

DOCTORAL THESIS

The effect of antifoams upon recombinant protein production in yeast

Sarah Routledge

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**THE EFFECT OF ANTIFOAMS UPON
RECOMBINANT PROTEIN PRODUCTION IN
YEAST**

by

Sarah Jayne Routledge

Doctor of Philosophy

Aston University

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ASTON UNIVERSITY

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PROTEIN PRODUCTION IN YEAST**

Sarah Jayne Routledge

Ph.D

2012

Thesis Summary

Foaming during fermentation reduces the efficiency of the process leading to increased costs and reduced productivity. Foaming can be overcome by the use of chemical antifoaming agents, however their influence upon the growth of organisms and protein yield is poorly understood. The objective of this work was to evaluate the effects of different antifoams on recombinant protein production. Antifoam A, Antifoam C, J673A, P2000 and SB2121 were tested at different concentrations for their effect on the growth characteristics of *Pichia pastoris* producing GFP, EPO and A_{2a}R and the yield of protein in shake flasks over 48 h. All antifoams tested increased the total GFP in the shake flasks compared to controls, at higher concentrations than would normally be used for defoaming purposes. The highest yield was achieved by adding 1 % P2000 which nearly doubled the total yield followed by 1 % SB2121, 1 % J673A, 0.6 % Antifoam A and lastly 0.8 % Antifoam C. The antifoams had a detrimental effect upon the production of EPO and A_{2a}R in shake flasks, suggesting that their effects may be protein specific. The mechanisms of action of the antifoams was investigated and suggested that although the volumetric mass oxygen transfer coefficient (k_{La}) was influenced by the agents, their effect upon the concentration of dissolved oxygen did not contribute to the changes in growth or recombinant protein yield. Findings in small scale also suggested that antifoams of different compositions such as silicone polymers and alcoxylated fatty acid esters may influence growth characteristics of host organisms and the ability of the cells to secrete recombinant protein, indirectly affecting the protein yield. Upon scale-up, the concentration effects of the antifoams upon GFP yield in bioreactors was reversed, with lower concentrations producing a higher yield. These data suggest that antifoam can affect cells in a multifactorial manner and highlights the importance of screening for optimum antifoam types and concentrations for each bioprocesses.

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Creation of the RCP-TEV-GFP construct was carried out in collaboration with Dr James Barwell, Aston University and flow cytometry work in collaboration with Prof Chris Hewitt, Loughborough University.

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ABBREVIATIONS

A _{2a} R	Adenosine _{2a} receptor
AQP	Aquaporin
β1-AR	Beta1 adrenergic receptor
BCA	Bicinchoninic acid
BHK	Baby hamster kidney cells
B _{max}	Maximum binding capacity
BMGY	Buffered complex glycerol medium
BMMY	Buffered complex methanol medium
BMY	Buffered complex medium
BSA	Bovine serum albumin
cAMP	Cyclic adenosine 3', 5' monophosphate
CGRP	Calcitonin gene-related peptide
CHO	Chinese hamster ovary cells
CHLG	Chlorophyll synthase
CLR	Calcitonin receptor like receptor
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DO	Dissolved oxygen
DO solution	Drop out solution
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
ECL	Extracellular loop
EG	Ethylene glycol
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoetin
GFP	Green fluorescent protein
GPCR	Guanine nucleotide-binding protein coupled receptor

G-protein	Guanine nucleotide-binding protein
Gs	G-protein stimulatory
h	Hour
HEK	Human embryonic kidney cells
HF	High fidelity
HGH	Human growth hormone
IC ₅₀	Half maximal inhibition concentration
ICL	Intracellular loop
K _d	Concentration of ligand required to bind half of the receptors
kDa	Kilo Dalton
<i>k_La</i>	Volumetric mass oxygen transfer coefficient
L	Litre
LB	Luria-Bertani
MDCK	Madin-Darby canine kidney epithelial cells
MFC	Mass flow controller
Min	Minute
Mol	Mole
Mg	Milligram
mL	Millilitre
nm	Nanomolar
Ni-NTA	Nickel nitrilotriacetic acid
OD	Optical density
OTR	Oxygen transfer rate
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween 20
PCR	Polymerase chain reaction
PDB	Protein data bank
PEG	Polyethylene glycol
pH	Negative logarithm of the hydrogen ion concentration
<i>P. pastoris</i>	<i>Pichia pastoris</i>

RCP	Receptor component protein
rpm	Revolutions per minute
s	Second
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SDS	Sodium dodecyl sulphate
Sf	<i>Spodoptera frugiperda</i>
T	Time
TAM	Thermal activity monitor
TEMED	N,N,N',N'-tetramethyl- ethane-1,2-diamine
TEV	Tobacco etch virus
TM	Transmembrane domain
Tris	Tris(hydroxymethyl)aminoethane
Tween 20	Polyoxyethylene sorbitan monolaurate
v/v	Volume/volume
WT	Wild type
w/v	Weight/volume
YPD	Yeast peptone dextrose
YPDS	Yeast peptone dextrose sorbitol
YNB	Yeast nitrogen base

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1. INTRODUCTION

1.1 Overview

This project is concerned with the optimization of recombinant protein production in yeast. Research was specifically conducted into areas of process optimization which have previously received little attention and could become important in obtaining higher yields of recombinant proteins. The work described here focuses on the influence of additives, known as antifoams, on the yield of a range of protein targets in the yeast *Pichia pastoris*. Investigation into the mechanisms of action of antifoams, which were found to have an effect upon yield of protein and growth of the organism, were carried out. The data generated should lead to a more informed approach when choosing antifoams for fermentation processes.

1.2 *P. pastoris* as a host for recombinant protein production

The production of recombinant proteins is essential for the development of drugs as well as the engineering of antibodies[1], the identification of functions and interactions of proteins[2] and also in the production of enzymes[3]. Valuable proteins such as insulin[4] and human growth hormone[5] have been produced recombinantly on an industrial scale and have enabled treatment and understanding of many diseases.

Recombinant protein production involves the amplification of a DNA sequence encoding the target protein[6] followed by insertion into a vector and transfer to the host cell which will produce the protein[7]. The combination of the DNA encoding the gene of interest, usually from another organism, and the vector DNA results in recombinant DNA; the protein produced by the cells containing this DNA is known as recombinant protein[7].

The vector into which the target DNA is inserted can be replicated inside a host cell to produce clones[8]. The choice of vector depends upon the host organism and the protein to be produced. Vectors typically contain a promoter which can affect the level of gene

expression, and antibiotic resistance or nutrient selection genes to aid selection of the organisms containing the target DNA[8] and expressing the recombinant protein of interest[9]. This work uses the methylotrophic yeast *Pichia pastoris* as an expression system for recombinant protein production.

1.2.1 *P. pastoris* general properties

P. pastoris is an ascomycetous budding yeast of the family *Saccharomycetaceae*[10]. It is Crabtree-negative[11] and methylotrophic, and has been isolated from tree sap, including sap from algarrobo trees[12]. It is strictly aerobic and had its genome sequenced in 2009[13]. A thin-section electron microscopy image of a *P. pastoris* cell is shown in Fig. 1.1.



Figure 1.1: Thin-section electron microscopy image of a *P. pastoris* cell

G, Golgi stack; *N*, nucleus; *ER*, peripheral ER membranes; *M*, mitochondrion; *V*, vacuole. Taken from Rossanese et al 1999[14].

P. pastoris is usually in a vegetative haploid state, and mating occurs when nitrogen is limited to form diploid cells. It is a homothallic yeast and cells of the same strain mate easily. It is thought that *P. pastoris* may have more than one mating type which switches at high frequency, and that mating occurs only between haploid cells of the opposite mating type. This has been shown with the related yeast, *Pichia methanolica*[15]. The diploid cells are able to remain in this state or can proceed to meiosis, where they produce asci containing four haploid spores. *P. pastoris* is most stable in the vegetative haploid state, unlike *S. cerevisiae* where haploid cells are unstable and mate to form diploid cells[15].

Sugars are utilized by all yeasts through a common glycolytic pathway. These metabolic pathways of non-conventional yeasts such as *P. pastoris* were assumed to be identical to those of *S. cerevisiae*, however slight variations exist[16]. As well as glucose, glycerol can be utilized by *P. pastoris* as a carbon source under aerobic conditions[16, 17]. This involves glycerol phosphorylation by a cytosolic glycerol kinase to 3-phosphoglycerol, which is then oxidized by a mitochondrial FAD-dependent glycerol phosphate ubiquinone oxidoreductase to produce dihydroxyacetone phosphate which is used in pyruvate synthesis and gluconeogenesis[18]. *P. pastoris* has a high glycerol uptake rate and 4 genes have been discovered that encode H^+ /glycerol symporters[19]. This makes glycerol an effective substrate to accumulate biomass whilst repressing the alcohol oxidase 1 (*AOX1*) promoter before induction to produce recombinant protein[20].

Koichi Ogata found that methanol could be used as a sole carbon source by some species of yeast such as *Candida*, *Hansenula*, *Pichia* and *Torulopsis* in 1969[21-23]. In the 1970's due to the low cost of acquiring methanol from methane these species were appealing for the production of single cell protein for animal feed[22, 23]. Phillips Petroleum Company developed the methods and media in order for *P. pastoris* to grow to high cell densities on methanol. However the price of methane rose due to the oil crisis and another source of animal feed protein, soy beans, became cheaper. Consequently interest was lost in the species until the 1980's when a new use was found. Phillips together with The Salk Institute of Biotechnology used *P. pastoris* as an expression system for heterologous protein production, utilizing the *AOX1* gene and promoter[24].

A large proportion of methanol transport into *P. pastoris* is thought to occur by passive diffusion[25, 26]. Methanol is then metabolized by *P. pastoris* as the sole carbon source beginning with oxidation of methanol to formaldehyde catalysed by the alcohol oxidase (*AOX*) enzyme also producing hydrogen peroxide. This occurs within the peroxisome which prevents damage to the cell by sequestering the toxic hydrogen peroxide[22] illustrated in Fig. 1.2. The *AOX1* and *AOX2* genes code for *AOX*, but *AOX1* is responsible for around 90% of the enzyme in the cell[22, 27]. Methanol strongly induces the *AOX1* promoter and can therefore be utilized to initiate the expression of the desired protein whether or not it is toxic. Higher expression levels can be obtained by using the *AOX1* promoter rather than *AOX2*.

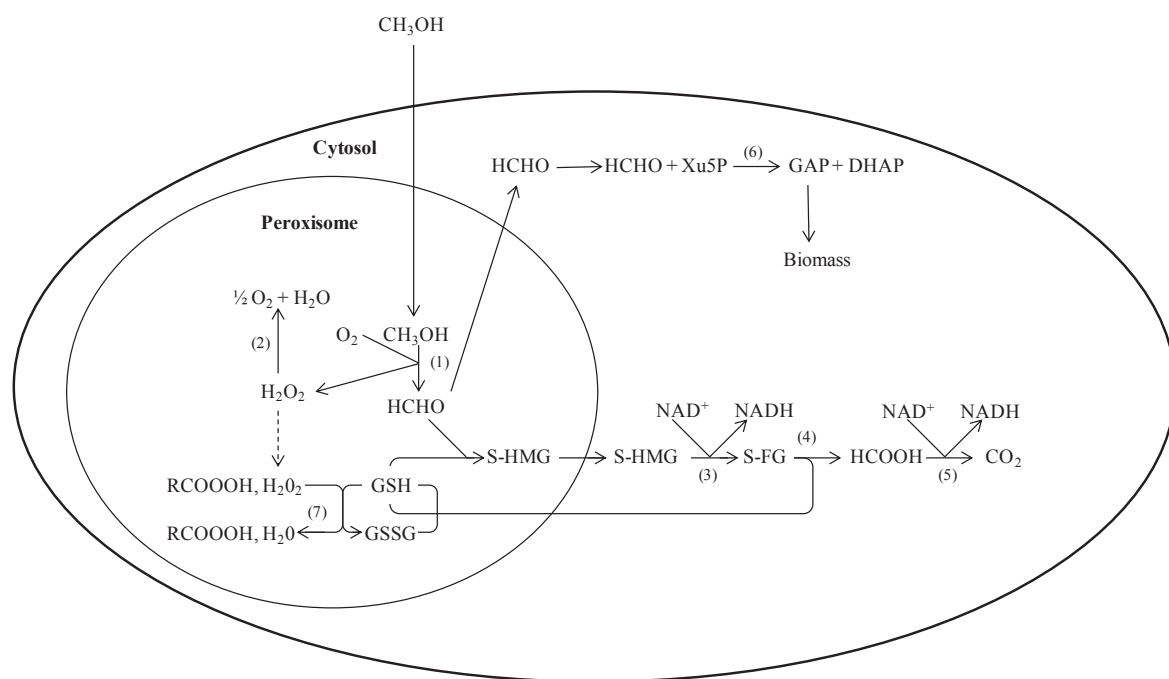


Figure 1.2: Methanol metabolism in methylotrophic yeasts

Adapted from Gellissen, G (2002)[28](1) alcohol oxidase, (2) catalase, (3) glutathione-dependent formaldehyde dehydrogenase, (4) *S*-formylglutathione hydrolase, (5) formate dehydrogenase, (6) dehydroxyacetone synthase, (7) *Pmp20*. Abbreviations: *S*-HMG, *S*-hydroxymethyl glutathione; *S*-FG, *S*-formylglutathione; GSH, reduced glutathione; GSSG, oxidized glutathione; *Pmp20*, peroxisome membrane protein with glutathione peroxidase activity; Xu5P, xylulose-5-phosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dehydroxyacetone phosphate.

Initial growth on glucose or glycerol prevents transcription and can be used to increase biomass before switching to methanol[22, 27, 29-31]. Growth on glycerol is preferred to glucose, however, as even in the presence of methanol, glucose represses transcription of the of *AOX1* promoter[9].

Three strains are available: (i) the wild type methanol utilization plus phenotype (Mut^+) as well as strains with either (ii) deletion in the *AOX1* gene (methanol utilization slow, Mut^s) which instead utilizes *AOX2* resulting in slower methanol metabolism and growth which may in some cases be desired over more rapid growth, or (iii) deletion in both genes (methanol utilization minus, Mut^-) which prevents growth upon methanol[27, 29]. However the disadvantages of the *AOX1* promoter are that two carbon sources are needed for adequate protein yield, large volumes of methanol must be stored which can be a fire hazard, and some products intended for use in foods may not be suitable for expression with methanol. Some reports have suggested methanol-free expression of recombinant proteins with *AOX1* promoter control[32]. Other promoters present in *P. pastoris* such as glyceraldehyde 3-phosphate dehydrogenase (*GAP*), formaldehyde dehydrogenase (*FLDI*) and isocitrate lyase (*ICLI*) are less widely used to drive protein expression[29]. Protease deficient strains are also available[33].

Hundreds of proteins have now been expressed using *P. pastoris*[22, 27]. Some examples of human proteins expressed are shown in Table 1.1.

Table 1.1: Examples of functional human proteins expressed in *Pichia pastoris*. Adapted from Cereghino and Cregg (2000)[34] and Macauley-Patrick et al (2005)[29]

Protein	Expression level	Application
β 2-Adrenergic receptor	25 nmol/g	Structure/function studies
Human aquaporin 10 (hAQP10)	30 mg/L[35]	Functional studies
Adenosine _{2a} receptor	200 pmol/mg	Structure/function studies
Bile salt-stimulated lipase	300 mg/L	Therapeutics
Caspase-3	1 ug/g	Functional studies
CD38	45 mg/L	Functional studies
Endostatin	20 mg/L	Therapeutics
Erythropoietin (EPO) receptor	200 mg/L	Functional studies
Granzyme B	1 mg/L	Structure/function studies
Green fluorescent protein (GFP)	1.2 g/L[36]	Optimization studies
Human growth hormone	500 mg/L[37]	Therapeutics
Interleukin-17	0.35 mg/L	Structure/function studies
Leukemia inhibitory factor	17 mg/L	Functional studies
Mast cell tryptase	6.5 mg/L	Functional studies
Monocyte chemoattractant protein-3	1 mg/L	Functional studies
Neural cell adhesion molecule (NCAM)	50 mg/L	Functional studies
Pancreatic α -amylase	20 mg/L	Structural studies
Serum albumin	3 g/L	Therapeutics
Tissue factor extracellular domain	10 mg/L	Functional studies
Tumor necrosis factor	10 g/L	Functional studies
Vascular endothelial growth factor (VEGF165)	40 mg/L	Therapeutics

1.2.2 Comparison of *P. pastoris* with other host systems for recombinant protein production

Bacterial hosts such as *Escherichia coli* were the first to be employed for the purposes of recombinant protein production[38] and a wide range of host expression systems can now be used to achieve sufficient yields of protein. Mammalian cells such as Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, Madin-Darby canine kidney epithelial (MDCK) cells, mouse myeloma (NS0) and human embryonic kidney (HEK) cells[39, 40] are commonly used for protein expression. Insect cell lines used are ovarian cells from *Spodoptera frugiperda* (Sf) and eggs from *Trichoplusia ni* known as high-five cells[41]. Whilst in principle these higher eukaryotic host cells provide the most authentic environment for the production of human recombinant proteins, they are relatively expensive and slow to culture. Microbial eukaryotes in the form of yeast therefore provide an attractive compromise since they have a well established history in biotechnology, especially in the brewing and baking industries. Their growth characteristics are well known and have also been exploited as effective expression systems for recombinant proteins. *Saccharomyces cerevisiae* in particular is well studied and frequently utilized, although more recently other species such as *Kluyveromyces lactis*, *Hansenula polymorpha*, *Yarrowia lipolytica* and *P. pastoris* have been successfully used[39].

P. pastoris is easy to culture to high cell densities and capable of producing high yields of proteins such as 12 g/L of tetanus toxin fragment C and 2.5 g/L of invertase[23, 42]. Advantages of *P. pastoris* over other expression systems such as prokaryotic *E. coli* are that post translational modifications are carried out such as disulfide bond formation, proteolytic processing, glycosylation and folding[22, 23, 42]. Proteins that may be inactive or end up in inclusion bodies in bacteria are biologically active when produced by *P. pastoris*[22]. Furthermore, proteins expressed by *P. pastoris* are not contaminated with endotoxins, as is sometimes the case with those produced by bacteria. Secretion of proteins into the culture medium means more efficient purification and as much as 30% of the total protein in the medium can be the target protein. The secretory pathway of *P. pastoris* is also similar to that of mammalian cells but with the advantage of quicker production of protein and reduced equipment and media costs compared to that required by mammalian

and insect cells[23, 42]. A summary of the characteristics of each system is shown in Table 1.2.

Table 1.2: Comparison of protein expression systems. Adapted from GenWay Biotech Inc (2009)[43]

Characteristics	<i>E. coli</i>	Yeast	Insect Cells	Mammalian Cells
Cell doubling time	30 min	90 min	18-24 h	18-24 h
Extracellular expression	Secretion to periplasm	Secretion to medium	Secretion to medium	Secretion to medium
Yield (mg L ⁻¹ culture)	50-500	10-200	10-200	0.1-100
Protein folding	Usually needed	Often folded; refolding may be needed	Properly folded	Properly folded
N-linked glycosylation	No	High mannose	Simple, no sialic acid	Complex
O-linked glycosylation	No	Yes	Yes	Yes
Phosphorylation	No	Yes	Yes	Yes
Acetylation	No	Yes	Yes	Yes
Acylation	No	Yes	Yes	Yes
Cost	Low	Low	Moderate	High

1.2.3 Comparison of *P. pastoris* with *S. cerevisiae*

Saccharomyces cerevisiae is a budding yeast and a member of the family *Saccharomycetaceae*[44]. It is Crabtree-positive[45] and its genome was the first of the eukaryotes to be fully sequenced and annotated[46]. It is able to utilize glucose and is capable of both aerobic and anaerobic respiration. One of the notable features of *S. cerevisiae* is that it is able to control the switch from fermentation to respiration by changing to a mixed respiro-fermentative metabolism once the external glucose concentration is greater than 0.8 mM, leading to the production of ethanol. Therefore, high glucose levels lead to ethanol production whereas low levels do not[47].

S. cerevisiae is a popular tool for recombinant protein production with a well-understood biochemistry and a range of promoters available[48]. However, *P. pastoris* has advantages over other species of yeast including *S. cerevisiae*, summarized in Table 1.3. Due to similar

growth requirements to *S. cerevisiae*, new protocols for growth were not needed for *P. pastoris*, and benefits such as the ability to reach higher cell densities and the presence of the *AOX1* gene make the species more popular for protein production[23]. In addition, less hyperglycosylation is observed in *P. pastoris* than in *S. cerevisiae*[49] which can lead to improper folding and hyperantigenicity which therefore interferes with therapeutic use. An absence of mannose residues in 1,3-terminal linkages in glycoproteins secreted by *P. pastoris* make them better suited for possible therapeutic use than those secreted by *S. cerevisiae* where there are many of these linkages[42, 50].

Table 1.3: Comparison of advantages and disadvantages of *Pichia pastoris* and *Saccharomyces cerevisiae*

Properties	<i>P. pastoris</i>	<i>S. cerevisiae</i>
Strongly inducible promoter	✓	✓ [‡]
Grows to high cell densities	✓✓	✓ ^{‡‡}
High product yield	✓	✗
Longer high mannose chains	✗	✓
Shorter high mannose chains	✓	✗
Frequent hyperglycosylation	✗	✓
Common hyperantigenicity	✗	✓
Stable plasmids	✓	✗
Secretion of proteins into media	✓	✗
Annotated genome	✓	✓

[‡] A range of promoters of varying strengths are available

^{‡‡} Due to its respiro-fermentative metabolism, biomass yields are typically lower than *P. pastoris*

Strains of *P. pastoris* have been developed where the glycosylation pathway has been engineered to produce recombinant antibodies with human N-glycosylation patterns[49] as well as strains able to secrete human glycoproteins, such as erythropoietin, with fully complex terminally sialylated N-glycans[51]. The construction plasmids of *P. pastoris* have also been found to be more stable than those of *S. cerevisiae* making the strains more reliable[52]. Overall *P. pastoris* is a very attractive host for protein production that is now being widely exploited[22, 23, 27, 29, 34]. During the last 15 years, 80% of all recombinant genes reported in the literature were expressed by either *E. coli* or *P. pastoris*[53].

1.3 Protein production formats

Recombinant proteins can be produced in *P. pastoris* in a variety of formats. Small systems include microtiter plates for culturing small volumes, deep well plates which can culture several milliliters and shake flasks which can be used to grow a few millilitres up to litres of culture. Larger scale production is performed in bioreactors which can range in volume from 250 mL up to thousands of litres of culture.

1.3.1 Small scale protein production

Colony screening and optimization tests are frequently performed in deep well plate or shake flask formats[54] as shown in Fig. 1.3. The manageable size allows a range of conditions or colonies to be tested simultaneously. Technology such as the PreSens shake flask and plate readers allow online monitoring of dissolved oxygen (DO) and pH in these systems, providing additional information enabling the precise control of the culture.

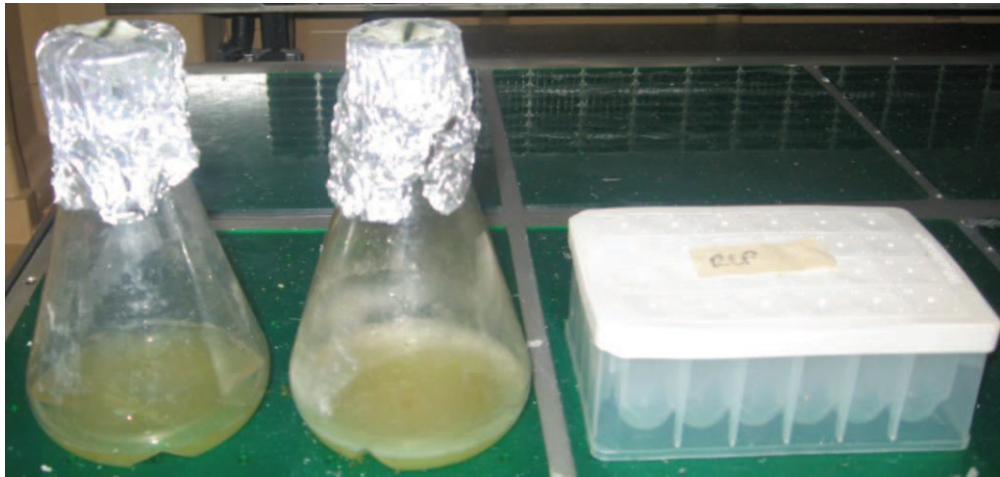


Figure 1.3: Small scale protein production formats

Shake flask cultures (left) and a deep well plate with 24 wells (right) containing cultures of P. pastoris in a shaking incubator. Photographed at Aston University.

The Microbioreactor 24 (Pall Corporation) allows growth of cultures in 24 ‘mini bioreactors’ with control over the shaker speed, temperature, DO and pH and provides online monitoring. It has been successfully used to develop a DoE model[36] and is also well-suited to screening colonies and is shown in Fig.1.4.



Figure 1.4: *Micro-24 micro-bioreactor*

Equipment details are given in Chapter 2. Image taken from Wolf Labs website:

http://www.wolflabs.co.uk/Applikon_Micro_24_Bioreactor.htm

1.3.2 Large scale protein production

Deep well plates and shake flasks are useful in screening for high-yielding clones and for maintaining basic cultivation parameters such as temperature. However, high yields of protein are often not achieved. Despite recent developments allowing pH and DO monitoring in shake flasks and plates, there remain restrictions in volume, particularly in plates as evaporation is a common problem[54], and even more importantly oxygen transfer rates are limited. The inability to adequately control pH and DO in these formats is a substantial limitation. In contrast, scale-up to bioreactors provides tight control and allows online monitoring of culture conditions, thereby facilitating the development of effective feeding strategies and further optimization of a given bioprocess to produce much greater quantities of protein [29]. An example of the set-up in the laboratory at Aston University is shown in Fig. 1.5.

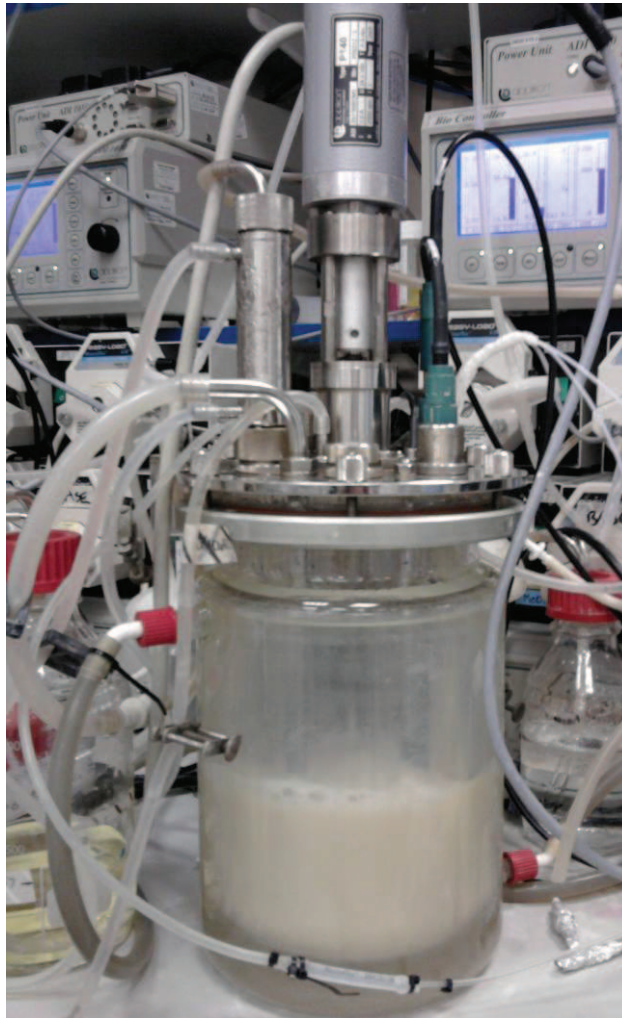


Figure 1.5: 3 L bioreactor set up

3 L stirred tank bioreactor (Applikon Biotechnology) set up and photographed at Aston University. Equipment details are given in Chapter 2.

In order to grow, aerobic yeast such as *P. pastoris* require a sufficient concentration of dissolved oxygen in the medium. One of the major differences from scaling from deepwell plates or shake flasks to bioreactors is the difference in oxygen transfer rate. This can be controlled much more effectively in a bioreactor with the stirrer speed and introduction of gases. The rate of oxygen transfer depends upon the k_La , or volumetric mass oxygen transfer coefficient, and upon $C_{1,\infty} - C_1$, where C_1 is the dissolved oxygen concentration and $C_{1,\infty}$ is the oxygen saturation concentration in the liquid phase at the gas-liquid interface[55]. The k_La is a measure of how much oxygen is transferred into the medium

over a certain amount of time[56]. The k_La of a system can be influenced by several factors such as properties of the medium like viscosity, the presence of organisms and their by-products. Additions to the medium such as antifoams also have an effect[56, 57]. It has been observed that low concentrations of antifoam can reduce the k_La but at higher concentrations the k_La may rise[58, 59]. To ensure optimum oxygen transfer within a system, the effect of differing concentrations of the antifoam to be used should be assessed, although this is not typically done.

1.3.3 Bioprocess optimization in recombinant protein production

The consistent attainment of high yields of recombinant proteins in host expression systems including *P. pastoris* is often an obstacle to furthering research. For this reason, considerable effort has been directed into process optimization either through varying one factor at a time[36, 60, 61] or by using ‘design of experiments’ (DoE) approaches which enable analysis of several factors simultaneously[36, 62].

Many optimization and scale-up methods are still based upon the inefficient and time-consuming approach of varying one parameter at a time while maintaining the others. This is particularly undesirable when a range of conditions need to be investigated, such as the temperature, pH, DO, medium composition and presence of additives. However, studies have successfully identified key process parameters using this approach[63]. A more systematic approach such as the DoE model is becoming more widely used. DoE allows the investigation of numerous parameters while reducing the number of experiments to build a predictive model and generate an equation linking the parameters to yield[36]. This has recently been demonstrated effectively by Holmes *et al* who successfully increased the specific yield of *P. pastoris* producing green fluorescent protein in the induction phase[36].

Recombinant protein production experiments in *P. pastoris* and *S. cerevisiae* are most commonly optimized in bioreactors by varying feeding and induction strategies[64-66]. For *P. pastoris*, induction with various methanol concentrations[67] and mixed feeds of glycerol and methanol, or methanol and sorbitol have been investigated[36, 68-70], often

with the aim of optimizing the specific growth rate, μ [67, 70]. It has also been demonstrated that strict maintenance of the culture pH[71] and varying the temperature during the induction step are important factors in increasing protein yields[72], both of which are simple to accomplish using a bioreactor. The optimum pH, temperature and DO required can vary with each strain and the protein being produced. For example, Jamshad and colleagues found that the growth temperature for the production of the human tetraspanin, CD81, in *P. pastoris* was 30 °C[73] whilst in another study Dragosits and colleagues demonstrated that decreasing temperature from 30 °C to 20 °C was optimal for the production of the antibody fragment, Fab 3H6[74]. A careful analysis of how the culture responds to the growth conditions is vital in achieving high yields of protein.

Investigation into the effects of various additives upon fermentation cultures of yeast has helped to optimize some processes. It has been discovered that addition of DMSO and histidine improved the yields of recombinant proteins[63, 75]. It was also found that addition of GPCR-specific ligands enhanced the yield of the receptors[63, 76-78]. The addition of tween-80 and oleic acid was found by Tang *et al* to improve yields of recombinant protein[79]. In contrast, the addition of antifoam components to bioprocesses is not typically optimized[80, 81].

1.4 Foaming

Recombinant proteins are often produced on a large scale in bioreactors for therapeutic use or research purposes after small scale screening for optimum conditions. Foaming is a problem that is particularly acute in bioreactors due to gassing used to maintain appropriate DO concentrations. It can lead to reduced process productivity since bursting bubbles can damage proteins[82], result in loss of sterility if the foam escapes the bioreactor[83] or more catastrophically, can lead to over-pressure if a foam-out blocks an exit filter. Examples of undesired foam formation can be seen in fermentation processes used for paper, food, beverage and drug production such as the synthesis of antibiotics[84]. Unwanted foaming can also occur during water purification, blood transfusions, and in the dyeing of fabrics[84, 85].

Foam is made up of liquid lamellas which are full of gas. Foams with high liquid content are unstable, while dry polyhedral foams are more stable and usually formed due to mechanical stresses[86]. To prevent the formation of foam, mechanical foam breakers, ultrasound or, most often, the addition of chemical antifoaming agents (or “antifoams”)[83] are routinely employed in bioreactors and large shake flasks. There is a well-established literature on antifoams, highlighting their importance in bioprocesses[83]. Antifoams can be classified as either hydrophobic solids dispersed in carrier oil, aqueous suspensions/emulsions, liquid single components or solids[87-89]. Many antifoams are commercially-available, with 19 being sold by Sigma-Aldrich alone. While little information is routinely given about their composition, their specific antifoam properties have been thoroughly investigated. These include their effects on foam height with time, their influence on the volumetric oxygen mass transfer coefficient (k_{La}) of the system, their gas hold-up characteristics and their globule size and distribution in relation to their action upon foams.

In bioreactors, foam probes can be used to administer antifoams, where a current is produced when foam touches the probe and is detected by a controller. The controller then causes antifoam to be added to the vessel. However, if the volume of the culture increases during the fermentation, this could cause constant stimulation of the probe leading to excessive amounts of antifoam being added to the vessel when it is not required and also damaging the equipment[86]. Consequently, antifoams are often added manually to processes on a laboratory scale.

Some simple methods of determining the ability of antifoams to reduce foam are the Bartsch shaking test[90] and the Ross-Miles pouring test[91]. Several mechanisms of action for these agents have been suggested which include bridging-dewetting, spreading fluid entrainment and bridging-stretching[92]. For oil-based antifoams, bridging-dewetting and bridging-stretching mechanisms are known to occur and are illustrated in Fig. 1.6. Bridging-dewetting (Fig 1.6A) occurs if the contact angle, θ_{aw} , of an oil drop with the surface of the foam film is $> 90^\circ$ and the bridging coefficient, B , is > 0 ; the drop shape alters and becomes a bi-convex lens (Fig 1.6 A (c)). The film is then ruptured (Fig 1.6A

(d)). However, if $B < 0$ there is no shape change and a stable bridge is formed. With bridging stretching (Fig 1.6B), the oil particle bridges the foam film surface (Fig 1.6B (a) and (b)). This leads to the formation of an oil bridge which stretches over time, becoming an unstable film. When the film ruptures, the entire foam structure is destroyed (Fig 1.6B (c) and (d))[84].

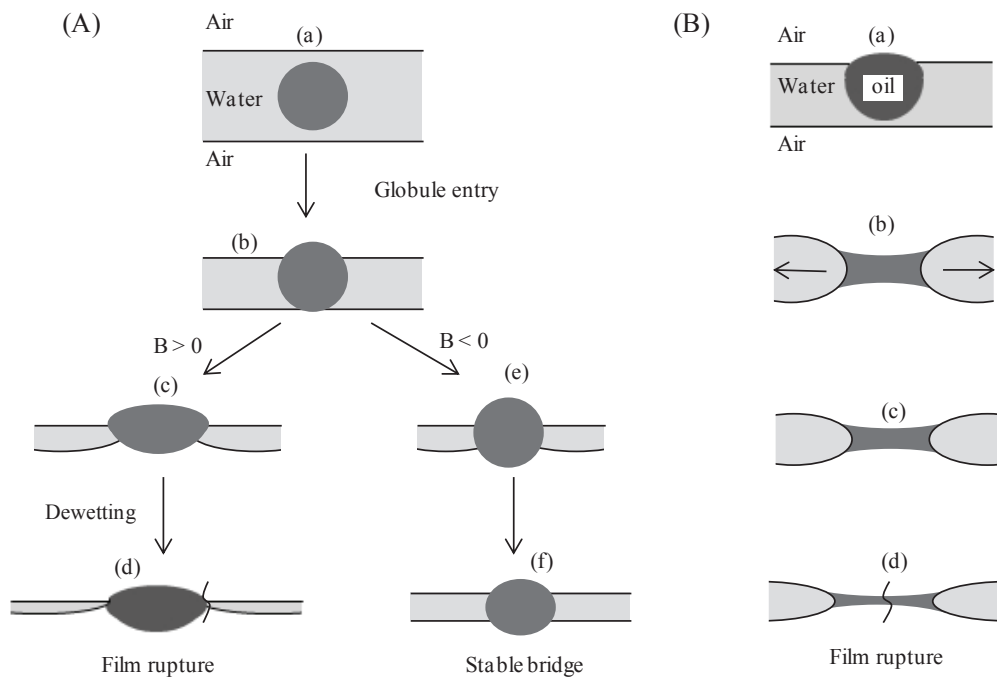


Figure 1.6: Bridging-dewetting and bridging-stretching antifoam mechanisms

(A) Bridging-dewetting, where the contact angle of an oil drop causes it to become a bi-convex lens, rupturing the film, and (B) bridging-stretching where the oil particle bridges the foam film surface forming an oil bridge, which stretches forming an unstable film, eventually rupturing the foam. Adapted from Denkov and Marinova 2006[84]

Much of the literature available on antifoams in fermentation processes documents their effects upon the dissolved oxygen and the $k_L a$ in a system, as these factors are well known to be affected by these agents[56-59, 93-97].

1.5 Antifoams in bioprocesses

Both positive and negative effects have been observed, for example Al-Masry[93] suggested that a silicon-based antifoam negatively affected the mass transfer coefficient, gas hold up and gas velocity within the media and Joshi suggested that antifoams could damage the equipment. However it was found by Koch *et al*[96] that antifoams without silicon oil did not greatly affect the oxygen transfer rate. Such studies have been performed in various growth media in both the absence and presence of cultures of prokaryotic and eukaryotic microbes[83, 88, 92, 93, 95, 96, 98]. In contrast, literature on the biological effects of antifoams on recombinant protein production by microbial host cells is more limited.

1.5.1 A review of the antifoam literature

Table 1.4 shows an analysis of representative examples of this body of work. This illustrates that antifoams may exert a variety of effect upon fermentation processes, depending upon the type of antifoam, medium, and process conditions within the bioreactor and the concentrations of antifoam used.

Varley *et al*[83] tested an unspecified antifoam at unspecified concentrations, which is of little use when deciding upon the most suitable antifoam for a process, and only provides useful information to those looking to alter the process to avoid the use of antifoam. The article highlights the fact that even with alterations to the process conditions, headspace would still be required in the vessel reducing the volume of culture, and therefore a system without antifoam is still not operating at optimum conditions.

Table 1.4: Summary of the effects of antifoam addition to microbial cell factories

Data from the references cited were analyzed for details of the experimental set-up (vessel, concentration of antifoam used) and whether the addition of the given antifoam (or other additive as part of the same study) affected the yield or growth characteristics of the cells. The yield data are reported according to the units specified in the respective reference. OTR is oxygen transfer rate, $k_{1,a}$ is the volumetric mass oxygen transfer coefficient and μ is the specific growth rate (h^{-1})

Microbial cell factory	Vessel	Antifoam	Effect on protein yield?	Effect on growth rate of cells?	Data to support a mechanistic rationale?	Other observations	Reference
Prokaryotes							
<i>Escherichia coli</i> K-12 producing β -galactosidase fusion protein	Bioreactor (2 L and 60 L working volume)	S184	Reduces specific activity	No change below 250 ppm No data reported above 250 ppm	No	OTR reduced in early stages of cultivation	[96]
		SLM54474	Reduces specific activity	Decreases with increasing concentration	No	Minimal effect on OTR and $k_{1,a}$	[96]
		VP1133	No effect	No change below 250 ppm No data reported above 250 ppm	No	OTR reduced in early stages of cultivation	[96]
		SE9	Increases volumetric activity	Low μ at 555 ppm High μ at 5000 ppm	No	OTR reduced in early stages of cultivation	[96]
		PEG8000	Increases titre at 0.5 %	No effect	No	Increased membrane permeability hypothesized	[99]
<i>Geobacillus thermoleovorans</i> secreting α -amylase		Tween-20	Increases titre at 0.3 %	No effect	No	As above	[99]
		Tween-40	Increases titre at 0.3 %	No effect	No	As above	[99]
		Tween-60	Increases titre at 0.3 %	No effect	No	As above	[99]
		SDS, Tween-80 and cholic acid	Increases titre by a factor of 2	No effect	No	As above	[99]
		TritonX-100	Reduces titre	Not reported	No		

<i>Bacillus subtilis</i> secreting α -amylase	Bioreactor	PEG600	Increases productivity (U mL ⁻¹ h ⁻¹) by a factor of 1.5 at 20 %	Not reported	No	Cells become more hydrophilic	[100]
<i>Bacillus amyloliquefaciens</i> secreting α -amylase	Bioreactor	PEG600	Reduces productivity (U mL ⁻¹ h ⁻¹) by a factor of 2 at 20 %	Not reported	No	Cells become more hydrophobic	[100]
Eukaryotes							
<i>Schizosaccharomyces pombe</i> secreting human transferrin	Not given; probably shake flasks	Dextran sodium sulphate (0.001 – 0.1 %)	Increases specific yield by a factor of 7	No effect	No	Anionic residues hypothesised to be important for exocytosis	[101]
		SDS (0.001 – 0.1 %)	Not reported	Cells do not grow at >0.001 %	No		[101]
		Deoxycholate	Increases specific yield (< 0.01 %)	Not reported	No		[101]
		Tween 20	Decreases specific yield	Growth defect at >0.001 %	No		[101]
		TritonX-100 (0.002 %)	No effect	No effect	No		[101]
		PEG8000	Increases specific yield at 0.1 %	Growth defect at 1 %	No		[101]

Research conducted by Koch *et al*[96] was thorough and investigated several possible effects of four antifoams from different groups on recombinant *E. coli* cultures. As well as evaluating influence upon gas hold up, bubble velocity and k_La as carried out in several other studies, it also investigated the biological effects of the antifoams, observing cell growth and recombinant protein yield. It illustrates the fact that different antifoam types have different effects upon factors in fermentation cultures, and therefore the importance of understanding these influences when choosing a suitable antifoam. For these reasons, Koch concluded that more research needs to be carried out into this area, including upon other principal organisms commonly used in fermentations such as species of yeast.

Many studies were concerned mainly with the effects of the antifoams on the mechanical aspects of fermentations, such as foam height over time. Other variables such as the k_La , and gas hold up, were investigated by Al-Masry[93], Varley *et al*[83], Koch *et al*[96] and Çalik *et al*[95]. The study by Denkov *et al*[92] provided valuable insight into the mechanisms of antifoam action by evaluating the properties of antifoams. However, few studies monitored the biological effects of the antifoams, suggesting that this area is not always considered. The question of toxicity effects of the antifoams was not raised in any of the articles reviewed and would be useful for those using fermentation for drug production.

Holmes *et al*[82] conducted research into the effects of four antifoams of different types on the recombinant protein yield and growth of *P. pastoris* and *S. cerevisiae* in YPD and SD^{URA} media, respectively. Various concentrations of antifoams were tested up to 8%. The findings varied among the differing types of antifoam. Growth of the organisms was increased by the addition of an alcoxylated fatty acid ester on vegetable base, and protein yields at concentrations of antifoam greater than 1% was decreased, although growth increased with certain antifoams[82].

Table 1.4 includes a small part of the wide range of antifoams and compositions available just from the studies reviewed. New antifoams are being developed, demonstrated by Çalik *et al*[95], and without understanding their effects it becomes more and more difficult to

select an appropriate antifoam to achieve optimum conditions during fermentation. Table 1.4 also shows that antifoams were evaluated with several different types of media. Comparison between different media was only investigated by Holmes *et al*[82], Etoc *et al*[98] and Çalik *et al*[95], and more thorough investigation of their influence would be useful in optimization.

Another key factor that was not thoroughly evaluated was the effect of differing concentration of antifoams upon the process being investigated. Only two studies reviewed attempted to discern the effects of concentration. It is possible that results obtained from the other studies could be very different under alternative concentrations, and an optimum for each of the variables could have been determined. It has been shown by Holmes *et al*[82] that concentration of antifoam has a definite influence upon the growth of organisms and recombinant protein yield during fermentation, and therefore concentrations are significant factors to consider and should be included in future research. Increased knowledge of the optimum concentration and type of antifoam to be added to a process is important, and will allow any possible beneficial effects upon growth and product yield to be exploited.

Overall, there have been few studies on the effects of antifoams in fermentation processes, and because of the numerous possible effects they may exert, information covering each area is brief and not always complete. Holmes *et al*[82] and Etoc *et al*[98] demonstrated that antifoams can adversely and positively influence these parameters, but further research into this area is required to verify the findings and to encompass the wide range of antifoam types and concentrations that may be used. Furthermore, more concise data is essential for each group of antifoams and for the most commonly used antifoams in fermentation processes to allow an informed decision to be made in order to achieve the optimum set up for the run. This will help to reduce costs and allow the user to select an antifoam that has an appropriate effect upon their process, whether that be destroying foams alone, changing the k_La , reducing damage to equipment, altering the growth of organisms or influencing product yield. Adequate information about antifoam action upon the protein yield and the growth of the cells during fermentation is not available in the literature suggested by the review of published antifoam data. For these reasons, an evaluation of the effects of

several antifoams on different protein targets was undertaken in the project presented in this thesis.

1.6 Target proteins

A range of soluble and membrane proteins have been expressed using *P. pastoris* for use as therapeutics, or targets in the development of drugs as well as for functional studies[29, 34]. Several targets were considered for optimization studies in this project.

1.6.1 Green fluorescent protein

Green fluorescent protein (GFP) is used as a fluorescent marker and was first isolated from the coelenterate, *Aequoria Victoria* or Pacific jellyfish, and can also be isolated from *Renilla reinformis*, the sea pansy[102]. Its natural function in these organisms is currently not well understood[103]. The Nobel Prize for Chemistry 2008 was awarded to Osamu Shimomura, Martin Chalfie and Roger Tsien for their discoveries leading to its many uses[104]. In the jellyfish *Aequoria Victoria*, the two proteins aequorin and GFP interact to produce green light. Aequorin is a photoprotein composed of 196 amino acids (21 kDa) and consists of molecular oxygen, apoaequorin which is a chromophoric unit, and a covalently bound prosthetic group, coelenterazine[105, 106]. When Ca^{2+} interacts with three high-affinity binding sites[105], coelenterazine is oxidized to an excited coelenteramide[106]. When coelenteramide returns to its ground state, blue light at 460 nm is emitted as well as CO_2 [105-107]. GFP absorbs the light and re-emits green light at 509 nm[105, 107].

Aequoria Victoria GFP (avGFP) is composed of 238 amino acids and is 24 kDa with a β -barrel structure formed by 11 β -sheets surrounding a chromophore[108, 109]. The wild-type structure of GFP was determined in 1996 by Yang *et al*[110] and is shown in Fig. 1.7, but previously the structures of mutants had been reported[111]. The chromophore is formed by post-translational modification of three amino acids of the internal helix at positions 65-67 (numbering according to *Aequoria Victoria*)[108, 109].

The side chain of the first residue at position 65 can vary between natural GFP-like proteins, but the Tyr66 and Gly67 are conserved[108, 109]. The β -barrel protects the chromophore from the surrounding environment[108, 109] and prevents non-radiative deactivation[108]. The barrel is stabilized by numerous non covalent interactions giving the protein resistance to thermal and chemical denaturation as well as proteolysis[109].



Figure 1.7: The structure of GFP

A, the β -barrel with semitransparent surface shown from the side; B, the β -barrel shown from above; C and D, the chromophore and nearby residues with carbon atoms coloured grey, nitrogen atoms coloured blue and oxygen atoms coloured red. Taken from Chudakov et al 2010[109].

The chromophore of GFP is a *p*-hydroxybenzylideneimidazolinone[112] synthesized without cofactors or enzymes as modifications are made to the internal amino acids by the β -barrel, only requiring molecular oxygen[108, 109]. This feature allows functional expression of the protein in a range of systems[108]. The first stage involves folding of GFP into a semi-native conformation[112] followed by cyclization of the protein backbone

at positions 65-67 (Ser-Tyr-Gly in avGFP)[109] to an α -enolate form[108]. Then follows oxidation to cyclic imine[108] and finally dehydration of C α -C β bond of Tyr66 with molecular oxygen[108, 109]. These reactions are illustrated in Fig. 1.8. A two-ring structure is formed which is able to absorb and emit light[109].

