from inclusion bodies is costly, time consuming and for some proteins their activity may be unable to be recovered [20, 140, 152]. For this reason many studies have looked at how to express soluble protein from *E.coli* [1-3, 8, 10, 12-15, 68, 81, 126]. Culture growth rate has been extensively demonstrated to be important in determining the solubility of the expressed proteins [12-15, 29, 81, 93]. At high growth rates protein expression can overwhelm chaperones and foldases required for correct folding leading to the accumulation of protein intermediates [10, 66, 80, 81]. Many studies have lowered the culture temperature of recombinant fermentations to restrict the expression rate [9, 12, 13, 48, 112]. Other methods of reducing inclusion body formation include using early induction [9, 65, 70], nutrient and oxygen restriction to restrict the growth rate [109, 126, 143, 226], and by directly reducing the expression rate by not fully inducing the recombinant proteins promoter [45, 81].

Trichostrongylus colubriformis is a parasitic nematode that infects the small intestine of sheep and has a significant economic impact on farming in New Zealand. Currently the only effective means of controlling *T.colubriformis* is through the regular use of anthelmintics. Sheep can develop immunity to *T.colubriformis* as a result of repeated natural infection [208]. Elevated immunoglobulin E (IgE) levels in sheep have been associated with protective

immunity to nematode infection [227]. *Aspin,* the protein studied in this chapter is an aspartyl protease inhibitor homologue produced by the nematode *Trichostrongylus colubriformis.* IgE recognising *Aspin* is more prominent in lambs that develop an effective immunity to gastrointestinal parasites [208]. It is hoped that *Aspin* can be used as a simple diagnostic test to identify animals whose immune system resists and rejects parasites. Previous attempts to express *Aspin* in a fermenter has resulted in the protein almost exclusively being produced as inclusion bodies (Robert Dempster, personal correspondence, Agresearch, Upper Hutt, NZ 2003). Although a procedure for the solubilisation of *Aspin* has been developed, the activity of the recovered protein is lower than soluble expressed protein. The objective of this section is to investigation a range of methods to increase the titre of soluble *Aspin*, develop parameters for production of *Aspin* in a small bioreactor and provide a basis of fermentation knowledge for the development of other soluble protein products in *E.coli.*

3.2 MATERIALS AND METHODS

3.2.1 Strain

The clone for this study which expressed the recombinant protein *Aspin* was kindly supplied by David Maas (Agresearch, Upper Hutt, New Zealand). *Aspin* was expressed in Rosetta-gami™ B(DE3) (Novagen, Germany). Genotype F⁻ *ompT* *hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm lac*Y1 *aphC* *gor*522::Tn*10*(Tc^R) *trx*B::kan (DE3) pRARE (Cm^R). The Rosetta-gami™ strain of *Escherichia coli* has a special mutation that promotes the formation of disulphide bonds [228]. All seed cultures were grown in the presence of 0.034g/L chloramphenicol, 0.015g/L kanamycin and 0.125g/L tetracycline. Production seeds were stored in Luria Broth with 200g/L glycerol at -80°C in the presence of all three antibiotics. The DNA encoding for *Aspin* was cloned into a Y2-4 expression vector under the control of the *araBAD* operon which is repressed in the absence of L-arabinose [22]. *Aspin* was fused to an E-tag and hexahistidine sequence to aid purification and identification. Shown in Figure 16 is recombinant *Aspin* affinity chromatography purified showing the three distinct proteins at 6, 22 and 28kDa (personal correspondence, Richard Shaw, AgResearch, Upper Hutt, New Zealand).

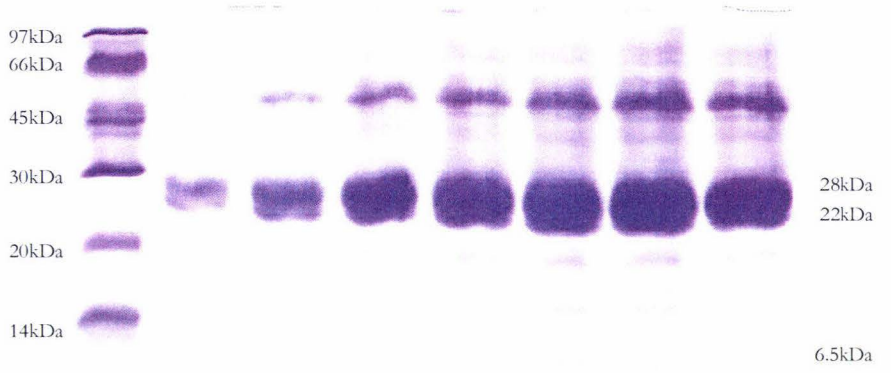

Figure 16: 15% SDS-PAGE gel of Talon column elusions of varying concentrations of *Aspin*. *Aspin* fragments are found between 20-30kDa and at 6.5kDa (personal correspondence, Richard Shaw, AgResearch, Upper Hutt, New Zealand). Lane 1 shows the low molecular weight marker.

3.2.2 Media and Materials

Reagent and chemicals were purchased from Sigma (St Louis, MO, USA), BioRad (Hercules, CA, USA), Merck (Darmstadt, Germany) and Difco (Sparks, MD, USA). All shaker flask and fermenter trials unless otherwise stated were grown in Terrific Broth which contained per litre 24g yeast extract (Merck), 12g soy-peptone (Merck), 4.8g potassium di-hydrogen orthophosphate, 2.2g di-potassium hydrogen orthophosphate and 5g glycerol. Fed-batch fermentation used a feeding solution of 315g/L of glycerol and 315g/L of yeast extract. Defined *E.coli* medium which was used in Fermentation 4 has been described previously [98].

3.2.3 Environmental Screening Trial

A Plackett Burman trial was used to test seven environmental variables for their effect on soluble *Aspin* production. Plackett and Burman is a partial factorial experimental design which is commonly used to identify the most important variable when large numbers of experiments are prohibitive [229]. Designs require one more experiment than the number of tested variables and the results of trials can be assessed in a simple spreadsheet using an F-test to determine the significance of each factor. However, no information on possible interaction between factors can be extrapolated. Seven environmental factors including; culture temperature, pH, yeast extract and salt concentration, induction time and L-arabinose concentration were tested. 1mL of inoculum culture was added to sixteen 250mL shaker flasks with 50mL of Terrific Broth and 0.015g/L of kanamycin. Cost prevented the addition of all three antibiotics to all cultures, therefore only kanamycin was added to provide some selective pressure. All cultures were grown in duplicate for 16 hours in shaking incubators at 180rpm under the conditions outlined in Table 6. After 16 hours cell density, total protein, recombinant protein content and the specific immunological activity were measured.

3.2.4 Shaker Flask Trials

The effect of varying the L-arabinose concentration and post-induction temperature was tested in shaker flask trials. 1mL of overnight inoculum culture was added to each 250mL shaker flask with 25mL of Terrific Broth and 0.015g/L of kanamycin. All cultures were grown at 37°C and 180rpm in a shaking incubator for 8 hours. For the post-induction temperature trial the flask temperatures were then adjusted in duplicate to 10, 16, 19, 23, 28, 37°C, before 2g/L of L-arabinose was added to each flask. For the inducer

concentration trial all cultures were reduced to 30°C post-induction and induced in duplicate with 0.05, 0.1, 0.5, 1, 2.5, 5, 7.5, 10g/L of L-arabinose. Total protein, recombinant protein content and final cell density were measured after 12 hours.

3.2.5 Protein Extraction

20mL of broth was centrifuged at 3000g for 15 minutes. The pellet was reconstituted in 1.5mL of extraction buffer (10g/L sodium phosphate, 22g/L sodium chloride adjusted to pH 8.0 with 5M NaOH) in eppendorf tubes. Samples were suspended in an ice bath and sonicated at 50% power in 1-minute bursts for 5-minutes using an ultrasound horn (Son-IM XL; Misonix Inc, Farmingdale, NY, USA). Cell debris and inclusion bodies were pelleted by centrifuging at 13,000rpm on a bench top centrifuge for 5 minutes. 1mL of supernatant was added to 0.050mL of TALON® metal affinity resin (Clontech Laboratories Ltd, Palo Alto, USA) and agitated for ≥1 hour. Unbound material was removed by centrifuging at 13,000rpm for 1 minute. The resin was washed three times in extraction buffer and the bound material eluted using 0.100mL of 37.2g/L Na_2EDTA. The pelleted inclusion bodies were solubilised in 1mL of solubilising buffer (described in Chapter 1) for 1 hour. The cell debris was then removed from the solubilised inclusion bodies by centrifuging the solution at 13,000rpm for 30 minutes. Protein concentration and content was measured using the BCA method and SDS-PAGE as outline in Chapter 1.

3.2.6 Analysis

The culture cell density and specific growth rate were calculated according to the methods outlined in Chapter 1. An ELISA was used to measure the immunological activity of samples. All samples were diluted to 0.001g/L total protein with PBS (8g NaCl, 0.2g $KH_2PO_4.12H_20$, 0.2g KCl per litre) and coated onto a 96 well ELISA plate. After incubation for 2 hours at 37°C unbound material was poured off and the plate washed 3 times with washing buffer (0.5g/L Tween 20 in PBS). Binding sites were then blocked for 5 minutes with blotto (50g/L non-fat milk powder in PBS) to prevent non-specific binding and rinsed 6 times in washing buffer. Purified IgE specific to *Aspin* was then added to the wells and incubation overnight. After washing, the wells were probed with monoclonal xB6/xD3 (provided by Richard Shaw, AgResearch Ltd, Upper Hutt, New Zealand), then a goat anti-mouse horse radish peroxidase (HPR) conjugate (Gibco; Invitrogen Corporation, Carlsbad, CA, USA). The colour was developed using 3,3',5,5'-tetramethyl-benzidine (TMB) substrate with the absorbance measured at 630nm and 450nm with a microtiter plate reader (MR5000; Dynatech Laboratories Inc, Chantilly, VA, USA).

3.2.7 Fermenter Setup

All fermenter trials where performed using a 3300mL glass jar fermenter (BioFlo 3000, New Brunswick Scientific., Edison, NJ, USA) as described in Chapter 2. The fermenter was fitted with pH and dissolved oxygen probes (Mettler Toledo, OH, USA). Temperature control was achieved through a base mounted water filled jacket. Agitation was provided by two centrally mounted six bladed Ruston turbines and aeration through a sparging ring at the base of the fermenter. Unless otherwise stated the dissolved oxygen

(DO) concentration was controlled at 30% (v/v) of air saturation by using a sequential cascade of agitation between 50-800rpm, aeration between 2-10 litres per minute (L/min) with pure oxygen blended into the sparging gas at high-cell-densities. The pH was controlled at 6.8 using 10% (v/v) phosphoric acid and 5M sodium hydroxide. Antifoam 289 (Sigma Chemical Co, MO, USA) was added automatically to control the foaming according to a conductivity probe located 5cm above the culture. All experiments unless otherwise stated had an initial medium volume of 1400mL and were controlled at 37°C. Fermentations were initiated with 100mL of culture grown for 16 hours in a 2000mL shaker flask at 37°C and 180rpm to an optical density of approximately 1.5.

3.3 RESULTS AND DISCUSSION

3.3.1 Factor Screening Trials

A Plackett Burman trial was used to test seven environmental variables for their effect on soluble *Aspin* production. Table 7 shows the F-test significance of each variable on the various production characteristics. Reducing the culture temperature from 37°C to 30°C had the largest effect on the specific production of soluble *Aspin* and the specific immunological activity of the recombinant protein. It has been widely reported that reducing the post-induction temperature increases the expression of active soluble protein [1, 8, 12, 18, 21, 81, 126]. At lower growth rates more chaperones and cofactors are available to prevent the accumulation of intermediates which have been suggested to cause inclusion bodies [16]. Due to the high significance, the post-induction temperature was chosen for further investigation

The concentration of yeast extract in the growth media had a strong negative effect on the soluble *Aspin* titre and the specific activity of the recombinant protein (Table 7). Yeast extract provides a readily accessible source of amino acids and trace metals [105, 106]. Cultures grown in yeast extract rich media such as TB have been found to have higher growth rates [3]. For recombinant proteins which have a tendency to form inclusion bodies, an increased growth rate often causes increased protein aggregation. Some papers have reported that media high in yeast extract are better for the expression of soluble protein in high-cell-density cultures, however these

reports consider the volumetric titre and increases can be attributed to increases in biomass [1, 3]. A small increases in final biomass was observed here at the higher yeast extract concentration (data not shown), though differences are likely to be larger in a fermenter as they are usually nutrient limited as opposed to oxygen limitation as found in shaker flasks [135].

Table 6: Plackett and Burman trial assessing the significance of various environmental factors on the volumetric titre of *Aspin*.

Experiment	Variables[a]							
	Temperature	Inducer Concentration	Induction Time	Yeast Concentration	Salt Concentration	pH	Dummy	*Aspin* titre [g/L]
1	L	L	L	H	H	L	L	0.3490
2	H	L	L	L	L	L	H	0.2439
3	L	H	L	L	H	H	H	0.7382
4	H	H	L	H	L	H	L	0.3715
5	L	L	H	H	L	H	H	0.4692
6	H	L	H	L	H	H	L	0.4344
7	L	H	H	L	L	L	L	0.6909
8	H	H	H	H	H	L	H	0.2595
Σ(H)	1.3094	2.060	1.854	1.449	1.781	2.013	1.711	
Σ(L)	2.2473	1.497	1.703	2.107	1.776	1.543	1.846	
Difference	-0.9379	0.5635	0.1514	-0.6582	0.0056	0.4701	-0.1350	
Effect	-0.2345	0.1409	0.0378	-0.1645	0.0014	0.1175	-0.0338	
Mean square	0.1100	0.0397	0.0029	0.0541	0.0000	0.0276	0.0023	
F-test	48.2394	17.4145	1.2567	23.7539	0.0017	12.1186		
Significant	+	+	-	+	-	+		

[a] The low and high levels are respectively; temperature (30, 37°C), inducer concentration (0.2, 2g/L), induction time (4, 8h), yeast concentration (5, 24g/L), phosphate salt concentration (0, 60mM), pH (6.8, 7.2). A dummy variable was used to assess the degree of variability. A 95% confidence interval was used for the test of statistical significance.

The concentration of inducer (L-arabinose) had a strong effect on both the amount of soluble *Aspin* produced and on the specific activity of expressed *Aspin* (Table 7). Increasing the L-arabinose concentration from 0.2g/L to 2g/L led to an increase in the titre of soluble *Aspin* and in the titre of total inclusion body protein. There is uncertainty surrounding the kinetics of induction of the *ara*BAD promoter. Previously the *ara*BAD promoter has been shown to be able to be modulated by the concentration of L-arabinose [22]. For proteins prone to aggregate, partial induction of the *ara*BAD promoter has been used to circumvent inclusion body formation [78, 81]. However Khlebnikov et al. [230] and Morgen-Kiss et al. [231] report that the concentration of arabinose effects the percentage of cells that are fully induced (vs. uninduced) rather than the level of expression in individual cells. The concentration of inducer was chosen for further investigation to determine the optimal concentration for soluble *Aspin* production.

The timing of induction had no statistically significant effect on either the titre of *Aspin* or specific activity (Table 7). Early induction has been shown to increase soluble protein production of some recombinant proteins by limiting culture growth rate [9, 65, 70]. Lim and Jung [70] found that induction in early logarithmic phase gave a five-fold improvement in soluble interferon-α production compared to when the culture was induced in late log phase. Other studies have found that inducing cells late in exponential growth phase led to the greater volumetric titre as cell densities are increased [65-67]. Early induction was observed to have little overall effect on the growth rate of cells and all the *Aspin* cultures achieved similar final cell densities (data not shown).

Medium supplementation with inorganic phosphate has been previously reported to increase cell growth and recombinant protein production [98, 109]. Ryan et al. [109] tested various levels of phosphate and found that both cell growth rate and β-lactamase production was increased when culture phosphate levels were supplemented to 128mM of phosphate. Shown in Table 7, the concentration of phosphate salt had no effect on the production or activity of *Aspin*. Terrific Broth is rich in peptone and yeast extract contain approximately 10% phosphate. For the low cell densities achieved in shaker flasks sufficient phosphate is likely to be available in the culture medium. However for high-cell-density fermentation phosphate supplementation may be still required.

The solubility of recombinant proteins expressed in *E.coli* have been reported to be effected by pH [140]. Reducing the pH from 7.2 to 6.8 led to an increase in both the volumetric and specific titres of soluble *Aspin* (Table 7). This is at odds with Strandberg and Enfors [140] who found that the amount of recombinant β-galactosidase expressed as inclusion bodies increased with decreasing pH. Culture pH effects protein folding and final conformation as different amino acid residues are exposed under various oxidative conditions. Expression of recombinant proteins have optimal pH ranges, above or below these ranges reduced titres are obtained [109]. As the optimal pH conditions are dependant on complex interaction between amino acid sequences, comparisons between clones expressing different proteins are not generally possible and empirical means must be used to determine the optimal pH of new proteins.

Table 7: F-test of the significance of various environmental factors on the final cell density, specific titre and immunological activity of *Aspin* produced.

Response	Significance environmental conditions[a]					
	Temperature	Inducer concentration	Induction time	Yeast Concentration	Salt Concentration	pH
Aspin [g/L]	0.048 (-)	0.017 (+)	0.001 (+)	0.023 (-)	0.000 (+)	0.012 (-)
Aspin [g/g DCW]	0.013 (-)	0.047 (+)	0.002 (-)	0.065 (-)	0.000 (+)	0.032 (-)
Activity [U/g *Aspin*]	0.022 (-)	0.011 (+)	0.001 (+)	0.012 (-)	0.001(-)	0.040 (-)

[a] Values greater than 0.048 indicate statistic significance (95% confidence interval). The sign in the bracket indicate whether changing from the low to the high value has a negative or positive effect.

