An Upstream Platform for the Production of High Grade Heterologous Proteins in the Yeast *Pichia pastoris*

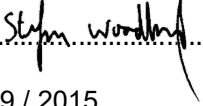A thesis submitted to University College London for the degree of Doctor of Philosophy

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I, Stefan Alexander Woodhouse, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Pichia pastoris is a methylotrophic yeast and well established expression system for the production of therapeutic proteins and industrial enzymes. It is characterised by its tightly regulated promoters, natural ability to secrete recombinant proteins and potential to grow to very high cell densities in order to combat low product titres. These high cell densities, in combination with the requirement of methanol as an inducing agent, lead to problems, however, with cell viability being negatively influenced, resulting in the release of host cell proteins, which include proteases that both reduce product quality and complicate purification.

This thesis introduces a simple fermentation strategy that reduces the amount of methanol required during fermentation by utilising the non-repressing substrate sorbitol as a co-feed with methanol during induction. This allowed cell growth profiles to remain similar to those seen with current protocols, with final cell densities of 120-140 g/L dry cell weight (DCW) being attained. Cell viability and product yields were unaffected by the new strategy, but protease release was reduced due to a shift in host cell protein impurity profiles. The scalability of fermentations was also greatly increased due to a 60% reduction in heat generation during induction. Finally, a methodology for the determination of cellular robustness was successfully developed using adaptive focussed acoustics: this was used to demonstrate that methanol induction did not negatively impact cellular robustness, and that reduced growth rates were a key requirement of enhanced cellular robustness. These results have all been demonstrated with two strains of *P. pastoris*, an in-house GS115 strain expressing secreted embryonic alkaline phosphatase (SEAP) and an industrially utilised strain, CLD804 expressing aprotinin.

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Abbreviations and symbols

(w/v)	Weight per volume
(v/v)	Volume per volume
2-D	Two-dimensional
α-MF	Alpha mating factor secretion signal
μ	Growth rate / micro
μ_{max}	Maximum growth rate
A	Surface area
ADH1	Alcohol dehydrogenase (promoter)
AFA	Adaptive focussed acoustics
AOX	Alcohol oxidase
Asn	Asparagine
ATPS	Aqueous two-phase system
B	Biotin
BCA	Bicinchoninic acid assay
BMGY	Buffered minimal glycerol-complex medium
BMMY	Buffered minimal methanol-complex medium
BMSY	Buffered minimal sorbitol-complex medium
BSA	Bovine serum albumin
BSM	Basal salt medium
C	Cell density
C-mol	Carbon mole (ratio)
CER	Carbon dioxide emission rate
D	Dextrose
DAS	Dihydroxyacetone synthase (promoter)
DCW	Dry cell weight
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DTT	Dithiothreitol
EBA	Expanded bed adsorption (chromatography)
EDTA	Ethylenediaminetetraacetic acid
ENO1	Enolase (promoter)
FLD1	Formaldehyde dehydrogenase (promoter)
FTC-casein	Fluorescein isothiocynate labelled casein
GAP	Glyceraldehyde 3-phosphate dehydrogenase (promoter)
Glc	Glucose
GUT1	Glycerol kinase (promoter)
GY	Glycerol
h	Heat transfer coefficient
H_2O	water
hGH	Human growth hormone
IEF	Isoelectric focussing
ICL1	Isocitrate lyase (promoter)
LC-MALDI-TOF	Liquid chromatography matrix-assisted laser desorption/ionisation time of flight
LDS	Lithium dodecyl sulphate
M	Methanol or Molar

MALDI-TOF	Matrix-assisted laser desorption/ionisation time of flight
Man	Mannose
MES	2-(N-morpholino)ethanesulphonic acid
Mut$^{(+,s,-)}$	Methanol utilisation phenotype positive, slow or negative
N/A	Not applicable
OD$_{600}$	Optical density at 600 nm
ODU	Optical density unit
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
PAGE	Polyacrylamide gel electrophoresis
PAP	Placental alkaline phosphatase
PBS	Phosphate-buffered saline
PHO1	*P. pastoris* acid phosphatase (secretion signal)
PHO89	Phosphatase-responsive gene (promoter)
PTM1	*Pichia* trace metals (1 L preparation)
Q	Heat
RO	Reverse-osmosis (water)
RPM	Rotations per minute
S	Sorbitol
SDS	Sodium dodecyl sulphate
SEAP	Secreted embryonic alkaline phosphatase
Ser	Serine
t	Time
TBS	Tris-buffer saline (buffer)
TCA	Tricarboxcylic acid (cycle)
TE	Tris & EDTA (buffer)
TFA	Trifluoroacetic acid
THI11	Thiamine biosynthesis (promoter)
Thr	Threonine
TPCK	Tosyl phenylalanyl chloromethyl ketone
V	Volume
vvm	Volume per volume per minute
WCW	Wet cell weight
YNB	Yeast nitrogen base

Chapter 1:Introduction

1.1 Background

Pichia pastoris is a methylotrophic yeast that has been a popular expression system for recombinant proteins in the biotechnology industry for many years, with the number of proteins expressed increasing from a mere handful in the late 80's (Schmidt, 2004) to hundreds now being successfully produced and many more in development (Ahmad, Hirz, Pichler, & Schwab, 2014). Having first been developed by the Philips Petroleum Company in the 1960s as an economical source for single-cell protein (SCP) animal feed, media and protocols were devised to run a continuous process that could reach high cell densities. This, however, was not successful due to the 1970s oil crisis increasing the cost of methane (a precursor of methanol), making this method of producing SCP un-affordable (Wegner, 1990). Therefore, in the following decade Phillips Petroleum contracted the Salk Institute Biotechnology/Industrial Associates Inc. (SIBIA, La Jolla, CA, USA) to develop *P. pastoris* as a heterologous protein expression system. Research by the SIBIA team led to the identification and isolation of the alcohol oxidase gene and its promoter. This discovery, along with the sale of the *P. pastoris* expression system patent to Research Corporation Technologies (Tucson, AZ, USA) and the licencing of component sales right to Invitrogen Corporation (Carlsbad, CA, USA) in 1993, led to *P. pastoris* becoming the widely used and successful expression system it is today (Cereghino & Cregg, 2000). The *P. pastoris* system owes this success to a multitude of factors:

1. As a simple eukaryote it is able to reliably perform eukaryotic protein modifications, such as glycosylation, without hyperglycosylating as other yeasts do (Montesino, García, Quintero, & Cremata, 1998)

2. It naturally secretes recombinant proteins whilst secreting very few endogenous ones, thus simplifying recovery and purification of products (Solà et al., 2004)

3. The ability of cells to grow on a simple, defined media contributes to lower purification costs

4. It is very well characterised, and genetic modification is simplified by the ready availability of protocols (Gonçalves et al., 2013)

5. Cells have a preference for respiratory growth as opposed to fermentative growth, meaning that there's little risk of fermentation products such as ethanol or acetic acid being produced and jeopardising fermentations (Cereghino et al., 2002)

1.2 The alcohol oxidase (AOX) promoter

The alcohol oxidase promoter system is the most commonly used in *P. pastoris* and is part of the reason for the success of the expression system. Two genes encode alcohol oxidase, AOX1 and AOX2, the use of which correspond to different AOX phenotypes. The methanol utilisation positive phenotype (Mut+) is most common and present when both AOX genes are active, and the AOX1 promoter is used for protein expression (Cereghino & Cregg, 2000). Under this promoter, heterologous protein expression is tightly regulated and can be highly induced with the addition of methanol to the media. This is because alcohol oxidase is the first enzyme produced in the methanol utilisation pathway (figure 1-1), meaning that the AOX1 promoter is activated by methanol, resulting in AOX forming 30% of the soluble protein in cells during induction (Invitrogen Corporation, 2009). This is in contrast to the undetectable levels of AOX when cells are cultured on other carbon sources such as glycerol of glucose in a batch fermentation (Couderc & Baratti, 1980) and ~1% during

fed-batch growth (R A Brierley, Bussineau, Kosson, Melton, & Siegel, 1990). Due to the

AOX genes both being active, methanol can be easily metabolised and cells grow at

the wild-type rate which is good for research purposes but can be problematic at

large-scale because of the high methanol feeding rates required (Bazan et al., 2009; J.

L. Cereghino & Cregg, 2000; Hanumantha Rao et al., 2011; Macauley-Patrick, Fazenda,

McNeil, & Harvey, 2005). As this is the most common phenotype, cultivation methods

discussed will be with regard to Mut[+] unless otherwise specified.

The Mut[s] (slow methanol utilisation) exists when the AOX1 gene is disrupted and

methanol metabolism is controlled by the AOX2 gene. As the phenotype name
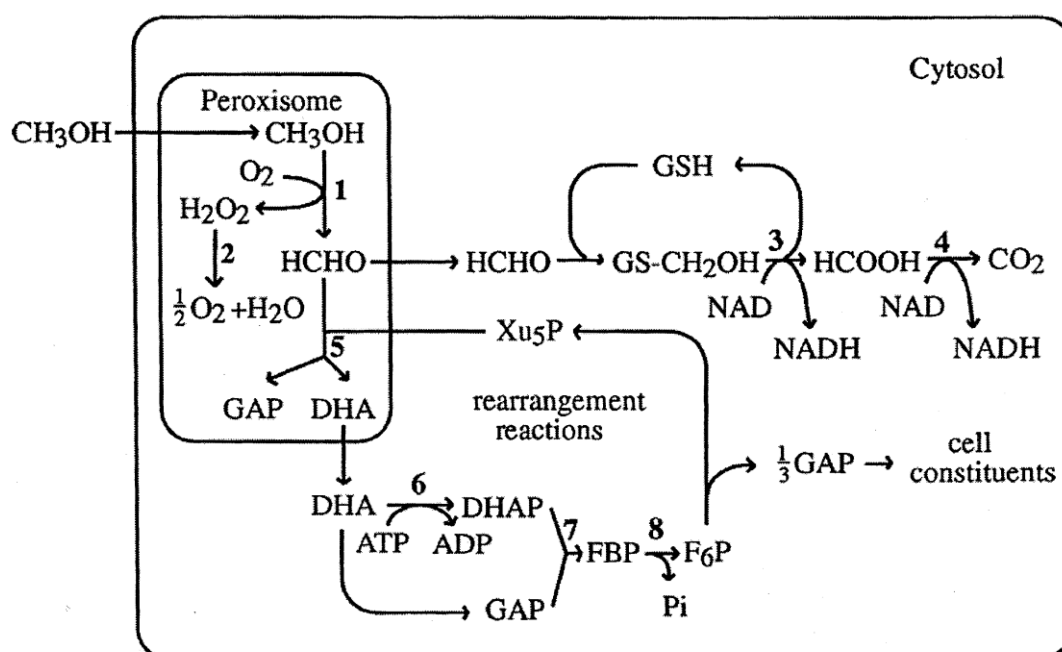
Figure 1-1 *The methanol utilisation pathway in P. pastoris. Enzymes involved are*
1 = alcohol oxidase; 2 = catalase; 3 = formaldehyde dehydrogenase; 4 = formate
dehydrogenase; 5 = dihydroxyacetone synthase; 6 = dihydroxyacetone kinase; 7 =
fructose 1,6-biphosphate aldolase; 8 = fructose 1,6-bisphosphatase. (J. L.
Cereghino & Cregg, 2000)

suggests, methanol metabolism is significantly slower in this phenotype due to the

lower activity of AOX2, with only 10% of alcohol oxidase normally being produced by

it (Pla et al., 2006). Although this phenotype is less regularly used due to its lower

induction phase growth rate, it can lead to higher heterologous protein production as

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the product does not have to compete with AOX synthesis (Chiruvolu, Gregg, & Meagher, 1997) and is still used in many phenotype comparison studies (Krainer et al., 2012; Pedro et al., 2015; Pla et al., 2006).

Finally the Mut[-] phenotype is created when both AOX promoters are disrupted/deleted, meaning that methanol metabolism cannot occur and cells are unable to grow on the carbon source. This phenotype is used when very low growth rates are required to successfully express the protein of interest. An additional benefit is that significantly less methanol (35 times less) is required during fermentation (Macauley-Patrick et al., 2005).

1.3 Current Pichia pastoris culturing methods

For the cultivation of Mut[+] *P. pastoris*, cells are grown on simple media such as basal salt medium (BSM) supplemented with a non-methanol based substrate, most commonly glycerol (Pla et al., 2006). This is to allow the cells to go through lag and exponential phase whilst diverting no resources to protein production, as glycerol inhibits the AOX promoter (R A Brierley et al., 1990). Once glycerol has been depleted during batch phase, a short glycerol fed-batch phase is often utilised to further increase biomass and smooth the transition into induction phase, although this is not always necessary and can be skipped (Gurramkonda et al., 2009). Following the cell biomass expansion on glycerol, a transition phase can be employed: this involves a starvation period whereby any residual glycerol is consumed to ensure that there is no inhibition of the AOX promoter during the induction phase (Inan & Meagher, 2001a; Zhang et al., 2005). Once all residual glycerol has been consumed, the promoter is induced by starting a methanol fed-batch phase with gradually increasing methanol feed rates allowing cells to first adapt (adaptation period) before higher feed rates are utilised to support greater growth and protein production rates. During

adaptation phase cells begin to develop organelles called peroxisomes; these contain the enzymes that the cells require to metabolise methanol (figure 1-1). As the cells cannot metabolise methanol until this adaptation occurs, methanol can accumulate in the bioreactor and reach toxic levels for the cells, hence low initial methanol feeding rates are required. The methanol induction phase is the longest phase and ends once biomass levels are too great to sustain a healthy culture or the bioreactor reaches its maximum capacity. The entire fermentation lasts approximately four days and a fermentation overview can be seen in figure 1-2 wherein the fermentation phases can be seen as well as the specific AOX activity (note that only the start of methanol fed-batch phase is shown). In a Mut^S fermentation the initial glycerol batch and fed-batch phases are carried out in the same manner as a Mut$^+$ fermentation, as the phenotypes do not differ in their metabolism of carbon sources other than methanol. Due to Mut^S's poor methanol utilisation however, the methanol fed-batch phase involves much
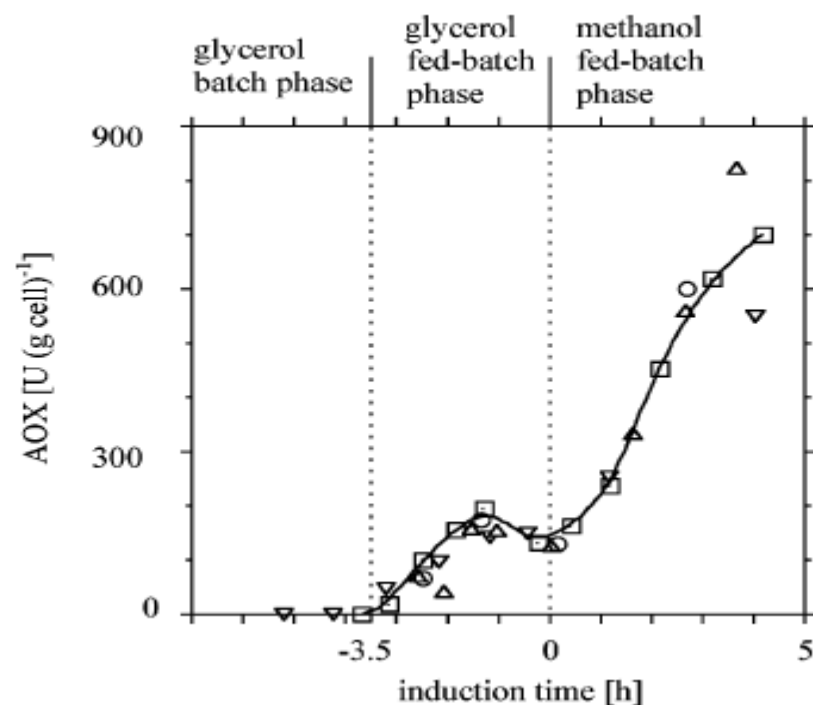
Figure 1-2 *Specific AOX activity during each stage of a methanol fed-batch fermentation* (Jahic et al., 2006)

lower methanol feed-rates and lasts for much longer (approximately 100 hours) (Invitrogen Corporation, 2002). Methanol's role in fermentations is both major and

complex due to its use as both the main carbon source and inducer. This means that steady methanol levels are of great importance, especially as methanol needs to be kept at the correct concentration in the media to maintain high productivity. Too much methanol is toxic to the cells and will result in cell death due to the accumulation of formaldehyde and hydrogen peroxide (both by-products of methanol metabolism) whereas too little will induce degradation of heterologous protein products.

In terms of actual fermentation conditions, there are no strict values for certain parameters due to *P. pastoris*' ability to grow in a large variety of environments. Although optimal growth occurs at 30 °C for example, low temperature cultivations can also successfully performed and fermentations can be run anywhere between pH extremes of pH 3.0 – 7.0; it is possible to cultivate cells outside of this pH range, but protease activity and product stability may be affected (Invitrogen Corporation, 2002; Potvin, Ahmad, & Zhang, 2012). *P. pastoris* also has a high tolerance for variation in dissolved oxygen concentration, however for optimal protein expression it should be maintained above 20% using a combination of agitation increases and a higher percentage of oxygen in the air inlet. Finally, the typical medium used for *P. pastoris* fermentation is a basic salt medium called basal salt medium (BSM) supplemented with PTM1 trace salts, the components of which can both be found in section 2.1.7.

1.4 **Improving product yields obtained in *Pichia pastoris***

In order to improve product yields of an expression system, two overall methodologies can be used:

1. Culturing methods can be adapted and improved in order to increase the amount of product that can be obtained from existing cell lines

2. Cell lines can be modified using microbiological techniques to improve cellular productivity

1.4.1 Culturing methods

The improvement of *P. pastoris* culturing methods is a large part of the research involving the yeast. Due to its preference for fermentative growth *P. pastoris* can grow to incredibly high cell densities with fermentation products such as ethanol not being a problem (Çelik & Çalık, 2012), thus high product titres can be attained simply by having a greater number of cells producing during fermentation than in a typical cell culture. This also compensates for the low cellular productivity of *P. pastoris*, which is similar to *Escherichia coli* (*E. coli*) and much lower than Chinese hamster ovary cells (CHO), the most frequently utilised microbial and mammalian cell lines respectively. This is demonstrated by equivalent product titres being attainable with all 3 strains (dependent on the complexity of the product) but with a typical *P. pastoris* fermentation having a final cell count of 1.8×10^{10} cells/mL (150 g/L DCW) compared to 1×10^{10} cells/mL (100 g/L DCW) in *E. coli* and 2×10^{6} cells/mL in CHO (Kunert, Gach, & Katinger, 2008; Soini, Ukkonen, & Neubauer, 2008). The disadvantage of these high cell densities is the effect that they have on the culture conditions and therefore on the cells' viability. As the cell density increases the oxygen requirement of the culture also rises along with the viscosity. The problem with this is that it becomes more difficult to supply the cells with the oxygen they require and so cell death increases which in turn adversely effects the culture conditions (Li et al., 2007). Although the number of overall impurities increases in the broth, e.g. cell debris, the most published issue is the release of reasonably high concentrations of host cell proteases (Ahmad et al., 2014; Cereghino & Cregg, 2000; Gonçalves et al., 2013). As proteases not only contaminate the fermentation broth, making purification more difficult (as both the protease and product are proteins) they can also potentially

degrade the product, ergo reducing the effect of proteases is a key issue in optimising the productivity of a *P. pastoris* fermentation.

1.4.1.1 Cultivation conditions - pH

A simple initial approach to reducing the problem of protease degradation of protein products is to utilise *P. pastoris*' ability to grow under a wide range of cultivation conditions, more specifically, a wide pH range. In this method the cultivation pH is chosen in order to minimise protease activity and maximise product stability. This means that each heterologous protein to be expressed must undergo an initial analysis to determine which pH is ideal as the values can vary hugely. This effect is widely acknowledged and many detailed studies have been done on the subject. Çalik et al. investigated the effect of pH on a *P. pastoris* strain (Mut[+] phenotype) expressing human growth hormone (hGH). In this study it was found that with increasing pH protease activity decreased overall and that the optimal pH for maximising AOX activity and therefore also maximising hGH production, was pH 5 despite the highest cell densities being recorded at pH 6. This was presumed to be a result of the decreased cell numbers at optimal pH whereby amino acid demand for heterologous protein synthesis meant that cell synthesis was reduced (Calik et al., 2010). Another study, conducted by Files et al. analysed the effect of pH on the amount of their specific recombinant protein (cystatin C) in its active form (Files et al., 2001). pH was shown to have a major effect on active cystatin C yield and did not depend on other parameters such as cell number or glycerol feed rate. Despite dry cell weight increasing with pH within the analysed range of 5 to 7, cystatin activity reached a maximum at pH 6 and then gradually dropped off with further pH increases, although not reaching the minimal yield found with pH 5. A later study by Jahic et al. showed similar results where a low pH increased correctly folded product concentration from 40% to 90% (Jahic et al., 2003). These studies all show that even a pH difference of

one can have a major effect on product yields, thus demonstrating the advantage of *P. pastoris*' tolerance of a large pH range.

1.4.1.2 Culturing conditions – temperature

The ability of *P. pastoris* to grow at a wide range of temperatures can also be taken advantage of when trying to reduce the impact of product proteolysis. Lower cultivation temperatures have been shown to improve product yields in a number of studies. This could be due to a number of effects, either the improvement of product stability at lower temperatures, reduced protease release due to greater cell viability or product folding problems at higher cultivation temperatures (Dragosits et al., 2009; Macauley-Patrick et al., 2005). Using a reduced induction temperature of 20 °C the yield of porcine interferon alpha was improved (in combination with a mixed feed strategy) by up to 2.1-fold of the yields obtained under standard cultivation conditions (Gao et al., 2015). In a smaller scale study (3 L) with pure methanol feeds and a reduced induction temperature of 22 °C, yields of poly vinyl alcohol dehydrogenase were improved 10-fold compared to a standard cultivation strategy (Jia et al., 2013). Similar results have been recorded across a range of reduced temperatures, with the lowest being 15 °C, and a wide spectrum of recombinant products, although all suffer one key disadvantage of having a severely reduced growth rates during low temperature induction (Hong, Meinander, & Jönsson, 2002; Jahic et al., 2003; Z. Li et al., 2001; Woo, Liu, Stavrou, & Neville, 2004).

1.4.1.3 Culturing methods - other

Culturing methods that can also be employed include oxygen limited fed-batch (OLFB), controlling the specific growth rate with excess methanol (Kobayashi et al., 2000) and running the process under higher pressure. The principle behind these techniques being to increase cell viability by either increasing the oxygen transfer rate

or reducing the specific growth rate (Jahic, Veide, Charoenrat, Teeri, & Enfors, 2006; Macauley-Patrick et al., 2005).

1.4.1.4 Culture supplements

It is not only cultivation conditions that can be exploited in order to reduce proteolysis; additions in the form of amino acid supplements can be made (Clare, Romanos, et al., 1991). Peptone and casamino acids are commonly used, competing as substrates for protease action and can also repress protease induction via nitrogen limitation. The disadvantage of this though is the relatively high cost of peptone which makes this method cost-prohibitive, especially when scaled up and can complicate downstream processing (Jahic et al., 2006; Werten et al., 1999). Despite the limitations adding amino acid rich supplements has been a successfully adopted technique in many *P. pastoris* fermentation studies. Early work showed that the yield of an anticoagulant and antimetastatic protein called ghilanten could be improved by enriching the culture with the aforementioned supplements (Brankamp et al., 1995). More recently however the technique has been applied in conjunction with alternative fermentation methods, whereby casamino acids were added every 24 hours at a concentration of 1 g/L. Although the purpose of the study was not to determine the effectiveness of amino acid rich supplements they were still used as the primary method for reducing proteolysis of their angiostatin protein product (Xie et al., 2005).

More conventional and arguably intuitive supplements that can be used in order to reduce proteolysis are protease inhibitors. Although the use of specific protease inhibitors could be un-economical at industrial scales it has been shown to be a highly effective method at small scale (Macauley-Patrick et al., 2005). Shi et al. found that the addition of three specific protease inhibitors during fermentation could reduce total protease activity by up to 53% when using saturating levels of the serine protease

inhibitor (Shi et al., 2003). Other protease inhibitors such as EDTA, a metalloprotease inhibitor, have also been used in combination with other supplements, reducing protease activity by 94% when producing Ovine Interferon – τ (Sinha, Plantz, Inan, & Meagher, 2005).

1.4.1.5 Mixed feeding strategies

Glycerol co-feeding

The methods that have been discussed so far can all be employed with the standard fermentation media and protocols that Invitrogen recommends, however with alterations to media compositions and feeds cell viability can potentially be further improved. Minor alterations can be made in the form of reducing salt concentration in the media (Brady et al., 2001), keeping a pure methanol feed but increasing the methanol concentrations used (as previously mentioned) or in some cases even decreasing them (Hong et al., 2002). The principle behind a reduction in methanol concentrations is due to the impact that the alcohol has on cell viability. Using and propidium iodide staining in combination with flow cytometry, Hohenblum, Borth, & Mattanovich (2003) showed that cell viability dropped from ~100% to below 70% during the course of methanol induction, demonstrating not only the value of flow cytometry as an analytical tool but also the significant detrimental effect methanol has on cell viability.

Major alterations to fermentation protocols consist of altering feed and media types. Mixing the methanol feed with another carbon source is a method often used in order to increase *P. pastoris* productivity (Cos et al., 2006), the earliest example of which being a mixed glycerol feed, although mixing feeds had already been done even earlier for non-recombinant methylotrophic yeasts (R A Brierley et al., 1990). This strategy is often employed when cultivating a Mut[s] strain due to its inability to efficiently

metabolise methanol and so the glycerol provides an additional carbon source for growth, reducing the induction time, increasing the final cell density and resulting in a higher overall volumetric productivity (Files et al., 2001). The reason this method has only traditionally been used for Mut^s phenotypes is because of glycerol's inhibitory effect on the AOX1 promoter and although higher cell masses are still attained with Mut$^+$, protein expression is significantly reduced (Hellwig et al., 2001; Orman, Pınar, & Ozdamar, 2009). Despite this, work has been carried out to try and take advantage of a mixed glycerol feed with Mut$^+$ strains. Initially work with Mut$^+$ involving mixed feeds relied on keeping the glycerol concentration at an absolute minimum to avoid any repression of the AOX1 promoter. This means that glycerol is continuously used purely for anabolism and does not accumulate, compensating for any possible carbon shortage when running in methanol limited conditions (M., J., M., K., & S-O., 2002; L. B. Trinh, Phue, & Shiloach, 2003). Zhang et al. (W. Zhang et al., 2003) found that the ratio for optimal methanol-glycerol mixing could be determined much the same as it could with a Mut^s strain and this hypothesis has since been confirmed (Woo et al., 2004). In this last investigation a Mut+ strain was used to produce an immunotoxin, glycerol was added to the methanol feed using a 4:1 methanol glycerol ratio in conjunction with adding yeast extract during induction as well. The reasoning behind this was that the glycerol would boost immunotoxin production whereas the yeast extract would aid growth, this resulted in a 50% increase in overall protein production. Further improvements in the use of glycerol as a co-feed during induction are expected as recent metabolic flux analysis deduced how glycerol was utilised by *P. pastoris* during mixed feed induction which could inform the design of later feeding strategies (Jordà, De Jesus, Peltier, Ferrer, & Albiol, 2014).

Sorbitol co-feeding

With even repressing carbon sources showing promise as co-feeds during induction, efforts have been made to find non-repressing carbon sources that could yield the same positive results on growth without the problem of potentially inhibiting AOX1 when using the Mut+ phenotype. Sorbitol was first discovered by researchers as a non-repressing substrate for Mut+ *P. pastoris* through communication with the Philips Petroleum Company (Sreekrishna et al., 1997). By performing numerous fermentation cycles the cells were primarily grown on sorbitol supplemented with yeast extract and peptone and induced by adding methanol to the sorbitol feed when required. These initial experiments were producing matrix metalloproteinases, run at a small (4L) scale and seemed promising, if only due to the reduced methanol consumption over the period of the fermentation. This work was continued and attempts were then made to verify sorbitol's benefits over glycerol as a mixed feed component. In this study a direct comparison was made between a mixed feed glycerol and mixed feed sorbitol fermentation using a Mut+ strain engineered to secrete recombinant sea raven antifreeze protein (srAFP). The fermentation utilising a sorbitol mixed feed was found to have a much higher specific protein production rate than the equivalent fermentation with glycerol mixed feeding (60 v.s. 45 g srAFP /μg cells/h) which was assumed to be due to the repressive effect of glycerol even at low concentrations. With sorbitol being present within the broth at the end of the fermentation at 5 g/L it was shown that sorbitol could be used and optimised as a feed without the concern of having to limit its feed rate for fear of it accumulating in the media (Thorpe, Anjou, & Daugulis, 1999).

The benefit of a mixed feed with sorbitol was further demonstrated in both a Mut+ and Muts system producing a specific lipase, by reducing the cellular stress of heterologous protein expression in the form of the unfolded protein response normally associated with *Rhizopus oryzae* lipase production in *P. pastoris*. This was shown to improve productivity, although one aspect of the results is disputed as

sorbitol and methanol were shown to be utilised simultaneously whereas all further research (as well as previous mixed glycerol feed research), demonstrated that varying carbon sources are consumed sequentially (Ramón, Ferrer, & Valero, 2007). After further confirmations of the benefit of sorbitol (Jungo et al., 2007) more detailed studies were done to highlight the specific benefits of sorbitol co-feeding. Celik and colleagues (2009) investigated initial sorbitol concentrations and their effect on batch cell densities, showing maximum growth at initial sorbitol concentrations of 50 g/L. They also investigated oxygen transfer characteristics which was continued by Calik et al. (2009) and optimal feeding ratios of sorbitol and methanol were determined by having an exponential methanol feed with an initial sorbitol batch in induction phase. Growth rates increased with greater methanol feeding rates, but the same problem arose as before in that cells are then directed more into biomass generation than product formation.

More recently studies have shown the benefit of sorbitol on cell mortality, with an 8.8% decrease in cell death and protease release by the end of fermentation (Wang et al., 2010) as well as others citing an increases in product yields (Gao et al., 2015; Niu et al., 2013) although little consistency has been seen in results due to varying culturing techniques.