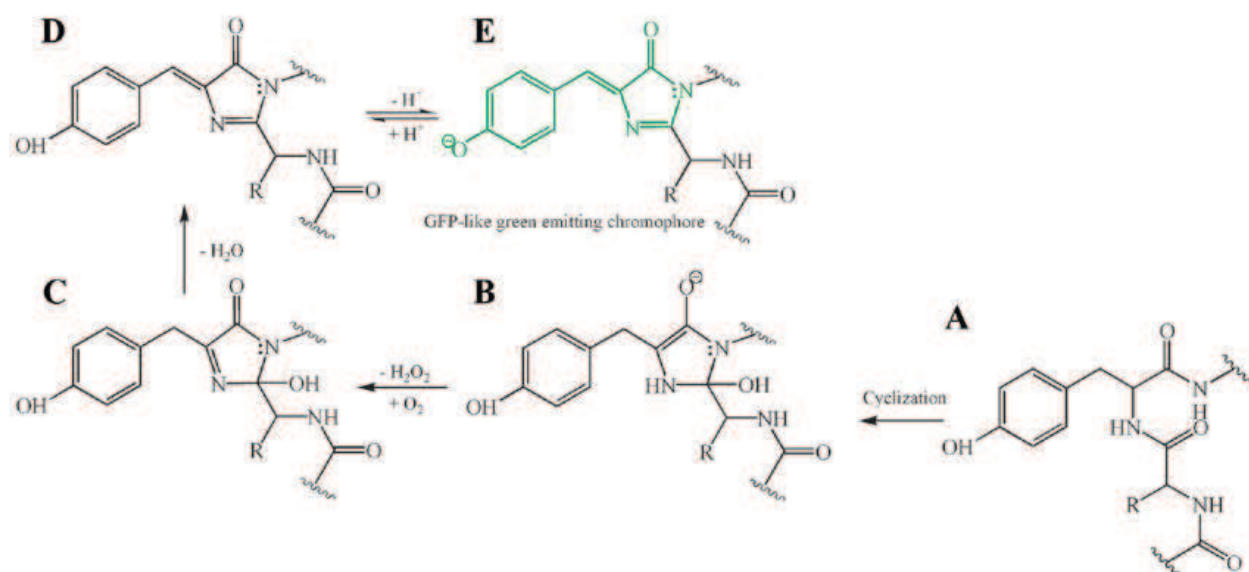


Figure 1.8: Mechanism for chromophore formation of GFP

A-B, cyclization of the tripeptide to an α -enolate form; B-C, oxidation to cyclic imine; C-D-E, dehydration of the C α -C β bond of Tyr66 forming a chromophore able to emit green light in a deprotonated state. Adapted from Stepanenko 2011[108].

Wild type GFP has a major excitation peak at 395 nm and an emission peak at 509 nm[102]. Fluorescence of GFP is stable. It can be detected under UV light [113], and imaging and quantification are simple. It can be detected in living cells[114], and can be fused to the amino or carboxy terminus of proteins for example aquaporins, to study their functions within cells, as well as levels of expression[113]. For these reasons, GFP is one of the most utilized fluorescent proteins as a marker[102, 113, 115, 116], and due to the fluorophore being gene encoded, GFP cDNA has been cloned[113] and several variants have been produced with differing spectra, optimized expression and pH sensitivity, which convey advantages for use under a wider range of conditions[117].

GFP has been recombinantly expressed in *E. coli*[107], *Caenorhabditis elegans*, *Drosophila melanogaster*, mammalian cells such as HeLa[118] as well as yeasts such as *P. pastoris*[36] and *S.cerevisiae*[119]. Fluorimetry and confocal microscopy are the most common methods of assaying GFP[36, 107, 111, 118].

1.6.2 Erythropoietin

Erythropoietin (EPO) is a glycoprotein hormone which is involved in the regulation of red blood cell production. It induces the proliferation, differentiation and maturation of bone marrow erythroid precursors into erythrocytes during instances of low blood oxygen[120, 121]. It is also involved in the neurosystem as a neuroprotective and is able to protect other organs[120]. EPO is composed of 161 amino acids with a weight of 30 kDa in its mature form[122]. It is a member of the cytokine family that includes interleukins 2-7, growth hormone and leptin[121]. EPO binds to the EPO receptor on the surface of erythroid progenitor cells with high affinity[120, 121]. Binding triggers signal transduction by ligand mediated receptor dimerization on the cell surface[121]. The crystal structure of EPO bound to the extracellular binding domains of the EPO receptor was determined at 1.9 Å by Syed *et al* in 1998[123] and is shown in Figure 1.9.



Figure 1.9: The structure of EPO

Heterotrimeric assembly of EPO (green) with 2 molecules of EPO receptor (blue and pink). Reproduced from the Protein Data Bank entry 1eer, accession number P01588 [124].

Normally, EPO is expressed in low levels by the kidney or liver of adults therefore many human donors are required to provide enough material for treatment. The use of recombinant human EPO has been approved by the FDA[122], and is used to treat blood disorders and anaemia associated with renal failure, cancer, HIV infection and chronic inflammatory disease[120, 122]. CHO cells are currently used to provide larger quantities of EPO[120, 122], however protein production using mammalian cells is often costly and inefficient. *E. coli* has been used to produce EPO, but prokaryotes cannot glycosylate proteins and the glycosylation of EPO is important in the prolongation of its biological half-life[122]. *S. cerevisiae* has also been used to produce EPO but hyperglycosylates proteins. *P. pastoris* is a more attractive host and has been used to produce EPO with the benefit of performing post-translational modifications without hyperglycosylation and at higher cell densities[121, 122]. The native polypeptide form of human EPO (18 kDa) was

produced in *P. pastoris* for the first time by Celik *et al* in 2007[122]. Commonly used assays for EPO quantification are ELISAs[125] and Western blots[120].

1.6.3 G protein-coupled receptors and accessory proteins

G protein- coupled receptors (GPCRs) are important membrane proteins and are involved in most physiological processes in the body[63]. They are targeted by over 30% of all drugs [68]. The superfamily consists of more than 800 human proteins making it one of the largest with many ligands such as hormones, lipids, neurotransmitters, ions and photons[64]. Other compounds may also bind to GPCRs; agonists, which are ligands that bind to a receptor and alter the receptor state which causes a biological response; antagonists, which reduce the action of agonists and may act at the same receptor macromolecule as the agonist; inverse agonists, which bind to receptors and reduce the fraction of them in an active conformation[126].

Agonists activate a heterotrimeric guanosine triphosphate-binding protein, or G-protein consisting of an α , β and γ subunit. [127]. The binding of an agonist to a GPCR leads to a conformational change which activates the associated G-protein. GTP is exchanged for GDP on the α -subunit causing the G-protein to dissociate from the receptor and also the dissociation of the α -subunit from the $\beta\gamma$ -complex. These subunits, the α -GTP and the $\beta\gamma$, are then able to interact with other protein targets such as enzymes or ion channels and a vast range of intracellular signals are activated to cause a physiological response[127-129] illustrated by Figure 1.10. The Nobel Prize in Physiology or Medicine was awarded to Alfred. G. Gilman and Martin Rodbell in 1994 for their work on G-proteins[130].



Figure 1.10: GPCR signalling pathways

A variety of ligands bind to the numerous GPCRs leading to G protein activation which in turn causes a range of physiological responses. Reproduced from http://www.ibibiobase.com/projects/db-drd4/G_protein.htm[131]

There are numerous classification systems for GPCRs, the most commonly used being the A-F system[71, 74] and the GRAFS system[72]. The A-F system describes GPCRs found in both vertebrates and invertebrates whereas the GRAFS system classifies human GPCRs. The A-F system splits the receptors into families according to sequence similarity. Family A is the rhodopsin-like receptors and is the largest group. It includes rhodopsin and adrenergic receptors as well as many olfactory receptors[71, 131]. Family B is the secretin receptor family and includes gastric inhibitory peptide, calcitonin, calcitonin receptor like and glucagon receptors[132]. Family C is the metabolic glutamate family, including the GABA receptor. Family D is the fungal mating pheromone receptors, E the cyclic AMP receptors and F the frizzled/smoothed receptors[71, 131]. In the GRAFS system there are five main families based upon evolutionary similarities; glutamate, rhodopsin, adhesion, frizzled/taste2 and secretin families. The largest of these is the rhodopsin family consisting

of 701 receptors[72]. Rhodopsin from natural sources was the first GPCR to have its structure solved in 2000 by Palczewski *et al*[133].

GPCRs contain 7 transmembrane domains (7TM bundle) which are α -helical[72] with 3 extracellular (ECL1-3) and 3 intracellular (ICL1-3) loops. The amino terminus (N-terminus) is extracellular and has the potential to bind ligands in some GPCRs, while the carboxy terminus (C-terminus) is intracellular and interacts with G-proteins and arrestins[64]. These structures are illustrated in Fig 1.11. Recently, breakthroughs have been made in determining the crystal structures of GPCRs[134]. However, only relatively few structures have been solved so far despite their importance. Bottle necks include difficulty in expressing great enough quantities for further studies and production of stable conformations[135]. In order to maintain their structure and function, GPCRs must remain in a lipid or hydrophobic environment which can interfere with NMR and crystallography. This also makes purification challenging as a poor choice of detergents can destroy the surrounding lipid membrane. Some of the structures that have been determined have been achieved by causing mutations in order to make the GPCRs more thermostable and increase expression levels. Intracellular loops have been replaced with T4 lysozyme and antibodies and nanobodies have been employed to stabilize the proteins[68]. Current GPCR crystal structures elucidated by X-ray crystallography include: β_1 -adrenergic, β_2 -adrenergic, adenosine 2_a , dopamine D₃, CXCR4, histamine H₁ rhodopsin and opsin[68, 136]. The histamine H1 receptor is the first recombinant GPCR to have been produced using yeast, *P. pastoris*, and have its crystal structure resolved[137].



*Figure 1.11: A generic Family A GPCR within the plasma membrane localized to a lipid raft
Protein features are described in the panels. Taken from commons.wikimedia.org by Repapetilto.*

1.6.3.1 Human Adenosine _{2a} receptor

The human adenosine _{2a} receptor (hA_{2a}R) is one of four GPCRs which are activated by adenosine; A₁, A_{2a}, A_{2b} and A₃. When activated, hA_{2a}R causes the levels of intracellular cAMP to increase[73]. In humans, adenosine is involved in the nervous and cardiovascular systems[138]. It also has a role in reducing inflammatory damage and inhibiting activated

immune cells. These anti-inflammatory effects therefore make this GPCR an attractive target for therapeutics, which could potentially aid in the treatment of heart disease and cancer[76, 139, 140]. Antagonists to hA_{2a}R include ZM241385, theophylline[76] and caffeine[73]. It has been observed that the interaction of caffeine with the A_{2a}R can lead to a reduced risk of Parkinson's disease in coffee drinkers and suggests that therapeutics could be developed targeting adenosine receptors to treat neurological disorders such as Huntington's disease, Parkinson's disease, asthma, seizures and pain[73, 140].

A_{2a}R is a 47 kDa protein in the rhodopsin-like or family A group of GPCRs. A truncated form of hA_{2a}R was produced in *E. coli* and a glycosylation deficient form produced in *P. pastoris* in milligram quantities[76] as well as in *S. cerevisiae*[139]. However, the crystal structure of hA_{2a}R bound to the antagonist ZM241385 determined by Jaakola *et al* in 2008 was produced by insect cells[73] (Figure 1.12). One of the main challenges of GPCR crystallization is the instability of the protein upon purification. GPCRs can adopt several conformations, some of which are more thermally stable than others. Certain domains within the GPCRs are also more flexible than others, and as a result, Jaakola *et al* replaced the majority of the third cytoplasmic loop with lysozyme from T4 bacteriophage and the C-terminal tail was removed to improve the chance of crystallization. Sodium chloride and cholesteryl hemisuccinate were also used during purification as well as a saturating concentration of antagonist to further aid stability[73].

Jaakola *et al* found that hA_{2a}R's structure is different from other crystallized GPCRs such as β_1 -adrenergic, β_2 -adrenergic and rhodopsin as the ECL2 does not have the α -helix and β -sheet structures. Instead there is a random coil with three disulphide linkages with ECL1, two of these being unique to hA_{2a}R. A fourth disulphide bond in ECL3 helps to maintain a rigid structure opening the ligand binding cavity. They also found that ZM241385 is bound in a different position to other crystallized GPCRs, nearly perpendicular to the membrane plane[73].



Figure 1.12 Crystal structure of $hA_{2a}R$ bound to the antagonist ZM241385

Viewed perpendicular to the plasma membrane. A shows the transmembrane part colored brown with ZM241385 in light blue and the four lipid molecules bound to the receptor in red. The four disulfide bonds are yellow. The extracellular loops (ECL) are green and the intracellular loops (ICL) are blue. B. The structure is rotated 180° around the x-axis. Image reproduced from Jaakola et al 2008[73].

1.6.3.2 β_1 -adrenergic receptor

The β_1 -adrenergic receptor (β_1 -AR) is part of a group of at least four subtypes of adrenergic receptor; α_1 , α_2 , β_1 and β_2 [141]. β_1 -AR and β_2 -AR are biochemically and functionally similar[142], and couple to the same effector protein; they both mediate the catecholamine-induced activation of adenylyl cyclase through the GTP binding protein Gs[142]. Similarity between them is greatest in the membrane spanning domains which are thought to be

involved in forming a pocket to bind ligands[141]. They mediate the physiological responses to the hormone adrenaline and the neurotransmitter noradrenaline. β_1 -AR binds with high affinity to both adrenaline and noradrenaline whereas β_2 -AR only binds with high affinity to adrenaline[143]. β_1 -AR is expressed at high levels in the heart[143] and is known to influence heart rate[144]. Stimulation in the human atrium and ventricle leads to an increase in the force of the contraction and also shortens the length of the contractile cycle as well as increasing cAMP and activation of cAMP-dependent protein kinase A[145]. It is also expressed at high levels in the brain and noradrenergic stimulation of β_1 -AR is known to regulate memory function[143]. It is expressed elsewhere in the body such as the cochlear and vestibular labyrinth[144].

1.6.3.3 Receptor component protein

It is thought that GPCRs act as monomers interacting with G proteins leading to signal transduction, however recent research suggests that other proteins are required for the function of numerous GPCRs. These proteins can be other receptors that form dimers, or accessory proteins that function as chaperones[146]. It has been found that in order to be functional, the calcitonin gene-related peptide (CGRP) receptor must consist of at least three proteins; CLR, RAMP1 and RCP[146-148].

CLR is a family B (secretin-like) GPCR named the calcitonin-receptor-like receptor, RAMP1 is the receptor activity modifying protein and is required for trafficking of CLR to the cell surface and ligand specificity, and RCP, the receptor component protein, which has been identified as an interaction partner for the CGRP receptor[146-148] illustrated in Fig 1.13. CGRP is a 37 amino acid neuropeptide secreted by the nerves of the central and peripheral neurosystem[147, 148]. It is important in heart function and is a vasodilator that affects the rate and force of heart beats and can quickly lower blood pressure. In addition, it is involved in neurogenic vasodilation and inflammation, as well as migranes[146-148]. CGRP binds to its receptor, and this binding stimulates cAMP production[146].

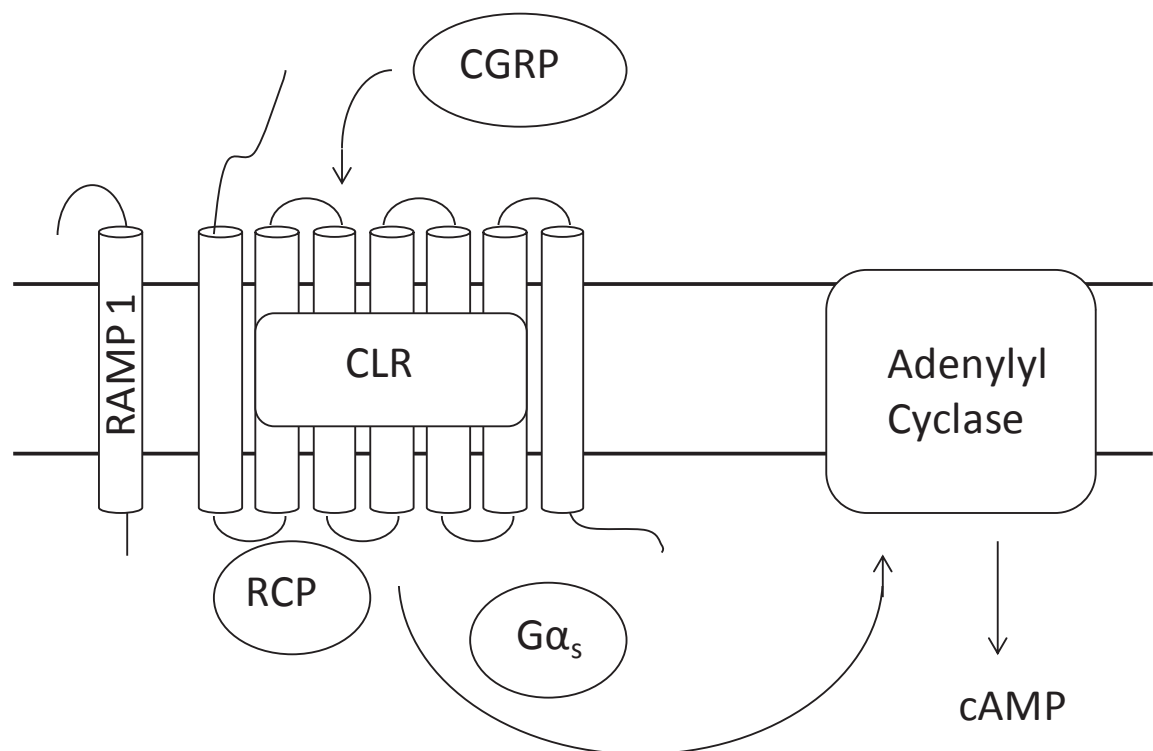


Figure 1.13: The proposed CGRP receptor complex consisting of CLR, RAMP1 and RCP

Binding of CGRP to the CGRP receptor consisting of RAMP1, CLR and RCP leads to cAMP production.

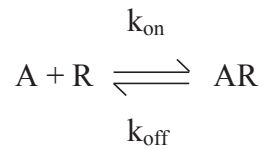
Adapted from Prado 2002[147].

RCP consists of 148 amino acids and is a hydrophilic protein. It is found in the heart and colocalizes with CGRP in the cerebellum, spinal cord and cochlea[148]. RCP couples the receptor to the cellular signal transduction pathway and its expression correlates with CGRP potency *in vivo*[146]. Little is currently known about RCP therefore overexpression and purification of this protein are vital in allowing structural and functional studies and the elucidation of a three-dimensional structure. At present, *E.coli* has been used to produce RCP[148].

1.6.3.4 Ligand binding assays

In order to determine whether a GPCR is correctly folded after expression, a common method is to perform a ligand binding assay with a radiolabelled ligand[76, 139]. This can

be performed using membrane suspensions[149]. Ligand binding assays are usually based upon the law of mass action[150, 151]:



where A is the ligand and R is the receptor. When the ligand and receptor collide in the required orientation to bind, the number of binding events per unit of time is the k_{on} which is the association rate constant. The ligand and receptor remain bound for an amount of time dependent upon the affinity of the ligand and receptor for each other. This is the dissociation rate constant, k_{off} . An equilibrium is reached when the rate at which ligand-receptor complexes form is equal to the rate at which ligand-receptor complexes dissociate. This ratio of association and dissociation indicates the affinity of the ligand for the receptor which is the equilibrium dissociation constant, K_d . K_d is the concentration of ligand that is required to bind half of the receptors. The smaller the K_d , the higher the affinity the receptor has for the ligand, while a high K_d means a low affinity[150]. At equilibrium the total receptor density $[R_T]$, the ligand concentration $[A]$ and the equilibrium dissociation constant determine the concentration of receptor-ligand complexes:

$$[AR] = \frac{[R_T] \times [A]}{[A] + K_d}$$

where $R_T = [R] + [AR]$ and $K_d = k_{\text{off}}/k_{\text{on}}$. This equation is the Hill-Langmuir binding isotherm. In equilibrium, when a single ligand species binds to a single uniform population of receptors under the law of mass action, a hyperbolic curve describes binding as a function of the molar ligand concentration[149, 151], an example of which is shown in Fig 1.14. The B_{max} is the maximum binding capacity, or the total number of receptors.



Figure 1.14: Saturation binding curve of a radioligand to a receptor

Saturation binding curve showing specific binding of a radioligand for human muscarinic M_3 receptor. The K_d is estimated to be 0.05 nM and the B_{max} 1.4 pmol/mg protein. Reproduced from Hulme and Trevethick 2010[149]

Three types of binding experiments may be conducted:

1. Kinetic binding experiments, where the binding of one or more concentrations of radioligand is measured at increasing time points to estimate the association rate constant, k_{on} and the dissociation rate constant k_{off} ;
2. Saturation binding experiments where the binding of an increasing concentration of radioligand is measured at equilibrium. The dissociation constant, K_d and the concentration of specific binding sites for the radioligand, R_T , usually termed B_{max} can be determined from these experiments;
3. Competition binding experiments where the binding of one or more set concentrations of a radioligand is measured at equilibrium in the presence of increasing concentrations of an unlabelled ligand. The data determine the binding constant of a compound for the unliganded receptor and the co-operativity between the compound and the radioligand for binding to the receptor. The inhibitor constant K_i can be calculated:

$$K_i = IC_{50} / (1 + [L] / K_d)$$

This is the Cheng-Prusoff equation where IC_{50} is the half maximal inhibitory concentration, L is the concentration of free radioligand and K_d is the dissociation constant of the radioligand for the receptor[149].

For membrane preparations of $A_{2a}R$ produced using *P. pastoris* by Fraser[76], the B_{max} was 8.5 ± 0.1 pmol/mg membrane protein and the K_d varied slightly for three different preparations; $K_d = 0.35 \pm 0.02$; 0.79 ± 0.06 ; 1.39 ± 0.12 nM. After purification of the receptor, the B_{max} was 18.1 ± 0.5 nmol/mg protein. The theoretical B_{max} for pure protein is 21.3 nmol/mg protein. The K_d for the purified protein was 2.2 ± 0.19 nM[76].

1.6.4 Human growth hormone

Human growth hormone (HGH), also known as somatotropin[152], is synthesized by the acidophil cells of the anterior pituitary as a prehormone[153]. It has a hydrophobic leader peptide of 20 amino acids which is removed by the pituitary during secretion[153], leaving an anionic non-glycosylated HGH consisting of 191 amino acids and a molecular weight of 22 kDa[5, 152]. HGH is an antagonist and binds to one human growth hormone receptor[154]. It is involved in protein synthesis, cell proliferation, metabolism, lipolysis and hypoglycemia[153, 155]. The structure of HGH bound to one human growth hormone receptor is shown in Fig 1.15.



Figure 1.15: Human growth hormone bound to one receptor

Human growth hormone (red) bound to one human growth hormone receptor (green). Image reproduced from Protein Data Bank entry 1a22, accession number P01241[154].

HGH has many therapeutic uses, particularly the treatment of hypopituitary dwarfism, children with growth hormone deficiency, girls with Turner's syndrome and adults with growth hormone deficiency or HIV infection[5, 152, 153, 155, 156]. It has also been used to treat bone fractures, bleeding ulcers and burns[152]. Its use in other applications such as children with short stature and maintaining health in older adults and the critically ill is also being investigated[5]. Originally, the only sources of HGH to treat hypopituitary dwarfism were human cadavers until production of recombinant HGH began using *E. coli* by Goeddel in 1979 and later *S. cerevisiae* by Tokunaga in 1985[153]. It has been produced mainly in *E. coli*[5, 157], but has also been produced by *Bacillus subtilis*, *Pseudomonas* species, *S. cerevisiae* and *P. pastoris*[5, 152, 153, 155, 156]. Assays for HGH include polyclonal radioimmunoassays (RIA) which have limited sensitivity, and monoclonal antibody nonisotopic assays which are more sensitive[158]. ELISAs and Western blots may also be performed in order to quantify the protein[37].

1.6.5 Chlorophyll synthase, CHLG

CHLG is an enzyme involved in the last stages of chlorophyll synthesis and catalyses the esterification of chlorophyllide *a* which is required for amassing chlorophyll-protein complexes[159]. It is a membrane protein of approximately 30 kDa naturally produced in plants and cyanobacteria[160]. It has not previously been expressed in *P. pastoris*, but has been expressed in *E. coli*[159]. The photoautotrophic growth of phototrophs is dependent upon chlorophyll formation. Chlorophyll is involved in the absorbance of light and energy transfer resulting in photosynthetic electron transfer to NADP^+ and the synthesis of ATP[161]. In order for plants to synthesize chlorophyll, over 16 enzymatic steps are carried out. The final steps involve reduction of divinyl protochlorophyllide at the C8 vinyl group to form monovinyl protochlorophyllide and then between C17 and C18 to form chlorophyllide *a*. Chlorophyllide *a* is converted to chlorophyllide *b* by chlorophyllide *a* oxygenase. Chlorophyll synthase, encoded in the CHLG gene, finally esterifies chlorophyllide *a* and *b* with geranyl-geranyl pyrophosphate or phytol pyrophosphate. The hydrophobic carbon hydride side-chains formed allow the assembly of stable chlorophyll[161]. A schematic of the process is shown in Fig.1.16. CHLG is thought to be involved in the co-regulation of the steps to synthesize chlorophyll[161] and therefore production of large quantities of this protein could allow photosynthesis to be better understood.

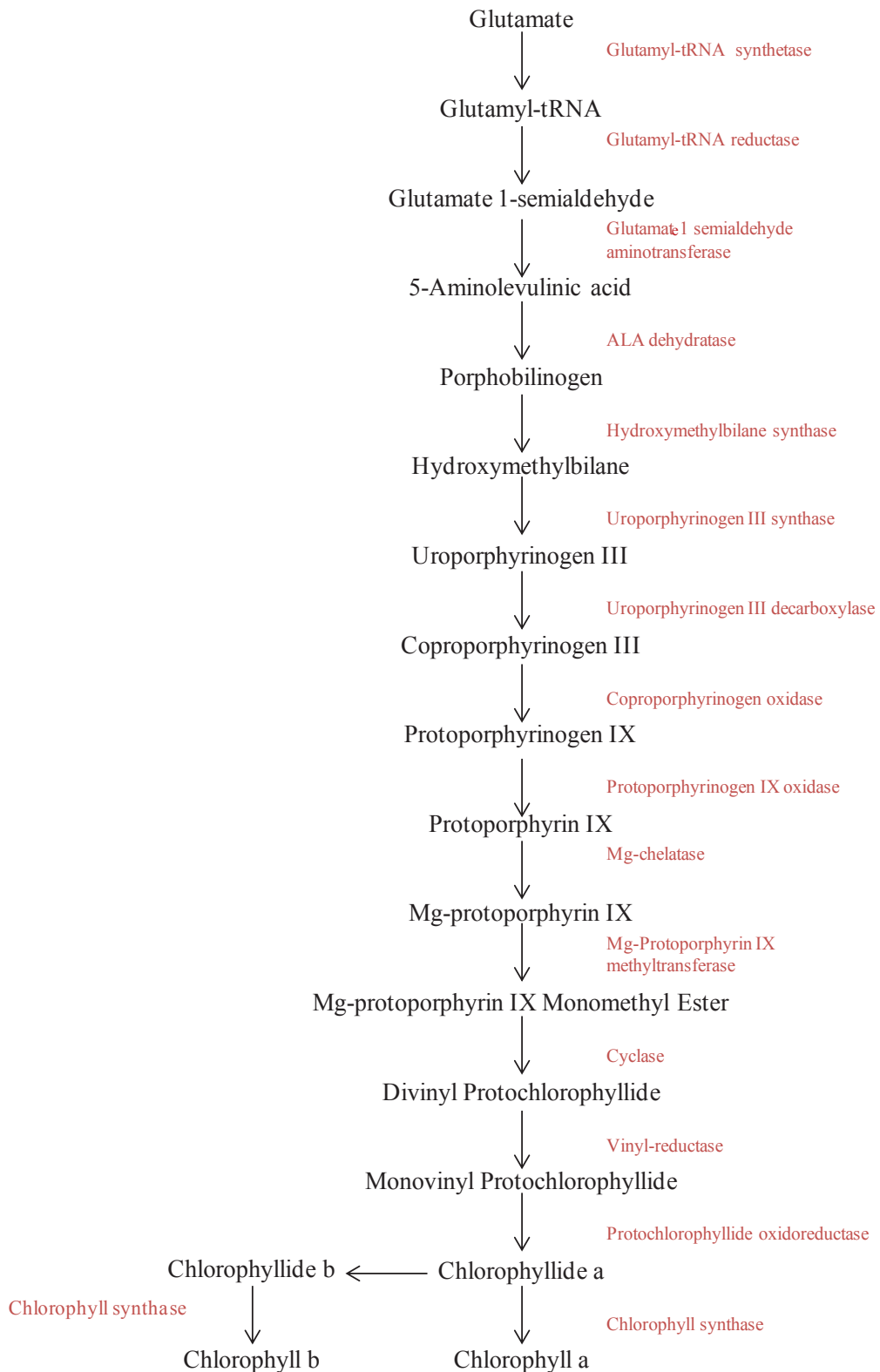


Figure 1.16: Chlorophyll biosynthesis pathway

Details each reaction step and enzyme involved. Adapted from *Plant Physiology*, Institute of Biology, Humboldt University, Berlin[162].

1.6.6 Aquaporins

Aquaporins are small pore-forming membrane proteins of around 24-30 kDa and are part of the major intrinsic protein (MIP) family[163] (See Figure 1.17). So far 13 are known to be expressed in humans and are also found in each kingdom, especially in plants[163-165].



Figure 1.17: Aquaporin conformation

Diagram of aquaporin conformation in the cell membrane to form an hourglass structure including extracellular loops (A, C, E), transmembrane domains(1-6) and intracellular loops (B, D). Taken from Agre. P et al (2002[166])

In 2003 Peter Agre received the Nobel Prize in Chemistry for his discovery of aquaporin 1 in 1992. The protein was serendipitously found during research into red cell Rh group antigens, when an unknown 28 kDa polypeptide was noticed during polypeptide purification. Before this discovery, water was thought to diffuse across the lipid bilayer. Now it is known that water transport can occur both by osmosis and via water channels[166, 167]. Aquaporins are highly selective and even protons are not transported. Those that are permeable to water only are termed aquaporins, and those that are permeable to both water and glycerol, such as human aquaporins 3, 7, 9 and 10 are named aquaglyceroporins[163, 164, 166]. See Table 1.5. While certain members such as AQP2 are relatively well understood[168-170], many others have yet to be expressed in sufficient quantities for further biochemical study.

Table 1.5: Summary of known aquaporin characteristics. Adapted from Offermanns and Rosenthal (2008[165] and Bowen (2005)[171]

Name	Gene	Alias	Site of Expression	Possible functions
Aquaporin 0	AQP0	MIP	Eye lens fiber cells	Fluid balance within the lens
Aquaporin 1	AQP1	Aquaporin-CHIP	Brain choroid plexus Red blood cells Kidney proximal tube Eye ciliary epithelium Alveolar epithelial cells	Production of cerebrospinal fluid Osmotic protection Concentration of urine Production of aqueous humor Alveolar hydration state
Aquaporin 2	AQP2	WCH-CD, aquaporin-CD	Kidney collecting duct Inner ear	Controls antidiuretic hormone activity
Aquaporin 3	AQP3		Kidney collecting duct Trachea epithelial cells Eye Urinary bladder Skin Gastrointestinal tract	Reabsorption of water into blood Secretion of water into the trachea
Aquaporin 4	AQP4	MIWC	Kidney collecting duct Brain ependymal cells Brain hypothalamus Lung bronchial epithelium Retina Inner ear Skeletal muscle	Reabsorption of water CSF fluid balance Osmosensing function Bronchial fluid secretion
Aquaporin 5	AQP5		Salivary glands Lacrimal glands Respiratory tract	Saliva production Tear production
Aquaporin 6	AQP6	AQP2L	Kidney	Low water permeability
Aquaporin 7	AQP7	AQP7L, AQPap, AQP9	Adipose tissue Testis and sperm	Transports glycerol from adipocytes
Aquaporin 8	AQP8		Testis Pancreas Liver Colon	
Aquaporin 9	AQP9		Leukocytes Liver Brain Reproductive system Skin Gastrointestinal tract	
Aquaporin 10	AQP10		Gastrointestinal tract	
Aquaporin 11	AQP11		Testis Liver Kidney	
Aquaporin 12	AQP12		Pancreas	

1.2 Objectives

Membrane proteins implicated in disease such as the aquaporins and A_{2a}R are often poorly expressed, and sufficient quantities are essential to allow further studies. Soluble therapeutic proteins including erythropoietin and human growth hormone are also required in large quantities. Methods of optimizing the production of these relevant proteins will therefore be useful in order to achieve the high yields required. GFP is an effective tool as it is easy to assay and is ideal for testing optimal conditions. Small scale screening was therefore considered in the presence of antifoams with these proteins, within the following objectives:

1. To analyze the effect of antifoams on recombinant protein cultures in a shake flask format (Chapter 3).
2. To understand the mechanisms underlying any observed effects observed in shake flasks (Chapter 4).
3. To analyze the effect of antifoams on recombinant protein cultures in a bioreactor format and to understand the mechanisms underlying any observed effects (Chapter 5).

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

Key pieces of laboratory equipment used for this study are detailed below.

2.1.1.1 Bioreactor equipment

The parts listed were used with 3 L stirred tank autoclavable bioreactors (Applikon) using an ADI 1010 controller and BioXpert version 2 software.

1. Jacketed 3 L glass vessel (Applikon).
2. Head plate to fit a 3 L vessel (Applikon).
3. BioXpert software (Applikon) installed onto a PC.
4. Thermo Circulator ADI 1018.
5. Tandem off-gas analyser.
6. Gas supply unit ADI 1026 (Applikon).
7. 60%:40% oxygen: nitrogen gas cylinder (BOC).
8. Dissolved oxygen (DO) probe (Applikon).
9. DO probe electrolyte and membranes (Applikon).
10. pH probe (Applikon).
11. pH 4 and pH 7 buffers (Fisher).
12. Optek probe (optical density).
13. Optek controller.
14. 250 mL glass sample bottle (Fisher).
15. Air compressor (Bambi, 75/150).
16. Recirculating chiller (Grant, LTL1).
17. Peristaltic pump ($\times 2$) for acid and base addition (Easyload Masterflex).
18. Peristaltic pump for feeds (Masterflex, C/L).
19. Filters (Sartorius, midistart 2000 0.2 μ m PTFE).
20. Silicon tubing (Fisher).

21. Tubing connectors and Y connectors (Fisher).
22. Clamps (Fisher).
23. Needles (Fisher).
24. Plastic syringes (Fisher, 5 mL, 20 mL and 50 mL).
25. 0.5 L glass liquid addition bottles ($\times 3$; Applikon).
26. 1 L glass liquid addition bottle (Applikon).
27. Tin foil (Fisher).
28. 20 mL sample tubes (Fisher).

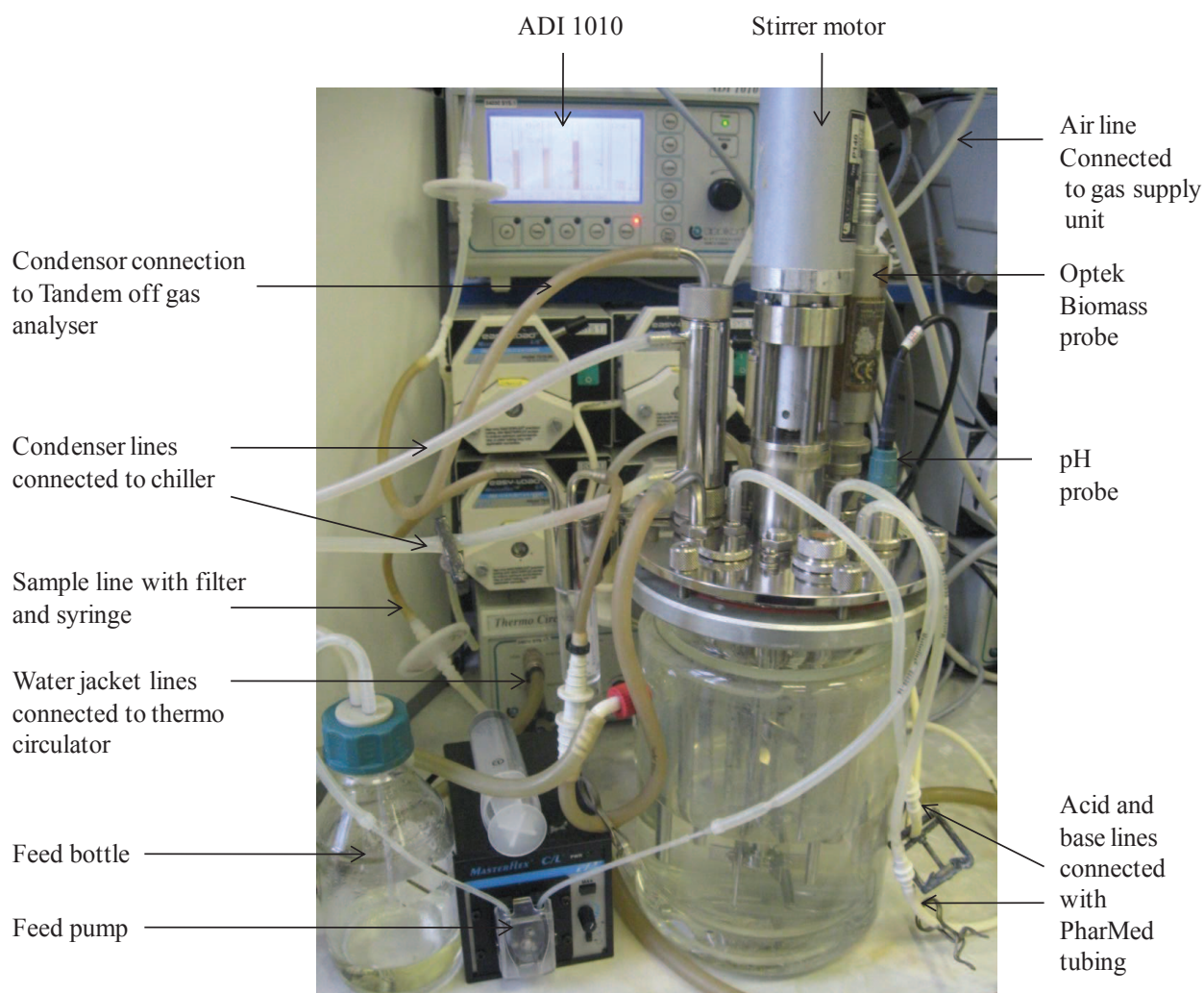


Figure 2.1: 3 L stirred tank bioreactor (Applikon Biotechnology)

Set up and photographed at Aston University with associated equipment as indicated in the figure.

2.1.1.2 Microbioreactor 24

The Microbioreactor24 (Pall Corporation) was used for screening experiments and is effectively 24 ‘mini bioreactors’. The pH, DO, and temperature can be controlled for each of the 24 wells. The single-use cassettes contain 24 wells with a working volume of 3-7 mL. Sensor spots on the base of the wells allow DO and pH to be monitored. A port in the base allows gases to be sparged into the wells to control the pH and DO. Control is regulated by Microbioreactor 24 software on a laptop and the individual settings for each well can be applied and each well monitored throughout the run. CO₂ and O₂ supplied by gas cylinders connected to the control unit allows two-way DO control, while N₂ gas passing through a pressurized bubbler containing 15% v/v ammonium hydroxide produces vapour to control the pH. A shaking platform upon which the cassette is clamped by a vacuum provides agitation from 500-800 rpm. The equipment is shown in Fig 2.2.

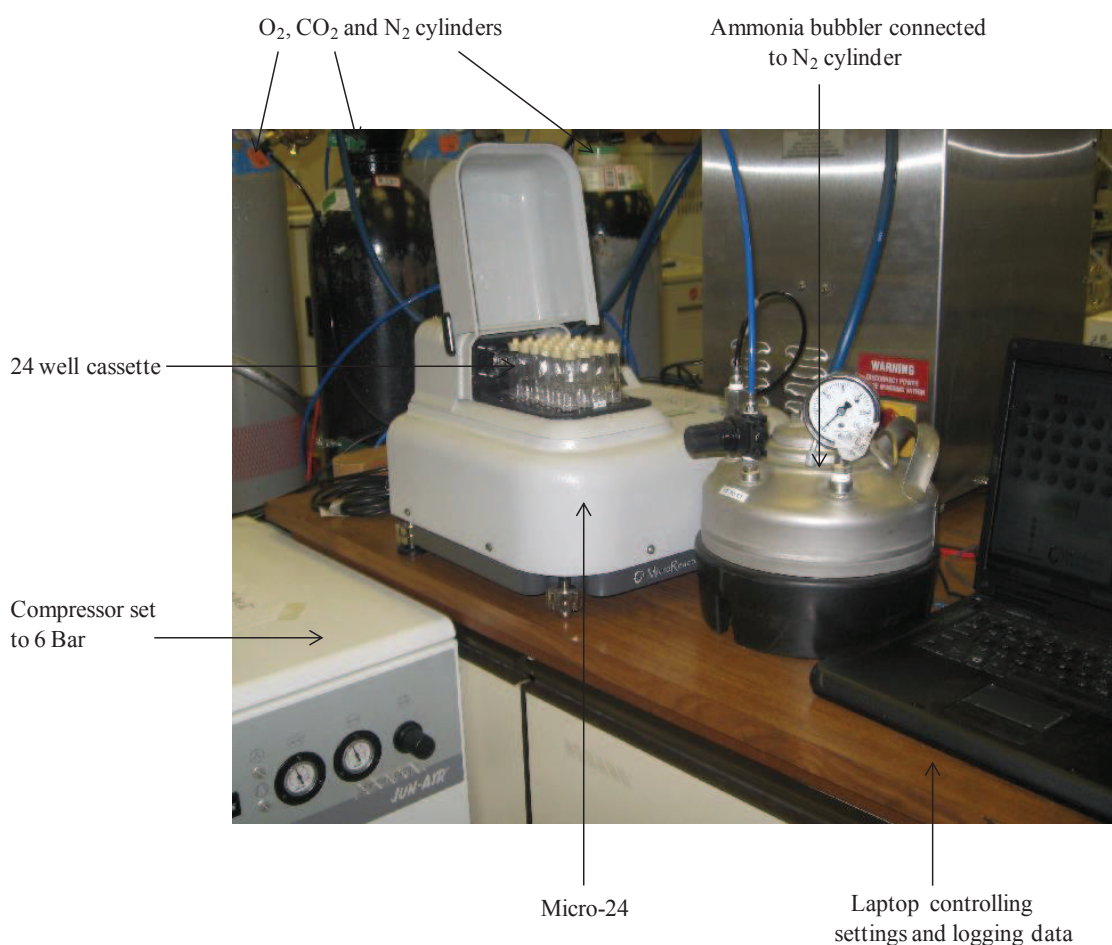


Figure 2.2: Micro-24 micro-bioreactor

Set up with associated equipment as indicated on the figure. Photographed at Aston University.

2.1.1.3 Thermal activity monitor (TAM)

The thermal activity monitor (2277 TAM, TA Instruments) is a differential calorimeter[172] and can be used to detect heat changes between samples. Before use, the equipment required calibration to the experimental conditions. The investigations were conducted at 30°C, therefore the TAM's water bath was set to 30°C. An electrical calibration was performed using Digitam software in the medium to be used at 30°C to set a baseline for the readings. The zero reading on the TAM was adjusted to match that of the software reading. The electrical calibration was begun and the maximum reading also adjusted in the TAM to match that of the electrical signal being applied by the software. The electrical signal was turned off and allowed to drop back to zero. The reading was adjusted a final time on the TAM to ensure the baseline was correctly set before beginning the experiment. A graphical display of the electrical calibration is shown in Fig.2.3.

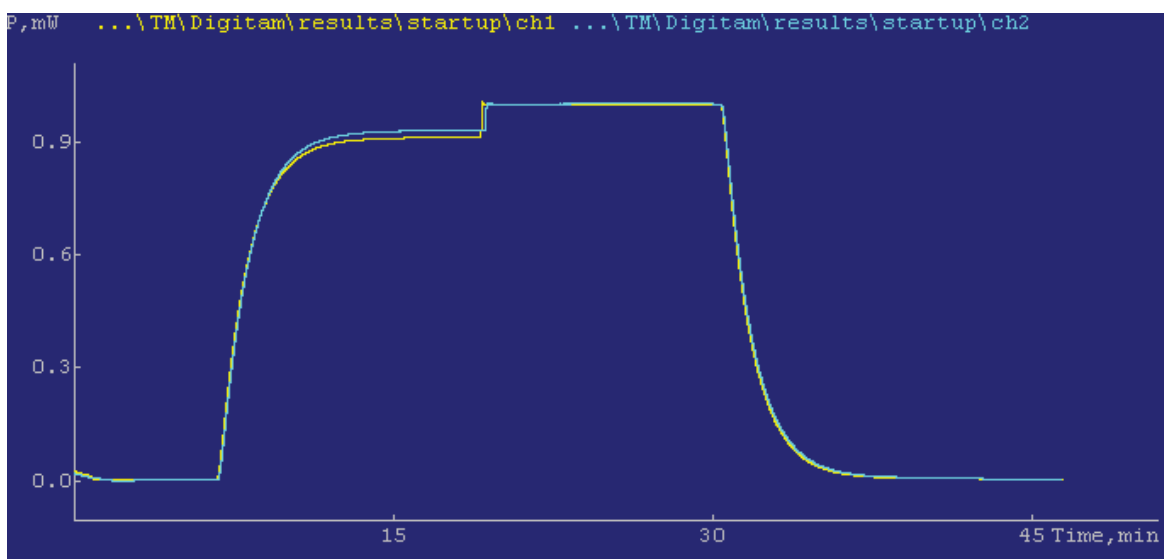


Figure 2.3: Electrical calibration of a thermal activity monitor (TAM) using Digitam software

The zero setting is adjusted on the TAM before the software applies 997 μ W. The reading on the TAM is adjusted to this value and allowed to drop back to zero before a final adjustment to set the baseline for experiments to be conducted at 30°C.

2.1.1.4 Centrifuges

Centrifuges used for these investigations were a Beckman Coulter Optima TLX Ultracentrifuge, a Beckman Coulter Allegra 25 R Centrifuge, a Sigma 1-13 benchtop centrifuge and Beckman Coulter Avanti J-20 XP floor centrifuge.

2.1.2 Media

Various media were used for the culture of *P. pastoris*, *S. cerevisiae* and *E. coli* and are detailed below.

2.1.2.1 *P. pastoris*

2.1.2.1.1 BMGY (Buffered glycerol-complex medium)

Composed of 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 4×10^{-5} % biotin, 1% glycerol. 10 g of yeast extract and 20 g peptone were dissolved in water to a total volume of 700 mL. The solution was autoclaved at 121°C for 20 min then cooled to room temperature. The following was then added; 100 mL 1 M potassium phosphate buffer pH 6.0, 100 mL 10 x YNB, 2 mL 500 x biotin, 100 mL 10 x glycerol. BMGY was stored at 4°C.

2.1.2.1.2 BMMY (Buffered methanol-complex medium)

Composed of 1% yeast extract, 2% peptone, 100mM potassium phosphate pH 6.0, 1.34% YNB, 4×10^{-5} % biotin, 0.5% methanol. 10 g of yeast extract and 20 g peptone were dissolved in water to a total volume of 700 mL. The solution was autoclaved at 121°C for 20 min then cooled to room temperature. The following was then added; 100 mL 1 M potassium phosphate buffer pH 6.0, 100 mL 10 x YNB, 2 mL 500 x biotin, 100 mL 10 x methanol and stored at 4°C.

2.1.2.1.3 YPD (Yeast peptone dextrose)

Composed of 1% yeast extract, 2% peptone, 2% dextrose, 2% agar. 20 g peptone, and 10 g yeast extract were dissolved in water to a total volume of 900 mL. For plates 20 g agar was

also added. The solution was autoclaved at 121°C for 20 min then cooled to room temperature before adding 100 mL 10 x glucose and stored at 4°C.