3.3.2 Inducer Concentration

The effect of varying the L-arabinose concentration was tested in shaker flask trials. Shown in Figure 18 maximum protein production corresponding to the highest level of culture induction was achieved using 2.5g/L of L-arabinose. Various levels of L-arabinose between 0.1-2g/L have been used in the production of a wide range of proteins [16, 22, 45, 46, 223]. Choi et al. [46] used 1g/L of L-arabinose to induce the production of human granulocyte colony stimulating factor (hG-CSF) to a level of 6-9g/L in a high-cell-density fermentation. Considering that trials were performed at low-cell-density in shaker flasks, the concentration of L-arabinose required are relatively high. Choi et al. [46] used a strain of *E.coli* which is unable to metabolise L-arabinose (ara$^-$). The high requirement for L-arabinose by the *Aspin* strain can likely be attributed to it being able to metabolise L-arabinose.

Future trials should look at whether extending the period over which inducing agent is added increases the level of expression.

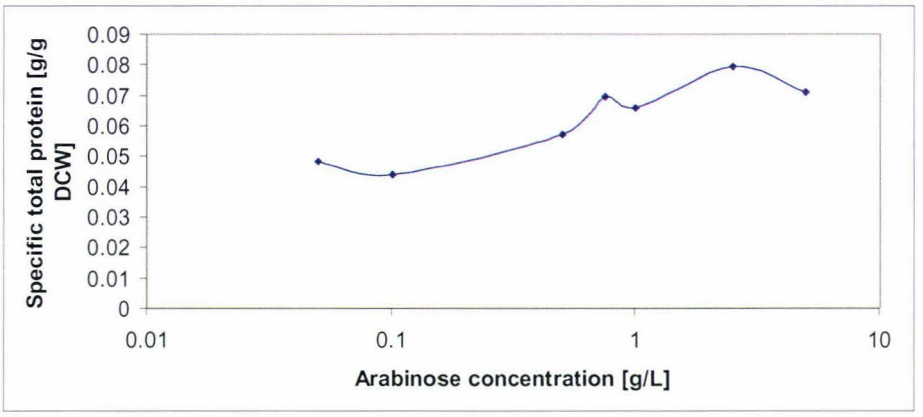

Figure 17: Effect of various concentrations of L-arabinose on the cell specific total protein titre. Fourteen 250mL shaker flasks were inoculated with 1mL of overnight seed culture in 25mL of Terrific Broth. Cultures were incubated for 4 hours at 37°C and 180rpm in a shaking incubator. The temperature was reduced to 30°C and the cultures induced in duplicate with 0.05, 0.1, 0.5, 1, 2.5, 5, 7.5, 10g/L of L-arabinose respectively. Cultures were tested for total protein production 12 hours after inoculation.

Shown in Figure 18 a three fold increase in the titre of soluble *Aspin* was observed when the L-arabinose concentration was increased from 0.05g/L to 0.75g/L. Above 0.75g/L the soluble fraction decreased, with additional *Aspin* expressed primarily in the insoluble form. As discussed previously, there is uncertainty as to the kinetics of arabinose induction of the *ara*BAD promoter. However the decrease in soluble *Aspin* above 0.75g/L does not appear to be consistence with reports of the arabinose concentration effecting the population average of induced versus non induced cells [230]. *Aspin* is intracellularly expressed in this study and therefore would be relatively protected from the culture population properties. A more plausible explanation is that the cellular expression of *Aspin* is modulated by the concentration of arabinose. This is consistent with several studies which

have found that soluble recombinant expression using the *ara*BAD was higher at concentration below fully induction [16, 45]. Lim et al. [45] observed a halving in the interferon-α production when the concentration of L-arabinose was reduced from 2 and 0.4g/L, however the fraction of interferon-α in the soluble increased from less than 5% to 80%. Increases in soluble protein as a result of restricted induction has been observed with other promoters and has been suggested to be caused by lower expression rates freeing up cellular resources that facilitate protein folding [81]. A reduction in recombination protein was observed when the culture was induced with 5g/L of L-arabinose likely as a result of hyper-production. High concentrations of arabinose are known to cause hyper-production of recombinant protein (>30% of total protein), which can lead to destruction of ribosomes, production of heat shock proteins and cell death [22]. The *ara*BAD promoter has been previously shown to be strongly regulated with a low level of basal expression [16, 22]. In the absences of L-arabinose, 0.053g/g DCW of soluble *Aspin* was produced (data not shown). For systems in which the reduction of basal expression is important, the addition of glucose to the growth medium causes strong repression of the *ara*C gene which regulates the *ara*BAD promoter [16].

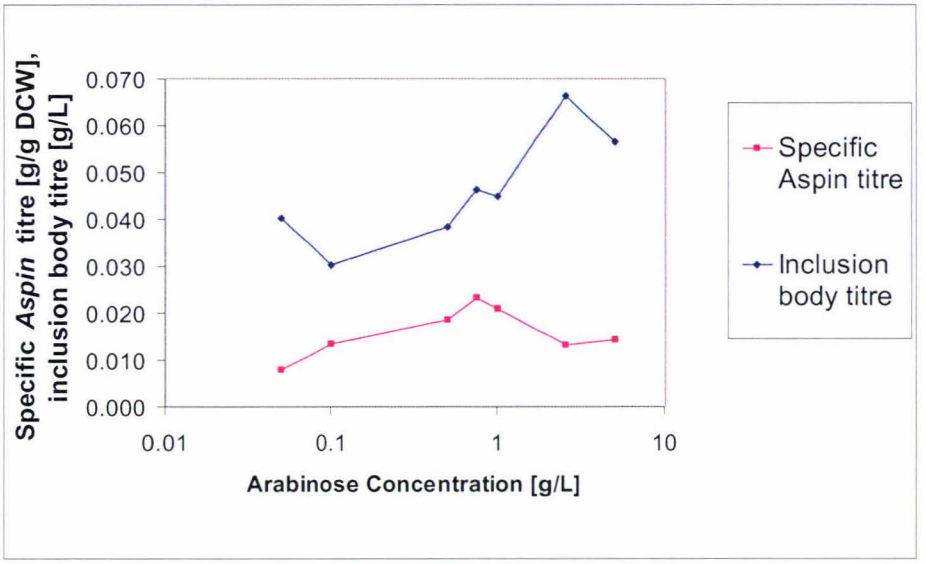

Figure 18: Effect of various concentrations of L-arabinose on the soluble and insoluble titre of *Aspin*. Fourteen 250mL shaker flasks were inoculated with 1mL of overnight seed culture in 25mL of Terrific Broth. Cultures were incubated for 4 hours at 37°C and 180rpm in a shaking incubator. The temperature was reduced to 30°C and the cultures induced in duplicate with 0.05, 0.1, 0.5, 1, 2.5, 5, 7.5, 10g/L of L-arabinose respectively. Cultures were tested for soluble and insoluble protein production 12 hours after inoculation

3.3.3 Temperature

For recombinant proteins which have a tendency to form insoluble aggregates in *E.coli* decreasing the culture temperature has been shown to reduce protein aggregation [3, 48, 66, 68, 81, 126]. Reducing the post-induction temperature from 37°C to 28°C led to a doubling in the specific titre of soluble *Aspin* (Figure 19). This is consistent with Schein and Noteborn [14] who tested a number of *E.coli* strains and plasmid constructs and found that recombinant protein solubility could be increased by reducing the culture temperature to between 20-30°C. Figure 19 shows the production of *Aspin* in inclusion bodies almost halved when the post-induction temperature was decreased from 37°C to 19°C. Seddi et al. [15] found that at a culture temperature of 37°C bovine procarboxypeptidase A was exclusively

expressed in an insoluble form, but by reducing the temperature to 15°C the soluble fraction could be increased to 30%. Lowering the temperature slows cell biosynthesis reducing the accumulation of protein intermediates which cause inclusion bodies [14-16, 80, 81]. Lowering cultivation temperatures also reduces stress responses during induction and product degradation through reduced protease activity [13]. At post-induction temperatures below 28°C the titre of soluble *Aspin* was less than the level achieved using a post-induction temperature of 37°C, as overall protein synthesis was reduced.

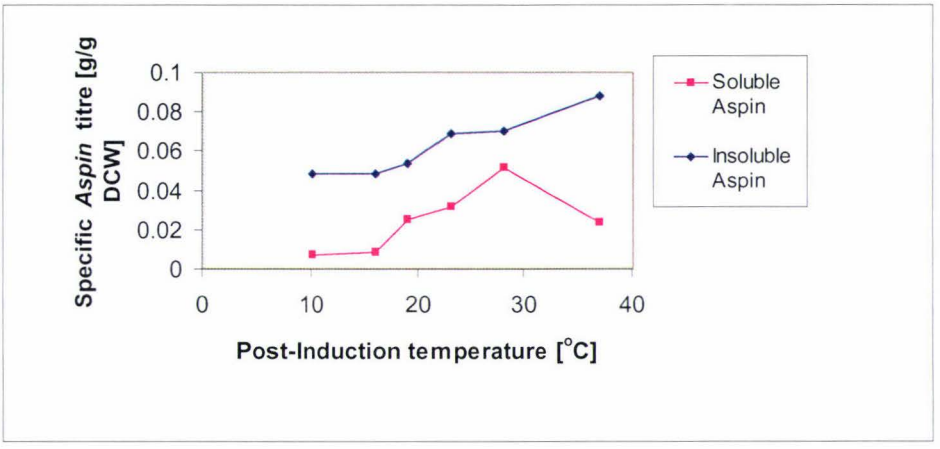

Figure 19: Effect of temperature on the specific soluble and insoluble titres of *Aspin*. Twelve 250mL shaker flasks were inoculated with 1mL of overnight seed culture in 25mL of Terrific Broth. Cultures were incubated for 4 hours at 37°C and 180rpm in shaking incubators. The culture temperatures were adjusted in duplicate to 10, 16, 19, 23, 27, 37°C respectively and induced with 2g/L L-arabinose. Cultures were tested for soluble and insoluble protein production 12 hours after inoculation.

An additional experiment was run with a post-induction temperature of 42°C (data not shown). As expected the production of *Aspin* was lower than when the culture was induced at 37°C, however the size of the effect was unexpected. At 42°C 0.034g/g DCW of soluble *Aspin* was produced which was lower than observed in the absence of inducing agent. The final cell-density protein was also the lower than in all the other trials. Increasing the

culture temperature has the effect that it increases the plasmid copy number and rate of recombinant protein synthesis [48]. However the reduction in specific soluble *Aspin* titre is likely a result of increases in inclusion body formation as well as reduced biomass yields. Hoffman and Rinas [48] previously found that culturing at 42°C encouraged the formation of inclusion body protein in *E.coli*.

3.3.4 Fermenter Trials

The first fermentation took the simplest approach using the fed-batch protocols developed in Chapter 1. As little fermentation knowledge existed for the *Aspin* clone an on-line (pH-stat) feeding program was used as opposed to an exponential feeding program. The fermentation was maintained at pH 7.0 and 37°C to simulate previous fermentations for which data was incomplete. The culture was grown for 24 hours at which time 21.9g DCW/L had accumulated. The volumetric titre of soluble *Aspin* was 0.008g/L with a low specific titre of 0.00037g/g DCW. It was suspected at this stage that the high culture post-induction growth rate was the likely the cause of the low titre of soluble *Aspin*. The pH-stat feeding regime maintains the culture at a high growth rate by maintaining the cell on the brink of starvation [127]. It has been widely reported that at high post-induction growth rates recombinant eukaryotic proteins expressed in *E.coli* tend to aggregate and form inclusion bodies [3, 13, 72, 210, 225]. 2.6g/L of inclusion body protein was produced at a specific titre of 0.118g/g DCW, however the content of *Aspin* was unable to be quantified due to the large number of impurities. Gribskov and Burgress [210] demonstrated that even endogenous proteins to *E.coli* if expressed at high rate can accumulate in inclusion bodies, suggesting that inclusion body formation is a consequence of high expression rates regardless of the system or protein used. There are a number of ways of

reducing the growth rate of fermentation cultures including; by reducing the post-induction temperature, the feed rate of nutrients and dissolved oxygen supply [65, 72, 94, 123, 220]. All three methods were investigated.

A second fermentation looked at the effect of reducing the culture growth rate by lowering the temperature post-induction to 28°C. An exponential feeding regime as apposed to a pH-stat was used as it allowed greater control of the growth rate. It also reduces the fluctuations in fermentation conditions caused by the cycles of culture starvation and feeding experienced in a pH-stat [217]. The exponential feeding regime used the equation outlined in Figure 7 to control the growth rate initially at $0.3h^{-1}$. Measured data taken from the fermenter showed that it was unable to control the temperature at 28°C and instead the temperature decreased to 25°C two hours after inoculation and remain there for the remaining period of the fermentation. At harvest, after 24 hours the final biomass concentration was 22.6g DCW/L and 0.085g/L of soluble *Aspin* was produced which is a ten fold improvement on the first fermentation. Shown in Figure 20, the culture metabolism is reduced significantly after eight hours as indicated by a reduction in the requirement for agitation to maintain the culture DO. Reducing the metabolic rate slows the production of proteins freeing up chaperones and foldases which are required to correctly folded proteins [10, 66, 80, 81]. Incorrectly or incompletely folded proteins have been suggested to cause the formation of inclusion bodies [80, 81]. A number of authors have used reduced temperature to increase the solubility of eukaryotic protein expression in *E.coli* [3, 8, 9, 12-15, 68, 75, 81]. Chao et al. [8] found that the four and half fold increase in D-hydantoinase production could be achieved by reducing the post-induction temperature from 37°C to 25°C

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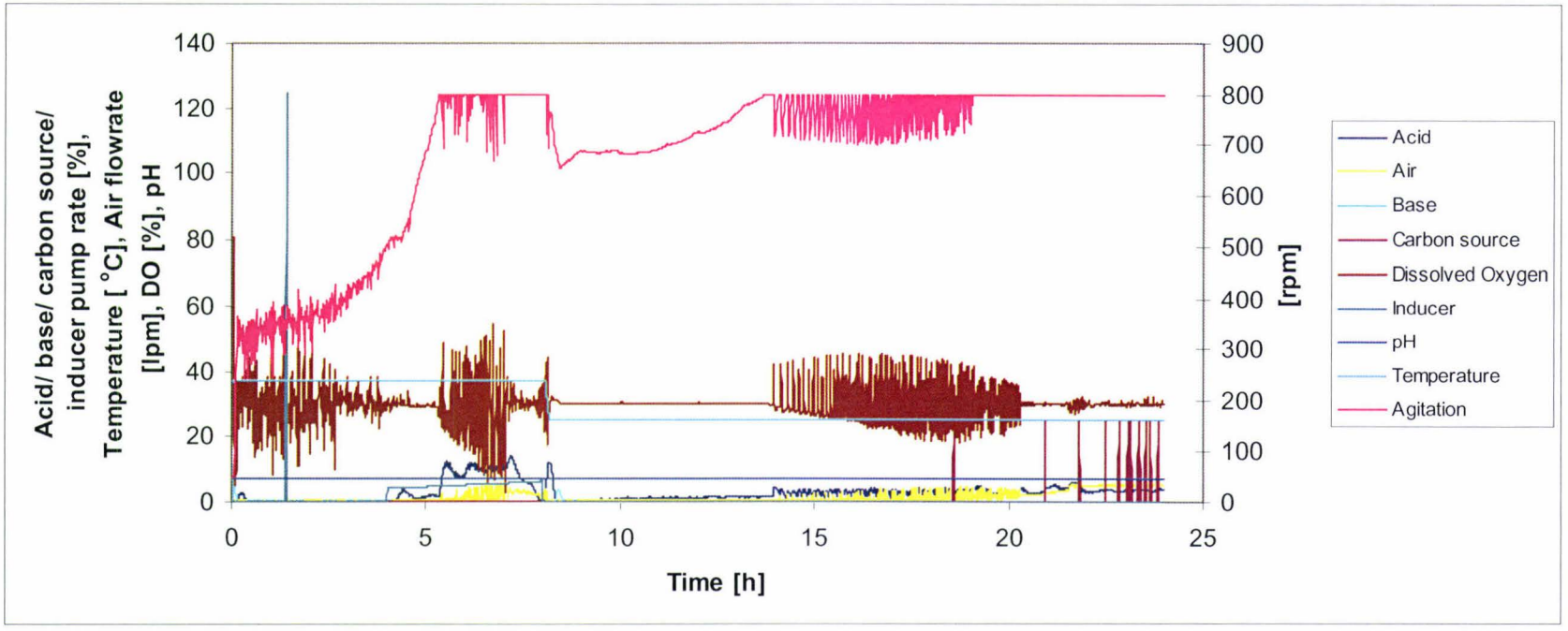

Figure 20: Fermentation output of various measured parameters of the second *Aspin* fermentation. Measured variables include; acid pump speed [%], air [l/min], base pump speed [%], carbon source pump speed [%], dissolved oxygen [%], inducer pump speed [%], pH, temperature [°C] and agitation speed [rpm]. The fermenter was inoculated with 100mL of cells at OD~2 into 1.4L Terrific broth. An exponential feeding regime was begun after 2 hours with a solution of 315g/L of glycerol and 315g/L yeast extract at a rate of $0.3h^{-1}$ according the formula outlined previously [87]. The DO was controlled at 30% through agitation between 200-800rpm and aeration 0.5-2 L/min, temperature 37°C and pH 6.8. The culture temperature was reduced to 25°C after eight hours and induced with 2g/L of L-arabinose.

A third fermentation looked at reducing the post-induction growth rate by nutrient limitation. An exponential feeding regime was set to control the growth rate initially at $0.3h^{-1}$. Prior to induction the feed rate was reduced to control the growth rate at $0.03h^{-1}$. The temperature was reduced to 25°C and the culture induced with 2g/L of L-arabinose. The fermentation achieved a final cell density of 14.8g DCW/L with 0.023g/L of soluble *Aspin* produced after 24 hours. This titre represents a three fold increase in soluble *Aspin* compared to the first fermentation, but only a third of that achieved in the previous fermentation and still a tenth of that achieved in the best shaker flask experiment. Shown in Figure 21, soluble *Aspin* production decreased as post-induction growth rate was increased. At the same time the titre of inclusion bodies increased with increasing growth rate. This is consistent with Shin et al. [126] who tested the expression of soluble human glucagon and human growth hormone at various growth-rates in high-cell-density cultures of *E.coli*. They found that protein initially expressed in soluble form, increasingly aggregated as inclusion bodies as the growth rate was increased [126]. The lower titre of soluble *Aspin* is possibly due to control at the lower rate being not as accurate as desired, with the actual measured growth rate found to be $0.046h^{-1}$.post-induction. This is lower than the first fermentation however higher than the second fermentation which had post-induction growth rates of $0.052\ h^{-1}$ and $0.037\ h^{-1}$ respectively. The lack of control could be a result of yeast extract in the feed solution containing approximately 30% carbohydrates which supplies an alternative carbon sources to the glycerol which is assumed in the exponential feeding model to be the limiting substrate [107]. The use of chemically defined media has been reported to provide better control and product consistency as batch to batch variation is reduced and nutrient availability can be accurately determined [1].