Alternative co-substrates

Other non-repressing substrates for the purpose of a methanol co-substrate have been discovered and investigations have been carried out to find which would be appropriate as part of a mixed feed induction regime. Studies using Mut[s] and Mut[-] phenotypes have found a number of potential candidates for use as co-feed with methanol during induction: Alanine, mannitol and trehalose for Mut[-] (Inan & Meagher, 2001a); acetate and lactic acid for Mut[s] (Xie et al., 2005). All Mut- experiments were shown to have a higher than normal productivity, although growth on trehalose was

particularly slow. Lactic acid was shown to be the most favourable candidate for mixed feed with specific productivity increasing 2.5-fold in comparison to a glycerol and methanol feed. Recently however, an alternative to the whole traditional cultivation strategy was revisited in the form of a glucose fed-batch fermentation whereby glucose is used as the sole carbon source. The study was only an initial insight, high biomass levels were attained and through metabolic flux analysis the potential for high recombinant protein production was also recognised (Heyland, Fu, Blank, & Schmid, 2010) although work has recently been carried out to understand the metabolomics of methanol glucose co-feeding (Jordà et al., 2013).

1.4.1.6 Media Composition

Finally, it should be noted that BSM is not the only defined media used for the cultivation of *P. pastoris*. As BSM precipitates above pH 5.5 it can be problematic, as many recombinant proteins are produced between pH 5.5 and 7.0. In addition to this, being able to vary culture pH is one of the benefits of *P. pastoris*, which is limited by the use of a media that can precipitate at relatively low pH. A small alteration to the recipe of BSM, in the form of replacing the phosphate source with sodium glycerophosphate can prevent precipitation (W. Zhang, Sinha, & Meagher, 2006). Other alternatives have been described by d'Anjou and Daugulis (Anjou & Daugulis, 2000) and the FM22 media developed by Stratton and colleagues (Stratton, Chiruvolu, & Meagher, 1998), both of which are designed to attain high cell densities whilst overcoming the problems of BSM. The key difference between the media types is nitrogen source however, in BSM and FM22 nitrogen is added in the form of ammonium hydroxide when controlling pH whereas d'Anjou media already contains it (Cos et al., 2006).

1.4.2 Cell engineering methods

The use of *P. pastoris* using the AOX promoter and optimising production / culturing methods as discussed above, has led to many improvements in cellular productivity, but work has also been done improving the system from a microbiological stand point. Before detailed modifications to the cell line are discussed, simple approaches which can be used in conjunction with the above culturing methods should be addressed.

1.4.2.1 Protease-deficient strains

As has been previously mentioned, minimising the action of released host cell proteases is a key objective in maintaining high product titres and quality. In order to combat the problem of protease action one approach has been to create protease-deficient strains. The use of these strains, such as SMD1163 (his4 pep4 prb1), SMD1165 (his4 prb1) and SMD1168 (his4 pep4), has proven to be an effective approach to reducing the degradation of certain recombinant proteins (J. L. Cereghino & Cregg, 2000). These strains have mutations in their his4, pep4 or prb1 genes, with the latter two coding for proteinase A and proteinase B respectively; important vacuolar proteases which also activate other vacuolar proteases such as carboxypeptidase Y (P. Li et al., 2007). White and colleagues (1995) used SMD1168 at a 3.3L scale using the AOX1 promoter (Mut[+]) to successfully express thrombomodulin whereas other researchers have found it to be invaluable in producing insulin-like growth factor-1 (R.A. Brierley, Davis, & Holtz, 1997) and ghilanten (Brankamp et al., 1995). This strain was further modified to disrupt the kex1 gene which codes for proteases associated with degrading murine and human endostatin, again the strain was found to be effective at producing high quality product, in this case endostatin (Boehm, Pirie-Shepherd, Trinh, Shiloach, & Folkman, 1999). More recent studies have used SMD1168 to assess new promoters (Stadlmayr et al., 2010) and others have

modelled SMD1168's metabolic pathways (Heyland et al., 2010). The disadvantage however with these protease deficient strains is their comparatively poor viability, growth rate and ease of transformation in comparison to wild-type strains making their use impractical unless proteolysis cannot be reduced in any other way (J. L. Cereghino & Cregg, 2000; Jahic et al., 2003).

Alternative modifications to reduce to the action of proteases can be made to the actual amino acid sequence of the recombinant protein being expressed, the principle being to alter the segment of the protein that is the recognised binding region for the proteases. This works if the binding region is a linker between the domains of a fusion protein that is not vital to the activity of the protein (Macauley-Patrick et al., 2005). Shortening the linker region has been shown to be effective in a lipase fusion protein, reducing the effect of proteolysis on the recombinant protein. However, the activity of the product was reduced by this modification and once the initial shake flask experiments were scaled up to a bioreactor culture, product concentrations were 10-fold lower (Gustavsson et al., 2001).

It should be noted that the methods above both focus on the issue of proteolysis in order to improve product yields, this is not the only approach though and cellular productivity i.e. actual quantities of recombinant protein produced by each cell, and product quality i.e. quality of recombinant protein the moment it's secreted, can also be improved through microbiological techniques.

1.4.2.2 Secretion signals

In order to increase cellular productivity a number of methods can be used, the most common of which being to add or change the secretion signal utilised to export the recombinant protein from the cell. Causing the protein to be produced extracellularly is advantageous not only because it removes the necessity of disrupting the cells in

order to obtain the product, complicating downstream processing, but *P. pastoris* also secretes very few endogenous proteins making the recovery process especially simple. Due to potential folding and stability issues, heterologous proteins chosen to be modified for secretion are normally secreted in their native host as the ability of *P. pastoris* to secrete a recombinant protein is highly dependent on the protein's complexity (J. L. Cereghino & Cregg, 2000; Maccani et al., 2014).

When modifying a strain to secrete a protein of interest the corresponding gene must be cloned in *P. pastoris* vectors to align them in the correct reading frame of either their original secretion signal (if the protein is produced extracellularly by its native host) or with one of a number of alternative secretion signals. The most popular one of these is the *S. cerevisiae* α-factor prepro-signal (or mating factor, α-MF) due to its consistent success rate and even sometimes being a better signal than the protein's natural one. One instance wherein the α-MF signal out-performed the native secretion signal was when used in the production of lipase Lip1 used in industrial processes. In this study the native signal allowed for secretion but for unknown reasons reduced expression levels, this problem was resolved by using the α-MF signal (Brocca, Schmidt-Dannert, Lotti, Alberghina, & Schmid, 1998). Most recently it has been used in conjunction with the glyceraldehyde 3-phosphate dehydrogenase (GAP) promoter (which will be discussed in more detail in section 1.4.2.4) in order to secrete active hydrophobin HFB1 and attain a high initial product purity of 70% in the broth when treated with a foam-separation technique (Kottmeier et al., 2012).

The other secretion signal that is commonly used is the *P. pastoris* acid phosphatase (PHO1) signal which has been used to secrete antibody fragements, although not with the same level of success as with the α-MF signal (Eldin et al., 1997; Luo et al., 1997). Another instance of the PHO1 signal sequence being used was when it was utilised to secrete Mir1 cysteine proteinase, however it was again not successful and the inactive

protein was retained in the host cell membrane. This was thought to possibly be due to excessive glycosylation as there were no regions of hydrophobicity that would have prevented secretion, using the α-MF signal may have prevented this (Pechan, Ma, & Luthe, 2004). The α-MF signal is not infallible though and there have been instances in which it has failed and researchers have often noticed variation in the number of N-terminal amino acids in secreted proteins using the sequence (Brocca et al., 1998; Raemaekers, Muro, Gatehouse, & Fordham-skelton, 1999). In order to overcome these difficulties other signals have been trialled with varying levels of success. The native phytohaemagglutinin (PHA-E) signal sequence was shown to be successful in processing and secreting Galanthus nivalis agglutinin (GNA) and more successfully than α-MF (Raemaekers et al., 1999). This work was continued and GNA was successfully produced at a 200L scale, using PHA-E, at a titre of 80 mg/L (Baumgartner et al., 2003). Another successful signal sequence that has been found is a viral preprotoxin signal sequence which was tested by secreting green fluorescent protein (GFP), this was found to work not only in *P. pastoris* but also in three other distantly related yeasts (Eiden-Plach et al., 2004). The issue with all sequences though, is that there is no rule for what will succeed and there is therefore a lot of trial and error experimentation involved in finding a successful secretion signal.

1.4.2.3 Gene dosage

Once a successful signal has been found there are also ways of improving the productivity of the gene. This can be done by increasing the gene dosage which simply means increasing the gene copy number in the expression cassette in order to maximise the overall quantity of heterologous protein expressed. This was shown to be possible in early studies wherein a methodology was created for the isolation of strains containing multicopy gene inserts (Clare, Rayment, Ballantine, Sreekrishna, & Romanos, 1991); in aiming to produce high yields of mouse epidermal growth factors

(mEGF), Clare et al. (Clare, Romanos, et al., 1991) produced a *P. pastoris* strain with 19 successfully integrated copies of the mEGF gene. This high gene dosage combined with the use of α-MF for secretion and casamino acid addition to reduce proteolysis caused mEGF concentrations in the culture to reach 450 mg/L, in comparison with previous experiments where only 7 mg/L were achieved. More recently it was demonstrated that the technique was also effective with other promoters (which will be discussed further on) and it was shown that when 22 copies of the lacZ (coding for β-galactosidase) gene were successfully inserted, the level of β-galactosidase activity increased 17-fold, relative to having a single insert of the gene (Sunga & Cregg, 2004). Furthermore, the initial investigations on the production of Hepatitis B surface antigen were also disputed when an investigation showed that increasing the gene copy number had a direct correlation with increasing protein production (Vassileva, Chugh, Swaminathan, & Khanna, 2001).

In contrast to these findings, some researchers have found that a greater copy number either didn't affect protein expression or even decreased it. It was shown that when using the GAP promoter no change was observed in levels of human trypsinogen expression and when using the AOX1 promoter secreted levels were actually decreased. This was thought to be because of rate-limiting folding at high heterologous protein expression levels inducing the unfolded protein response, leading to protein retention in the cells, an effect that has been previously observed (Hohenblum, Gasser, Maurer, Borth, & Mattanovich, 2004).

1.4.2.4 Alternative promoters – GAP

Although the majority of this review considers optimisation of *P. pastoris* utilising the AOX promoter or a derivative of it, there are other promoters that can and have been utilised to positive effect. The previously mentioned GAP promoter is the most common alternative promoter to be used: discovered in 1997 by Waterham and

colleagues, it is a constitutive promoter which means that it is unregulated and heterologous protein is therefore continually produced, although the level of production varies depending on the carbon source. The benefit of this promoter is that methanol is no longer needed to induce protein production and therefore at an industrial scale, large volumes of the explosive material don't need to be stored anymore (Waterham, Digan, Koutz, Lair, & Cregg, 1997; A. L. Zhang et al., 2009). Although initial results were promising, concern over the potential cytotoxic effects of constitutive protein production meant that it has not been often; however this is only applicable when the protein itself is toxic to cells (Cos et al., 2006). The benefit of the GAP promoter is that despite having comparatively low levels of protein synthesis in the same time frame as the AOX promoter, a continuous fermentation will yield more product overall than a fed-batch fermentation (five to six fold higher). In addition to this, the continuous media exchange means that proteolytic activity is reduced as sensitive proteins or the problem proteases are removed from the culture (Goodrick et al., 2001; Vassileva et al., 2001). Since these initial studies it has been demonstrated that the GAP promoter can be used to successfully produce a great variety of proteins. Li et al. (Z. Li et al., 2001) used the promoter in combination with the α-MF to successfully produce herring antifreeze protein which was properly folded and fully functioning whereas Kottmeier and colleagues (Kottmeier et al., 2012) used the same combination to produce high levels of hydrophobin HFB1. Other studies have shown similar results with different heterologous proteins (Hohenblum et al., 2003; Menéndez, Hernández, Banguela, & País, 2004). The main reasoning behind these studies has been to remove the problem of methanol storage and transport, but in order to increase product yields further, other investigations have been done to determine the effect of using both the GAP and the AOX1 promoter in combination. A comparison was done between using solely the GAP promoter and using both promoters to express granulocyte-macrophage colony-stimulating factor

(hGM-CSF) whilst the cells grew on defined medium. In both instances the optical density of the culture followed roughly the same trend (although higher with both promoters), however the use of both promoters lead to a doubling of final secreted protein concentration from ~90 mg/L under purely constitutive expression and ~180 mg/L under both constitutive and induced expression (J. M. Wu, Lin, Chieng, Lee, & Hsu, 2003). In addition to this work, a further study was done to demonstrate that two different heterologous proteins can be sequentially expressed and recovered using a two promoter system. hGM-CSF was again used in combination with the GAP promoter and secreted human serum albumin (hSA) was associated with the AOX1 promoter. As the study was carried out at a small scale (100mL) induction was carried out by changing the culture medium, this meant that the proteins could be analysed in the spent medium (yeast extract peptone dextrose [YPD] and yeast extract peptone mannitol [YPM] respectively). Both proteins were successfully expressed and recovered from the medium and reached concentrations between 20 and 25 mg/L. In the same study the ability to express an intracellular protein (β-galactosidase) constitutively and a secreted protein (hSA) by induction, sequentially, was also assessed and deemed equally successful (Wu et al., 2003). Other, less commonly utilised constitutive promoters are summarised in table 1-1.

Table 1-1 *A summary of the most utilised and recently discovered alternative constitutive promoters. Adapted from Ahmad et al. 2014*

Constitutive Promoter	Corresponding gene	Regulation
GAP	Glyceraldehyde-3-P dehydrogenase	Constitutive expression on glucose, to a lesser extent on glycerol and methanol
TEF1	Translation elongation factor 1	Constitutive expression on glycerol and glucose
PGK1	3-Phosphoglycerate kinase	Constitutive expression on glucose, to a lesser extent on glycerol and methanol
GCW14	Potential glycosyl phosphatidyl inositol (GPI)-anchored protein	Constitutive expression on glycerol, glucose and methanol
G1	High affinity glucose transporter	Repressed by glycerol, induced upon glucose limitation
G6	Putative aldehyde dehydrogenase	Repressed by glycerol, inducted upon glucose limitation

1.4.2.5 Alternative promoters – FLD1, ICL1 and THI11

Since the discovery of the GAP promoter, three more have been used successfully to express heterologous protein; the formaldehyde dehydrogenase (FLD1) promoter, isocitrate lyase (ICL1) promoter and more recently the thiamine biosynthesis (THI11) promoter. Formaldehyde dehydrogenase is a key enzyme in the methanol utilisation pathway and the FLD1 gene was isolated and sequenced by Shen and co-workers (Shen, Sulter, Jeffries, & Cregg, 1998). They demonstrated that the FLD1

promoter is strongly induced by methanol or methylamine, due to both metabolic pathways having formaldehyde as an immediate, using β-lactamase as a reporter. This means that methanol no longer needs to be used as a carbon source and studies have been done with methylamine as the sole nitrogen source and therefore the inducer of FLD1, and sorbitol as the carbon source. Sorbitol was again used due to its non-repressive effect on alcohol oxidase (as it is also used in the metabolic pathway) and using this combination of nutrients, *Rhizopus oryzae* lipase was produced at comparable levels to AOX1 regulated expression (Resina, Serrano, Valero, & Ferrer, 2004). The promoter has also been found to have an alternative use as a marker for transformation when *P. pastoris* FLD1 hosts are selected on methylamine plates (Sunga & Cregg, 2004). The ICL1 promoter was isolated in 2003 and an initial, proof-of-principle study was done using a dextranase gene from *P. minioluteum,* wherein dextranase was successfully produced when the cells were grown on a glucose-based complex carbon source (Menendez, Valdes, & Cabrera, 2003). Recently an investigation was done to find new promoters for expression of heterologous proteins in *P. pastoris*, of the 24 assessed THI11 appeared most promising, having high transcription levels and seemingly being the only promoter that could be controlled by an addition (thiamine) independently of the key nutritional components such as the carbon or nitrogen source (Stadlmayr et al., 2010). A summary of the inducible promoters discussed and recently discovered ones can be found in table 1-2.

1.4.2.6 Product Quality – glycosylation patterns

The above discussion has been focussed on improving productivity by increasing the amount of product secreted by the cells, however this only addresses the issue of product quantity, not quality. Part of the appeal of *P. pastoris* as an expression system is its ability to correctly fold mammalian proteins and perform post- translational modifications, unlike for example *E. coli* which does not naturally produce any

complex proteins and therefore doesn't have the cellular machinery to perform these modifications. In terms of correct folding, this becomes apparent with proteins with high numbers of disulphide bonds. Due to the interior of *E. coli* being a reducing environment, disulphide bonds are unable to form whereas highly disulphide-bonded proteins such as coagulation protease factor VII have been successfully produced in *P. pastoris* (White, Kempi, & Komives, 1994). This means that a heterologous protein product expressed by *P. pastoris* for use in humans will be more similar to its native structure and function. This not only makes it a more effective product but also less likely to be recognised as a foreign object by the body's immune system, as this takes into account post-translational modifications such as glycosylation patterns in determining whether a protein is native or not. Despite being able to produce proteins with similar properties to mammalian cells though, the post-translational modifications that *P. pastoris* makes can still be different enough to the native patterns to trigger an immune response or cause functionality problems.

Table 1-2 A summary of commonly used and recently discovered inducible promoters. Adapted from Ahmad et al. 2014

Inducible Promoter	Corresponding Gene	Regulation
AOX1	Alcohol oxidase 1	Inducible with methanol
DAS	Dihydroxyacetone synthase	Inducible with methanol
FLD1	Formaldehyde dehydrogenase 1	Inducible with methanol or methylamine
ICL1	Isocitrate lyase	Repressed by glucose, induced in the absence of glucose / by addition of ethanol
PHO89	Putative Na+ / phosphate symporter	Induced upon phosphate starvation
THI11	Thiamine biosynthesis gene	Repressed by thiamine
ADH1	Alcohol dehydrogenase	Repressed by glucose and methanol, induced by glycerol and ethanol
ENO1	Enolase	Repressed by glucose, methanol and ethanol, induced by glycerol
GUT1	Glycerol kinase	Repressed by methanol, induced by glucose, glycerol and ethanol

Glycosylation is one of the most common and complex post-translational modifications that *P. pastoris* can perform and in some respects excel at. Glycosylation can be N-linked or O-linked, involving the transfer of oligosaccharides to specific amino acid recognition sequences, being Asn-X-Ser/Thr in the former and unknown in the latter. Although the mechanism of O-linked glycosylation is poorly understood the behaviour *P. pastoris* in performing them is known (Cregg et al., 2000). In higher-

eukaryotes the process involves the addition of a variety of sugars to proteins whereas *P. pastoris*, like other yeasts, can only add mannose residues and these are not necessarily added to the same sequences as in mammalian cells, even on the same proteins. It is only in N-linked glycosylation where *P. pastoris* shows its advantage. In order to perform these modifications the oligosaccharide core that was added is trimmed to $Man_8GlcNAc_2$ and in higher eukaryotes a number of addition and trimming reactions are performed in order to produce glycans of either high-mannose type, a mixture of different sugars called complex type or a combination of the two called hybrid type. In contrast, *S. cerevisiae* simply elongates the glycan chains by adding an unspecific number of mannose residues (up to 150) in a process called hyperglycosylation (Cregg et al., 2000). The benefit of *P. pastoris* is that although it has been known to hyperglycosylate proteins, the typical length of an outer chain on a secreted protein is 8 or 9 mannose residues long although in characterisation experiments chains of up 18 residues have been seen (Montesino et al., 1998). In addition to this, core-related structures undergo α-1-6-linked mannosylation and have seemingly no terminal α-1-3-linked mannoses which are hyper-immunogenic (Trimble et al., 1991).

Despite these advantages expressed proteins can still be antigenic, and although this has been exploited in improving augment vaccine immunogenicity (Lam et al., 2005), for non-vaccine pharmaceutical products this needs to be avoided and even if non-antigenic the bulk of the side chains can prevent correct protein folding. In order to overcome these problems the glycosylation patterns of can be humanised. Considering that the effect of altering oligosaccharide structures on an expressed protein can range from being negligible to rendering it completely inactive (Helenius & Aebi, 2001; Varki, 1993) it is unsurprising that minor alterations to the glycosylation machinery of *P. pastoris* can lead it to produce proteins with near to human structures. An initial study by Callewaert et al. (Callewaert et al., 2001)

showed that if a mannosidase (in this case T. reesei 1,2-α-D-mannosidase) is co-expressed with the heterologous protein of interest, the product is a humanised. With this simple methodology there was a greater than 85% reduction in the number of α-1,2-linked mannose residues and the majority of N-glycans were human-type, high-mannose oligosaccharides ($Man_5GlcNAc_2$). Another successful approach to humanisation has been to genetically engineer the *P. pastoris* secretory pathway, eliminating the α-1,6-mannosyltransferase gene and introducing α-1,2-mannosidase in order to mimic the higher eukaryotic N-glycan processing pathway. This method lead to high yields of proteins with the human-type, 5-mannose residue oligosaccharide N-glycans (Choi et al., 2003) and a similar approach with the localisation of more higher eukaryotic proteins had similar results with homogenous, human-like N-glycan structures being produced (Hamilton et al., 2003). More recent studies have replicated these results by again deleting the gene (OCH1) responsible for producing high mannose-type N-glycans (Bobrowicz et al., 2004; Vervecken et al., 2004; D. Zhang, Xu, Xin, Zhu, & Jin, 2011). These successes and the work of Potgieter and colleagues (Potgieter et al., 2009) have now been capitalised on and a glycoengineered *P. pastoris* fermentation was carried out at a 30L and 1,200L scale producing monoclonal antibodies. Titres of 1.6 g/L were achieved and the process was shown to be robust and commercially viable (Ye et al., 2011) which is vital considering the pressure of trying to find ways of manufacturing monoclonal antibodies more economically (Farid, 2007).

1.4.3 Downstream processing of *Pichia pastoris*

Although the primary focus of this research is the production of high-grade proteins using upstream processing techniques, the clarification of cells and the subsequent recovery and purification of the protein product have to be considered in order to understand the implications of modifying the upstream process. The downstream

processing of *P. pastoris* is already a problematic procedure and any alterations made to the cultivation of *P. pastoris* and protein production will have an impact on the rest of the protein recovery process. Although researchers have heavily focussed on solving the upstream challenges of *P. pastoris*, two primary issues with the recovery and purification of *P. pastoris* cultures have been identified:

1. The incredibly high cell densities reached (over 200 g/L dry cell weight) (Heyland et al., 2010) which can overburden traditional clarification techniques such as centrifugation and filtration

2. The high salt concentrations resulting from the use of BSM which can negatively impact on protein purification by causing eventual protein aggregation and a subsequent lack of binding to chromatography ligands (Brady et al., 2001)

These problems are unique to *P. pastoris* due to the way in which it is cultured. When a product is produced extracellularly in other cell lines e.g. CHO, the fermentation broth can be directly processed in primary recovery steps, such as diafiltration and centrifugation, before the product is further purified by chromatography and filtration operations. In contrast to this, the high cell densities (approximately 100x more biomass than CHO) of *P. pastoris* do not allow for direct processing of the feed stock; instead the fermentation broth must first be diluted. This greatly increases the volume that needs processing, which in turn increases either equipment scale or processing time, both of which lead to increased costs (Kunert et al., 2008).

In order to overcome these problems a number of techniques have been suggested. A simple way to optimise current primary recovery techniques has been suggested in the form of ultra scale-down mimicking of centrifugation in order to determine the optimal operating parameters at large scale. Using two different recombinant strains it was discovered that different strains had different impacts on centrifuge

performance, with *P. pastoris* X-33 having better dewatering levels than an industrially utilised glycoengineered *Pichia* strain (Lopes et al., 2012).

Expanded bed absorption (EBA) chromatography has been assessed as an alternative to traditional recovery / purification techniques due to its capacity to handle high solid concentrations. An early study compared EBA to a centrifugation, filtration, packed-bed chromatography methodology for clarification of an endostatin (Trinh et al., 2000). It was found that the ion exchange EBA step was quicker and more effective than the traditional methodology at processing the 400 g/L biomass, taking 8 hours rather than 16 hours and yielding 29% rather than 18% endostatin in the final eluant. Later work then compared EBA to another technique that can process high cell densities, aqueous two-phase systems (ATPS) extraction, which can handle cell suspensions up to 50% wet weight as opposed to EBA's 10-12.5%. Both methods delivered similar results in terms of product purity but had their own merits in different areas. The obvious advantage of EBA is the simplicity of the unit operation, however due to highly viscous culture reducing the settling velocity of the absorbant too greatly the culture has to be diluted in advance. Although ATPS extraction requires an additional process step to recover the product from fraction it has been separated into, the ability for it to handle such high cell densities is an advantage (Thömmes et al., 2001).

Finally flocculation and acid precipitation techniques, traditionally used in waste-water treatment, have been successfully applied to improve the efficiency of traditional centrifugation and filtration techniques although the results are not as dramatic as with EBA and ATPS extraction (Roush & Lu, 2008).

1.5 Project significance and objectives

As has been mentioned in the above literature review *P. pastoris* has a large number of advantages as an expression system with its innate secretion signals, well understood genetics, ability to grow to high cell densities and ability to perform some post-translational modifications. Despite these advantages there is still reluctance in industry to adopt *P. pastoris* as their expression system for a multitude of reasons. One key challenge is the use of high cell densities in the culturing of *P. pastoris* which are required to compensate for the expression system's lower cellular productivity. These high cell densities, in combination with the use of methanol as an inducing agent, lead to decreased cell viability, limited scalability due to metabolic heat generation and strain on primary recovery unit operations. These factors are compounded and result in poor product yields.

In this thesis a *P. pastoris* culturing methodology is proposed to simply and effectively improve the expression system in terms of product yield and scalability. Each of the following chapters has a specific objective in order to do this:

- Chapter 3: To establish a simple mixed feed induction strategy with sorbitol as a co-feed with methanol and assess the impact of the protocol on cell's growth characteristics and to determine whether the results are scalable and strain dependent

- Chapter 4: To develop a deterministic model which can predict the scale limitations of *P. pastoris* fermentations based on metabolic heat generation and bioreactor cooling capacities. This model can then be used to determine the impact of the mixed feed induction strategy on process scalability

- Chapter 5: To determine the affect of the mixed feed induction strategy on cell viability, product yield and the potential impact that this will have on downstream processing

- Chapter 6: To develop a small scale, offline technique to determine cellular robustness during fermentation in order to assess the impact of methanol induction on cellular robustness

Chapter 2:Materials and methods

2.1 Materials

2.1.1 Chemicals

All chemicals were purchased from Thermo Fisher Scientific (Hemel Hemptstead, UK) and of analytical grade unless otherwise specified.

2.1.2 *P. pastoris* strains

The yeast strains used in this study have been: *P. pastoris* GS115 wild-type, *P. pastoris* GS115 Mut+ SEAP and *P. pastoris* CLD804 Mut+ Aprotinin. The wild-type strain was purchased from Invitrogen (Paisley, UK) *P. pastoris* GS115 Mut+ SEAP was created in-house by Randone (2014) and *P. pastoris* CLD804 Mut+ Aprotinin was provided by Fujifilm Diosynth Biotechnologies (Billingham, UK).

2.1.3 Media components

500X B – Prepared by dissolving 20 mg biotin in 100 mL reverse osmosis (RO) water. The solution was then filter sterilised and stored at 4 °C.

10X D – Prepared by dissolving 40 g dextrose in 200 mL RO water. The solution was then heat sterilised in an autoclave and stored at room temperature.

10X GY – Prepared by adding 100 mL glycerol to 900 mL RO water. The solution was then heat sterilised in an autoclave and stored at room temperature.

10X M – Prepared by adding 50 mL methanol to 950 mL RO water. The solution was then filter sterilised and stored at room temperature.

50% methanol sorbitol C-mol/C-mol mixed induction feed – Prepared by dissolving 71.62 g D-sorbitol in minimal RO water aided by gentle heating before topping up to

100 mL total volume. 100 mL pure methanol was added to this solution and mixed thoroughly before being filter sterilised and stored at room temperature.

10X S – Prepared by dissolving 200 g D-sorbitol in minimal RO water before topping up to 1 L to volume. The solution was then filter sterilised and stored at room temperature.

10X YNB – Prepared by dissolving 16.8 g yeast nitrogen base with ammonium sulphate (YNB) in 200 mL RO water. The solution was then filter sterilised and stored at 4 °C.

2.1.4 Yeast extract peptone dextrose (YPD) agar medium

YPD agar medium was prepared by dissolving 10 g of yeast extract, 20 g of bacteriological peptone and 20 g of agar powder in 900 mL RO water. The mixture was then heat sterilised in an autoclave and 100 mL 10X D was added aseptically. The solution was distributed evenly in petri dishes, which were then sealed with parafilm and stored at 4 °C for later use.

2.1.5 Buffered minimal glycerol-complex (BMGY) medium

BMGY medium was prepared by dissolving 10 g of yeast extract and 20 g of bacteriological peptone in 700 mL RO water, the resulting solution was then heat sterilised in an autoclave. Once the mixture had cooled 100 mL 1M potassium phosphate buffer, 10X YNB, 2 mL 500X B and 100 mL 10X GY we added aseptically before storing the media at 4 °C for later use.

Buffered minimal methanol / sorbitol complex medium (BMMY / BMSY) were prepared using the same methodology as BMGY with 10X M and 10X S replacing 10X GY in BMMY and BMSY respectively.

2.1.6 Basal salt medium (BSM)

The components listed in table 2-1 were dissolved in RO water at half of the final volume, before being adjusted to pH 5 with ammonia and topped up with RO water to the final media volume).