2.1.2.1.4 Stock solutions

2.1.2.1.4.1 10 xYNB (13.4% Yeast nitrogen base with ammonium sulphate without amino acids)

134 g of yeast nitrogen base was dissolved in water to a total volume of 1 L and filter sterilized. The medium was stored at 4°C.

2.1.2.1.4.2 500 x biotin (0.02%)

20 mg biotin was dissolved in water to a total of 100 mL and filter sterilized. It was then stored at 4°C.

2.1.2.1.4.3 10 x glucose (20%)

200 g glucose was dissolved in water to a total of 1 L water and autoclaved at 121°C for 20 min then cooled to room temperature. The solution was stored at 4°C.

2.1.2.1.4.4 10 x methanol (5%)

5 mL methanol was mixed with 95 mL water and filter sterilized. The solution was then stored at 4°C.

2.1.2.1.4.5 10 x glycerol (10%)

100 mL of glycerol was mixed with 900 mL of water. It was filter sterilized and stored at room temperature.

2.1.2.1.4.6 1 M potassium phosphate buffer pH 6.0

A 1 M solution of K_2HPO_4 was made by dissolving 174.18 g in water to a total volume of 1 L. A 1 M solution of KH_2PO_4 was made by dissolving 136.06 g in water to a total volume of 1 L. Next 132 mL of 1M K_2HPO_4 was mixed with 868 mL KH_2PO_4 and the pH set to 6.0

using a pH meter and phosphoric acid. The solution was autoclaved and stored at room temperature.

2.1.2.2 *E. coli*

2.1.2.2.1 LB (Luria-Bertani)

20 g LB powder was dissolved in water to a total volume of 1 L. For plates 20 g agar was added and the solution autoclaved at 121°C for 20 min then cooled to room temperature and stored at 4°C.

2.1.2.3 *S.cerevisiae*

2.1.2.3.1 CSM

1.7 g YNB without amino acids, 5 g ammonium sulphate and 20 g agar was made up to 1 L with deionized water and autoclaved at 121°C for 20 min then cooled to room temperature. 100 mL 10 x Drop Out (DO) solution –URA and 50 mL glucose was added.

2.1.2.3.2 10 x DO solution (-URA)

L-Adenine hemisulphate salt	200 mg
L-Arginine HCl	200 mg
L-Histidine HCl monohydrate	200 mg
L-Isoleucine	300 mg
L-Leucine	1,000 mg
L-Lysine HCl	300 mg
L-Methionine	200 mg
L-Phenylalanine	500 mg
L-Threonine	2,000 mg
L-Tryptophan	200 mg
L-Tyrosine	300 mg
L-Valine	1500 mg

Made up to 1 L with deionized water.

2.1.2.3.3 2 x CBS

10 g ammonium sulphate, 6g potassium phosphate, 1 g magnesium sulphate and 10 g glucose were dissolved in deionized water up to 998 mL and autoclaved at 121°C for 20 min then cooled to room temperature. 1 mL trace elements and 1 mL vitamin solution were then added.

2.1.2.3.4 Trace elements

EDTA (di-sodium)	15.0 g
Zinc sulphate heptahydrate	4.5 g
Manganese chloride tetrahydrate	1.0 g
Cobalt (II)-chloride hexahydrate	0.3 g
Copper (II)-sulphate penthydrate	0.3 g
Di-sodium molybdenum dihydrate	0.4 g
Calcium chloride dihydrate	4.5 g
Iron sulphate-heptahydrate	3.0 g
Boric acid	1.0 g
Potassium iodide	0.1 g

Made up to 1 L with deionized water and filter sterilized.

2.1.2.3.5 Vitamin solution

D-biotin	0.005 g
Ca D(+) panthothenate	1.0 g
Nicotinic acid	1.0 g
Myo-inositol	25.0 g
Thiamine hydrochloride	1.0 g
Pyridoxol hydrochloride	1.0 g
p-amino benzoic acid	1.0 g

Made up to 1 L with deionized water and filter sterilized.

2.1.2.4 Fermentation media

2.1.2.4.1 Basal salts medium

1 L basal salts medium (BSM)[173] was prepared by dissolving 26.7 mL 85% phosphoric acid, 0.93 g calcium sulphate, 18.2 g potassium sulphate, 14.9 g magnesium sulphate heptahydrate, 4.13 g potassium hydroxide and 40.0 g glycerol in water to a total of 1 L. The solution was autoclaved after being added to the bioreactor.

2.1.2.4.2 PTM₁ trace salts

1 L PTM₁ trace salts was prepared by dissolving 6.0 g cupric sulphate pentahydrate, 0.08 g sodium iodide, 3.0 g manganese sulphate monohydrate, 0.2 g sodium molybdate dihydrate, 0.02 g boric acid, 0.5 g cobalt chloride, 20.0 g zinc chloride, 65.0 g ferrous sulphate heptahydrate, 0.2 g biotin and 5.0 mL sulphuric acid in water to a total of 1 L. The solution was filter sterilized and stored at room temperature.

2.1.2.5 Antibiotics

Antibiotics were added to media to provide selection pressure for growth of correctly transformed cells which would contain the gene for resistance to the antibiotic.

2.1.2.5.1 Ampicillin

500 mg ampicillin was dissolved in 10 mL sterile distilled water to give 50 mg/mL and stored at -20°C

2.1.2.5.2 Kanamycin

500 mg kanamycin was dissolved in 10 mL sterile distilled water to give 50 mg/mL and stored at -20°C.

2.1.2.5.3 Zeocin

250 mg zeocin was dissolved in 10 mL sterile distilled water to give 25 mg/mL. It was wrapped in foil due to light sensitivity and stored at -20°C.

2.1.2.6 Reagents

2.1.2.6.1 BEDS solution

BEDS solution was prepared containing 10 mM bicine-NaOH pH 8.3, 5% v/v dimethyl sulphoxide (DMSO), 3% v/v ethylene glycol, and 1M sorbitol and made up to 1 L with deionized water.

2.1.2.6.2 Breaking buffer pH 7.4

Breaking buffer was prepared containing 5.5% w/v glycerol, 2 mM EDTA 0.5 M pH 7.4, 100 mM NaCl, 50 mM NaH₂PO₄ 1 M, 50 mM Na₂HPO₄ and made up to 1 L with deionized water.

2.1.2.6.3 Buffer A pH 7

Buffer A was prepared containing 20 mM HEPES, 50 mM NaCl, 10% w/v glycerol and made up to 1 L with deionized water.

2.1.2.6.4 Lysis buffer pH 8

Lysis buffer was prepared containing 50 mM NaH₂PO₄, 300 mM NaCl and 10 mM imidazole and made up to 1 L with deionized water.

2.1.2.6.5 Wash buffer pH 8

Wash buffer was prepared containing 50 mM NaH₂PO₄, 300 mM NaCl and 20 mM imidazole and made up to 1 L with deionized water.

2.1.2.6.6 Elution buffer pH 8

Elution buffer was prepared containing 50 mM NaH₂PO₄, 300 mM NaCl and 300 mM imidazole and made up to 1 L with deionized water.

2.1.2.6.7 SDS gels

The compositions of 12% SDS separating gels and 4% stacking gels are given in Tables 2.1 and 2.2.

Table 2.1: Composition of 12% SDS separation gel

12% SDS separating gel	Volume per gel
Polyacrylamide 30%	2.25 mL
Tris-HCl 1.5 M pH 8.8	1.5 mL
SDS 10%	60 μ l
Ammonium persulphate 20%	20 μ L
TEMED	2.25 μ l
Water	1.8 mL

Table 2.2: Composition of 4% SDS stacking gel

4% SDS stacking gel	Volume per gel
Polyacrylamide 30%	0.35 mL
Tris-HCl 1.5 M pH 6.8	1.5 mL
SDS 10%	25 μ l
Ammonium persulphate 20%	10 μ L
TEMED	2.5 μ l
Water	1.55 mL

2.1.2.6.8 Laemmli sample buffer

4 x Laemmli sample buffer[174] was made by mixing 2.4 mL 1M Tris pH 6.8, 0.8 g SDS stock, 0.01% bromophenol blue, 1ml β -mercaptoethanol, 4 mL 100% glycerol and 2.8 mL deionized water.

2.1.2.6.9 Binding buffer

Binding buffer was prepared containing 50 mM sodium phosphate, 0.5 mM EDTA, pH 7.4 and made up to 1 L with deionized water.

2.1.2.7 Molecular biology reagents

2.1.2.7.1 RCP primers

Forward primer:

5' ATTAACAAGAATTCTTATGGAAGTGAAGGATGCCAATTCTGCG 3'

Reverse primer:

5' AACTGATTGGCCGGCTGGGCCGCTGCTGGGTCCTCTTCGTC 3'

2.1.2.7.2 GFP primers

Forward primer:

5' ATTAACAAGCGCCGCTTATGAGTAAAGGAGAAGAAGAACTTTTCACTGG 3'

Reverse primer:

5' AACTGATTTCTAGAATTTTGTAGAGCTCATCCATGCCATGTGT 3'

2.1.2.7.3 TEV primers

Forward primer:

5' CTCGAAAATCTTTATTTTCAAGGTCCGC 3'

Reverse primer:

5' GGACCTTGAAAATAAAGATTTTCGAGGTAC 3'

2.1.2.7.4 HGH primers

Forward 1:

5' CACCATATTGAAGGGAGATTCCCAACTATACCACTATC 3'

Forward 2:

5' GGAATTCCACCATCACCATCACCATATTGAAGGGAG 3'

Reverse 1:

5' GCTCTAGAATCTAGAAGCCACAGCTGCCCTCCAC 3'

2.1.2.7.5 *AOX1* sequencing primers

Forward primer

5' GACTGGTTCCAATTGACAAGC 3'

Reverse primer

5' GCAAATGGCATTCTGACATCC 3'

2.1.2.7.6 Vectors

pPICZ α A and pPICZ α B expression vectors were used for *P. pastoris* cloning work. The vector components for pPICZ α A, B and C are shown in Fig. 2.4.



Figure 2.4: pPICZ α A,B,C expression vector

Contains α -factor, restriction enzymes present in the multiple cloning site and carboxy-terminal tags. Taken from Invitrogen's Pichia pPICZ α A,B,C expression manual[175].

2.1.2.7.7 Restriction enzymes

Several restriction enzymes were used for digestion of DNA and were obtained from New England BioLabs Inc. Enzyme details and reaction conditions were taken from the New England BioLabs Inc. website (<http://www.neb.com/nebecomm/enzymefinder.asp>).

2.1.2.7.7.1 *EcoRI*-HF

Source is an *E. coli* strain that carries the cloned *EcoRI* gene from *E. coli* RY13. Reaction temperature 37°C. A high fidelity enzyme was used to reduce star activity.

2.1.2.7.7.2 *XbaI*

Source is an *E. coli* strain that carries the *XbaI* gene from *Xanthomonas badrii*. Reaction temperature 37 °C.

2.1.2.7.7.3 *SfiI*

Source is an *E. coli* strain that carries the *SfiI* gene from *Streptomyces fimbriatus*. Reaction temperature 50°C.

2.1.2.7.7.4 *NotI*

Source is an *E. coli* strain that carries the cloned and modified (K150A) *NotI* gene from *Nocardia otitidis-caviarum*. Reaction temperature 37°C. A high fidelity enzyme was used to reduce star activity.

2.1.2.7.7.5 *KpnI*

Source is an *E. coli* strain that carries the *KpnI* gene from *Klebsiella pneumoniae* OK8. Reaction temperature 37°C.

2.1.2.7.7.6 *SacII*

Source is a *Streptomyces lividans* strain that carries the *SacII* gene from *Streptomyces achromogenes*. Reaction temperature 37°C.

2.1.2.8 Antifoams

Five antifoams were evaluated in this work; Antifoam A (Sigma) a 30% emulsion of silicone polymer; Antifoam C (Sigma) a 30% emulsion of silicone polymer; J673A (Struktol) an alkoxyated fatty acid ester on a vegetable base; P2000 (Fluka) a polypropylene glycol; SB2121 (Struktol) a polyalkylene glycol. Antifoams were added to medium at concentrations between 0.001% v/v up to 10% v/v.

2.1.3 Strains and culture conditions

Yeast, and *E. coli* cells were used for different procedures. Yeast was used for recombinant protein production, *E. coli* for producing plasmid DNA.

2.1.3.1 *Escherichia coli*

E. coli was used to amplify plasmid DNA for transformation into *P. pastoris* cells. LB media and incubation temperatures of 37°C provided optimum growth conditions.

2.1.3.1.1 DH5 α

Competent cells for high efficiency transformation of plasmids were obtained from laboratory stocks at Aston University. Genome information: F⁻ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ 80dlacZ Δ M15 Δ (lacZYA-argF)U169, hsdR17(r_K⁻ m_K⁺), λ -. An Hoffman-Berling 1100 strain derivative (Meselson68) and nalidixic acid resistant[176-179].

2.1.3.1.2 XL10 Gold

Competent cells were obtained from Stratagene for high efficiency transformation of large plasmids. Genome information: endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac Hte Δ (mcrA)183 Δ (mcrCB-hsdSMR-mrr)173 tet^R F'[proAB lacI^qZ Δ M15 Tn10(Tet^R Amy Cm^R)]. Tetracycline, chloramphenicol and nalidixic acid resistant[179].

2.1.3.2 *Pichia pastoris*

Two strains of *P. pastoris* were used in this study; X33 and SMD1163.

2.1.3.2.1 X33

Pichia pastoris X33 is the wild type strain with no deletions to the *AOX1* gene and can be selected for by zeocin. The X33 strain used was from laboratory stocks at Aston University, and is also available from Invitrogen. Growth conditions were chosen from those recommended by Invitrogen; medium buffered to pH 6.0, 30°C and 220 rpm[173]. BMGY medium was used for the initial biomass accumulation stage before transferring to BMMY where methanol induces protein expression. Large-scale fermentations were carried out in basal salts medium.

2.1.3.2.2 SMD1163

SMD1163 is a protease deficient strain. Genome information: *his4 pep4 prb1*. The *PEP4* gene encodes proteinase A, which is a vacuolar aspartyl protease needed for the activation of other vacuolar proteases such as carboxypeptidase Y and proteinase B. The *PRB1* gene encodes proteinase B[22].

2.1.3.3 *Saccharomyces cerevisiae*

Two strains of *S. cerevisiae* were used in this study; WT and TM6*.

2.1.3.3.1 Wild type

The wild type BY4741 strain was used and hA_{2a}R in the pYX212 vector obtained from Dr N. Fraser was transformed into these cells by Dr. R. Darby. Uracil selection was used to identify expressing colonies.

2.1.3.3.2 TM6*

The strain used was KOY-TM6* containing the gene encoding a chimeric hexose transporter, TM6*[180]. hA_{2a}R in the pYX212 vector obtained from Dr N. Fraser was transformed into these cells by Dr. R. Darby. Uracil selection was used to identify expressing colonies.

2.2 Methods

2.2.1 Molecular Biology Techniques

2.2.1.1 Primer design

Expasy was used to search for target DNA sequences and the information provided used in order to design primers which ranged from 24 to 42 bases long. Primers were ordered from Invitrogen.

2.2.1.2 DNA amplification - PCR

DNA was amplified by PCR, the reactions set up on ice with:

1 μ L forward primer (100 pM)

1 μ L reverse primer (100 pM)

1 μ L PFU polymerase (Stratagene)

5 μ L PFU polymerase buffer (Stratagene)

1 μ L dNTPs (10 mmol)

1 μ L template (100 ng)

Made up to 50 μ l with sterile deionized water

PCR reactions were carried out at:

95°C 2 min (denaturation)

30 cycles of the following steps were then carried out:

95°C for 30 s

55°C for 30 s (annealing)

72°C for 1 min (extension)

Lastly a final extension step was carried out at 72°C for 10 min.

Negative controls were performed by setting up reactions without template.

2.2.1.3 Restriction digests

Where possible, double digests were carried out using the most appropriate buffer and conditions. Standard conditions are detailed below and all steps carried out on ice.

2 μ g DNA

0.5 μ L (10 units) restriction enzyme (New England BioLabs)

0.5 μ L 100 x BSA (100 μ g/mL) (New England BioLabs)

6 μ L NE buffer (New England BioLabs)

Deionized water to 50 μ L

The reaction mixture was incubated at the optimum temperature for the restriction enzyme for 1 h. For insert DNA, 1 μL antarctic phosphatase (New England BioLabs) (to catalyze the removal of 5' phosphate groups from the DNA to prevent self-ligation) and 5 μL antarctic buffer was added. The reaction was left for 30 min at 37°C. All reactions were then heat inactivated by incubating at 65°C for 20 min.

2.2.1.4 Ligations

Standard ligation reactions were carried out on ice with the following reagents:

100 ng vector DNA

Insert DNA (3:1 molar excess with respect to vector DNA)

1 μL T4 ligase (New England BioLabs)

2 μL T4 ligase reaction buffer (New England BioLabs)

Deionized water to 20 μL

The reaction mixture was incubated at 21°C for 3 h. Alternatively, the mixture was left on the bench overnight.

2.2.1.5 Agarose gel electrophoresis

1% agarose gels were run to verify the presence of the required DNA after amplification. 1 x TAE buffer was made by measuring 20 mL 50 x TAE buffer and mixing with 980mL water. 1.5g agarose was added to 150 mL 1 x TAE buffer and heated in a microwave until dissolved. Once cooled, 3 μL ethidium bromide was added and after mixing the gel was poured. Combs were inserted and the gel left to set for around 30 min. Once completely set, the combs were removed and the gel transferred to a tank with the remaining 1 x TAE buffer added. Samples were set up by adding 1 μL 10 x BlueJuice loading buffer (Invitrogen) to 5 μL samples before dispensing into the wells of the gel. A well was also set up with 2 μL DNA ladder (Hyperladder IV, Bionline). The gel tank was connected to a power source and 100V was applied for approximately 30 min. Once run, the gel was stained with 4 μL ethidium bromide and visualized under a UV light box.

2.2.1.6 Transformation

DNA was transformed into two different strains of competent cells, *E.coli* DH5 α cells and XL10 Gold cells. Different protocols were used for the different strains.

2.2.1.6.1 *E.coli* DH5 α transformation

The DH5 α cells stored at -80°C were defrosted on ice and using cold cut off tips, 50 μ L dispensed into cold eppendorf tubes. To each tube 1 μ L DNA was added and kept on ice for 20 min. The cells were then heat shocked at 37°C for exactly 1 min 15 s and kept on ice for a further 5 min. Then 900 μ L cold LB without antibiotic was added to the tubes which were then incubated at 37°C for 50 min to allow growth and time for antibiotic resistance genes to be expressed. The tubes were then centrifuged at 14,000 rpm for 5 min and 800 μ L supernatant discarded. The pellets were resuspended in the remaining supernatant and this was spread onto agar plates with appropriate antibiotic added. The plates were allowed to dry before incubating at 37°C overnight.

2.2.1.6.2 *E.coli* XL10 Gold transformation

XL10 Gold cells stored at -80°C were defrosted on ice and 4 μ L β mercaptoethanol added per 200 μ L cells to allow more efficient uptake of DNA. Using cold cut off tips, 50 μ L were dispensed into cold eppendorf tubes. To each tube 1 μ L DNA was added and kept on ice for 20 min. The cells were then heat shocked at 42°C for exactly 30 s then kept on ice for 5 min. Then 900 μ L cold LB without antibiotics was added to the tubes which were incubated at 37°C for 1 h. The tubes were then centrifuged at 14,000 rpm for 5 min and 800 μ L supernatant discarded. The pellets were resuspended in the remaining supernatant and this was spread onto agar with appropriate antibiotic added. The plates were allowed to dry before incubating at 37°C overnight.

2.2.1.7 Minipreps

Minipreps were set up using reagents from a GenElute plasmid miniprep kit (Sigma-Aldrich) and the recommended protocol followed[181]. The transformed *E.coli* cultures were used to recover the plasmid DNA and allow sequencing. One colony was taken from

the appropriate transformed *E.coli* plate and used to inoculate 5 mL LB with the desired antibiotic. The culture was allowed to grow overnight at 37°C and centrifuged the next day at 5,000 rpm for 4 min. The supernatant was discarded and the pellet resuspended in 200 µL cell resuspension solution by vortexing and then transferred to eppendorf tubes. Following resuspension 200 µL cell lysis solution was dispensed into each tube and 10 µL alkaline protease solution also added. The tubes were immediately mixed by inverting 4 times and before 5 min the reaction was stopped to prevent nicking of the plasmid DNA by alkaline protease. The reaction was stopped by adding 350 µL neutralizing solution and mixed by inverting 4 times. The tubes were centrifuged at 14,000 rpm for 10 min and the cleared cell lysate decanted into spin columns inserted into collection tubes, avoiding the white precipitate. The columns were centrifuged at 14,000 rpm for 1 min and the flow through in the collection tubes discarded. Column wash solution was diluted with 95% ethanol and 750 µL added to each tube before centrifuging at 14,000 rpm for 1 min. The wash was repeated with 250 µL column wash solution and a further centrifugation step at 14,000 rpm for 2 min. The spin columns were transferred to new collection tubes and plasmid DNA was eluted by adding 100 µL nuclease-free water and centrifuging at 14,000 rpm for 1 min. The plasmid DNA was stored at -20°C.

2.2.1.8 DNA quantification

The concentration of DNA purified from minipreps was determined by using a Nanodrop 1000 spectrophotometer (Thermo Scientific). ND2000 software was used and 2µL water loaded into the Nanodrop to blank it. Once the software had blanked against it, the nanodrop was blanked again with 2 µL water as a medium blank. Next 2 µL of DNA was loaded and the software calculated the absorbance corresponding to the concentration of DNA present in the sample. An example of the software reading a DNA sample is shown in Fig. 2.5.

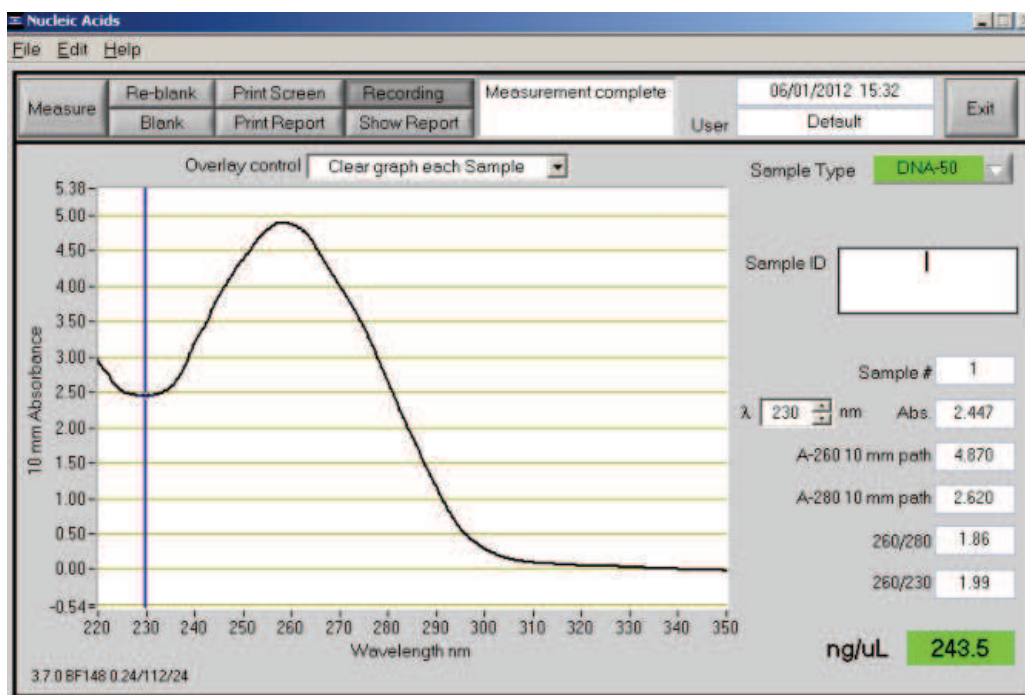


Figure 2.5: Nanodrop DNA quantification

Screenshot of Nanodrop software reading for a DNA sample with a peak at 260 nm.

2.2.1.9 DNA sequencing

DNA samples were prepared for sequencing at Birmingham University's genomics laboratory by mixing 4.5 μ L miniprep DNA and 1.5 μ L primer with 9 μ L deionized water. 10 μ L of this mixture was sequenced at Birmingham University. The data were analyzed using Chromas Lite version 2.01 and the sequences were aligned using Biology Workbench version 3.2 or T-Coffee software version 8.99.

2.2.1.10 Maxipreps

Once the DNA had been validated by sequencing, maxipreps were carried out to obtain larger and purer quantities of plasmid DNA. Reagents from a PowerPrep HP plasmid maxiprep kit (OriGene) were used, and the recommended protocol followed[182]. A colony was taken from the transformed *E.coli* plates and used to inoculate 5 mL LB medium with the appropriate antibiotic and cultured at 37°C overnight. From these cultures, 0.5 mL was used to inoculate 200 mL LB medium with antibiotic and the flasks incubated at 37°C 220 rpm overnight. Next 50 mL from each flask was dispensed into tubes and centrifuged at

40,000 rpm for 5 min. The supernatant was discarded and another 50 mL poured in, repeating the process until all the culture was pelleted.

Columns were set up for each pellet with a beaker beneath to catch flow through. 30 mL equilibration buffer was added to each column and left to drain by gravity. The pellets were resuspended in 10 mL cell suspension buffer using a 1 mL pipette. Then 10 mL cell lysis solution was added and the tubes inverted 5 times before centrifuging at 4,600 rpm for 10 min. The supernatant was decanted into the empty columns avoiding the white precipitate. Once the columns had drained, 60 mL wash buffer was added and again left to drain. 15 mL elution buffer was dispensed into each tube, and the flow through caught in tubes to which 10 mL isopropanol was added. These samples were spun at 15,000g for 30 min and the supernatant discarded before adding 4 mL 70% ethanol to dissolve the pellet. A further 1 mL 70% ethanol was added and the sides of the tubes scraped to remove all pellet. The tubes were centrifuged at 5,000 rpm for 7 min and the supernatant discarded. The pellets were resuspended in 500 μ L sterile deionized water and transferred to eppendorf tubes and stored at -20°C.

2.2.1.11 Linearization of DNA

DNA to be transformed into yeast was linearized by mixing 5-10 μ g DNA, 1.5 μ L *PmeI* (New England Biolabs), 0.5 μ L 100 x BSA, 5 μ L NEbuffer 4 and up to 50 μ L with deionized water. The reaction mixture was heated to 37°C for 2 h, then 65°C for 20 min.

2.2.1.12 Preparation of electrocompetent *P. pastoris* cells

5 mL of YPD was inoculated with *P. pastoris* X33 or SMD1163 cells at 30°C 220 rpm overnight. The culture was diluted with YPD to an OD₅₉₅ of 0.15-0.2 in 50 mL medium. The culture was incubated at 30°C, 200 rpm until the OD₅₉₅ reached 0.8-1. The culture was centrifuged at 5,000 rpm for 5 min at room temperature. The supernatant was discarded and the pellet resuspended in 9 mL ice-cold BEDS solution with 1 mL 1M dithiothreitol (DTT). The mixture was incubated for 5 min at 100 rpm in a 30°C incubator on a low shake speed. The culture was then centrifuged at 5,000 rpm for 5 min at room temperature and the pellet

resuspended in 250 μL of BEDS without DTT. 40 μL volumes were aliquoted and stored at -80°C for up to 6 months.

2.2.1.13 Electroporation of *P. pastoris* cells

P. pastoris competent cells and the DNA to be transformed were defrosted on ice. 1M sorbitol and YPD were put on ice with 1.5 mL eppendorf tubes and electroporation cuvettes. 40 μL cells were transferred into the cold cuvette and 4 μg DNA added. The cuvette was incubated on ice for 2 min. The cuvettes were placed into the electroporation equipment and electroporated at 1,800 V with 15 ms pulse length. The cells were immediately suspended in 0.5 mL 1 M sorbitol and 0.5 mL YPD. The cells were transferred into the eppendorfs and incubated at 30°C on the lowest shaking speed for 1 h 30 min. The culture was then plated onto agar with increasing concentrations of antibiotic to select for colonies containing the plasmid DNA and incubated at 30°C for up to 8 days.

2.2.1.14 Transformation of *S. cerevisiae* cells - lithium acetate

S. cerevisiae cultures were incubated in 5 mL 2 x CBS At 30°C overnight. 1 mL of culture was centrifuged at 14,000 rpm to obtain a pellet. The supernatant was discarded. 15 μL 100 mM lithium acetate was added to the pellet and vortexed before centrifugation at 14,000 rpm. The supernatant was removed and a further 15 μL 100 mM lithium acetate was and again vortexed before centrifugation at 14,000 rpm. Single stranded salmon testes carrier DNA was heated to 100°C for 5 min then put on ice for 2 min. 240 μL polyethylene glycol (50% PEG 3350), 36 μL 1 M lithium acetate and 25 μL salmon DNA was added followed by 0.5 μg plasmid DNA. The mixture was incubated for 30 min at 30°C . The cells were then heat shocked for 20 min at 42°C and centrifuged at 6,000 rpm for 1 min. The supernatant was removed with a pipette and 500 μL sterile deionized water added. The cells were gently resuspended and 20 μL dispensed onto CSM –URA agar plates. The plates were incubated at 30°C until colonies began to form.

2.2.2 Yeast cultures and analysis

2.2.2.1 Small scale formats

Precultures were set up using 250 mL baffled shake flasks containing 50 mL BMGY medium inoculated with 1 colony from the desired YPD agar plate. The flask was incubated at 220 rpm overnight. The required volume of log phase cells taken from this culture in order to inoculate the experimental cultures to an OD₅₉₅ of 1 was centrifuged at 5,000 rpm for 10 min and the pellet resuspended in the required volume of BMMY medium. 6 mL deepwell plates, 200 µL multiwell plates and a range of shake flasks were used. Antifoam investigations were conducted in 100 mL non-baffled shake flasks and 2 L non-baffled shake flasks.

2.2.2.2 Bioreactors

A commonly-used bioreactor for recombinant protein production is the stirred-tank reactor with agitation provided by impellers[183]. 3 L stirred-tank bioreactors (Applikon) were used for all large-scale bioreactor experiments.

2.2.2.2.1 Preparation of the bioreactor

1 L BSM was made and poured into the glass vessel. The head plate was attached and secured with bolts. Silicon tubes were attached to each of the ports on top of the head plate and clamped and the ends wrapped in foil. The pH probe was added after calibrating using pH 4 and pH 7 buffers. The DO probe was also added as well as the Optek probe for biomass readings. A 0.2 µm PTFE gas filter was added to the inlet gas sparger and left unclamped to allow pressure equalization and avoid vessel damage during autoclaving. A 250 mL glass sample bottle was attached to the sample port and a length of silicon tubing with a filter attached to the fork. The bioreactor was then autoclaved and sterilized at 121°C for 20 min with a slow cool cycle. A 0.5 L glass liquid addition bottle containing 50% (v/v) phosphoric acid and a 0.5 L glass liquid addition bottle of 28 % ammonium hydroxide were prepared for pH control. PharMed tubing was used and filters attached to the caps.

2.2.2.2.2 Connecting the bioreactor to the control unit

The bioreactor was removed from the autoclave and placed next to the control unit. The DO probe was connected to the ADI 1010 controller and allow to polarize for a minimum of 6 h. The pH probe was also connected to the ADI 1010 controller and the Optek probe connected to the Optek controller. 5 mL 50% (w/v) glycerol was added to the port for the temperature probe (the thermowell tube) and the temperature probe inserted. The chiller was turned on and connected by silicone tubing to the condenser. The silicone tubing attached to the condenser was connected to the off-gas analyser. The compressor and the 60%: 40% oxygen: nitrogen cylinder were connected to the gas supply ADI 1026 unit. The gas supply was attached to the sparger line on the bioreactor with a length of silicon tubing from the ADI 1026 unit with a filter also added. The stirrer motor was attached to the head plate and acid and base bottles set up with the pumps connected to the controller equipment. The foil was removed from the acid and base lines on the bioreactor and sprayed with 70% ethanol before connecting to the lines on the acid and base bottles. The water jacket lines were connected to the thermo circulator ADI 1018 unit. The feed bottles were also connected to the bioreactor by inserting the tubing from the bottles into a peristaltic pump and connecting the tubing to the feed line tubing on the bioreactor, spraying the line ends with 70% ethanol.

2.2.2.2.3 Culture conditions

Recombinant protein production experiments in this work using bioreactors were with *P. pastoris*. The settings used for GFP and EPO experiments were pH 5, 30 °C, 30% DO and a minimum stirrer speed of 700 rpm. For A_{2a}R experiments, the temperature was reduced from 30°C to 22°C during induction. The DO set point was set to 30% and the stirrer was put into a cascade of 700-1,250 rpm to maintain the 30% setpoint. Once the stirrer was no longer able to maintain the setpoint, the mass flow controller (MFC) increased the proportion of air drawn from the 40%: 60% nitrogen: oxygen cylinder. The air flow into the bioreactor was set to 2 L/min and the cylinder pressure was set to 2.5 bar. The flow rate of exit gas to the Tandem off-gas analyzer was set to approximately 0.4 L min⁻¹ by adjusting the clamp on the open end of the forked tubing. 5 mL PTM₁ trace salts was added using syringe and needle in an sterile manner through the septum and into the vessel. The pH was

then adjusted to pH 5 by entering this setpoint into the ADI1010 controller, causing the pumps to add the required volume of acid or base. The bioreactor was left running at these settings for approximately 1 h before inoculating.

2.2.2.2.4 Starting a bioreactor run

A seed culture was prepared by inoculating 50 mL BMGY medium with one colony of the desired *P. pastoris* strain from an agar plate. The culture was incubated at 30°C, 220 rpm overnight. The next day, the volume of seed culture required to inoculate 1 L basla salts medium to the desired OD. Immediately before adding the required volume of culture to the bioreactor, BioXpert software was set up and the run details entered. Antifoam was added to the required volume in a sterile manner through the septum, then the bioreactor was inoculated with the culture. The run was immediately started on the BioXpert software and DO control turned on. The various run parameters were graphically displayed throughout the run by the software.

2.2.2.2.5 Glycerol batch and fed batch phase

The fermentation cultures initially began growing on the 40 g/L glycerol already present in the BSM. Once this had been consumed, usually around 20 h and indicated by a DO spike[184], a fed batch phase was begun. 50% w/v glycerol with 12 mL PTM₁ trace salts/L was fed into the vessel at a flow rate of 12 mL/h for 4 h to increase biomass prior to induction. After 4 h, the fed was stopped and the culture starved for 1 h to ensure all glycerol in the medium was consumed before inducing.

2.2.2.2.6 Induction phase

Induction was begun after starvation using 20% v/v methanol with 12 mL PTM₁ trace salts/L. It was initially fed in at 4 mL/h for a minimum of 3 h, then increased to 8 mL/h overnight. Early the next morning the feed was increased to 12 mL/h and remained at this flow rate for the remainder of the fermentations which were approximately 100 h in total.

2.2.2.3 Optical density measurement

Optical densities of yeast cultures were measured using a spectrophotometer set to a wavelength of 595 nm, using x 100 dilution with water.

2.2.2.4 Dry cell weights

Dry cell weight measurements were carried out by drying 1.5 mL eppendorfs (Fisher) in an oven at 100°C for 24 h followed by drying in a dessicator for 48 h. The tube weight was determined using a microbalance (Denver Instruments). 1 mL of culture was dispensed into the tubes and spun at 14,000 rpm for 10 min. The supernatant was removed and the tubes dried in an oven at 100°C for 48 h followed by a dessicator for 48 h. The tubes were again weighed.

2.2.3 Protein analysis

2.2.3.1 Membrane preparations

Membrane preparations were made from *P. pastoris* cells. 20 mL culture samples were centrifuged at 14,000 rpm and the supernatant discarded. 500 µL pH 7.4 breaking buffer was added. The mixture was transferred to breaking tubes and 1.5 mL glass acid washed beads were added. Protease inhibitor cocktail IV (Calbiochem) was added at 1:2,000 to each tube and immediately put on ice. A cell lyser (TissueLyser LT, Quiagen) was used to disrupt the cells at 50 Hz for 15 min. The tubes were pierced and the solution collected after centrifugation at 5,000 rpm for 5 min. The pellets were resuspended in the supernatant and transferred to fresh 1.5 mL eppendorfs. The tubes were spun at 15,000 rpm for 15 min and the supernatant centrifuged again at 66,000 rpm for 1 h. The supernatant was removed and the membrane pellets resuspended overnight at 4°C in pH 7 buffer A. Membrane preparations were stored at -80°C.

2.2.3.2 BCA Assay

The total protein content of culture supernatants (2 µl) at 48 h post-induction was analyzed by bicinchoninic acid (BCA) assay. 4.9 mL of BCA solution (B9643, Sigma) was mixed with 100 µL 4% mM copper (II) sulfate solution (C2284, Sigma). 198 µL of this solution

was used to assay each independent supernatant sample in duplicate using a plate reader (BioTek Instruments) at 570 nm. To determine the concentration of protein in the samples, a bovine serum albumin standard (Sigma) was used to plot a standard curve (Fig 2.6). The data were analyzed using a one-way ANOVA ($P < 0.0001$) and a Dunnett's multiple comparison test.

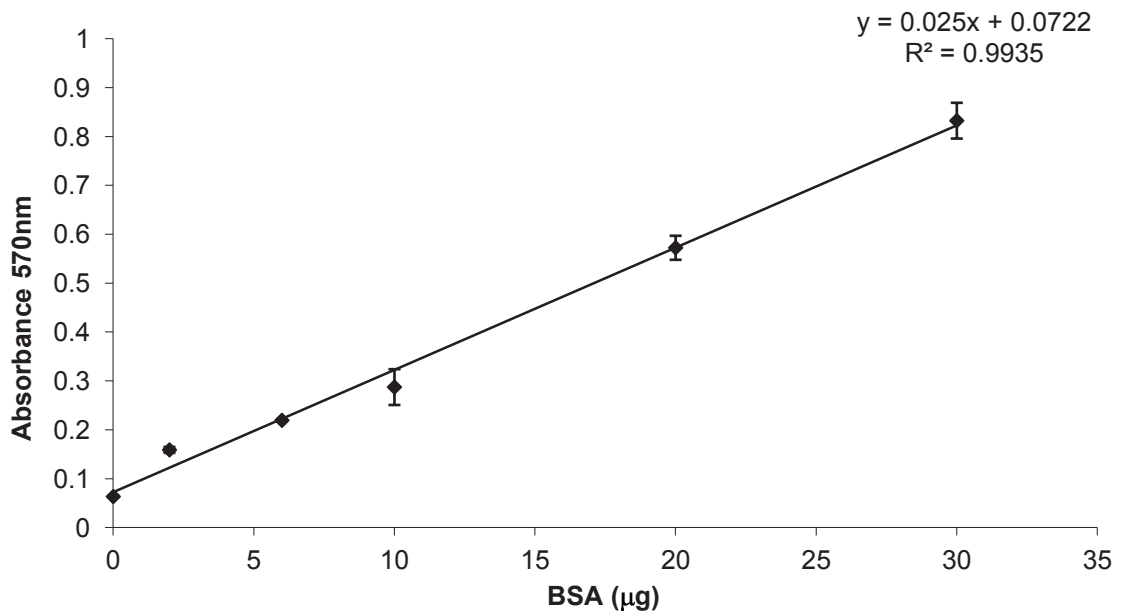


Figure 2.6: BCA standard

Example BCA standard with increasing concentrations of BSA measured at 570 nm.

2.2.3.3 Fluorescence measurements

Culture supernatants (100 µL) were assayed at 24 h and 48 h post-induction for GFP fluorescence in black 96 well plates using a Spectramax Gemini XS plate reader with an excitation wavelength (λ_{exe}) of 397 nm, and emission wavelength (λ_{em}) of 506 nm. Triplicate determinations were performed for each independent sample including blanks with BMMY or BSM. All samples and blanks were buffered to pH >7.0 using 50 µL 1 M potassium phosphate pH 7.5. Data were collected at 25 °C. To determine the concentration of GFP in each of the samples, a recombinant GFP standard (Vector Laboratories Ltd) was used to construct a standard curve relating RFU to protein concentration (Fig. 2.7), as described previously[36]. All data were analyzed using a one-way ANOVA to test for a

significant difference between any of the means. In all cases $P < 0.001$ indicated a high degree of significance. A Dunnett's multiple comparison test was then performed to compare each treatment mean (e.g. addition of various antifoam concentrations) and the control mean (e.g. 0% antifoam).

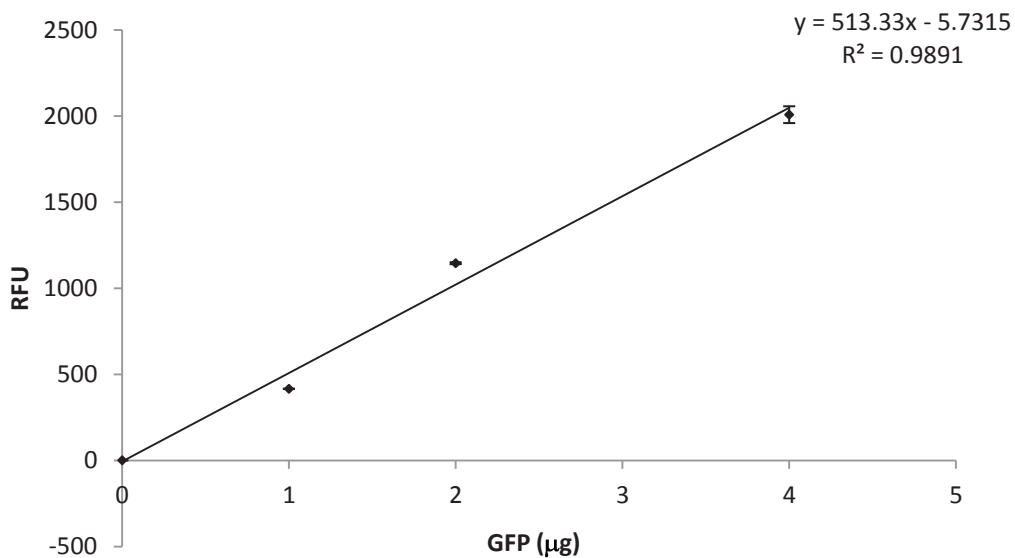


Figure 2.7: GFP standard

Increasing concentration of recombinant GFP standard measured at excitation wavelength (λ_{exc}) of 397 nm, and emission wavelength (λ_{em}) of 506 nm by Spectramax Gemini XS fluorescence plate reader

2.2.3.4 ELISA

A GFP ELISA kit (RandD Systems) was used to detect GFP present in sample supernatants. Sample supernatant was diluted 1:5,000 using Assay Diluent and 100 μL added to the wells of the Anti-GFP antibody coated plate. A dilution series of recombinant GFP standard was performed and 100 μL was added to wells. All samples were assayed in duplicate. The plate was incubated at 37°C for 2 h. The wells were then washed with 250 μL 1 x wash buffer three times. Excess wash buffer was removed by tapping the plate on paper towel. Then 100 μL of anti-GFP antibody previously diluted to 1:1,000 using Assay Diluent was added to each well, and the plate incubated at room temperature for 2 h on an orbital shaker. The wells were washed 3 times as previously. 100 μL of secondary antibody previously diluted to 1:1,000 using Assay Diluent was added to each well and the plate was incubated for 1 h on an orbital shaker. The wells were washed 3 times as previously, then

100 μ L Substrate Solution warmed to room temperature was added to the wells. The plate was incubated at room temperature until a colour change occurred, varying from 2-30 min. The enzyme reaction was stopped by adding 100 μ L Stop Solution to each well. The results were read using a spectrophotometer at 450 nm. A GFP standard curve was plotted to determine the concentration of GFP in the samples.

2.2.3.5 Flow cytometry

Shake flask cultures of *P. pastoris*, as described above, were used to generate samples for flow cytometry analysis. The antifoams used were Antifoam A at 0.6%, Antifoam C at 0.6%, J673A at 0.8%, P2000 at 0.6% and SB2121 at 0.6% (v/v). Triplicate flasks were used for each antifoam. 48 h samples were diluted 1:1,000 in phosphate buffered saline to a final concentration of 10^6 - 10^7 cells/mL, as determined using a haemocytometer. Fluorescent measurements were made using a Beckman Coulter (High Wycombe, UK) flow cytometer with $\lambda_{\text{exc}} = 488$ nm from an argon-ion laser at 15 mW. Diluted samples were additionally stained with 10 μ L propidium iodide (PI; 1 mg/mL in water). All solutions were passed through a 0.2 μ m filter, immediately prior to use, to remove particulate contamination. The optical filters were set up so that PI fluorescence was measured at 630 nm and GFP fluorescence was measured at 525 nm. The data were analyzed using a one-way ANOVA ($P < 0.0001$) and a Dunnett's multiple comparison test.

2.2.3.6 GFP purification

GFP was purified using its His₆ tag with Ni-NTA agarose resin and columns (Qiagen). 5 mL pH 8.0 lysis buffer and 1 mL 50% of Ni-NTA was added to 15 mL supernatant in the lysis buffer in duplicate for each sample to be purified and incubated at 4°C for 1 h. The mixture was then loaded into a capped column before removing the cap and collecting the flow through. The column was washed twice with 4 mL pH 8.0 wash buffer and the wash fractions were collected. The protein was then eluted 4 times with pH 8.0 elution buffer and the fractions combined and stored at 4°C before analysis by Western blot.

2.2.3.7 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was used to separate the proteins in sample supernatant and membrane preparations. 12% SDS separating gels were made with 4% SDS stacking gel. Ammonium persulphate was made up fresh and TEMED added last. Isopropanol was added to the top of the separating gel while it set to level it and was washed away with deionized water before adding the stacking gel. 5 µl of 4 x Laemmli buffer was added to 15 µl of protein sample and heated to 70°C for 10 min. Samples were loaded into the gel with a prestained protein ladder (Fermentas) and 1 x SDS running buffer was added. 150 V was applied for 1 h.

2.2.3.8 Western Blots

Western blots were performed on the SDS-PAGE gels. Protein was transferred onto nitrocellulose membranes which were then blocked with PBS and 5% milk powder (Marvel) for 1 h at room temperature with agitation. The membranes were then washed with phosphate buffered saline with 0.2% Tween 20 (PBST). PBS with 5% milk powder was again added with a 1:5,000 mouse anti-His₆ monoclonal antibody (Serotec). The membranes were incubated for 1 h before washing with PBST. PBS with 5% milk powder and 1:5,000 secondary rabbit antibody were added and the gels incubated for 1 h. The gels were washed and EZ-ECL chemiluminescence solution (Biological Industries) was added. The gels were exposed to white light for 15 min and viewed with a Chemidoc system (UVItech). In order to compare between Western blots, ImageJ software was used.

2.2.3.9 Silver stain

Silver stains were performed on SDS page gels using reagents from a SilverQuest kit (Invitrogen). The gel was rinsed in ultrapure water and fixed in 100 mL fixative (40% v/v ethanol, 10% v/v acetic acid) overnight at 4°C. The fixative was decanted and the gel washed in 30% v/v ethanol for 10 min. The ethanol was decanted and the gel washed in 100 mL sensitizing solution for 10 min. This was decanted and the gel washed in 30% v/v ethanol for 10 min. The gel was then washed in 100 mL ultrapure water for 10 min. The water was decanted and 100 mL staining solution was added for 15 min. This was decanted and the gel washed in ultrapure water for 20 – 60 s and the water decanted. The gel was

incubated in 100 mL developing solution for 4 – 8 min until bands appeared. Once the desired band intensity had been reached, 10 mL stopper solution was added. The gel was agitated gently for 10 min then washed in ultrapure water for a further 10 min.

2.2.4 Radioligand binding

100 µg of membrane protein was used in all radioligand binding reactions. Tubes were set up for total and non-specific binding for each sample. Total binding determination comprised 50 nM ZM241385 labelled with tritium ($[^3\text{H}]$ ZM241385), 1 µL adenosine deaminase (0.1u), 100 µg membrane protein and binding buffer up to 275 µL. Non specific determination were set up comprising 50 nM $[^3\text{H}]$ ZM241385, 10 nM unlabeled ZM241385, 1 µL adenosine deaminase, 100 µg membrane protein and binding buffer up to 275 µL. Binding curves were performed with 100 µg of membrane protein with increasing concentrations of radiolabeled ligand. The concentrations of ligand are shown in Table 2.3.