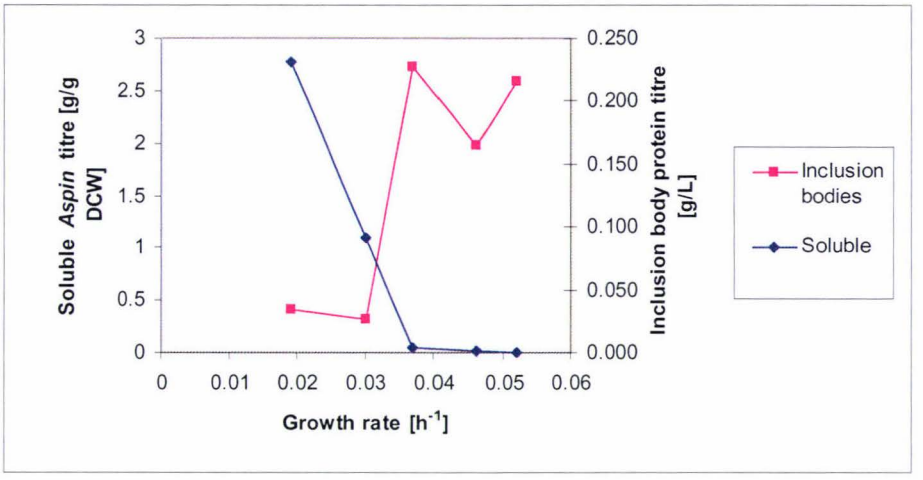

Figure 21: Effect of the post-induction growth rate on the soluble *Aspin* titre and inclusion body production for the fermentation cultures. All the fermentations were inoculated with 100mL of overnight seed into 1.4L of Terrific Broth at 37°C, pH 6.8. The DO was controlled at 30% through agitation between 200-800rpm and aeration 0.5-2 L/min except for two of the fermentations which used constant aeration of 0.8 L/min and agitation at 350 and 250rpm respectively. The cultures were all induced with 2g/L of L-arabinose after eight hours and harvested after 24 hours. Three of the fermentations used an exponential feeding regime which was begun after 2 hours with a solution of 315g/L of glycerol/ yeast extract at a initial rate of $0.3h^{-1}$ according the formula outlined in previously [87].

Fermentation four used the defined medium described previously [98] to allow more accurate control of the growth rate. The same feeding parameters were used as outlined for the previous fermentation. Growth was much slower than observed with complex media. This is a common observation with cultures grown on synthetic media as cells need to synthesize their own amino acids which consumes a proportion of their metabolic capacity [22, 105]. The culture took 20 hours to reach a cell density of 10g DCW/L at which time it was induced with 2g/L of L-arabinose. The post-induction growth rate was accurately controlled with the actual measured rate being $0.033h^{-1}$. The culture was grown for 28 hours at which time 13.1g DCW/L had accumulated. The volumetric titre of soluble *Aspin* was 0.027g/L, with a specific titre of 0.0021g/g DCW. This titre could be due to the medium not providing sufficient nutrients for optimal growth and protein expression. It is widely reported that recombinant protein titres are

generally lower when cultures are grown on chemically defined media [1, 3, 74, 96, 216, 218]. Kweon et al. [1] found that the expression of soluble *Phytolacca insularis* protein grown on a chemically defined medium could be improved two-fold by the addition of 5g/L of yeast extract which is a complex medium component. Despite the lower titres, in a commercial production environment chemically defined media are more commonly used as product consistency is more important than the attainment of high titres as it provides greater business certainty [107].

The highest level of *Aspin* achieved in the fermenter was still under a half of that achieved in a shaker flask so a fifth fermentation attempted to emulate shaker flask conditions. The dissolved oxygen concentration in a shaker flask was measured by placing the fermenter dissolved oxygen probe in a shaker flask with 50mL of Terrific Broth which had been allowed to equilibrate for 2 hours in a shaking incubator at 37°C and 180rpm. A variety of agitation and aeration conditions were tested with 350rpm and 0.8 L/min of aeration found to produce in an un-inoculated fermenter a similar dissolved oxygen concentration to the shaker flask. To further emulate the conditions found in a shaker flask no feeding or pH adjustment was used during the fermentation. The post-induction temperature was dropped to 23°C, 8 hours after inoculation to further reduce the growth rate. The final cell density after 24 hours was 0.910g DCW/L and the post-induction growth rate $0.019h^{-1}$. Predictably, the titre was similar to that achieved under the same condition in a shaker flask with 0.222g/L of soluble *Aspin* produced. This result is at odds with Strandberg and Enfors [140] who found that inclusion body formation was more common in the uncontrolled environment found in shaker flasks [140]. This discrepancy is possibly due to *Aspin* being more stable at lower pH. In the later part of uncontrolled fermentations the pH decreases as the cells produce metabolites such as ammonium (Figure 22). As observed in the environmental screening trials, reducing the pH in turn

improved the expression of soluble *Aspin*. Further trials should look at characterising the effect of pH on production to determine the optimal pH for expression. The high product titre may also be due to the dissolved oxygen concentration in the later part of the fermentation hovered near zero (Figure 22). The lowering of the growth rate by restriction of the oxygen supply has previously been used to increase recombinant protein titres [109, 143, 226]. Unutmaz et al. [226] observed a 60 fold increase in α-lytic protease production when the dissolved oxygen level was reduced from 30% to near zero. Post-induction, the growth rate was $0.019h^{-1}$ which was the lowest of all the previous fermentations. This resulted in the highest specific titre of soluble *Aspin* and lowest titre of inclusion body protein at 0.176g/g DCW and 0.416g/L respectively.

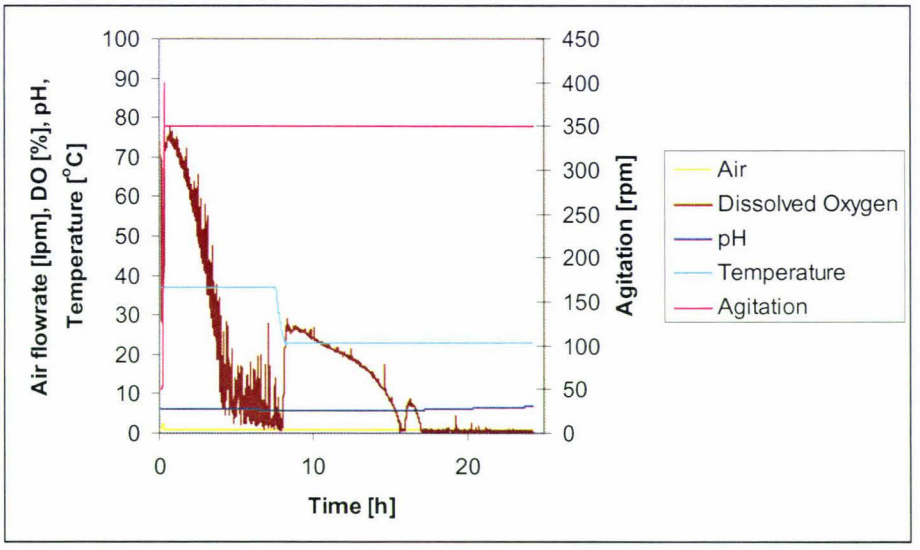

Figure 22: Fermentation output of various measured parameters for the fifth *Aspin* fermentation. The fermenter was inoculated with 100mL of cells at OD~2 into 1.4L Terrific Broth. No pH or DO control was used. Agitation was held constant at 350rpm and aeration 0.8 L/min. The culture temperature was initially held at 37°C and then reduced to 25°C after eight hours and induced with 2g/L of L-arabinose.

A sixth fermentation looked at the effect of further reducing the growth rate. The same fermentation parameters were used as described for the previous fermentation except the agitation rate was reduced to 250rpm and the post-induction temperature to 19°C. The final cell density after 24 hours was 0.92g DCW/L and 0.322g/L of inclusion body protein produced. Surprisingly the post-induction growth rate was measured to be $0.03h^{-1}$ higher than the previous experiment. Shown in Table 8 the soluble *Aspin* titre was similar to the second fermentation with 0.084g/L produced. The low titre of soluble *Aspin* is likely due to the increase in growth rate. This variability in control appears to be a result of the inoculation flask for this experiment having a lower cell density than the previous fermentation meaning that the growth wasn't limited by the oxygen supply at induction. Variations in conditions at induction could be reduced through the use of standard cell densities for when to inoculate and induce. This result highlights the sensitivity of the *Aspin* strain to the growth-rate and the need for accurate process control.

Shown in Table 8, in the first three fermentations in which cell densities greater than 10g DCW/L were achieved, the specific titres of soluble *Aspin* were low. As discussed earlier, this is likely a result of the inability to reduce the growth rate significantly enough to free up chaperones and foldases required for correct protein folding. Fermentation 5 demonstrated that using a chemically defined medium, the growth rate could be more accurately controlled than using a complex medium. Unfortunately the final cell density and soluble *Aspin* titre was poor. This study did not have sufficient time to experiment with other chemically defined media however if *Aspin* is to become a commercial reality it is recommended that further work should look to optimise a chemically defined medium which balances expression with product consistency.

Table 8: Summary of growth conditions and production characteristics of the *Aspin* fermentations. The shaker flask experiment was inoculated with 1mL of overnight seed culture in 25mL of Terrific Broth and incubated for 4 hours at 37°C and 180rpm in a shaking incubator. At induction the temperature was reduced to 28°C and 2g/L of L-arabinose added. The production characteristics were measured after 12 h. All the fermentations were inoculated with 100mL of overnight seed into 1.4L of Terrific broth except fermentation 4 which used defined *E.coli* medium. The DO was controlled at 30% through agitation between 200-800rpm and aeration 0.5-2 L/min, temperature 37°C and pH 6.8. The culture temperatures were reduced to there respective post-induction temperatures after eight hours and induced with 2g/L of L-arabinose. For fermentations 2-4 an exponential feeding regime was begun after 2 hours with a solution of 315g/L of glycerol and 315g/L of yeast extract at a initial rate of $0.3h^{-1}$ according the formula outlined in previously [87]. Fermentation 5 and 6 used a constant aeration of 0.8 L/min and agitation of 350 and 250rpm respectively.

Fermentation Conditions			Results			
Fermentation	Post-Induction Temperature [°C]	Post-induction Growth Rate [hr^{-1}]	Final Cell Density [g/L]	Inclusion body protein titre [g/L]	Soluble *Aspin* titre [g/L]	Specific soluble *Aspin* titre [g/g DCW]
Best shaker flask	28	[a]	0.91	0.448	0.239	0.259
Fermentation 1 (pH-stat)	28	0.052	21.9	2.585	0.008	0.0004
Fermentation 2 (exponential fed)	25	0.037	22.6	2.721	0.085	0.004
Fermentation 3 (exponential fed)	25	0.046	14.8	1.982	0.023	0.002
Fermentation 4 (exponential fed)	25	0.033	13.1	[b]	0.027	0.002
Fermentation 5 (no feeding)	23	0.019	0.96	0.416	0.222	0.231
Fermentation 6 (no feeding)	19	0.030	0.92	0.322	0.084	0.091

[a] post-induction growth rate not measured

[b] no inclusion body pellet recovered

Prior to this study all previous attempts to produce *Aspin* in a fermenter had produced titres of exclusively insoluble protein (personnel communication,

Robert Dempster, Agresearch, Upper Hutt, NZ). The soluble protein titre of 0.220g/L achieved in the best fermentation is relatively high compared to other published data. Two papers have been published showing soluble recombinant eukaryote proteins expressed in *E.coli* at levels between 0.2-0.5g/L [3, 69], but more commonly lower titres of 0.001-0.040g/L of culture are obtained [2, 9, 15, 68].

3.4 CONCLUSION

A major problem with expressing eukaryote proteins in *E.coli* is that they commonly aggregate to form inclusion bodies. The objective of this section was to investigation a range of methods to increase the titre of soluble *Aspin*, develop parameters for production of *Aspin* in a small bioreactor and provide a basis of fermentation knowledge for the development of other soluble protein products in *E.coli*. The production of soluble *Aspin* was found to be highly dependant on the rate of expression. At high rates of expression chaperones and foldases which cells use to fold proteins can be overwhelmed, leading to the accumulation of proteins intermediates which form inclusion bodies [81]. Several methods of reducing the rate of protein expression were investigated including by reducing the culture temperature, concentration of inducer, feeding rate, and limiting the supply of oxygen. The titre of soluble *Aspin* doubled when the post-induction temperature was reduced from 37 to 28°C. 0.75g/L of L-arabinose was found to be optimal for soluble *Aspin* production, above this concentration additional recombinant protein was produced solely in inclusion bodies. Using fed-batch techniques in the fermenter elevated cell densities were achieved however *Aspin* was almost exclusively produced in inclusion bodies. It was suggested that this was due to a lack of control over the growth rate of the culture and it was recommended that future experimentation should look at developing a chemically defined medium to improve process control. A final titre of 0.220g/L of soluble *Aspin* was produced in the fermenter induced with 2g/L of L-arabinose, with the post-induction temperature reduced from 37°C to 23°C and with a low dissolved oxygen concentration maintained. This titre is relatively high compared to other published data for soluble recombinant proteins expressed in *E.coli* [3, 69].

4 Pichia pastoris

4.1 INTRODUCTION

The yeast *Pichia pastoris* is capable of growing to high cell densities on inexpensive, defined media and can secrete proteins extracellularly simplifying downstream processing [4, 25-28]. *P.pastoris* also has the ability to perform some eukaryotic post-translational modifications such as glycosylation [33, 83]. However, proteins produced in *P.pastoris* are not always folded correctly, may be over glycosylated and titres are variable, with reports of recombinant protein levels varying from milligrams to grams per litre [4, 26]. Yeast cells also grow more slowly than bacteria such as *E.coli*, with typical cultures taking several days [29].

The optimal temperature for *P.pastoris* culture is 30°C. Above 30°C recombinant protein expression is reduced with expression ceasing at 32°C [4]. *P.pastoris* can grow over a wide range of pH which has been widely utilised to reduce the action of proteases [116]. The most common promoter which encodes for alcohol oxidase (AOX), is induced through the addition of methanol and repressed in the presence of glycerol or glucose [53]. *P.pastoris* cultures are grown first on a glycerol/ dextrose medium to accumulate biomass and then transferred to an equivalent medium with methanol in place of the glycerol/ dextrose to induce expression. Common media include minimal glycerol medium (MGY) [27, 53, 84, 86], buffered glycerol medium BGY [26, 52, 82, 83, 113-115], buffered glycerol-complex medium BMGY [5, 26, 82-84, 86, 115] and basal salt media [5, 52, 114-117]. *P.pastoris* is capable of achieving very high cell densities (>150g/L dry cell weight) in fed-batch culture and is limited often by the capacity of equipment to supply oxygen and remove heat [4]. During expression, the dissolved oxygen concentration

is extremely important as oxygen is required for the first step of methanol catabolism with formaldehyde accumulating in its absence [113].

The *P.pastoris* clone which was used as a model here expresses recombinant bovine granulocyte colony stimulating factor (G-CSF), a cytokine that enhances neutrophil functions against bacteria, yeast and fungi [27, 46]. Recombinant bovine G-CSF is used as an immunostimulate in animal vaccine trials at the Wallaceville Animal Research Centre. A recombinant source is used as it is difficult to purify the large amounts required for experiments (milligram quantities) from natural sources and to ensure it is of high purity. Previously all G-CSF used in trials was produced from shaker flask cultures however only limited amounts of recombinant protein could be produced using this method. The objective of this chapter is to investigate methods to increase the titre of G-CSF and develop parameters for production in a small scale bioreactor. This section also aims to provide a basis of fermentation knowledge for the development of other recombinant protein products produced in *P.pastoris*.

4.2 MATERIAL AND METHODS

4.2.1 Strain

The clone studied in this chapter which expressed the recombinant protein G-CSF was kindly supplied by Neil Wedlock (Agresearch, Upper Hutt, New Zealand). The host was *Pichia pastoris* SMD1168 Mut^+ His^+ strain (Invitrogen, CA, USA). The gene sequence for G-CSF was cloned into the pPICZ (Invitrogen) plasmid which contains a *poly*-histidine tag and c-myc epitope to aid characterisation and purification [232]. The recombinant plasmid was incorporated into the cell genome of *P.pastoris* by homologous recombination. The pPICZ plasmid contains a resistance gene for the antibiotic Zeocin™ to aid the selection of transformed cells. Recombinant protein expression was controlled by the AOX1 promoter which is repressed in the absence of methanol. Production seeds were stored in yeast extract peptone dextrose medium (YEPD) with 20% (v/v) glycerol at -80°C in the presence of 0.010g/L of Zeocin.

4.2.2 Media

Reagent and chemicals were purchased from Sigma (St Louis, MO, USA), BioRad (Hercules, CA, USA), Merck (Darmstadt, Germany) and Difco (Sparks, MD, USA). Shaker flask trials were carried out in enhanced yeast extract HySoy dextrose medium (YEHD) which comprised of per litre; 42g yeast extract (Merck), 35g soy-tone (Merck), 17g dextrose (sterilised separately by filtration through 0.22μm membrane) and 0.25mL antifoam 289 (Sigma).