Table 2-1 *Recipe for fermentation basal salt medium (BSM)*

Component	Amount added /L Final BSM Volume
Ortho-phosphoric acid, 85%	26.7 mL
Calcium Sulphate	0.93 g
Potassium Sulphate	18.2 g
Magnesium Sulphate 7H$_2$O	14.9 g
Potassium Hydroxide	4.13 g
Glycerol	40.0 g

2.1.7 PTM1 trace salts

The components listed in table 2-2 were dissolved in minimal RO water before adding RO water up to the final volume of 1 L and filter sterilising before storage at 4 °C

Table 2-2 *Recipe for PTM1 trace salts solution*

Component	Amount added /L Final PTM1 Volume
Cupric Sulphate 5H_2O	6 g
Sodium Iodide	0.08 g
Manganese Sulphate H_2O	3 g
Sodium Molybdate 2H_2O	0.2 g
Boric Acid	0.02 g
Cobalt Chloride	0.5 g
Zinc Chloride	20 g
Ferrous Sulphate 7H_2O	65 g
Biotin	0.2 g
Sulphuric Acid	5 mL

2.2 *Pichia Pastoris* Cultivation

2.2.1 *P. pastoris* cell bank creation

For the creation of master cell banks, cells were spread on YPD agar plates and grown at 30 °C for 24 hours. The largest colony that had grown was then used to inoculate 5 mL BMGY in a 50 mL falcon tube and again grown for 24 hours. Two 1 L baffled shake flasks containing 50 mL BMGY were subsequently inoculated with 1 mL from the 5 mL cell culture each. One flask was then sealed for the duration of the culturing process whereas the other was sampled every 2 hours to monitor cell growth. The flasks were incubated at 30 °C and agitated at 250 rpm in an orbital shaker. Once cells

had reached mid-exponential phase ($OD_{600} \approx 15$), 30 mL sterile glycerol was added to the sealed shake flask and mixed well. The resulting mixture was then aliquoted into 1 mL cryovials before being stored at -20 °C for 24 hours and then transferred to -80 °C for long term storage.

Working cell banks were made by the same methodology as previously explained except that the first two steps were omitted and the shake flasks containing 50 mL BMGY were instead inoculated with a previously created 1 mL master cell bank vial.

2.2.2 *P. pastoris* shake flask fermentation

2.2.2.1 Comparing growth on different carbon sources

A seed flask was grown by inoculating a 1 L baffled shake flask containing 150 mL BMGY with 1 mL of *P. pastoris* GS115 working cell bank and incubating the flask at 30 °C and agitating at 250 rpm in an orbital shaker. Once cells had reached mid-exponential phase ($OD_{600} \approx 30$) ~5 mL of cell culture was pelleted by centrifugation at 14,000 rpm for 5 minutes. The cells were then resuspended in 150 mL BMGY / BMMY / BMSY to an OD_{600} of 1 in duplicate flasks for each condition. After 24 hours growth the cultures were supplemented with 1.5 mL pure glycerol / pure methanol / 25M sorbitol solution. Samples were taken every 2 hours to monitor growth via OD_{600} measurements.

2.2.2.2 Comparing growth on a range of mixed feed ratios

The methodology described in section 2.2.2.1 was used to culture *P. pastoris* CLD804 cells in a seed flask before being harvested, pelleted and resuspended to an OD_{600} of 1 in complex media. In contrast to the previous methodology, the media in which cells were resuspended contained ratios (C-mol/C-mol) of either 100% methanol (BMMY), 80% methanol 20% sorbitol, 60% methanol 40% sorbitol, 40% methanol 60%

sorbitol, 20% methanol 80% sorbitol or 100% sorbitol (BMSY). Samples were taken every 2 hours to monitor growth via OD_{600} measurements.

2.2.3 *P. pastoris* fermentation

2.2.3.1 20 L fermentation

An initial seed culture was generated by inoculating a 500 mL baffled shake flask, containing 50 mL BMGY, with 1 mL of working cell bank. This culture was then incubated for 24 hours at 30 °C and agitated at 250 rpm in an orbital shaker. This cell culture was subsequently transferred into a 2 L baffled shake flask containing 450 mL BMGY and incubated under the same conditions for 24 hours.

A 20 L (total volume) Bio Bench bioreactor from Applikon (Delft, Netherlands) was used for pilot scale fermentations and was filled with 7.5 L BSM and steam sterilised in situ at 121 °C for 15 minutes. Once reactor temperature had dropped back to 30 °C, the BSM was supplemented with 31.9 mL of PTM1 and the reactor was inoculated once dissolved oxygen (DO) levels had returned to 100%. Inoculation was carried out by transferring cells to a sterile drainable flask and introducing them to the reactor via a silicon tube connected to the head plate. This was done to attain an initial cell density of $OD_{600} \approx 1$.

Fermentations were run according to the Invitrogen protocol (Invitrogen Corporation, 2002) or a modified version of it to incorporate a mixed induction feed regime wherein the mixed feed was fed at the same volumetric flowrate as methanol is in the unaltered protocol. Cells were cultured at 30 °C and an initial pH of 5; this was slowly increased over time to 6.5 in the case of GS115 but maintained for CLD804. DO was maintained at 30% via a cascade control system whereby impeller speed would rise from an initial speed of 400 rpm to a maximum of 1010 rpm in order to

increase oxygen transfer to the broth, when this was no longer sufficient, gas blending was employed to increase the proportion of oxygen in the inlet air to a maximum of 100% oxygen, at a rate of 1 vvm.

An initial glycerol batch phase was run until the carbon source was exhausted at approximately 18 hours post-inoculation, this was indicated by a DO spike (as indicated by DO briefly hitting 100% and growth stalling). This was followed by a glycerol fed-batch phase wherein 50% (w/v) glycerol (supplemented with 12 mL PTM1/ L) was added at a rate of 18.15 mL / hr / L BSM. This feed was maintained for 6 hours and followed by a brief starvation period to ensure the removal of any glycerol from the culture that would inhibit recombinant protein production. Induction was carried out using pure methanol or a 50% methanol sorbitol C-mol/C-mol mixed feed, both supplemented with 14 mL PTM1 / L. Induction occurred in 3 feeding phases: adaptation, intermediate and growth phase. Adaptation phase involved an induction feed rate of 3.6 mL/ hr/ L BSM and was carried out until cells were deemed to have adapted to the methanol containing feed. This was assessed in two ways: Firstly by a stabilisation of DO readings and secondly by seeing base being added to the bioreactor (indicating cellular metabolism). This stage took between 2 to 6 hours. Following adaptation, the feed rate was increased to 7.3 mL / hr / L BSM for 2 hours and then maintained at 10.9 mL / hr / L BSM for the remainder of the fermentation. Samples were taken throughout the fermentation to monitor growth and further analytics detailed in later sections.

As methanol accumulation was a concern for both reasons of safety (due to the large volumes involved) and toxicity to cells, the methanol levels were checked daily. This was done by briefly shutting off the feed pump and observing how long it took for a DO spike to occur. If it was immediate, methanol was determined to be limiting in the bioreactor and therefore of no risk of accumulating.

2.2.3.2 1 L fermentation

A seed culture was generated by inoculating a 1 L baffled shake flask containing 150 mL BMGY with 1 mL of working cell bank. This culture was then incubated for 24 hours at 30 °C and agitated at 250 rpm in an orbital shaker.

A bank of four 1 L (total volume) HT Multifors from Infors AG (Bottmingen, Switzerland) were used for bench-top fermentations and filled with 550 mL of BSM each before being sterilised in an autoclave. The BSM was supplemented with 2.4 mL PTM1 before inoculation, by aseptic injection into the reactor via a septum on the head plate. Inoculation was also carried out via this septum and to a starting cell density of $OD_{600} \approx 1$, once DO levels had returned to 100%.

The fermentations were carried out using the same conditions and protocol as described in section 2.2.3.1 with the exception of the cascade control which had the impeller speed increase from 300 rpm to 1100 rpm in order to increase oxygen transfer.

2.3 Analytical Techniques

2.3.1 Determination of Cell Density

Cell density was first determined by measuring optical density of the cell culture at a wavelength of 600nm (OD_{600}). A spectrophotometer was first blanked using a cuvette containing water before cell culture samples were measured. If samples had an OD_{600} > 1, they were serially diluted with water to an have an OD_{600} between 0.2 and 1. These readings were taken on the first stable value in order to avoid sample sedimentation affecting the result. Triplicate samples were taken.

In order to gain a more accurate quantification of cell density, cell weights were also measured. This was done by first pipetting 1 mL of well mixed cell culture (mixed by aspirating) into pre-weighed 1.5 mL Eppendorf sample tubes, spinning down the samples in a bench-top centrifuge at 14,000 rpm for 3 minutes and removing the supernatant by pipette (this supernatant was stored at -20°C for later analysis). The tubes were then weighed and had their empty weight subtracted in order to determine the wet cell weight in g/L. Sample tubes were then dried at 100°C for approximately 24 hours (once weight had stopped changing) and reweighed in order to determine the dry cell weight in g/L. These dry cell weights were then used to determine the maximum growth rate (μ_{max}) via the equation:

$$\mu = \frac{\ln C_2 - \ln C_1}{t_2 - t_1}$$

Where: C = cell density (g/L)

t = time at which cell density reading was taken (h)

2.3.2 SDS-PAGE Electrophoresis

NuPAGE® Novex 4-12% Bis-Tris 15 well 1 mm pre-cast gel kits from Invitrogen (Paisley, UK) were used to qualitatively assess Aprotinin release throughout *P. pastoris* CLD804 fermentations and provide an insight into protein impurity profiles before two-dimensional gel electrophoresis. Samples were prepared in PCR sample tubes wherein 2 μL of fermentation supernatant was added to 2.5 μL NuPAGE® LDS (lithium dodecyl sulphate) sample buffer, 1 μL reducing agent and 3 μL ultra-pure H_2O. Sample volume was increased or reduced depending on the product band intensity but kept constant when lanes were compared. Loaded sample volume was maintained at 10 μL by increasing or decreasing the volume of water added by the change in fermentation supernatant added. The mixtures were then heated at 90°C for 15 minutes.

A 5 µL Mark 12™ unstained protein ladder (Invitrogen, Paisley, UK) was first loaded onto the gel followed by the 10 µL samples. Gaps were left between ladder and samples and between different fermentations. Running buffer was prepared by diluting 20X MES (2-(*N*-morpholino)ethanesulphonic acid) buffer in RO water to a final volume of 1 L. Gels were run at a constant voltage of 200 V and predicted current of 125 mA for 35 minutes.

Gels were then removed from their casing, washed in RO water, then fixed in a 100 mL solution containing 50% methanol and 7% acetic acid (by volume with RO water) for one hour. Following this the gels were washed again in RO water and left to stain in 60 mL SYPRO® Ruby (Thermo Fisher Scientific, Hemel Hempstead, UK) overnight in containers omitting all light. Gels had any excess staining solution rinsed off with RO water and were washed in a 100 mL solution containing 10% methanol and 7% acetic acid (by volume with RO water) for 30 minutes, again protected from light. A final RO water rinse was done before the gels were scanned at excitation / emission wavelengths of 280/610 nm using the Typhoon™ 9410 Variable Mode Imager (Amersham Biosciences, Little Chalfont, UK).

2.3.3 Protein Quantification

2.3.3.1 Secreted Embryonic Alkaline Phosphatase (SEAP)

The QUANTI-Blue™ Colorimetric Enzyme Assay Kit from Invitrogen (Paisley, UK) was used to detect and quantify SEAP present in the supernatant of fermentation samples. Due to the very small quantities of SEAP in the samples it had to be determined how long the assay had to be developed for, in order to get the greatest sensitivity without degradation to the product by protease activity. QUANTI-Blue™ detection medium was first made by dissolving one sachet in 100 mL RO water, warming the resulting solution for 10 minutes at 37 °C, filter sterilising the solution and storing it at 4 °C

until use. 20 μL samples were loaded into a clear 96-well plate in triplicate as well as a standard curve. The standard curve was created by performing serial dilutions of placental alkaline phosphatase (PAP) which was supplied as part of the NovaBright™ Phospha-*Light*™ kit (Thermo Fisher Scientific, Hemel Hempstead, UK) at a concentration of 300 μg/L and also loaded in 20 μL samples in triplicate. 180 μL of QUANTI-Blue™ detection medium was then added to the samples before the plate was incubated at 37 °C for 6 hours in a Tecan Safire[2] plate reader (Tecan, Switzerland). During this time regular absorbance readings were taking at 620 nm in order to determine the progression of the reaction. The time point chosen for the quantification of SEAP was the latest time point at which the highest SEAP concentration's absorbance was still increasing linearly i.e. not subject to signal overexposure or protease degradation.

2.3.3.2 Aprotinin

The amount of aprotinin in fermentation samples was determined by SDS-PAGE electrophoresis (as described previously) and analysing the aprotinin band by densitometry using ImageJ (National Institutes of Health, USA). In order to ensure accurate readings, samples were diluted to the point at which the product band was no longer over-exposed (0.5 μL of fermentation sample was typically loaded).

2.3.3.3 Total Soluble Protein

Total secreted protein was quantified using two assays: Bradford Assay (Lonza Biologics, Slough, UK) and the Pierce™ BCA Protein assay kit (Thermo Fisher Scientific, Hemel Hempstead, UK). For both assays standard curves were first created by performing serial dilutions of 2 g/L bovine serum albumin (BSA) stock solution and loading the standards (dependent on the volume of sample determined) in

triplicate onto a clear, flat bottomed, 96-well plate. The amount loaded was kept consistent with the amount of sample loaded.

For the Bradford Assay, 5 – 20 µL samples were loaded (depending on experiment and sensitivity required) in triplicate onto the 96-well plate. All samples and standards then had 200 µL of filtered Bradford reagent added and were transferred to a Tecan Safire² plate reader after 5 minutes incubation at room temperature. Absorbance readings were taken at 595 nm and sample values were compared to the standard curve in order to determine protein concentration.

For the BCA assay, a working reagent was first prepared by mixing 50 parts reagent A with 1 part reagent B having calculated the total volume required per plate (approximately 15 mL). 25 µL samples were loaded in triplicate onto the 96-well plate and 200 µL working reagent was then added. The plate was sealed (to prevent evaporation) and incubated at 37 °C for 30 minutes. Following this incubation time the plate was transferred to a Tecan Safire² plate reader and absorbance was measured at 562 nm, with sample values being compared to the standard curve in order to determine protein concentration.

2.3.4 DNA Quantification

Total DNA release was quantified using the Quant-iTTM PicoGreen® dsDNA reagent assay kit from Invitrogen (Paisley, UK). DNA standards were first created from stock DNA solution by performing serial dilutions with ultra-pure water, 100 µL of each was then loaded in triplicate onto a clear, flat-bottomed, 96-well plate. To create the working reagent for the assay, pure PicoGreen® dsDNA reagent was diluted 200-fold in TE buffer; this had to be done in low-light levels due to the photo-sensitivity of the reagent. 100 µL of samples were then also loaded in triplicate onto the 96-well plate and 100 µL of working reagent was then added to all wells before the plate was

wrapped in foil in order to exclude light, and incubated for 5 minutes at room temperature. The plate was then analysed using a Tecan Safire^2 plate reader with fluorescence settings at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. DNA concentration in the samples was then determined by comparing the values to the standard curve.

2.3.5 Protease Quantification

Protease release into the cell culture supernatant was determined using the Pierce® fluorescent protease assay kit. 5 mg/mL FTC-casein (fluorescein isothiocyanate labelled casein) stock solution was first created by dissolving 2.5 mg of FTC-casein in 500 μL ultra-pure water and preparing 24, 20 μL aliquots. These were then used to make a working reagent by diluting one aliquot per 96-well plate, 500x in BupH™ TBS buffer. Trypsin stock solution was created by dissolving 50 mg of lyphilised TPCK Trypsin in 1 mL of TBS buffer and making 20 μL. A range of trypsin standards were then made by performing serial dilutions with TBS buffer. Samples were prepared for analysis by first adding 50 μL of sample per well in triplicate, of a clear flat-bottomed 96-well plate before adding 50 μL of 1% to each to correct the pH to 7.2. The standard curve was prepared by loading the 100 μL of TBS diluted trypsin in triplicate to the plate. Finally 100 μL of working reagent was added to each well and the plate was incubated at room temperature for 5 minutes before being analysed using a Tecan Safire^2 plate reader with fluorescence settings at an excitation wavelength of 480 nm and an emission wavelength of 520 nm

2.3.6 Proteomic Analysis

2.3.6.1 Two-Dimensional Electrophoresis

In order to determine the protein impurity profiles from fermentation end-point supernatant samples 2-D electrophoresis was used. 1 mL samples of supernatant was first treated with 0.4 µL molecular biology grade benzonase and 5mg of cOmplete mini EDTA-free protease inhibitor tablets and mixed by brief vortexing before being incubated on ice for 2 hours. Having determined the protein concentration of replicate samples by one of the previously described methods (see section 2.3.3.3), samples were split into volumes containing 200 µg of protein each, which were then purified using the 2-D Clean-Up Kit (GE Healthcare, USA) using the low sample load protocol. After being air-dried for a minute, the protein pellets were then resuspended in 125 µL DeStreak Rehydration Solution (GE Healthcare Life Sciences, Little Chalfont, UK), which had 6 mg DTT (dithiothreitol) and 5 µL IPG buffer added per 1 mL solution, and vortexed briefly to ensure complete suspension of the pellet. The samples were then centrifuged at 14,000 rpm for 2 minutes in order to ensure that no insoluble impurities were loaded onto the first dimension. For each sample, 125 µL were loaded into 7 cm ceramic strip holders, ensure no bubbles and that the floor of the well was evenly covered with sample. The 7 cm Immobiline DryStrips pH 3-10 NL (IPG strips) [GE Healthcare Life Sciences, Little Chalfont, UK] that were used for the first dimension, first had the backing plastic removed and were then inserted positive end first, gel side down, into the strip holders, ensuring no bubbles became trapped underneath. Finally the strips were covered with 300 µL of overlay fluid to ensure the strips did not dry out during isoelectric focussing (IEF) and sealed with a plastic lid. The standard focussing protocol for 7 cm Immobiline DryStrips pH 3-10 NL was used.

Once IEF had been completed (8,000 volt hours is reached) the IPG strips were removed from the strip holders and washed using ultra-pure H_2O. The strips were then transferred into 15 mL tubes containing 5 mL LDS sample buffer with 5 mg DTT and incubated at room temperature with gentle agitation for 15 minutes. The strips were then removed from the solution and washed again in ultra-pure H2O before being transferred to 15 mL tubes containing 5 mL LDS sample buffer with 125 mg iodoacetamide, and incubated at room temperature with gentle agitation for 15 minutes. The strips were then washed again in ultra-pure H_2O before being loaded onto NuPAGE® Novex® 4-12% Bis-Tris ZOOM® 1 mm protein gels and sealed in with 400 μL of agarose and a 5 μL Mark 12™ unstained protein ladder was used for each. Running buffer was prepared by diluting 20X MES buffer in RO water to a final volume of 1 L. Gels were run at a constant voltage of 200 V and predicted current of 125 mA for 50 minutes. The gels were then stained with SYPRO® Ruby and visualised as described in section 2.3.2.

2.3.6.2 Mass Spectroscopy

Supernatant from samples taken from the end of fermentations was analysed by LC-MALDI-TOF/TOF (liquid chromatography matrix-assisted laser desorption/ionisation time of flight mass spectrometry run in parallel) mass spectroscopy. For this samples were stored at -20 °C in ultra-pure water and outsourced to the University of Kent, School of Biosciences for analysis. An aliquot of supernatant equivalent to 100 μg of protein was solubilised in solution of 20 μL 8M urea, 100 mM ammonium carbonate and 20 mM DTT. This solution was incubated at room temperature for 1 hour before being carboxyamidomethylated by the addition of 10 μL of 100 mM iodoacetamide and incubation at room temperature for further 15 minutes. The solution was then diluted with 65 μL ultra-pure water. 5 μL of 0.2 mg/mL modified sequence-grade trypsin (Promega, Madison, USA) was added to

the solution before incubated at room temperature for 18 hours. In order to stop trysin activity, 10 μL of 10% trifluoroacetic acid (TFA) was added and samples were then stored at -20 °C before analysis.

The samples were analysed by reverse-phase LCMS on an Agilent 1100 HPLC system coupled to a micrOTOF-Q II electrospray mass spectrometer (Bruker, Coventry, UK). The tryptic peptides were separated on a Aeris 150 x 2.1 mm XB-C18 column (Phenomenex, Macclesfield, UK) using a 0.1% formic acid, water/0.1% formic acid, acetonitrile gradient of 5 to 50% B over 45 minutes. Peptides were subjected to MS/MS fragmentation utilising a top 5 data dependant analysis protocol and data analysed using Bruker's Compass Data Analysis 4.1 to prepare mascot generic data files which were then submitted to a Matrix Science mascot MS/MS ion search.

2.3.7 Sonication

2.3.7.1 Ultrasonic Disintegration

In order to measure non-secreted cellular products in fermentation samples, cells were disrupted using ultrasonic disintegration. The Sonosep Sonicator was used to treat 1 mL samples for 6 seconds at an amplitude of 15 μm. Samples were kept on ice during sonication to avoid potential overheating of the suspension.

2.3.7.2 Adaptive Focused Acoustics

The Covaris E210 Focused-ultrasonicator (Covaris, Inc., Woburn, MA, USA) was used as a tool to assess cellular robustness during fermentation and was characterised as described in chapter 6. Prior to sonication, the water bath used for transduction was cooled to and maintained at 10°C (± 1 °C) using a water chiller in addition to being degassed for 1 hour. Cell culture suspensions of 1-3 mL were loaded into 6 mL

borosilicate glass vials and suspended in the chilled water above a 200196 ultrasonic transducer. Duty cycle and cycles per burst were maintained at 20% and 1,000, corresponding to the percentage of a cycle that the sonicator is producing energy and the frequency of these cycles, respectively. The settings which were varied throughout the characterisation were the sample volume and intensity. Finally the device was used in power-tracking mode wherein the device optimises operating frequency using an electrical feedback loop in order to maximise power input into the sample vessel.

In order to determine cellular robustness, 20 mL fermentation samples were taken and pelleted by centrifugation. The pellets were then resuspended in phosphate-buffered saline (PBS) to an optical density of 150. 1 mL aliquots of the sample were made in 6 mL borosilicate glass vials before each was subjected to range of sonication times of 0, 30, 90, 180, 300, 450 and 600 seconds at a duty cycle of 20%, cycle per burst of 1,000 and intensity of 10 on power tracking mode equation to a power input of ~93 W. Protein release was measured in order to quantify cellular disruption after sonication.

2.3.8 Particle Size Distribution

The Mastersizer 2000 (Malvern Instruments, UK) was used to determine the particle size distribution of cell culture samples in order to assess cell viability by determining average cell size and whether cell debris was present. During operation a refractive index of 1.5 was assumed for *P. pastoris* cells and the dispersant was assumed to be water. Samples were added dropwise to 100 mL ultra-pure H_2O in a Hydro 2000 SM dispersion unit until a light obscuration of 15% was reached.

2.3.9 Determination of Cellular Heat Generation

In order to determine the heat generated by cells during various stages of the fermentation, the cooling system of the bioreactors was disengaged and temperature readings were taken via the built in temperature probe. The equilibration time during which the residual cooling effect of the stagnant cooling water was warmed by the cells was discounted in the calculation of rate of temperature rise, and readings were taken every minute (manually) over a period of 30 min.

2.3.10   Determination of Cell Culture Viscosity

The viscosity of cell culture samples was assessed using a DV-II Viscometer (Brookfield Viscometers, Harlow, UK) with a CP-42 spindle being used for cell culture applications. 1 mL of cell culture was loaded into the viscometer cup which was then reattached and an initial speed rotational of 5 rpm was used to evenly distribute the sample between the spindle cone and the cup. Once torque readings had stabilised, shear stress and shear rate readings were taken at a range of rotational speeds from 10 rpm to 200 rpm.

2.3.11   Optical Microscopy

Cell culture suspensions were observed using a Leica DMRA2 microscope (Leica, Cambridge, UK) with image capture and cell size analysis being done using a Leica Q500MC image processing and analysis system.

2.3.12   Transmission Electron Microscopy

Detailed yeast morphological analysis was done by transmission electron microscopy (TEM). 0.2 mL cell culture samples were pelleted by centrifugation and the supernatant removed. The cell pellets were then resuspended in 1 mL fixing solution

containing 2.5% (v/v) glutaraldehyde and 20 mmol/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer and stored at 4 °C, before being sent to the UCL department of Biosciences for analysis. Cells were then immobilised in 1% (w/v) agar and further fixed in 1% (w/v) osmiumtetroxide in 75 mmol/L cacodylate buffer., before being dehydrated on ice using an ethanol series and stained with 1% (w/v) uranylacetate (in 70% ethanol) (Lünsdorf et al., 2011). After an 8 hour polymerisation period, cells were penetrated with epoxy. Ultrathin sections were cut from the imbedded cells and analysed using a Jeol 1010 electron microscope (Jeol, Welwyn Garden City, UK). Peroxisome counting was done with anonymised samples to eliminate any bias.

Chapter 3: Development of a mixed feed fermentation strategy

3.1 Introduction

In Chapter 1, approaches to improving the overall cell viability and productivity of *P. pastoris* cultures were reviewed and discussed. These approaches were split into two major categories: cultivation strategies and strain engineering methods. The latter methodology was discounted as a means of improving *P. pastoris* as a platform for producing recombinant protein, because any improvements made would be cell line specific and any existing cell lines couldn't benefit from this. These changes would also have to be demonstrated to be effective with any new cell lines created, as variation in product characteristics means that the benefit of host cell modifications on productivity will vary as well. Cultivation strategies on the other hand can be applied to most cell lines and can be easy to implement. Of all alternative cultivation strategies, the use of a methanol sorbitol mixed induction feed showed the most promise as it could be applied to all *P. pastoris* Mut+ strains, which constitute the majority of industrially utilised strains (Ahmad et al., 2014; James M Cregg, 1993).

Sorbitol was chosen as a co-feed with methanol during induction due to it being reported to be a non-repressing carbon source, unlike glycerol and glucose which inhibit protein product via the AOX1 promoter (Inan & Meagher, 2001a). A number of strategies for the implementation of sorbitol co-feeding have been suggested, utilising metabolic flux analysis (Celik, Calik, & Oliver, 2010; Niu et al., 2013) and complex feeding strategies. The main focus of these has been final product concentration only, this is insufficient when considering a whole bioprocess as the number and amount of impurities produced can vary and complicate downstream processing (Gronemeyer, Ditz, & Strube, 2014). This chapter will focus on development of the mixed feed regime, using both *P. pastoris* GS115 producing secreted embryonic alkaline phosphatase (SEAP) and an industrially utilised strain *P. pastoris* CLD804 which produces Aprotinin, in order to assess whether the results are

strain dependent. Later chapters will then discuss the implications of the implementation of the newly developed mixed feed regime. Therefore, the key objectives of this chapter are:

1) To determine an optimal methanol to sorbitol ratio based on growth and productivity of cells

2) To develop a simple and scalable feeding strategy utilising the optimal ratio of carbon sources obtained

3.2 Results and Discussion

3.2.1 Effect of a mixed induction feed on growth

3.2.1.1 Growth on different carbon sources

In order to gain an understanding of how *P. pastoris* behaves on different substrates, a study was carried out in shake flasks. *P. pastoris* GS115 Cells were cultivated in complex media containing glycerol, methanol or sorbitol as the sole carbon source, with an equal amount of carbon present in each flask and all flasks being inoculated with an equal concentration of starter culture grown on glycerol containing BMGY (figure 3-1). This starter culture had been harvested after 15 hours of growth in order to obtain an inoculum in which cells were in exponential phase, thus minimising the lag phase in the growth comparison and ensuring comparability between all subsequently inoculated flasks. It can be seen from figure 3-2 that cells were able to rapidly metabolise glycerol, leading to a comparatively high growth rate of μ = 0.29 1/h (due to the low cell densities involved, cell growth rates were calculated using OD_{600} readings with the methodology described in section 2.3.1). This meant that BMGY could support a cell density of OD_{600} = ~50 before the carbon

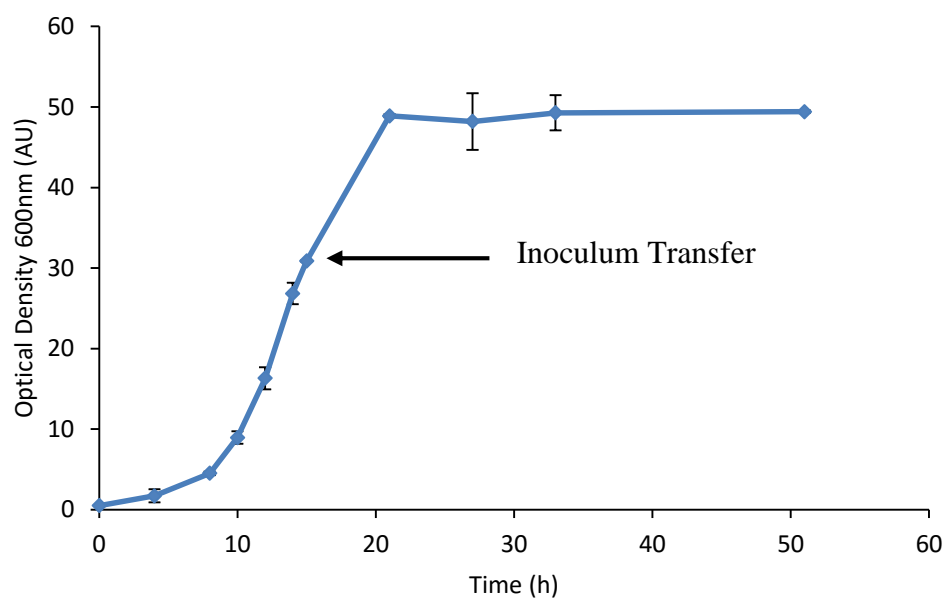**Figure 3-1** *Growth curve of P. pastoris GS115 producing SEAP starter culture in a baffled shake flask containing 150 mL BMGY*
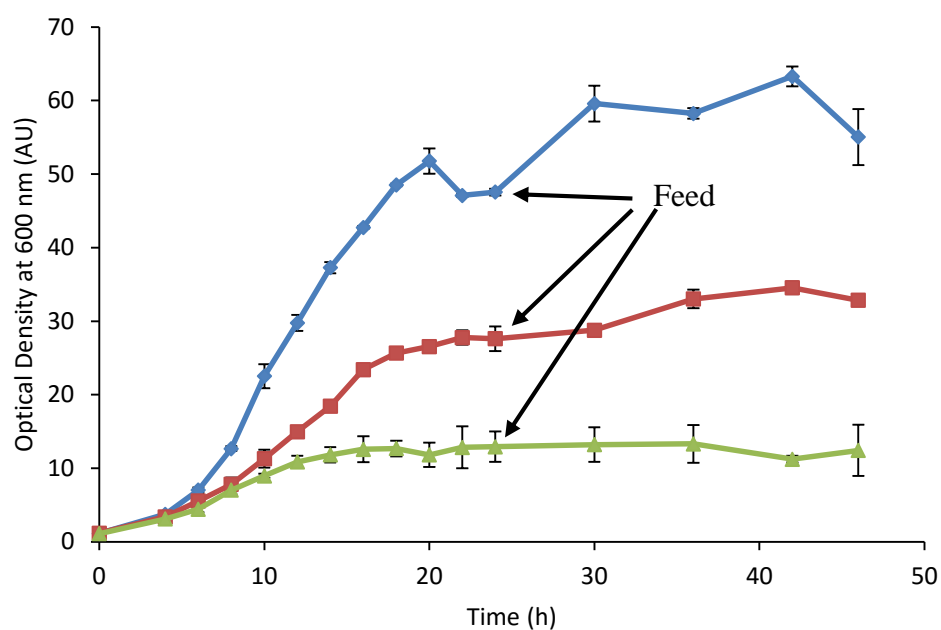
Figure 3-2 *Growth curve of P. pastoris GS115 producing SEAP in baffled shake flasks, post-inoculum transfer to 150 mL complex media containing glycerol (◆), methanol (■) or sorbitol (▲) as a carbon source. All flasks received 1.5 mL of additional carbon source 24 h post-inoculation.*

source was exhausted, which happened after 20 hours post-inoculation. Additional glycerol supplementation allowed cells to reach a maximum density of $OD_{600}= \sim60$ before the carbon source was again exhausted. By comparison, the use of methanol as a carbon source led to a lower exponential growth rate of $\mu = 0.15$ 1/h which resulted in a cell density of $OD_{600} = \sim30$ to be reached and sustained during stationary phase. Finally, it was found that sorbitol was metabolised slowest of the three carbon sources leading to a significantly lower growth rate $\mu = 0.12$ 1/h and final cell density of $OD_{600} = \sim12$ during stationary phase, which was sustained throughout the remainder of the shake flask fermentation. These findings show that sorbitol can support cell growth, albeit at lower growth rates and final cell densities than pure methanol would provide. Although no direct comparison of growth rates could be found in literature, these findings are in agreement with previous studies which showed the cell yield on sorbitol is lower than on glycerol (Thorpe et al., 1999) and results in lower growth rates (Resina et al., 2004).