Table 2.3: $[^3\text{H}]$ ZM241285 concentrations used to produce a binding curve for $A_{2a}R$ in the presence of antifoams

Concentration of $[^3\text{H}]$ ZM241285	Volume of $[^3\text{H}]$ ZM241285 (µl)	Volume of 10 nM ZM241385 (µl)	Membrane protein (µg)	Binding Buffer
0.5 nM	1 µl of 50 nM stock	10	100	Up to 100 µl
1 nM	2 µl of 50 nM stock	10	100	Up to 100 µl
2.5 nM	6 µl of 50 nM stock	10	100	Up to 100 µl
5 nM	10 µl of 50 nM stock	10	100	Up to 100 µl
10 nM	0.5 µl of 1 µM stock	10	100	Up to 100 µl
20 nM	1 µl of 1 µM stock	10	100	Up to 100 µl

The tubes were then incubated at room temperature for 1 h 30 min then centrifuged at 14,000 rpm for 5 min. The pellets were rinsed with 500 µL water twice then 100 µL solouene added in a fume cupboard. The tubes were incubated at room temperature overnight. 1 mL scintillation fluid was dispensed into the tubes and they were mixed by vortexing. Each tube was placed into a counting vial and loaded onto the radioactivity counter. Non specific counts were subtracted from the total counts to obtain the counts for each sample.

2.2.5 Bartsch Test

The Bartsch test was conducted following a protocol adapted from that outlined by Denkov *et al*[185]. A 500 mL graduated glass cylinder was filled with 166 mL BMMY medium and in all cases except for the control, the appropriate antifoam was added to 0.001% v/v and 1% v/v. The cylinder was sealed using parafilm and shaken up and down ten times. The height of the foam was recorded every 30 s for 15 min and repeated 5 times for each antifoam. The activity of the antifoam agent was determined by measuring the total volume of the whole system and subtracting the volume of media.

2.2.6 Mass spectrometry

2.2.6.1 Lipid extraction

Lipid was extracted from *P. pastoris* cells using the method outlined by Spickett *et al* 2001[186]. Cultures were centrifuged at 14,000 rpm and the supernatant discarded. The cell pellets weighed approximately 40 mg. 0.5 mL methanol at 50°C was added to the pellets and the cells incubated in a sonicating water bath for 15 min. 0.5 mL chloroform was then added and the cells sonicated for a further 15 min. 0.5 mL of 0.88% KCl was added and the mixture was vortexed. The cells were centrifuged at 14,000 rpm for 2 min to separate the organic and aqueous layers. The organic layer at the bottom of the tube was transferred to a fresh tube and dried under a stream of nitrogen gas. The lipid extracts were stored at -80°C until analysis.

2.2.6.2 Electrospray mass spectrometry

An AB Sciex QTrap 5500 mass spectrometer was used to analyze yeast lipid samples with Analyst software. 500 µL 100% methanol was added to each dried lipid sample and a 1:1,000 dilution performed with methanol. Settings were a 5 µL/min flow rate of sample with a syringe diameter of 3.26 mm. An ion spray voltage of 5,500 V was used for samples read in positive scanning modes and -4,500 V for samples read in negative scanning modes. Precursor ion and neutral loss scanning modes were used.

3. THE EFFECT OF ANTIFOAMS UPON SMALL SCALE RECOMBINANT PROTEIN PRODUCTION BY *P. PASTORIS* IN SHAKE FLASKS

Antifoams are commonly added to cultures in both shake flasks and bioreactors without first investigating the effects they could exert upon the cells and the proteins being produced. There has never been a systematic study of these effects upon cells producing recombinant protein, especially at concentrations above 0.1%. In order to establish whether antifoams affect recombinant protein yield in *P. pastoris* X33 cultures, and if so to investigate the underlying mechanisms, a small scale experimental system was chosen to evaluate the effects of the antifoam. Recombinant protein production was carried out in *P. pastoris* under the control of the methanol-inducible *AOXI* promoter. The 20 mL BMMY cultures were grown in 100 mL non-baffled flasks in the presence of five different antifoams at concentrations of 0% - 1%. Experiments were carried out at 30°C in triplicate for each of the conditions examined.

3.1 Target protein selection

Several protein targets were considered for small scale investigation of the effects of antifoams in shake flasks, some of these being pre-existing strains (green fluorescent protein, adenosine _{2a} receptor, erythropoietin, CHLG, β 1-AR and the aquaporins) and others being strains generated in this project (human growth hormone, receptor component protein).

3.1.1 Green fluorescent protein

Green fluorescent protein (GFP) is an ideal protein for investigation as it is simple to assay and is stable at a range of temperatures and pH. The construct created by Dr William Holmes[36] was used for all antifoam experiments and is shown in Fig 3.1.

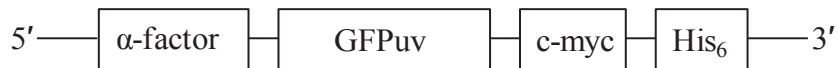


Figure 3.1: Schematic for X33-GFP construct

pPICZαA-GFPuv construct with AOX1 promoter(not shown) upstream of the α-factor, GFPuv and c-myc and His₆ tags[36].

The construct was created using the pPICZαA vector with the GFPuv sequence inserted into the multiple cloning site (*EcoRI* and *XbaI*). An α-factor is on the amino-terminus (N-terminus). On the carboxy-terminus (C-terminus) is a c-myc tag and a His₆ tag. The vector was transformed into *P. pastoris* X33 cells[36] to create strain X33-GFP.

3.1.2 Erythropoietin

The erythropoietin (EPO) construct was created by Dr Nagamani Bora, Aston University. The DNA was transformed into *P. pastoris* X33 cells[187] and denoted X33-EPO. A schematic illustrating the construct is shown in Fig 3.2.

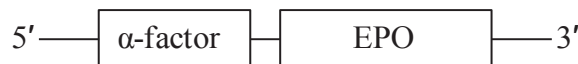


Figure 3.2: Schematic for X33-EPO construct

EPO construct designed by Dr. Nagamani Bora with an α-factor upstream of the EPO coding sequence.

3.1.3 β 1-AR

The β 1-AR construct was designed by Dr. Antoine Gautier (Laboratory of Molecular Biology, Cambridge) and was created in the pPIC9K expression vector and was transformed into SMD1163 cells and denoted SMD- β 1-AR. A schematic illustrating the construct is shown in Fig 3.3.

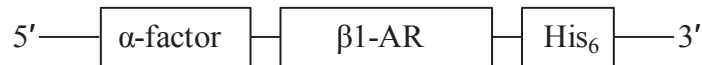


Figure 3.3: Schematic for SMD- β 1-AR construct

Design of the β 1-AR construct created by Dr. Antoine Gautier. An α -factor is upstream of the β 1-AR coding sequence with a His₆ tag downstream of the sequence.

3.1.4 Adenosine_{2a} receptor

The adenosine_{2a} receptor (A_{2a}R) construct used was designed by Fraser[76] and is a glycosylation deficient version of the protein with the N-linked glycosylation site at Asn154 mutated to a Gln. The A_{2a}R protein is tagged with a FLAG tag and His₁₀ tag on the amino-terminus. It was transformed into *P. pastoris* SMD1163 cells to create strain SMD-A_{2a}R. The design is shown in Fig 3.4.

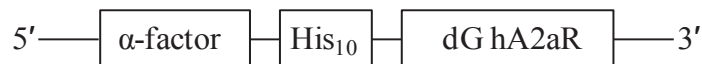


Figure 3.4: Schematic for SMD-A_{2a}R construct

Glycosylation-deficient A_{2a}R sequence designed by Fraser. Consists of an α -factor, FLAG and His₁₀ tag. The cassette was cloned into the multiple cloning site of the pPICZ α A construct. Image adapted from Fraser 2006[76].

3.1.5 Aquaporins

The aquaporin 7 construct was designed by Dr. Kristina Hedfalk, Göteborg University. It was created in the pPICZ α B expression vector and transformed into *P. pastoris* X33 cells to create strain X33-AQP7. The design is shown in Fig. 3.5.



Figure 3.5: Schematic for X33-AQP7 construct

X33-AQP7 sequence designed by Dr. Kristina Hedfalk. An α -factor is upstream of the AQP sequence and His₆ and c-myc tags downstream of the sequence.

3.1.6 CHLG

The CHLG construct was designed by Dr. Mohammed Jamshad, Aston University, and the CHLG sequence was transformed into the pPICZB vector (*KpnI* and *NotI*) and denoted X33-CHLG. It consists of a Kozak sequence upstream of the CHLG sequence and a His₆ tag and a stop codon downstream as shown in Fig. 3.6.



Figure 3.6: Schematic for X33-CHLG construct

Construct design of CHLG in pPICZB. Consists of a Kozak sequence upstream of the CHLG sequence and His₆ tag and stop codon downstream.

The maxiprep DNA obtained from Dr. Mohammed Jamshad was transformed into *P. pastoris* X33 electrocompetant cells to create strain X33-CHLG. Western blots with anti-His antibodies carried out upon membrane preparations from shake flask samples induced for 48 h in BMMY. Faint bands were observed between 25 and 35 kDa suggesting the presence of the 30 kDa CHLG.

3.1.7 Receptor component protein construct

A construct containing the receptor component protein, RCP, tagged with GFP for ease of detection with a TEV recognition site to allow cleavage of the RCP from the tags was designed in collaboration with Dr James Barwell, Aston University. The full sequence is given below for the multiple cloning site showing the positions of *AOXI*, RCP-TEV-GFP, c-myc tag and His₆ tag in Fig. 3.7. The expression vector pPICZ α B was used and the RCP sequence inserted into the multiple cloning site.

A)



B)

Coding sequence for pPICZ α B-RCP-TEV-GFP:

```

ATGAGATTTCCTCAATTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAG
ATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTTGCTGTTTTGCCAT
TTTCCAACAGCACAAATAACGGGTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAAAGAAGAAGGGGTATCTC
TCGAGAAAAGAGAGGCTGAAGCTGCAGGAATTCTTATGGAAGTGAAGGATGCCAATTCTGCGCTTCTCAGTAACTACGAG
GTATTTCACTACTAACTGATCTGAAAAGAGCAGCGTAAAGAAAAGTGGAAAGAATAAACACAGCTCTGGGCAACAGAACTT
GAACACTATCACCTATGAAACGTTAAAATACATATCAAAAAACACCATGCAGGCACCAGAGTCTGAAAATTGTCAGAGAAT
TTCTCACAGCATTGAAAAGCCACAAGTTGACCAAAAGCTGAGAAGCTCCAGCTGCTGAACCACCGCCTGTGACTGCTGTG
GAGATCCAGCTGATGGTGAAGAGAGTGAAGAGCGGCTCACGGAGGAGCAGATTGAAGCTTCTCCACACCGTCACCA
GCATTCGCTGCAGAGCCAGAGGCTGAGCAGAAGAAGAATACAAACAGCAATGTGGCAATGGACGAAGAGGACCCAGC
AGCGGCCAGCCGGCCGCTCTCGGATCGGTACCTCGAAAATCTTTATTTCAAGGTCCGCGGGCGCCGCTTATGAGTAAAGG
AGAAGAACTTTTACTGGAGTTGTCCCAATCTTGTTGAATTAGATGGTATGTTAATGGGCACAAATTTCTGTCACTGG
AGAGGGTGAAGGTGATGCAACATACGGAAAACCTACCCTAAATTTATTTGCACTACTGGAAAACCTACCTGTTCCATGGC
AACACTTGCTACTACTTCTCTTATGGTGTTCATGCTTTTCCCGTTATCCGGATCATATGAAACGGCATGACTTTTCAAG
AGTGCCATGCCCGAAGGTTATGTACAGGAACGCCTATATCTTCAAAAGATGACGGGAACTACAAGACGCGTGTGAAGT
CAAGTTTGAAGGTGATACCCTTGTTAATCGTATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGAAAACATTCTCGGACA
CAAACCTCGAGTACAACATAACTCACACAATGTATACATACGGCAGACAAAACAAAAGAATGGAATCAAAGCTAACTTCA
AAATTCGCCACAACATTGAAGATGGATCCGTTCAACTAGCAGACATTATCAACAAAATCTCCAATTGGCGATGGCCCT
GTCCTTTTACCAGACAACCATTACCTGTCGACACAATCTGCCCTTTTGGAAAGATCCCAACGAAAAGCGTGACCACATGGTC
CTTCTTGAGTTTGTAACTGCTGCTGGGATTACACATGGCATGGATGAGCTCTACAAAATTCTA GAACAAAAACTCATCTCA
GAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATCAT
  
```

AOX1 RCP TEV GFP c-myc 6x His

Translated:

```

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLFPFSNSTNNG
LLFINTTIIASIAAKEEGVSLEKREAEAAGILTEVKDANSALLSNYEVFQLLTDLKEQRKESG
KNKHSSGQQNLNTITYETLKYISKTPCRHQSPDIVREFLTALKSHKLTKAEKLLQLLNHRPVT
AVEIQLMVEESEERLTEEQIEALLHTVTSILPAEPEAEQKKNTNSNVA MDEEDPA AAQPAV
SDRYL ENLYFQC PRRPL MSKGEELFTGVVPILEVELDGDVNGHKFSVSGEGGDATYGKLT
KFICTTGKLPVWPPTLVTTFSYGVQCFSRYPDH MKRHDFKSA MPEGYVQERTISFKDDGN
YKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVIYITADKQKNGIKANFK
IRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHVLSLSTQSALS KDPNEKRDM VLLEFVTA
GITHGMDELYK ILE QKLISEEDLNSAVD HHHHHH
  
```

Figure 3.7: pPICZ α B-RCP-TEV-GFP construct design

A) Schematic illustrating position of the α -factor, RCP, TEV, GFP, c-myc and His₆ tags. B) Sequence for the coding region cloned into the SfiI and EcoRI sites of the pPICZ B vector.

A schematic illustrating the cloning strategy for pPICZ α B-RCP-TEV-GFP is show in Fig. 3.8.

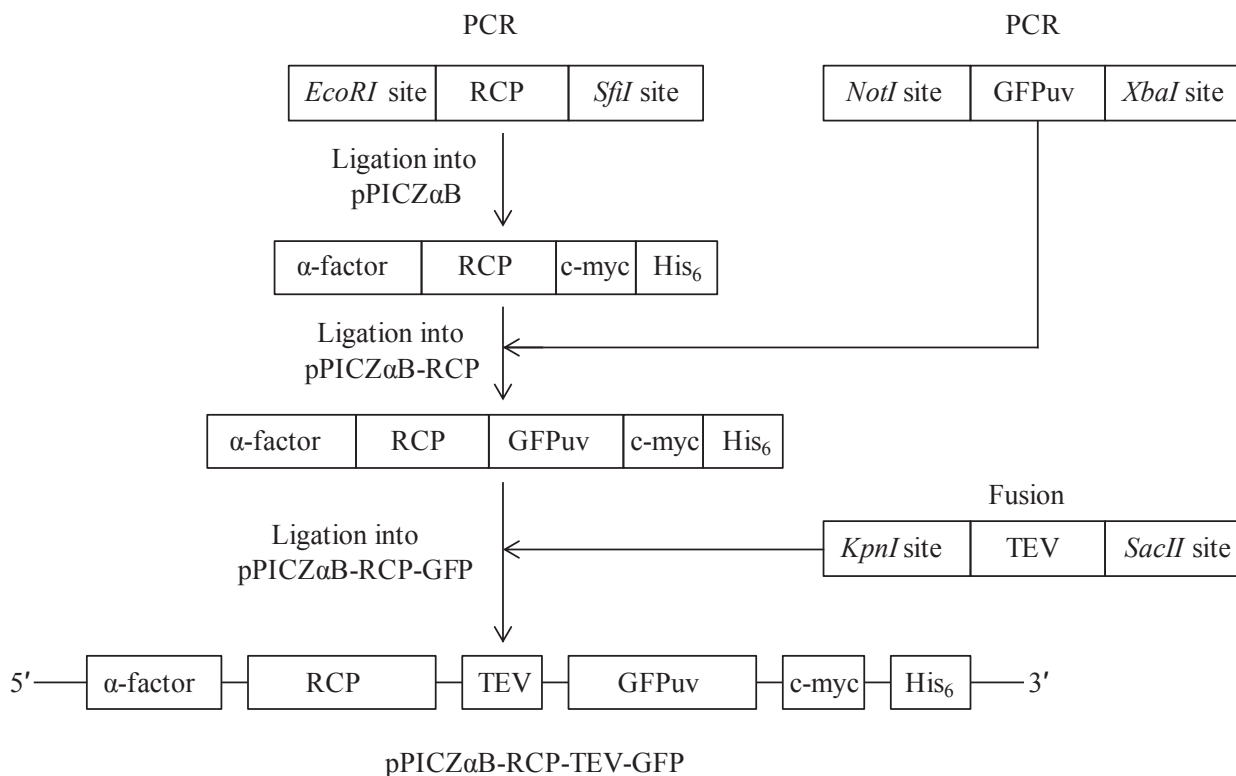


Figure 3.8: Schematic outlining the cloning strategy for the creation of pPICZ α B-RCP-TEV-GFP

PCR was used to amplify RCP and GFPuv sequences with restriction sites. The sequences were ligated into the pPICZ α B vector. TEV primers were fused and the sequence was ligated into the pPICZ α B-RCP-GFP construct to create pPICZ α B-RCP-TEV-GFP.

The RCP sequence was initially amplified using PCR with the forward and reverse RCP primers (Chapter 2), with *EcoRI* and *SfiI* restriction sites. An agarose gel, illustrated in Fig 3.9A, showed a band at just below 500 bp. As the PCR product was expected to be 450 bp this suggested that the RCP sequence had been successfully amplified and restriction digests followed by ligation reactions were performed on the insert and vector to introduce RCP into pPICZ α B. The vector was then re-digested with *SfiI* and *EcoRI* to determine whether the insert had been ligated into the vector. The agarose gel shown in Fig 3.9B shows the insert at just under 500 bp, suggesting that the insert had been successfully ligated.

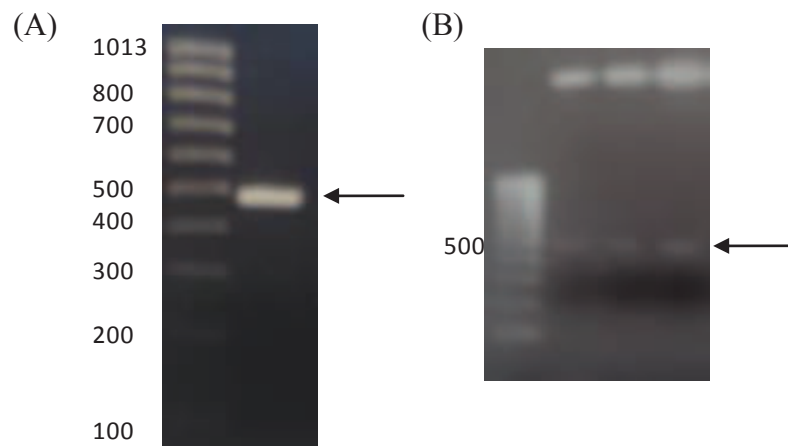


Figure 3.9: Agarose gels showing the amplified RCP sequence at approximately 450 bp (A) and the insert shown after re-digestion of the vector to check for successful ligation in three independent samples (B) Molecular mass markers are indicated in bp.

The GFPuv sequence was amplified by using PCR using primers with *Not I* and *Xba I* restriction sites. The agarose gel for the reaction is shown in Fig. 3.10 and shows the expected GFP sequence at 700 bp. The GFPuv sequence was inserted by digestion of the insert and pPICZαB-RCP vector with *NotI* and *XbaI*, followed by ligation. The vector was then re-digested with *Not I* and *Xba I* to determine whether the insert had been ligated into the vector.

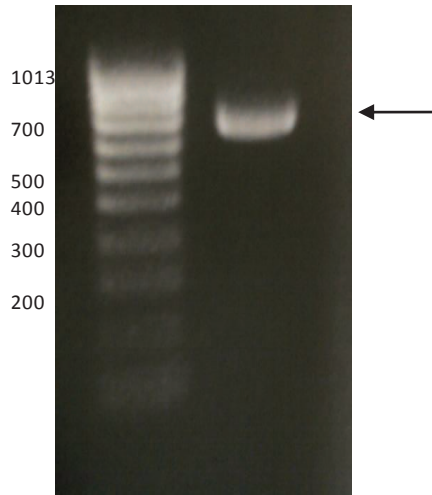


Figure 3.10: Agarose gels showing PCR amplification of GFP

Amplification of GFP_{uv} at 700 bp. Molecular markers are shown in the first wells and indicate the size of the fragment in bp.

A TEV recognition site to allow cleavage of the RCP from the GFP and His₆ tags following purification was created by fusing two primers with *KpnI* and *SacII* restriction sites. The fused TEV primers were inserted by digestion of the pPICZαB-RCP-GFP vector and insert followed by ligation.

This DNA was then transformed into XL10 Gold competent *E. coli* cells by heat shock to amplify the material. The cells were grown on LB agar with increasing concentrations of zeocin and colonies picked from the plates and grown in LB medium overnight. The DNA was extracted and sequenced using Birmingham University's Functional Genomics and Proteomics Unit's DNA sequencing service. The sequence alignment can be found in Appendix 8.1. Once the desired sequence had been verified, the DNA was transformed first into electrocompetant *P. pastoris* X33 cells, however due to poor colony growth, the DNA was then transformed into electrocompetant SMD 1163 *P. pastoris* cells to create strain SMD-RCP-TEV-GFP. Colony screening was performed using the Microbioreactor 24.

3.1.8 Human growth hormone

A construct containing human growth hormone (HGH) was created based on the construct and procedure designed by Calik *et al* 2008[152]. The construct consists of a His₆ tag on the amino-terminus, Factor Xa, HGH, followed by a stop codon shown below. The Factor Xa allows cleavage of the tags for the isolation of pure HGH. The design is shown in Fig 3.11.

The HGH sequence was amplified from vector pHGH1 (patent 4,342,832) purchased from the American Tissue Culture Collection (ATCC) by performing a two-step PCR reaction. The first reaction (Fig 3.13A) introduced His₂ and Factor Xa onto the 5' end followed by HGH, a stop codon and an *XbaI* site. The second PCR reaction added an *EcoRI* recognition site and His₄. The HGH forward and reverse primers were used (Chapter 2).

A)



B)

Coding sequence for pPICZ α A-HGH:

```

ATGAGATTTCCTTCAATTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAG
ATGAAACGGCACAATAATCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTCGATGTTGCTGTTTTGCCAT
TTTCCAACAGCACAAATAACGGGTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAAAGAAGAAGGGGTATCTC
TCGAGAAAAGAGAGGCTGAAGCTGAATTCACCATCACCATCACCATATTGAAGGGAGATTCCCAACTATACCACTATCT
CGTCTATTCGATAACGCTATGCTTCGTGCTCATCGTCTTCATCAGCTGGCCTTTGACACCTACCAGGAGTTTGAAGAAGCC
ATATCCCAAAGGAACAGAAGTATTCATTCTGCAGAACCCCCAGACCTCCCTCTGTTTCTCAGAGTCTATCCGACACCCT
CCAACAGGGAGGAAAACACAACAGAAATCCAACCTAGAGCTGCTCCGCATCTCCCTGCTGCTCATCCAGTCGTGGCTGGAG
CCCGTGCAGTTCCTCAGGAGTGTCTTCGCCAACAGCCTAGTGTACGGCGCCTCTGACAGCAACGCTATGACCTCCTAAAG
GACCTAGAGGAAGGCATCCAAACGCTGATGGGGAGGCTGGAAGATGGCAGCCCCGGACTGGGCAGATCTCAAGCAGA
CCTACAGCAAGTTCGACACAACTACACAACGATGACGCACTACTCAAGAACTACGGGCTGCTCTACTGCTTCAGGAAG
GACATGGACAAGGTCGAGACATCTCGCATCGTGCAGTGCCGCTCTGTGGAGGGCAGCTGTGGCTTCAGATTCTAGA
ACAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATCAT

```

AOX1 6xHis Factor Xa HGH Stop c-myc

Translated:

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MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPPFSNSTNNG
LLFINTTIIASIAAKEEGVSLEKREAEA EFHHHHHH EGR FPTIPLSRLFDNA MLRAHRLHQI
AFDTYQEFEEAYIPKEQKYSFLQNPQTS LCFSESIPTPSNREETQKQSNLELLRISLLLIQSW
LEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEGIQTL MGRLEDGSPRTGQIFKQYKSKFD
TNSHNDDALLKNYGLLYCFRKD MDKVETFLRIVQCRSVEGSCGF Stop ILEQ KLISEEDI NSA
VD HHHHHH

```

Figure 3.11: pPICZ α A-HGH construct design

A) Schematic illustrating position of the α -factor, Factor Xa, HGH and stop codon. B) Sequence for the multiple cloning site of pPICZ A vector containing HGH.

As the PCR product was expected to be approximately 613 bp and bands were seen between 600 and 700 bp on both agarose gels, this suggested that the HGH sequence had been successfully amplified in both reactions and restriction digests followed by ligation reactions were performed on the insert and vector to introduce HGH into pPICZαB. The DNA gels for the PCR reactions are shown in Fig. 3.12B and C.

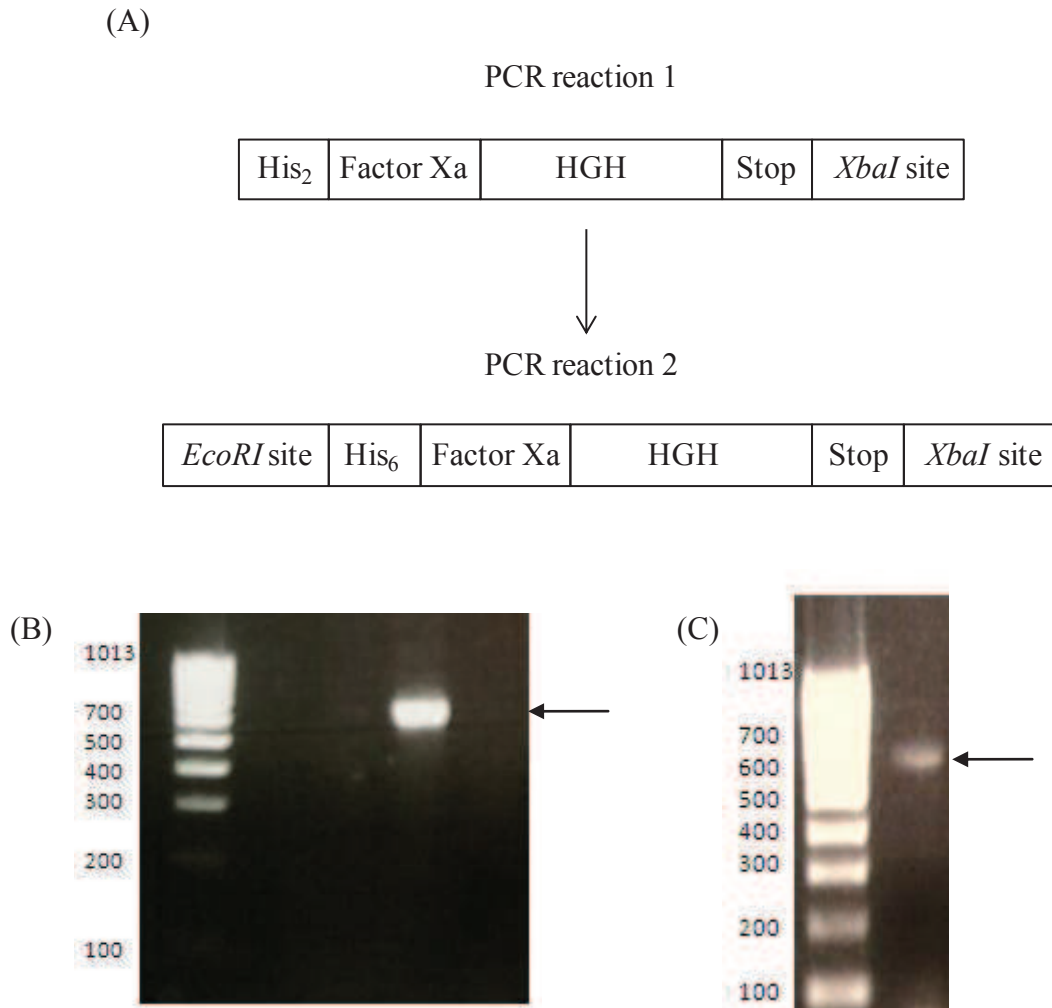


Figure 3.12: PCR strategy for HGH amplification and agarose gels

(A) Two-step PCR reactions to amplify HGH. The first reaction added His₂ and a Factor Xa site to the 5' end and a stop codon and XbaI site to the 3' end. The second reaction added an EcoRI site and His₆ to the 5' end. Adapted from Calik et al 2008[152]. Agarose gels showing the PCR reactions for (B) the first step and (C) the second step amplifying the HGH sequence and adding a His₆ tag and Factor Xa site with EcoRI and XbaI restriction sites. Molecular markers are indicated.

The vector was then re-digested with *EcoRI* and *XbaI* to determine whether the insert had been ligated into the vector. The agarose gel shown in Fig 3.13 shows the insert at 600 bp, suggesting that the insert had been correctly ligated.

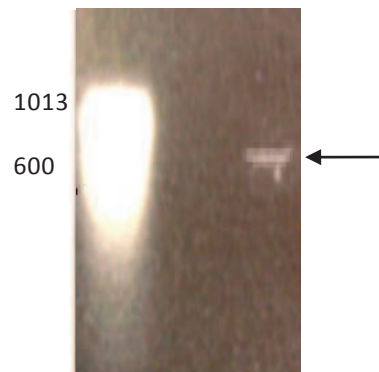


Figure 3.13: Agarose gel showing PCR amplification of HGH

The HGH insert shown at approximately 600 bp after re-digestion of the vector to check for successful ligation. A molecular marker in the left hand well indicates the size of the fragment.

This DNA was then transformed into XL10 Gold competent *E. coli* cells by heat shock to amplify the material. The cells were grown on LB agar with increasing concentrations of zeocin and colonies picked from the plates and grown in LB medium. The DNA was sequenced at Birmingham University. The sequence alignment can be found in Appendix 8.2. Once the desired sequence had been verified, the DNA was transformed into electrocompetant SMD1163 *P. pastoris* cells to create strain SMD-HGH.

3.2 Target protein implementation

The GFP, EPO and A_{2a}R constructs were taken forward for antifoam investigations. Each of these proteins have reliable quantification methods and are measured in a different manner. GFP is simple to assay by fluorimetry, and EPO can be detected by ELISA or immunoblotting techniques. Differences in the concentration of A_{2a}R can be measured by radio ligand binding, and the effect of the antifoams upon activity of this protein can also be determined. GFP and EPO are soluble proteins, while tests upon A_{2a}R allow analysis of

the effects of antifoams upon a membrane protein. In contrast it was concluded that the aquaporins are difficult to assay and a Western blot would be relied upon for quantification. The same is true for HGH. The RCP-GFP construct is large as it contains a fusion protein. Therefore, on the basis of a stable strain, good assay and ease of growth, the GFP, EPO and A_{2a}R constructs were taken forward for antifoam evaluations. In the first instance, GFP was used to establish all experimental procedures.

3.2.1 Growth characteristics of *P. pastoris* strain X33-GFP in BMGY medium

In order to ensure cells were actively growing in log phase prior to any experimental determination, three separate colonies were analyzed. *P. pastoris* X33-GFP from two different plates and a glycerol stock was streaked onto fresh YPD plates denoted a, b and c, respectively. Colonies from each were used to inoculate BMGY and growth curves were monitored over 44 h to determine the growth profiles of each colony as shown in Fig. 3.14.

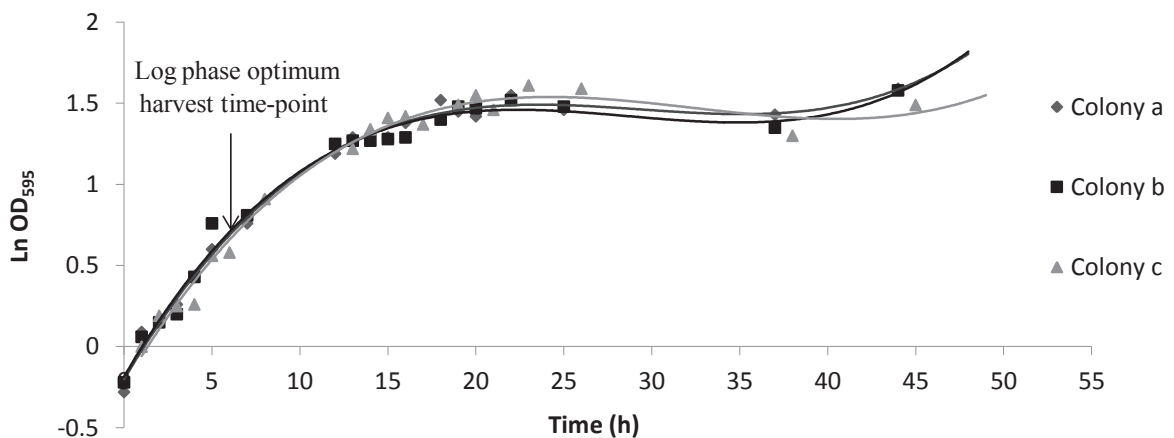


Figure 3.14: Growth curve for *P. pastoris* in BMGY medium

Growth curves for colonies a, b and c monitored over 44h. The natural log of the OD at 595 nm was plotted against time.

As suggested by Fig. 3.14, the optimum time point for transfer of the colonies to BMMY is between 5 and 10 h while they are in log phase and this information was taken into account when cultures for the following investigations were set up.

3.3 Antifoams

Five antifoams were selected for evaluation based on their widespread use and range of characteristics. They are usually added to fermentation processes in minimal quantities[188, 189], therefore concentrations above 0.1% were examined in this study. Information about each of the antifoams evaluated is given in Table 3.1 The precise contents of each of the agents is known only by the manufacturers, and attempts to use mass spectrometry to gain more information were unsuccessful due to their chemical characteristics. Samples of J673A were sent to Alta Bioscience based at Birmingham University, however a peak was not obtained with the MALDI-TOF equipment.

Table 3.1: Compositions of antifoams for evaluation

Antifoam	Composition	Manufacturer
Antifoam A	30% emulsion of silicone polymer	Sigma
Antifoam C	30% emulsion of silicone polymer	Sigma
J673A	Alkoxylated fatty acid ester on a vegetable base	Struktol
P2000	Polypropylene glycol	Fluka
SB2121	Polyalkylene glycol	Struktol

Antifoams A and C contain silicone polymers, which consist of repeating units of dimethyl silicone[190]. They are both 30% emulsions of the same antifoam concentrate with slight differences in components. P2000 is a polypropylene glycol, which is a polymer of propylene units. An example of these structures are shown in Fig. 3.15.

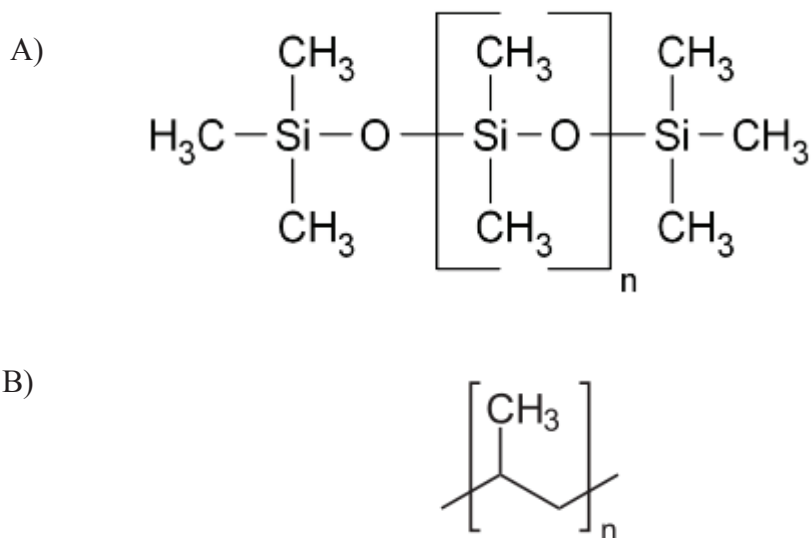


Figure 3.15 Silicone polymer and polypropylene glycol structures

A) The structure of silicone polymers consisting of repeating dimethyl silicone units. Image reproduced from chemwiki.ucdavis.edu[190]. B) The structure of polypropylene glycol. Image taken from <http://commons.wikimedia.org/wiki/File:Polypropylen.svg>. The repeating units are contained within brackets, where n represents the number of repeats.

The antifoams were initially characterized for to their ability to destroy foam, as well as any possible influence upon the viability of the cells at the concentrations used in this study.

3.3.1 The effect of antifoams upon foam destruction

In order to compile a picture of the properties of each antifoam, the original purpose of the agents, foam destruction, was evaluated. Simple methods of determining the ability of antifoams to reduce foam are the Bartsch shaking test[90] and the Ross-Miles pouring test[91]. The Bartsch test involves filling a graduated measuring cylinder with the medium used for the process and adding the antifoam to be investigated. The cylinder is sealed and shaken a set number of times. The foam height is then measured over time and provides a quick and simple method of determining the efficiency of the antifoam agent of destroying the foam. A Bartsch shaking test was conducted with each of the antifoams at 0.001% v/v, which is a low concentration that is often added to bioreactor cultures to reduce foam. The

test was repeated 5 times for each antifoam and the foam height was monitored over 15 min. The data are shown in Fig. 3.16. The test demonstrated that in the absence of an antifoaming agent, initial foam destruction was quick until a stable foam was formed. Foam height reduced slowly and in the 15 min testing time did not reach zero, demonstrated by Fig. 3.16A. The most effective agent for foam reduction was J673A, where less foam was formed after initial shaking, and destruction was rapid. Antifoam A had the least activity of the agents tested, and its related antifoam, Antifoam C, also required several minutes to completely destroy all of the foam present. All antifoams were effective at foam destruction and most foam was destroyed within one minute (Fig. 3.16B). The findings are summarized in Table 3.2.

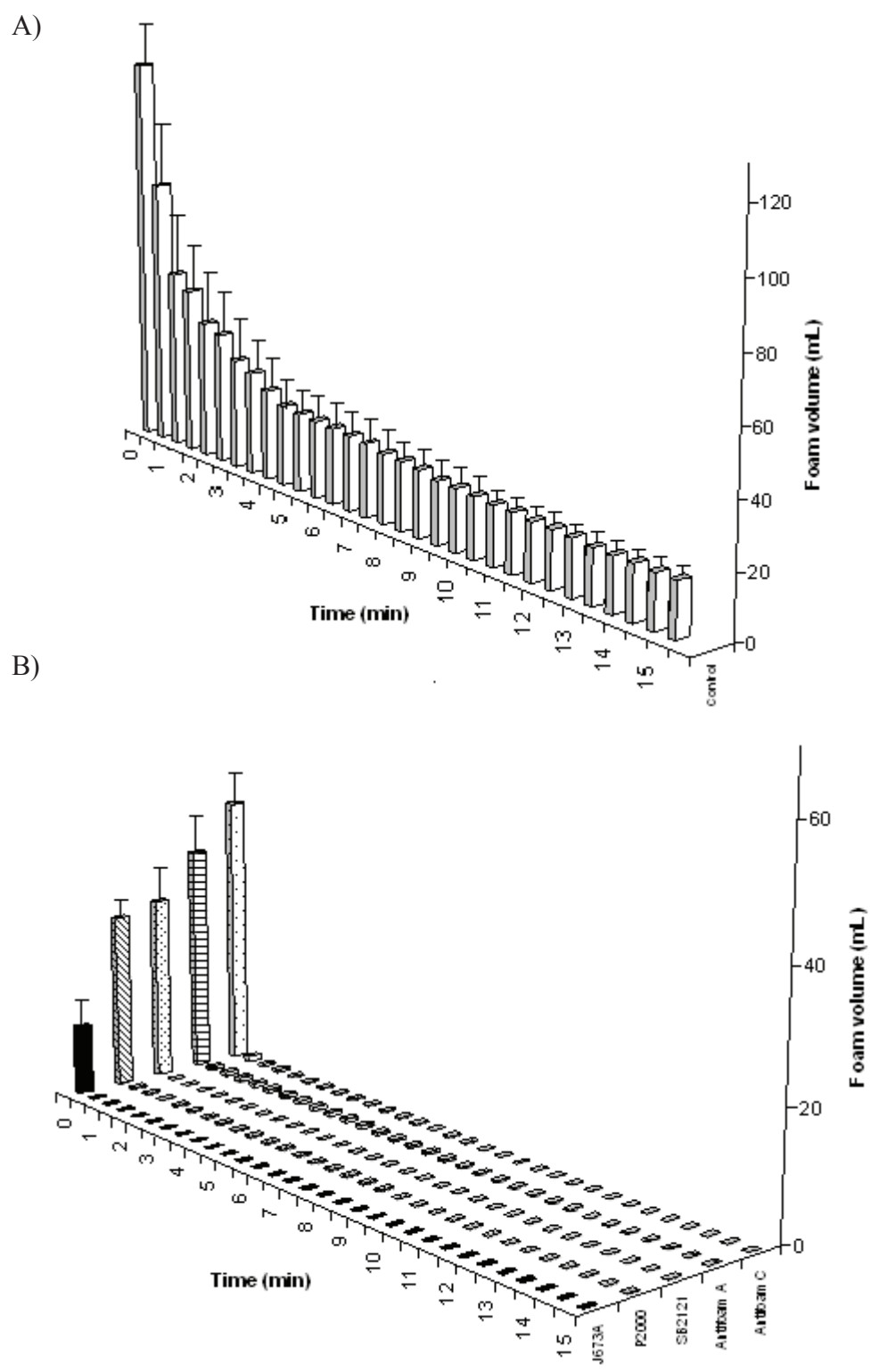


Figure 3.16: Antifoam Bartsch test
 The antifoams were tested for their foam destruction capacity. Foam volume (mL) was recorded for 0% v/v antifoam (A) and 0.001% of each antifoam (B) in BMMY medium over a 15 min time course (n = 5).

Table 3.2.: Summary of Bartsch test findings

Agent	Time for foam destruction < 15 min	Stable foam formed	Description
Control (0% antifoam)	No	Yes	Quick initial reduction but stable foam reduced very slowly
Antifoam A	Yes	No	Rapid foam destruction but least effective agent
Antifoam C	Yes	No	Rapid foam destruction
J673A	Yes	No	Rapid foam destruction, most effective agent with lowest initial foam height
P2000	Yes	No	Rapid foam destruction
SB2121	Yes	No	Rapid foam destruction

A Bartsch test was also conducted with antifoams at 1% v/v and all foam was destroyed within 10 seconds. This investigation demonstrates the effectiveness of each of the agents for their principal use, and differences are apparent in the foam destruction capacity of each type of antifoam. If their mechanisms of action upon foam differ, it is likely that they also exert different effects upon the cultures they are added to.

3.3.2 The effect of antifoams upon cell viability

The effects of antifoams upon the viability of the cells themselves is not widely reported. As the concentrations of antifoams added to the cultures in these investigations are much higher than would normally be used, the influence of these agents upon the viability of the *P. pastoris* cells was evaluated.

3.3.2.1 Capacitance

The effect of antifoams upon capacitance was monitored in shake flasks as a method for assessing the viability of the cells. A Foagale capacitance probe loaned by Applikon Biotechnology Ltd. was used in shake flask cultures. The probe pulses a known charge into

the flask, and if cells are intact, the charge is maintained around their surface and is detected by the probe. If the cells are dead and the surface is disrupted, a difference in charge is detected. In the shake flask set up, the signal was found to be noisy making comparison between conditions difficult. It is likely that the confines of the flask and low levels of media caused interruption of the signals sent to and from the probe and no meaningful data was able to be gathered from this system. Future experiments could be carried out in a bioreactor where the signal might be more stable. An example of some of the data is shown in Fig. 3.17.

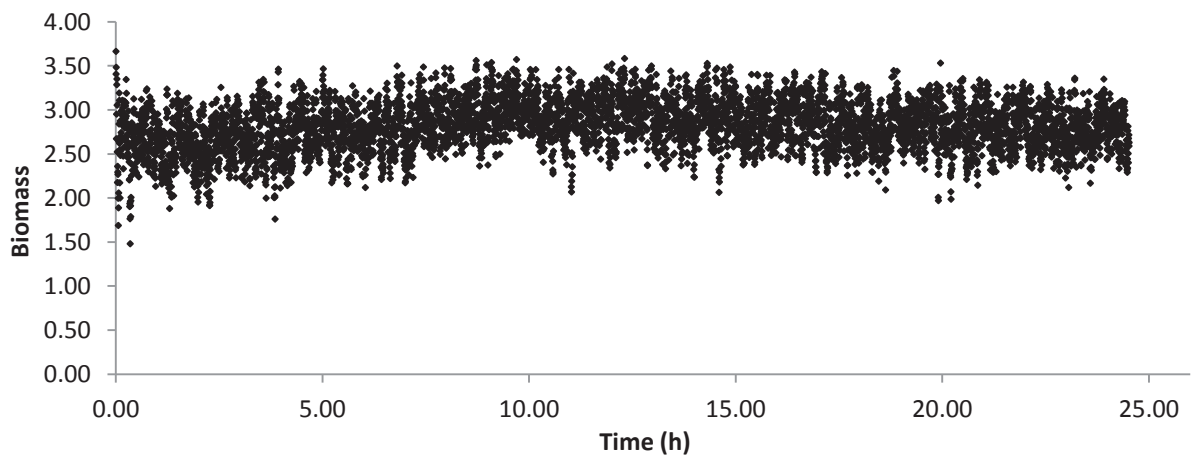


Figure 3.17: Biomass of *P. pastoris* with 0.8% Antifoam C monitored by capacitance probe
*Biomass of *P. pastoris* X33 GFP in BMMY in the presence of 0.8% Antifoam C in a 250 mL shake flask measured by a Fogale capacitance probe over 24 h.*

3.3.2.2 Propidium iodide staining

An alternative method of assessing cell viability was conducted by performing propidium iodide exclusion and flow cytometry. In this assay, dead cells are stained red[191] and appear in population C (Fig. 3.18) while live cells fluoresce green due to GFP production and appear in population B. The *P. pastoris* cells producing GFP were cultured in the 100 mL non-baffled shake flasks to be used for the protein production investigation (section 3.4) in triplicate for each antifoam condition. Samples were taken after 48 h and following a haemocytometer count, were diluted with PBS to be in the region of 10^6 - 10^7 cells/mL. 2

mL of the diluted samples were taken and 10µl propidium iodide added. The data shown in Figure 3.18A, generated using WinMDI software, suggest that there are no dead cells present in cultures containing 0 % antifoam. Fig. 3.18B shows that the same result was obtained in the presence of 0.6 % Antifoam A. This result was seen for all antifoams tested.

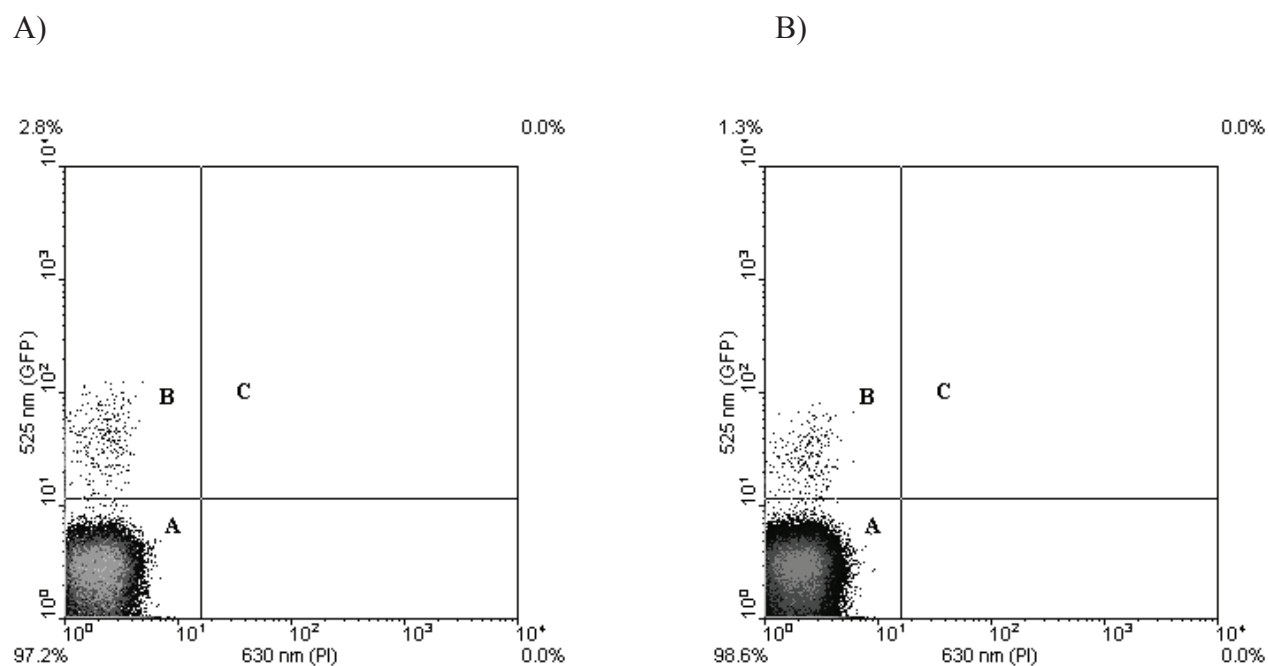


Figure 3.18: Viability of cells determined by propidium iodide staining

Viable cells without antifoam (A) and with 0.6% Antifoam A (as a representative example; B) are shown. Population A, which is not cellular, comprises events that are related to electronic and particulate noise. Population B comprises cells with enhanced green fluorescence due to the expression of GFP. Population C is where any dead cells (stained red with propidium iodide) would be observed.

3.4 The effect of antifoams upon GFP production

The effect of the antifoams upon GFP production by *P. pastoris* was investigated as it is simple to assay and is a robust protein, stable at a range of temperatures and pH. The agents were added at concentrations from 0–1 % v/v for GFP shake flask experiments. These concentrations are higher than the 0.1 % routinely used for de-foaming purposes and it has

been reported previously that antifoams at these concentrations can influence the yield of protein[82]. The total amount of GFP in these 20 mL cultures (the total yield) was measured by fluorimetry 48 h post-induction. Measurements were read in triplicate for three biologically independent experiments (n = 9) and compared to readings for a recombinant GFP standard at known concentrations to calculate the yield of protein. The data were analyzed by a one-way ANOVA ($P < 0.0001$) and a Dunnett's multiple comparison test where * = $P \leq 0.05$ and ** = $P \leq 0.01$.

3.4.1 Antifoams do not induce changes in GFP fluorescence

In order to determine whether any of the antifoams themselves affected the fluorescence of GFP, 1 % antifoam was incubated in BMMY for 48 h to mimic the experimental set-up. This was then spiked with 200 μg , a similar concentration of recombinant GFP standard to that obtained in test cultures. There was no significant difference between the fluorescence of GFP in the presence or absence of any of the antifoams suggesting that they did not influence the sample readings, as shown by Fig. 3.19. The fluorescence values of the antifoams themselves were also measured at 1 % after 48 h and found to be minimal, similar to the buffer control readings.

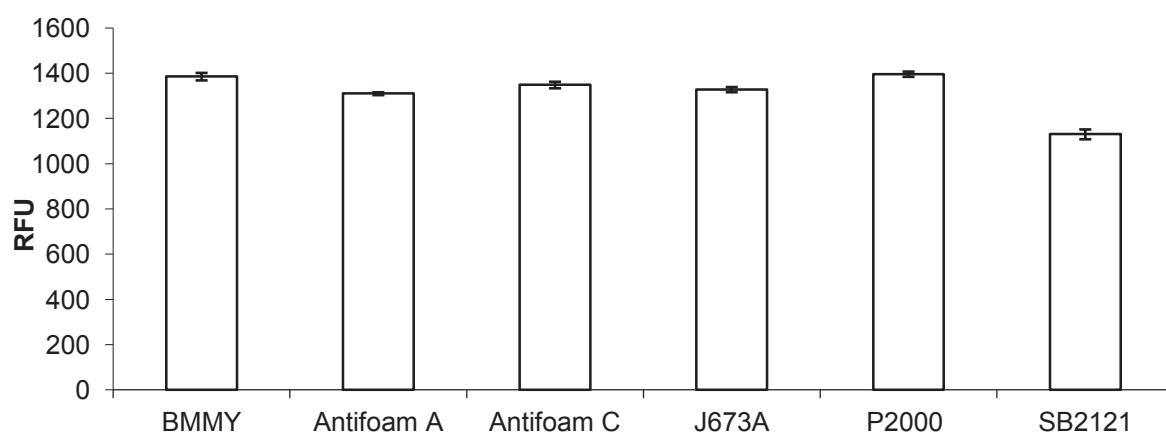


Figure 3.19: Effect of antifoams upon recombinant GFP fluorescence

Effect of antifoams added at 1% v/v upon the fluorescence of 200 μg recombinant GFP standard in BMMY medium after 48 h in triplicate shake flasks. GFP readings were measured using fluorimetry and samples were measured in triplicate for each sample. Error bars represent the standard errors.

3.4.2 Antifoam addition affects total GFP yield in shake flasks

The shake flask data show that all of the antifoams tested increased the total yield of GFP in the 20 mL shake flasks after 48 h above concentrations of 0.6%. The total yield of GFP as a function of Antifoam A addition rose significantly at concentrations of 0.6 % and above (Fig. 3.20A) with no further increases above 1 % (data not shown). A similar but more pronounced trend was observed for Antifoam C (Fig. 3.20B), which is unsurprising since Antifoam C is a 30 % emulsion of the same antifoam concentrate as Antifoam A, but with different non-ionic emulsifiers[192]. Fig. 3.20C shows that addition of 1 % J673A increased the total yield of GFP compared to the control without antifoam from 246 μg to 394 μg , representing one of the largest effects of the antifoams evaluated. At concentrations above 1 %, the total yield of GFP decreased (data not shown). Addition of P2000 (Fig. 3.20D) also resulted in a significant increase in total yield at or above 0.6 %, while addition of SB2121 (Fig. 3.20E) increased total yield at concentrations above 0.4 %. In both cases the largest improvement was obtained on addition of 1 % of the antifoam, again almost doubling the yield. Overall, all five antifoams tested increased the total yield of GFP at concentrations in the range of 0.4–1 % v/v. The highest yield was achieved by adding 1 % P2000 (422 μg GFP) followed by 1 % SB2121 (396 μg GFP), 1 % J673A (394 μg GFP), 0.6 % Antifoam A (373 μg GFP) and 0.8 % Antifoam C (348 μg GFP). All five yields were significantly higher than the corresponding yields from the 0 % control, as shown in Fig. 3.20.

The cultures at the optimum concentrations of antifoam were imaged using a fluorescence microscope (Fig 3.21). There are clear differences in the intensity of GFP present in the cultures compared to the control, especially in the case of 1% J673A, 1% P2000 and 1% SB2121. These cultures appear to demonstrate that there is also a greater amount of intracellular GFP present when compared to the control and Antifoam A and C cultures.

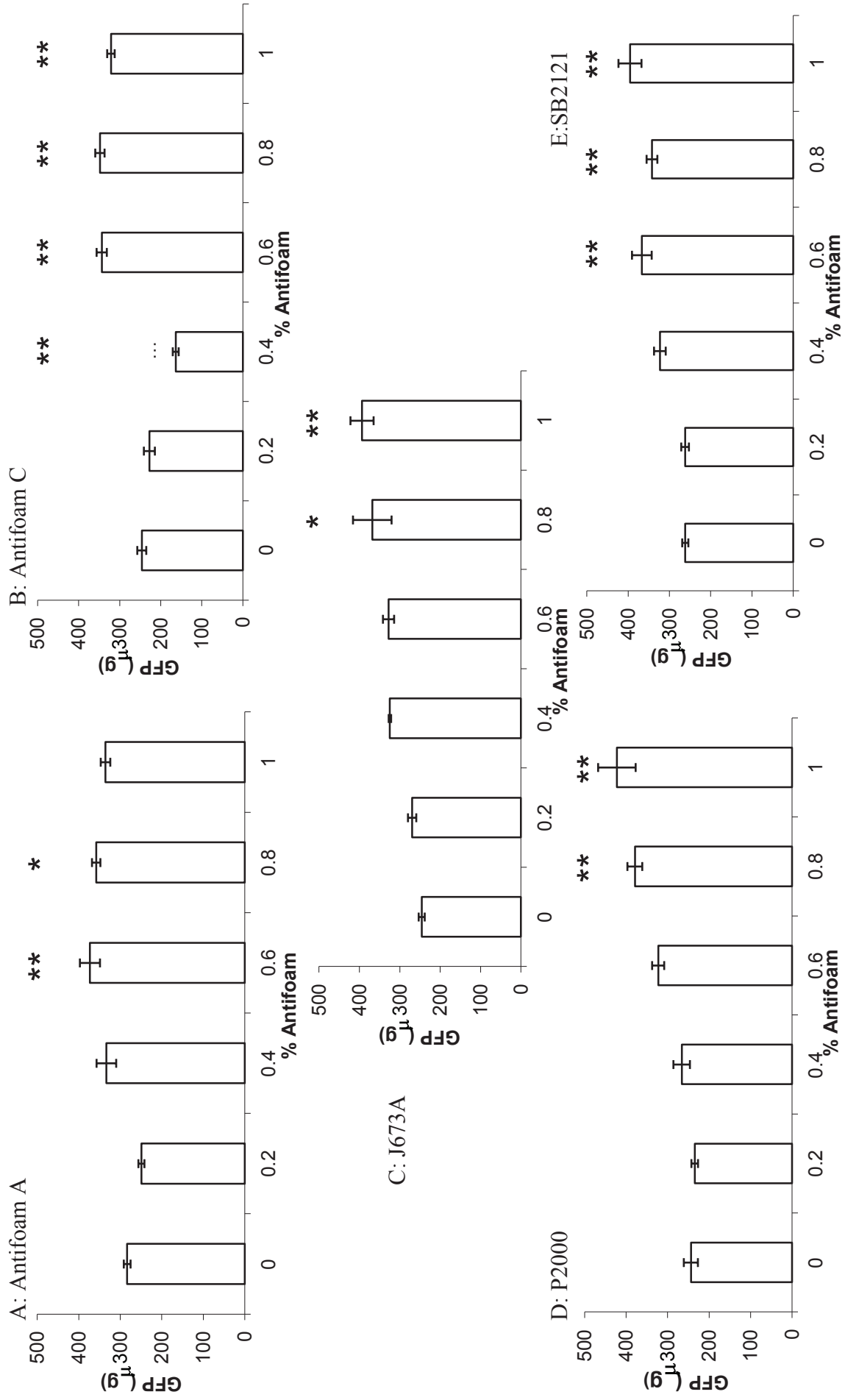
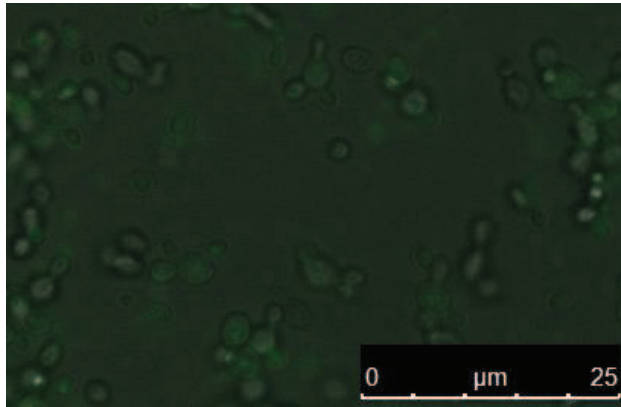
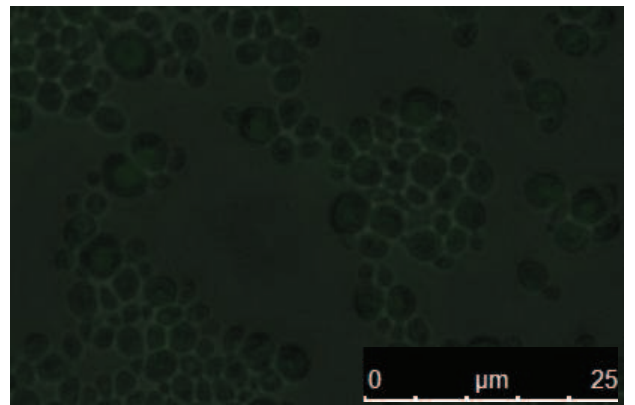


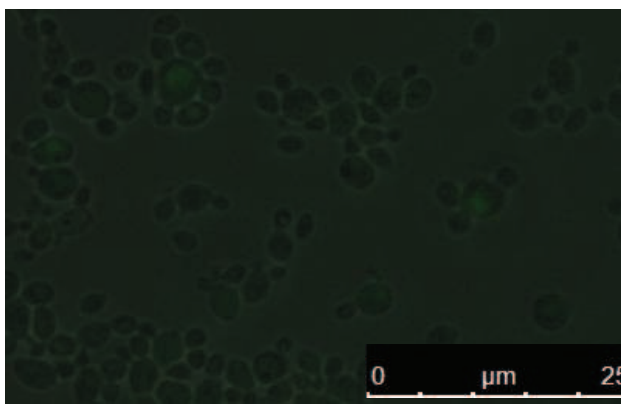
Figure 3.20: Antifoam addition increases the total yield of GFP in 20 mL *P. pastoris* cultures Bar charts showing the total yield of GFP (μg) at 48 h in 20 mL *P. pastoris* cultures following addition of Antifoam A (A), Antifoam C (B), J673A (C), P2000 (D) and SB2121 (E) at concentrations from 0-1%. The error bars show the respective standard errors. In all cases $n = 9$.



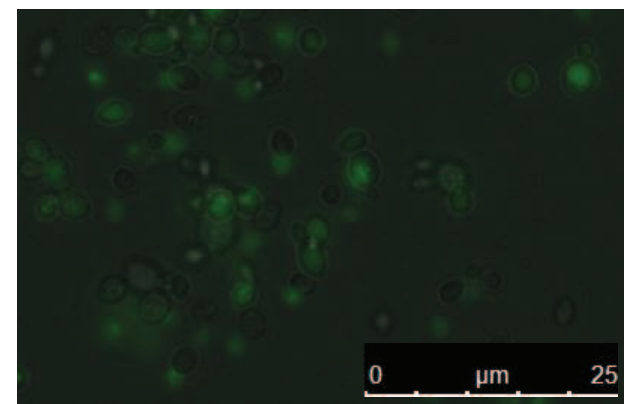
A) 0% Antifoam



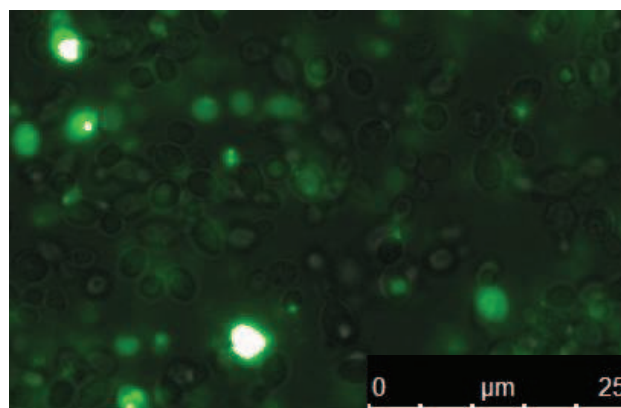
B) 0.6% Antifoam A



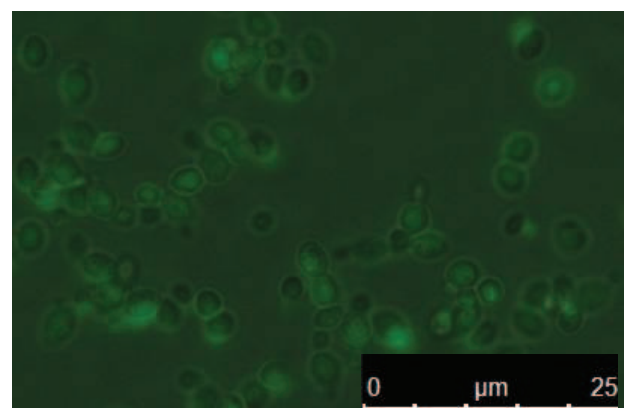
C) 0.8% Antifoam C



D) 1% J673A



E) 1% P2000



F) 1% SB2121

Figure 3.21: Fluorescence microscopy images of *P. pastoris* producing GFP

P. pastoris producing GFP in the presence of antifoams viewed under a fluorescence microscope at 100 x magnification. Both intracellular and extracellular GFP is observed. A Leica Microsystems DMI4000B microscope with a Leica CCD camera and Leica application suite AF software were used.

3.4.3 The effects of antifoam addition are due to changes in culture density for P2000 and SB2121

To account for any changes in the growth characteristics of the cells on addition of the antifoams, the total yield was normalized to the optical density of the cultures to obtain the specific yield ($\mu\text{g OD}_{595}^{-1}$) by dividing the total GFP yield data by the optical density data shown in Table 3.3.

Table 3.3: Effect of antifoams upon optical density of *P. pastoris* cultures. Increasing concentrations of antifoam were added to shake flask cultures and the optical density was measured at 595 nm after 48 h in BMMY medium

	0%	0.2%	0.4%	0.6%	0.8%	1%
Antifoam A 24 h	15.6	16.4	14.5	16.4	14	19.3
Antifoam A 48 h	24.1	21.2	25.3	23.9	22.2	27.3
Antifoam C 24 h	15.3	18	8.8	15.2	13.8	20.3
Antifoam C 48 h	23.8	23.3	14.7	22.9	20.6	22.1
J673A 24 h	12.5	14.7	17.2	22.8	15.9	20
J673A 48 h	24.4	19.9	24.5	25.8	22	24
P2000 24 h	12.9	14.3	16.4	14	8.3	12.4
P2000 48 h	20.4	25.2	30.1	23.9	29.4	31.9
SB2121 24 h	11.1	17.9	20.1	27.7	22	19.5
SB2121 48 h	20.6	28.5	28.5	32.6	31.4	25.8

OD₅₉₅ was demonstrated to be a reliable measure of cell density in these experiments by comparing the number of cells counted using a haemocytometer at a given OD₅₉₅ in the absence and presence of a range of concentrations of the different antifoams used in the study: there was no statistically significant difference in cell number between cells harvested at a given OD₅₉₅ in the absence or presence of all antifoam concentrations tested at 48 h. Typical values were 8.8×10^7 cells/mL at an OD₅₉₅ of 24.8 in the absence and presence of 0.5 % SB2121. However, it was clear that both OD and haemocytometer measurements are associated with some variability. The data are shown in Fig 3.22.

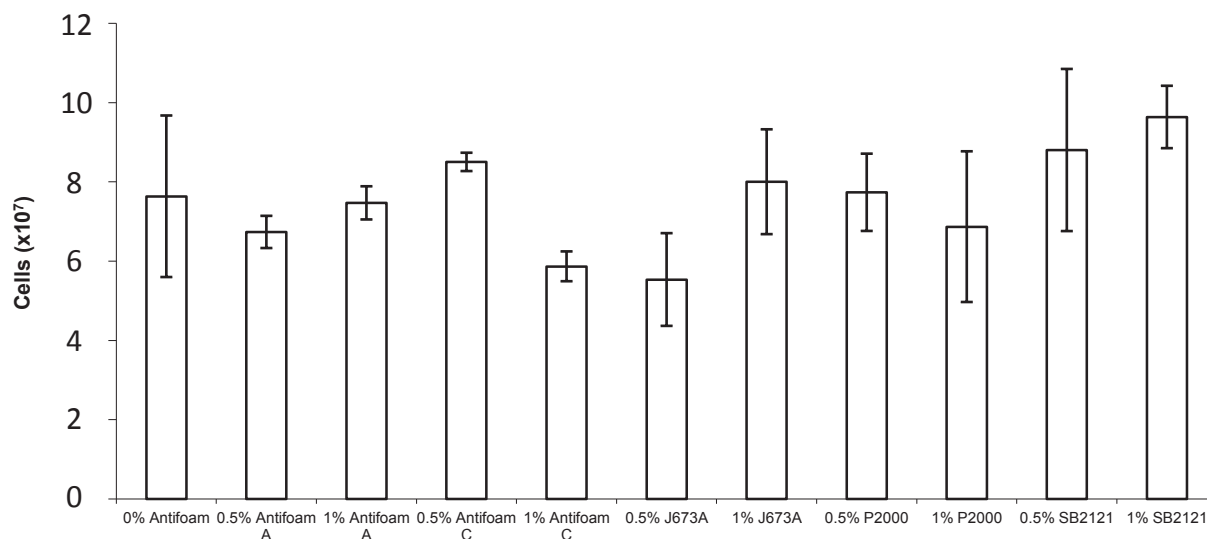


Figure 3.22: Haemocytometer counts for *P. pastoris* cultures

Comparison of the number of *P. pastoris* X33 GFP cells at 48 h in the presence of antifoams counted by haemocytometer. Error bars represent standard error and $n = 3$.

For Antifoam A, Antifoam C and J673A, the specific yield data were similar in trend to the total yield data (Fig. 3.23A–C): addition of these antifoams in the range 0.2–1 % v/v caused a significant increase in specific yield compared to the control cultures with no antifoam. For cultures containing P2000 or SB2121, however, there was no statistically significant difference in the specific yield at each antifoam concentration compared with the control except for 1% P2000 where $P < 0.05$ (Fig. 3.23D–E). This suggested that the enhancements in total yield due to P2000 or SB2121 addition might be attributable to changed growth characteristics of the cells. An increase in OD₅₉₅ (at 48 h, see Table 3.3) was observed with increasing antifoam concentration for both antifoams (Fig.3.23D–E; 48 h data), which was less pronounced for Antifoam A, Antifoam C and J673A (Fig.3.23A–C). The cell counts shown in Fig 3.22 also showed that for SB2121 more cells were present in the cultures at both 0.5% and 1%, however these increases were not significant when compared to the control. These data suggest more than one mechanism of antifoam action: one possibly due to changed culture density (P2000, SB2121) and a second due to increased cellular production levels of recombinant GFP (Antifoam A, Antifoam C, J673A).

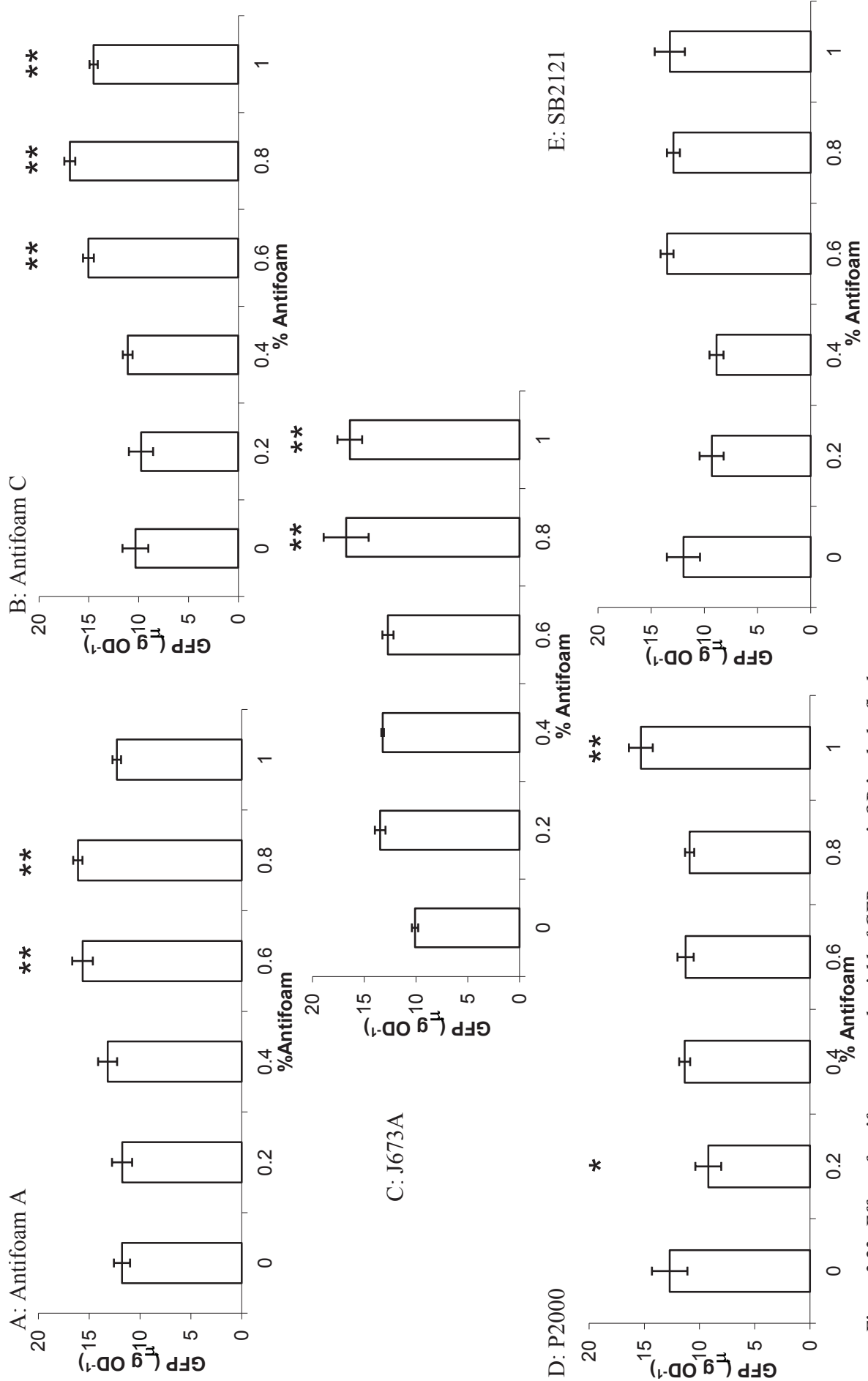


Figure 3.23: Effect of antifoams upon the yield of GFP per unit OD in shake flasks
 Bar charts show the yield of GFP produced by *P. pastoris* cells in the presence of (A) Antifoam A, (B) Antifoam C, (C) J673A, (D) P2000 and (E) SB2121

3.4.4 The effect of antifoams upon the growth rates of log phase *P.*

pastoris X33-GFP cells

As there appeared to be differences in the growth of the cells in the presence of antifoams P2000 and SB2121, growth curves were plotted for the log phase of the *P. pastoris* cultures in the presence in the optimum concentrations of antifoams from the previous shake flask experiments. Flasks were set up as before with 20 mL BMMY culture and incubated at 30°C for 24 h. The cultures were set up in triplicate and the OD read every 2 h. The natural log of the OD data was taken and plotted over time to allow the specific growth rate, μ , to be calculated. The data are shown in Table 3.4.

Table 3.4: Growth rates calculated from the log phase of *P. pastoris* X33-GFP in the presence of optimum concentrations of antifoam where $n = 2$.

Antifoam	μ (h ⁻¹)
0% Antifoam	0.13
0.6% Antifoam A	0.12
0.8% Antifoam C	0.09
1% J673A	0.19
1% P2000	0.15
1% SB2121	0.14

The growth rates for the log phase cells show that the control culture with 0% antifoam had a μ of 0.13 h⁻¹. 0.8% Antifoam C had a lower μ of 0.09 h⁻¹, suggesting that cultures in the presence of this antifoam grew more slowly than those without antifoam. 0.6% Antifoam A had a similar μ to the control of 0.12 h⁻¹ whereas 1% P2000 and 1% SB2121 were slightly higher, with $\mu = 0.15$ h⁻¹ and $\mu = 0.14$ h⁻¹, respectively. The highest growth rate was achieved with 1% J673A where $\mu = 0.19$ h⁻¹. A similar trend can be seen with the growth rates of the antifoam cultures to the total yields of GFP produced. The best yielding antifoams for GFP production, J673A, P2000 and SB2121 also had higher growth rates than the control. Higher growth rates could suggest that nutrients are taken up from the medium more rapidly, enabling the cell to grow more quickly and produce more recombinant protein. Infact, the antifoams may also affect the lipid composition of the yeast

cell membrane[193, 194], possibly allowing more efficient diffusion of methanol into the cell resulting in faster growth and a greater level of recombinant GFP production[66].

Overall, all of the antifoams tested increased the total recombinant protein yield, and this was achieved at higher concentrations than would normally be used for defoaming purposes. The best effect was observed when 1% P2000 was added to the culture after 48 h, and the total yield of GFP in 20 mL was increased from 244 μg to 422 μg . When the GFP yield was normalized by dividing by the OD to obtain the yield of GFP per unit OD at 48 h, it was found that the effects of antifoam addition were not due to changes in the growth of the cultures, except for P2000 and SB2121, suggesting at least two mechanisms of action of the antifoams for improving the yield of GFP. During the log phase of growth of the cells, the highest growth rates were seen with cultures containing 1% J673A, 1% P2000 and 1% SB2121. For P2000 and SB2121, these increases in growth rate could contribute to improved cell densities, agreeing with the normalized yields findings. For J673A, as the normalized yield data suggested that the improvements in total yield were not due to changes in growth, the high growth rate could indicate that the properties of the membrane lipids in this culture were altered by the antifoam, allowing nutrients to be taken up more rapidly which were utilized for recombinant protein production rather than to increase biomass.

3.5 Effect of antifoams in scale up into 2 L shake flasks

Antifoams are more commonly used in larger scale systems such as bioreactors. Before scaling up into a bioreactor, the antifoams were evaluated in 500 L BMMY medium in 2 L non-baffled shake flasks to determine whether their effects could be observed on a larger scale. The lowest yielding antifoam concentration that had a significant effect upon GFP production in the smaller shake flasks (0.8% Antifoam C) and one of the best yielding antifoams (0.8% J673A) were evaluated in this system. The results were determined by measuring the samples in triplicate using a fluorimeter, therefore $n = 3$ for each data set. The data are shown in Fig. 3.24.

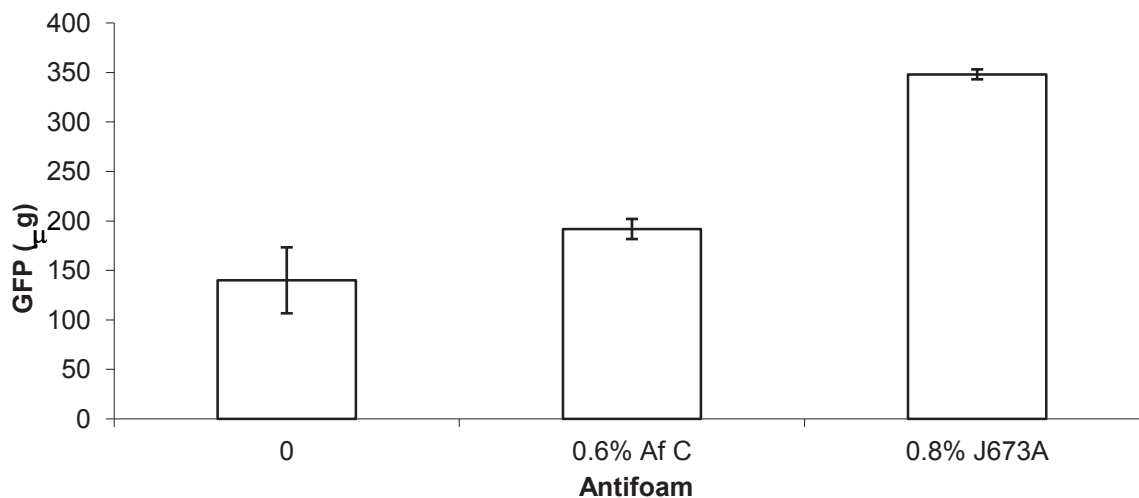


Figure 3.24: GFP production in 2 L flasks in the presence of antifoam

Effect of antifoams upon GFP production by P. pastoris after 48 h in 2 L shake flasks containing 500 mL BMMY medium where n = 3.

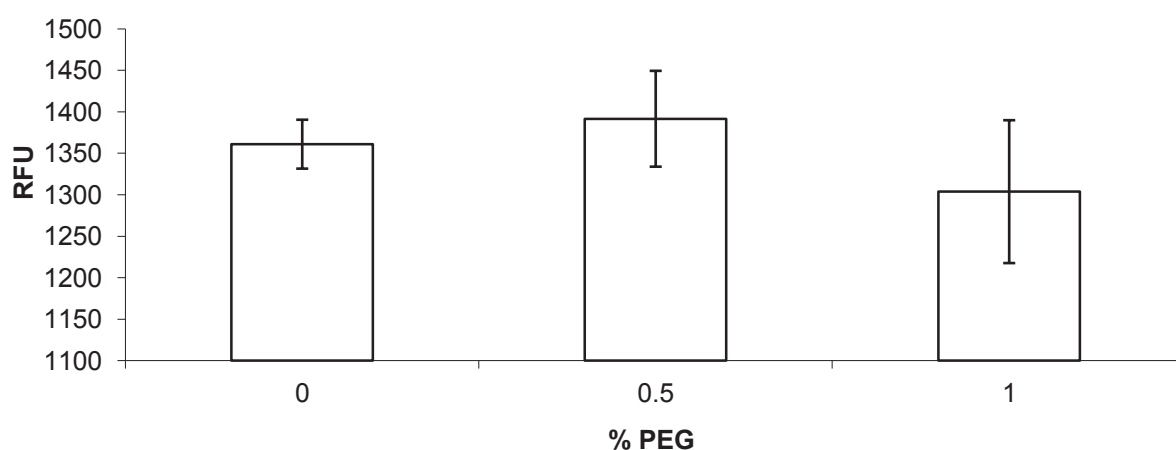
Fig 3.24 demonstrates that the effects of the antifoams can be applied to larger scale shake flasks with the same trend of effects as previously observed. Antifoam C at 0.6% produced a lower yield of GFP in 100 mL shake flasks than 0.8% J673A, but still produced a higher total yield than the control. J673A at 0.8% produced a much higher total yield of GFP than the control which was again almost double, and also the 0.6% Antifoam C cultures in both the small scale and large scale shake flasks. These data suggest that the effects of the antifoams are not specific to small shake flasks, and could be replicated in larger cultures to boost recombinant protein yields.

3.6 Effect of polyethylene glycol and ethylene glycol upon GFP yield

Some of the antifoams that increased the yield of GFP in shake flasks contain components that have similar compositions to other agents such as ethylene glycols and polyethylene glycols. P2000 is a polypropylene glycol and SB2121 is a polyalkylene glycol. An experiment was conducted to see if other similar agents not designed for defoaming

purposes could have the same effects as their antifoam counterparts. A polyethylene glycol (PEG) and an ethylene glycol (EG) were added at 0.5% and 1% to 20 mL BMMY in 100 mL non-baffled shake flask cultures of *P. pastoris* producing GFP and incubated for 48 h. The results are illustrated in Fig. 3.25.

A)



B)

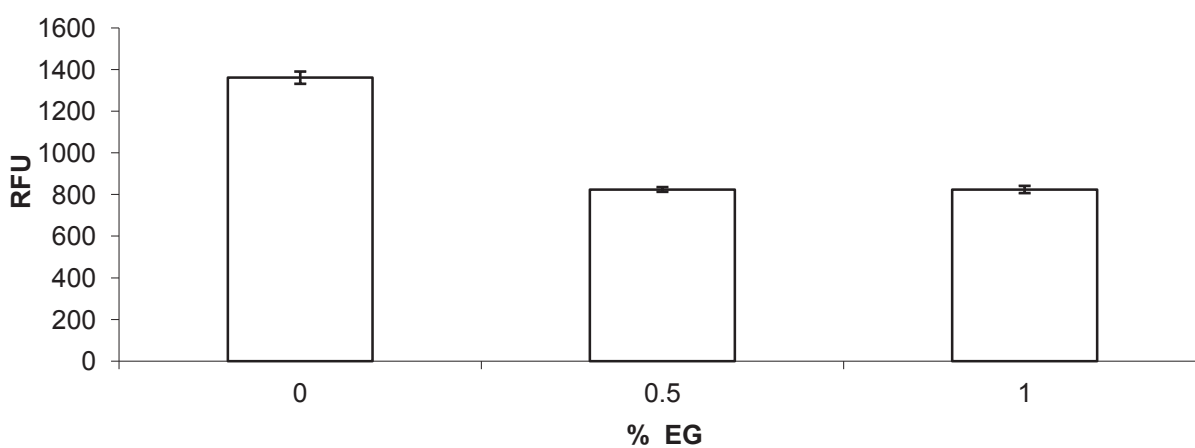


Fig. 3.25: The effect of PEG and EG upon GFP yield

The effect of A) polyethylene glycol and B) the effect of ethylene glycol upon GFP production after 48 h by *P. pastoris* in 20 mL BMMY in 100 mL shake flasks. In all cases $n = 9$.

The two other agents tested do not appear to have the same effects upon GFP yield as the antifoams at the concentrations tested, and in all cases except for 0.5% polyethylene glycol, decreased the yield. These findings imply that there could be a relationship between the ability of an agent to destroy foam, hence its use as an antifoaming agent, and the ability to affect recombinant protein production. It is possible that there is a common component or mechanism of action of the antifoaming agents, which as well as destroying foam, can at certain concentrations lead to an improvement in total recombinant protein yield.

3.7 Effect of antifoams upon GFP purification

There is a general concern that addition of high concentrations of antifoam can interfere with downstream processes and purification of the proteins. As all of the antifoams increased the yields of GFP at higher concentrations than would normally be added, an investigation was conducted to determine whether they inhibited the purification of GFP. Shake flask cultures were set up in duplicate in 100 mL non-baffled shake flasks with 20 mL *P. pastoris* culture producing GFP in the presence of the optimum concentration of antifoam from the shake flask experiments. After 48 h, 15 mL of each culture was purified using a one-step Ni-NTA nickel affinity column. A silver stain was performed on some of the samples before and after purification (Fig 3.26A). The GFP present in the supernatant before purification, in the wash and in the elution was measured by fluorimetry. The data were converted to percentages of the total GFP content to allow comparison, shown in Fig. 3.26B.

Compared to the control, a greater percentage of GFP was present in the elution of the samples containing 1% J673A and 1% SB2121. The other antifoam samples contained a similar percentage of GFP compared to the control, suggesting that antifoams, at these concentrations and for this method of purification, do not inhibit purification of GFP. The presence of 1% J673A and SB2121 also appeared to improve the final percentage of GFP obtained in the elution.

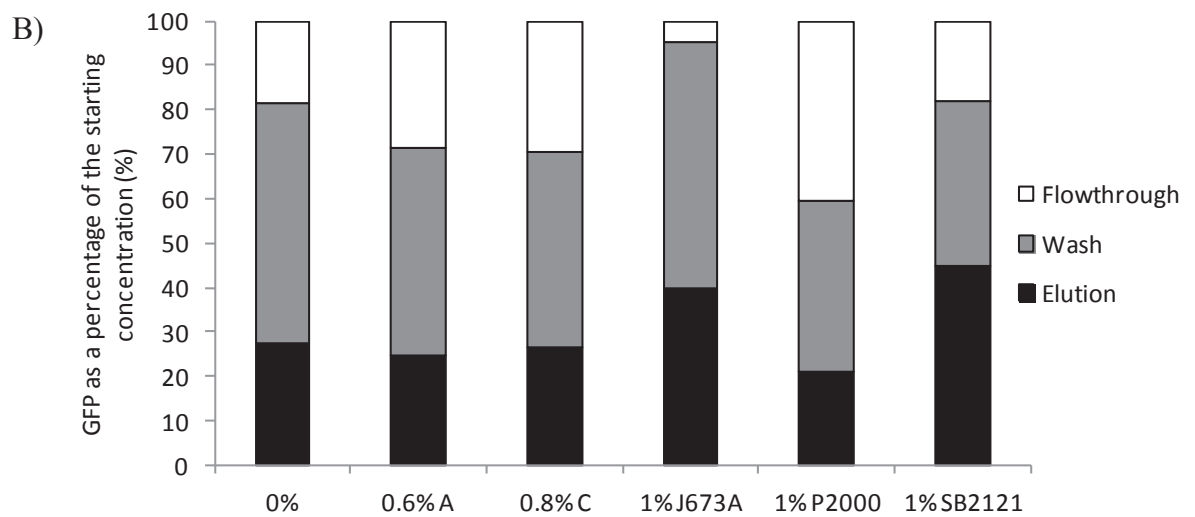
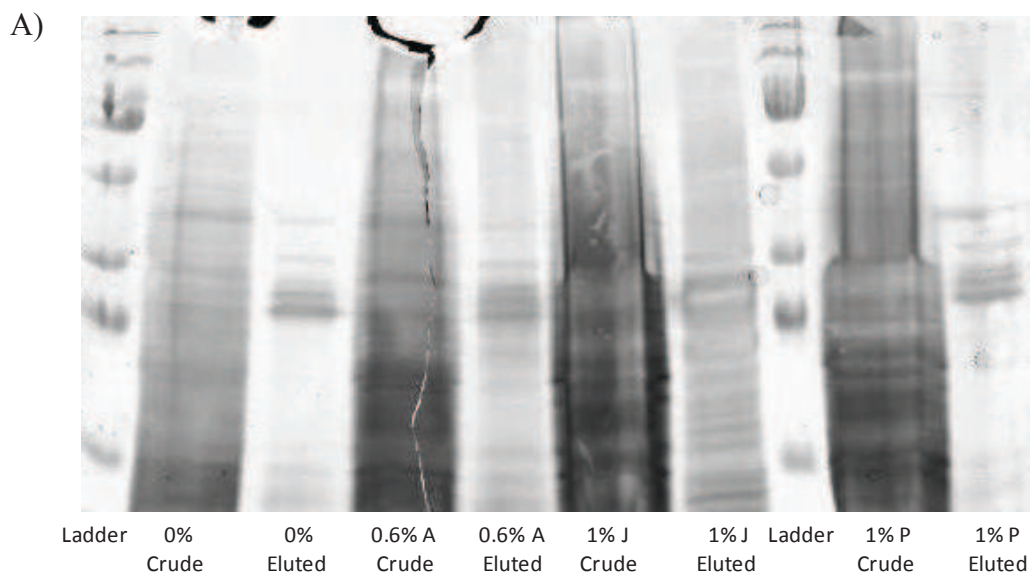


Figure 3.26: The effect of antifoams upon the purification of GFP using nickel affinity columns

A) Silver stain with crude and eluted samples; B) Comparison between the percentage of GFP present in each stage of the purification process. A represents Antifoam A, J represents J673A and P represents P2000. In all cases $n = 6$.

3.8 Effect of antifoams upon human erythropoietin production

In order to determine if the effects of the antifoam were specific to GFP, or whether they could be applied to another soluble protein, their effects upon the production of erythropoietin (EPO) were evaluated. The same system was used and antifoams at 0.6% and 1% were added to the 100 mL non-baffled shake flasks with 20 mL BMMY media containing *P. pastoris* producing secreted EPO. These concentrations were chosen as previously with GFP, at 0.6% all of the antifoams significantly increased the total protein yield, and at 1% some of the highest yields were obtained. Flasks were again set up in triplicate for each condition. Samples were collected at 48 h and measurements were attempted by performing an ELISA, however there was found to be unacceptable variability between samples read on different plates. This method was therefore deemed to be unreliable, and hence Western blots were performed on the samples. Western blots are not an ideal method to compare the quantity of protein produced under different conditions, therefore future experiments would also include a standard of known quantity on each to more accurately compare between blots. Nonetheless, the band intensity was measured using Image J software. The data were then averaged as illustrated in Fig. 3.27.

Fig 3.27 suggests the best condition for the production of EPO in shake flasks was without any antifoam addition and that the antifoams at these concentrations did not improve the yield of protein in comparison. The highest yield in the presence of the antifoams was observed with 1% J673A which was not significantly lower than the control, followed by 0.6% J673A. The most detrimental effect upon EPO production was seen when 0.6% Antifoam C was added to the flasks. Except for J673A, the other antifoam conditions tested had a pronounced detrimental effect upon the protein yield, emphasizing the importance of optimizing the antifoam and concentration to be used during the protein production process.

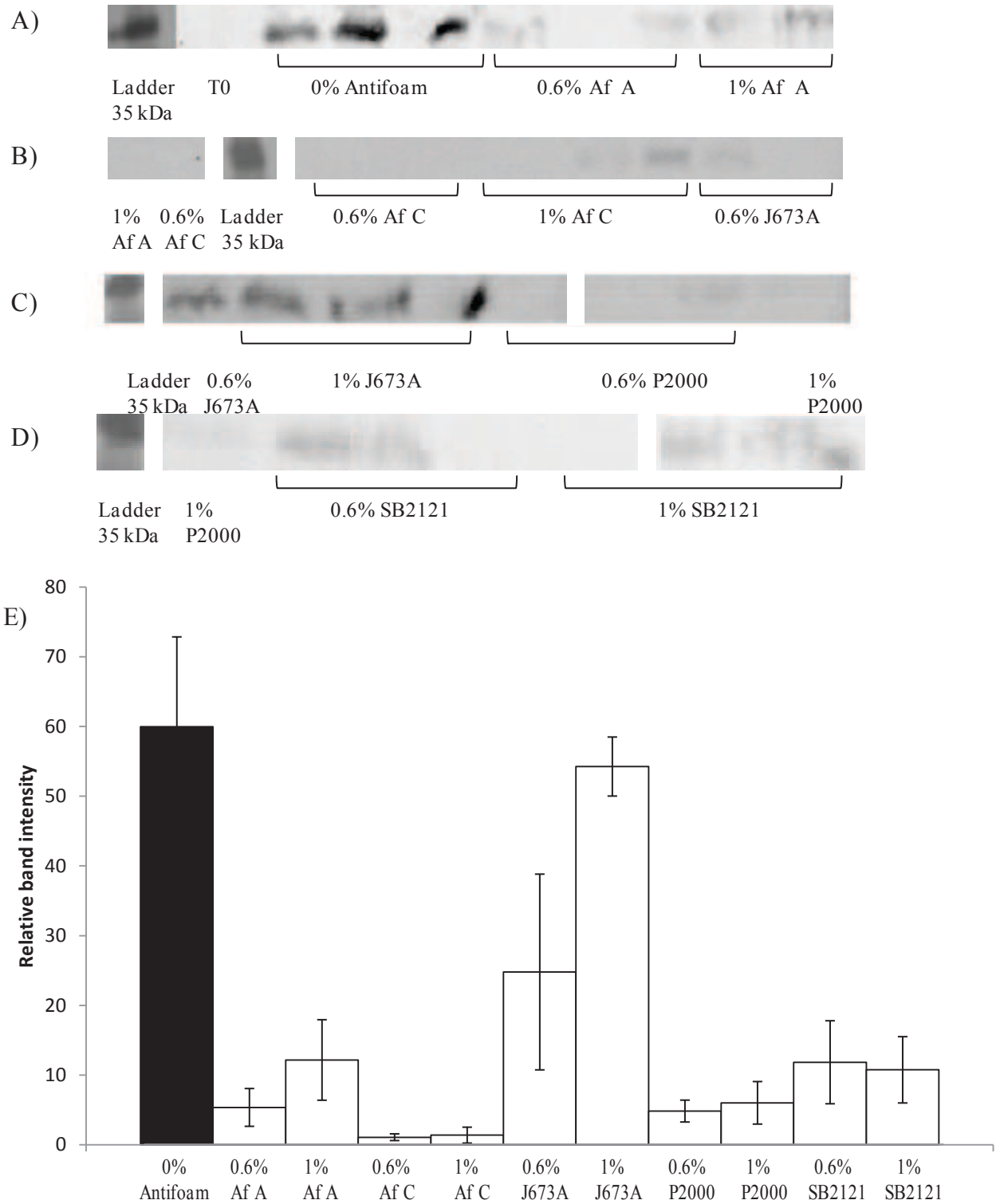


Figure 3.27: The effect of antifoams upon EPO production in shake flasks

The Western blots from four SDS-page gels are shown in A) to D). E) shows a bar chart representing the relative amount of EPO produced. The black bar shows the relative amount of EPO in cultures without any antifoam present and the white bars show the effects of antifoam addition at 0.6% and 1% of the panel of antifoams. In each case $n = 3$.