Fermentations were carried out in Basal Salt Medium containing per litre: 26.7mL of 85% (w/v) phosphoric acid, 0.93g calcium sulphate.$2H_2O$, 18.2g potassium sulphate, 14.9g magnesium sulphate.$7H_2O$, 4.13g potassium hydroxide, 40g glycerol, 0.5mL antifoam 289 and 4.35mL of *Pichia* trace metal (PTM_1) solution. Both media were sterilised at 121°C for 30 minutes. PTM_1 solution contains per litre; 6g copper sulphate.$5H_2O$, 0.08g sodium iodide, 3.0g manganese sulphate.H_2O, 0.2g sodium molybdate.$2H_2O$, 0.02g boric acid, 0.5g cobalt chloride, 20g zinc chloride, 65g ferrous sulphate.$7H_2O$, 0.2g biotin, 5mL sulphuric acid which were all filter sterilised separately. Prior to inoculation the medium pH was adjusted to 5.0 by the addition of 30% (w/v) ammonium hydroxide.

4.2.3 Inoculum Development

Frozen cell suspension (1mL) was thawed at room temperature and added to 25mL of enhanced YEHD medium in a 250mL baffled shaker flask. The cultures were incubated for 24 hours on a rotary shaker at 200rpm, and 30°C. For fermentation studies 6mL of the culture was then transferred to a 2000mL baffled shaker flask containing 150mL of enhanced YEHD and cultured for a further 24 hours.

4.2.4 Medium Trials

Medium trials were carried out using commonly used shaker flask media for *P.pastoris*. 1mL of seed was added to 25mL of minimal glycerol medium (MGY), buffered minimal glycerol medium (BMG), buffer glycerol-complex

medium (BMGY), yeast extract peptone dextrose medium (YEPD), enhanced yeast extract Hy-Soy dextrose medium (YEHD) in separate 250mL shaker flasks. The different media were made according to protocol outlined in Pichia Fermentation Process Guidelines (Invitrogen). All cultures were maintained at 30°C, 200rpm in a shaking incubator for 24 hours. Each culture was then centrifuged at 3000g for 5 minutes and the pellet resuspended to an optical density of 5 in the same medium with glycerol or dextrose replaced with 0.5% (v/v) of methanol. Cultures were grown for a further three days with 0.5% (v/v) of methanol added each day.

4.2.5 Environmental Screening Trial

A Plackett Burman trial (discussed in Chapter 2) was used to test five environmental variables for their effect on soluble G-CSF production. The effect of culture temperature, resuspended optical density of cells at induction, methanol concentration, antibiotic concentration, and pH were investigated. Ten 250mL shaker flasks containing 25mL of enhanced YEHD medium were inoculated with 1mL of production seed and grown at 30°C and 200rpm in a shaking incubator. After 24 hours the cultures medium was removed by centrifuging at 3000g for 5 minutes. Pelleted cells were resuspended in enhanced YEHD with methanol replacing the dextrose in the medium and grown for a further three days under the conditions outline in Table 10.

4.2.6 Optical Density Trial

200mL of enhanced YEHD medium was inoculated with 1mL of frozen seed in a 2000mL shaker flask. This culture was placed in a shaking incubator at 30°C and 200rpm for 48 hours. The culture was then centrifuged at 3000g for 5 minutes. Pelleted cells were resuspended in sixteen 250mL shaker flasks to OD of 0.25, 0.5, 1, 2, 4, 8, 10, 12 in YEHD medium with 0.5% (v/v) methanol replacing the dextrose. Cultures were grown for three days at 30°C, 200rpm with 0.5% (v/v) methanol added each day.

4.2.7 pH Trial

25mL of enhanced YEHD medium was inoculated with 1mL of seed in a 250mL shaker flask. This culture was placed in a shaking incubator at 30°C and 200rpm for 24 hours. The culture was then centrifuged at 3000g for 5 minutes. Pelleted cells were resuspended in fourteen 25mL cultures at optical densities of 5 in YEHD medium with 0.5% (v/v) methanol replacing the dextrose and supplemented with 0.1M phosphate buffer. The cultures were adjusted in duplicate to pH 3, 4, 5, 6, 7, 8, 9 with 2M NaOH and 2M HCl. Cultures were grown for three days at 30°C, 200rpm with 0.5% (v/v) methanol added each day. After 72 hours the cells were removed by centrifuging at 3,000g for 5 minutes and the supernatant adjusted to pH 8.0.

4.2.8 Methanol Concentration Trial

25mL of enhanced YEHD medium was inoculated with 1mL of production seed in a 250mL shaker flask. This culture was placed in a shaking incubator at 30°C and 200rpm for 24 hours. The culture was then centrifuged at 3000g for 5 minutes. Pelleted cells were resuspended in ten 25mL cultures to an optical density of 5 in enhanced YEHD medium with; 0.125, 0.25, 0.5, 1.0 and 2.0% (v/v) methanol replacing the dextrose. Cultures were grown for three days with the same amount of methanol added to the culture each day.

4.2.9 Fermentation

Fermentation protocols used were based on the Pichia Fermentation Process Guidelines (Invitrogen). Fermentation trials where performed using a 3.3L glass jar fermenter (BioFlo 3000, New Brunswick Scientific., Edison, N.J.) previously described in Chapter 1. All experiments unless otherwise stated, had an initial medium volume of 1.25L and were inoculated with 150mL of seed at an OD of approximately 5. Sparging was provided at a constant rate of 2 L/min and agitation was manually controlled between 500-1000rpm to maintain the DO above 30%. At high cell-densities pure oxygen was blended into the sparging gas to maintain the dissolved oxygen at 30% using a PID controller. The temperature was controlled at a 30°C and pH at 5.0 through the addition of 28% (w/v) ammonium hydroxide. Fed-batch feeding of cultures was carried out according to protocol outline previously [87]. Cultures were grown till the glycerol in the initial medium was completely exhausted as indicated by a rapid increase in DO (18-20 hours). A continuous feed of 50% (v/v) glycerol containing 1.2% (v/v) of PTM_1 solution was then started at 18mL/h per hour for four hours to accumulate biomass. The glycerol feed was stopped for a half hour starvation period before induction

to ensure the complete depletion of glycerol in the medium. Induction was initiated using a continuous feed of methanol containing 1.2% (v/v) of PTM_1 solution at an initial rate of 3mL/h. This was increased over 8 hours to 12mL/h at which level it was retained at for the remainder of the culture (3 days). Cells were harvested by centrifugation at 10,000rpm for 10 minutes with the supernatant stored at 4°C.

4.2.10 Cell Density

Culture growth was monitored by measuring optical density at 595nm using a Genesis 2C spectrophotometer (ThermoSpectronic, NY, USA). The dry cell weight (DCW, [g/L]) was calculated from a calibration curve prepared using six cultures at various cell concentrations, sampled in triplicate. The cell dry weight and specific growth rate were calculated according to the protocol outlined in Chapter 1.

4.2.11 Protein Assays

Prior to protein extraction all cultures were adjusted to pH 8.0 using 5M NaOH and 2M HCl to prevent premature elution from the chromatography resin. 0.100mL of Talon™ resin was centrifuged at 14,000rpm for 1 minute in a 1.5mL micro-centrifuge tube. The supernatant was carefully pipetted off and the pelleted beads resuspended in 1mL of extraction buffer (described in Chapter 2). The beads were again centrifuged at 14,000rpm for 1 minute and the supernatant removed. 1mL of culture supernatant was then added, the beads agitated using an micro-centrifuge tube shaker and incubated for 30

minutes on a rotating tray. The beads were centrifuged at 14,000rpm for 1 minute and the supernatant removed. The resin was then washed three times in extraction buffer and the G-CSF eluted using a 0.100mL of 37g/L Na_2EDTA. Total protein and SDS-PAGE was performed as outlined in Chapter 1.

4.3 RESULTS & DISCUSSION

4.3.1 Media Trials

As shown in Table 9 the cultures grown on the two chemically defined media MGY and BMG grew to much lower cell densities and produced lower levels of recombinant protein than those grown in complex media. Cultures grown on chemically defined media have previously been found to produce lower biomass and recombinant protein titres than those grown on complex media, as cells need to synthesis their own amino acids [82, 83, 116]. The titre of G-CSF was especially low for the culture grown on MGY with no corresponding band visible on a Coomassie blue stained SDS-PAGE gel. The low titre is likely due to proteolysis of the protein of interest. Werten et al. [116] observed that recombinant mouse collagen expressed in *P.pastoris* on chemically defined media could barely be detected as proteases degraded the protein. The addition of complex medium components such as peptone, yeast extract and casamino acids reduces degradation by extracellular proteases by providing excess substrates for proteolysis [82].

An improvement in the titre of recombinant protein was observed between the culture grown on MGY and BMG medium which have the same composition; however BMG contains a phosphate buffer. Buffering the medium helps to reduce fluctuations in pH which may cause disruption to normal metabolic processes. Choi and Jimenez-Flores [83] also found that recombinant protein expression in *P.pastoris* was improved by using a buffered medium. Surprisingly, the buffered medium BMG had a lower final cell density compared to that found in MGY. The media YEPD and BMGY also have similar compositions except that BMGY contains a phosphate buffer. They showed the same characteristic as MGY and BMG with the buffered

medium BMGY having a lower final cell density but higher G-CSF titre. This is likely due to expression and growth having different optimal pH. Both BMGY and BMG were buffered at pH 6 which may cause lower growth. The accumulation of biomass in *P.pastoris* fermentations are generally carried out at pH 5.0 [114, 116, 130] however a wide range of pH between 3-8 have been used during recombinant protein expression [6, 28, 53, 116, 129, 130].

The complex medium, enhanced YEHD produced the highest titre of recombinant protein. The increase in titre is likely due to enhanced YEHD containing approximately twice the quantity of yeast extract and one and a half times the quantity of peptone of either YEPD or BMGY. Yeast extract is rich in amino-acids, peptides, vitamins, growth factor and trace elements. Growth and expression is usually higher on media rich in yeast extract, as cells do not need to synthesise their own amino acids and growth factor [105, 106]. The type of peptone digest is also important to recombinant protein titre, as the amount of various amino acids and growth factors vary between digests. YEHD uses a peptone derived from soy beans, where BMGY and YEPD used peptone derived from a meat. The increase in titre using soy peptone is consistent with Yan et al. [86] who tested recombinant protein production in *P.pastoris* on various peptone digests and found that production was greatest using soy peptone [86]. Due to the high titre of G-CSF, enhanced YEHD was selected for subsequent shaker flask trials.

Table 9: Final biomass, specific and volumetric titres of G-CSF produced after 3 days on various media. Minimal Glycerol (MGY), Buffered Minimal Glycerol (BMG), Buffer Glycerol-complex medium (BMGY), Yeast Extract Peptone Dextrose medium (YEPD), enhanced Yeast Extract Hy-soy Dextrose medium (YEHD). Dextrose media (YEHD). Cultures were inoculated with 1mL of overnight seed at OD~10 in 25mL of media. All cultures were grown in 250mL baffled shaker flasks at 30°C, 200rpm in a shaking incubator for 24 hours. Each culture was then centrifuged at 3000g for 5 minutes and the pellet resuspended to an optical density of 5 in the same medium with glycerol or dextrose replaced with 0.5% (v/v) of methanol. Cultures were grown for a further three days with 0.5% (v/v) methanol added each day.

Media	Production characteristics		
	Final cell density [g CDW/L]	Volumetric G-CSF titre [g/L]	Specific G-CSF titre [g/ g DCW]
MGY	2.48	0.000[a]	0.000[#]
BMG	2.07	0.010	0.005
BMGY	5.20	0.013	0.003
YEPD	4.90	0.011	0.002
Enhanced YEHD	5.08	0.026	0.005

[a] G-CSF titre could not quantify as protein was not visible on Coomassie blue stain of SDS-PAGE gel. A faint band was visible using a silver stain.

4.3.2 Environmental Screening Trial

Shown in Table 10 varying the temperature of the culture during expression had no effect on the production of G-CSF. *P.pastoris* is most commonly cultured at 30°C [4, 5, 26, 104, 115, 117]. However, reducing the temperature has been shown to improve recombinant protein titres [5, 117, 233]. Hong et al. [5] found that the activity of laccase expressed in *P.pastoris* was much higher at 16-19°C than at 30°C. Culturing at lower temperature slows the rate of protein production, freeing up cofactors and foldases ensuring that proteins are correctly folded. Also, on lowering the culture temperature, proteolytic activity is reduced and less low molecular weight proteins are released simplifying downstream processing [5].

The optical density at induction had a strong effect on the expression of recombinant protein (Table 10). Damaso et al. [233] investigated a number of culture parameters and also found that induction cell density had the greatest effect on the production of xylanase from *Thermomyces lanuginosus* in *P.pastoris*. The concentration at which cells are resuspended at induction effects the methanol to cell concentration as well as the final cell density that is achieved [233]. Resuspending at higher cell-densities helps slow growing cultures to reach their maximum cell density more quickly. This increases productivity, however if suspended at too higher concentrations then recombinant protein production stops prematurely as the culture conditions become limiting. Induction at lower cell densities means that expression occurs during exponential growth at which stage cell metabolic activity is at its greatest. Earlier expression is used for *P.pastoris* strains which can metabolise methanol more quickly which enables them to express while still accumulating biomass. Due to its high significance, the optical density at induction was chosen for further investigation.

As shown in Table 10 the concentration of methanol in the expression medium had a moderate effect on the expression of G-CSF. A range of methanol concentrations between 0.5-1% (v/v) has been previously used in shaker flasks [52, 82-84, 86]. In *Pichia pastoris* cultures methanol serves both as a carbon source and an inducer agent. During induction the concentration of methanol must be maintained within a relatively narrow range to avoid growth and expression inhibition. Guarna et al. [52] observed that recombinant protein production decreases above 1.0% (v/v) methanol while other researchers have observed growth inhibition at concentrations above 3% (v/v) [5, 52, 82]. Due to its moderate significance the methanol concentration was chosen for further investigated.

Culture pH had the greatest effect on the volumetric titre of G-CSF. The pH of fermentations effects enzyme mediated reactions within the cells as well as the folding and final conformation of expressed proteins. *P.pastoris* can be grown over a wide range of pH from 3-7 with minimal effect on the growth rate [4]. This characteristic has been extensively used to optimise expression conditions and protecting secreted proteins from proteolysis. Reducing the culture pH to between 3-5 post-induction has been found to inactivated neutral proteases reducing proteolysis of the expressed product [5, 83, 113, 115, 117, 141]. Others have found that recombinant protein expression is highest at pH between 6-7 [4, 53, 130]. Due to its high significance pH was also selected for further investigated.

No trials had previously been performed on this clone to assess plasmid stability over multiple passages (Neil Wedlock, personal communication, 2003). Therefore it was decided that the stability of this clone should be tested as part of this study. Currently the antibiotic Zeocin is used at a concentration of 0.010g/L in shaker flask cultures to provide selective

pressure on the recombinant. However this added considerable expense to process and was suggested maybe unnessessary. For this study it could be important as when culture size is scaled up from shaker flask to fermenter the culture age increases, increasing the chance of the sequence of foreign DNA being rejected [50]. A single passage without Zeocin was tested here and was found to have no effect on the titre of G-CSF. This is consistent with a previous study that investigated the integration of foreign DNA into the *Pichia pastoris* chromosome and observed that clones were stable for more than 60 passages in the absence of selective pressure [234].

Table 10: Plackett and Burman trial assessing the significance of various environmental factors on the volumetric titre of G-CSF.

Run	Variable[a]							Response
	Temperature	Optical Density	pH	Methanol Concentration	Antibiotics	Dummy	Dummy	G-CSF [g/L]
1	H	H	H	H	L	L	H	0.026
2	L	H	H	L	H	H	H	0.002
3	H	L	L	L	H	L	H	0.007
4	L	L	L	H	L	H	H	0.008
5	H	H	L	L	L	H	L	0.008
6	L	H	L	H	H	L	L	0.016
7	H	L	H	H	H	H	L	0.015
8	L	L	H	L	L	L	L	0.011
Σ(H)	0.0567	0.0723	0.0744	0.0644	0.0597	0.0528	0.0633	
Σ(L)	0.0563	0.0407	0.0386	0.0486	0.0533	0.0602	0.0497	
Difference	0.0004	0.0316	0.0358	0.0159	0.0064	-0.0074	0.0135	
Effect	0.0001	0.0079	0.0090	0.0040	0.0016	-0.0018	0.0034	
Mean square	0.0000	0.0001	0.0002	0.0000	0.0000	0.0000	0.0000	
F-test	0.0012	8.4270	10.7926	2.1184	0.3427		0.0000	
Significant	-	+	+	-	-			

[a] The low and high levels are respectively; temperature (25, 30°C), resuspended optical density of cells at induction (1, 10), methanol concentration (0.5, 1g/L), antibiotic concentration (0, 0.010g/L), and culture pH after induction (3, 6). Two dummy variables were used to assess the degree of variability. A 95% confidence interval was used for the test of statistical significance..

4.3.3 Optical Density at Induction

P.pastoris cultures are generally resuspended in methanol media at an optical density of 1-10 [28, 52, 84, 85, 129, 235]. Shown in Figure 23 the recombinant protein expression increased as the cell density at induction was increased. Both the specific and volumetric titre of G-CSF were at a maximum when the culture was resuspended at an optical density of 8. *P.pastoris* strains with the Mut^+ phenotype as used in this study are commonly induced at OD 1-2 [5, 28, 83]. This discrepancy in the optimum optical densities is likely due to the G-CSF strain having a slower growth rate than that commonly observed for other Mut^+ strains. Large variations in growth rate between *P.pastoris* clones have been previously observed [103]. Increasing the density of cells at induction leads to higher final cell densities which increases the volumetric titre of protein. However, as shown in Figure 23 above an optical density of 8 both the volumetric and specific production of G-CSF decreased rapidly. This reduction in recombinant protein titre when resuspended at high cell densities is due to the shaker flask culture oxygen concentration becoming limited, meaning that recombinant protein production must compete with cell maintenance for limited resources. Previously it has been reported that the maintenance of the dissolved oxygen concentration is critical to the production of recombinant proteins in *P.pastoris* [103]. The high optimal cell density at induction observed for this clone is closer to that used for Mut^s strains. In contrast to Mut^+ strains, a number of studies using the slow methanol metabolising strains (Mut^s) resuspend their cells at an optical density of 10 to aid their cultures in reaching high-cell-densities. Brucato et al. [235] tested both Mut^s and Mut^+ strains of *P.pastoris* expressing recombinant rabbit tissue factor (*rr*TF) and observed that only Mut^s stains produced active *rr*TF when suspended in induction media at an

OD of 10. Unlike Mut^+, Mut^S strains grow much slower with little risk of reaching limiting conditions in shaker flasks.