3.2.1.2 Growth on varying methanol to sorbitol carbon source ratios

With this work completed, focus was turned to determining an ideal carbon source feed ratio both with respect to cell growth and recombinant protein production. In order to do this, *P. pastoris* CLD804 cells were grown in complex media containing a range of methanol sorbitol ratios and grown for a period of 27 hours to allow cells in all conditions to reach stationary phase. Comparison between the different feed ratios was based on keeping the mass of carbon constant between each substrate mixture which was calculated knowing that the carbon mole (C-mol) ratio of sorbitol : methanol = 1 : 1.0553 (determination of the ratio can be found in Appendix A and feed preparation in section 2.1.3). This work not only served to determine the effect of varying sorbitol concentrations on growth, but as a new cell

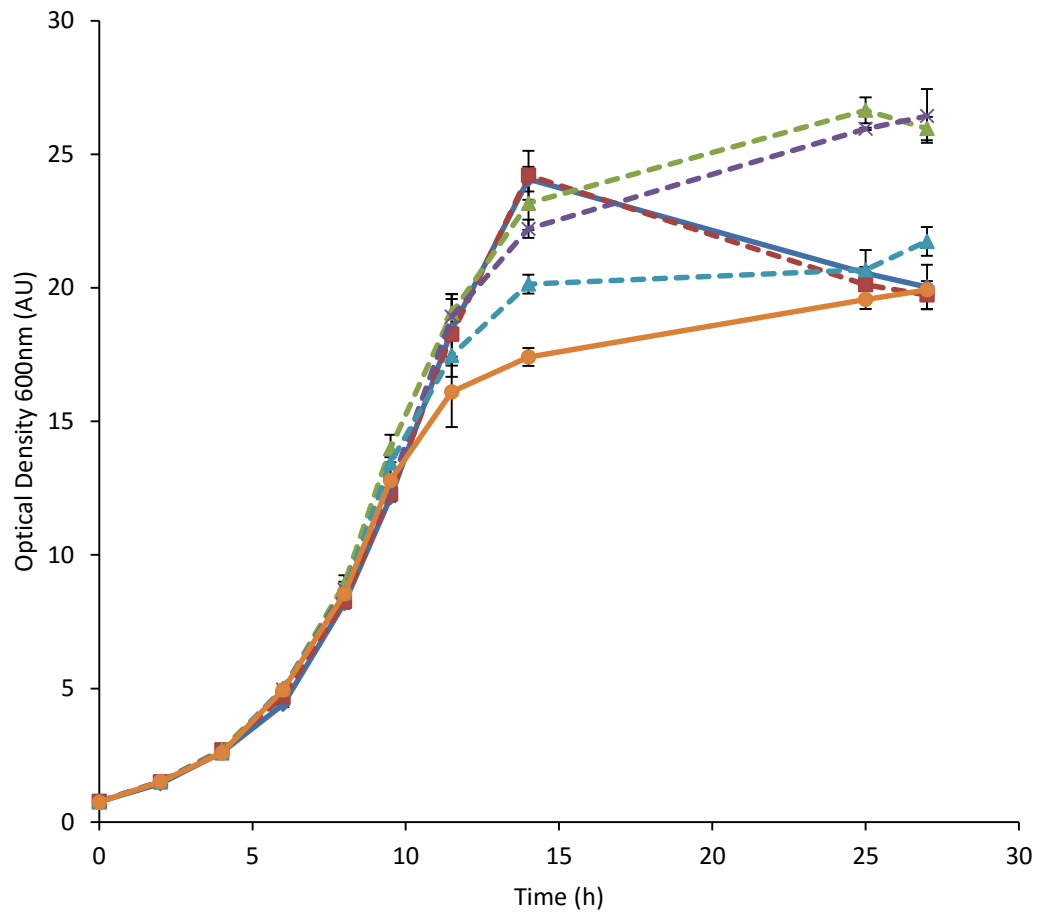**Figure 3-3** *Growth curves of P. pastoris CLD804 producing Aprotinin in 150 mL complex media containing pure methanol (solid line, ◆), 80 % methanol 20 % sorbitol C-mol/C-mol (dashed line, ■), 60 % methanol 40 % sorbitol C-mol/C-mol (dashed line, ▲), 40 % methanol 60 % sorbitol C-mol/C-mol (dashed line, x), 20 % methanol 80 % sorbitol C-mol/C-mol (dashed line, ▲) and pure sorbitol (solid line, ●) as a carbon source.*

line was used (*P. pastoris CLD804*) it also was designed to demonstrate that the effect is the the same, regardless of cell line. The results confirmed the findings in figure 3-2 obtained using *P. pastoris* GS115, with pure methanol again leading to a cell density of OD_{600} = ~25 at the start of stationary phase and cells reaching an OD_{600} of = 12 whilst growing on pure sorbitol over the same period of time. The incremental increases in methanol concentration corresponded to incremental increases in the maximum cell density reached until a ratio of 80% methanol 20 % sorbitol C-mol/C-mol was reached, which yielded the same cell density as pure methanol. As the cells were not given extra carbon source after 24 hours growth, the more rapidly growing flasks (80% methanol 20% sorbitol C-mol/C-mol and pure methanol) had exhausted their carbon supply after 25 hours unlike all other, slower growing flasks which had continued to grow. This correlated with the previous study an increased sorbitol concentration in flasks led to cell growth being supported for longer. This meant that a ratio containing between 40% and 60% methanol C-mols was optimal for growth as high cell densities could be reached and sustained for longer than with pure methanol.

3.2.2 Effect of a mixed feed on product yield

This positive result in terms of cell growth would not however be beneficial without product yield also being increased or at least being equivalent between a pure methanol carbon source and mixed carbon sources. This work was also used to confirm that sorbitol does not induce or inhibit recombinant protein production via the AOX1 promoter, as has been often cited in literature (Inan & Meagher, 2001b; Thorpe et al., 1999; Xie et al., 2005). As *P. pastoris* CLD804 produces high levels of Aprotinin (a commercially used protease inhibitor historically sold under the trade name Trasylol (Schneeweiss, Seeger, Landon, & Walker, 2008)), product
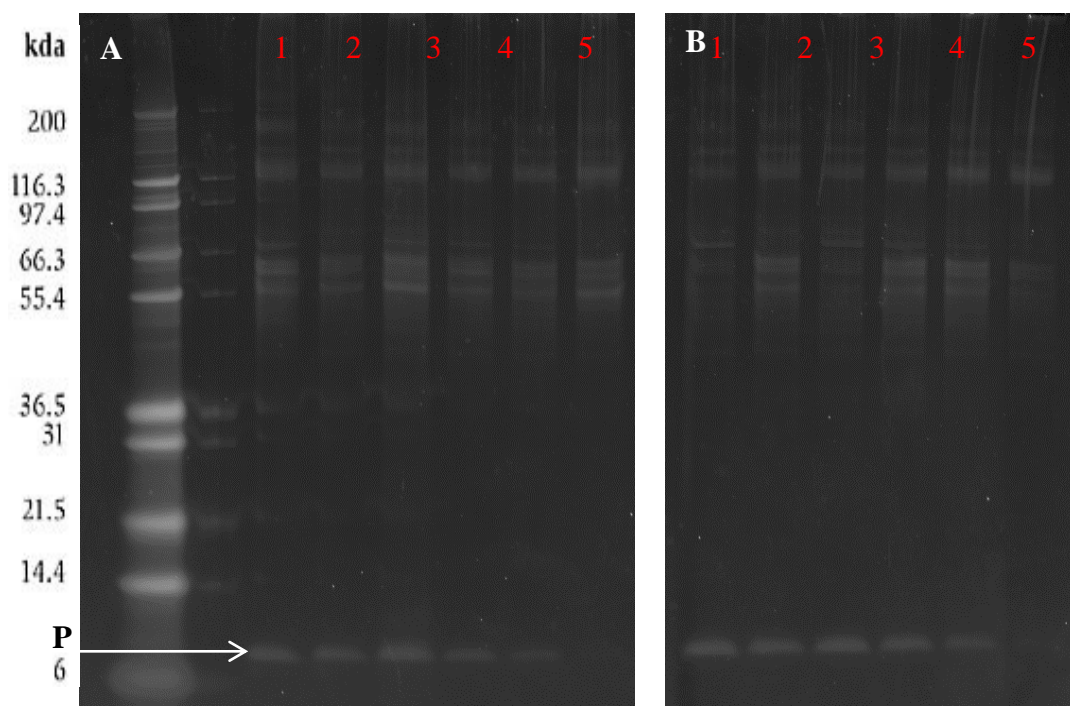
Figure 3-4 *SDS-PAGE gel showing samples taken from P. pastoris CLD804 cultures shown in figure 3-3, 15 hours post induction with lanes in A containing loaded with 4 µL of sample and B with 5 µL of sample but the same total load volume. In both A and B samples were from flasks where pure methanol (1), 80 % methanol 20 % sorbitol C-mol/C-mol (2), 60 % methanol 40 % sorbitol C-mol/C-mol (3), 40 % methanol 60 % sorbitol C-mol/C-mol (4), 20 % methanol 80 % sorbitol C-mol/C-mol (5) and pure sorbitol (6) were used as a carbon source. Note: Aprotinin product band indicated by P.*
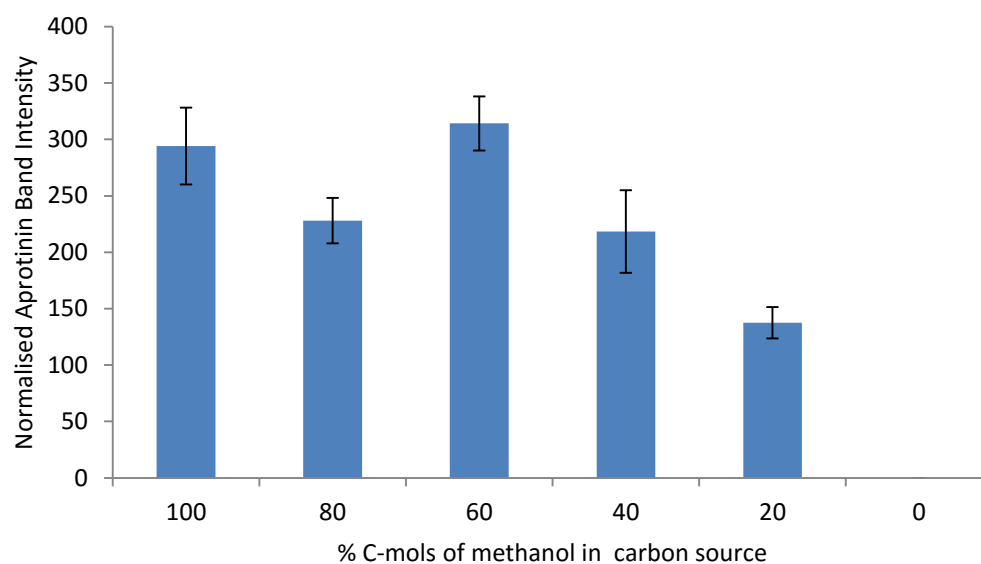
Figure 3-5 Densitometric analysis of the Aprotinin bands in figure 3-4 A & B. Band intensity was normalised firstly by cell density and then by loaded sample concentration. Error bars are a result of the standard deviation between product band density in A & B.

concentrations could easily be visualised using SDS-PAGE. Samples were taken at 15 hours post-inoculation for protein analysis as this was the highest cell density reached by all flasks before cell death started to occur. Total protein secretion was found to be equivalent between each condition as determined by Bradford assay (see Appendix A), SDS-PAGE analysis however showed there to be distinct variation between samples. It was first confirmed that not only did sorbitol not induce recombinant protein production, as shown by the absence of an Aprotinin band with pure sorbitol as the carbon source, but that it also did not inhibit it, with all methanol containing samples producing similar product bands. The intensity of these bands was then quantified by densitometry analysis which showed that methanol concentrations of 40 % C-mol or above were favourable for recombinant protein secretion. Combining this information with the previous growth data it was assessed that between 40 % and 60 % C-mol of methanol yielded the greatest specific yield calculated by band intensity per OD unit (figure 3-5). It was therefore decided that a mixed induction feed ratio of 50% methanol sorbitol C-mol/C-mol would be an appropriate ratio to assess the potential benefits of utilising a mixed induction feed. This aligns well with previous work found in the literature in which a methanol sorbitol mixed feed was assessed, albeit via chemostat culture, wherein a mixed feed ratio of 43% methanol to 57% sorbitol C-mol/C-mol was found to be optimal (Jungo, Schenk, Pasquier, Marison, & Von Stockar, 2007).

3.2.3 Scale studies

Fermentations during this project were carried out at 1 L (total volume) scale in four parallel bioreactors in order to provide reproducible and comparable data. High cell densities are the cause of many of the challenges associated with *P. pastoris* processes, whilst also being necessitated due to its typically low specific yields (Curvers et al.,

2001). It was therefore necessary to demonstrate that high cell densities achieved in larger fermentations could be reproduced at a scalable mL working volume.

For this scale comparison study the strain GS115 was used due to its use in previous work in the department and prevalence in literature. A scale-down analysis was first done in order to ensure that cells were experiencing similar conditions in both a 1 L and a 20 L bioreactor. Impeller tip-speed was decreased by the decrease in scale, with maximum tip speed being reduced from 3.97 m/s to 2.19 m/s (see Appendix A for calculations), and so sheer effects from mixing were not a problem at 1 L scale. Due to the lower tip-speed the level of mixing was also assessed. In both bioreactors however, fully turbulent conditions were found to be present, according to Reynolds number. Power input to volume ratio was used as the key scale down criteria to ensure that cells were experiencing similar conditions in both bioreactors. The P/V ratio was found to be equivalent in both bioreactors, with P/V = 12,400 W/m³ at 20 L scale and P/V = 11,460 W/m³ at 1 L scale.

 Standard methanol induced fermentations were run at both scales and their growth profiles compared to each other and to literature data. The fermentations chosen for this comparison all used the AOX1 promoter system and were not protease deficient strains, so that growth characteristics were comparable. They were also grown with a similar strategy involving an initial glycerol batch phase, followed by pure methanol induction phase. As can be seen in figure 3-6, cells grown in the 1 L parallel bioreactors attained a cell density of 148 g/L DCW, which exceeded both the density attained by the same strain in 20 L (116 g/L DCW), and densities attained by other studies, the highest of which reached a maximum cell density of 129 g/L DCW. In addition to this, multiple other fermentation characteristics were compared and assessed as seen in table 3-1, although productivity was omitted as a point of comparison due to the wide range of expression levels seen, which depends on the

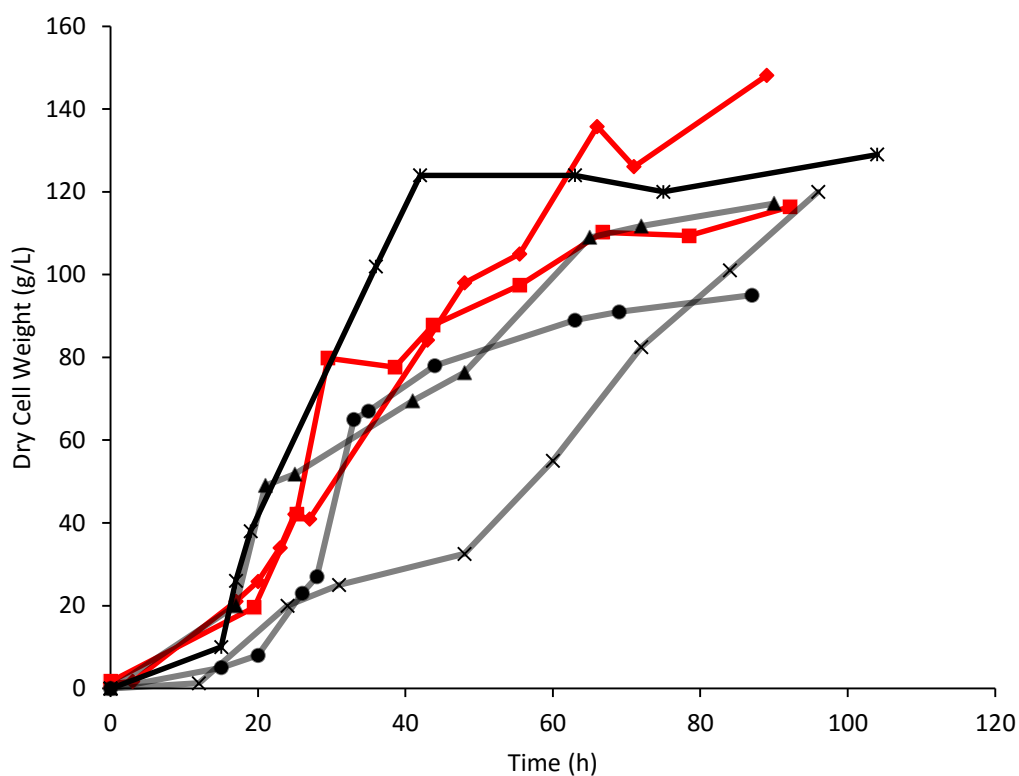*Figure 3-6* *Comparison of growth profiles of P. pastoris seen in literature. With P. pastoris GS115 SEAP fermentations at 1 L (♦) and 20 L (■) scale being compared to 30 L (● , Curvers et al. 2001), 20 L (▲ , Werten et al. 1999), 5 L (✳ , Ayed et al. 2008) and 2.5 L (X , Hong et al., 2002). Note: no induction time is shown due to variation between protocols, refer to table 3-1 for further details.*

type of product expressed; not the scale it was expressed at. It was concluded that the growth profiles were not affected by scale but instead were dictated by the methanol feeding regime and other fermentation conditions such as temperature, whereby a lower methanol feeding rate and lower temperatures led to lower cell densities being obtained and vice versa (Hong et al., 2002). Having successfully demonstrated that the 1 L bioreactors were appropriate for the investigation, a fermentation strategy was chosen and developed in order to provide replicable data that could be used to assess and compare different induction feeds. Due to previous work at UCL using the Invitrogen protocol (Invitrogen, 2002) for *P. pastoris* cultivation, it was deemed an appropriate starting point for the development of a strategy as the results could be compared to historical data as well as literature that is based on similar protocols. In addition to this, it allowed work to be done within limitations of the system being used: without a means of measuring residual methanol within the bioreactor during fermentation and with feed rates having to be measured and altered manually using scales, complex feeding strategies were not feasible. Therefore fermentations were carried out by either using this protocol with pure methanol or with the 50% methanol sorbitol C-mol/C-mol mixed feed, as this would provide the same amount of carbon for the same set volumetric flow rate. Fermentations using both *P. pastoris* GS115 and *P. pastoris* CLD804 were carried out in order to establish whether the modified feeding strategy with a mixed feed would be successful and transferable between different strains. Due to the unreliable nature of optical density readings at high cell densities (figure 3-7A), dry cell weights were also taken in order to accurately assess the growth profiles on each feeding strategy (figure 3-7B).

The application of a mixed induction feed was discovered to yield a similar overall growth profile to cells growing on pure methanol during induction, with this effect being strain independent (growth rates shown in table 3-1) and final cell densities attained being within 10 g/L DCW of each other for both GS115 and CLD804 (figure

80

3-7). Although final cell densities were equivalent between feed types, some growth characteristics did vary. It was found that for both strains the adaptation phase, in which *P. pastoris* cells change their organelles in order to successfully metabolise methanol, was eliminated during induction by mixed feed (figure 3-8). This was thought to be due to the difference in how methanol and sorbitol are metabolised by cells. In order to metabolise methanol, cells need to create peroxisomes which allow methanol to be oxidised by AOX (Gould, McCollum, Spong, Heyman, & Subramani, 1992; Veenhuis, Van Dijken, & Harder, 1983). This process takes time (~6 hours) and during it, care has to be taken not to overload cells with the otherwise toxic carbon source. In contrast, sorbitol is metabolised via the same route as glycerol and so no adaptation phase is required. This means that cells can continue growing on a mixed feed, utilising sorbitol for growth, whilst they adapt their internal structure for methanol metabolism. It was also found that the maximum specific growth rate of cells on the mixed induction feed was 35% lower than their growth rate on pure methanol, dropping from 0.02 1/h to 0.013 1/h. This meant that a relatively constant growth rate was maintained throughout mixed feed induction, unlike on methanol where cells showed typical lag, exponential and stationary phase behaviour. Despite these differences however, the final cell density attained was approximately the same, and so the mixed induction feed protocol was deemed to be successful in terms of cell growth. The results also indicate that there could be an improvement in cell viability and as a consequence, productivity. This is in part due to the potential reduction in cellular stress which is associated with adaptation to methanol, which can lead to translational arrest (Edwards-Jones et al., 2015) but also because of a reduction in cell sheer sensitivity as a consequence of lowered growth rates (Overbeck, Kampen, & Kwade, 2015). These effects will be fully

Table 3-1 *A comparison of the growth characteristics of P. pastoris in fed-batch fermentations at a range of scales, comparing this project's fermentation's with both methanol and mixed feed to traditional methanol induced fermentations. M = methanol induction feed & MS = 50% methanol sorbitol C-mol/C-mol induction feed.*

Total Volume (L)	Working Volume (L)	Strain	Product	Media	Induction Feed	μMax Exponential Growth (1/h)	μMax Growth During Induction (1/h)	Final DCW (g/L)	Fermentation Time (h)	Reference
20	15	GS115	SEAP	BSM	M	0.15	0.010	116	92	This study
1	0.6	GS115	SEAP	BSM	M	0.17	0.021	148	89	This study
1	0.6	GS115	SEAP	BSM	MS	0.18	0.013	142	89	This study
1	0.6	CLD804	Aprotinin	BSM	M	0.14	0.020	134	93	This study
1	0.6	CLD804	Aprotinin	BSM	MS	0.15	0.013	124	91	This study
80	40	X-33	ScFv-Fc fusion protein	BSM	M	N/A	0.022	93	N/A	Wang et al., 2012
30	20	GS115	Human chymotrypsinogen B	BSM	M	0.18	0.017	95	87	Curvers et al., 2001
20	15	GS115	rGelatin	BSM	M	0.22	0.021	117	90	Werten et al., 1999
5	3.75	KM71H	hIFNα2b	BSM	M	0.19	N/A	129	104	Ayed et al., 2008
2.5	1.8	SMD1168	Laccase	BSM	M	0.23	0.035	120	96	Hong et al., 2002

Figure 3-7 *A = A comparison of the growth curves of P. pastoris GS115 producing SEAP with a methanol (━━) or 50% methanol sorbitol C-mol/C-mol (━ ━) mixed induction feed vs. P. pastoris CLD804 producing Aprotinin with a methanol (━━) or 50% methanol sorbitol C-mol/C-mol (━ ━) mixed induction feed. B = A comparison of the growth curves of P. pastoris GS115 producing SEAP with a methanol (━━) or 50% methanol sorbitol C-mol/C-mol (━ ━) mixed induction feed vs. P. pastoris CLD804 producing Aprotinin with a methanol (━━) or 50% methanol sorbitol C-mol/C-mol (━ ━) mixed induction feed.*
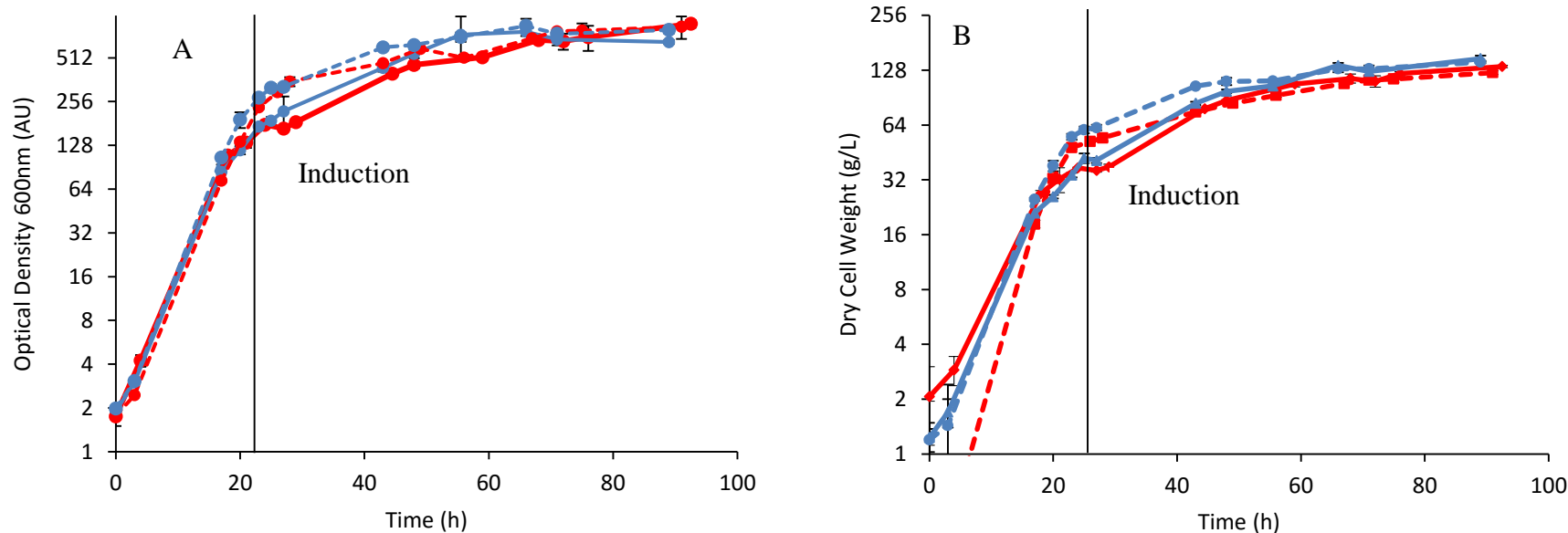
Figure 3-8 *A = A comparison of the growth curves on a base 2*

logarithmic axis in order to show growth phases of P. pastoris GS115 producing SEAP with a methanol (▬) or 50% methanol sorbitol C-mol/C-mol (▬ ▬) mixed induction feed vs. P. pastoris CLD804 producing Aprotinin with a 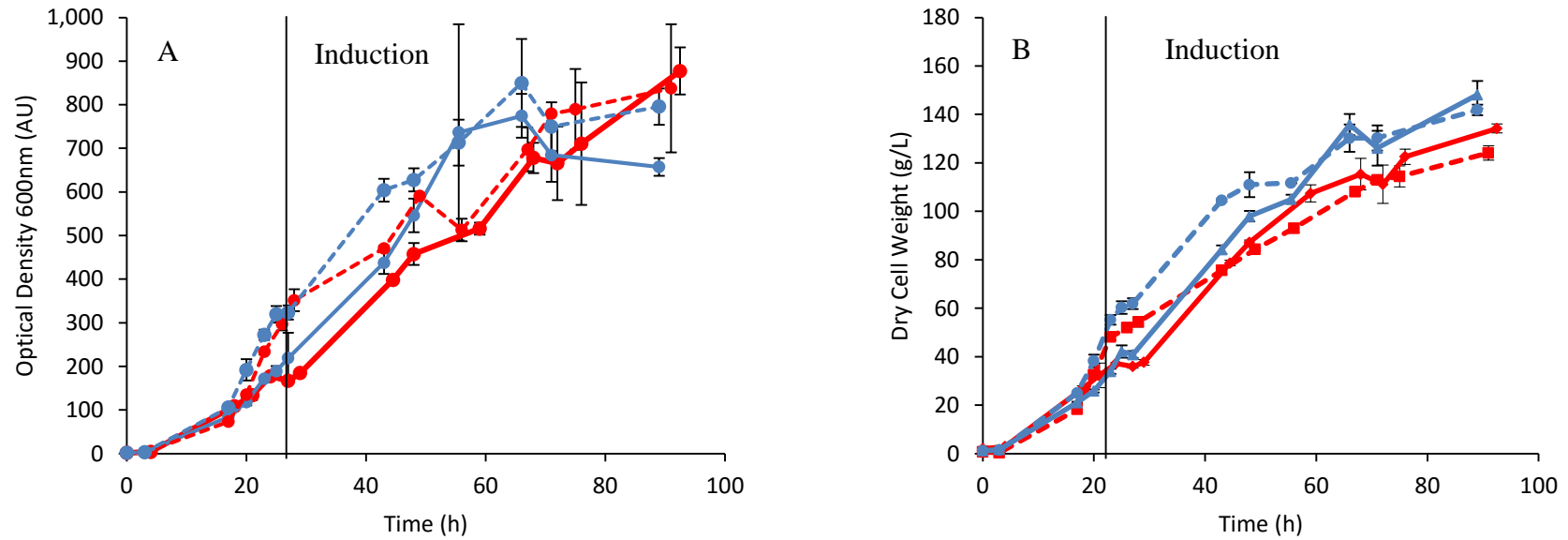methanol (▬) or 50% methanol sorbitol C-mol/C-mol (▬ ▬) mixed induction feed. B = A comparison of the growth curves of P. pastoris GS115 producing SEAP with a methanol (▬) or 50% methanol sorbitol C-mol/C-mol (▬ ▬) mixed induction feed vs. P. pastoris CLD804 producing Aprotinin with a methanol (▬) or 50% methanol sorbitol C-mol/C-mol (▬ ▬) mixed induction feed.

assessed in chapter 5 wherein cell viability is analysed and chapter 6 wherein cellular robustness during fermentation is characterised.