3.9 Effect of antifoams upon adenosine_{2a} receptor production

A_{2a}R, a membrane-bound protein, was produced in the same set up as previously described with 0.5% and 1% of each antifoam added to 20 mL BMMY cultures. Single point radio-ligand binding assays with A_{2a}R antagonist [³H]ZM241385 were performed on 100 µg of membrane protein for each of the samples in duplicate (n= 6). The results are shown in Fig. 3.28.

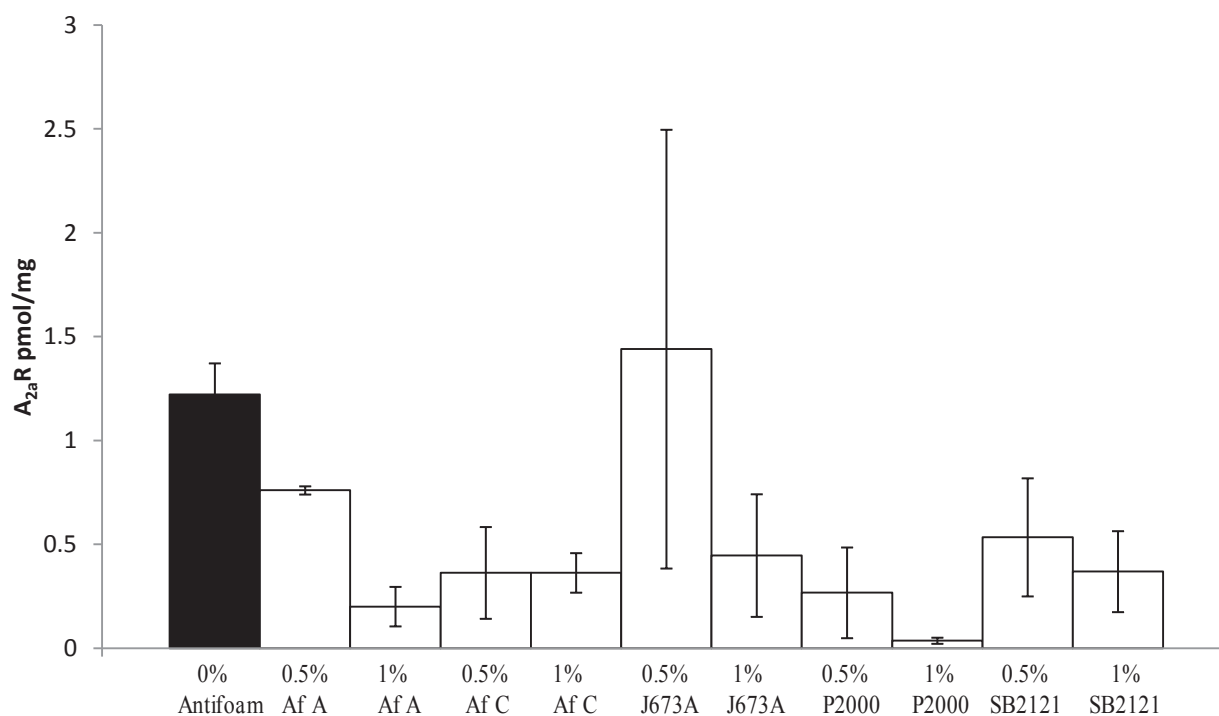


Figure 3.28: The effect of antifoams upon A_{2a}R production in shake flasks

Af A represents Antifoam A and Af C represents Antifoam C. The black bar represents the yield of A_{2a}R produced per mg of membrane protein in the 20 mL shake flask cultures, and the white bars show the yields for the cultures containing antifoams at 0.5% v/v and 1% v/v. For each data set, n = 6.

The data suggest that overall, adding antifoams at these concentrations is detrimental to the production of A_{2a}R in shake flasks. There are also large variations in results for some of the antifoam-containing cultures. However, 0.5% J673A has the least limiting effect upon the yield of the protein of the antifoams tested. The lowest yield was achieved in the presence of 1% P2000.

3.10 Summary

The Bartsch test demonstrated that each antifoam has a different effectiveness upon the destruction of foam which may be influenced by their different compositions and therefore different mechanisms of foam destruction. These antifoams when incubated with *P. pastoris* cells, did not reduce the viability of the cells, even at high concentrations. All of the antifoams tested in shake flasks increased the total yield of GFP at concentrations higher than would usually be used in bioreactor cultures for defoaming purposes. The best effects were observed with 1% P2000 which increased the total GFP yield from 244 μg to 422 μg . The least effective antifoam was Antifoam C, but this antifoam still significantly increased the total GFP yield compared to the control. When the total yield data was normalized to obtain the yield of GFP per unit OD, antifoams P2000 and SB2121 appeared to have caused the increases in protein yield due to changes in the growth of the cells. Although there was no statistical correlation between foam destruction capacity and either total or specific yield, J673A and SB2121 were the most effective at foam destruction and among the best at increasing GFP yield, whilst Antifoam A was the least effective and produced lower yields than J673A and P2000.

When the growth rates of *P. pastoris* cells in the log phase were measured in the presence of the concentration of antifoam that produced the highest yield of GFP, Antifoam C grew more slowly than the control without antifoam, Antifoam A grew at the same rate, P2000 and SB2121 had slightly higher growth rates, and J673A had a higher growth rate, 0.19 h^{-1} compared to 0.12 h^{-1} for the 0% antifoam culture.

Investigation into the effects of other similar agents to the antifoams, PEG and EG, showed that they appeared to have a detrimental effect overall upon the total yields of GFP. This implies that there is something specific to antifoams which produces the effects upon the growth or recombinant protein yields of *P. pastoris* cells producing GFP. The effects of antifoam J673A and Antifoam C were investigated in 2 L shake flasks, and it was found that their effects had the same trend as in 100 mL shake flasks, suggesting that the effects of the antifoams can be scaled up. The effect of the antifoams at their optimum concentration for GFP production on the purification of the protein was investigated, as

these concentrations are high and are thought to inhibit purification. It was found that the presence of antifoams was not detrimental to the purification of GFP using nickel affinity column purification, and in addition, 1% J673A and 1% SB2121 appeared to improve the concentration of GFP present in the elution.

When the antifoams were tested on the production of EPO in shake flasks at the concentrations that yielded more GFP than the controls, it was found that the highest total yield was achieved without antifoam present. The least detrimental antifoam was 1% J673A. When the same investigation was conducted with A_{2a}R, again the cultures without antifoam appear to produce the highest yields. Again the least detrimental antifoam was J673A but this time at 0.5%. Due to the large variation in counts obtained for the samples containing 0.5% J673A, it is unclear whether this condition was more effective at increasing the yield of A_{2a}R than the control and this data set would need to be repeated. It is possible that too few antifoam concentrations were tested, and that the optimal concentration of antifoam is dependent upon the protein. Lower concentrations may have increased the yields compared to the control, and could be examined in future work. The variations observed could also be attributed to pipetting error leading to variations in the concentrations of ligand present in each of the tubes. To reduce this problem the number of replicates should be increased to ensure the data obtained from this sensitive technique is more robust. Alternatively, larger volumes and amounts of membrane protein could be used if available, such as 500 µg rather than 100 µg.

The investigations in this Chapter have shown that antifoams can affect the production of recombinant protein and the growth of *P. pastoris* cells in shake flasks. For the production of recombinant GFP, the effects were beneficial at high concentrations and in some cases almost doubled the total yield of GFP obtained. They appear to have at least two mechanisms of action; one that improves the production or secretion of GFP and another that influences the growth of the cells. For other proteins, a detrimental effect was observed. This highlights the importance of screening for optimal antifoams and concentrations in bioprocesses, as the addition of antifoams has the capacity to enhance the yield of protein, or become detrimental.

4. MECHANISMS OF ANTIFOAM ACTION

The antifoams tested in Chapter 3 had a range of effects in the shake flask evaluations depending upon the type and concentration as well as on the protein being produced. It was noted that the antifoams appeared to have at least two mechanisms of action leading to increases in yield of GFP; one that suggested the improvements were due to a change in the growth of the cells, and the other an improvement in the production level and/or secretion of the protein. These possible mechanisms were further investigated in shake flask format, by examining the effects of the antifoams upon secretion and retention of GFP, membrane composition and changes in the growth characteristics of the cells as measured by the effects of the antifoams upon dissolved oxygen (DO) and the volumetric mass oxygen transfer coefficient (k_La)

4.1 The effect of antifoams upon GFP secretion

GFP was chosen as the target protein for secretion and retention investigations as many of the observations of Chapter 3 were made using this protein. GFP is also easy to detect due to its fluorescent properties, making it the ideal choice for flow cytometry experiments.

4.1.1 Effect of antifoams on the total yield of GFP determined by flow cytometry and fluorometry

An investigation into whether antifoam addition might have a physical influence on the cells was conducted. The amount of GFP retained in the cell was measured (by flow cytometry) and that secreted into the culture medium (by fluorimetry). The *P. pastoris* cells were cultured in the same shake flask system used for the protein production investigation discussed in Chapter 3. Antifoam concentrations of 0.6% were chosen as these were the lowest concentrations to increase the total yields of GFP in the shake flask experiments, except for J673A where 0.8% increased the total yield compared to the control. Samples

were taken after 48 h and following a haemocytometer count, were diluted with PBS to be in the region of 10^6 - 10^7 cells/mL. 2mL of the diluted samples were analyzed using the flow cytometer. These data represent the GFP retained in the cells, while the GFP secreted in the shake flask experiments was measured by fluorimetry. In order to compare these two different data sets, each was normalized to its respective 0% antifoam control, which was consequently set to 1. The values shown in Fig. 4.1 are for determinations at 48 h (n = 3). The significance of the changes in retained and/or secreted GFP compared to the respective 0% antifoam control was analyzed by a one-way ANOVA ($P < 0.0001$) and a Dunnett's multiple comparison test where * = $P \leq 0.05$ and ** = $P \leq 0.01$.

Fig. 4.1 shows that addition of Antifoam A, Antifoam C, J673A and P2000 caused a statistically significant increase ($P < 0.01$) in the amount of GFP secreted into the medium compared with the 0 % antifoam control. The amount of protein retained in the cells was also greater suggesting that antifoam addition enhanced the ability of the cells to produce recombinant GFP. For P2000 however, more GFP was retained inside the cells compared with the 0 % antifoam control. This is consistent with the growth of the cells being affected by P2000 addition rather than resulting in improved secretion efficiency, and also suggests that there has been some metabolic change to the cells compared to the control. Data for SB2121 was similar to that for P2000. These data also correlate with the fluorescence microscopy pictures in Fig 3.22 of Chapter 3, where GFP can be clearly seen retained inside the cells of cultures containing P2000 and SB2121.

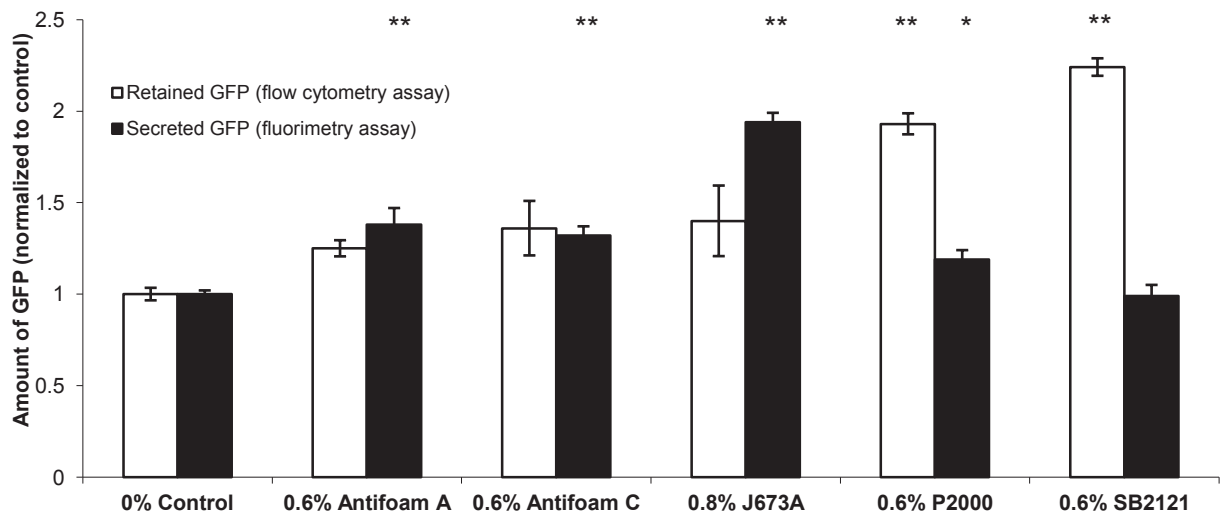


Figure 4.1: The effect of antifoams upon GFP secretion and retention

The amount of GFP retained in the cell analyzed by flow cytometry (white bars), and that secreted into the culture medium analyzed by fluorimetry (black bars). For the retained dataset $n = 3$ and secreted dataset $n = 9$.

It is possible that the antifoams were not only increasing the recombinant proteins being produced by *P. pastoris*, but could have an effect upon total protein secretion. A bicinchoninic acid (BCA) assay is a commonly used method to determine total protein. This assay was performed on cultures incubated with 0%, 0.5% and 1% antifoams for 48 h to allow investigation of the range of concentrations. Samples were measured in triplicate and analyzed by a one-way ANOVA ($P < 0.0001$) and a Dunnett's multiple comparison test where $* = P \leq 0.05$ and $** = P \leq 0.01$ and $*** = P \leq 0.001$). The data are illustrated in Fig 4.2.

The antifoams did not cause any change in the total concentration of all proteins in the supernatant measured by BCA assay for cultures containing antifoams at representative concentrations of 0 %, 0.5 % and 1 %, except for 0.5 % Antifoam C ($P < 0.05$) and 1 % SB2121 ($P < 0.01$). In the presence of these two antifoam concentrations, a decrease of 13–14 % was observed in the total protein concentration of the supernatant compared to 0 % antifoam-containing control cultures. At time point T0, as expected before induction, there was significantly less protein in these samples compared to the control ($P < 0.001$). These data suggest that as the antifoams did not increase the total protein being produced by *P.*

pastoris, the antifoam effects might be specific to the recombinant protein production pathway.

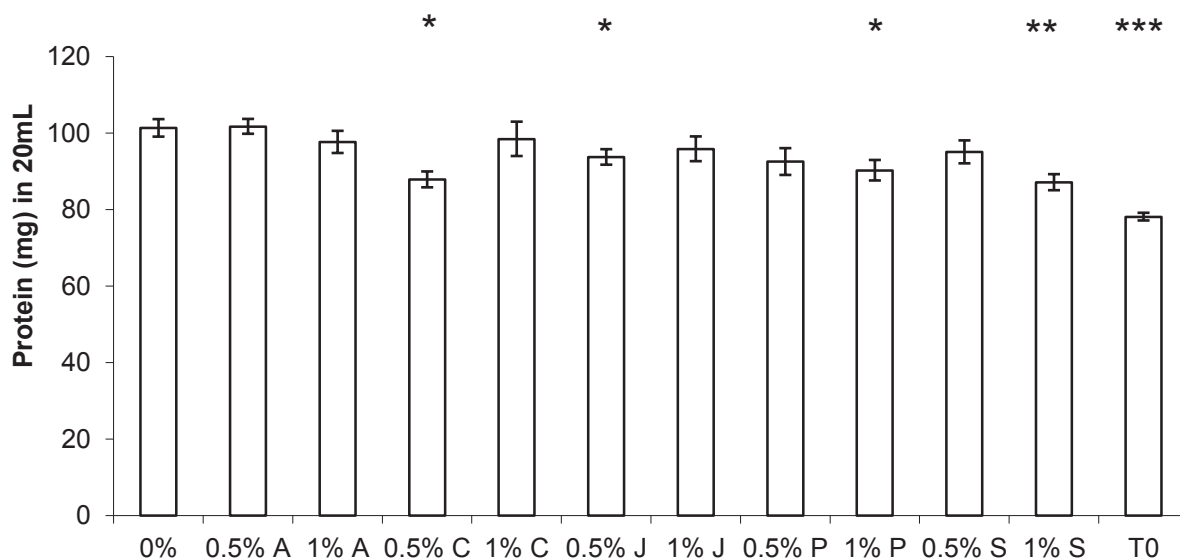


Figure 4.2: Effect of antifoams upon total protein secretion

The effect of antifoams at 0.5% and 1% v/v upon total protein secretion by *P. pastoris* producing GFP after 48 h measured by BCA assay. A represents Antifoam A, C represents Antifoam C, J represents J673A, P represents P2000 and S represents SB2121 and T0 represents time zero. In each case n = 6.

AOX1 expression in the presence of methanol can account for up to 30% of the total soluble protein[22], however this did not appear to be the case when the yields of GFP produced under the control of this promoter in the shake flask investigations of Chapter 3 were compared with the total yields of protein produced. 20 mL cultures without antifoam produced 250 µg of GFP, however the total yield of all protein was in the region of 100 mg. This suggests that the soluble GFP accounts for only 0.25 % of all protein in the medium. This implies that in the shake flask experiments the methanol induction was not optimal, or the methanol became limiting during the investigation. Induction in shake flasks therefore could be optimized in future work.

4.1.2 Effect of antifoams upon lipid composition of *P. pastoris* membranes

Further to the observations that antifoams may influence the ability of the *P. pastoris* cells to secrete recombinant GFP, the lipid composition of the cells incubated in the presence of optimum concentrations of the antifoams for 48 h was compared using electrospray mass spectrometry. Preliminary data analysis suggests that there was a relative change in the phosphatidylcholine composition of the lipids of membrane samples of 48 h *P. pastoris* cells incubated with 1% P2000 compared to control membrane lipids. The data also suggest a relative change in the phosphatidylinositol composition of membrane lipids from each of the antifoam-containing cultures compared to those without antifoam. Further analysis is required to determine the precise effects of the antifoams upon the lipid composition of *P. pastoris* cultures incubated with antifoam.

4.2 Effect of antifoams on the growth characteristics of cells producing GFP

The precise effects of antifoams upon cells is not well documented. Several growth characteristics of *P. pastoris* were therefore investigated in the presence of the panel of antifoams.

4.2.1 The effects of antifoams upon cell metabolism

The shake flask results from Chapter 3 showed that *P. pastoris* producing GFP in the presence of some of the antifoams produced differing amounts of protein and grew differently to the control. This implies that there may have been changes to the metabolism of the cells, which can be detected as changes in the heat output rate by microcalorimetry.

The thermal activity monitor (TAM) is a sensitive differential calorimeter developed in order to study biological systems[172] and is shown in Fig. 4.3. Calorimetry involves the measurement of heat. Differential calorimeters are able to measure the difference in heat between the sample and a reference[195]. In *P. pastoris* cultures, the temperature difference

between cultures containing 0% antifoam and the optimum antifoam concentration was measured by incubating the cells at 30°C and setting the reference temperature of the TAM to 30°C, maintained by a water bath. A constant flow of sample from the shake flask cultures through the TAM allowed differences in the heat between the reference and the samples to be detected and displayed graphically by DigiTam software.

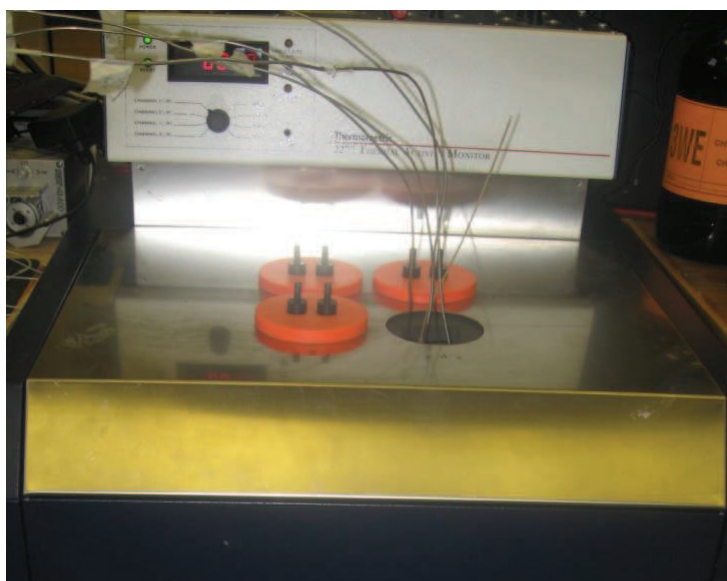


Figure 4.3: Thermal activity monitor (TAM)

A TAM set up and photographed at Aston University. A water bath maintains the reference temperature while lines draw culture through the calorimeter and allow differences in heat to be monitored.

The antifoams were added at their optimum concentration to BMMY shake flask cultures of *P. pastoris* producing GFP in a setup identical to that of Chapter 3. The flasks were incubated at 30°C, 220 rpm with the lines from the TAM drawing the culture into the calorimeter using a pump set to a flow rate of 30 rpm for all experiments. The TAM water bath was set to 30°C and the baseline calibrated. The data were recorded over 20 h, as *P. pastoris* cells began to block the lines once the optical density increased after this time point.

Two control shake flasks were set up from the same culture with cells in the exponential phase without antifoam and run simultaneously to illustrate the fact that there was no difference in heat output between the two channels and cultures set up using the same initial culture. Antifoams were also added to BMMY without culture to demonstrate that the antifoams themselves did not produce any additional heat output. Data were logged using Digitam software. Control data are shown in Fig 4.4.

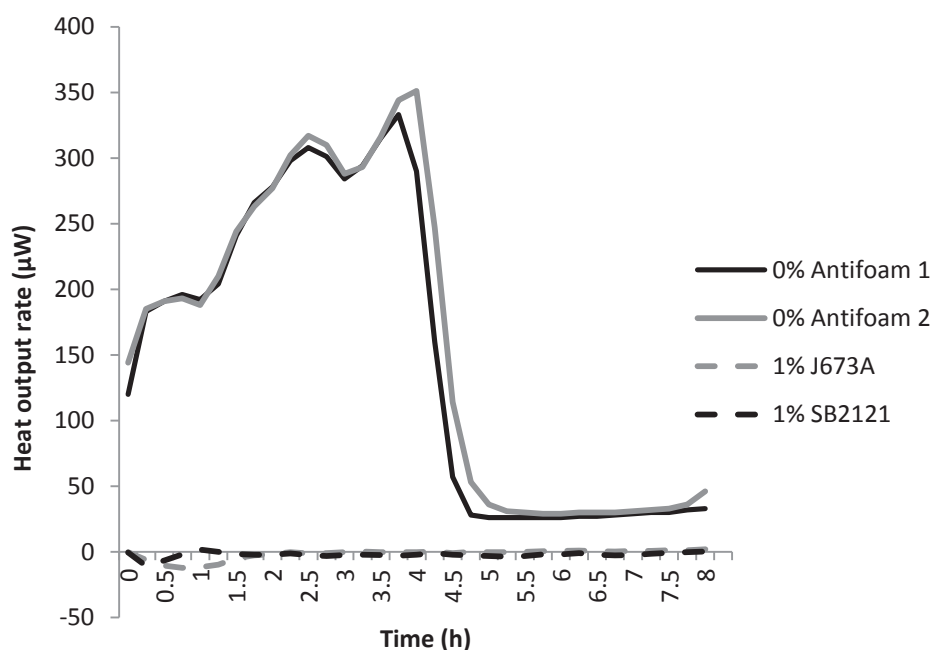


Figure 4.4: Heat output rate produced by control cultures

TAM heat output rate data for cultures of P. pastoris in BMMY without antifoam, represented by 0% Antifoam 1 and 2, and data for BMMY with two antifoams, 1% J673A and 1% SB2121 without culture.

The control data show that for the two cultures containing no antifoam, 0% Antifoam 1 and 0% Antifoam 2, there is minimal difference in the heat output data. This shows that cultures set up at the same time and from the same preculture produce a reproducible heat output trace. For BMMY medium containing the optimum concentration of antifoams, there was no heat output, demonstrated in Fig 4.4 by 1% J673A and 1% SB2121, illustrating the fact that any differences in heat output profiles for cultures with antifoams is not due to heat output of the antifoams themselves.

Test cultures containing antifoams were then set up and two flasks containing cultures from the same culture were monitored simultaneously; one culture without antifoam and the other with the optimum concentration of antifoam. The data for each antifoam are shown in Figures 4.5 and 4.6. The TAM data for Antifoam A run 1 (Fig 4.5A) show that as the cells grew, more heat was given out when compared to the control culture, suggesting a difference in the metabolism of the cells. However, run 2 (Fig. 4.5B) shows a different trace; less heat was given out by both cultures compared to the first run, and the control culture produced slightly more heat than the culture containing Antifoam A. The traces for run 2 again suggest that there was a difference in metabolism between the cultures, which is again different from the first run. These differences between runs may be due to the different cultures being used for each run which would contain a different population of cells in different phases of growth, therefore heat output would not be identical between cultures.

0.8% Antifoam C run 1 (Fig. 4.5C) showed a greater heat output for the culture without antifoam, again suggesting a difference in metabolism. Run 2 (Fig4.5D) shows an almost identical heat output trace for cultures with and without Antifoam C, implying that during this run, the metabolism of the cells was not affected by the antifoam.

For J673A cultures, both run 1 and 2 (Fig 4.5E and F) showed a greater heat output for cultures containing 1% J673A. This strongly suggests that the metabolism between the different cultures had been affected by the presence of this antifoam. The P2000 culture shown in run 1 (Fig.4.6A) produced more heat than the control, however in the second run (Fig.4.6B), the traces were similar. The SB2121 run 1 culture (Fig.4.6C) produced more heat, but produced less heat than the control in the second run (4.6D). It also grew much more slowly than the cultures in the first run, suggesting that most of the cells were in late log phase or stationary phase.

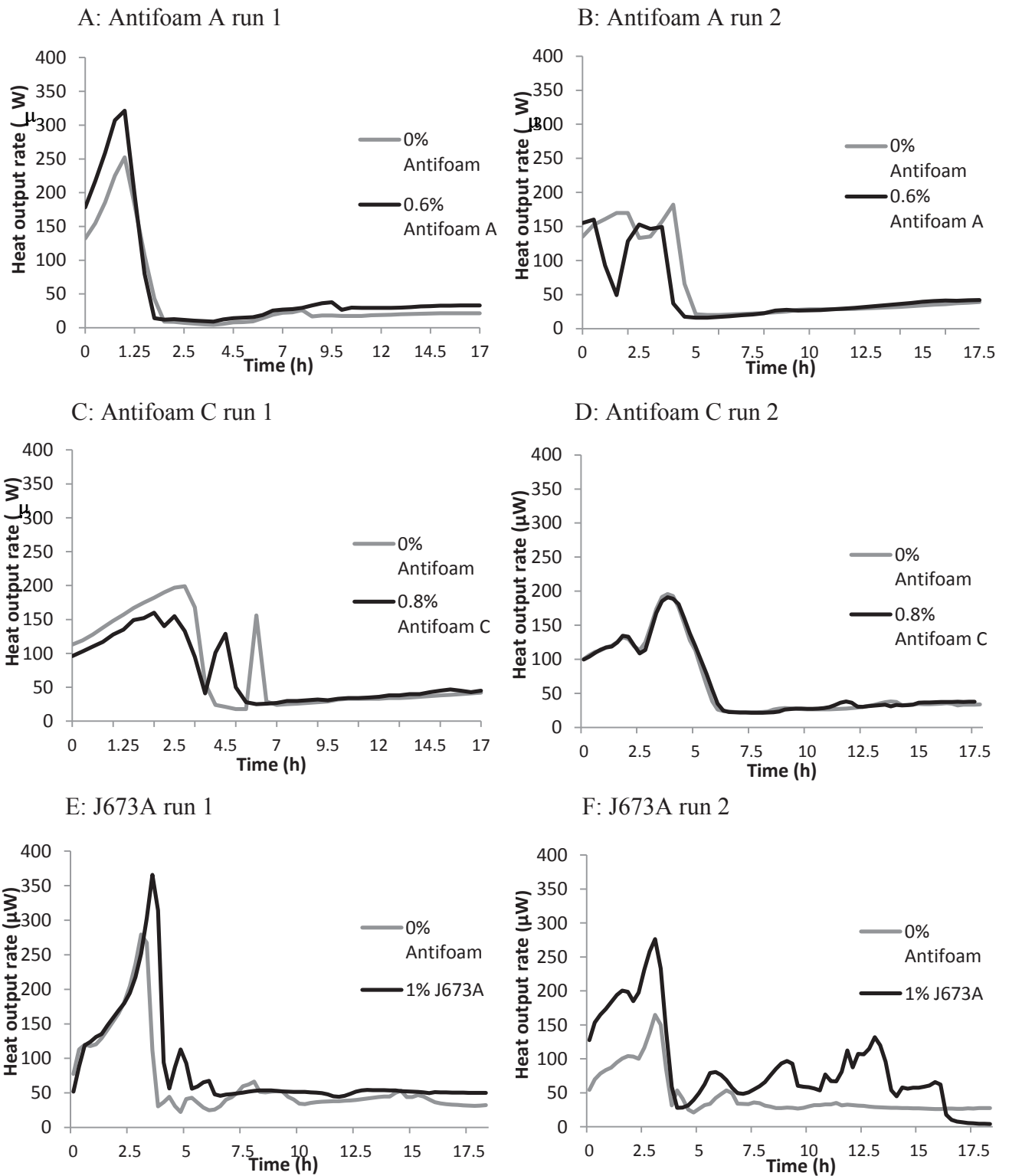


Figure 4.5: Heat output rate produced by antifoam containing cultures

Heat output rate of shake flask cultures of *P. pastoris* producing GFP with and without optimum antifoam concentrations measured by on-line flow microcalorimetry. A: Antifoam A run 1; B: Antifoam A run 2; C: Antifoam C run 1; D: Antifoam C run 2; E: J673A run 1; F: J673A run 2

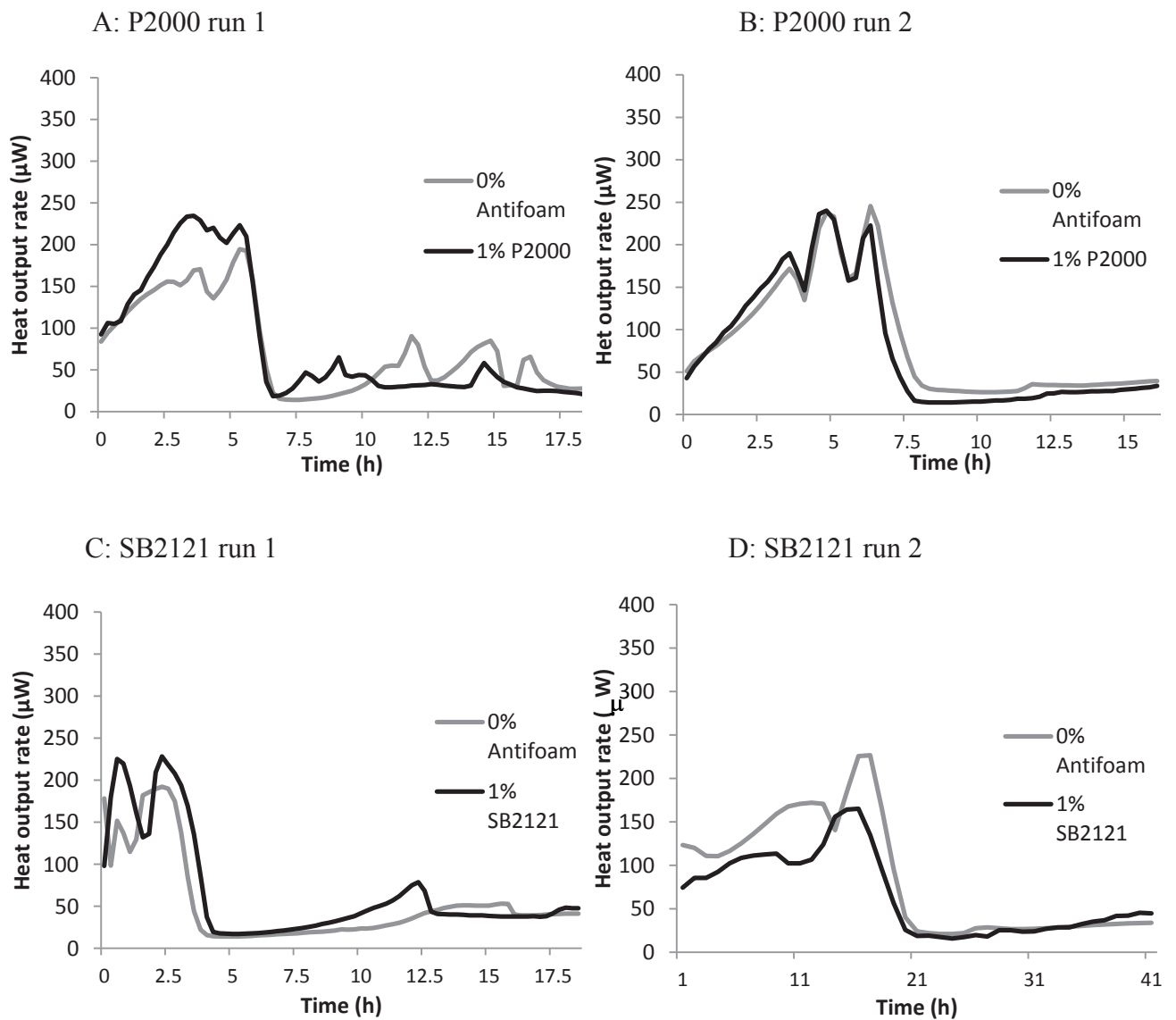


Figure 4.6: Heat output rate produced by antifoam containing cultures

Heat output rate of shake flask cultures of *P. pastoris* producing GFP with and without optimum antifoam

concentrations measured by on-line flow microcalorimetry. A: P2000 run 1; B: P2000 run 2; C: SB2121 run 1; D: SB2121 run 2.

Overall, the data suggest that although there was variability between runs, when comparisons were made between runs with cultures set up from the same initial culture, the antifoams in most cases did affect the metabolism of the cells. This suggests that the cells are growing differently which could be linked to the increases in yield of GFP for these cultures. The antifoams appeared to cause the cells to produce more heat in several cases, seen most clearly with J673A. Less heat was observed with some SB2121 and Antifoam C cultures, and in some cases did not greatly change the heat output as seen with some P2000 and Antifoam A cultures. It might be that cells in a certain growth phase are more affected by the presence of the antifoams. For example, if a greater number of the cell population were entering stationary phase, perhaps the antifoams had less effect. Although cells were taken from cultures in log phase, it is possible that they contained cells that were in late log or stationary phase which could explain differences between traces. Because of the variation in the data which may be due to the population of the cells present, it is unclear whether increases or decreases in heat output can be directly linked to an increase in productivity.

4.2.2 Effect of antifoams upon dissolved oxygen availability in cultures

Antifoams can affect the DO available in the medium, and this effect has been observed in several studies[59, 93, 196]. Therefore, it was investigated whether the antifoams affected the DO in the shake flasks and thereby altered the growth of the cells.

4.2.2.1 Measurements in a multiwell format

In the first instance, the effect of each antifoam concentration upon DO was measured with a PreSens SensorDish Reader (SDR). This equipment was loaned by Applikon Biotechnology and is able to detect oxygen and pH in a multiwell plate format. A 24 well OxoDish was selected with sensor spots at the bottom of the wells containing luminescent dye shown in Fig 4.7A. The dye is excited by the SensorDish reader upon which the multiwell plate sits, and the luminescence lifetime is detected through the transparent bottom of the plate. The luminescence lifetime depends upon the oxygen partial pressure in

the medium and is monitored wirelessly by the software (Fig. 4.7B), converting it into percentage oxygen in the medium using calibration data and an internal conversion formula. Real time measurements can be viewed throughout the experiment and displayed in the form of a graph[197].

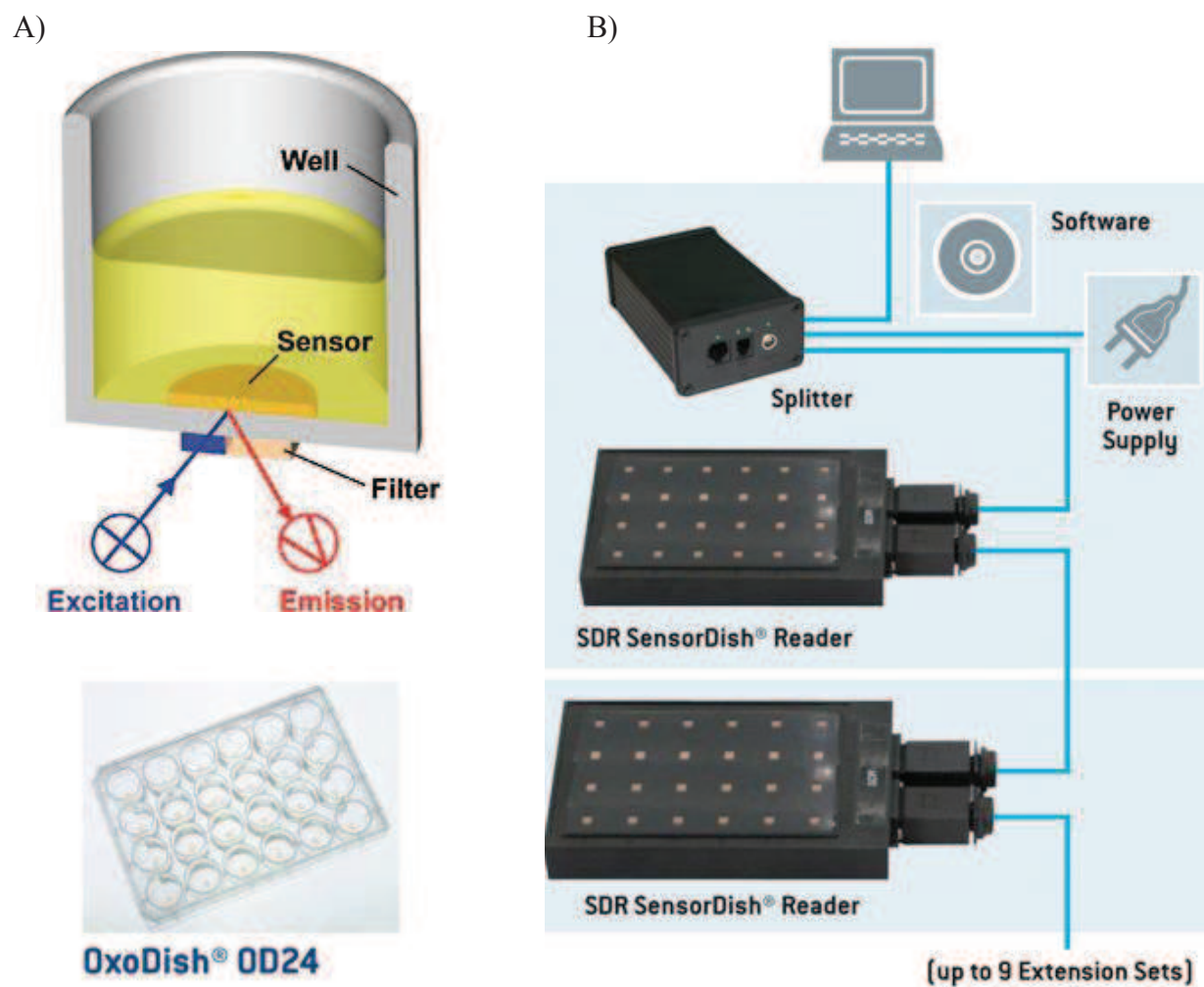


Figure 4.7: PreSens SensorDish reader (SDR) equipment

A) A well from an OxoDish containing DO and pH sensor spots allowing online measurement of dissolved oxygen partial pressure and pH; B) SDR main units. Images adapted from the Presens SFR web page[197].

P. pastoris cultures producing GFP containing the first antifoam to be tested, Antifoam A, were set up in the multiwell plate in BMMY medium and incubated at 30°C for 48 h. The

results obtained while using this system were variable; some wells did not appear to detect the oxygen in the medium as accurately as others. Using this multiwell format, there was also a problem with evaporation of the samples due to the small volumes used which may have interfered with the readings. Some of the data generated for Antifoam A in BMMY are shown in Fig. 4.8. This graph also highlights some of the problem wells, where the DO remained low before suddenly increasing.

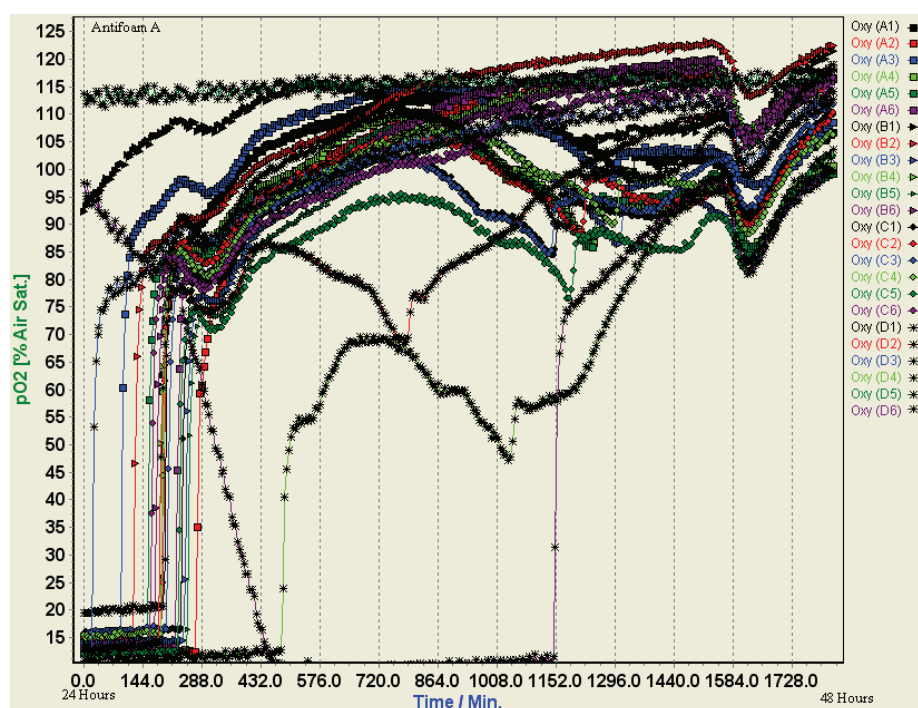


Figure 4.8: PreSens SensorDish reader data

Data generated for a 24 well setup with cultures of *P. pastoris* in BMMY producing GFP in the presence of various concentrations of Antifoam A.

It was decided that for more comparable conditions to those that had been used for the shake flask experiments of Chapter 3, another piece of equipment with the same technology would be used in a shake flask format. It was anticipated that this would avoid the problems of evaporation seen in the multiwell setup.

4.2.2.2 Measurements in a shake flask format

The effect of the addition of each of the antifoams on DO was assessed in shake flask cultures with the PreSens Shake Flask Reader (SFR, loaned by Applikon Biotechnology), and flasks with DO patches allowing online monitoring. A flask containing pH and DO sensor spots is illustrated in Fig. 4.9A, and the SFR in Fig. 4.9B. Similar to the SDR, the sensor patches in the flasks contain luminescent dyes which are excited by the SFR. Again, the luminescence lifetime depends upon the oxygen partial pressure and is monitored wirelessly by the software, which converts it into percentage oxygen in the medium using calibration data and an internal conversion formula. The data can be displayed graphically in real time[198].

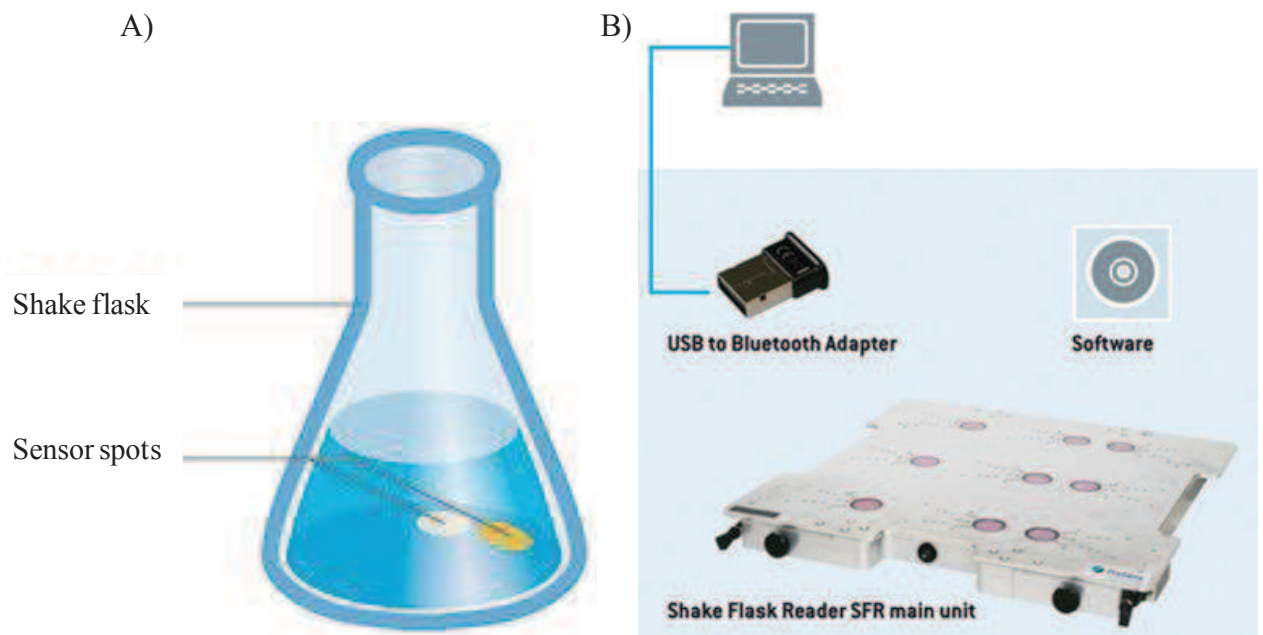


Figure 4.9: PreSens shake flask reader equipment

A) A shake flask containing DO and pH sensor spots allowing online measurement of dissolved oxygen partial pressure and pH; B) SFR main unit. Images adapted from the Presens SFR web page[198].

The experimental set up was the same as originally used for the shake flask experiments, except 125 mL shake flasks with sensor spots were used as the closest match to 100 mL flasks. Cultures of *P. pastoris* producing GFP were set up in 25 mL BMMY medium in the sensor flasks and clamped upon the SFR in a shaking incubator set to 30°C. The DO in the flasks was monitored over 48 h.

Figure 4.10 shows that there was no difference in DO in the flasks in the presence or absence of antifoam for each antifoam. After approximately 12 h for each culture condition, the DO in the flasks became limiting. Since functional GFP can be expressed by anaerobic bacteria and in media containing 0.1 ppm dissolved oxygen[199], there was no concern that this would influence the data obtained. For the cultures containing Antifoam A, C and J673A, methanol was added before it was fully consumed and became limiting. For the cultures containing P2000 and SB2121, methanol addition was delayed in order to observe the effects on the DO of the cultures where methanol was limiting. The DO decreased as the cells metabolized the methanol present in the medium and rose once they had consumed it as was seen with P2000 and SB2121 cultures. DO then remained high in the P2000 and SB2121 cultures until additional methanol was added at which point the DO immediately decreased and utilization continued. Methanol concentrations for the P2000 and SB2121 cultures were confirmed by gas chromatographic analysis. Some of these data are shown in Fig.4.11. Overall, there was no difference in the DO content of cultures containing antifoam and those without. This suggested that the antifoams either did not have any effect upon DO in shake flasks, or if they did have an effect, it was too small to be detected by this equipment. It is possible that in low volumes, effects upon DO may also be small and thus did not have any noticeable effect upon the growth of the cells. Alternatively, the antifoams may have increased the DO in the flasks and the cells in these cultures rapidly consumed any extra oxygen in order to produce more recombinant protein. In that case, the DO detected in the flasks would appear to be the same as that of the control.

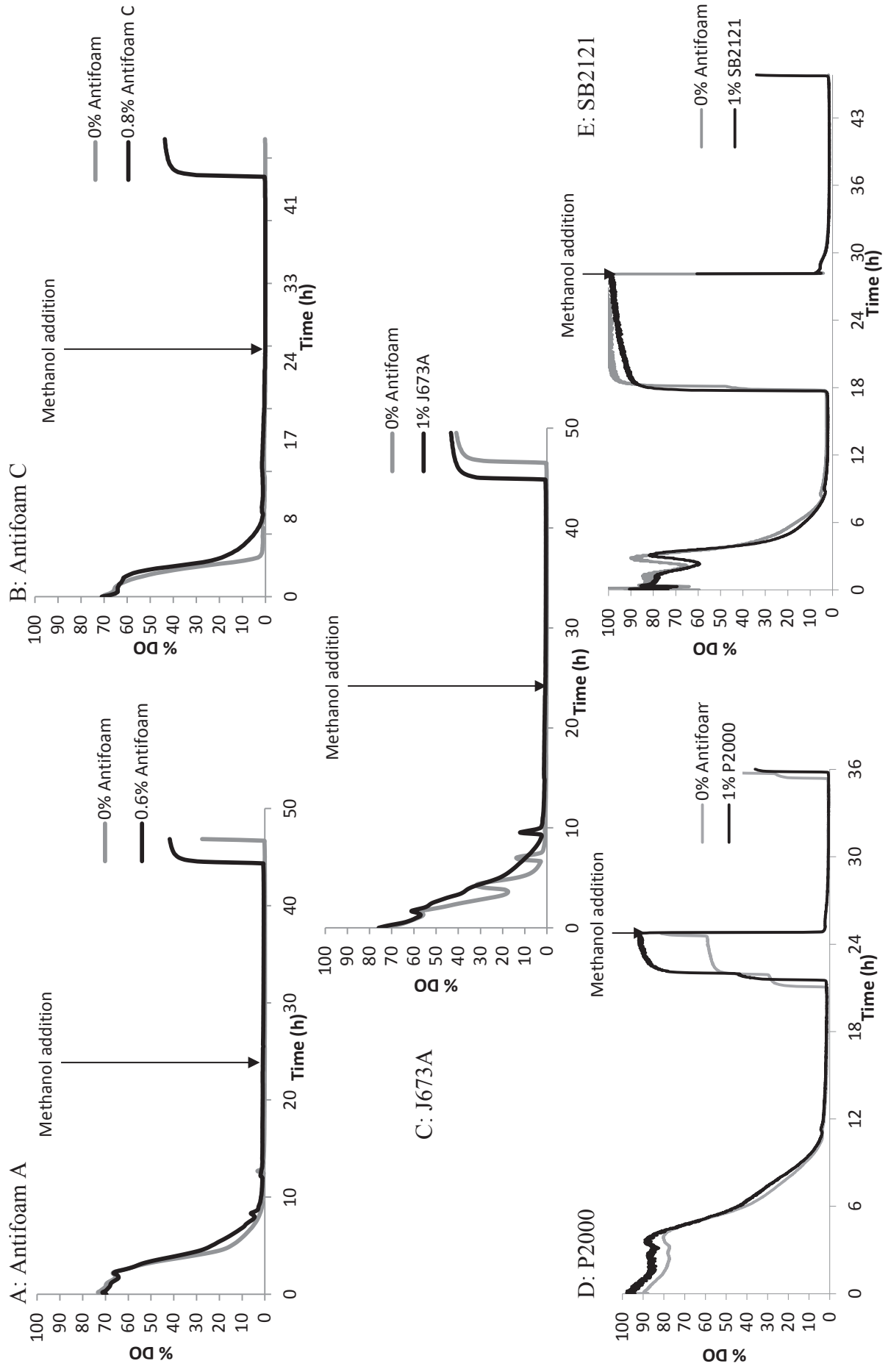


Figure 4.10: DO traces for shake flask cultures in the presence of antifoam. The DO traces for 125 mL shake flasks with 25 mL cultures of *P. pastoris* producing GFP in BMMY medium in the presence of antifoams at their optimum concentrations for improving the total yield of GFP. A) 0.6% Antifoam A; B) 0.8% Antifoam C; C) 1% J673A, D) 1% P2000 and E) 1% SB2121.

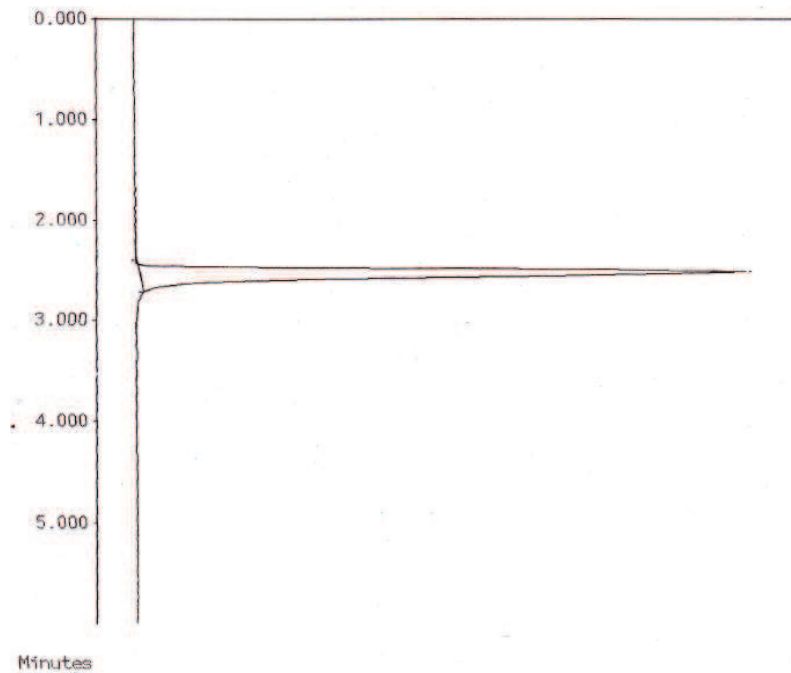


Figure 4.11: Methanol chromatography trace for *P. pastoris* shake flask culture supernatant

Chromatography trace for supernatant from a sample of *P. pastoris* in BMMY incubated with 1% P2000 for 6 h. Methanol concentrations were calculated by measuring the area under the graph. No peaks were observed for samples at 24 h suggesting that no methanol was present.

4.2.3 Effect of antifoams upon A_{2a}R production in shake flask cultures of *S. cerevisiae* strains with different requirements for DO

As discussed previously, antifoams are widely believed to exert their action by influencing the oxygen available in the medium, as measured by DO and k_La . As there did not appear to be any difference in the DO in shake flasks in the presence or absence of concentrations of antifoams examined, another approach was used. Strains were therefore selected for which data suggest that recombinant protein yields are related to high or low DO conditions. The *S. cerevisiae* TM6* strain has been observed to produce more A_{2a}R in high DO conditions and the WT strain found to produce more A_{2a}R in low DO conditions in preliminary small scale screening performed by Z. Bawa, Aston University (unpublished data), shown in Table 4.1.

Table 4.1: Effect of DO upon $A_{2a}R$ production by WT and TM6* *S. cerevisiae* strains. Data provided by Z. Bawa, Aston University

DO (%)	WT (fmol/mg protein)	TM6* (fmol/mg protein)
30	442.13	4.59
50	14.01	35.47

These strains were incubated with 0.6% Antifoam A which was found to increase the k_{La} and 1% Antifoam A was found to lower the k_{La} . Samples were taken once the glucose in the culture had decreased to 30 mmol. The yield of $A_{2a}R$ produced was assayed by radioligand binding. The data are shown below in Fig 4.12.

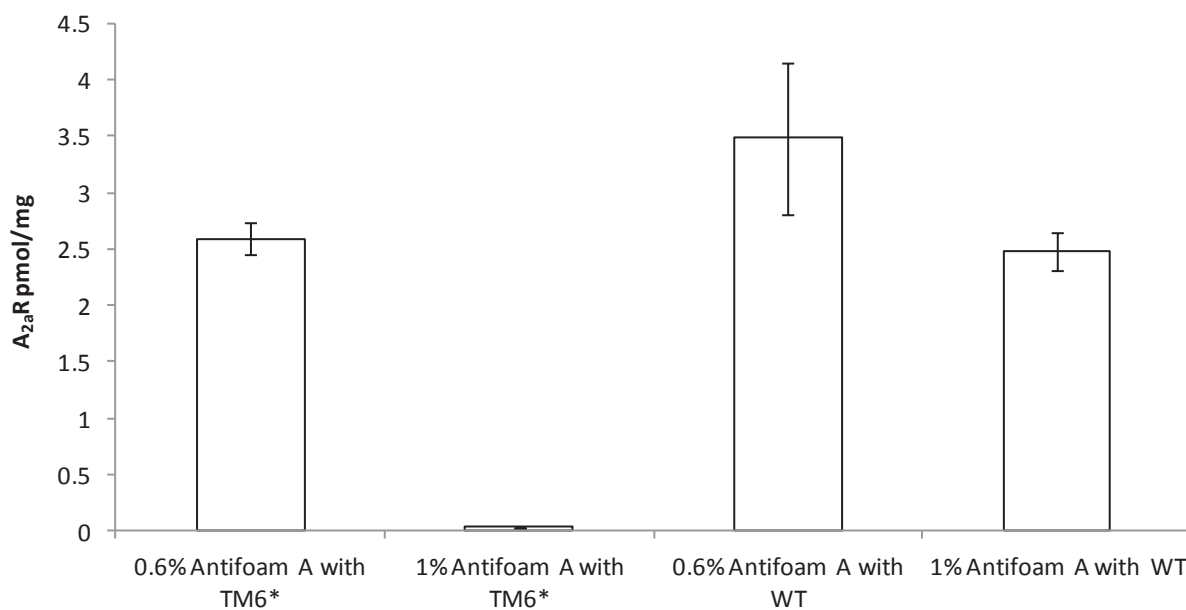


Figure 4.12: $A_{2a}R$ production in *S. cerevisiae* in the presence of antifoams

Effect of Antifoam A at 0.6% and 1% v/v upon the production of $A_{2a}R$ in two strains of *S. cerevisiae*, TM6* and WT in 100mL shake flasks. In all cases (except 1% Antifoam A with TM6* where $n = 1$) $n = 6$.

The data shown demonstrate that in the presence of 1% Antifoam A, the TM6* strain produced significantly less $A_{2a}R$ than the 0.6% Antifoam A culture. 0.6% Antifoam A in

shake flasks was found to increase the k_{La} , and TM6* has previously been found to produce more A_{2a}R under conditions with high DO, suggesting that the antifoam may have caused an increase in protein yield by altering the rate of oxygen transfer in the culture medium. However, with the WT strain, there was also less A_{2a}R produced in the culture containing 1% Antifoam A although this strain had been observed to produce a better yield of protein in low DO conditions. The difference in yield was not as pronounced as the differences seen for TM6*. This could suggest that the DO in the flask is a more essential parameter in order for the TM6* strain to produce recombinant proteins, whereas it may be less critical to the growth and protein production of the WT strain. It could alternatively suggest that the antifoam had another unknown effect which caused the difference in yields and the change to the k_{La} were not substantial enough to alter the DO conditions in the flask to the optimum levels required to produce higher protein yields. Again as with the DO shake flask experiments, it is possible that if there were any changes to the DO in these cultures influenced by the antifoams, it could be small and therefore not large enough to have resulted in any noticeable effect. The DO present in the flasks may already have been sufficient for the growth of the cells and the production of recombinant protein, and any changes to this did not have any further influence. Since a control without antifoam was not performed, it is unknown whether the concentrations of antifoam tested improved or reduced the yield compared to 0%. Follow up experiments including this control need to be performed to fully interpret the results.

4.2.4 Effect of antifoams upon the k_{La} characteristics of cultures in shake flasks

As P2000 and SB2121 affected the density of the cultures as shown in Chapter 3, there is a possibility that the oxygen transfer rate in the system was affected by antifoam addition which may explain the increase in GFP yields. The volumetric mass oxygen transfer coefficient, k_{La} , is a measure of how readily oxygen dissolves into the medium, whilst the DO is the quantity oxygen that has dissolved into the medium and is affected by the k_{La} . As this coefficient is widely recognized to be affected by antifoams[59, 93, 94, 200], the k_{La} was therefore measured in shake flasks in the presence of 0-1% v/v of the antifoams. The