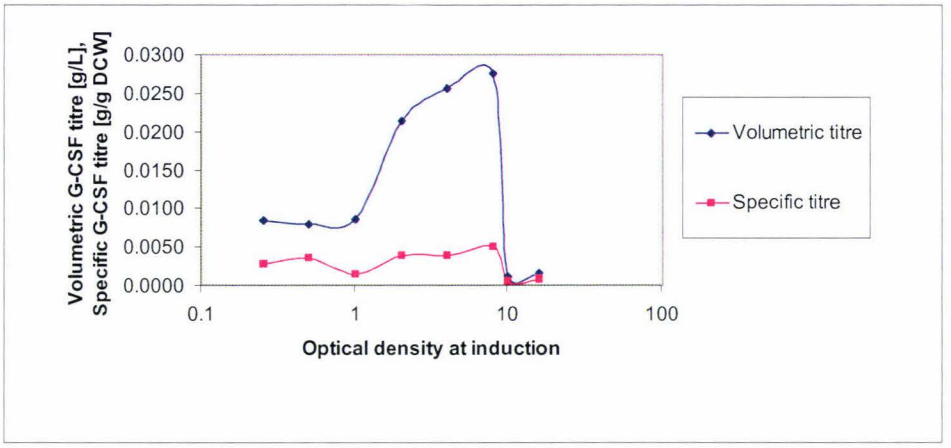

Figure 23: Effect of resuspending cells to various optical densities prior to induction on the volumetric and specific titres of G-CSF. 200mL of enhanced YEHD medium was inoculated with 1mL of frozen seed in a 2L shaker flask. This culture was placed in a shaking incubator at 30°C and 200rpm for 48 hours. The culture was then centrifuged at 3000g for 5 minutes. Pelleted cells were resuspended in sixteen 250mL shaker flasks to OD of 0.25, 0.5, 1, 2, 4, 8, 10, 12 in YEHD medium with 0.5% (v/v) methanol replacing the dextrose. G-CSF production was measured three days after induction.

4.3.4 pH Trial

A range of pH were tested to determine the optimum for G-CSF production. As shown in Figure 24 the production of G-CSF is greatest at pH 6 however between pH 6-8 the level of production is relatively stable. Most recombinant proteins in *P.pastoris* are expressed at pH 5.0 [6, 53, 103, 114, 116, 129, 130]. However several studies have used higher pH to improve protein activity and stability [76, 82, 83, 87, 113]. Files et al. [4] found that recombinant human cystatin-C activity was greatest when expressed at pH 6.0. Choi and Jimenez-Flores [83] found that by maintaining the pH at 6.0 the level of glycosylation of β-casein was increased as well as the proteins activity [83]. Above pH 8.0

both the specific and volumetric titre of G-CSF dropped sharply. This is likely due to growth inhibition by the oxidative conditions. Above pH 7 *P.pastoris* growth has been shown to be inhibited [4, 83]. Adrio et al. [82] found that expression of deacetoxycephalosporin C synthase was highest when induced at pH 6-7 but dropped by 60% at pH 8.0. Shown in Figure 24, the production of G-CSF was also lower below pH 5.0. This reduction in titre below pH 5.0 could be due to flocculation of the yeast. Flocculation accompanied by a significant reduction in culture growth has been previously observed in *P.pastoris* at pH between 3.3-4.5 [117]. Subsequently no published literature could be found for recombinant protein expression in *P.pastoris* using pH between 3.5-4.5. However reducing the culture pH to 3 during *P.pastoris* fermentation has been extensively used [5, 83, 113, 115, 117, 141]. At pH 3 neutral proteases are inactivated reducing degradation of the protein of interest. Proteolysis is unlikely to have a large effect on the production of G-CSF in this study, as the strain used SMD1168 has been selected for its deficiency in a number of proteases [5, 15]. Also the medium used is rich in protein which provides sacrificial substrate for proteolysis.

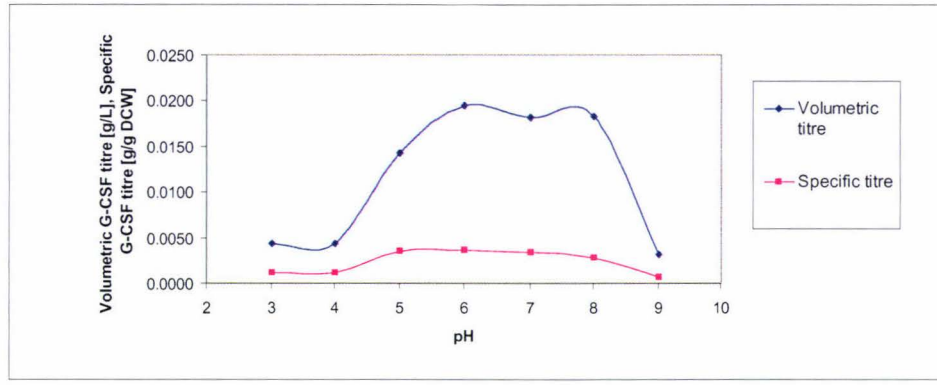

Figure 24: Effect of various pH on the volumetric and specific titres of G-CSF after 3 days. 25mL of enhanced YEHD medium was inoculated with 1mL of seed in a 250mL shaker flask. This culture was placed in a shaking incubator at 30°C and 200rpm for 24 hours. The culture was then centrifuged at 3000g for 5 minutes. Pelleted cells were resuspended in fourteen 25mL cultures at an optical density of 5 in YEHD medium with 0.5% (v/v) methanol replacing the dextrose and supplemented with 0.1M phosphate buffer. The cultures were adjusted in duplicate to a pH 3, 4, 5, 6, 7, 8, 9 with 2N sodium hydroxide and 2M hydrochloric acid. Cultures were grown for three days at 30°C, 200rpm with 0.5% (v/v) methanol added each day.

4.3.5 Methanol Concentration

Shown in Figure 25 both the volumetric and specific titre of G-CSF increased with increasing methanol up to 1.0% (v/v) per day. 0.5-1.0% (v/v) methanol per day is widely used for shaker flasks cultures of *P.pastoris* [5, 52, 82-86]. Above 1% (v/v) methanol per day the volumetric titre of G-CSF decreased rapidly with 40% less recombinant G-CSF produced when induced with 2% (v/v) methanol. Guarna et al. [52] observed a similarly decline in recombinant protein production when the methanol concentration exceeded 1% (v/v). The reduction in recombinant protein production is likely due to the accumulation of toxic metabolic products. The first step in the metabolism of methanol in *P.pastoris* is the oxidation to formaldehyde by the enzyme alcohol oxidase (AOX). In shaker flasks the next step in methanol

metabolism is often limited by the supply of oxygen causing the accumulation of formaldehyde and other metabolic products such as hydrogen peroxide which are toxic to cells [5]. It is therefore important to ensure the maximum oxygen transfer possible is achieved in shaker flask through using optimum shaker culture volumes (10-20% of total volume), the use of loose foil as lids and by ensuring the agitation rate is as high as possible. Concentration above 2% (v/v) methanol were not tested here as they have previously been shown to inhibit growth of *P.pastoris* [5, 82]. Some studies have found that recombinant protein titres increase at lower methanol concentrations [5, 56, 104]. Hong et al. [5] found that culture methanol concentration had a marked effect on the production of active heterologous laccase, with the recombinant protein titre increasing five fold when lowered from 1.0% to 0.5% (v/v). Reducing the concentration of methanol leads to reduction in the culture growth rate. Lower growth rates maybe desirable for the production of some recombinant protein products [5, 56].

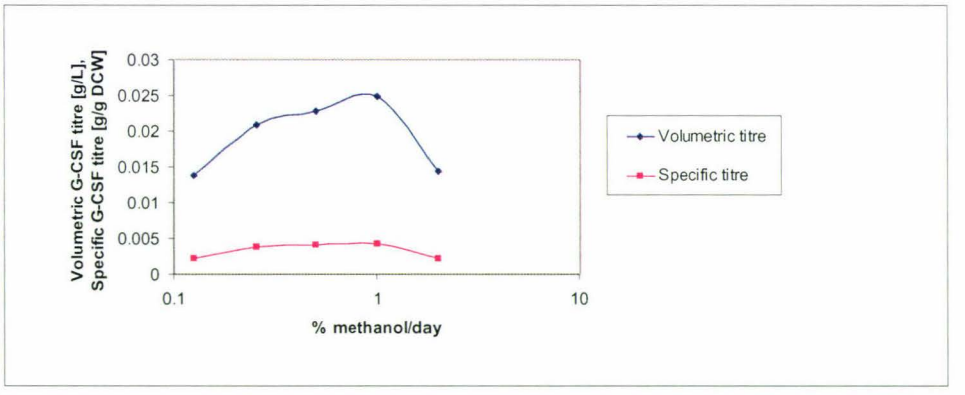

Figure 25: Effect of various concentrations of methanol on the volumetric and specific titre of G-CSF after 3 days. 25mL of enhanced YEHD medium was inoculated with 1mL of seed in a 250mL shaker flask. This culture was placed in a shaking incubator at 30°C and 200rpm for 24 hours. The culture was then centrifuged at 3000g for 5 minutes. Pelleted cells were resuspended in ten 25mL cultures to an optical density of 5 in enhanced YEHD medium with; 0.125, 0.25, 0.5, 1.0, 2.0% (v/v) methanol replacing the dextrose. Cultures were grown for three days with the same amount of methanol added to the culture each day.

An additional experiment was performed to test the effect of induction by direct addition of methanol to the growth medium. This was compared with the standard shaker flask procedure where cells are removed by centrifugation and resuspended in fresh medium containing methanol (induction medium). The experiment was carried out as the latter technique is more time consuming and involves the transfer of the cells to and from centrifuge tubes increasing the chances of contamination. Unfortunately the latter technique of removing the growth medium prior to induction produced significantly more recombinant protein (results not shown). Both dextrose and glycerol are known to repress recombinant protein expression regulated by the AOX promoter [83]. The lower protein titre using direct addition of methanol may either be due to some residual dextrose remaining in the medium or due to the depletion of essential amino acids and trace elements which are replenished when the medium is changed.

4.3.6 Fermentation

Fermentations were initially grown in batch culture until the exhaustion of the glycerol in the media as indicated by a sharp increase in pH and DO. This occurred after approximately 15 hours with the cell density reaching 20g DCW/L. A feed made up of 50g/L of glycerol containing 1.2% (v/v) *Pichia* trace metal (PTM_1) solution was then started at an initial rate of 12mL/h. The slow feed was used to increase the cell-density while gradual depressing the AOX promoter by maintaining the actively growing cells in a semi-starved state. Maintaining the culture in a semi-starvation for up to six hours a prior to induction has been found to increase gene expression [103]. As the culture grew the feed was progressively increased to 24mL/h however every half an hour the feed was stopped till the dissolved oxygen spiked to ensure that the glycerol did not accumulate in the culture.

Table 11: Fermentation protocols used for *P.pastoris* G-CSF.

Stage	Fermentation characteristic[a]		
	Time	Feeding rate	Feeding Solution
1) Batch phase	0-18hr	No feeding	-
2) Glycerol fed-batch	18-22hr	12-24mL/hr	50g/L glycerol[b]
3) Glycerol depletion	22-22.5hr	No feeding	-
4) Methanol induction	22.5-23hr	1mL pulse	methanol
5) Methanol fed-batch	>23hr	2-12mL/hr	methanol[b]

[a] Protocol based on Pichia Fermentation Process Guidelines (Invitrogen).

[b] Feed solutions contained 12mL/L *Pichia* Trace Metal (PTM_1) solution.

After 4 hours of glycerol feeding the culture reached a cell density of approximately 34g DCW/L. The glycerol feed was stopped and a half an hour depletion period was implemented to ensure that the cells consumed all the residual glycerol in the broth. All residue glycerol must be removed prior to methanol feeding as it is known to repress the AOX promoter reducing the level of recombinant expression [26, 56, 103]. A pulse of 0.1% (v/v) methanol was then added to initialise induction. Pulses of methanol were used during the initial stages of induction as apposed to a continuous feed to avoid repression from the accumulation of methanol while the alcohol oxidase promoter was not fully induced. During this initial period of induction the pH stabilised and the requirement for ammonium ceased. The stabilisation of the pH has been suggest to be a result of cells stopping growing while the synthesis of alcohol oxidase is switched on [103]. Once the methanol was consumed as indicated by a rapid rise in DO (after 30 minutes – 1 hour), a feed of methanol contain 1.2% (v/v) of PTM_1 solution was

started. PTM_1 solution contains trace metals and biotin which have been found to aid the achievement of final cell densities in excess of 100g DCW/L [55]. Shown in Table 11 are the fermentation protocols used for the production of G-CSF. These protocol were based on the Pichia Fermentation Process Guidelines (Invitrogen)

Four fermentations were carried out testing a number of different rates of methanol feeding between 3-12mL/h, however the culture consistently stopped growing once it reached a cell density of 40g DCW/L with less than 10% of the recombinant protein production achieved in shaker flask cultures. This result was unexpected as *P.pastoris* has been widely reported to be capable of growing to high cell densities in excess of 150g DCW/L [4, 26, 87, 110, 113]. Under microscopic inspection the cell appeared to have irregularities in their cell wall which were not observed in shaker flask cultures. These irregularities could be a result of stress from toxic conditions or due to nutrient deficiencies in the medium. To determine whether the inability to sustain growth was due to the medium components or death of the cells, 10mL of stationary culture was centrifuged to separate the cells from medium. The cells were then resuspended in fresh medium and the suspect medium filter sterilised before a new frozen stock of *P.pastoris* was added. Both cultures were grown overnight in 25mL universal bottles at 30°C and 250rpm. After 48 hour, the fermenter cells in new medium had grown however the new cells in the suspect fermenter medium had not. This suggests that the cells are still viable and that the medium is likely to be either deficient or toxic to cell growth.

Initially the hypothesis of a deficiency in the culture medium was investigated with the complex medium enhanced YEHD used for a fermentation under the same feeding conditions used previously. YEHD contains a high

concentration of yeast extract and peptone which supplied free amino acids and trace metals which may be deficient in the defined medium. The culture grew more quickly than using the basal salt medium as expected as cells grown on complex media such as YEHD do not have to synthesise their amino acids. Consistent with the previous fermentations, growth ceased once the culture cell density reach 40g DCW/L, thus suggesting that deficiencies in the media were unlikely to have caused the termination of growth. To further ensure that this was the case an attempt was then made to revive the cells in four separate 10mL cultures of filter sterilised fermenter medium. New inoculum was added with additional carbon (glycerol), nitrogen (ammonium solution), amino acids (yeast extract) and trace metals (PTM_1 solution) to the separate cultures. None of theses culture showed signs of cell growth after two days of incubation at 30°C and 250rpm further suggesting that it unlikely a nutrient deficiency was the cause of the low cell density in the fermentations.

Possible materials which could inhibit growth include cell metabolites, the product or accumulated nutrients. In the absence of oxygen *P.pastoris* is known to accumulate metabolites such as formaldehyde and hydrogen peroxide, which are toxic to cells. In all the fermentations the dissolved oxygen was maintained at 30% of air saturation to ensure that these metabolites did not accumulate. The fermentation product G-CSF has previously been expressed in *P.pastoris* with no reports of inhibitory behaviour. It is most likely that media or feed components contributed to the inhibition of growth. A mass balance based on the cellular composition of yeast was performed to compare the elements supplied in the Basal Salt medium with those required to sustain a cell density of 100g DCW/L [236]. Shown in Figure 26 less than 1% of the required amount of sodium was supplied in the basal salt medium. Sodium is however unlikely to be deficient as during autoclave of the fermenter phosphate buffered saline (PBS) was

added to the fermenter at concentrations that are more than sufficient to cover the cellular requirement to reach 100g/L (0.368g sodium per run). Interestingly both magnesium and sulphur were supplied in 9 and 50 times in excess of their respective requirements. 50% of these excesses are supplied by the magnesium sulphate in the initial medium. Magnesium sulphate is a common additive to media to prevent the precipitation of salts during fermentation [45, 71, 101]. A fermentation run with half the concentration of magnesium sulphate was carried under the procedure outlined previously however produced no improvement in biomass or recombinant protein titre. Further work should look at optimising the medium and reducing the concentration of possible inhibitor components. The most likely inhibitory compound is methanol. Mut^+ strains of *P.pastoris* as used here, can only tolerate up to 0.5g/L of residual methanol [103]. This means that at high growth rates unless the balance between supply and demand is accurate either inhibitory concentrations or starvation can occur in a very short period of time. As outlined previously, pulse feeding of methanol was used to ensure that methanol did not accumulate during initial induction. A slow methanol feed was then begun after the initial methanol pulse was consumed. However, methanol may still have accumulated to inhibitor levels. Brierley et al. [56] found that for some Mut^+ strains of *P.pastoris* it took up to 4 hours for the AOX promoter to be fully induced. A retest for the Mut^+ phenotype was performed according to the protocols previously outline [232] with the G-CSF clone passing the requirements (data not shown). However the result of test result which can be displayed on a continuum was very close to the lower limit which would designate it a Mut^s strain. As discussed in the section on investigating the optimum optical density at induction, the G-CSF strain exhibited traits similar to Mut^s strains. Protocols for the fermentation of Mut^s commonly differ in that the methanol feed rates are much lower, with a maximum feed rate of 3mL/hr [5, 116]. Due to a slow initiation of induction and slow metabolism rate, methanol may have accumulating to inhibitory levels in the first few hours of induction.

Figure 26: Comparison between chemical compounds in the basal salt medium with 150mL of dextrose feed and 200mL of methanol feed with the cellular composition of a yeast culture at 100g/L DCW [236].