3.3 Conclusions

In this chapter, shake flask investigations successfully demonstrated that sorbitol could not only be used as a carbon source to provide sustainable growth equivalent to methanol but also confirmed that it is a non-repressive carbon source, with even 20 % methanol 80 % sorbitol C-mol/C-mol ratios resulting in product secretion. Overall growth rates were reduced by increasing sorbitol concentrations with pure sorbitol resulting in a 28% reduction in cell density compared to pure methanol, at the end of the exponential growth phase. Based on shake flask growth profiles and product secretion levels, it was determined that a mixed feed ratio of 50% methanol sorbitol C-mol/C-mol would be an ideal starting point for an investigation into the benefits of a mixed feed strategy.

It was shown that cell densities equivalent to large scale could be attained using a working volume of 600 mL in 1 L bench-top bioreactors running in parallel. A mixed feeding strategy was successfully devised based on an existing protocol (Invitrogen Corporation, 2002) and the ideal mixed feed ratio determined by the shake flask studies, and utilised at 1 L scale using both *P. pastoris* GS115 SEAP and *P. pastoris* CLD804 Aprotinin. Similar growth characteristics were seen between both feeds and strains, with all reaching equivalent final cell densities. It was noted however, that the use of a mixed feed resulted in the removal of the adaptation phase that is normally seen when cells change their composition in order to metabolise methanol as well as a lower maximum growth rate.

Chapter 4: Reducing scale limitation of *Pichia pastoris* fermentations by the application of a mixed induction feed to minimise heat generation

4.1 Introduction

During the culturing of the majority of microbial cells, temperature control is a vital feature of the bioreactors, to keep the cells in an environment that is both conducive to growth and protein production. This normally requires the balance of maximising growth with higher temperatures (around 37 °C for infectious organisms such as *Escherichia coli* or 30 °C for yeasts such as *Pichia pastoris*) and increasing productivity with lower temperatures (Dragosits et al., 2009; Semba et al., 2008). As cells generate metabolic heat during growth, fermentations need to be cooled in order to prevent overheating and for this balance to be maintained. One of the crucial disadvantages of *Pichia pastoris* that hinders its application in an industrial setting is the heat generated by cells when methanol is metabolised during induction. This is problematic because it reduces the scalability of fermentations with bioreactors becoming heat transfer limited at a much smaller scale than during other fermentations (Junker, 2004).

It has previously been shown that the application of sorbitol can reduce heat generation and proposed that this could help with *P. pastoris* scale limitations (Curvers et al., 2001; Jungo et al., 2007; Niu et al., 2013). The aim of this chapter is to highlight the issue of scalability in *P. pastoris* cultivation and show how the application of the methanol sorbitol mixed induction feed protocol introduced in chapter 3 can meet this challenge, with the use of predictive modelling and confirming findings with small-scale data.

4.2 Results and Discussion

4.2.1 Assessing the impact of a mixed induction feed on heat generation

The differences in heat generation during different stages of fermentation of 1 L fermentations of *P. pastoris* CLD804 were observed under non-temperature controlled conditions and the temperature rise was noted. This was initially done for a fermentation induced with pure methanol using the standard Invitrogen protocol (Invitrogen Corporation, 2002) in order to observe the impact of induction on the bioreactor's heat output (figure 4-1). It was observed that not only did heat output increase dramatically with the introduction of methanol, but that it was also independent of cell density with the rate of temperature increase not varying between each day of induction. During growth on glycerol temperature rose at a rate of 4.76 K/h/L compared to elevated average rate of 16.2 K/h/L during growth on methanol. As cell density increased from 18.4 g/L on glycerol to 109 g/L on day 1 of induction and 139 g/L on day 3 of induction, the specific heat generation rate is seen to drop from 0.26 to 0.15 K/L/h/g DCW as a consequence of induction, and from 0.15 to 0.11 K/L/h/g DCW during growth on methanol.   This effect is due to heat output being directly linked to the oxygen uptake rate (OUR) of the cells (Potgieter et al., 2009), which is in turn linked to the carbon source feed rate, which was fed at a limiting rate and did not vary during induction. The same experiment was carried out with a 50% methanol sorbitol C-mol/C-mol mixed induction feed (as described in chapter 3) with readings being taken during each day of induction. The use of this alternative induction feed resulted in a reduced average rate of temperature increase of 6.52 K/h/L with the rate again being independent of cell density, with
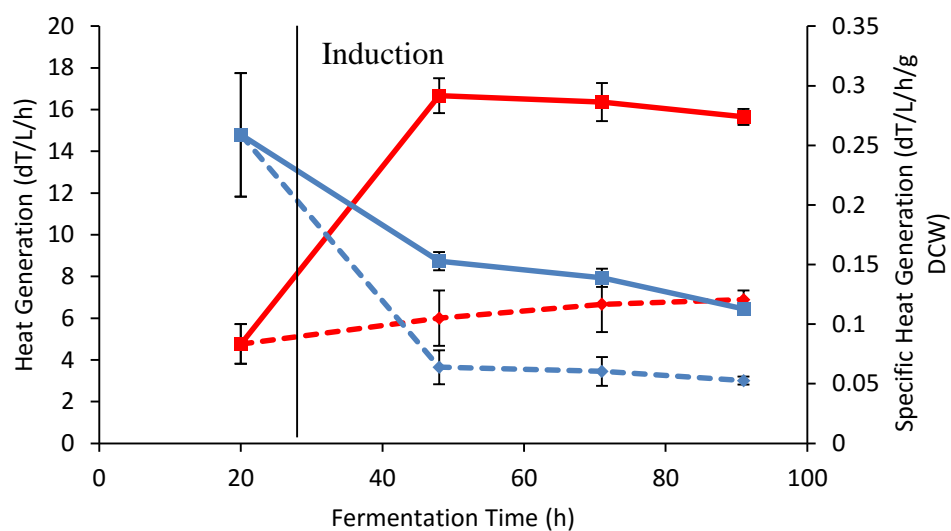
Figure 4-1 *Comparison of heat generation (■, ♦) and specific heat generation (■, ♦) from P. pastoris CLD804 during a fermentation with a pure methanol induction feed (solid line) and a 50% methanol sorbitol C-mol/C-mol mixed induction feed. Error bars account for equipment measurement error.*

specific heat generation dropping to 0.064 K/L/h/g DCW on day 1 of induction and 0.053 K/L/h/g DCW by day 3. Although these results were generated with *P. pastoris* CLD804, the same overall effect is experienced by other strains, although not with the same level of heat generation. Although all Mut+ strains of *P. pastoris* are able to metabolise methanol using AOX1 which will result in an elevated OUR, the rate at which they metabolise methanol, as well as the methanol feeding rate and overall fermentation conditions (eg. temperature (Wu, Wang, & Fu, 2012)) will affect how much the OUR is increased by.

4.2.2 Using OUR to predict heat generation

The heat generation of aerobically cultured cells is directly correlated to the OUR of the cells, therefore it was hypothesised that the mixed induction feed must result in an OUR ~40% of that seen during growth on pure methanol, in correlation with the 60% drop in heat generation seen in section 4.2.1. It is likely for this to be the case because of the difference in how methanol is metabolised compared to glycerol or sorbitol. During glycerol or sorbitol metabolism, the key source of oxygen requirement is the TCA (tricarboxylic acid) cycle, as well as being the main cause of carbon dioxide release. In contrast, methanol first needs oxidising in the cells' peroxisomes in a process that naturally requires oxygen and produces carbon dioxide. This process results in formaldehyde formation which is further metabolised, eventually linking into the same metabolic pathway that glycerol and sorbitol take, leading to biomass formation or ending in the TCA cycle (Gao et al., 2012; Niu et al., 2013). As oxidation during carbon source metabolism is the main source of heat generation in cells, these additional oxidation steps required in methanol uptake result in significantly increased heat generation when compared to sorbitol, which requires no such additional oxidation steps before entering the TCA cycle. In order to demonstrate this difference, fermentations were run with *P. pastoris* CLD804; OUR

and CER (carbon dioxide emission rate) were calculated using data provided by the bioreactors, the online gas analyser and the equations given in Appendix B. Both OUR (figure 4-2) and CER (figure 4-3) increased during induction on methanol compared to growth on glycerol. Both fermentations showed a steady increase in OUR and CER during glycerol batch and fed-batch phases before dropping during methanol adaptation phase at the start of induction. The end of adaptation was characterised by a spike in both OUR and CER as cells successfully metabolise the accumulated methanol. As the metabolism of sorbitol does not require any changes to metabolic pathways there is a less substantial drop in OUR during adaptation with the mixed feed and a smaller spike due to less methanol accumulating in the bioreactor. OUR reached a maximum of 70 mmol/L/h during glycerol batch phase in both fermentations, and an average of 310 mmol/L/h during induction with pure methanol. This is in contrast to induction with the mixed feed, wherein OUR was an average of 117 mmol/L/h during induction, which is ~40% of the OUR seen during pure methanol induction, correlating well with the ~60% reduction in heat generation established in section 4.2.1 by the application of the mixed induction feed. This is also in agreement with literature, wherein OUR was also reduced by the inclusion of sorbitol during induction feeding, although this reduction varied between studies from 30% to 40% due to different feeding strategies and operating conditions(Jungo et al., 2007; Niu et al., 2013). CER followed the same trend in both fermentations with the difference during induction being less pronounced (averages of 133 mmol/L/h with pure methanol and 109 mmol/L/h with mixed feed) which is due to the same amount of carbon being metabolised in both conditions, but with the distribution of carbon source going to biomass generation and protein production varying resulting in different CERs (Niu et al., 2013). Initial variation in both OUR and CER after adaptation phase is seen due to feed rate adjustments.
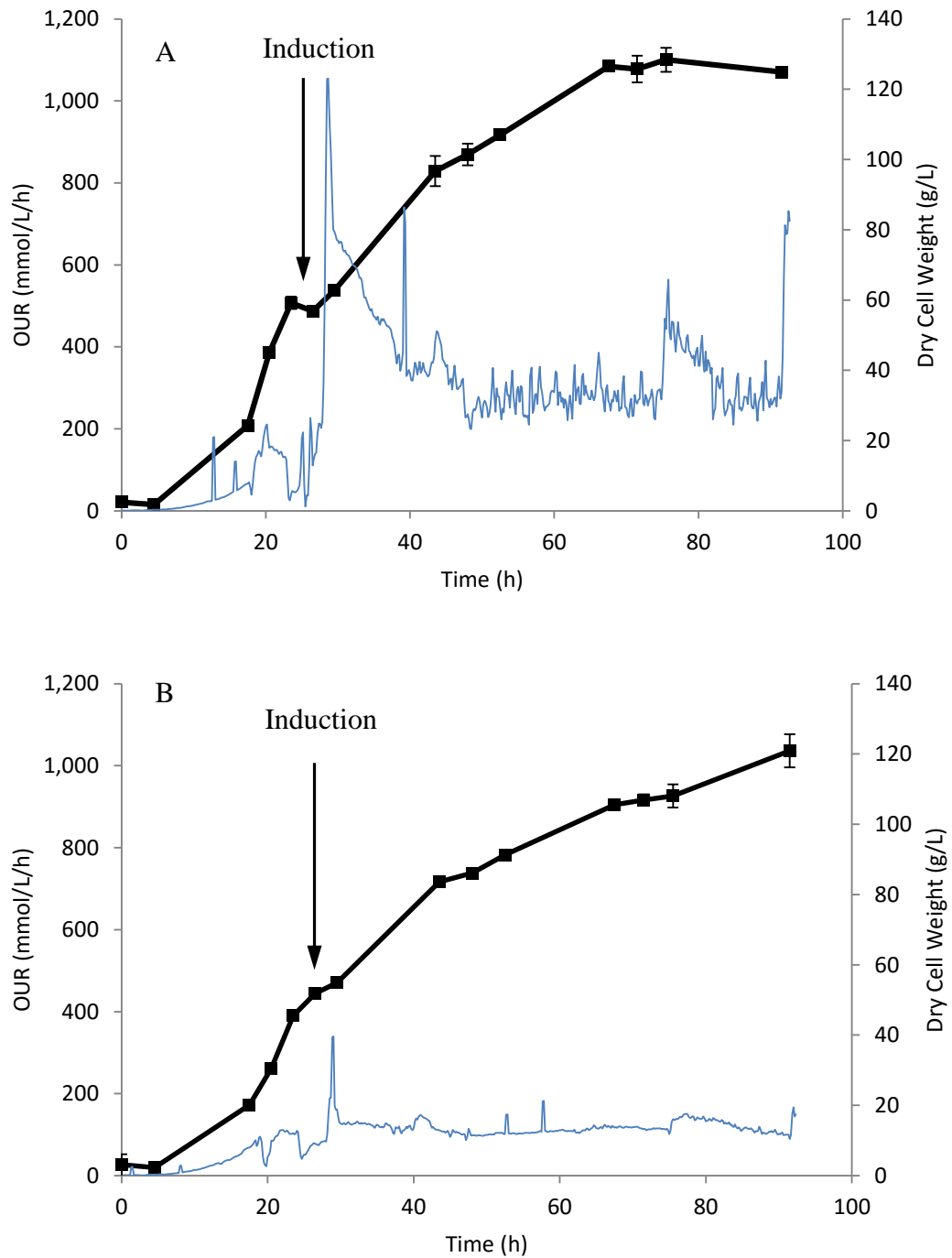
Figure 4-2 *Growth of P. pastoris CLD804 in 1 L bioreactors with OUR (-) in mmol/L/h shown by a 30 minute moving average on the primary y-axis and dry cell weight (■) in g/L shown on the secondary y-axis. A = methanol induction feed and B = 1:1 methanol sorbitol mixed induction feed*
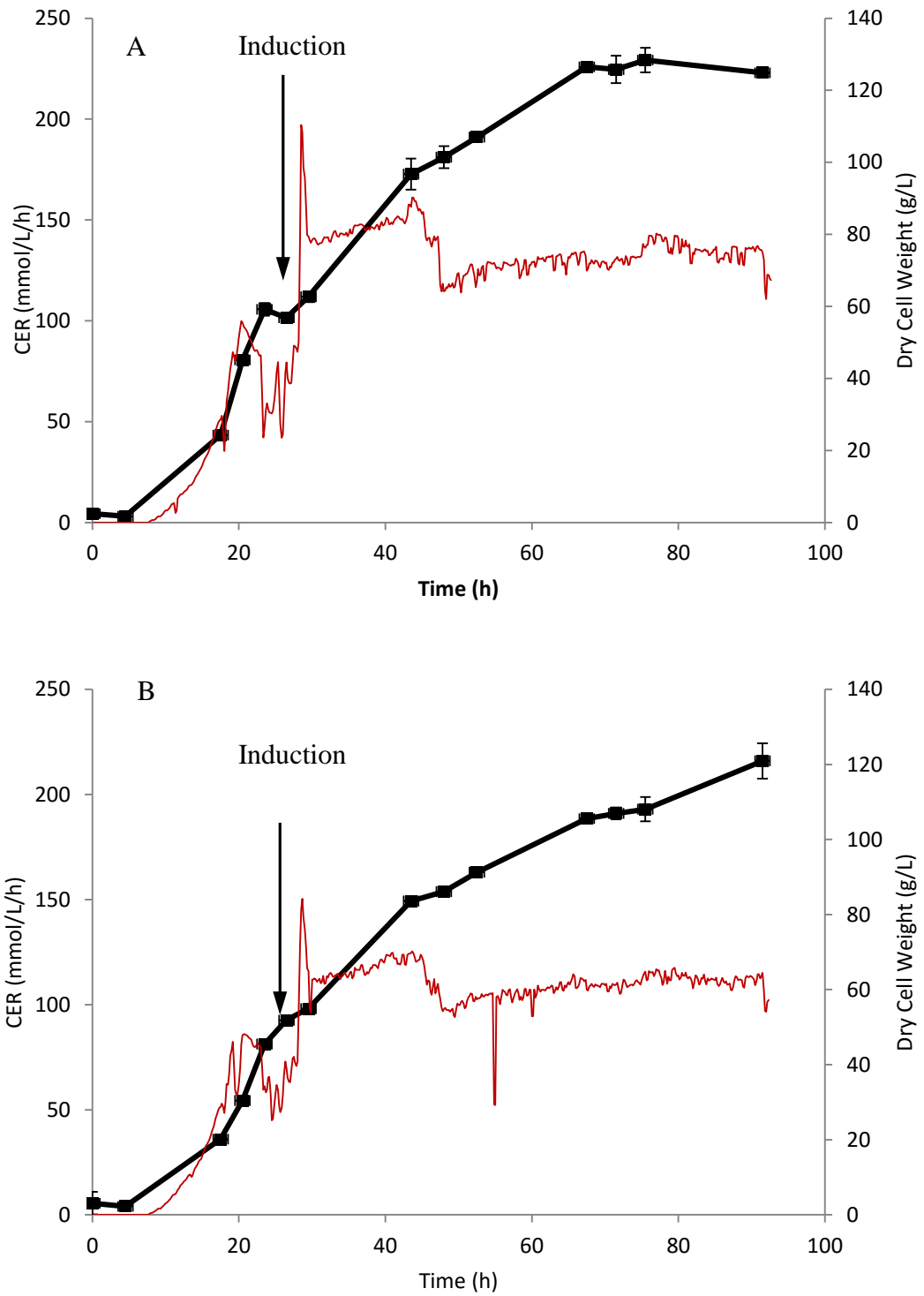
Figure 4-3 *Growth of P. pastoris CLD804 in 1 L bioreactors with dry cell weight (■) in g/L shown on the primary y-axis and CER (-) in mmol/L/h shown by a 30 minute moving average on the secondary y-axis. A = methanol induction feed and B = 1:1 methanol sorbitol mixed induction feed.*

4.2.3 Developing a model to predict heat transfer limitations on scale up

With this confirmation that the mixed induction feed could reduce heat generation (as measured, and predicted by OUR readings) work was done to predict what impact this would have on the scalability of *P. pastoris* fermentations. In order to do this, the amount of heat generated during a fermentation of any scale had to be calculated, given that approximately 460 kJ of heat is released for every mole of oxygen consumed during fermentation (Doran, 1995). This information could then be used to calculate the heat generation of a fermentation and then be compared to the cooling capacity of the bioreactor at the same scale. Heat generated (derivation in appendix B):

$$Q_{gen} = 127.78 \,.\, OUR \,.\, V$$

Where: Q_{gen} = heat generated by cell culture (W)

OUR= oxygen uptake rate (mmol/L/h)

V = bioreactor working volume (m^3)

Cooling capacity of the bioreactor was calculated using the following equation:

$$Q = h \,.\, A \,.\, \Delta T$$

Where: Q = heat that can be removed from the bioreactor (W)

h = overall heat transfer coefficient (W/m^2/^{o}C)

A = surface area available for heat transfer (m^2)

ΔT = heat transfer temperature gradient (^{o}C)

These equations were then used to construct a simple model which allowed the maximum working volume of a bioreactor to be calculated, given a maximum OUR

seen during induction. The model was first tested with the OURs seen during the previously described fermentations (310 mmol/L/h for methanol and 117 mmol/L/h for mixed feed), to give an indication of how scalable the existing feed regime would be (figure 4-4). A cooling water temperature of 4°C was assumed as it was the minimum that can be obtained in an industrial setting, as well as an exit temperature of 9°C (Philip Milburn, personal communication). It was discovered that when running at 30°C there would be few problems scaling up the process, with the mixed feed regime never becoming heat transfer limited and the methanol induced fermentations only having potential heat transfer problems at 5,500 L working volume. More problematic were fermentations run at 25°C, this is because the lower temperature difference between the cooling water and the cell culture means that the bioreactors have a reduced cooling capacity. These 25°C simulations again did not cause any heat transfer limitations during scale up for the mixed feed, but during methanol induction the risk of overheating was already seen at a working volume of 2,000 L. It should be noted that simulations run at lower temperatures are not representative of the same induction feeding rates as those at 30°C. This is because OUR is seen to be increased by decreasing temperatures (Wu et al., 2012), and so processes become heat transfer limited at a smaller scale than predicted. The model is still however applicable for a set OUR at all temperatures, but care has to be taken when using a particular feed regime and reducing the temperature as heat generation will not remain constant.

These results already demonstrate a great benefit inherent to the mixed induction feed, but the OURs didn't necessarily represent an industrial setting. After communication with
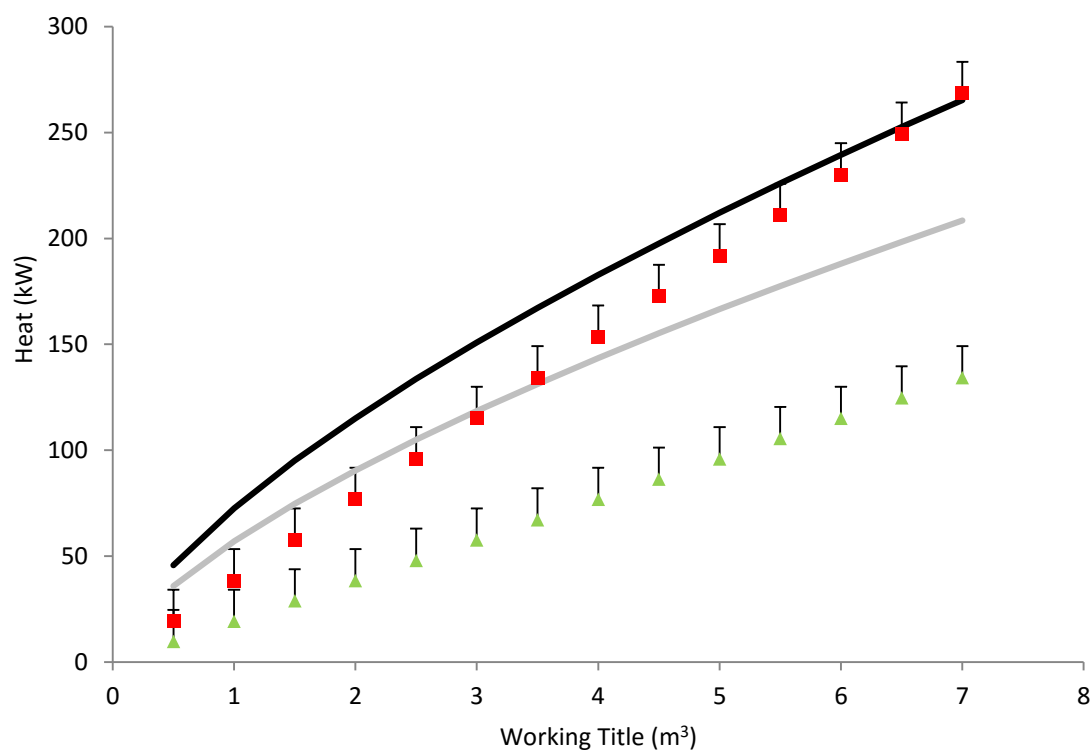
Figure 4-4 *Heat generated by P. pastoris fermentations during methanol induction (■)
and 50% methanol sorbitol C-mol/C-mol mixed induction (▲), compared to the cooling
capacity of bioreactors at 30ºC (▬) and 25ºC (▬). The vertical error bars
correspond to the maximum heat output from the impeller motor.*
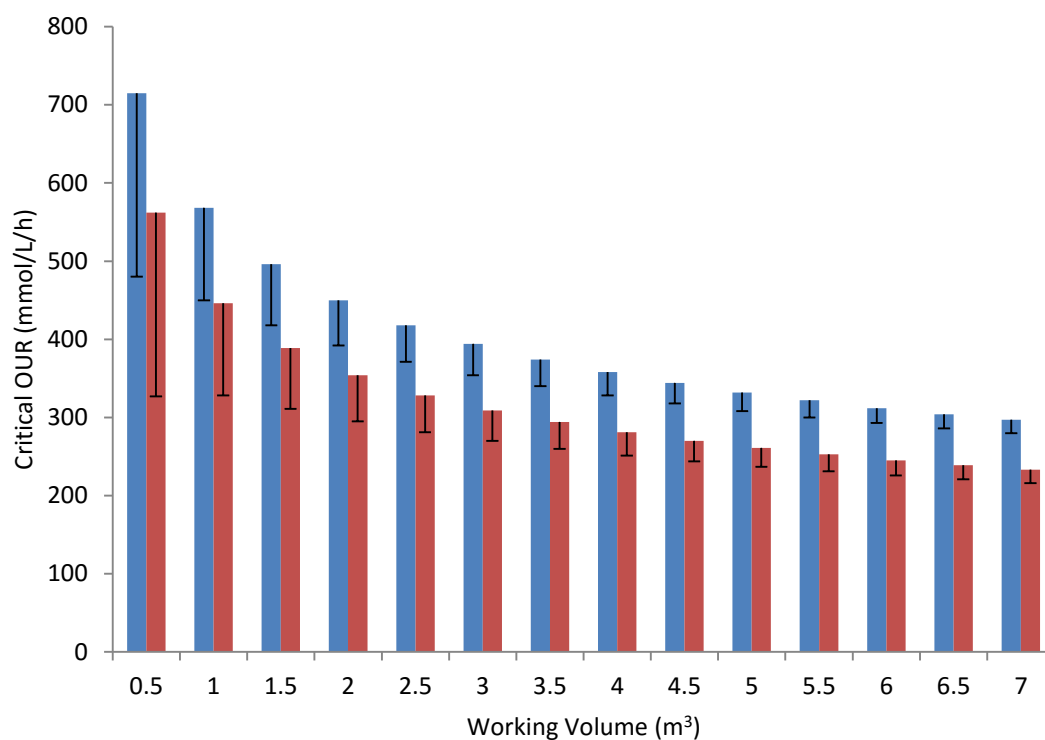
Figure 4-5 *Critical OUR values indicating at what working volume P. pastoris fermentations become heat transfer limited, at an operating temperature of 30°C (■) and 25ºC (■). Error bars represent the impact of maximum motor heat input (15 kW) on the critical OUR.*

Fujifilm Diosynth Biotechnologies, it was established that fermentations typically become heat transfer limited at a working volume of ~3,500 L as a consequence of fermentations regularly having OURs of 350 mmol/L/h with processes with OURs of 500 mmol/L/h or above being impossible to run at working volumes in excess of 7,000 L. This was verified using the model as processes with OURs of 500 mmol/L/h became potentially heat transfer limited at a working volume of 500 L. To counter the problems of higher OURs at large scale, fermentations are run at 30°C in order to improve heat transfer. With the application of a mixed feed however, even fermentations with OURs of 500 mmol/L/h on methanol could be run at large scale as the predicted OUR would be approximately 200 mmol/L/h, if the same feeding regime is used with a 50% methanol sorbitol C-mol/C-mol induction feed instead of pure methanol.

4.3 Conclusions

These fermentations successfully demonstrated the increase in heat generation by the addition of methanol during the induction phase of fermentation with temperature rising at approximately three times the rate it did on glycerol feed. With the introduction of the 1:1 methanol sorbitol mixed feed (discussed in chapter 3), the heat output during induction was reduced by ~60%. These temperature differences were found to be independent of cell density with heat output remaining relatively constant throughout induction for both induction feed types; instead, heat generation was determined to be directly related to the oxygen uptake rate (OUR). This allowed for the creation of a simple model to predict at what scale bioreactors would become heat transfer limited, given an average induction phase OUR. With OURs being halved

with the introduction of a mixed feed, even the most demanding fermentations were predicted be scalable up to a working volume of 5,500 L at 25 °C and 7,000 L at 30 °C.

Chapter 5:Impact of sorbitol as a co-induction feed with methanol

5.1 Introduction

It was previously discussed in chapters 1 and 3 that the application of a mixed induction feed cultivation strategy was to improve the viability and productivity of *P. pastoris* cultures. In chapter 3 the development of a simple, scalable fermentation strategy was described and a 50% methanol sorbitol C-mol/C-mol induction feed ratio was determined to optimise cell growth and product yield in shake flask cultures. Further to this, the mixed induction feed was determined to be scalable, with the feeding regime leading to successful 1 L fermentations which were also found to be replicable at 20 L scale. The impact of the new induction regime on overall scalability was then discussed in chapter 4, with the reduction in heat generation caused by the mixed feed, allowing *P. pastoris* fermentations to reach much larger scales.