PreSens SFR was again used for this investigation with 125 mL non-baffled shake flasks with 25 mL BMMY at 30°C without any inoculum. The dynamic method of k_La measurement based upon the method outlined by Bandyopadhyay and Humphrey[201] was used.

The experiment was carried out by firstly sparging the flask with compressed air until the DO reached saturation at approximately 80% air saturation. This reading was taken to be the 100% DO saturation in the flask. The k_La measurements were carried out by starting at 100 % DO and flushing with nitrogen until the DO dropped to 0 % (See Figure 4.13).



Figure 4.13.: SFR set up for k_La measurements

125 mL shake flasks with sensor spots containing 25 mL P. pastoris producing GFP in BMMY were clamped above the detectors with lines allowing sparging with compressed air and N₂. Photographed at Aston University.

The flask was then supplied with air and the DO gradually rose to 100 % saturation, whereupon it was again flushed with nitrogen to reduce it to 0 % before reconnecting the

air. The time points were recorded by the SFR software and DO was plotted as a function of time as shown by Fig. 4.14. As several antifoam concentrations were tested, they were added cumulatively in a step-wise fashion to the flask once the DO was at 100% saturation, followed by a flush with nitrogen.

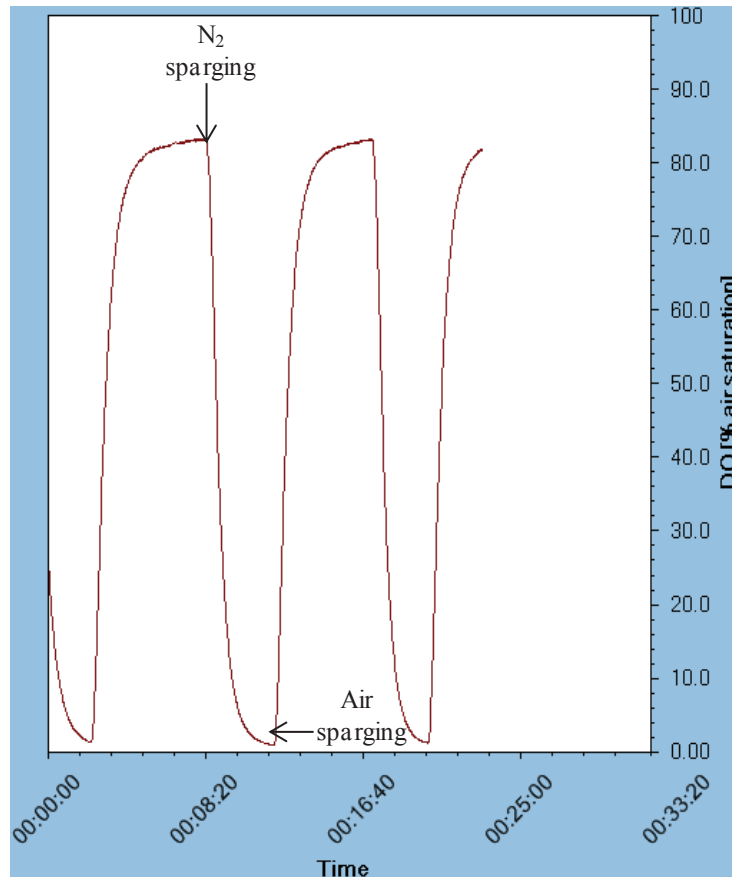


Figure 4.14: k_La measurements logged by SFR software

SFR software logging changes in DO in the presence of antifoam while sparging with compressed air to increase the DO and with N_2 to reduce it to 0%.

The data generated for the upwards slope of the plot where oxygen dissolved into the medium were used to calculate the k_La using the following formula:

$$k_La (t_2 - t_1) = \ln \left(\frac{c_{1,\infty} - c_{1,t1}}{c_{1,\infty} - c_{1,t2}} \right)$$

where t_1 and t_2 are consecutive time points, $c_{1,\infty}$ is the oxygen saturation concentration and c_1 is the oxygen concentration at each time point. $c_{1,\infty}$ was calculated by using the following constants:

- X_{O_2} (Fraction of O_2 in air) = 0.2095
- T (Temperature) = 30°C or 303 K
- P (Approximate pressure in the flask) = 1 bar
- R (Gas constant) = 8.3144 J/(K x mol)
- M_{O_2} (O_2 partition coefficient) = 30

Combining $C = n / V$, where C is the concentration of gas in the head space, n is the number of moles and V is the volume, and the universal gas law $PV = nRT$, gives $C = P / (RT)$. This enables the concentration of air in the gas phase, or all gases, to be calculated. The gas concentration of O_2 in the gas phase was then calculated by multiplying the concentration of gas in air by X_{O_2} . Dividing the gas concentration of O_2 by M_{O_2} gave the maximum liquid oxygen saturation concentration, $c_{1,\infty}$ at 100% DO. DO percentage values at a particular time point were converted to oxygen concentrations by dividing by 100 and multiplying by $c_{1,\infty}$. The k_La data for the shake flasks with BMMY media in the presence of antifoam are shown in Table 4.2. The data were normalized to the control (0% antifoam) to allow comparison between the antifoams.

Table 4.2: The effect of antifoam addition to the k_La in shake flasks with BMMY medium. The data highlighted in yellow represent no significant change from the control k_La , green a significant increase and red a significant decrease in the % change of k_La . In all cases $n = 2$.

Antifoam	% Antifoam					
	0%	0.2%	0.4%	0.6%	0.8%	1%
Antifoam A	100	98.6	290	380.8	78.1	55.4
Antifoam C	100	43.2	89.5	138.6	269.4	98.8
J673A	100	66.6	58.7	58.5	58.4	108.2
P2000	100	63.9	115.9	116.2	34.7	32.3
SB2121	100	70.2	102.8	65.1	45.8	43.8

Addition of 0.4% and 0.6% Antifoam A caused a large increase in k_{La} compared to the control, whereas addition of the related antifoam, Antifoam C, led to an initial reduction in k_{La} , which increased on addition of antifoam up to 0.8% and then returned to control levels at 1%. After an initial decrease in k_{La} was caused by J673A addition up to 0.4%, it remained relatively constant up to 1%. Addition of P2000 at all concentrations tested caused relatively minor changes to the k_{La} . SB2121 addition did not substantially increase the k_{La} at any of the concentrations tested. Most of the concentrations investigated caused a decrease in the k_{La} . In shake flasks, the k_{La} was higher at mid-range values and decreased with increasing concentration. Overall there was no statistically significant correlation between increased k_{La} and total yield.

4.3 Antifoams as potential carbon sources

It is possible that the antifoam agents could provide a carbon source for the *P. pastoris* cells which could explain the boost to productivity observed with the GFP cultures. J673A, for example, contains fatty acids on a vegetable oil base and it is thought that vegetable oils may be metabolized as a carbon source[202], which could explain why J673A addition enhanced the yield of GFP. In order to determine whether the antifoams could be metabolized, shake flask cultures were set up as before in 100 mL non-baffled shake flasks with 20 mL culture producing GFP. A culture was set up in BMGY medium and then the cells were transferred to medium without any glycerol or methanol with 1mL of each antifoam (5% v/v) denoted BMAY. Controls were set up in medium that did not contain glycerol, methanol or antifoams which was denoted BMY. All shake flasks were set up in triplicate. The OD_{595} was measured after 24 h and the data are shown in Fig 4.15.

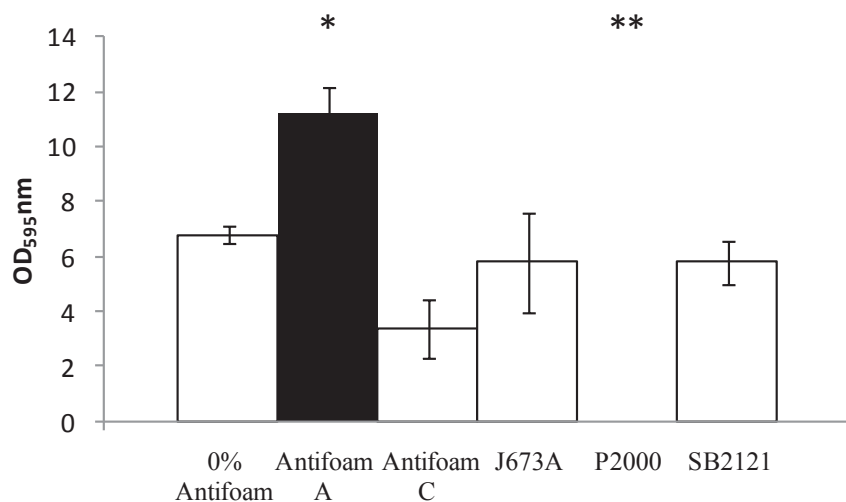


Figure 4.15: Antifoams as potential carbon sources at 5% v/v

The effect of 1 mL of antifoam in medium without either glycerol or methanol present upon *P. pastoris* growth in shake flasks after 24 h. In each case $n = 3$.

The data in Fig 4.15 show that Antifoam A can be metabolized by the *P. pastoris* cells in the absence of other carbon sources. Compared to the control cultures without any antifoam added, the OD was significantly higher. The related antifoam, Antifoam C, however led to a reduction in the OD in comparison with the control. Antifoams J673A and SB2121 did not have much effect upon the growth of the cells compared to the control. Cultures containing P2000 did not grow at all.

Further shake flask experiments were set up taking the best and worst yielding conditions. This time 2 mL (10% v/v) Antifoam A were added and 2 mL P2000 to see if the effects on the OD would be more pronounced and if higher concentrations of antifoam were required to obtain any growth for P2000. The data are shown in Fig 4.16.

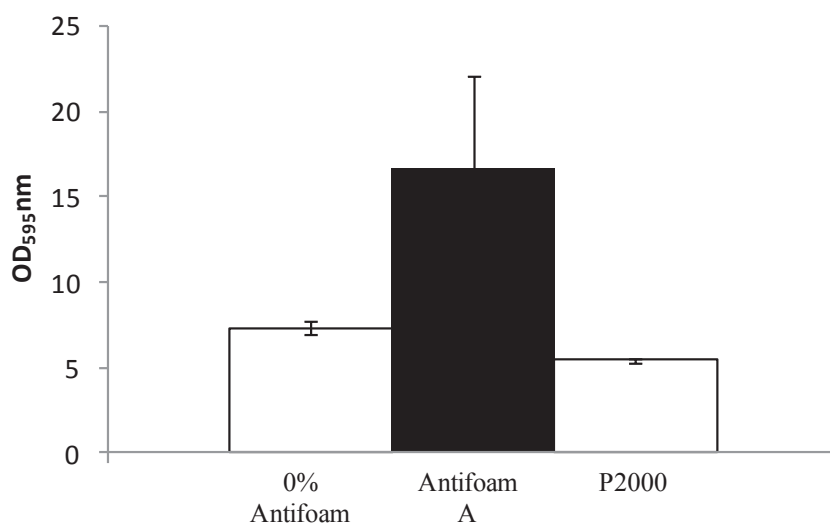


Figure 4.16: Antifoams A and P2000 as potential carbon sources at 10% v/v

The effect of 2 mL Antifoam A and P2000 upon P. pastoris growth in shake flasks in medium without either glycerol or methanol present after 24 h. In each case n =3.

Fig 4.16 shows that at higher volumes of Antifoam A, the OD has increased to double that of the control. This suggests that the volume of antifoam A added previously was depleted in the first set of experiments and became limiting to the growth. Antifoam A can clearly be metabolized in the absence of other carbon sources, and at 2 mL, the OD in this experiment of 16 is almost comparable to the OD's of 24 h shake flask cultures of BMGY after this period, which are usually between 18-22. The cultures containing 2 mL P2000 grew in this investigation, suggesting that the concentration of antifoam required to allow growth varies between antifoams. It appears that although the antifoams may be metabolized at concentrations of 10% v/v, the highest concentration used in the initial shake flask experiments was 1% v/v therefore for all of the antifoams, possibly except for Antifoam A, this concentration would be unlikely to substantially affect the growth of the cells if methanol became limiting. In the case of these antifoams, a different mechanism may be responsible for the changes in the yield of protein.

4.4 Summary

Antifoams have been demonstrated to affect the ability of *P. pastoris* cells to secrete recombinant GFP and can have various effects depending upon the type and concentration. Investigation into their effects upon the secretion of GFP by *P. pastoris* cells showed that in the case of cultures containing Antifoam A, Antifoam C and J673A, significantly more protein was secreted into the medium than the cultures without antifoam. Cultures containing P2000 and SB2121 retained significantly more GFP than the control, and produced more GFP, both retained and secreted, overall. When total complement of protein produced by cultures containing the antifoams was measured, it was found that it had not significantly been increased. This suggested that the antifoams influenced only the recombinant protein secretion pathway for GFP and had no effect upon total protein produced by *P. pastoris*.

Preliminary mass spectrometry data for glycerophospholipids suggested that there may have been a change to the relative compositions of phosphatidylcholine for 1% P2000 containing cultures and phosphatidylinositol composition for each antifoam-containing culture compared to controls. Microcalorimetry data suggested that the antifoams did affect the metabolism of the cells, illustrated by increases or reductions in heat output compared to control cultures. Due to varied data however, it is unclear whether the increases in yield of GFP can be linked with either an increase or a decrease in heat output. Investigation into the effects of antifoams upon DO suggested that there was no difference in the DO in the flasks both with and without antifoam, implying that effects were either too small to be detected, or that the cells were rapidly utilizing any extra oxygen causing the traces to appear similar. Effects upon the k_{La} were more noticeable, with Antifoam A and C at two concentrations increasing the k_{La} and other antifoams and concentrations either causing no change or decreasing it. While there was no correlation with the total GFP yields suggesting that the DO was already sufficient for the cells to grow, the antifoams had very different effects upon the k_{La} which should be taken into account along with the recombinant protein to be produced, as some proteins may be sensitive to DO concentrations, such as EPO[187] and antibody Fab fragments[203].

When the ability of *P. pastoris* cells to metabolize the antifoams in the absence of other carbon sources was examined, it was found that at 5% v/v only cultures containing Antifoam A were able to grow better than the control. P2000 cultures did not grow at all, however, when the concentration was increased to 10%, the cells were able to grow and Antifoam A-containing cultures grew almost as well as cultures in medium containing methanol or glycerol. However, in the shake flask experiments, antifoams were added up to 1%, therefore except possibly for those containing Antifoam A, the concentration would not be high enough in these cultures to allow the cells to grow any better should methanol have become limiting.

It seems that the antifoams have several mechanisms of action, and although the exact mechanism for the increases in GFP yield could not be pinpointed from these investigations, it is most likely that a combination of effects led to the changes to recombinant protein yield.

5. THE EFFECTS OF ANTIFOAMS UPON RECOMBINANT PROTEIN PRODUCTION IN BIOREACTORS

After initial screening experiments for optimal conditions in shake flasks, bioreactors are usually employed in order to produce large quantities of the recombinant protein of interest. Antifoams are more commonly used in this system than in shake flasks, as stirring and the introduction of the gases required for the organisms to grow lead to foam formation which can compromise the sterility of the culture if a foam-out occurs. Foaming may also cause damage to the equipment if foam blocks a filter and over pressurization of the vessel results. The antifoams examined in Chapter 3 were therefore tested in bioreactors in order to determine whether their effects could be reproduced on a large scale to further boost protein yield. GFP and A_{2a}R were the target proteins for these investigations. Their effects upon the DO and k_La were specifically investigated as optimization of these parameters in bioreactors is often an objective in scale-up.

5.1. Effect of antifoams upon GFP production in bioreactors

A commonly-used bioreactor for recombinant protein production is the stirred-tank reactor with agitation provided by impellers[183]. A 3 L stirred-tank autoclavable bioreactor and equipment from Applikon Biotechnology were used for these investigations (See Chapter 2 Fig 2.1 for an image of the set up). A preculture of *P. pastoris* strain X33-GFP was grown overnight in BMGY. The bioreactor was set up and 1 L basal salts medium added before autoclaving. Basal salts medium was used for these experiments as it is more commonly used in large scale recombinant protein production as the composition is well defined, unlike BMGY or BMMY which contain yeast extract. The probes were connected and the DO probe allowed to polarize overnight. The next day, the temperature of the bioreactor was set to 30°C and the pH was set to 5.0 with 28% v/v ammonium hydroxide and 50% v/v phosphoric acid. The stirrer was put into a cascade ranging from 700 rpm to 1,250 rpm to maintain a DO setpoint of 30%. 1.5 L/min of compressed air was passed into the vessel. An oxygen cylinder containing 40%: 60% nitrogen: oxygen was also connected to the mass

flow controller unit at 2.5 bar to allow further control of the DO to maintain the 30% setpoint. These settings were maintained for 1 h and the DO calibrated to 100%. 4.35 mL PTM₁ trace salts was added aseptically and the required volume of antifoam was also added[204].

The bioreactor was then inoculated to an OD₅₉₅ of 0.5 in 1 L with the preculture. The run was started immediately after inoculation and BioXpert software was used to log the data. The batch phase continued until a DO spike was observed[66], indicating that the glycerol present in the growth medium had been metabolized (20–24 h after inoculation). A fed batch phase was then begun to increase biomass using 50% v/v glycerol with 12 mL PTM₁ trace salts per L of feed. After 4 h of fed batch, the culture was starved for 1 h to ensure all remaining glycerol was utilized. Induction with 20% v/v methanol was then performed to initiate recombinant protein production. Holmes *et al* found that the highest protein yielding induction feed for *P. pastoris* producing GFP was a 60% methanol 20% sorbitol feed[36]. Problems were encountered while using this feed, however, as the sorbitol accumulated in the tubing causing it to burst. The second most productive feed was 20% v/v methanol therefore this was used for each run. The induction phase was allowed to continue to around 96 h post-inoculation. A typical bioreactor trace is shown in Fig 5.1.

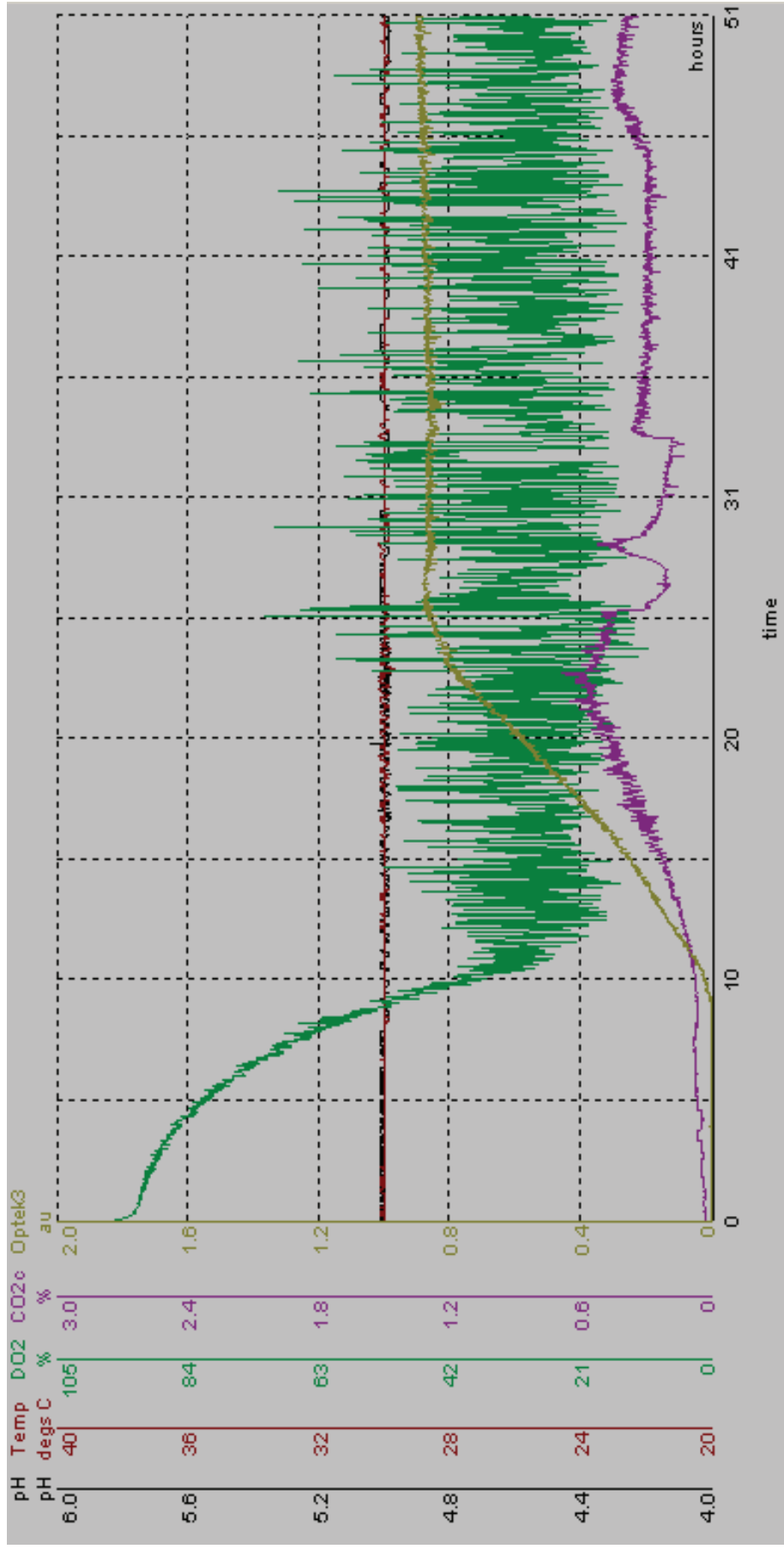


Figure 5.1: Example bioreactor trace plotted using BioXpert software

The black trace shows the pH maintained at the setpoint of 5 throughout the run. The red trace shows the temperature which was set to 30°C. The green trace represents the DO in the medium which is kept around the set point of 30 %. Once the concentration is lower than 30%, the stirrer speed increases and enriched air from the cylinder is sparged into the medium. Stirrer speed reduces as the DO concentration becomes higher, causing a continual fluctuation in DO around 30%. The pink trace shows the CO₂ produced by the culture and the yellow trace shows the density of the cells.

5.1.1 Effect of antifoams upon total GFP yield in bioreactors

Initial bioreactor experiments were conducted using the best yielding antifoam concentrations observed in the small scale shake flask evaluations described in Chapter 3. The antifoams were added to bioreactor cultures of *P. pastoris* producing GFP at 0.1% and at the optimum concentration in shake flasks as shown in Table 5.1.

Table 5.1: Concentrations of antifoam added to 1 L bioreactor cultures of P. pastoris producing GFP. Control concentrations were 0.1% and the optimum concentrations from shake flasks were used for the test concentrations.

Antifoam	Control Concentration	Volume Added	Test Concentration	Volume Added
Antifoam A	0.1%	1 mL	0.6%	6 mL
Antifoam C	0.1%	1 mL	0.8%	8 mL
J673A	0.1%	1 mL	1%	10 mL
P2000	0.1%	1 mL	1%	10 mL
SB2121	0.1%	1 mL	1%	10 mL

Antifoams were added immediately prior to inoculation. In the case of 0.1% Antifoam C and 0.1% P2000, foaming occurred during the run, and more antifoam was added (200 μ L and 500 μ L, respectively). All conditions were performed in duplicate, except for 0.1% and 0.6% Antifoam A which were performed in quadruplicate as some of these runs were preliminary tests, initially run for 51 h. GFP measurements were made as previously for the shake flask experiments using fluorimetry with a recombinant GFP standard and basal salts medium as a blank. The results are shown in Fig 5.2.

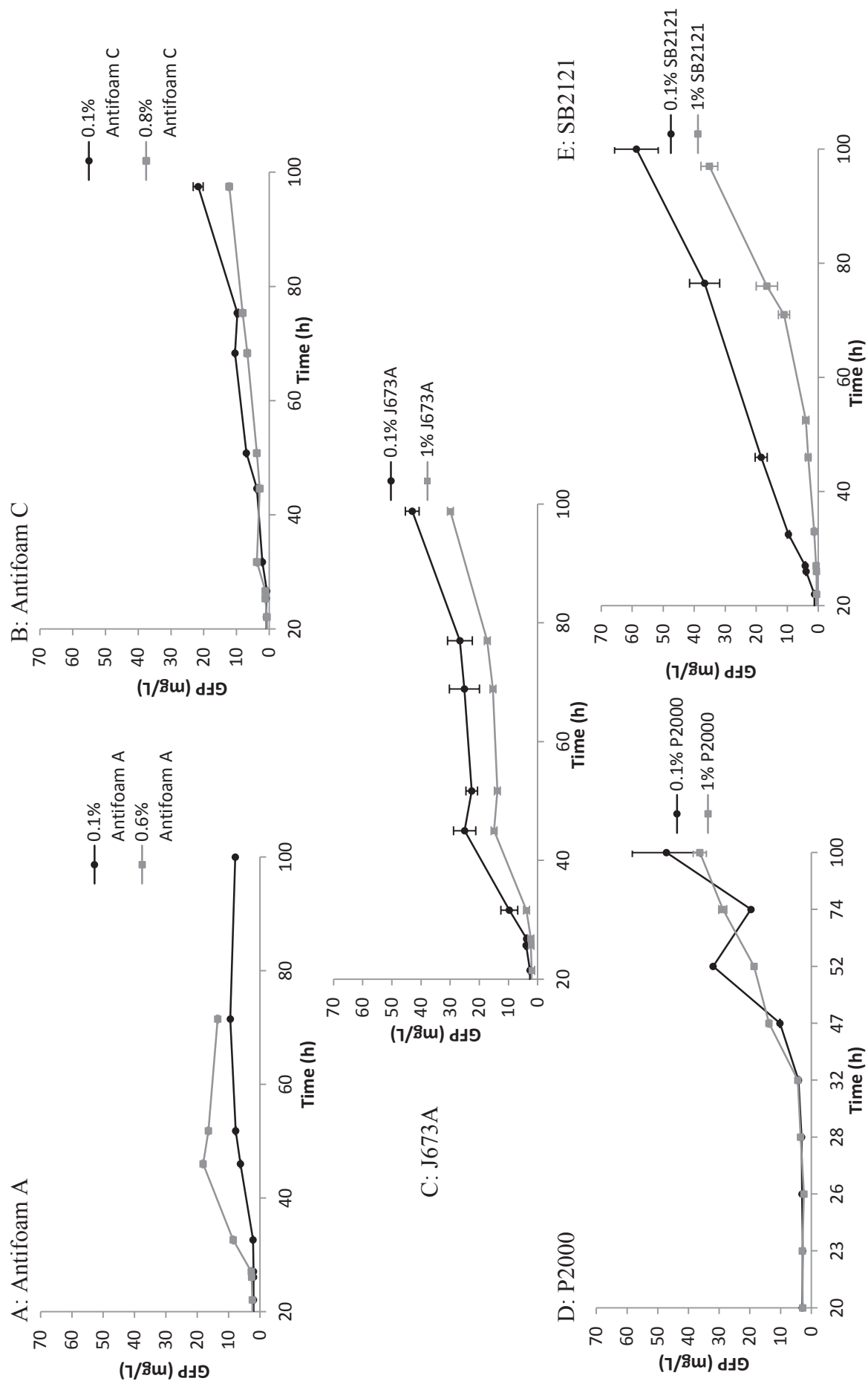


Figure 5.2: The effect of antifoams upon GFP production by *P. pastoris* in 3 L stirred-tank bioreactors during the induction phase. Antifoam A (A), Antifoam C (B), J673A (C), P2000 (D) and SB2121 (E) at concentrations from 0-1%. The error bars show the respective standard errors. In all cases n equals at least 6.

Fig 5.2 E shows that after 100 h the best antifoam condition was 0.1% SB2121 which resulted in a total yield of 59 mg/L GFP, followed by 0.1% P2000 (Fig 5.2 D) which produced a total yield of 47 mg/L GFP. The poorest yielding condition was 0.1% Antifoam A (Fig 5.2A) which produced a total yield of 8 mg/L (although it is unclear if 0.6% would have produced less GFP if the run had lasted 100 h as the 0.1% Antifoam A run did), followed by 0.8% Antifoam C which produced 12 mg/L GFP.

In the shake flask studies, a higher concentration of antifoam had produced a higher yield, whereas in the bioreactors a higher yield was seen at a low concentration of antifoam, except for Antifoam A. Although a different concentration effect was observed, the trend for the highest yielding and lowest yielding antifoams was still the same.

The yield of GFP produced by the bioreactor cultures containing Antifoam A decreased after 71.5 h for 0.1% and after 52 h for 0.6%. This suggests possible increased protease activity in the presence of this antifoam, especially as the effect occurred sooner with a higher concentration. Shake flask cultures were set up with a dilution series of protease inhibitors, however the *P. pastoris* cells did not grow in any of the cultures. Possible protease activity could be more easily verified by setting up a bioreactor culture with a protease deficient strain and comparing the effects of the presence of 0.6% Antifoam A on yields in the non-protease deficient strain used here.

To confirm the fluorimetry readings which suggest that the cultures with 0.1% antifoam produced a higher total yield of GFP, a Western blot was performed on 18 μ L sample supernatants for 0.1% SB2121 and 1% SB2121. The Western shown in Fig. 5.3 backs up the fluorimetry data with darker bands present for the 0.1% samples compared to the 1% samples.

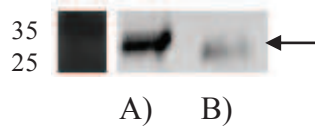


Figure 5.3: Western blot performed on bioreactor X33-GFP culture samples

A) 0.1% SB2121 and B) 1% SB2121. Protein ladder marker with bands at 25 and 35 kDa show that the sample bands are 29 kDa GFP. The band obtained for the 0.1% SB2121 sample is much darker than that of the 1% sample suggesting the presence of more protein.

5.1.2 Effect of antifoams upon specific yield of GFP

To determine whether altered growth of the cells in the bioreactor cultures explained the differences in yield, the dry cell weights for 1 mL of cells was measured and used to calculate the dry cell weight per L. The total yield of GFP per L was divided by the dry cell weight per L to obtain the yield of GFP per mg of cells. The data are shown in Fig 5.4.

The data show that with the exceptions of J673A and SB2121, more GFP was produced per mg of cells by the cultures containing higher concentration cultures than those with 0.1% v/v antifoam. SB2121 again produced the most GFP at 0.1% (1.9 μg GFP/mg cells) with a large difference of approximately double the GFP yield compared to the 1% cultures (0.8 μg GFP/mg cells). A difference in specific yield between the two concentrations suggests that this was not due to a difference in the growth of the cells, which is in contrast to the effects observed in the shake flask experiments. The cultures containing J673A also showed a large difference in the specific yield of GFP produced, with more GFP present per mg of cells in the 0.1% cultures. Again this suggests that the effects are not due to changes in the growth of the cells, which is also what was observed in the shake flask experiments. P2000 cultures showed little difference between the 0.1% and 1% cultures, however slightly more GFP was produced in the cultures containing 1% which is the opposite to the results for the total yield where 0.1% produced more total GFP. This result is similar to that seen for the shake flask investigation, where there was no significant difference between the high and low concentrations, suggesting that for these cultures the increase in total GFP yield could be due to changes in the growth of the cells.

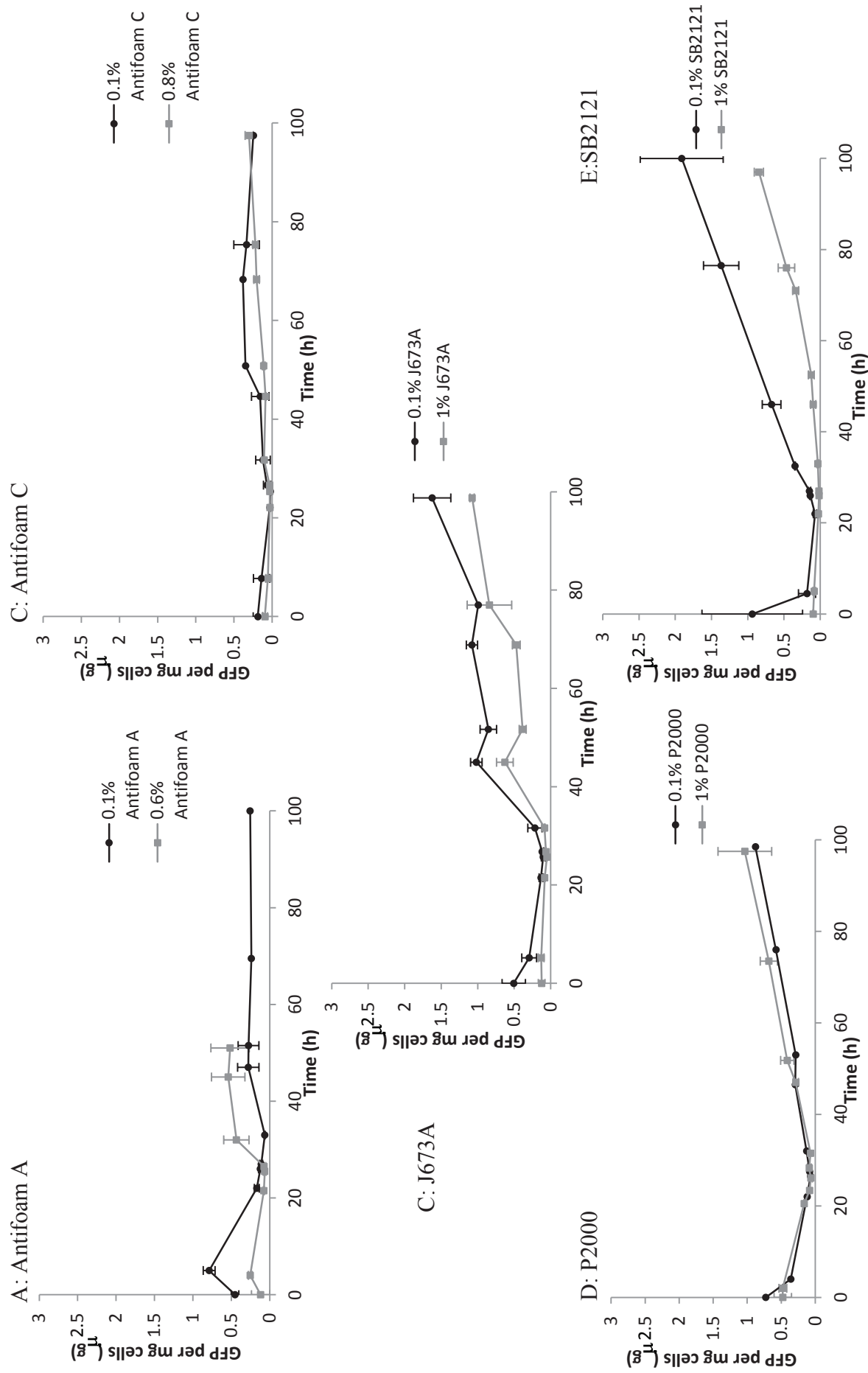


Figure 5.4: The effect of antifoams upon the specific yield of GFP by *P. pastoris* in 3 L stirred-tank bioreactors during the induction phase. Antifoam A (A), Antifoam C (B), J673A (C), P2000 (D) and SB2121 (E) at concentrations from 0-1%. The error bars show the respective standard errors. In all cases n equals at least 6.

Antifoam A at 0.6% produced more GFP per mg cells at 50 h compared to cultures containing 0.1% antifoam, which was the same trend as for the total yield. There was a slightly larger difference in the amount of protein produced therefore increases in total yield appear to be due to another mechanisms of action and not due to the growth of the cells. Bioreactor cultures containing Antifoam C normalized to the specific yield of GFP showed small differences at each concentration therefore the total yield increases were due to the growth of the cells. For this antifoam, the higher concentration produced more GFP per mg of cells than the 0.1% cultures.

The same trend is still observed for the best and worst producing antifoam effects for the specific yield as the total yield in bioreactors as well as the shake flask experiments, with the most productive antifoams being SB2121, J673A and P2000, and the poorest being Antifoams A and C. The antifoams can again be split into two different categories according to their mechanisms of action; those that caused increases to total GFP yields due to changes in growth as shown by P2000 and Antifoams A and C; and those that caused an increase in production or secretion of GFP per cell as demonstrated by antifoams SB2121 and J673A. The antifoams in each group are also interesting, as previously in the shake flasks P2000 and SB2121 had caused changes to growth, whereas in the bioreactors it appears that Antifoams A and C also have this effect rather than increasing the secretion or production per cell. Another interesting trend is that the two antifoams that produced a greater amount of GFP at 0.1% for both the total and specific yields (SB2121 and J673A) are in the group that seems to enhance the secretion or production of the protein. In contrast, P2000, Antifoam A and Antifoam C which caused a switch in concentration effect upon normalizing the total GFP yield to the per mg of cells yield (although differences were small) are also all in the same group which appears to have influenced the growth of the cells. This could suggest that antifoams that enhance the production or secretion of the GFP are more effective at this when added at low concentrations to bioreactors, but in all cases, except for Antifoam A, the total yield is improved by adding 0.1% antifoam rather than higher concentrations.

5.1.3 Influence of the culture medium on the results

One of the conditions that differed in the bioreactor experiments compared to the shake flask tests was basal salts medium was used instead of BMMY. To rule out the involvement of the medium on the ‘concentration switching’ effect seen with the bioreactor results, a shake flask experiment was set up as previously, this time using basal salts medium. Cultures were set up in basal salts medium containing 40 g /L glycerol. Flasks were then set up in basal salts medium containing 0.5% v/v methanol and were inoculated with the culture to an OD of 1. Either 0.1% or the optimum antifoam concentration observed in the BMMY shake flasks was added and the flasks were incubated for 48 h at 30°C, 220 rpm. Samples were measured in triplicate and analyzed by a one-way ANOVA ($P < 0.0001$) and a Dunnett's multiple comparison test where $* = P \leq 0.05$ and $** = P \leq 0.01$ and $*** = P \leq 0.001$). Data are shown in Fig. 5.5.

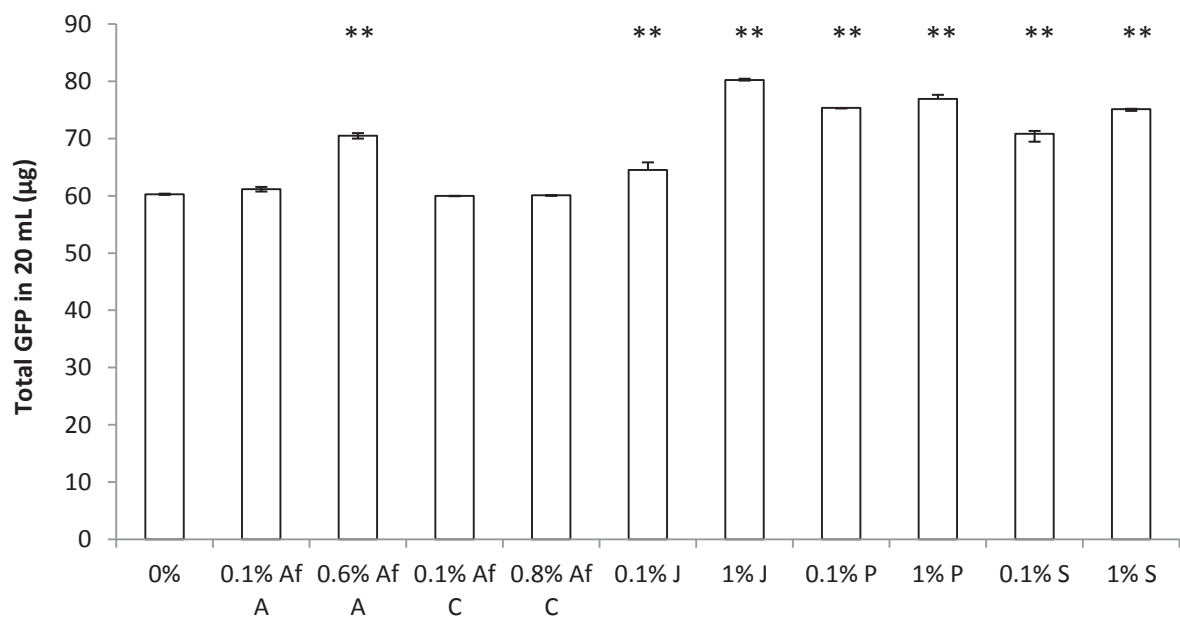


Figure 5.5: Effect of Antifoams upon *P. pastoris* X33 producing GFP in basal salts medium in shake flasks

Af A represents cultures containing Antifoam A, *Af C* represents Antifoam C, *J* represents J673A, *P* represents P2000 and *S* represents cultures containing SB2121. In each case $n = 9$.

Fig 5.5 shows that all 0.1% antifoam-containing samples produced a significantly greater yield of GFP than the control in basal salts medium, except for those containing Antifoam A and C. This was mirrored at higher concentrations of antifoams. In addition, the higher concentrations of antifoam also produced more GFP than the 0.1% cultures with the exception of 0.6% Antifoam C, which is similar to the trend seen for the BMMY shake flask results. Again, the best yielding antifoams were J673A, P2000 and SB2121. This suggests that the switching effect is not due to changing from BMMY medium to basal salts medium in the bioreactors.

Notably, the total GFP yields in basal salts medium in shake flasks were much lower than those achieved in BMMY medium. The 0% cultures in BMMY medium produced approximately 250 μg of GFP in 20 mL compared to 60 μg in basal salts medium. The maximum total GFP yield in BMMY was 422 μg compared to just 80 μg in basal salts medium. Interestingly, the ODs reached in basal salts medium were higher than achieved in BMMY. The average OD for cultures without antifoam was 49.4 compared to 22.66 in BMMY. Therefore, in basal salts medium the cells produced much less GFP per cell than in BMMY. This implies that basal salts medium is less suitable for recombinant protein production in small scale shake flask cultures. It is possible that as methanol was not constantly fed into the flasks as it would be in the bioreactors, this suggests that cultures growing in basal salts medium may require the constant feed of methanol to produce greater quantities of protein, whereas in BMMY the nutrients and methanol provided are enough for the cells to effectively produce recombinant protein.

5.1.4 Influence of the point of addition of the antifoam

In order to determine whether the antifoams had a better or worse effect upon the production of recombinant protein when added at the beginning of the fermentation or only when required to destroy foam, J673A was added to bioreactor cultures of *P. pastoris* producing GFP at concentrations of 0.1% and 1% only when foaming occurred. This was around 20 h after the start of the run for each culture, and the conditions were run in duplicate. Data are shown in Fig.5.6.

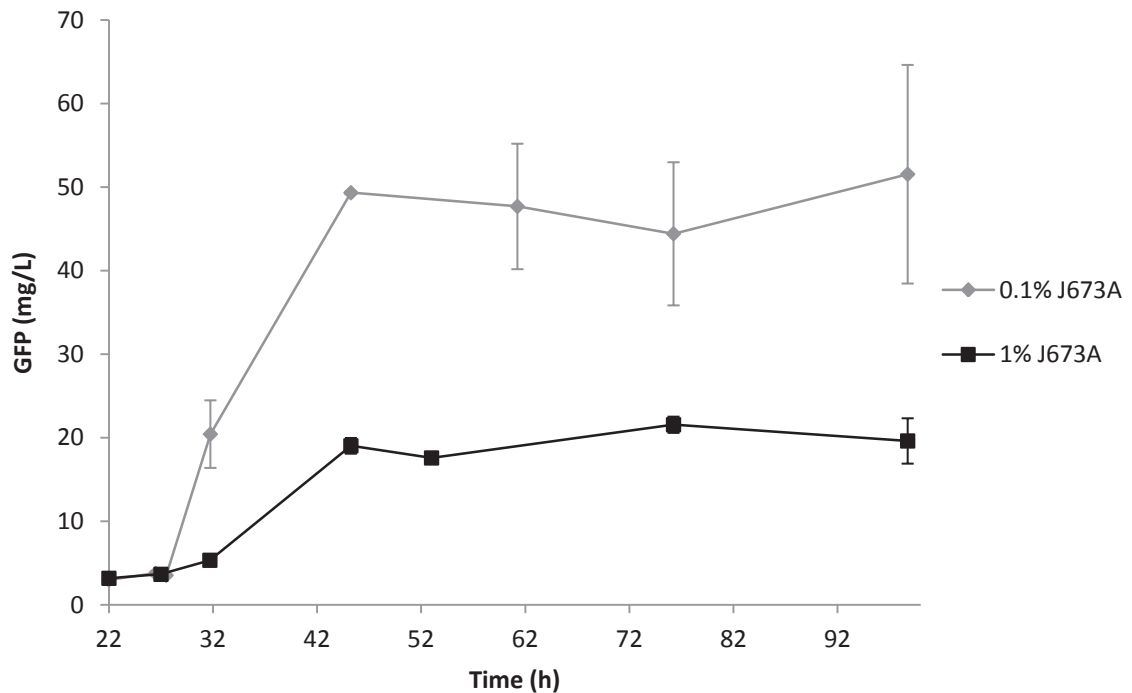


Figure 5.6: Effect of J673A addition to bioreactor cultures only when foaming occurred

*J673A at 0.1% and 1% J673A were added only when required to reduce foaming to cultures of *P. pastoris* producing GFP in 3 L stirred tank bioreactors. Foaming occurred around 20 h into the run and antifoams were added. Induction occurred at 27 h. In each case $n = 6$.*

When antifoam J673A was added to the bioreactor cultures once foaming occurred, the total yield when it was added to 0.1% was 50 mg, whereas at 1% the total yield was 20 mg. The same trend was observed as when the antifoam was added from the beginning of the run, with 0.1% producing the best effect. However, 43 mg was obtained when J673A was present throughout the run, suggesting that adding this concentration of antifoam later in the run had a better effect. When 1% J673A was present from the start of the run, a total yield of 30 mg was achieved, but when this concentration was added when foaming occurred, only 20 mg of GFP was produced. For this concentration, it appears to be more effective when added at the start of the run, although this could also reflect batch-to-batch variation.

5.2 Effect of antifoams upon oxygen availability in bioreactors

As demonstrated by the GFP bioreactor experiments, low concentrations of antifoam produced a better yield, whereas the opposite was true in the shake flasks where high concentrations produced more GFP. This change in concentration effect occurred upon scaling up from a shake flask to a bioreactor, where the most obvious difference between these two systems is the oxygen transfer characteristics of the system. The difference in the physical properties of the vessels such as the shape affects the k_La . In a shake flask, the oxygen in the flask is uncontrolled and as seen in Chapter 4 (Fig 4.10), the DO dropped to 0% as the cells consumed the methanol in the medium. In a bioreactor however, the DO can be maintained at the desired setpoint by introduction of air or oxygen-enriched gas, or by stirring. An investigation into the effects of the antifoams upon the k_La and the DO in bioreactors was therefore conducted in order to determine whether these parameters might explain the differences observed.

5.2.1 Effect of antifoams upon k_La in bioreactors

The antifoams in the shake flasks had an effect upon the k_La at different concentrations, although this did not seem to be correlated with recombinant protein yield. Their effects upon k_La in the bioreactors were expected to differ from that of the shake flasks. In this system, the dynamic method of k_La measurement outlined by Bandyopadhyay and Humphrey[201] was again used in the absence of cells.

1L of BMMY was dispensed into the bioreactor and the antifoams were added in a stepwise manner. The bioreactor settings were a stirrer speed of 700 rpm, temperature set to 30°C, and 1.0 L/min flow rate of air. A DO probe was connected and calibrated[205]. The DO data were recorded with a Picolog ADC-16 recorder every 300 ms via the DO measurements from the DO probe. The k_La measurements were carried out by starting at 100% DO, adding the required volume of antifoam then flushing with nitrogen until the DO had reached 0%. This was repeated twice for each antifoam concentration and the data were recorded via a laptop. These data were used to calculate the k_La , as explained in Chapter 4.

The results are shown in Table 5.2 with red indicating a decrease in k_{La} , yellow no significant difference and green indicating an increase.

Table 5.2: Effect of the presence of antifoams upon k_{La} in a 3 L stirred-tank bioreactor at increasing concentrations. Percentage increases and decreases are shown in relation to the control readings taken without antifoam present (100%). Green indicates an increase, yellow no change and red a decrease to k_{La} .

Antifoam	% Antifoam							
	0%	0.01%	0.1%	0.2%	0.4%	0.6%	0.8%	1%
Antifoam A	100	118.8	55.6	63.8	50.4	88.7	147.1	206.9
Antifoam C	100	338.5	140.3	207.5	238.4	232.7	265.3	366.2
J673A	100	64.5	51.7	204	90.7	99.6	130.9	103.9
P2000	100	270.4	140.8	94.7	68.7	193	211.5	769
SB2121	100	52.8	34.9	39.9	23.4	32.6	73.1	35.7

The only antifoam to not decrease the k_{La} at any of the concentrations tested was Antifoam C. The k_{La} was highest at 1% and lowest at 0.1%. SB2121 decreased the k_{La} at each concentration, with the highest being at 0.8% and the lowest at 0.4% which was the lowest k_{La} value out of all the conditions investigated. P2000 caused the biggest increase in k_{La} out of the antifoams at 1%. Antifoam A initially caused a decrease in the k_{La} which was then doubled at 1% and J673A caused a decrease in k_{La} at low concentrations and increased it at 0.8%. In most cases the antifoams reduced the k_{La} at low concentrations which rose again at high concentrations.