Media component	Amount [g]	Chemical composition [g]											
		C	N	P	S	K	Mg	Na	Ca	Fe	Cu	Mn	Mo
Phosphoric acid	22.6	0	0	7.232	0	0	0	0	0	0	0	0	0
Calcium sulphate	0.93	0	0	0	0.1767	0	0	0	0.2139	0	0	0	0
Potassium sulphate	18.2	0	0	0	3.276	8.19	0	0	0	0	0	0	0
Magnesium sulphate	14.9	0	0	0	4.023	0	2.98	0	0	0	0	0	0
Potassium hydroxide	4.13	0	0	0	0	2.891	0	0	0	0	0	0	0
Dextrose	50	20	0	0	0	0	0	0	0	0	0	0	0
Water	1000	0	0	0	0	0	0	0	0	0	0	0	0
Ammonium hydroxide	100	0	40	0	0	0	0	0	0	0	0	0	0
Cupric sulphate.$5H_2O$	0.0261	0	0	0	0.003393	0	0	0	0	0	0.006786	0	0
Sodium iodide	0.000348	0	0	0	0	0	0	5.22E-05	0	0	0	0	0
Manganese sulphate.H_2O	0.01305	0	0	0	0	0.00248	0	0	0	0	0	0.004307	0
Sodium molybdate.$2H_2O$	0.00087	0	0	0	0	0	0	0.000165	0	0	0	0	0.000348
Boric acid	0.000087	0	0	0	0	0	0	0	0	0	0	0	0
Cobalt chloride.$6H_2O$	0.002175	0	0	0	0	0	0	0	0	0	0	0	0
Zinc chloride	0.087	0	0	0	0	0	0	0	0	0	0	0	0
Ferrous sulphate.$7H_20$	0.283	0	0	0	0.03396	0	0	0	0	0.17829	0	0	0
Biotin	0.00087	0.000426	9.57E-05	0	0.000113	0	0	0	0	0	0	0	0
Sulfuric Acid	0.022	0	0	0	0.00726	0	0	0	0	0	0	0	0
Dextrose feed	75	30.00001	1.98E-06	0	0.007873	0.000513	0	1.42E-05	0	0.036855	0.001404	0.000891	7.2E-06
Methanol feed	200	75.00542	5.28E-06	0	0.020994	0.001368	0	3.79E-05	0	0.09828	0.003744	0.002376	1.92E-05
Total		125.0059	40.0001	7.232	7.549293	11.08536	2.98	0.00027	0.2139	0.313425	0.011934	0.007574	0.000374
% cell composition [100g/L]		99%	297%	425%	5807%	443%	993%	0%	107%	121%	199%	189%	187%

4.3.7 P.pastoris oFSH Fermentation

The protocols outlined in Table 11 for fermentation of the G-CSF clone were tested using another clone of *P.pastoris* expressing recombinant ovine follicle stimulating hormone (*o*FSH) to see whether the protocols could be used for other strains. 1250mL of Basal salt medium was grown batch wise for 25 hours. Glycerol feeding was initiated at a rate of 12mL/h. However, shown in Figure 27 the feed rate was quickly increased to 24mL/h after 1 hour and then 36mL/h thirty minutes later as brief pulses in the feed produced fast DO spikes. Once the culture reached 60g DCW/L (after 30 hours), the glycerol feed was stopped and the culture DO allowed to spike. Methanol feeding was started at 3.6mL/h for 24 hours, then increased to 5.4mL/h for four hour and 12mL/h for the rest of the culture. Figure 27 shows that the DO concentration rose for the first 3 hours of methanol induction (between hours 30-34 post-inoculation) as the culture adapted to methanol. After 34 hours the DO concentration steadily declined as the culture cell density increased. The culture was grown to a cell density of 71g DCW/L at which stage the culture was stopped as the laboratory supply of methanol ran out. Although both clones were the same strains (SMD1168), the growth rate of the later *o*FSH clone was much quicker and showed no signs of inhibition at 40g DCW/L. The successful growth of the *o*FSH clone is likely due to either the quicker growth rate preventing the accumulation of methanol to inhibitor level or just a comparative difference to the G-CSF clone which may exhibit abnormal behaviour. A range of different constructs of *P.pastoris* expressing G-CSF could be tested to assess whether the low yields in fermenter cultures is due to a clonal limitation. Unfortunately the titre of recombinant protein was unable to be quantified as the *o*FSH could not be isolated from the large number of other proteins produced.

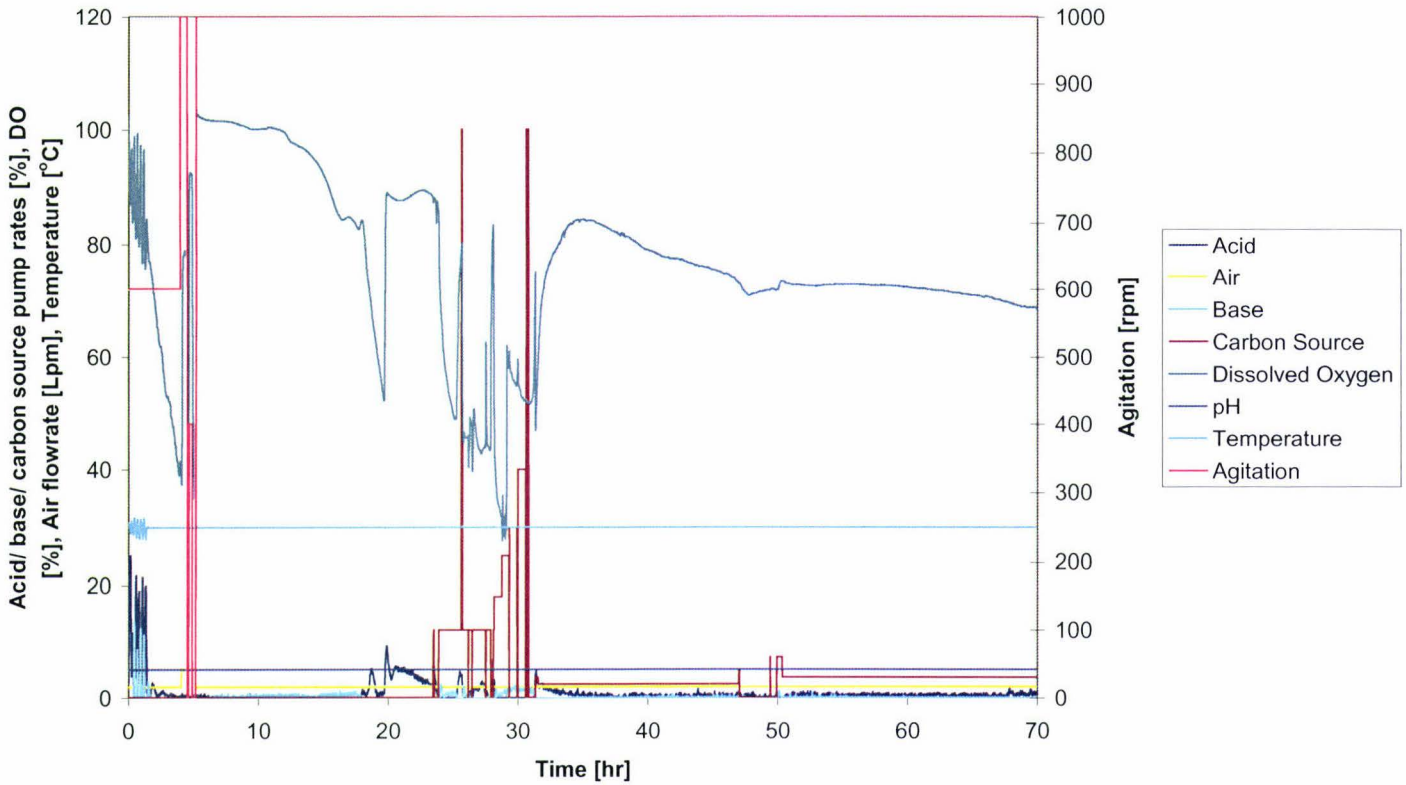

Figure 27: Typical *P.pastoris* fermentation profile of various measured variables. Acid/ base and carbon source (either glycerol or methanol) pump feed rate (1.5mL/%). Sparging airflow rate, culture dissolved oxygen concentration as a percentage of air saturation, pH, temperature and agitation rate. 1250mL of basal salt medium was inoculated with 150mL of overnight seed at OD of approximately 5. Agitation was manually stepped up to 1000rpm to maintain the DO above 30%, aeration was constant at 2 L/min with pure oxygen blended at high cell-densities to maintain the DO above 30%. The culture temperature was maintained at 30°C and pH at 5.0. Fed-batch feeding of the culture was carried out according to protocol outline previously [21]. Cultures were grown till the glycerol in the initial medium was completely exhausted as indicated by a DO spike (~18 hours). A continuous feed of 50% glycerol containing 1.2% (v/v) of *Pichia* Trace Metal (PTM_1) solution was then started at 1.8% (v/v) for four hours to accumulate biomass. The glycerol feed was stopped for a half hour starvation period before induction. Induction was initiated using a continuous feed of methanol containing 1.2% (v/v) of PTM_1 solution at an initial rate of 3mL/h which was increased over 28 hours to 12mL/h at which level it was retained at for the remainder of the culture.

4.4 RECOMENDATIONS

A maximum of 0.028g/L of G-CSF was produced in the shaker flask experiments. This is consistent with previous reports of recombinant protein yields in shaker flasks which are typically 0.010-0.030g/L of recombinant protein [6] and is an improvement on previous yields achieved at Agresearch (personnel communication, Neil Wedlock, Agresearch, Upper Hutt, New Zealand). However, fed-batch fermentation titres have been observed to be 10-20 times higher [104, 110, 117]. As discussed previously this is most likely due to the accumulated of methanol to toxic levels. Further investigation should look to maintain pulse feeding for a longer period and testing lower methanol feed rates below 3mL/h. Ultimately if large numbers of *P.pastoris* fermentations are to be performed, then the investment in an online methanol analyser would ensure that methanol was maintained below inhibitory concentrations and at a level that maintained culture induction [115].

4.5 CONCLUSION

The objective of this chapter was to investigate methods to increase the titre of G-CSF in *Pichia pastoris*, develop parameters for the production in a small scale bioreactor and provide a basis of fermentation knowledge for the production of other recombinant products in *P.pastoris*. *P.pastoris* is often selected by molecular biologists when a large amount of soluble, glycosylated protein is required. *P.pastoris* has the advantages of being able to handle larger proteins than *E.coli*, perform some post-translational modifications, grow on simple inexpensive media and proteins are generally secreted simplifying downstream processing. The production of G-CSF was found to be influenced by the medium composition, cell to methanol concentration and culture pH. Various media were tested with enhanced YEHD which contains a phosphate buffer and a high concentration of yeast extract producing the highest titre of G-CSF. G-CSF production was at a maximum when cells were resuspended to an optical density of 8 prior to induction and when 1% (v/v) of methanol was added per day. *P.pastoris* can be grown over a wide range of pH from 3-7 with minimal effect on the growth which has been widely utilised to optimise fermentation conditions [56, 87, 237]. Expression of G-CSF was highest at pH 6.0 with no benefits observed at high or low pH. A maximum volumetric titre of 28mg/L of G-CSF was produced in shaker flasks of enhance YEHD maintained at 30°C, pH 6.0 and 200rpm. Cells were resuspended to an optical density of 8 at induction with 1% (v/v) methanol added per day. This titre is an improvement on previous yields achieved at Agresearch and consistent with previous recombinant protein titres for shaker flasks of *P.pastoris* [6]. *P.pastoris* has been widely reported to be capable of growing to high cell densities in fed-batch cultures with corresponding increases of 10-20 times the recombinant protein produced in shaker flasks [104, 110, 117]. The growth of the G-CSF clone was found to be inhibited at 40g DCW/ L. This is likely due to the accumulation of toxic materials in the

culture medium. It was recommended that further experimentation should look at optimising the medium to reduce potential toxic components and also at testing a range of methanol feeding rates below 3mL/ h. The fermentation procedure developed was used to grow another clone *P.pastoris* oFSH. The latter clone showed no signs of growth limitations and reached a cell density of 71g DCW/L suggesting that the G-CSF clone may be exhibiting abnormal behaviour.

5 Insect cells

Insect cells can perform complex post-translation modifications, produce high active protein concentrations (up to 1g/L) and most proteins are extracellularly secreted simplifying downstream processing [31-33]. However, insect cells are extremely delicate, require high seeding ratios, can be infected by mammalian viruses and grow significantly slower than bacteria and yeasts cells [23, 31, 32]. Insect cell media are also more complex and expensive than prokaryote media, cells do not grow to as high cell densities and protein production is most commonly in the milligram per litre range [32]. Insect cell expression has enjoyed increased interest in recent years as a result of fears worldwide over the transmission of prion diseases in mammalian derived products [7]. The ability of insect cells to grow on serum free media (SFM) allows the production of complex eukaryotic proteins which are free of all mammalian material and potentially infection materials.

Follicle-stimulating hormone (FSH) is a glycoprotein gonadotropin secreted by the anterior pituitary gland. Development of ovarian follicles in sheep has been found to be largely controlled by the supply of FSH [7]. Purified ovine FSH produced at AgResearch has been used to induce super-ovulation in sheep. A recombinant source of FSH is sought for a number of reasons including; native FSH is difficult to purify in large quantities from pituitary glands. Pure preparations of FSH are also difficult to achieve without impurities such as luteinizing hormone (LH), which is considered to be responsible for observed variations in superovulation found with native FSH [7]. FSH extracted from pituitary glands may also contain infectious materials such as prion proteins. Insects cell have been chosen here to avoid the use of any animal products in the production process, minimising the risk of transmission of infectious diseases. Prior to this research all *o*FSH produced at Wallaceville Animal Research Centre was produced in T-flasks and small

shaker flasks. Combined with extremely low titres only small amounts of *o*FSH could be produced without the injection of considerable time and resources. The objective of this chapter is to investigate methods to increase the titre of *o*FSH and develop parameters for production in a small scale bioreactor to increase the availability of protein for future trials. This section also aims to provide a basis of fermentation knowledge for the development of other recombinant protein products produced in insect cells.

5.1 MATERIALS AND METHODS

5.1.1 Strain and Media

The insect cell line High Five™ (BTI-TN-5B1-4, Catalogue number B855-02, Invitrogen, Carlsbad, California, USA) expressing recombinant *ovine* follicle stimulating hormone (*o*FSH) was supplied by Dr Jun Lin (Agresearch, Upper Hutt, New Zealand). An *o*FSHβα fusion gene described previously [141] was cloned on the pMIB/V5-His vector (Invitrogen) for transfection. The pMIB vector contains genes that confer resistance to the antibiotics blasticidin and ampicillin and the gene of interest is constitutively expressed with a poly-histidine (6 x histidine) tag and a V5 epitope to simplify purification and detection. Stock cultures were stored at -80°C with 3 x 10^6 cells/mL in 42.5% (v/v) conditioned Express Five® serum free media (SFM), 42.5% (v/v) Express Five® SFM, 10% (v/v) dimethyl sulfoxide (DMSO) and 5% (v/v) fetal calf serum (FBS) in 1mL cryotubes. All experiments were performed in Express Five® Serum Free media (Invitrogen) supplemented with per litre; 10mL penicillin/ streptomycin, 100μL gentamycin and 10mL L-glutamine. Growth medium additionally contained 10% (v/v) bovine fetal calf serum (Invitrogen).

5.1.2 Adaptation of Cell line

The *o*FSH cell line was adapted and maintained by Jessica Tiffan (Agresearch, Upper Hutt, New Zealand). Frozen 1mL cryotubes were thawed by placing in a 37°C water bath. Cells were then transferred into 9mL of warmed growth medium in a centrifuge tube and centrifuged at 800g for 10 minutes to remove the DMSO. The pelleted cells were then resuspended in 6mL of

growth medium with 0.010g/L of blasticidin in a 15mL culture flask. The culture was then incubated at 27°C till the cells formed a confluent monolayer. For the first five passages the cells were resuspended 50:50 in SFM with 0.010g/L of blasticidin to adapt the cell to serum free media. Cells were incubated at 27°C in 50mL culture flasks for two days between passage splits. The cells were then passaged in exclusively SFM till the doubling time approximately stabilised to 24 hours (2-3 passages) before transferring the cells to a 250mL shaker flask. Shaker flasks cultures were placed in a shaking incubator at 10rpm and 27°C. Cell counts were performed every two-three days and the cell concentration diluted back to between 0.5 and 0.8 x 10^6 cells/mL. If during suspended culture the cells developed large clumps (greater than 10 cells) then 10 units of heparin (Invitrogen) was added. Cell lines were maintained for a maximum of 30 passages before the line was re-established from frozen stock.

5.1.3 Cell Density and Growth rate

Cell density and viability were measured by the trypan blue exclusion method using a hemocytometer [238]. 0.1mL of sterile trypan blue solution (0.4% (v/v) trypan blue in PBS adjusted to pH 7.2) was added to 0.9mL of culture. Cell clumps were broken up by repeated drawing through a 0.100mL pipet tip. 0.010mL was then loaded onto a hemocytometer and examined at 20 times magnification. Total cells and dead cells counts were performed with the dead cells staining blue. The culture specific growth rate was calculated according to the equation in Figure 28 which has been described previously [239]. This measurement of specific growth rate is only accurate during exponential growth [240].

$$\mu=(\log_e N_t-\log_e N_0)/t$$

Figure 28: Equation for the calculation of the specific growth rate (μ). N_t is the cell number at time t, N_0 is the cell number at time 0. t is the time period between 0 and t.

5.1.4 Environmental Factor Screening Trial

The significance of various environmental factors including temperature, cell density, agitation rate, culture volume, flask geometry and antibiotic addition were investigated using a Plackett Burman experimental design (discussed in Chapter 3). Eight 250mL shaker flasks were grown under the conditions outlined in Table 12. A total/ viable cell count and Western blot was performed after 48 hours [93].

5.1.5 Inoculation Cell Density Trial

The effect of the initial inoculation cell density was also tested in shaker flask trials. Five 25mL cultures in 250mL shaker flasks were resuspended at 0.4, 0.6, 0.8, 1 and 2 x 10^6 cells/mL. Cultures were grown at 27°C at 100rpm in a shaking incubator. Total/ viable cell counts were taken each day and a Western blot performed after 48 hours.