In this chapter the effect of a 50% methanol sorbitol C-mol/C-mol induction feed on cell culture viability and product yield will be discussed. Various studies have highlighted the potential of sorbitol to both reduce cellular stress (Ramón et al., 2007; Wang et al., 2010; Xie et al., 2005; Zhu et al., 2013) and ultimately product yields for a range of therapeutic proteins (Çalık et al., 2013; Gao et al., 2012; Jungo et al., 2007; Niu et al., 2013; Ramón et al., 2007; Wang et al., 2010; Xie et al., 2005). One of these studies also indicated that cell viability may be improved by the inclusion of sorbitol during induction feeding (Wang et al., 2010). These results however, were produced using different strains and cultivation methods, meaning that the impact of the mixed feed is not directly comparable. The following work will evaluate the impact that a 50% methanol sorbitol C-mol/C-mol induction feed has on the viability of two strains of *P. pastoris* (GS115 and CLD804) and the yield of their respective products, SEAP

and Aprotinin. Furthermore, the potential impact of these findings on downstream processing will be evaluated. Thus the key objectives of this chapter are:

1. Determine the impact of a 50% methanol sorbitol C-mol/C-mol induction feed on the product yield of SEAP and Aprotinin from *P. pastoris* GS115 and CLD804 respectively

2. Assess the impact of a the mixed induction feed on cell morphology with regard to cellular stress

3. Determine the impurity profile from methanol induction and mixed feed induction in order to assess the impact on downstream processing (DSP)

5.2 Results and Discussion

5.2.1 Determing product yields during mixed feed fermentation

Fermentations were carried out at a 1 L (total volume) scale with *P. pastoris* GS115 and CLD804 with both a pure methanol and a 50% methanol sorbitol C-mol/C-mol induction feed using the methodology described in section 2.2.3.1, the growth curves obtained can be found in figures 3-7 and 3-8. Samples were taken throughout these fermentations and used to monitor product release into the supernatant. Both strains were cultivated in the same way with the exception of their induction phase pH which was 6.5 for *P. pastoris* GS115 and 5 for *P. pastoris* CLD804. As discussed in section 1.4.1.1, the cultivation pH is chosen as a result of the product being secreted, as pH has no impact on growth between pH 3 and 7. For *P. pastoris* GS115 a pH of 6.5 was chosen due to SEAP being most stable in alkaline conditions as other alkaline phosphatases are (Harada et al., 1986) and a trade-off being found between cell growth and cell productivity (Randone, 2014). In contrast, Aprotinin which is
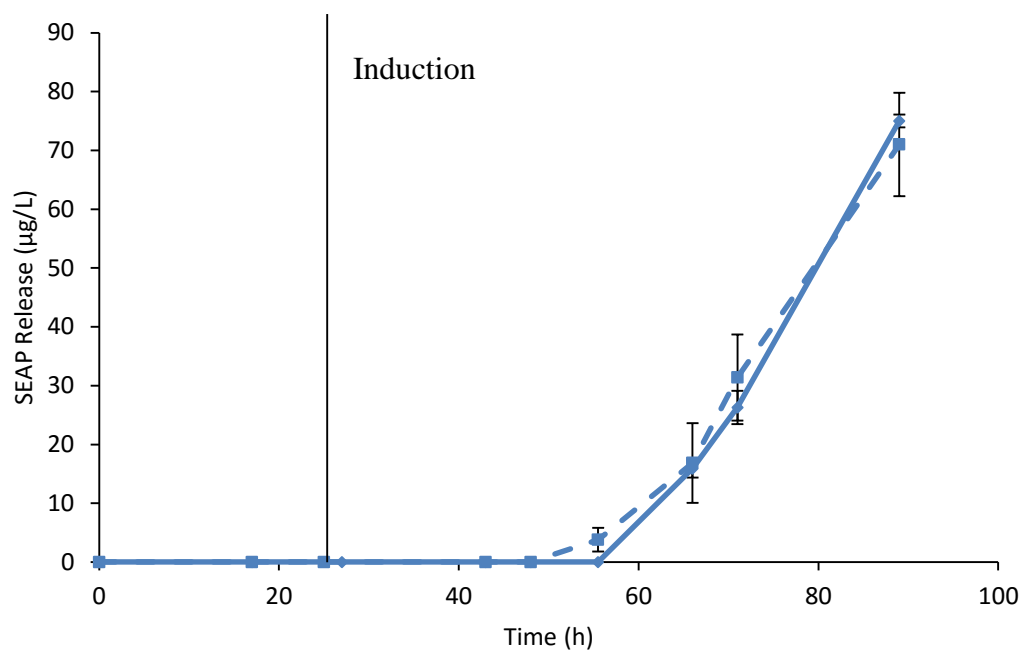
Figure 5-1 *SEAP release by P. pastoris GS115 throughout 1 L fermentations with a methanol induction feed (solid line) and a 50% methanol sorbitol C-mol/C-mol induction feed (dashed line)*

secreted by *P. pastoris* CLD804, is stable at a wide pH range(Fritz & Wunderer, 1983) so a pH of 5 was maintained throughout the fermentation.

P. pastoris GS115 only secreted minimal amounts of SEAP during the fermentation, reaching a maximum product concentration of 75 µg/L with methanol induction and 71 µg/L with mixed feed induction (figure 5-1). These low product levels were due to the low productivity levels of the strain, which was created in-house (Randone, 2014), some product not being secreted (see appendix C) and the nature of the assay used to detect it (see section 2.3.3.1 for assay methodology). Due to the

low expression levels of the strain, the assay had to be incubated for 6 hours at 37 ℃ in order for the reaction to progress enough to get a signal from the earlier fermentation time points (see appendix C). This meant that there could have been product degradation over time leading to the particularly low expression levels seen, however as all samples were exposed to the same incubation time, they are still comparable. Product secretion reached quantifiable levels approximately 25 hours after induction for both pure methanol induction and mixed induction, but with cells having a slightly higher productivity of 2.3 µg/L/h during methanol induction compared to 2.1 µg/L/h on the mixed induction feed. When cell density during fermentation was taken into account, the specific yield and specific productivity were found to have a negligible difference between induction feeds with specific yields of 0.5 µg/g and specific productivities of 0.015 µg/g/h.

P. pastoris CLD804, in contrast to GS115, secreted high levels of the product Aprotinin, with it being the most prominent protein species in the medium throughout the induction phase for both methanol and mixed feed induction (figure 5-2). No aprotinin was detectable in the medium before induction in either feeding regime, again supporting the evidence that AOX1 (alcohol oxidase 1) is a tightly regulated promoter (Looser et al., 2015). Densitometry analysis of the end-point product bands
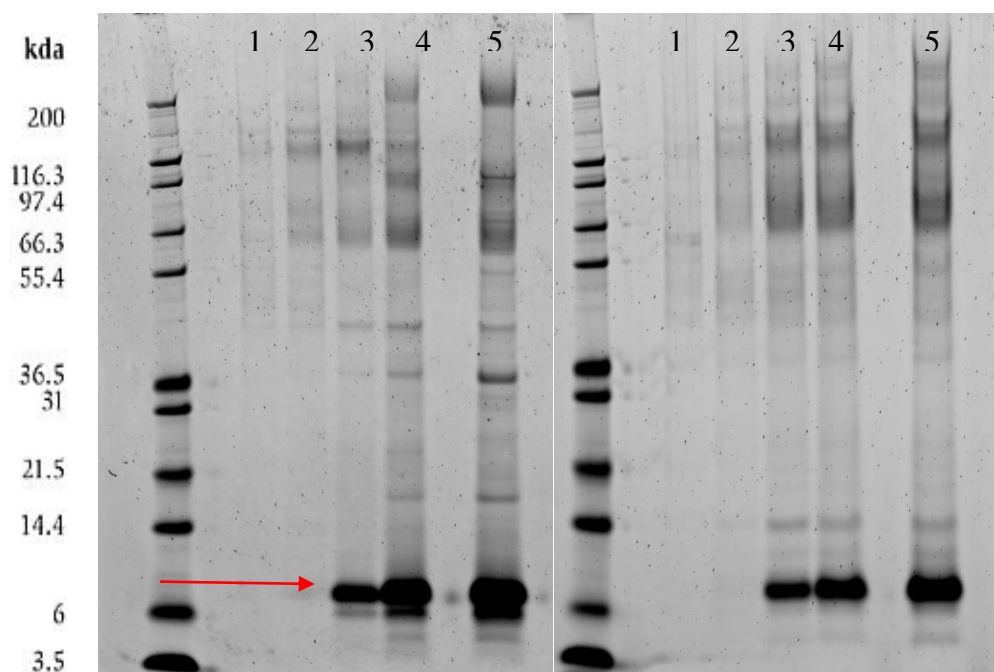
Figure 5-2 *SDS-PAGE showing Aprotinin secretion during P. pastoris CLD804 methanol induced fermentation (A) and 50% methanol sorbitol C-mol/C-mol induced fermentation (B). Lanes correspond to fermentation time points of 1 = 17 h, 2 = 23 h, 3 =43 h, 4 = 67 h and 5 = 91 h, where induction occurred at 24 h in each. The arrow indicates the product band (Aprotinin).*

revealed that a methanol induction led to a slightly higher product concentration than the mixed feed (band density of 11,600 compared to 10,200), but this difference was mitigated when final dry cell weight was taken into account, with methanol induction resulting in a normalised band density of 86.1 and mixed feed induction resulting in a normalised band density of 82.6. Using BSA standards it was determined that this amounts to approximate aprotinin concentrations of 1.2 g/L for methanol induction compared to 1 g/L with mixed feed induction. These results are both in contrast to previous literature which consistently found an improvement in productivity with the introduction of a mixed feed regime (Çalık et al., 2013; Gao et al., 2012; Jungo et al., 2007; Niu et al., 2013; Ramón et al., 2007; Wang et al., 2010; Xie et al., 2005). This discrepancy could be due to the optimisation of the feeding regimes present in the previous studies, as many different techniques were used in order to test the impact of sorbitol. Typical co-feeding techniques used were the maintenance of a set methanol concentration in the bioreactor whilst feeding sorbitol (Gao et al., 2012; Ramón et al., 2007; Wang et al., 2010; Xie et al., 2005) and adding sorbitol in large pulses during fermentation due to sorbitol accumulation not being linked to AOX1 inhibition (Çalık et al., 2013). Only few studies refer to the use of directly replacing methanol with sorbitol during induction on the basis of C-mols present in each carbon source, and although these also saw an increase in product yield, the improvement was minor (Jungo et al., 2007; Niu et al., 2013).

5.2.2 Determining cell quality during mixed feed fermentation

5.2.2.1 Assessing cell viability

Cell viability was the next factor to be assessed during fermentations. With sorbitol co-feeding having no impact on final product yields, it was important to understand
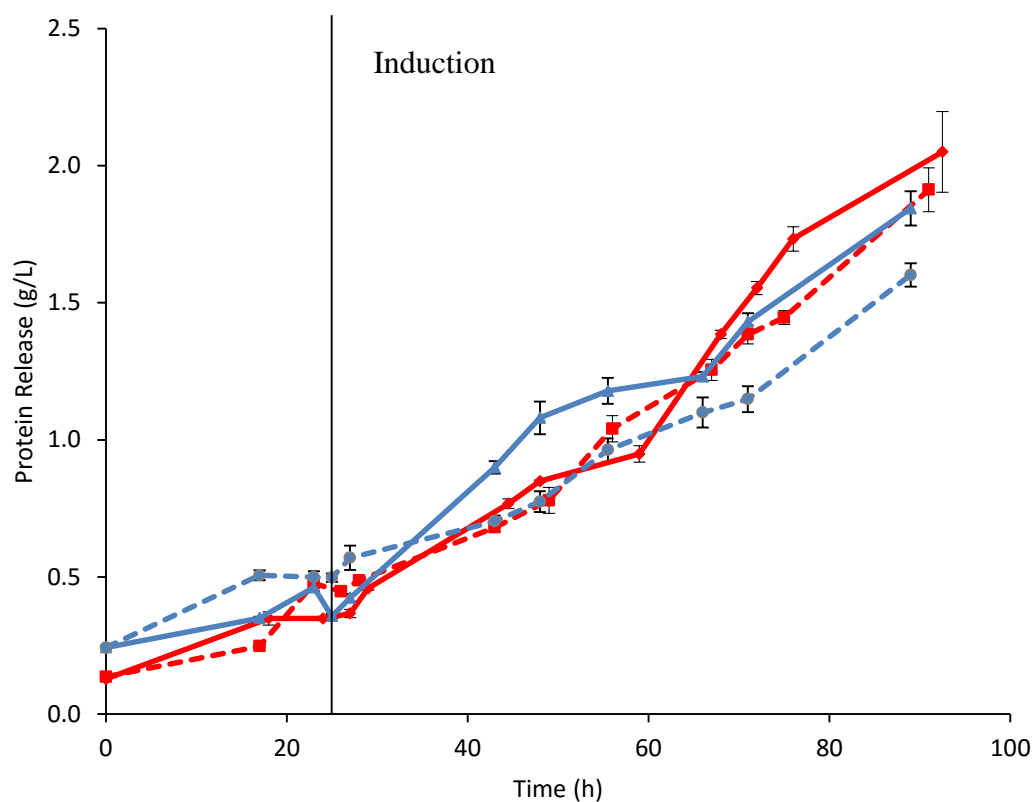
Figure 5-3 *A comparison of the protein release (as measured by BCA assay) by*

P. pastoris GS115 producing SEAP in 1 L parallel bioreactors, with a methanol (━━) or
50% methanol sorbitol C-mol/C-mol (━ ━) mixed induction feed vs. P. pastoris CLD804

producing Aprotinin with a methanol (━━) or 50% methanol sorbitol C-mol/C-mol (━ ━
) mixed induction feed.
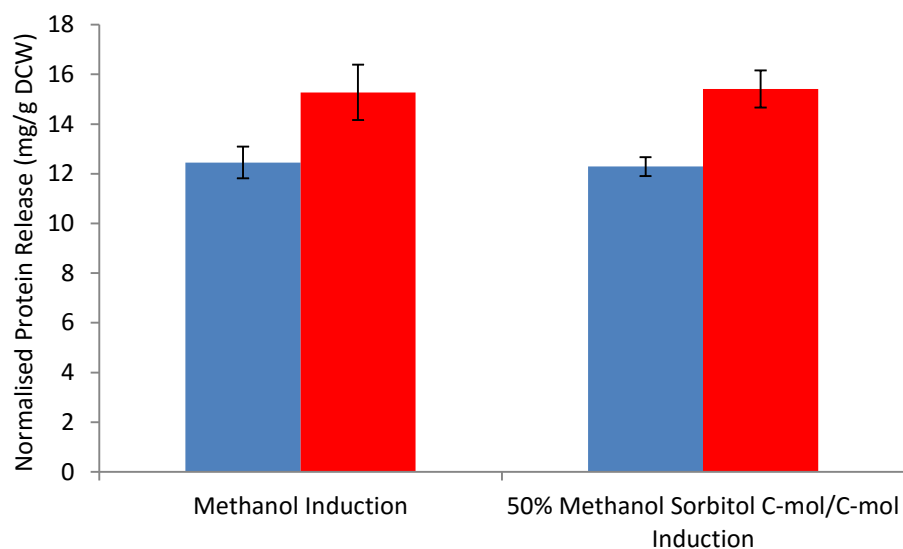
Figure 5-4 *A comparison of normalised protein release measured in mg of protein released per g of dry cell weight, between the end of methanol induced and 50% methanol sorbitol C-mol/C-mol induced fermentations of P. pastoris GS115 (*■*) and P. pastoris CLD804 (*■*) in 1 L parallel bioreactors. Error bars represent triplicate readings.*

how cell physiology was being affected in order to explain the discrepancy between results. Trypan blue staining using a Vi-CELL (Beckman Coulter, High Wycombe, UK) was initially used to determine cell viability, due to sample numbers being too high to do manual cell counts, however this method was replaced as a consequence of unreliable results due to *P. pastoris* cell sizes being too close to the edges of the operating window of the system (2 μm - 70 μm) Cell viability was therefore determined by a range of factors instead; including protein, DNA and protease release.

Protein release increased throughout all fermentations (figure 5-3), not correlating with cell density, with the rate of protein release increasing during induction phase (table 5-1). *P. pastoris* CLD804 released more protein overall than *P. pastoris* GS115 throughout each fermentation for both feed regimes. This difference in protein release is due to the greater amount of product secretion (as discussed in section 5.2.1). There was however, a negligible difference in normalised protein release between methanol and mixed induction feeds at the end of fermentation with *P. pastoris* GS115 releasing 12.3 ±0.6 mg/g DCW and P. pastoris CLD804 releasing 15.3 ±1.1 mg/g DCW (figure 5-4).

Although *P. pastoris* naturally secretes few proteins (Lopes et al., 2012), protein release is not the most accurate measure of cell viability. As DNA is only released upon cell death, it was used as an additional measure of viability. Residual DNA from inoculation degraded during glycerol batch and fed-batch phases and then increased throughout the fermentation for all strains and conditions reaching concentrations between 2,300 ng/mL and 2,800 ng/mL (figure 5-5). This showed that cell death occurred throughout induction phase, although final normalised DNA release showed little variation between feed types (figure 5-6).

Table 5-1 shows that both protein and DNA release rates increased during induction for both feed types, despite declining growth rates. This demonstrates that there is a
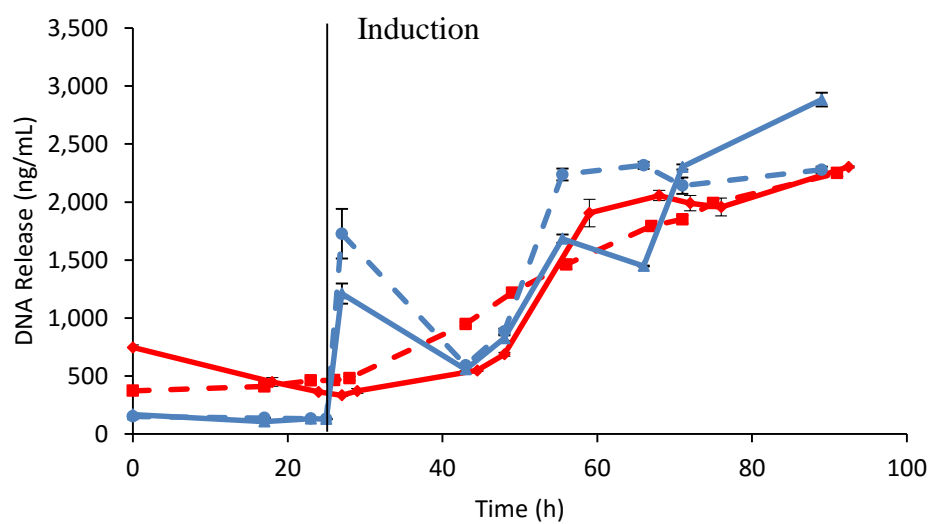
Figure 5-5 *A comparison of the DNA released (as measured by Picogreen assay) by P. pastoris GS115 producing SEAP with a methanol (━━) or 50% methanol sorbitol C-mol/C-mol (━ ━) mixed induction feed vs. P. pastoris CLD804 producing Aprotinin with a methanol (━━) or 50% methanol sorbitol C-mol/C-mol (━ ━) mixed induction feed, all in 1 L parallel bioreactors.*
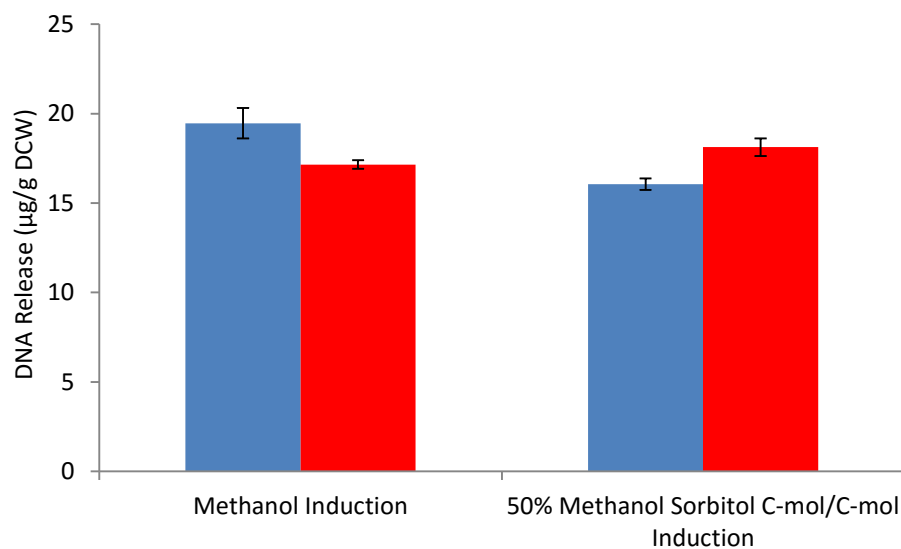
Figure 5-6 *A comparison of normalised DNA release, measured in µg of DNA released per g of dry cell weight, between the end of methanol induced and 50% methanol sorbitol C-mol/C-mol induced fermentations of P. pastoris GS115 (*■*) and P. pastoris CLD804 (*■*) in 1 L parallel bioreactors. Error bars represent triplicate readings.*

Table 5-1 *A comparison of protein and DNA release rates before and after induction for P. pastoris GS115 and P. pastoris CLD804, when induced by methanol or 50% methanol sorbitol C-mol/C-mol. Growth rates are given to show independence of cell growth and protein/DNA release rate.*

		P. pastoris GS115		*P. pastoris* CLD804	
		Methanol Induction	50 % Methanol Sorbitol C-mol/C-mol Induction	Methanol Induction	50 % Methanol Sorbitol C-mol/C-mol Induction
Growth Rate (1/h)	Pre-Induction	0.144	0.166	0.120	0.151
	Induction	0.021	0.013	0.020	0.013
Protein Release Rate (g/L/h)	Pre-Induction	0.010	0.010	0.009	0.012
	Induction	0.023	0.020	0.026	0.023
DNA Release (µg/L/h)	Pre-Induction	N/A	N/A	N/A	3.821
	Induction	41.694	32.530	28.322	26.313

drop in viability consistent with what previous studies have found (Hohenblum et al., 2003; Wang et al., 2010), however there is no indication that overall cell viability was improved by the inclusion of sorbitol in the induction feed. This result was confirmed by particle size distribution which showed no differences in the amount of cell debris present between feed types (see appendix C).

5.2.2.2 Assessing cellular stress

As cell viability was not improved by the application of the 50% methanol sorbitol C-mol/C-mol induction feed, the causes of this had to be determined. It has previously been shown that a major impact of methanol induction is an increase in cellular stresses due to its catabolism into formaldehyde and the induction of oxidative stress and unfolded protein response pathways (Edwards-Jones et al., 2015; Hohenblum et al., 2003; Vanz et al., 2012; Zhu et al., 2013). If sorbitol was to improve cell viability, it would be assumed that this is due to a reduction of the metabolic burden of methanol on the cells. In order to visualise the degree of cellular stress that cells were undergoing during fermentation, they were analysed by transmission electron microscopy (TEM) at each stage of fermentation. Peroxisomes were used as a measure for quantifying cellular stress as they are formed in direct response to the presence of methanol, with the number increasing or decreasing according to the cell's requirement. As mentioned in section 3.2.3 peroxisomes are the organelles in which methanol is broken down into formaldehyde and therefore a key source of cellular stress (Veenhuis et al., 1983).

P. pastoris CLD804 cells taken from the glycerol fed-batch phase were taken as a control sample, as the plentiful carbon source and lack of protein production lead to cells being in their least stressed state. In all 40 cells counted only one cell was found to contain a peroxisome. By contrast, cells taken 24 hours post-induction from both the methanol and the mixed feed fermentation contained large numbers of

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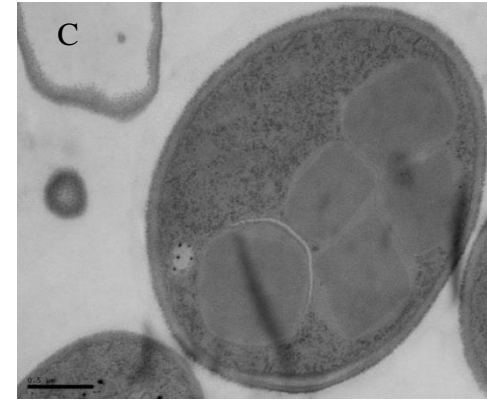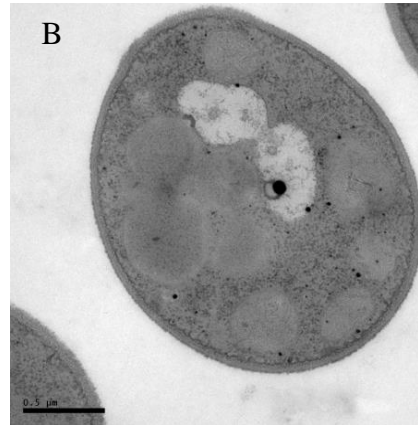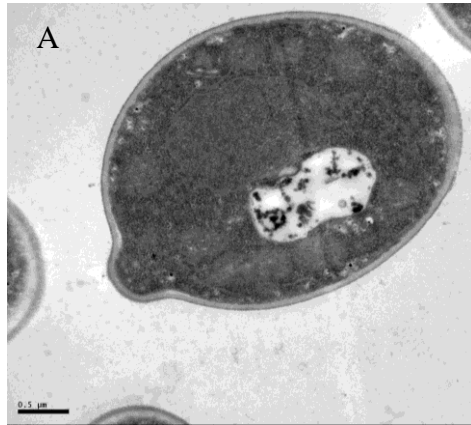
Figure 5-7 *Transmission electron microscope images at 30k magnification of P. pastoris CLD804 cells during A) glycerol fed-batch B) 24 h post methanol induction, example of young cell C) 24 h post methanol induction, example of old cell D) 24 h post mixed feed induction, example of young cell E) 24 h post mixed feed induction, example of old cell.*
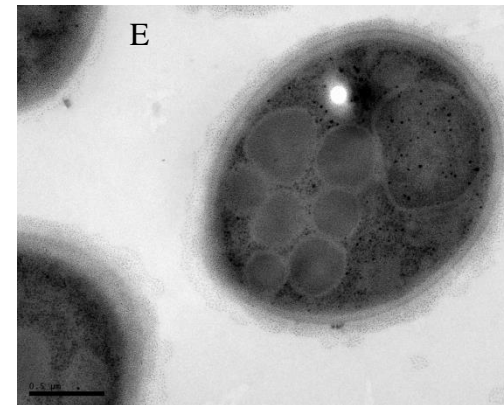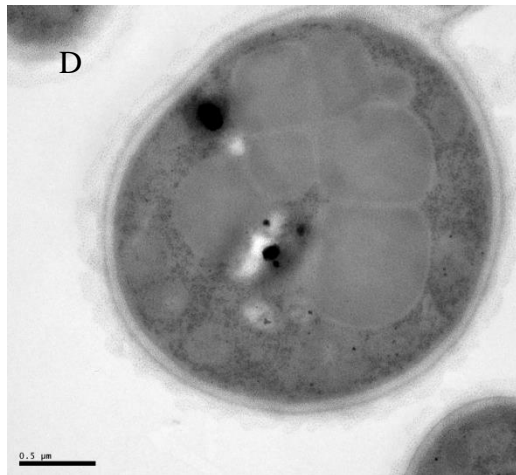
peroxisomes (figure 5-7), with an average of between 4 and 5 peroxisomes per cell. In both instances, approximately half the peroxisomes had the structural characteristics seen in cells during growth phase (small and round) with the remainder having stationary phase characteristics (large and cubic), which is consistent with what is to be expected at this stage of fermentation. This is due to peroxisomes increasing in size as the cells age, also becoming more cubic in shape; as the growth rate is comparatively low after one day of induction, with cells entering the decline phase, the proportion of older cells and therefore cubic peroxisomes increases (Veenhuis et al., 1983). Within the 40 cells visualised in each fermentation condition, 162 peroxisomes were counted during methanol induction and 198 were counted during mixed feed induction. This difference in peroxisome number is not statistically significant according to F-test analysis ($F = 2.41$ when $F_{crit} = 4.08$ when $\alpha = 0.05$), wherein the null hypothesis was that there was no difference between the two groups of samples.

This result supported the previous findings as cells produced an approximately equal number of peroxisomes in response to methanol, regardless of the presence of sorbitol, indicating that they are equally likely to be under metabolic stress.

5.2.3 Assessing the impact of a mixed induction feed on downstream processing

During previous parts of this investigation it was noted that although cell viability was unaffected by the presence of sorbitol during induction feeding, the type of impurities (specifically proteins) released appeared to vary (figure 5-2). Of the protein impurities released during fermentation, proteases are of particular interest, as vacuolar proteases released as a consequence of high cell density fermentation result
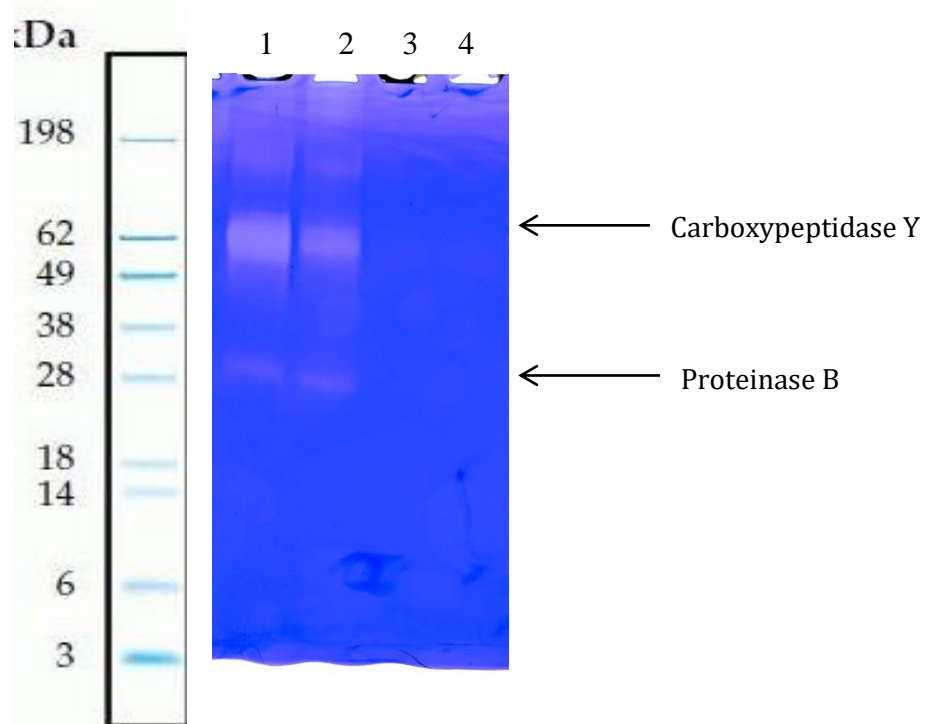
Figure 5-8 *Zymogram gel showing protease release from P. pastoris CLD804 during methanol induced fermentation (lane 1 = 75 h , lane 2 = 91 h) and 50% methanol sorbitol C-mol/C-mol induced fermentation (lane 3 = 75 h, lane 2 = 91h) in 1 L parallel bioreactors. Key proteases are putatively identified. Time points are hours post-inoculation.*
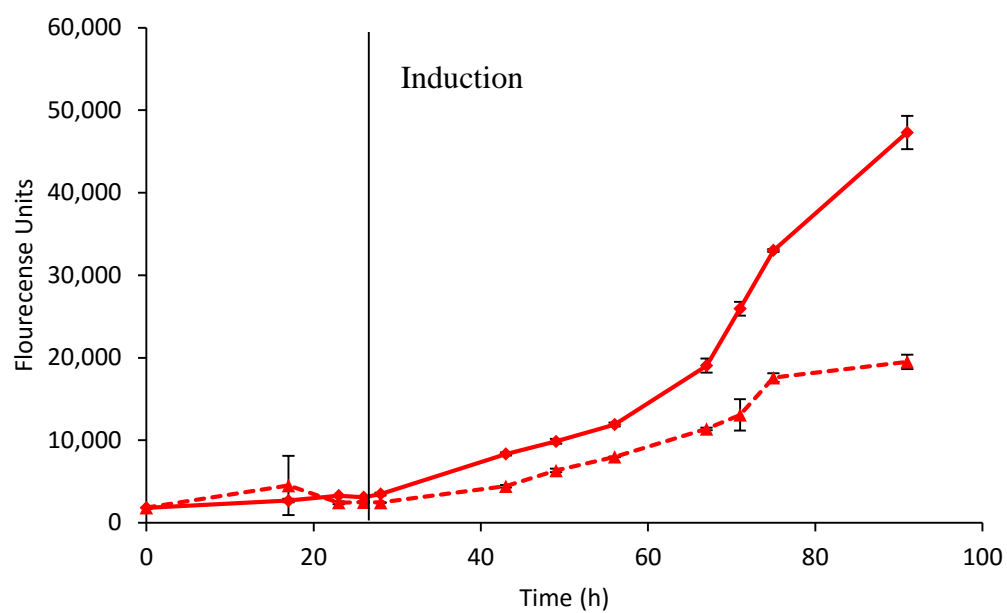
Figure 5-9 *A comparison of the protease released (as measured by Pierce fluorescence protease assay) by P. pastoris CLD804 producing Aprotinin with a methanol (▬) or 50% methanol sorbitol C-mol/C-mol (▬ ▬) mixed induction feed, in 1 L parallel bioreactors*

in product proteolysis, a common problem in *P. pastoris* fermentations (Zhang, Liu, & Wu, 2007). To assess any potential differences in protease release, a protease specific analysis was done in order detect which protein bands corresponded to proteases secreted during fermentation. Pure methanol induction resulted in the release of more proteases into the fermentation media (figure 5-8) than the mixed induction feed, with lanes 1 and 2 showing distinct bands that were putatively determined to be carboxypeptidase Y bands and proteinase B, compared to no clear bands being identifiable in lanes 3 and 4. These bands were identified by comparison to literature (Zhang et al., 2007) and this difference in release was confirmed by protease assay (figure 5-9). The release of these particular proteases is in correlation with previous studies which have found that methanol induction results in the release of a number of proteases, the most prominent of which being carboxypeptidase Y (Sinha et al., 2005). Vacuolar protease concentrations (linked to cell stress induced autophagy) are typically increased during induction by methanol, due to peroxisomal damage by reactive species released as a result of methanol oxidation (Vanz et al., 2012). The decrease in protease release by the application of the mixed induction feed indicates that there may be an improvement in product quality, which is indicated in figure 5-2, wherein the product band (aprotinin) is split into further lower molecular weight species.

To better understand these differences in proteins released during fermentation, end point fermentation samples were first analysed using 2-D gels. Initial results with GS115 showed that major metabolic host cell proteins, released due to cell death, were present in both samples (figure 5-10): these were identified by comparison to literature (Vanz et al., 2012). This was expected as the increase in metabolic proteins as a result of methanol induction (e.g. alcohol oxidase and formate dehydrogenase) would be present with both induction feed regimes. Aside from the key metabolic
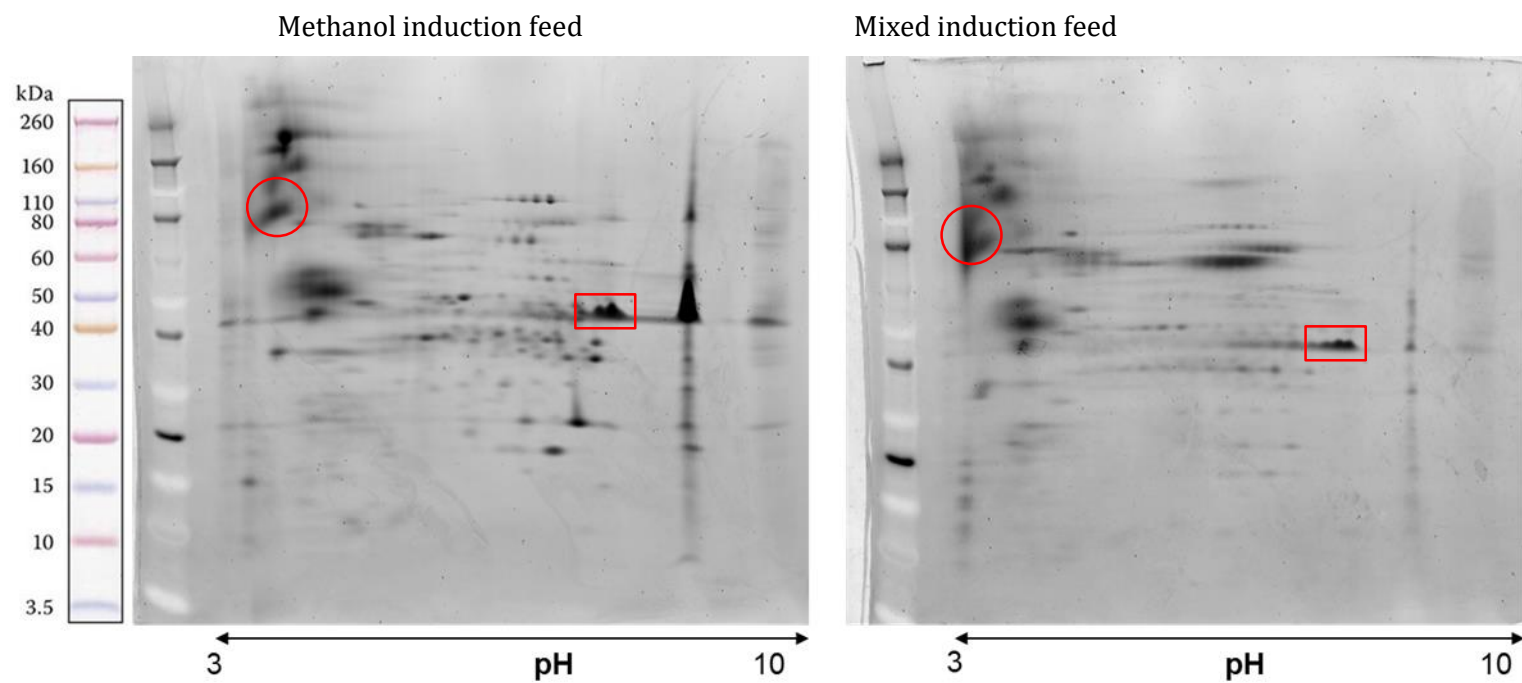
Figure 5-10 *2-D gels showing end point samples from P.pastoris GS115 fermentations with a pure methanol induction feed (left) and a 50% methanol sorbitol C-mol/C-mol mixed induction feed (right). Alcohol oxidase is highlighted by a circle and formate dehydrogenase by a square*

proteins, there was distinct variation in other, unidentified proteins which led to CLD804 samples being assessed. Repeated experiments with CLD804 samples did not however, give consistent results and so the protein profiles were instead determined using liquid chromatography mass spectrometry (LC-MALDI-TOF/TOF). To identify the proteins present in the samples, they were successfully trypsinised and purified to provide peptides for LC-MALDI-TOF/TOF analysis. The resulting peptide mass fingerprints were then compared to both the Swiss prot and NCBI nr (national centre for biotechnology information, non-redundant) protein databases for *Pichia pastoris* using Mascot software (www.matrixscience.com). This analysis did not yield any protein matches on either database with all Mascot scores being below the significance threshold (Mascot score = 67, $p < 0.05$) even with repeat digestions and alternative mass spectrometry methods (electrospray). As there were no matches it was determined that a compound in the sample peptide solutions must have introduced an unknown modification to the peptides outside of the tolerance of the analysis, rendering them unidentifiable. As a consequence, the peptides that were subjected to mass spectrometry after chromatography had to be compared between the feeds, rather than the proteins they came from (figure 5-11). Certain peptides were present in both samples, as would be expected, however there were also distinct variations in the peptides, and therefore proteins released, in the media at the end of fermentation, between the pure methanol and mixed induction feeds. In figure 5-12 parts of the overall peptide profile have been compared in order to demonstrate both the differences and the commonalities between the samples.

This work demonstrates that the inclusion of sorbitol in the induction feed does have an impact on the protein impurity profiles of *P. pastoris*, however further work needs to be done in order to determine exactly what proteins are up/down regulated by
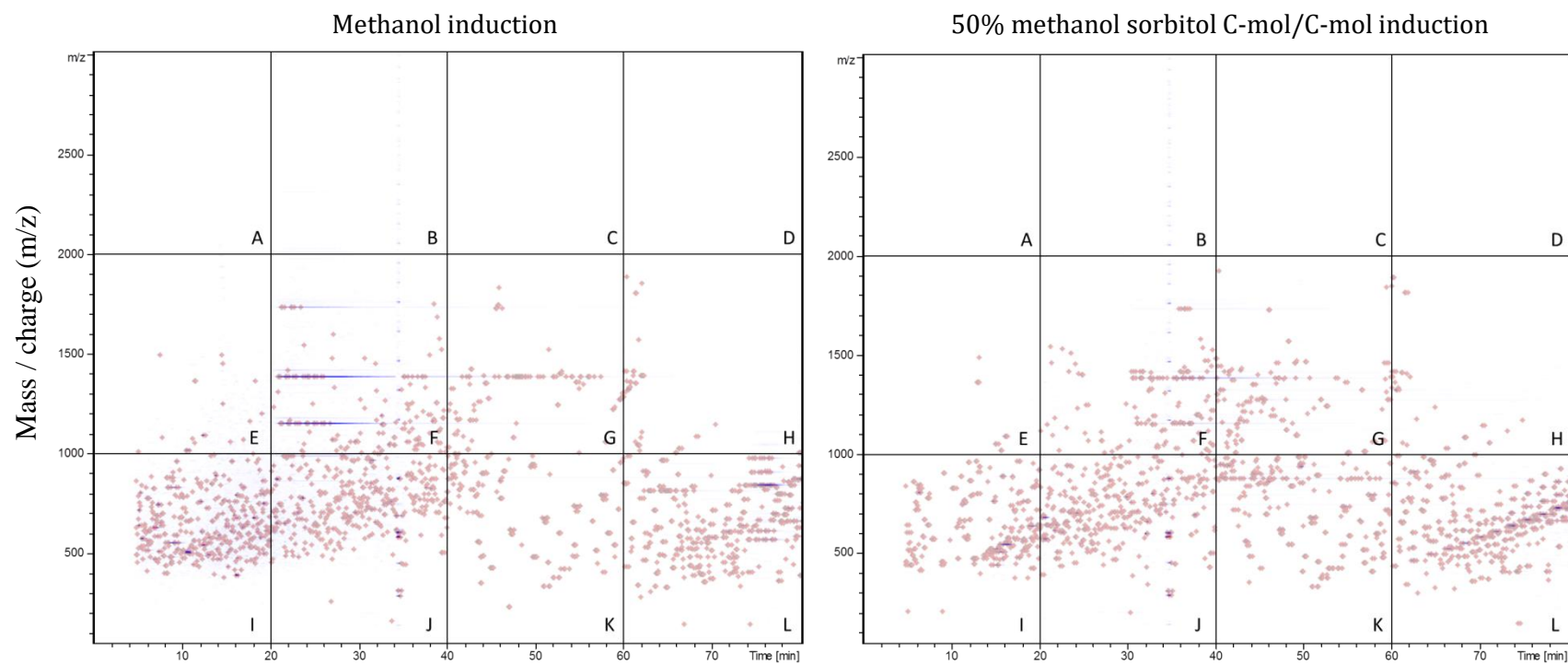
Figure 5-11 *Comparison of peptide profiles obtained from LC-MALDI-TOF/TOF analysis of samples from the end of P.pastoris CLD804 fermentations with a pure methanol induction feed (left) and a 50% methanol sorbitol C-mol/C-mol mixed induction feed (right).*

Methanol induction feed
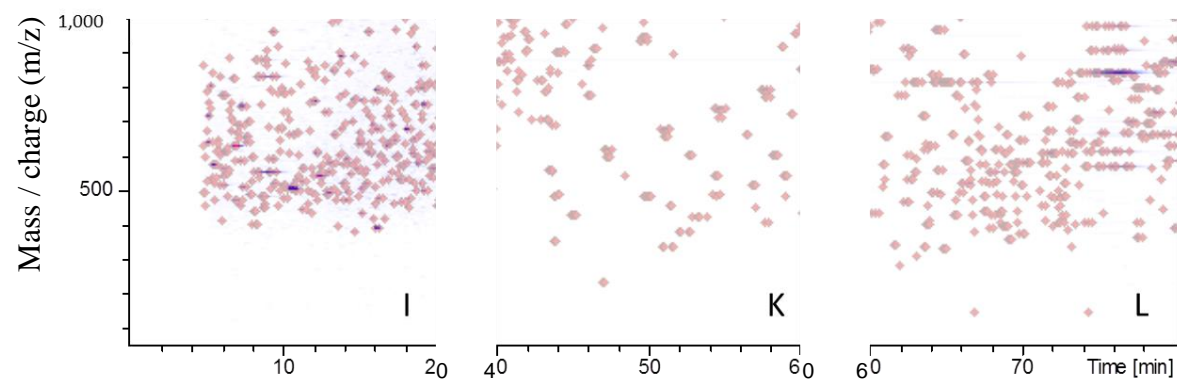
50% methanol sorbitol C-mol/C-mol induction feed
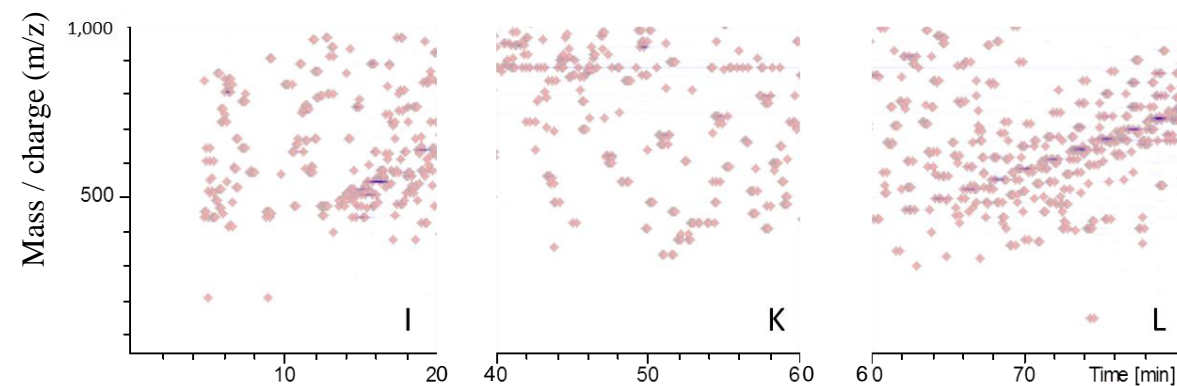
Figure 5-12 *Detailed comparison of sections of the peptide profiles from figure 5-10 showing the end of P. pastoris CLD804 fermentations with methanol induction feed (top) and 50% methanol sorbitol C-mol/C-mol mixed induction feed.*

these changes as well as the subsequent impact that they might have on product purification.

5.3 Conclusions

In this chapter a 50% methanol sorbitol mixed induction feed during 1 L total volume fermentations was not found to impact the product yield from *P. pastoris* GS115 or CLD804, with concentrations of SEAP and Aprotinin in fermentation supernatant not varying between pure methanol induction and mixed feed induction.

Cell viability was assessed offline throughout both fermentation regimes via protein and DNA secretion levels as a measure of cell death. Although cell death was found to increase during induction, no significant difference was found between induction feeds or cell lines. Cell death was, however, determined to be a result of induction as protein and DNA release rates were shown to be independent of cell growth rates.

The lack of impact that a mixed induction feed had on cell viability was a consequence of cellular stress not being reduced by the presence of sorbitol during induction. This was indicated by similarly elevated numbers of peroxisomes per cell during both induction feed types in comparison to the glycerol fed control cells.

Finally, protein impurity profiles were evaluated with pure methanol induction leading to an increase in protease release by the end of fermentation, potentially leading to a decrease in product quality. Mass spectrometry analysis found there to be a shift in the protein impurity profile with the use of a mixed induction feed, however the proteins affected were not successfully identified.

Chapter 6: Characterisation of the Covaris E450 sonicator and its use in establishing an automated approach to measure the cellular robustness of *Pichia pastoris*

6.1 Introduction

The determination and improvement of *Pichia pastoris* cell viability has been a focus of this study, with a range of techniques for the assessment of cell culture viability being discussed in detail in chapter 5. Limitations to the methods employed to determine cell viability have however been discovered. In this chapter a new, semi-automated, bench-top, offline method of determining cellular robustness is assessed, as it is hypothesised that cell viability is directly linked to cellular robustness.

In order to do this the E450 sonicator by Covaris Inc. (Woburn, MA, USA) which utilises adaptive focussed acoustics (AFA), had to be characterised to determine its applicability for this role. AFA involves the sonication of samples suspended in a water bath via a transducer with highly targeted acoustic waves. Using this technique up to 96 samples can be sonicated in series, at pre-determined times and intensities of the user's choosing. As a consequence, AFA has previously been successfully utilised as a scale down model for the disruption of both yeast and bacterial cells by homogenisation, in which protein and product release were used as measures of cellular disruption (Q. Li, Aucamp, Tang, Chatel, & Hoare, 2012; Wenger, DePhillips, & Bracewell, 2008). This will lead to a simpler and quicker method of determining cellular robustness than those proposed in previous studies (Overbeck et al., 2015).

The key objectives of this chapter are to:

1. Determine the parameters that have the greatest impact on the disruption of *P. pastoris* in order to develop a protocol for the determination of cellular robustness

2. Assess the applicability of AFA for determining cell viability by measuring cellular robustness throughout a *P. pastoris* fermentation

6.2 Results and Discussion

6.2.1 Characterising the Covaris E450 sonicator

An initial investigation was done to determine what factors most influenced cellular disruption. Cells were grown in shake flasks to the end of exponential phase ($OD_{600} \approx$ 40) before being distributed as 1 mL aliquots in 6 mL Covaris sample tubes. Cells were not induced so that protein release would be purely a result of disruption and not induction. Samples were sonicated over a range of intensities in order to determine at what stage cell disruption occurred. Duty cycle (the proportion of time that the transducer is producing acoustic waves) was kept at a constant 20 % as this has been previously shown to produce the greatest power input for all intensities and the number of cycles per acoustic burst was negated as a factor (maintained at a constant 1,000) as it has been shown to have little impact on cell disruption (Wenger et al., 2008). A power threshold was discovered, whereby no disruption was detectable, regardless of sonication time, below a certain power input. This effect was seen with both *P. pastoris* GS115 and CLD804 with consistent disruption only occurring at 70 W and above (figure 6-1). This effect is seen because of the way in which cell disruption occurs. When the acoustic waves generated by the transducer are focussed onto the sample being treated they compress the sample liquid before bubbles are created during rarefaction. These bubbles then collapse during the subsequent compression phase, releasing powerful shockwaves through the medium. When the kinetic energy of these shockwaves exceeds the strength of the cell walls, cellular disruption occurs (Chaplin & Bucke, 1990). With increased power input the number of cavitation bubbles increases, as well as the cavitation zone size,
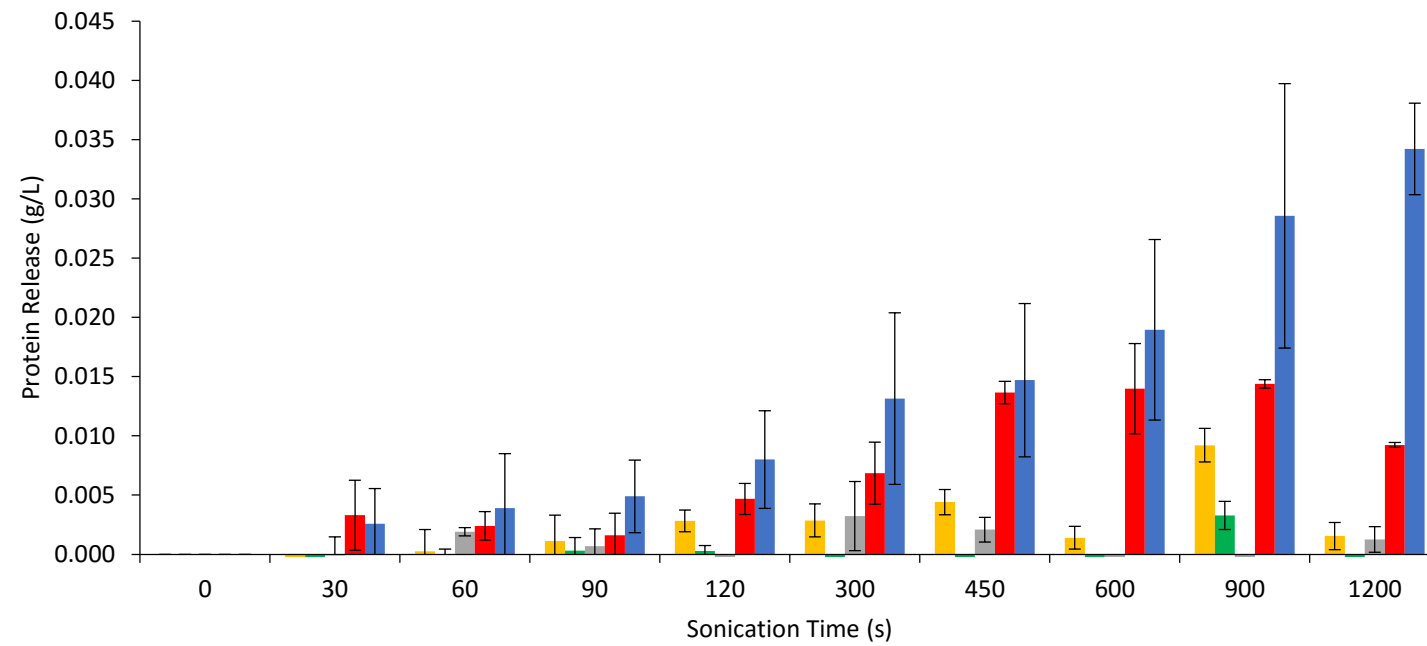
Figure 6-1 *P. pastoris GS115 cell disruption, as measured by protein release, at a range of sonication times and sonication intensities of 31 W ( ) 47 W ( ) 60 W ( ) 70 W ( ) 93 W ( ) in 1 mL samples. Error bars represent triplicate protein assay readings of each sample.*

due to the greater pressure formed by the greater amplitude sound wave; this in turn means that more energy is released in the form of shock waves with increasing sonication intensity (Lamminen, Walker, & Weavers, 2004). This means that at 70 W the average energy release from cavitation is enough to disrupt the *P. pastoris* cell wall. Below 70 W it is still possible for cellular disruption to occur as both power input and energy release by cavitation fluctuate, and so the longer the exposure time at lower power inputs, the more likely it is that disruption can occur. This effect is demonstrated by lower inputs still showing low levels of cell disruption despite being below the 70 W threshold.

As samples taken during fermentation would vary greatly in cell density, the influence of this on the capacity of the sonicator to disrupt cells had to be determined, in order to prevent cell density influencing the apparent robustness of cells being assessed. *P. pastoris* GS115 cells were cultured in shake flasks and grown to late exponential phase before being pelleted and resuspended in phosphate buffered saline (PBS) in order to mimic a range of cell densities. Protein release was again found to increase with sonication time but only increased with cell density up to an OD_{600} of 300 before decreasing rapidly (figure 6-2). This increase was predicted as an increased number of cells present in the sample would mean that more protein is released when these cells are disrupted. However, the drop in protein release with increasing cell density was unexpected and contradicted previous studies wherein sonication efficiency was found to be near to independent of cell density (Wenger et al., 2008).

This effect was further investigated using *P. pastoris* CLD804 cultured in the same manner. In addition to varying cell density, the impact of sample volume on sonication was also assessed. A key benefit of an offline robustness assay for fermentation would be to only require small volumes in order to make it viable for
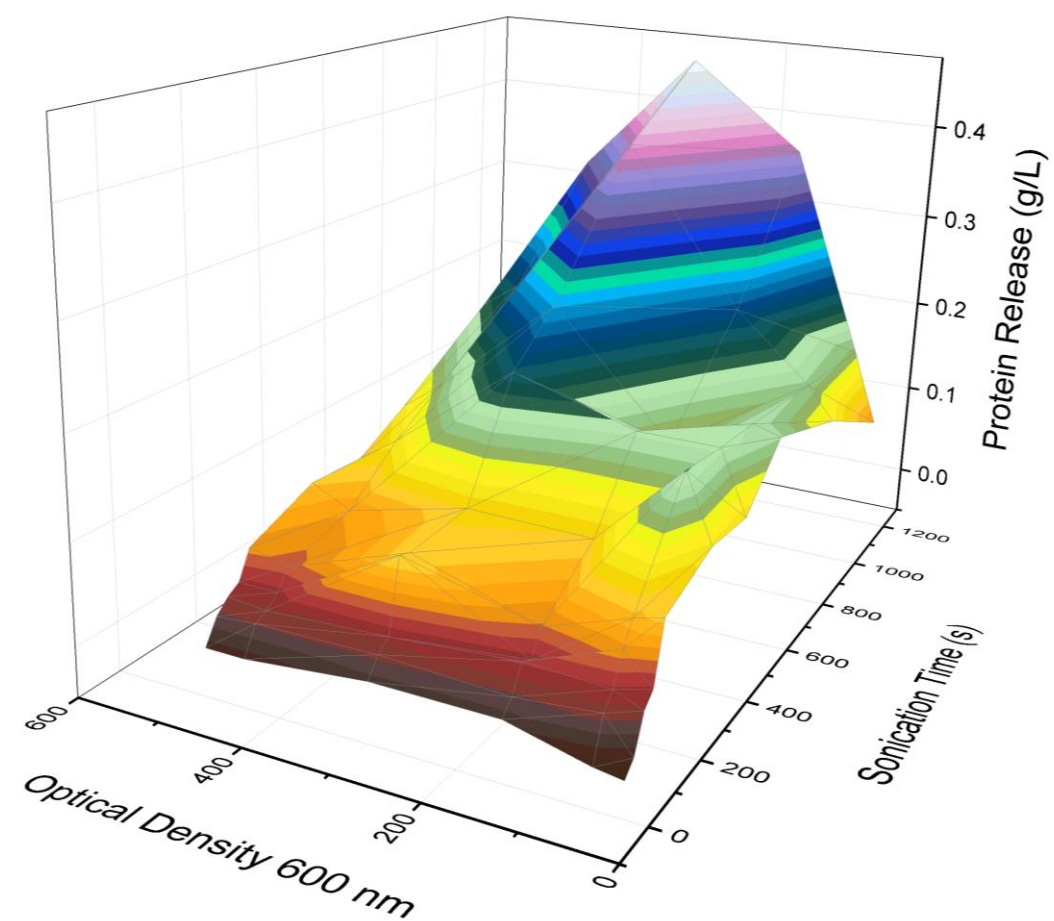
Figure 6-2 *P. pastoris GS115 cell disruption, as measured by protein release, at a range of sample cell densities and sonication times in 1 mL samples. Points plotted are averages of triplicate protein assay readings.*

even benchtop fermentations, and so demonstrating reliable results at a range of volumes was essential. With specific protein release showing an optical density of 150 to be the point at which cellular disruption starts to decline, optical densities of 50, 150 and 300 were chosen. Cell disruption was confirmed to be greatest at an optical density of 150, regardless of sample volume, with maximum cell disruption occurring after 10 minutes sonication (figure 6-3). Sample volume was however seen to increase the effectiveness of sonication with the rate of protein release rising from 0.014 to 0.017 to 0.023 mg/L/ODU/s for sample volumes of 1 mL, 2 mL and 3 mL respectively. Importantly, the clearest trends in protein release with sonication time were found at higher cell densities, with results at OD600 = 50 showing variable results across all sample volumes. Cell density plays an important role in the way in which disruption occurs for two reasons: firstly the sonicator is designed for the disruption of particles suspended in liquid, as higher cell densities are reached (figure 6-4), the proportion of liquid in the sample decreases (OD_{600} = 300 is a solids density of ~25%) meaning that there is physically less space for cavitation bubbles to form and subsequently collapse to cause disruption. Secondly, the high viscosity of high cell density cultures (Thömmes et al., 2001) means that mixing does not occur as readily during treatment, resulting in fewer cells being trapped in the focal zone, as well as the velocity gradients that cells are exposed to being lower due to the same sound wave intensity resulting in less liquid movement.