5.1.6 Agitation Rate Trial

The effect of agitation rate on the growth of cells and expression of recombinant ovine FSH was tested in shaker flasks at 80, 100, 120, 140 and 160rpm. Cells were inoculated into 25mL of Express Five medium in 250mL shaker flasks to a concentration of 1 x 10^6cells/mL and grown at 27°C in separate shaking incubators. Total/ viable cell counts were performed each day and a Western blot was performed after 48 hours.

5.1.7 Bioreactor

The cultivation of the High Five cells was carried out in a 3300mL glass jar bioreactor (described in Chapter 2). The temperature was controlled at 27°C and agitation provided by a three blade marine propeller at 150rpm. Culture DO was controlled at 50% of air saturation through the automated adjustment of the oxygen fraction in the headspace above the culture. The DO concentration was controlled by headspace sparging till approximately 100 hours at which stage direct sparging at 0.8 L/min through a base mounted sparging ring was used as headspace sparging was insufficient to control the DO. The bioreactor was also fitted with 0.22µm filter placed on the end of sampling port to allow supernatant to be removed while retaining cells. The bioreactor was inoculated with 400mL of exponentially growing cells at 0.15 x 10^6 cells/mL in 1000mL of Express Five® Serum Free media supplemented with per litre; 10mL penicillin/ streptomycin, 0.100mL gentamycin and 10mL L-glutamine. The entire bioreactor was wrapped in tin foil to exclude light as the medium is sensitive to ultraviolet. Culture feeding was begun after 120h with fresh medium fed at a rate of 0.02h^{-1}. Culture supernatant was removed to maintain a constant culture volume in the bioreactor.

5.1.8 Western Blot

Recombinant *o*FSH production was detected by Western blotting. Western blotting of the *o*FSH was kindly performed by Jessica Tiffan (Agresearch, Upper Hutt, New Zealand). Culture samples were centrifuged at 800g for 15 minutes to remove the cells. The supernatant was then run on a standard SDS-PAGE with a 4% stacking and 13.5% resolving gel according to the method outlined in Chapter 2. Proteins were then transferred electrophoretically to a mixed ester nitrocellulose membrane (Hybond-C; Amsterham Pharmacia Biotech) in alkaline transfer buffer (2% (v/v) methanol, 3g/L Tris, 14g/L glycine) using the Mini trans-blot (BioRad). The filter was then incubated in blocking solution (5g/L Tris-HCl, pH 7.5, 29g/L NaOH, 0.1% (v/v) Tween-20, 50g/L non-fat milk powder) for 30 minutes at 37°C. The filters were washed in TBS buffer (5g/L Tris-HCl, pH 7.5, 38g/L NaCl, 0.1% (v/v) Tween 20) and then incubated overnight at room temperature in rabbit antibody raised to *o*FSHα (supplied by Keith Henderson Agresearch, Upper Hutt, New Zealand) diluted 1/500 in blocking solution. Unbound primary antibody was removed by washing in TBS buffer and the filter incubated for 6 hours at room temperature in goat anti-rabbit IgG (Sigma) conjugated to horseradish peroxidase (HRP) diluted 1/2000 in blocking solution. Unbound secondary antibody was removed by washing in TBS buffer. The blot was developed by incubating in developing solution (100μL of 250mM 3-aminophthalhydrazide (Sigma), 44μL of 90mM p-carminic acid (Sigma), 17.85mL distilled water, 6μL of 30% (v/v) hydrogen peroxide). The filter was exposed to photographic film and developed to view the image. The relative density of bands was evaluated densitometrically from photographs using an imaging densitometer GS-800 (Bio-Rad) and Quantity One (Bio-Rad) analytical imaging software.

5.2 RESULTS AND DISCUSSION

5.2.1 Environmental Screening Trial

Shown in Table 12, the rate of agitation had the most significant effect on *o*FSH production with more recombinant protein expressed at the higher agitation rate of 100rpm. This is consistent with Taticek et al. [30] who tested a number of agitation rates between 60-180rpm and found that culture growth and cell productivity increased with increasing agitation. Oxygen transfer has been suggested to be the most common limiting factor in insect cell cultures [118]. Agitation rate, culture volume and the use of baffles in shaker flasks all interrelate in determining the oxygen transfer [232]. Agitation, due to it high significance was subsequently selection for further investigation.

For 250mL shaker flasks used in this trial 10-20% volume of medium to shaker flask volume is recommended for optimal oxygen and nutrient transfer [232]. Recombinant protein expression was tested with a culture volume of 25 and 50mL in a 250mL shaker flask. It was found that there was a moderate increase in *o*FSH expression at the higher tested culture volume. This is an unexpected result as generally higher titres are achieved at lower volumes as the surface area to volume increases oxygen transfer [30]. This discrepancy may be due to the baffle geometry causing improvement in surface agitation at the higher volume improving the culture oxygen transfer.

Table 12 shows that whether the flask had baffles or not had little effect on the production of *o*FSH, with flasks containing baffles having slightly increased levels of expression. Baffles break up the circular motion of the fluid in the shaker flask and create turbulence improving the oxygen transfer and distribution of nutrients. Insect cells are however very shear sensitive as they do not have cell walls, only a thin cell membrane to protect them against rupture [79, 90, 135]. Baffles increase the shear forces on cells and may damage sensitive protein. For this reason a balance must be established between the conflicting demands of nutrient transport and shear sensitivity.

Most recombinant protein expressed in *T.ni* are grown at 27°C [37, 79, 118, 134]. Reducing the culture temperature reduces the rate of protein expression which has been previously shown to improve the activity of recombinant proteins in prokaryotes [3, 68]. Reducing the cultivation temperature from 27°C to 23°C had a negative effect on the expression of *o*FSH with a lower titre when grown at 23°C for two days. The lower titre is likely a result of a reduction in the rate of expression at the lower temperature. Reuveny et al. [133] found that insect cells produced similar titres of recombinant protein in the temperature range of 22 to 27°C, but increasing the temperature from 22°C to 27°C led to earlier production and an increase in the proportion of recombinant protein secreted in the culture medium.

Cell density has previously been reported to have a major effect on the production of a wide range of recombinant proteins produced in insect cells [35, 37, 88]. Shown in Table 12, the inoculation cell density was found to have a significant effect on the expression of *o*FSH. The volumetric expression of *o*FSH was higher when inoculated at a cell density of 0.6×10^6 cell/mL compared to 0.3×10^6 cells/mL. Generally insect cells are inoculated at cell densities between $0.3\text{-}0.8 \times 10^6$ cells/mL [30, 37, 88, 118]. Inoculation

at higher cell densities allows the attainment of high cell mass more quickly. However, the specific recombinant production has been found to decrease with increasing cell density [31, 32, 34, 118, 241]. A balance must therefore be found between achieving high cell-densities and maintaining the specific titre of recombinant protein. Inoculation cell density was selected for further investigation due to its high significance.

Blasticidin was included in the environmental screening trial as concern had been expressed about the level of infected cells in the master seed (personnel communication, Jun Lin, Agresearch, Upper Hutt, NZ). Blasticidin is used to provide selective pressure to prevent the ejection of the foreign DNA from infected High Five cells. Shown in Table 12, the addition of blasticidin had no effect on the expression of *o*FSH suggesting that clone is relatively stable over a single passage in the absence of the antibiotic. However the effectiveness of blasticidin as a selection agent is questionable. An experiment was preformed by another scientist at Agresearch to test the concentration of blasticidin required to kill native High Five cells which did not contain the resistance gene. Blasticidin concentrations up to 0.1g/L, 5 times the recommended concentration [238] were tested with the cultures still containing viable cells (Jess Tiffen, personal communication 2003). Blasticidin is an extremely expensive component and some manufactures will not allow the use of antibiotic such as blasticidin in their production facilities. If *o*FSH is to become a commercial reality then the establishment of a pure and stable master seed is important to ensure product consistency and to reduce or eliminate the use of antibiotics.

Table 12: Plackett and Burman trial assessing the significance of various environmental factors on the volumetric titre of øFSH.

Experiment	Variable[a]							Response
	Temperature [°C]	Cell Density [10^6 cells/mL]	Volume [mL]	Baffles	Agitation [rpm]	Antibiotics [g/L]	Dummy	FSH [g/L]
1	L	L	L	H	H	H	H	0.0001
2	H	L	L	L	L	H	L	0.0001
3	L	H	L	L	H	L	L	0.0013
4	H	H	L	H	L	L	H	0.0004
5	L	L	H	H	L	L	H	0.0001
6	H	L	H	L	H	L	L	0.0001
7	L	H	H	L	L	H	L	0.0008
8	H	H	H	H	H	H	H	0.0063
Σ(H)	8.16	8.84	8.63	6.95	9.05	7.32	6.95	
Σ(L)	2.32	1.63	1.84	3.53	1.42	3.16	3.53	
Difference	5.84	7.21	6.79	3.42	7.63	4.16	3.42	
Effect	1.46	1.80	1.70	0.86	1.91	1.04	0.86	
Mean square	4.27	6.50	5.76	1.46	7.28	2.16	1.46	
F-test	2.92	4.44	3.94	1.00	4.98	1.48		
Significance	-	+	-	-	+	-		

[a] The low and high levels for each variable were respectively; temperature (23, 27°C), cell density (0.3, 0.6 x 10^6 cells/mL), shaker culture volume (25, 50mL), with and without baffles, agitation (60, 100rpm), antibotics (none, 0.010g/L) and a single dummy variable was used to assess the amount of variability. A 95% confidence interval was used for the test of statistical significance.

5.2.2 Inoculation Cell Density

Shown in Figure 29 the culture growth rate of High Five cells increased with increasing inoculation cell density up to 1 x 10^6 cells/mL. A few studies have found that inoculating at cell densities above 0.8 x 10^6 cells/mL prolongs growth and increases productivity [242, 243]. However more commonly insect cells are inoculated at cell densities between 0.5-0.8 x 10^6 cells/mL as in this range cells grow at an exponential rate [88]. The growth rates of the *o*FSH cultures were lowest at the two lowest tested inoculation cell densities, with the culture inoculated at 0.4 x 10^6 cells/mL only reaching a cell density of 1.53 x 10^6 cells/mL after 48 hours (data not shown). This existence of a minimum inoculum concentration below which growth is slow has been suggested to be result of a requirement of insect cells for carry over growth-promoting metabolites [88]. A number of studies have recommended that cell densities are maintained above 0.5 x 10^6 cells/mL to prevent significant lags in growth [30]. Shown in Figure 29 when inoculated at 2 x 10^6 cells/mL the growth rate was lower than at 1 x 10^6 cells/mL. No references to the use of inoculation cell density over 2 x 10^6 cells/mL were found. The lower growth rate at 2 x10^6 cells/mL is likely due to the larger seeding volume, meaning that less fresh medium was supplied limiting the availability of nutrients for growth.

Shown in Figure 29 the specific cellular titre of *o*FSH was at a maximum at the lowest tested cell density of 4 x 10^5 cells/mL, decreasing as the inoculation cell density was increased. This is consistent with previous observations of insect cell expression and has been attributed to a combination of the depletion of nutrients critical for expression and cell to cell contact inhibiting specific protein production [31, 34, 35, 37, 88, 118]. The volumetric titre of *o*FSH increased with increasing inoculation cell density up to 1 x 10^6 cells/mL. This is likely due to an increase in cell number despite the

reduction in specific *o*FSH expression. Chico and Jager [34] found that maximum expression of a recombinant β-trace occurred at 1 x 10^6 cells/mL which is very low compared to the maximum cell density that can be achieved by the cell line. At the highest tested cell density of 2 x 10^6 cells/mL, expression decreased possibly due to the large numbers of cells using up nutrients maintaining growth, meaning less was available for recombinant protein expression. This problem could be circumvented by replacing the medium once the cells reach a certain cell density. Medium replacement after cultures reach 1 x 10^6 cells/mL has been previously found to increase in recombinant protein expression [79].

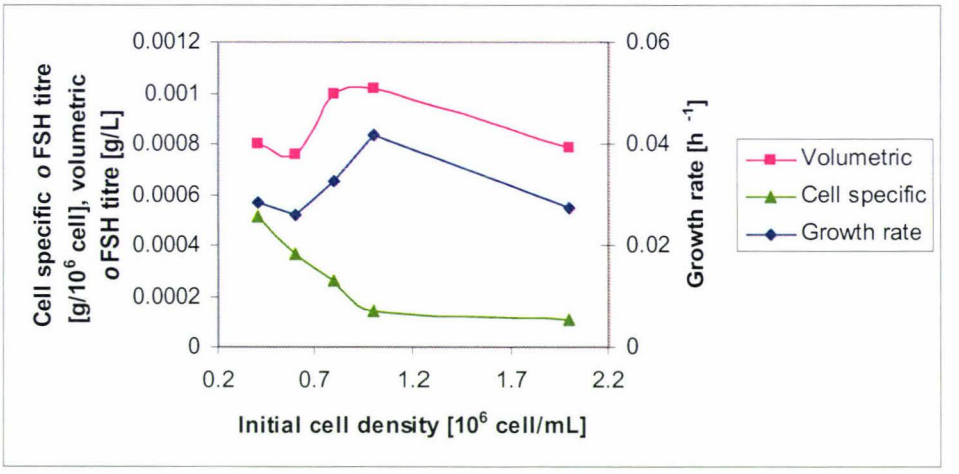

Figure 29: Effect of inoculation cell density on the growth rate of High Five cells, volumetric and cell specific expression of *o*FSH in Express Five. Six 250mL shaker flasks were inoculated with 0.4, 0.6 0.8, 1, 2 x 10^6 cells/mL in 25mL of Express Five. Cultures were then incubated at 27°C at 100rpm for 48 hours. The cell density and recombinant protein production was measured after 48 hours.

5.2.3 Agitation Rate

Shown in Figure 30, the growth rate of the High Five cells was found to increase with increasing agitation up to a maximum at 140rpm. Above 140rpm no significant change in growth rate was observed. Taticek et al. [30] investigated the effect of a range of agitation rates on *T.ni* cells and also found that the growth rate increased with increasing agitation rates up to 150rpm. The level of dissolved oxygen transfer has been suggested to be the limiting factor in insect cell culture in shaker flasks [118]. Increased agitation improved culture mixing and oxygenation which has been observed to lead to higher growth rates and the attainment of higher cell densities [30]. At agitations rates above 120rpm the cells were observed to aggregate together (data not shown). This has previously been suggested as a sign of insufficient adaptation to suspended culture. Saarinen et al. [37] maintained low levels of aggregation in suspended culture by increasing the agitation rate by a maximum of 5rpm at each passage and ensuring that the cells attained a doubling time of 24 hours before increasing the agitation rate further [37]. Further studies should look at whether further improvements in growth rate and expression can be made by slowly adapting the culture to suspension. Shown in Figure 30, above 140rpm no significant increase in cell growth rate was observed, likely as a result of oxygen transfer no longer being growth limiting. The experiment carried out at 160rpm had the lowest cell viability of all the cultures at 73%, with shear damage the likely cause (data not shown).

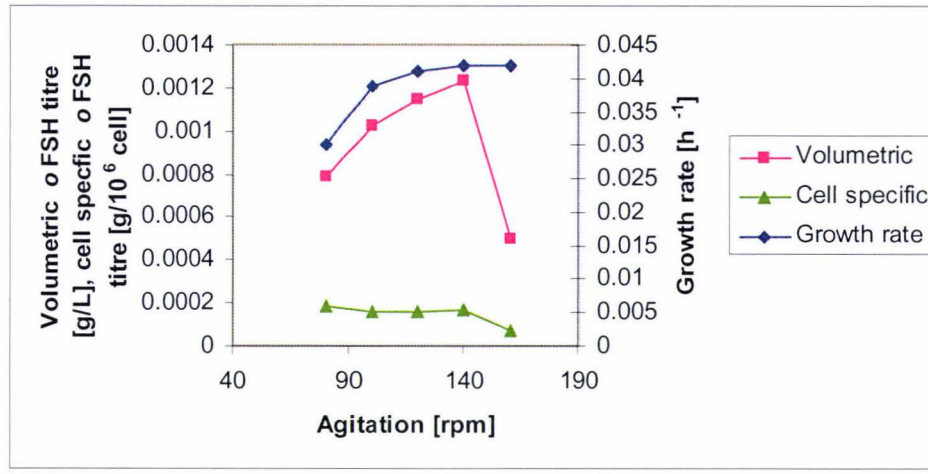

Figure 30: Effect of agitation on the growth rate of High Five cells, volumetric and cell specific expression of øFSH in Express Five. 250mL shaker flasks inoculated with 1 x 10^6cells/mL in 25mL of Express Five. Cultures were incubated at 27°C and agitated at 80, 100,120,140,160 rpm respectively for 48 hours. The cell density and recombinant protein production was measured after 48 hours.

The specific titre of *o*FSH was at a maximum at the lowest tested agitation rate of 80rpm and decreasing as the agitation rate was increased. It has been widely reported that the specific production of recombinant proteins in *T.ni* cells decreases with increasing cell density [31, 34, 35, 37, 88, 118]. Wickham et al. [31] observed a 6-fold reduction in specific recombinant protein production with an increase in cell density from 0.14-1.14 x 10^6 cells/mL. The decrease in specific recombinant titre has been suggested to be a result of nutrient depletion with medium supplement or replacement widely used to improve titres at the higher cell densities [36, 88]. The volumetric titre of *o*FSH increased with increasing agitation rate between 80-140rpm with 56% more *o*FSH produced at 140rpm than at 80rpm. Previous work done with this clone found that high agitation rates causes the secreted *o*FSH to aggregate (Figure 31), as observed by the formation of a band at 70kDa (personal communication, Jun Lin, AgResearch, Upper Hutt, NZ, 2003).