This shows that cell density is an important factor in obtaining accurate robustness results, as it not only affects the rate of cell disruption but also because lower optical densities lead to more error in results. From this, and knowing that sample sizes of down to 1 mL could be processed with no significant effect on results, a bench-top robustness assay could be developed.
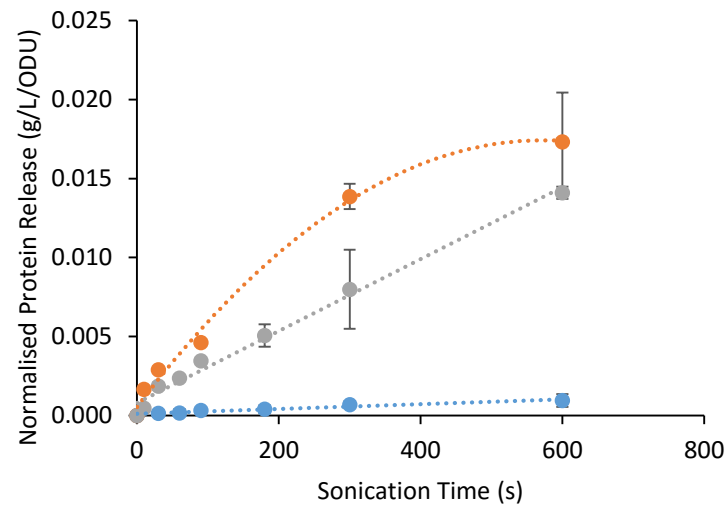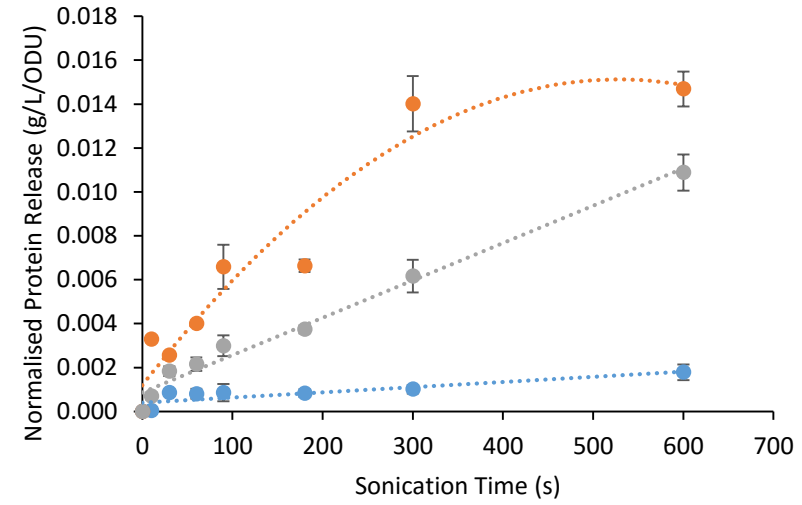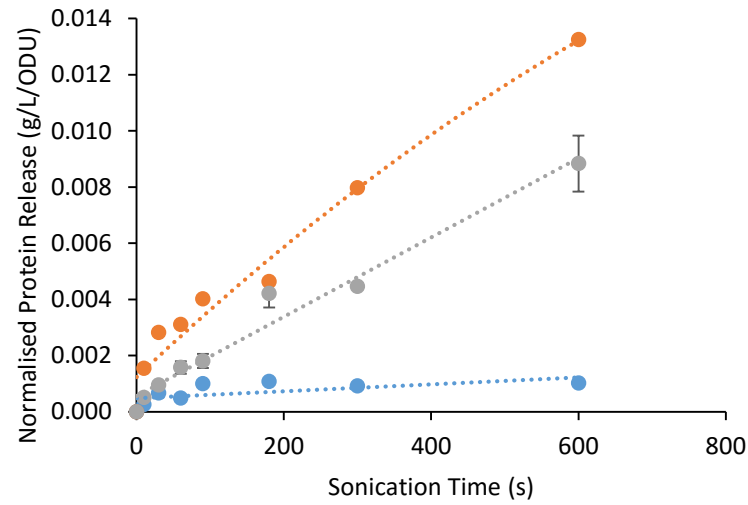
Figure 6-3 *P. pastoris CLD804 cell disruption, as measured by protein release, at sample volumes of A = 1 mL, B = 2 mL and C = 3 mL. Cell densities in samples were 50 (●), 150 (●) and 300 (●). Error bars represent triplicate protein assay readings of each sample.*

B
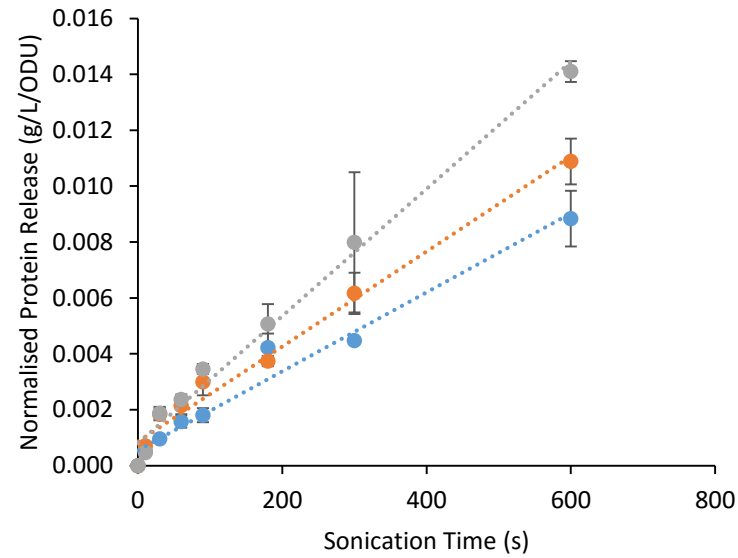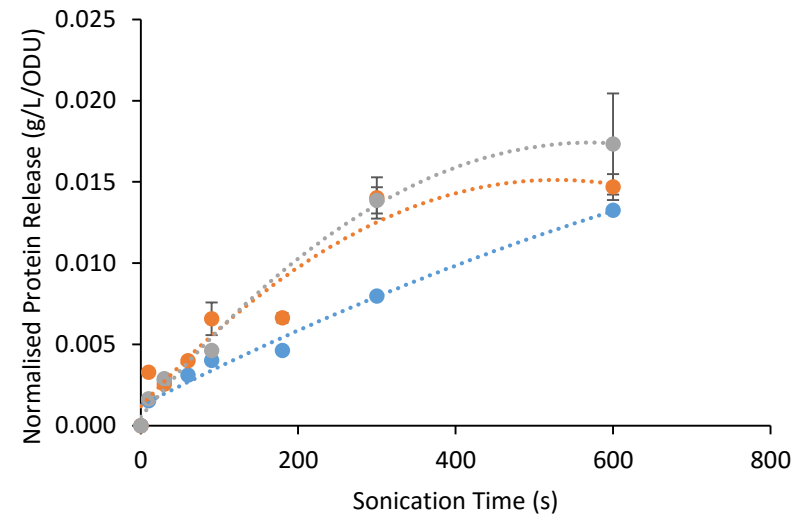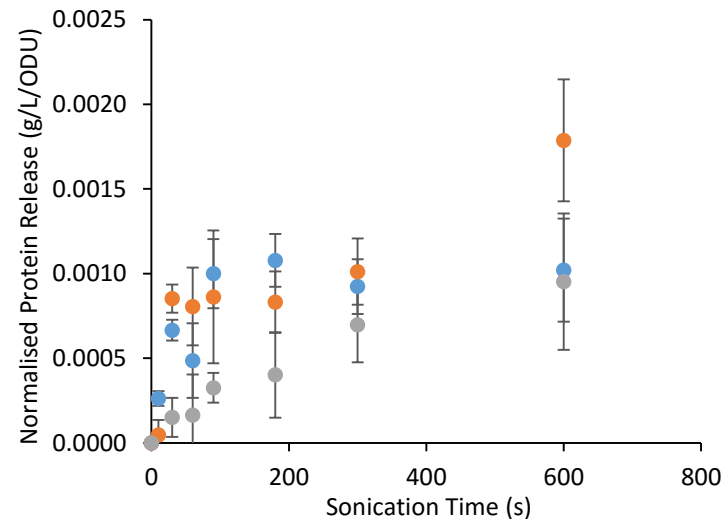
Figure 6-4 *P. pastoris CLD804 cell disruption, as measured by protein release, at sample optical densities of A = 50, B = 150 and C = 300. Sample volumes were 1 mL (●), 2 mL (●) and 3 mL (●). Error bars represent triplicate protein assay readings of each sample.*

6.2.2 Determining cellular robustness during fermentation

P. pastoris CLD804 was cultured in a 1 L bioreactor using the standard Invitrogen protocol (Invitrogen Corporation, 2002) with pure methanol induction (figure 6-5), during which samples were taken to assess cellular robustness during each stage of fermentation (full methodology in section 2.3.7.2). Cellular robustness was found to vary significantly throughout fermentation, with cells taken from the glycerol fed-batch phase (at 22 hours post-inoculation) having the highest rate of protein release by cell disruption, followed by cells from induction day 1 (48 hours) and with cells from adaptation phase (25 hours) and induction day 2 (72 hours)(figure 6-6). This is in contrast to the initial hypothesis that cells would be weakened by methanol induction and means that cellular robustness cannot be used as a measure of cell viability in *P. pastoris*. When cellular robustness was compared to cell growth rate however, it was noted that there was correlation (table 6-1); this is because of the way in which *P. pastoris* grows. As it's a multilateral budding yeast species (Kreger-van Rij, 1984), an increased growth rate means that a greater number of cells are likely to be budding or have multiple buds on them. This budding mechanism in turn weakens the cell wall as their morphology makes the budding more prone to shear and cells that have produced many daughter cells have budding scars, which also reduce the integrity of their cell wall. This effect has been previously noted in a study using *Saccharomyces cerevisiae* using compression tests to assess cellular robustness (Overbeck et al., 2015), but never using sonication nor *P. pastoris*.
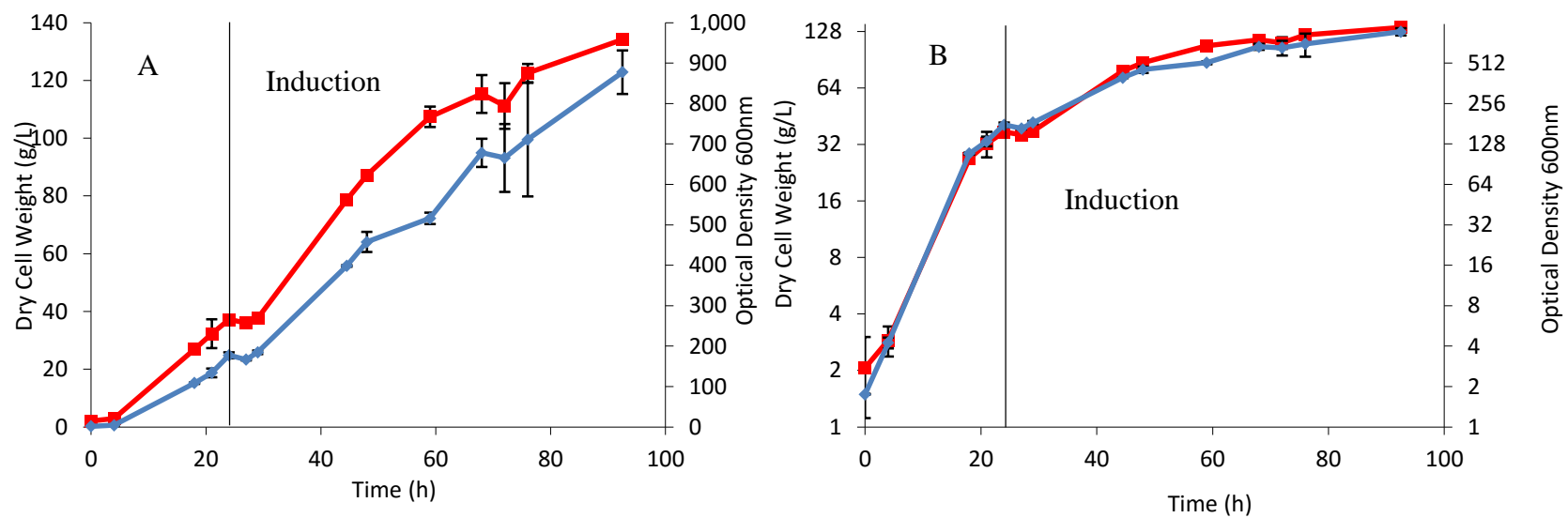
Figure 6-5 *Growth of P. pastoris CLD804 with pure methanol induction as determined by dry cell weight on the left axis (■) and optical density on the right axis (■). A= Regular axes, B = Logarithmic axes in order to show growth phase.*
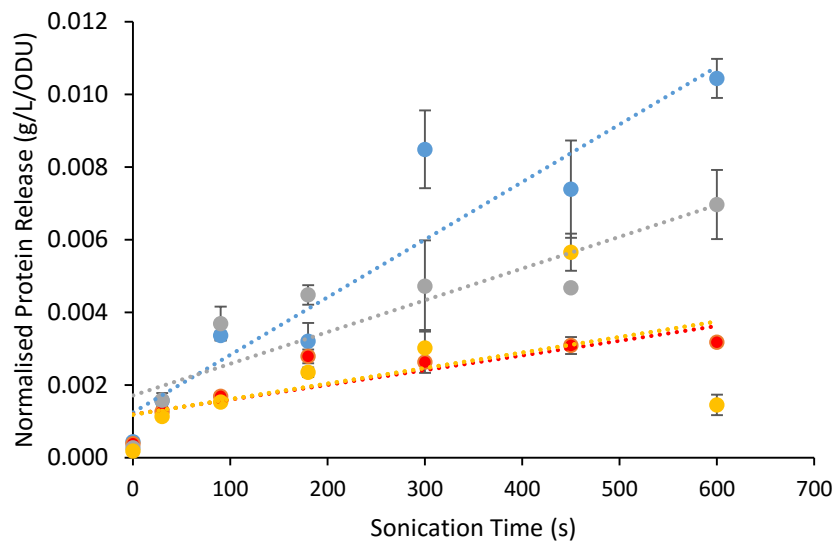
Figure 6-6 *P. pastoris CLD804 cell robustness from a 1 L fermentaiton, as measured by protein release, during glycerol fed-batch phase (●), adaptation phase (●), induction day 1 (●) and induction day 2 (●) where a higher protein release rate indicates lower cellular robustness. Error bars represents triplicate protein assay readings.*

Table 6-1 *A comparison of growth rate and specific protein release rate, as a result of sonication, as an inverse measure of robustness during each phase of P. pastoris CLD804 pure methanol induced fermentation.*

Phase	Time (h)	Growth Rate (1/h)	Indicated Specific Protein Release Rate (mg/L/ODU/s)
Glycerol fed-batch	22	0.054	0.028
Adaptation	25	0.002	0.009
Induction day 1	48	0.042	0.016
Induction day 2	72	0.010	0.010

6.3 Conclusions

In this investigation it was found that the cellular disruption of *P. pastoris* was dependent on overcoming a power threshold of approximately 70 W, below which little or no disruption occurs. In addition to this, cell density was found to have major impact on the effectiveness of sonication to disrupt cells, with cellular disruption being maximised at a cell density of OD_{600} = 150 before being severely hampered by increasing cell densities.

Using this information a methodology was developed for the offline, semi-automated determination of cellular robustness during fermentation. This showed that cellular robustness is not linked to cell viability as it was found to be lowest during growth on glycerol and greatest during adaptation phase and at the end of induction phase, with the latter seen as a low point in cell viability. It was concluded that this supported other studies that found that cellular robustness in other yeasts is directly linked to cell growth rate due to the detrimental effect of budding on cell wall integrity (Overbeck et al., 2015). This effect has never been demonstrated before with *P. pastoris* or with a sonicator, and so the use of AFA is suggested as a fast, novel method of determining cellular robustness during fermentation.

Chapter 7:Conclusions & Future Work

7.1 Conclusions

In this thesis a fermentation strategy utilising sorbitol as a co-feed with methanol was developed and assessed with the goal of reducing the amount of methanol required during *Pichia pastoris* fermentations in order to improve process scalability, cell viability and ultimately increase product yields.

In assessing the impact of sorbitol on cell growth using shake flask cultures it was determined that increasing sorbitol concentrations in the media, with corresponding decreasing methanol concentrations, caused a reduction in growth rate. This combined with product release data, wherein product secretion occurred in all methanol containing flasks, allowed an optimal methanol sorbitol ratio of 50% C-mol/C-mol to be determined, based on the highest normalised product release. Using this ideal mixed feed ratio a fermentation protocol, based on literature, was developed. Growth profiles attained in 1 L parallel bioreactors, by the use of a pure methanol induction feed and a mixed induction feed, were found to be equivalent with the exception of the adaptation phase, which was eliminated by the application of the mixed feed for both *P. pastoris* GS115 and *P. pastoris* CLD804.

To determine the scalability of the new feeding protocol a deterministic model, based on metabolic heat production and bioreactors cooling capacities was developed. This model was used to predict that the application of the mixed feed regime would result in a 60% reduction in heat generation during induction, allowing fermentations to reach a predicted maximum working volume of 7,000 L compared to 5,500 L with pure methanol induction. This reduction in heat generation was confirmed experimentally at 1 L scale using *P. pastoris* CLD804.

Cell viability and product release were not affected by the use of a mixed induction feed for both *P. pastoris* GS115 producing SEAP and CLD804 producing aprotinin. This was thought to be due to cellular stress, as estimated by the number of peroxisomes

generated per cell, not being reduced by the inclusion of sorbitol in the induction feed. Protease release by *P. pastoris* CLD804 however, was found to be decreased by the mixed induction feed as a result of shift in the protein impurity profile.

Finally a technique was developed to determine cellular robustness using the Covaris E450 sonicator. Cells were found to have a threshold power input for disruption, wherein an acoustic intensity of 70 W or more was required to damage them, regardless of sonication time. Sample density was also determined to be the most important factor to be controlled with cell densities above $OD_{600} = 150$ resulting in reduced sonication effectiveness. Using this information, samples were taken during fermentation and cells were tested for their robustness. Cellular robustness was not linked to cell viability but instead to cellular growth rate, with cells being most robust during adaption phase and the end of induction phase; this is due to budding reducing the ability of cells to withstand shear. This data correlated with other studies, validating the technique for the determination of cellular robustness in a semi-automated, small scale, offline manner.

7.2 Future work

In this investigation a mixed feed induction strategy utilising sorbitol as a co-feed with methanol was successfully devised, and the impact of this feed on *P. pastoris* fermentation characteristics was assessed. The application of this new fermentation strategy did not result an improvement in product yield or cell viability, but did improve the desirability of the *P. pastoris* expression system by improving scalability by reducing heat generation during fermentation. The research also indicated that the host cell protein impurity profile was affected by the introduction of the new feed regime which has potential implications on the purification of recombinant products produced in *P. pastoris*.

To improve monitoring of the fermentation process in future studies a reliable means of determining cell viability needs to be established. In addition to this, online monitoring of methanol concentrations in the bioreactor, as well as a means of easily quantifying residual sorbitol would allow the fermentation process to be fully characterised. This would allow for more informed scale up of the mixed feed fermentation process.

In order to build upon this work, the proteins released during mixed feed induction need to be identified to establish what impact they would have on product purification. The majority of proteins released during methanol induction are enzymes associated with methanol metabolism and so any major shift protein impurity profile would be a result of concentrations of these being reduced (Vanz et al., 2012). This would either be a result of reduced cell death, as less host cell proteins would be released, or due to a shift in metabolic pathways. The benefit of this would be product dependent as different impurities would co-purify with different products, however any overall reduction in protein release other than the product would be beneficial. The most important group of contaminating proteins that need to be reduced are the host-cell proteases, as these have the potential to degrade the product, which not only reduce product yields, but also interfere with chromatographic steps as product fragments are likely to co-purify with intact product and decrease overall product quality. Experiments involved in the optimisation of the feeding regime would need to focus on these factors, and protein impurity profiles would have to be done throughout in order to understand how sorbitol affects them.

It is predicted that these benefits would be seen by maintaining methanol levels to the minimum concentration required for induction (~0.5% w/v) with excess sorbitol which would also further increase the scalability of fermentations with the decrease

in heat generation. There would also be limited impact on product purification as sugar alcohols only bind to ion exchange chromatography resins at extreme pHs due to their lack of charge (Scopes, 1994). The caveat however, with these alternative methods, would be the increased cost of goods due to the greater cost of sorbitol when compared to methanol. During 50% methanol sorbitol C-mol/C-mol mixed induction feeding, sorbitol accounts for 67% of the cost (with molecular biology grade sorbitol costing 37.2 £/kg compared to 16.4 £/kg for methanol). This cost discrepancy however, is of minor significance considering the increased process scalability as well as the percentage of cost of goods that is attributed to media. For example, in a 2,000 L scale process, a mixed feed fermentation would result in an increased cost of goods of approximately 6.5%. When it is considered that 70% of costs are labour and indirect costs at this scale, increase in the cost of goods has a minor impact on the overall cost of the bioprocess (Lim et al., 2010; Pollock, Ho, & Farid, 2013). With pure methanol induced fermentations requiring multiple reactors to be operated in order to reach broth volumes above 5,000 L, the minor cost saving is removed by a large increase in capital and labour costs, meaning that the mixed feed fermentation will be more economical in the majority of circumstances.

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Appendix A

The following was used to determine the amounts of methanol and sorbitol required in the 50% methanol sorbitol C-mol/C-mol induction feed to ensure that equal amount of carbon were going to come from each substrate:

Methanol, M_r = 32.042

Mass of carbon in methanol as percentage of $M_r = \frac{12.011}{32.042} \times 100 = 37.48\%$

Sorbitol, M_r = 182.17

Mass of carbon in sorbitol as percentage of $M_r = \frac{72.064}{182.17} \times 100 = 39.56\%$

Thus, carbon mass ratio of sorbitol : methanol = 1 : 1.0553

Protein released by *P. pastoris* CLD804 into media growing in 150 mL complex media containing a range of methanol to sorbitol carbon source ratios, in 1 L shake flasks after 14 hours growth. Results are compared to pure complex media with no cells inoculated (M).
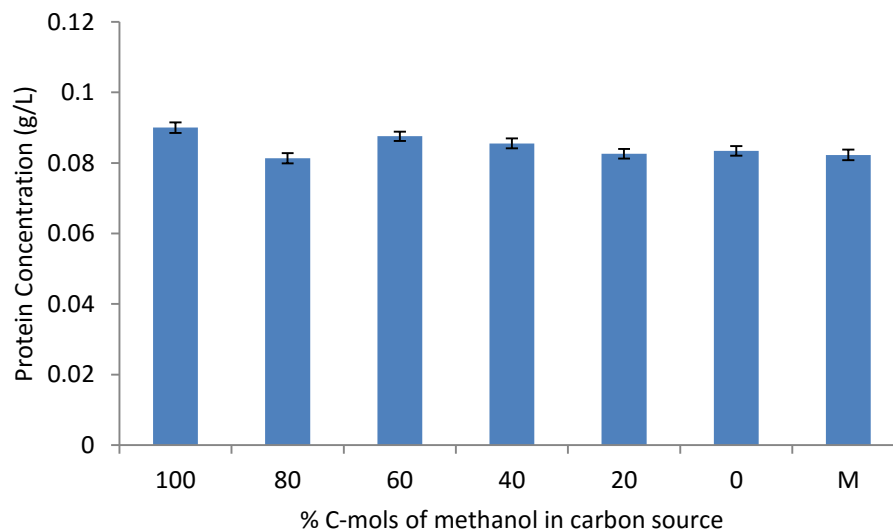
Scale-down calculations comparing an Applikon 20 L bioreactor to 1 L Infors bioreactors.

Defined parameters:

Density (ρ) =   995.7 Kg/m³
Viscosity (μ) =   0.004 Pa s
Number of impellers (n) =   3
20 L impeller diameter (d_i)=   0.075 m
20 L working volume (V) =   0.015 m³
1 L impeller diameter (d_i) =   0.038 m
1 L working volume (V) =   0.0007 m3

Equations:

Reynolds number (Re) = $\frac{\rho \cdot \mu \cdot Di}{N}$

Where N = impeller speed in (1/s)

Power input (P) = $n \cdot (n_p \cdot \rho \cdot N^3 \cdot D_i{}^5)$

Where the power constant (N_p) = 5.5 (Doran, 1995)

20 L

Impeller Speed (rpm)	Impeller Speed (1/s)	Tip speed (m/s)	Reynolds Number	Power Input (W)	P/V (W/m³)
500	8.3	1.96	11,668	22.56	2,333,672
600	10.0	2.36	14,002	38.99	2,800,406
700	11.7	2.75	16,336	61.91	3,267,141
800	13.3	3.14	18,669	92.41	3,733,875
900	15.0	3.53	21,003	131.58	4,200,609
1000	16.7	3.93	23,337	180.49	4,667,344
1010	16.8	3.97	23,570	185.96	4,714,017

1 L

Impeller Speed (rpm)	Impeller Speed (1/s)	Tip speed (m/s)	Reynolds Number	Power Input (W)	P/V (W/m³)
500	8.3	0.99	2,995	0.75	1,076
600	10.0	1.19	3,594	1.30	1,860
700	11.7	1.39	4,194	2.07	2,953
800	13.3	1.59	4,793	3.09	4,408
900	15.0	1.79	5,392	4.39	6,276
1000	16.7	1.99	5,991	6.03	8,610
1100	18.3	2.19	6,590	8.02	11,459

Appendix B

The oxygen uptake rate was calculated using the following equation:

$$\text{OUR} = \frac{Q}{V} \cdot \left(\frac{C_{O2}^{in} - C_{O2}^{out}}{100} \right) \cdot \frac{1}{R \cdot T}$$

Where:

Q = volumetric flowrate of air (L/s)

V = volume of fermentation liquid (L)

C = percentage oxygen in inlet / outlet air

R = gas constant

T = temperature (K)

Carbon dioxide emission rate (CER) was calculated the same way but with exit CO_2 concentration – inlet CO_2 concentration replacing the oxygen concentration difference.

The equation for heat generation was derived as follows:

$$Q_{gen} = 460{,}000 \cdot \frac{\text{OUR}}{1{,}000} \cdot \frac{1}{3{,}600} \cdot 1{,}000V$$

This simplifies to:
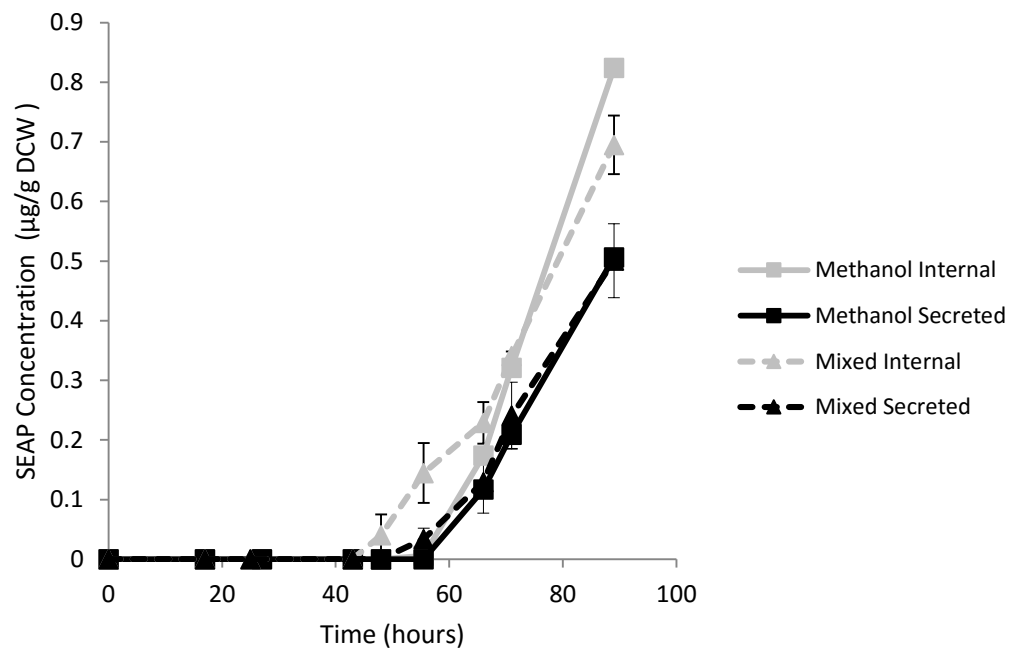
$$Q_{gen} = 127.78 \cdot \text{OUR} \cdot V$$

Where: Q_{gen} = heat generated by cell culture (W)

OUR = oxygen uptake rate (mmol/L/h)

V = bioreactor working volume (m³)

Appendix C

Comparison of internal retained SEAP by P. pastoris GS115 compared to secreted SEAP with both a pure methanol induction feed and a 50% methanol sorbitol C-mol/C-mol mixed induction feed.

SEAP assay (Quanti-blue) response curves with SEAP standard concentrations diluted from 300 µg/L to 0.48 µg/L in 5x serial dilutions.
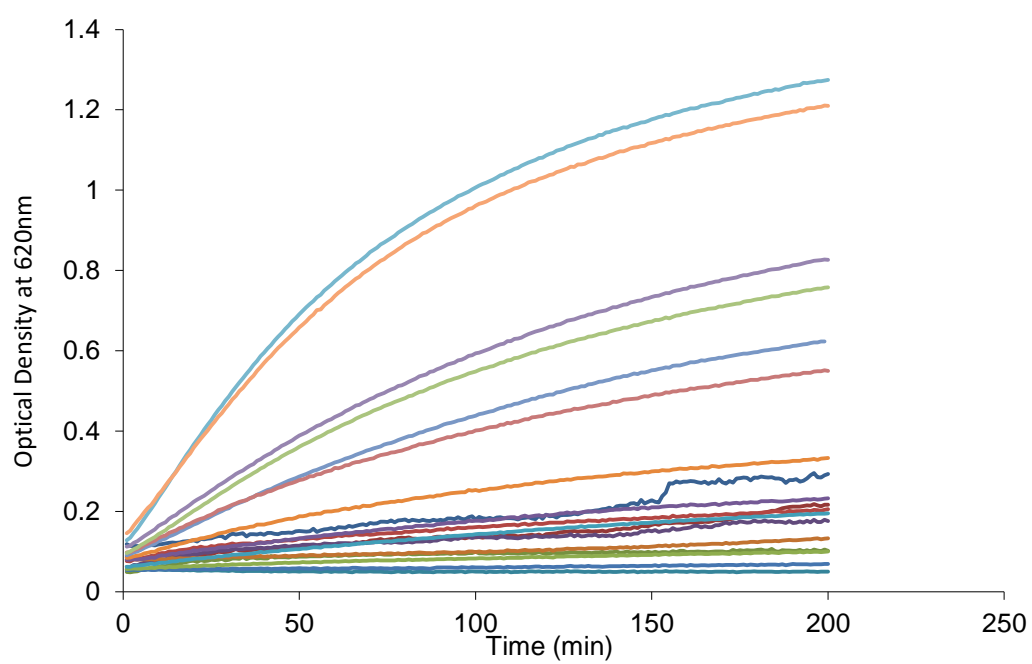
Particle size distribution during 1 L fermentations of *P. pastoris* CLD804 with a methanol induction feed and a 50% methanol sorbitol C-mol/C-mol mixed induction feed.