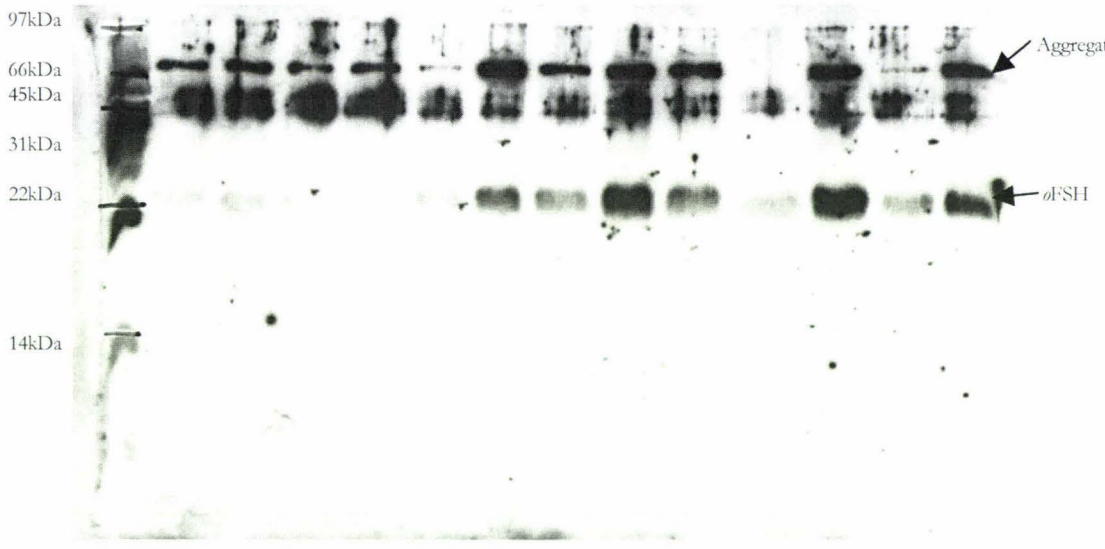

Figure 31: Western blot of aggregated *o*FSH protein at 70kDa. Proteins were separated on a 13.5% SDS-PAGE gel.

This has led previously to the optimisation of the culture condition at extremely low agitation rates of 10-60rpm to prevent losses to aggregation. However although the use of higher agitation rates increased the band of aggregated protein, it also led to an overall increase in titre of the non-aggregated *o*FSH (Figure 30). Above 140rpm both the specific and volumetric titre of *o*FSH decreased rapidly likely as a result of shear damage to the secreted protein. Joosten and Shuler [88] observed shear caused by an agitation rate of 150rpm had a detrimental on effect on the glycosylation of a secreted human placental alkaline phophatase (SEAP) expressed in *T.ni* cells.

A maximum of 0.0012g/L of *o*FSH was produced in shaker flask cultures when agitation was maintained at 140rpm, 27°C and the culture was inoculated with 1 x 10^6 cells/mL. This represents a significant improvement on previous shaker flask yields achieved at Agresearch (personal communication, Jun Lin, Agresearch Upper Hutt, NZ) and is consistent with a previous report for the production of recombinant bovine FSH produced in insect cells [7].

5.2.4 Bioreactor

Due to time constraints only one attempt was made to cultivate the High Five cells in a bioreactor. Time constraints also combined with a slow growing inoculum to prevent the bioreactor from being seeded at the previously determined optimal cell density. Shown in Figure 32, the concentration of oxygen required in the headspace to maintain the DO increased throughout the experiment till approximately 100 hours after inoculation, at which stage the required oxygen concentration reached 100%. Direct culture sparging

was begun after 118 hours to maintain the DO. The bioreactor culture grew until approximately 120 hours, when the cells went into a rapid decline. It was suspected the decline in the culture was likely the result of either a lack of cell adaptation to suspended culture, due to a reduction in the condition of the medium or due to shear damage from direct sparging.

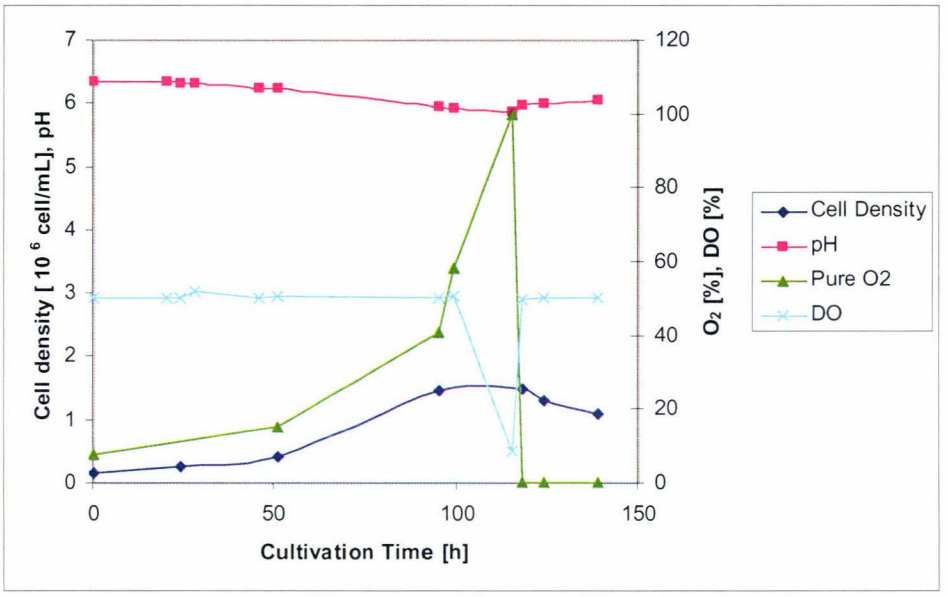

Figure 32: Profile of measured variables for the bioreactor culture of *o*FSH in High Five cells. The measured variables are; cell density [10^6 cells/mL], pH, pure oxygen in sparging gas [%] and culture DO [%]. 1L of Express Five medium was inoculated with 400mL of cells at 0.8 x 10^6 cells/mL. The DO was controlled at 50%, temperature 27°C, agitation 150rpm. Feeding with fresh media at a rate of $0.02h^{-1}$ was begun after 120 hours with supernatant removed to retain constant volume.

After 120 hours when the culture cell-density began to decline, a feed of fresh medium was begun to ensure the decline in growth was not due to a depletion of nutrients. Previously it has been shown that *T.ni* cells consume large amounts of amino acids in particular cystine and asparagine and that the addition of these amino acids is important for good cell growth [36], while others have found that supplementation of lipids are important to growth and

expression [36, 88, 134]. Express Five, the medium used in this fermentation contains all of these ingredients which have been suggested to be important for insect cell growth [238]. Shown in Figure 32 the feeding of fresh medium after 120 had no effect on the decline of the culture. This suggests that nutrient depletion is unlikely to have been the primary reason for the decline in cell density. At the same time as fresh medium was added culture supernatant was removed to maintain the culture volume. This helps to dilute any toxic metabolites or product which may have been inhibiting growth. Major metabolites produced by *T.ni* cells as they grow include ammonia and alanine. However surprisingly it has previously been found that insect cells are relatively unaffected by the accumulation of metabolites [145, 241].

Another possible explanation for the decline in the culture cell density, maybe that the cells were damaged by shear from the direct sparging of the bioreactor with air. Just prior to the culture going into decline, direct culture sparging was begun. Sparging was performed through a base mounted sparging ring described in Chapter 1 at the lowest controllable flow rate of 0.8L/min. Insect cells are relatively sensitive to shear, unfortunately most methods of providing sufficient oxygen transfer to maintain high-cell-density such as sparging cause shear [30, 79, 88, 145]. Previously it has been shown that damage to cells in culture occurs when bubbles created by sparging burst [137]. The design of the bioreactor used in this study (BioFlo 3000) was primarily for the culturing of robust micro-organisms [139], and likely inappropriate for insect cell culture. A number of things can be done to reduce damage caused by shear including reducting the aeration rate, keeping the bubble size as large as possible, using a bioreactor with a higher aspect ratio and by placing the cells in a packed bed external to main reactor [137]. Reducing the aeration rate in isolation would limit the ability to reach high-cell-densities in the bioreactor. Increasing the bioreactors aspect ratio would

require the modification of the current bioreactor design, which may conflict with its purpose as a multiple use vessel. Increasing the bubble size has been shown to reduce the damage to animal cells [137], however increasing the bubble size reduces the bubble surface area and oxygen transfer meaning that the sparging rate must be increased. If further research dictates that a large number of insect cell experiments are to be performed then the option of placing the cell in a packed bed external to reactor where the medium is pumped slowly through should be investigated. An external packed bed apparatus protects the cells from shear by removing them from the main reactor where they are exposed to sparging and agitation [244]. Removing the cell from the main reactor also means that higher agitation rates can be used increasing the mass transfer of nutrients and oxygen [244].

Shown in Figure 33, the production of recombination protein increased consistently for 140 hours. A maximum concentration of 0.00035g/L of *o*FSH was produced the bioreactor. This is approximately one-third of the concentration produced in the best shaker flask run. The low titre is likely a result of the low cell concentrations achieved being 3-6 times less than that found in the shaker flask. Shown in Figure 33, the specific titre of *o*FSH began at a maximum and decreased as the culture progressed and the cell density increased. A major problem with the production of recombinant proteins in *T.ni* cells is the reduction in specific titre with increasing cell density which has been often referred to as the "cell density effect" [31, 34, 35, 37, 88, 118]. The reduction in specific titre has been suggested to be a result of nutrient depletion and the most common strategy used to overcome the cell density effect is to partially or completely replace the medium [34, 79, 118]. The increase in specific *o*FSH production after 140 hours of culture is likely a result of the supply of fresh nutrients which was begun after 120 hours. The late decline in *o*FSH titre could be a result of proteolysis from cell

lysate. High-Five cells have been previously shown to contain a number of proteases which can degrade the protein of interest [245].

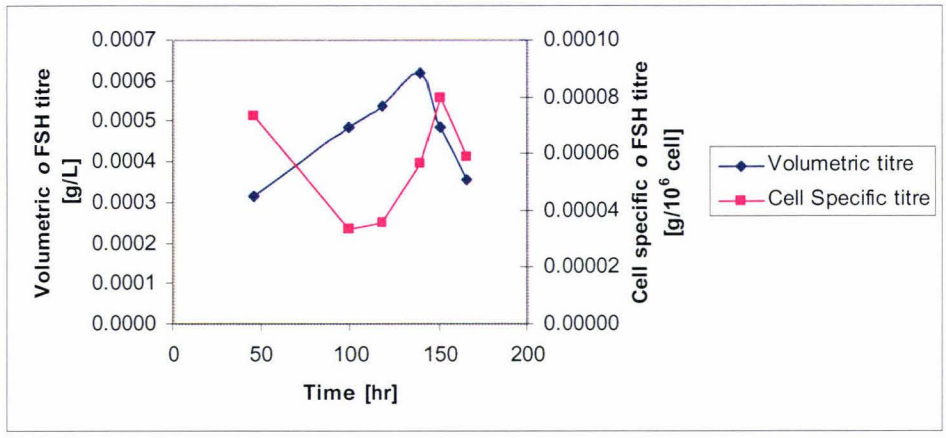

Figure 33: Profile of the specific and volumetric titres of *o*FSH for bioreactor culture of High Five cells. 1L of Express Five medium was inoculated with 400mL of cells at 0.8 x 10^6cells/mL. The DO was controlled at 50%, temperature 27°C, agitation 150rpm. Feeding with fresh media at a rate of $0.02h^{-1}$ was begun after 120 hours with supernatant removed to retain constant volume.

Another possible explanation for the decline in bioreactor culture is that cells declined due to lack of adaption to serum-free media. The High Five cell line is a sub-population of *T.ni* which was selected for its ability to grow in suspended culture [37]. Previously cell instability has been observed for this clone in shaker flasks with viability falling rapidly after 3-4 passages in suspended culture (personal correspondence, Jun Lin, 2003). Taticek et al. [30] also observed culture instability in suspended culture after a few passages and attributed this to insufficient adaptation to serum-free media (SFM). Further, they found that concentration of serum-free media needs to be increased in small increments of 5-10% dilution per passage to ensure long term stability of cultures. The current adaption protocol uses at Agresearch uses a 50% dilution per passage. Work was being carried out by another group at the same time as this study at further reducing the time required to adapt this clone to SFM (personal correspondence Jun Lin, 2003). However

this approach is likely to only further reduce the long term stability of the cell line. Before any more experimentation is carried out on this clone a concerted effort needs to be made resolve this issue and improve the stability of the cells in suspended culture.

5.3 RECOMMENDATIONS

Shown in Figure 32 the maximum cell density achieved in the bioreactor was extremely disappointing considering previous accounts of densities of up to 9 x 10^6 cells/mL of *T.ni* cells in perfusion culture [34, 37]. Future experimentation should look to achieve perfusion culture as there are several inherent advantages. In batch culture the maximum productivity has been reported to be restricted to cell densities of 0.5–1 x 10^6 cells/mL. This is much lower than reports for perfusion systems which place the maximum cell density range maintaining the cell specific productivity between 2–3 x 10^6 cells/mL with an inherent increase in volumetric titre of recombination protein [34, 118]. Perfusion culture can also be maintained for longer production periods and product can be removed continuously [244].

5.4 CONCLUSION

The objective of this chapter was to investigate methods to increase the titre of *o*FSH, develop parameters for production in a small scale bioreactor and provide a basis of fermentation knowledge for the development of other recombinant protein products produced in insect cells. The rate of agitation and inoculation cell density was found to have the greatest effect on the expression of *o*FSH. Expression was greatest when shaker flasks were inoculated at a cell density of 1 x 10^6 cells/mL, maintained at 27°C and agitated at a rate of 140rpm. 0.0012g/L of *o*FSH was produced which is a significant improvement over previous yields achieved at Agresearch (personal communication, Jun Lin, 2003) and consistent with a report for recombinant bovine FSH produced in insect cells [7]. A single attempt to grow the High Five cells in a bioreactor was achieve a lower cell density and a third of the volumetric titre of *o*FSH that was achieved in the optimised shaker flasks. This was likely caused by either the low inoculation cell density, insufficient adaption of the culture to suspension or due to shear damage in the bioreactor. It was recommended that future work should be focused on improving the stability of the High Five cells in suspension before further attempts to optimise the cell line or grow the cell line in a bioreactor were made.

CONCLUDING REMARKS

This research set out to maximise the titre of four recombinant protein products, develop protocols to express the proteins in a small scale bioreactor and provide a knowledge base for the development of other recombinant protein fermentations.

Production of *Eg*95 as inclusion bodies in *E. coli* was found to be influenced by the medium, feeding strategy, dissolved oxygen concentration and induction timing. *E.coli* growth and expression were higher in cultures containing the phosphate buffer system and high levels of yeast extract. 0.1 mM of IPTG was found to fully induce the *lac* promoter and the volumetric titre of *Eg*95 was greatest when induced in mid-exponential phase, i.e. when the specific growth rate was at it highest. Online feeding methods such as DO-stat and pH-stat that maintain the culture on the brink of starvation, produced a lower final cell density than an exponentially fed-fermentation. *E.coli* cultures were found to grow for longer when maintained at low dissolved oxygen concentrations. A maximum titre of 1.73g/L of *Eg*95 was produced in a fed-batch fermentation controlled at 37°C, pH 7.0 and 30% DO. This titre represented a 360% increase on the highest titre achieved prior to this research.

The recombinant protein *Aspin* was used to investigate the culture conditions for maximizing the production of soluble protein (as opposed to inclusion bodies) in *E.coli*. The production of soluble *Aspin* was found to be highly dependant on the rate of expression. At high rates of expression the cellular protein folding mechanisms were apparently overwhelmed, leading to

accumulation of proteins as inclusion bodies [10, 80, 81]. Increases in soluble *Aspin* titre were achieved by reducing the expression rate using a combination of a reduced culture temperature and dissolved oxygen. A batch fermentation induced with 2g/L of L-arabinose, the post-induction temperature reduced from 37°C to 23°C, with a low dissolved oxygen concentration maintained, achieved a soluble *Aspin* titre of 220 mg/L. This titre was relatively high compared to other published data for soluble recombinant proteins produced in *E.coli* [1-3].

The production of soluble G-CSF in *Pichia pastoris* was found to be influenced by the medium composition, cell-to-methanol ratio and pH. G-CSF expression was greatest when cells were grown in enhanced YEHD which contains high levels of yeast extract and peptone. The optimal feed rate of methanol for G-CSF expression were 1% (v/v) per day. Close control of the methanol concentration was important, as cell growth and expression were inhibited above 2% (v/v) methanol [82]. *Pichia pastoris* can grow across a range of pH, which can be used to favour protein folding or deactivate proteases [116, 246]. In shaker flasks, maximum expression occurred when cells were buffered at pH 6.0 and resuspended to an optical density of 8 prior to induction. A maximum titre of 28mg/L of G-CSF was produced in a shaker flasks of YEHD maintained at 30°C, pH 6.0, 200rpm with 1% (v/v) methanol added per day. This is consistent with previous reports for shaker flasks [4, 27, 83, 233]; however, previous reports of fermenter cultures of *P.pastoris* have reported up to twenty fold increases in volumetric titres compared to shaker flask yields [6]. Levels of G-CSF attained in shake flasks could not be achieved in a fermenter. Low levels of growth and expression in the fermenter were likely due to either a build-up of toxic materials or a clonal limitation. It is recommended that further work should look at testing lower

methanol feed rates and at optimising the medium to eliminate potential inhibiting reagents.

Constitutive expression of *o*FSH in High Five™ insect cells was influenced by the cell density and rate of agitation. Consistent with previous findings for recombinant protein expressed in insect cells, the specific titre of *o*FSH decreased with increasing cell density over the entire tested range of inoculation cell densities. The maximum volumetric titre of recombinant protein occurred when the culture was inoculated with 1 x 10^6 cells/mL and cultured at 27°C. The optimal agitation rate of 140rpm was a balance between the cultures requirement for oxygen and reductions in culture viability caused by shear. 0.0012g/L of *o*FSH was produced in the best shaker flask which represented an improvement on previous yields and was consistent with a report for bovine FSH produced in insect cells [7]. A single attempt to express *o*FSH in the bioreactor produced a low titre likely due to a below optimum inoculation cell density, insufficient adaption of the cells to suspension or shear damage. It was recommended that additional work should look at improving the stability of the clone before further optimisation or bioreactor experiments are carried out.

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