Compared to the k_{La} in shake flasks, in the bioreactors there were more conditions which caused an increase in k_{La} . The k_{La} was only significantly increased in the shake flasks at concentrations of 0.4% and 0.6% Antifoam A and 0.6% and 0.8% Antifoam C. These data show there are very clear differences in the effect of antifoams upon k_{La} in shake flasks and bioreactors. The bioreactor k_{La} data could not be correlated to that of the total GFP yield data in this study, as at least one other antifoam concentration is required to generate a further data point to enable analysis. These experiments could be performed at an

intermediate concentration, such as 0.5%, in the future to gain further insight into the effects of k_{La} upon protein yield in bioreactors.

5.2.2 Effect of antifoams upon DO control in bioreactors

Antifoams affect the DO in bioreactors[200]. To test whether their effects on the DO are substantial enough to influence the growth of the cells, J673A was added during the induction phase and the air supply was switched off. The stirrer remained on to prevent the cells from settling at the bottom of the bioreactor but was taken out of cascade and stayed at 700 rpm throughout the induction phase. J673A was added at 0.1% and 1% and the runs were performed in duplicate. Fig 5.7 illustrates the results.

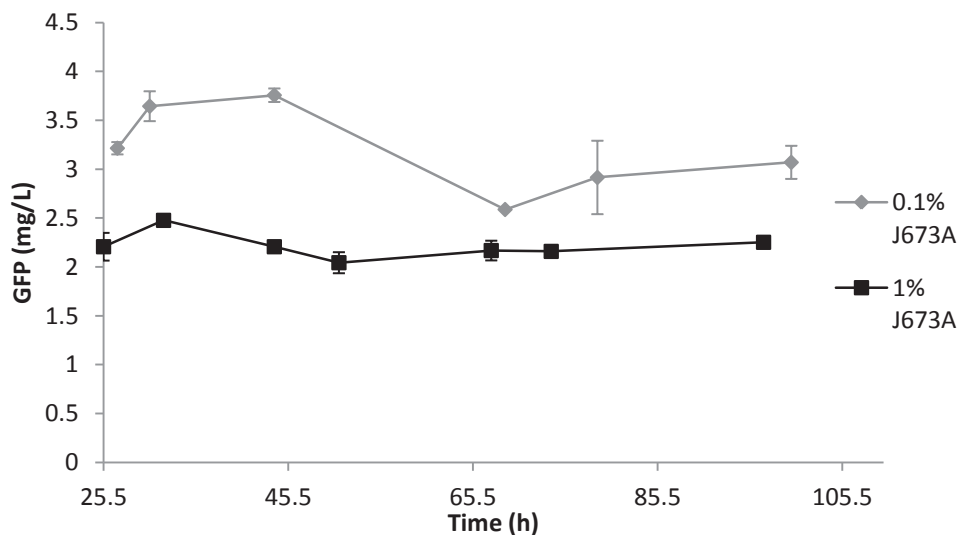


Figure 5.7: Effect of J673A upon GFP yield without DO control in bioreactors

Effect of 0.1% and 1% J673A upon GFP production by P. pastoris in 3 L stirred-tank bioreactors without DO control during the induction phase. The stirrer speed was set to 700 rpm throughout the run and the air supply was turned off. In each case n = 3.

0.1% J673A, again had the best effect out of the two concentrations, however the yields were extremely low compared to runs where DO was maintained at 30%. The yields for both concentrations without DO control did not change from the GFP yields produced at the beginning of induction. This suggests that if there is an effect upon the DO by the

antifoams, it is not sufficient for the cells to grow and produce high levels of recombinant protein.

As the cultures shown in Fig 5.7 did not grow sufficiently to produce high yields of GFP, J673A addition was tested at 70% DO. If DO is not limiting, the effects of the antifoam should be removed and less difference in the yield of GFP observed. 0.1% or 1% J673A were therefore added and the DO set point placed at 70%. The bioreactors were run for 78.5 h. The data are shown in Figure 5.8 with the J673A total GFP yield data at 30% DO from Fig 5.2 for comparison).

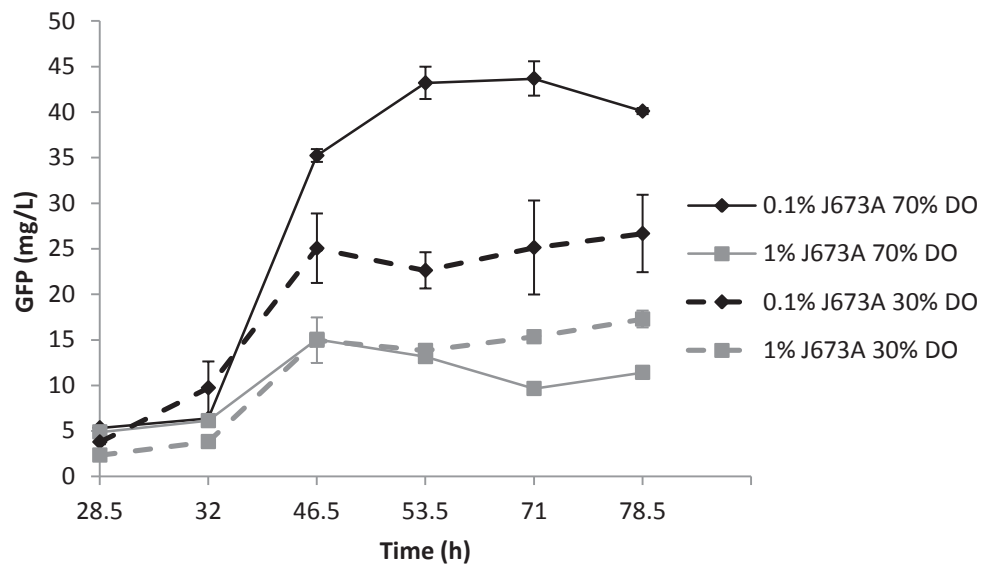


Figure 5.8: Effect of J673A at different DO settings in bioreactors

Effect of 0.1% and 1% J673A upon GFP production by P. pastoris at 70% DO and 30% DO in 3 L stirred tank bioreactors with data for 0.1% and 1% J673A at 30% DO for comparison.

Figure 5.8 suggests that even when the DO in bioreactors is high, there is still a significant difference between the total yield of GFP at high and low concentrations of J673A. Again, the same trend as previously observed was demonstrated; in the presence of 0.1% J673A, 40 mg/L of GFP was produced and 11 mg/L in the presence of 1% J673A. As there were still significant differences in the yield of protein at both antifoam concentrations, this

suggests that the effect of antifoams upon DO is not a major influence on the ability of the cells to produce GFP.

When the 70% DO data is compared to yields at 30%, it is demonstrated that 0.1% J673A added at both of these DO settings provides the largest GFP yields. The 70% cultures produced more GFP, suggesting that production of GFP is improved at higher DO settings which agrees with findings by Holmes *et al*[36]. However, the data for the 1% J673A cultures show that at 30% DO, more GFP was produced than at 70%.

The total yield of GFP was divided by the dry cell weights to obtain the specific yield of GFP per mg of cells. The data are shown in Fig 5.9.

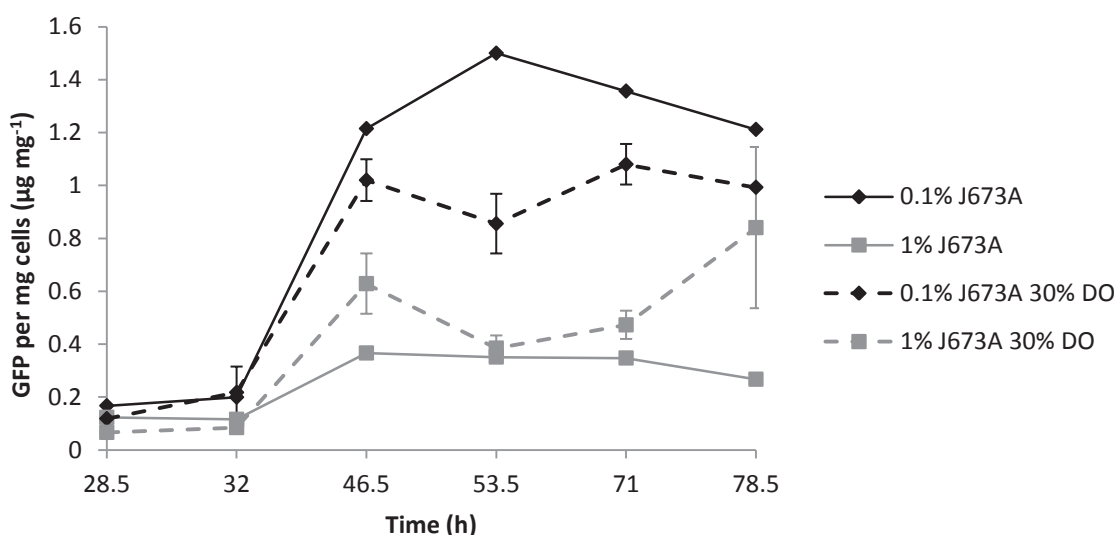


Figure 5.9: Effect of J673A upon specific yield at 70% DO

Effect of 0.1% and 1% J673A upon the specific yield of GFP per mg of P. pastoris cells at 70% DO in 3 L stirred tank bioreactors with data for 0.1% and 1% J673A at 30% for comparison. For all datasets n = 6.

The same trend is observed for the normalized data shown in Fig 5.9 for the 70% DO cultures implying that the difference in effects is not due to changes in the growth of cells at this DO setting. Again as was observed for the total yield, bioreactor cultures with 0.1% J673A at 70% DO produced a higher yield of GFP than at 30% DO, and 1% J673A cultures at 30% DO produced a higher yield than those at 70% DO. The investigations into the

effect of antifoams upon DO in bioreactors suggest that although antifoam can influence the k_{La} , their effects upon DO do not explain any improvements in yield of GFP.

5.3 Effect of antifoam upon total EPO yield in bioreactors

Bioreactors with *P. pastoris* producing EPO were set up containing 0.6% Antifoam C and 1% J673A as these were the lowest and highest yielding conditions, respectively, from the shake flask investigation. Western blots were performed upon the samples, however many bands were observed as demonstrated by Fig 5.10. Several blots were performed using new samples with similar results. This made it difficult to come to a conclusion as to effects of each antifoam. Previous attempts at performing ELISAs proved to be inaccurate, therefore the results from these experiments were inconclusive. EPO is highly glycosylated and the carbohydrate chains make up around 30% of its mass (approximately 32-37 kDa)[120]. It has been previously found upon production of recombinant EPO by *P. pastoris* that several glycoisoforms may be produced. These were visualized as smears or several bands when Western blots were performed[120]. The presence of differing amounts of glycans could explain the presence of multiple bands on the Western blots shown in Fig 5.10. It appears that production in a bioreactor provides conditions more favorable for the formation of several different glycoisoforms. This could be due to a change from BMMY medium to basal salts medium, or due to more efficient oxygenation and feeding when compared to shake flasks.

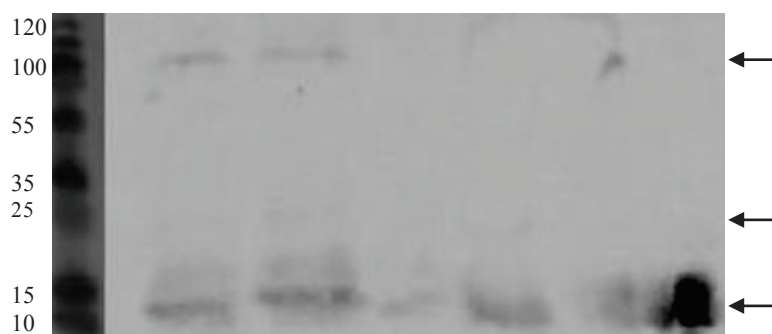


Figure 5.10: Western blot performed on bioreactor X33-EPO samples

Western blot performed on supernatant samples from *P. pastoris* producing soluble EPO in a 3 L bioreactor. Lanes contain samples in the presence of different antifoams, however due to the presence of numerous bands, the effect of the antifoams upon EPO production in bioreactors was inconclusive. Protein ladder marker indicates the size of bands in kDa.

5.4 Effect of antifoams upon total A_{2a}R yield in bioreactors

In the shake flask investigation, 0.5% J673A produced the best yield of A_{2a}R and 1% P2000 the lowest out of the antifoam conditions tested. Bioreactor cultures were set up with these concentrations of antifoam to discover whether the antifoams had the same effect in scale up. The settings used were a stirrer cascade of 700 – 1,250 rpm, pH 5, basal salts medium, and 1.5 L/min air flow rate. Temperature was 30°C during the batch and fed batch phases and dropped to 22°C during induction. The effect of reducing the temperature during induction was found by Fraser to double the yield of A_{2a}R produced[76]. He also found an improvement in yield upon adding the A_{2a}R antagonist, theophylline, however this was not added during these investigations to reduce the number of variables present for the antifoams to interact with. Membrane preparations were analyzed from 10 mL of culture at 23, 51 and 76 h post-innoculation. Radioligand binding assays were performed on the samples in triplicate. The results are shown in Fig 5.11 and illustrate data at 79 h post-innoculation where the protein yield was highest.

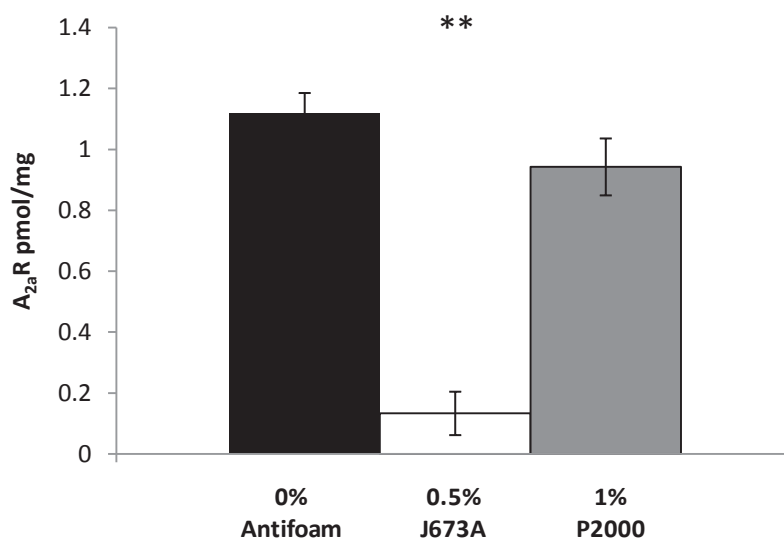


Figure 5.11: The effect of antifoams upon $A_{2a}R$ production by *P. pastoris* in bioreactors
 0% antifoam, 0.5% J673A and 1% P2000 bioreactor membrane preps from 79 h post-inoculation cultures analyzed by radioligand binding to determine $A_{2a}R$ yield. In each case $n = 4$.

These data show that the highest yielding condition tested was without antifoam added to the cultures. Of the antifoams, 1% P2000 added to the bioreactor produced a higher yield of $A_{2a}R$ than the best condition in the shake flask, 0.5% J673A. Again there is a switching of effects of the antifoams upon scale up into the bioreactors. When a t-test was performed on the culture with very little antifoam, the bioreactor equivalent of 0%, compared to the culture containing 1% P2000, there was no significant difference between the $A_{2a}R$ yields. Binding curves were also performed on these samples to determine the B_{max} and K_d for each condition. Each tube contained 100 μ g of membrane protein with increasing concentrations of radiolabeled ZM241285. Binding curves were performed with 3 readings per concentration of radiolabeled ZM241385 ligand. The curves were performed twice, with the repeat performed on a different day. The curves are illustrated in Fig 5.12 with the B_{max} and K_d for each antifoam condition.

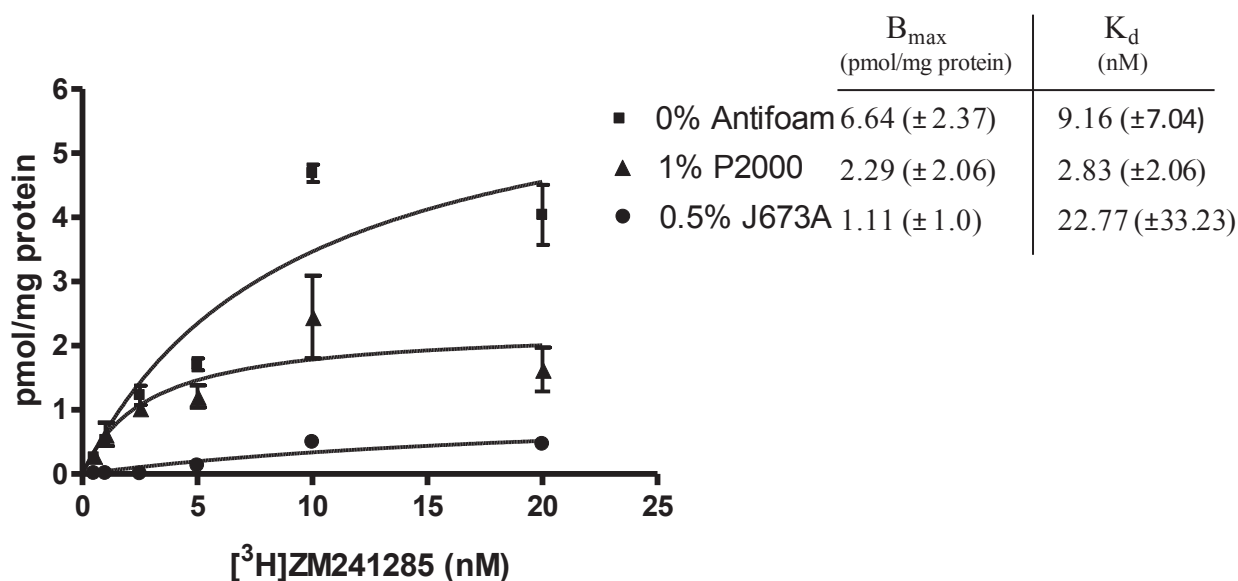


Figure 5.12: Binding curve for $A_{2a}R$ produced in the presence of antifoam

Saturation binding curve for production of $A_{2a}R$ by *P. pastoris* in the presence of 0%, 1% P2000 and 0.5% J673A bound to increasing concentrations of $[^3H]ZM241285$. B_{max} in pmol/mg protein and K_d in nM. Standard error is shown in brackets and $n = 4$. B_{max} $P = 0.048$ measured by one-way ANOVA.

The highest B_{max} was obtained for the $A_{2a}R$ samples without antifoam present which was 6.64 pmol/mg protein. The lowest B_{max} was seen in the presence of 0.5 % J673A which supports the data in Figures 5.11 at 1.11 pmol/mg protein. 1% P2000 had the highest B_{max} out of the two antifoam conditions tested, however this was 2.29 pmol/mg protein and lower than that achieved without antifoam. This is consistent with the data from Fig 5.11 that also showed a higher yield of protein was produced with the control culture. In experiments conducted by Fraser, for membrane preparations of $A_{2a}R$ produced using *P. pastoris*, the B_{max} was 8.5 ± 0.1 pmol/mg membrane protein. This value is higher than the B_{max} values achieved in these experiments, however theophylline was not added which is known to improve the yields[76] and this could account for the slightly lower values here.

The lowest K_d was seen with the 1% P2000 samples at 2.83 nM, and the highest with 0.5% J673A at 22.77 nM. The K_d for the 0% samples was 6.64 nM, suggesting that although the B_{max} was lower for the 1% P2000 samples, the affinity of the ligand for the receptor was slightly higher than in the presence of 0% antifoam. Fraser's membrane-bound $A_{2a}R$ K_d

values varied slightly between samples; $K_d = 0.35 \pm 0.02$; 0.79 ± 0.06 ; 1.39 ± 0.12 nM, but were lower than those achieved in these experiments.

The error values are high for these data, but the curves were performed on different days and variability was seen between these measurements. It has been noticed in the laboratory that the $A_{2a}R$ appears to degrade over time once membrane preparations have been isolated. This may explain why there was variation between binding curve results performed on different days.

5.5 Summary

In bioreactors, the opposite effects were observed than in the shake flask GFP experiments. While high concentrations had previously resulted in higher total yields of GFP in flasks, in bioreactors low concentrations led to higher total yields. The best antifoam was SB2121 at 0.1% which produced 60 mg/L. The trend for the best and worst antifoams however remained similar to that for the shake flask experiments. When the total GFP yield data were normalized, the trends were similar to the total yield data, with 0.1% antifoams still producing much higher yields of GFP per dry cell weight, except for Antifoam C. For this antifoam, the increase in total yield in bioreactors appears to be due to the growth of the cells. The change in medium from BMMY to minimal medium was also investigated; the trend in GFP yield for cultures grown in minimal medium in shake flasks however was the same as for BMMY so this does not account for changes in the trends when scaling up from shake flasks into bioreactors.

When P2000 and J673A were tested upon bioreactor cultures of $A_{2a}R$, it was found that whereas in shake flasks J673A had produced the least detrimental effect upon protein yield and P2000 had led to the lowest yield, the opposite was true in bioreactors. However, the control culture containing approximately 10 μ L of Antifoam A, still produced a higher yield of $A_{2a}R$ than both antifoam conditions. A binding curve showed that the K_d was lowest for 1% P2000 than the control, suggesting that although less protein had been produced, the affinity of the receptor for the ligand was higher for cultures in the presence

of this antifoam. J673A had a higher K_d than both the control and P2000 samples.

Bioreactor cultures producing EPO in the presence of antifoams were inconclusive as to the effect of the antifoams upon the yield due to smearing and the presence of several bands which could be attributed to several different glycoisoforms.

In bioreactors, the effect of the antifoams upon k_{La} was still variable depending upon the type and concentration. SB2121 was the only antifoam this time to decrease the k_{La} at every concentration tested. The data suggest that antifoams have a more positive effect upon k_{La} in bioreactors than they do in shake flasks. Bioreactors containing 0.1% and 1% J673A without DO control during the induction showed that DO is important for the growth of the cells and the production of recombinant protein. At 0.1% J673A, the k_{La} was decreased from the control k_{La} , and at 1% the k_{La} remained the same. These factors did not appear to influence the growth of the culture positively, therefore the effects of J673A at these concentrations did not benefit the growth of the cells. At 70% DO however, there was still a difference between the total yield of GFP produced, suggesting that the antifoam effect upon DO does not contribute significantly to the increases in yield observed.

Based on these findings and from shake flasks it is likely that another mechanism is responsible for these results or a combination of factors is involved.

6. DISCUSSION

The investigations described in this thesis have shown that antifoams can influence the yield of recombinant proteins produced by yeast, on both a small scale in shake flasks and on a large scale in bioreactors. There are a range of different types of antifoam available, some of which destroy foam more effectively than others. The concentrations at which antifoams affect protein yields is dependent upon the type of antifoam, the recombinant protein itself and the system it is produced in. The antifoams were found to affect the growth of the cells, the ability of the cells to secrete or retain protein and also the k_{La} .

6.1 The effect of antifoams upon recombinant protein yield

Antifoams can be split into two categories of fast and slow antifoams, depending upon their mechanism of foam destruction. Slow antifoams are often oils which destroy foam over a longer period of time. Fast antifoams are generally mixed agents which enter foam films within a short space of time and destroy them by the bridging-stretching mechanism[92]. It has also been observed that the most effective agents at destroying foam are those with the most efficient oil film spreading characteristics[206]. Due to the rapid destruction of the foam by all the agents investigated in the Bartsch test in Chapter 3, these antifoams fall into the category of fast antifoams.

One of the least effective agents Antifoam C, is a 30% aqueous emulsion of Antifoam A[192] which may have contributed to the reduced activity compared to the other agents. This does not however appear to influence the ability of this antifoam to increase the yield of protein, although out of the panel of antifoams, it possessed the weakest ability to increase the yield of protein. Antifoam J673A produced the best foam destruction results, which coincides with one of the best increases in protein yield observed out of these antifoams. This antifoam is an alkoxylated fatty acid ester on a vegetable oil base and it is known that vegetable oils can alter the structure of foams by increasing bubble size and reducing the stability of the foam[202]. Antifoam SB2121 also had a larger effect upon

increasing the total yields of GFP compared to Antifoams A and C and was better at destroying foam. P2000 however, produced the highest yield of GFP, but was worse at destroying foam than all the agents but Antifoam C. There is a trend between the ability of the panel of antifoams to destroy foam and increase the yield of GFP in shake flasks, except for antifoam P2000. A better understanding of the components present in each antifoam is required to determine the mechanisms and differences in foam destruction by each antifoam.

Other agents with similar properties such as PEG and EG appeared not to have a benefit upon recombinant GFP production. It seems that there is a common mechanism or a direct link between foam destruction and recombinant protein yield, which is supported by the Bartsch test data. It has also been demonstrated that the effects of the antifoams upon GFP production can be scaled up to larger volumes in shake flasks. This could therefore possibly be of benefit to those producing recombinant proteins in these systems without access to the much more costly large scale bioreactors and associated equipment.

6.1.1 The effects of antifoam upon GFP yields

In the 100 mL shake flask study of the effect of antifoams upon GFP production, addition of the five antifoams tested increased the total yield of secreted recombinant GFP produced by 20 mL *P. pastoris* cultures. Generally, the total GFP yield secreted into the culture medium was increased when antifoam was added at concentrations of at least 0.4% v/v compared with the 0% antifoam control. These concentrations are higher than those that would be used for defoaming purposes in bioreactors, which are normally below 0.1%, and which caused no effect in these experiments. The biggest effect was seen with addition of 1% P2000 to the cultures, and the total yield of GFP was increased from 244 µg to 422 µg. Addition of 1% J673A to a 20 mL culture yielded 394 µg GFP compared with 246 µg GFP when there was no antifoam present. This is notable as J673A is approved for industrial use.

When the specific yield of GFP was calculated for shake flask cultures of *P. pastoris* producing GFP, there was found to be no statistically significant difference in the specific yield at each antifoam concentration compared with the control for cultures containing P2000 and SB2121 (with the exception of 1% P2000). This finding suggested that the enhancements in total yield due to P2000 or SB2121 addition might be attributable to changes in the number of the cells since there was no change in the yield per cell. This suggested at least two methods of action were responsible for the effects of the antifoams upon GFP production. In bioreactor cultures, it was found that cultures containing Antifoam C and P2000 at 0.1% contained a greater number of cells which contributed to the increases in GFP yield compared to those containing higher concentrations. Interestingly, slightly more GFP appeared to be produced per mg of cells by cultures containing higher concentrations of antifoam, although the total yields were greater with 0.1% antifoam due to greater numbers of cells in these cultures.

6.1.2 The influence of antifoams upon culture growth rates

The growth rates for the log phase cultures in the presence of the antifoams suggest that cultures containing 0.8% Antifoam C had the slowest growth, whereas the highest yielding antifoams, J673A, P2000 and SB2121 also had higher growth rates, with J673A growing the fastest at ($\mu = 0.19 \text{ h}^{-1}$). A similar observation was made by Holmes *et al*, where J673A increased the growth of *P. pastoris* and J673A, P2000 and SB2121 increased the growth of *S. cerevisiae* producing a recombinant Fc fusion protein in shake flasks[82]. Increased growth rates have been found to lead to increased productivity[184, 207] which is true for 0.6% Antifoam A, 1% J673A, 1% P2000 and 1% SB2121 cultures which grew at similar or higher growth rates than the control cultures and produced a higher yield of GFP. However, some studies aiming to control growth rates in order to improve specific productivity (q_p) have found that maximal specific growth rates did not relate to maximal specific productivity[208-211]. It has also been found that high levels of protein expression may lead to a reduction in specific growth rate[69]. This could explain the results obtained for Antifoam C at 0.8% which grew at a lower growth rate than the control but still produced a

higher yield of protein. It seems that the relationship between growth rate and productivity varies depending upon the specific parameters of the cultures.

Antifoams have been shown to influence the permeability of yeast cells as well as the sterol distribution[193]. If the antifoams in the *P. pastoris* cultures producing GFP had affected the membrane permeability by altering the sterol composition and leading to an increase in diffusion of nutrients or methanol into the cells, this may have allowed the cells to grow at a higher rate, as an increasing substrate uptake rate has been correlated to increasing growth rates[184]. In addition, the cells may also have produced a higher yield of recombinant protein due to a higher nutrient uptake rate, as increases in specific uptake rate has been linked to increases in specific productivity[184, 212]. This suggests that certainly in the case of antifoams J673A, P2000 and SB2121, an increase in nutrient uptake caused by an alteration of the permeability of the membrane could have lead to a higher growth rate and as a result an increase in protein yield. TAM data suggests that in the presence of J673A there was a difference in metabolism compared to control cultures. This could be as a result of increased nutrient uptake thereby altering the metabolism of the cells. Antifoam A, and most notably Antifoam C, grew more slowly and TAM data for these antifoams was inconclusive. This may suggest a different mechanism for the increase in GFP yield for these antifoams.

6.1.3 The influence of antifoams upon secretion

Combining flow cytometry and fluorimetry data showed that the antifoams can influence the amount of GFP retained inside the cell as well as the amount secreted into the medium. Antifoam A, Antifoam C and J673A enhanced the GFP secreted compared to 0% antifoam suggesting that the increase in total yield observed could be due to this secretion effect. This is consistent with an earlier study which suggested that antifoams can affect cell permeability in yeast by perturbing sterol biosynthesis which then alters the permeability of the membrane[193]. It has recently been shown that alterations in the ergosterol biosynthesis pathway have been linked with increases in recombinant protein secretion and that surfactants may affect the membrane fluidity also leading to a greater amount of

secreted protein[194]. Antifoams P2000 and SB2121 produced a higher total yield of GFP compared to the other antifoams as discussed in Chapter 3, however the flow cytometry data showed that a greater proportion of this protein was retained inside the cell. This is also clearly visible in the fluorescence microscopy images of the cultures (Fig. 3.21), with P2000 and SB2121 cells appearing greener than the others. The increases in yield in the presence of Antifoam A, C and J673A could therefore be due to the fact that the GFP is secreted more efficiently from the cell as a result of increased permeability of the membrane. This suggests that the antifoam effect may be especially beneficial for the production of soluble proteins. Although more GFP was produced overall, it seems that for these antifoams, the main reason for increase in total GFP was due to an increase in the secretion of the GFP. The presence of the antifoam may have not only allowed more GFP to be secreted, but this increase in secretion may have in turn reduced a bottle-neck and allowed the cell to produce more recombinant protein as it did not accumulate inside the cell.

6.1.4 Influence of antifoams upon membrane composition

Yeast plasma membranes contain polar lipids such as glycerophospholipids and sphingolipids. Non-polar lipids consist of free fatty acids, diacylglycerols, triacylglycerols, sterols and steryl esters[213]. Ergosterol is a major component of yeast plasma membranes[214-216] and helps to maintain the structure of the membrane[215] as sterols are rigid hydrophobic molecules with a polar hydroxyl group[217]. Membrane fluidity is important for nutrient uptake and exchange of substrates[217], and affects the movement and activity of membrane proteins and insertion sites[218]. Fatty acids and sterols affect the fluidity of the membrane[218]. Preliminary analysis of electrospray mass spectrometry data suggested changes in relative phosphatidylcholine composition in 1% P2000 samples and changes in relative phosphatidylinositol composition for all antifoam-containing cultures compared to controls. These findings imply that the antifoams may have affected the lipid composition of the membranes which has been suggested previously[193]. However, a more detailed analysis of the data is required in addition to investigation of the antifoam

effects upon other lipid classes to confirm these findings and correlate them to improvements in secretion of recombinant protein or more efficient nutrient uptake.

6.1.5 Antifoams as a carbon source

There is evidence to suggest that vegetable oils may be metabolized as a carbon source[202], but there is no information regarding the ability of yeast to metabolize the other agents such as silicone polymers or polyalkylene glycols. The investigation into adding the antifoam agents into medium as a carbon source (denoted BMAY) revealed that *P. pastoris* could grow in medium without carbon sources (denoted BMY). It appeared that the cells in these cultures may have utilized the peptone and YNB for growth which became limiting resulting in lower ODs being reached than if either glycerol or methanol were present. Cultures containing 1 mL (5% v/v) J673A and SB2121 reached similar ODs as cultures in the medium without any antifoam added. This implies that these two antifoams were not beneficial to the growth of the cells at this concentration. Antifoam C and most significantly P2000 actually appeared to inhibit the growth of the yeast. Antifoam A was the only antifoam at this concentration that allowed the cells to grow more than the control implying that this antifoam can be metabolized and utilized by the cells for growth.

When P2000 and Antifoam A were added at 10% v/v, the OD for the culture containing Antifoam A increased up to 17, which is almost as high as cultures growing on glycerol, with ODs between 18-25. The P2000 culture grew almost as well as the control culture, suggesting that certain antifoams need to be added at higher concentrations to prevent inhibition of growth and for any useful effect upon growth to occur. It has been found that certain strains of bacteria, such as Sphingomonads, *Pseudomonas* sp. and *Stenotrophomonas maltophilia* can utilize polypropylene glycols as well as polyethylene glycols as a sole carbon source[219]. This was not found with the concentrations of P2000, which is a polypropylene glycol, in these experiments. However, the OD increased upon increasing the volume so it is possible that when added at high concentrations it could provide enough of a carbon source to be metabolized enabling the yeast to grow.

6.1.6 The influence of culture format

Bioreactor cultures of *P. pastoris* producing GFP had the opposite effect when antifoams were added at high concentrations. This main difference from scaling up from shake flasks to bioreactors is the oxygenation in the medium, which is discussed further in section 6.2. It is likely that the DO in bioreactors is already sufficient for the growth of the cells in both shake flasks and bioreactors, and although the antifoams did influence the k_{La} , this did not affect the way the cells grew. This suggests that other mechanisms are responsible, such as an alteration to membrane permeability leading to better secretion of protein or a faster uptake rate of nutrients.

6.1.7 Protein-dependent effects

When the effect of the antifoams upon other proteins was evaluated in shake flasks, it was found that of the concentrations tested, the control cultures produced a higher yield of protein for both EPO and A_{2a}R. The antifoams at these concentrations tested appeared to be detrimental to the production of these proteins, however J673A had the best effect in both cases. When the A_{2a}R investigation was carried out in bioreactors, it was found that P2000, which had previously had the most detrimental effect upon protein production produced a higher yield of A_{2a}R than J673A. In shake flask experiments for *S. cerevisiae* WT and TM6* strains producing A_{2a}R, a decrease in yield was observed for both strains when increasing the Antifoam A concentration from 0.6% to 1%. The effect of Antifoam A upon k_{La} may have contributed to these results in the case of the TM6* strain which has been found to produce a higher yield of A_{2a}R at high DO conditions. This evidence highlights the fact that it is important to evaluate the antifoams and different concentrations in each bioprocess system and for each recombinant protein, as the yield could be boosted or it could be severely decreased. If further concentrations had been tested upon EPO and A_{2a}R cultures, it is possible that a combination would have been discovered that did boost the yield. It is likely that as the control cultures produced the best yields in both the shake flasks and bioreactors, that the optimum antifoam concentrations for these two proteins is low.

6.2 The effects of antifoams upon the oxygenation of recombinant cultures

The shake flask investigations on oxygenation of the cultures showed that in the presence of antifoams, there was no overall difference in the DO present in the media compared to cultures without antifoam. This is most likely to be because the addition of the antifoams had not led to a noticeable effect upon the DO, but may possibly be due to the fact that the cells had consumed any extra dissolved oxygen made available. The bioreactor experiments conducted without any DO control during the induction phase clearly demonstrated that the cells could not grow under these conditions. This suggests that the effects, if any, of the antifoams upon DO are small and do not contribute substantially to the growth of the cells. This would agree with the shake flask DO findings, if as the trace may suggest, there was no change in the DO between control and antifoam cultures.

When the effects of the antifoams upon how readily the oxygen dissolves into the medium (the k_La , which therefore influences the DO available to cells) was investigated, a different effect was observed upon the k_La for each of the antifoams at different concentrations and between shake flasks and bioreactors. The k_La of a system can be influenced by several factors such as the properties of the medium like viscosity, the presence of organisms and their by-products. Additions to the medium such as antifoams also have an effect[57]. Changes to the k_La can be due to effects on kl (m/s) and on a (specific surface area m^{-1})[59, 200]. It has been suggested that antifoams enhance bubble coalescence and increase bubble size leading to a reduction in the specific surface area therefore lowering k_La [59, 83, 93, 94, 200]. However it has also been previously observed by Morao *et al* and Liu *et al* that at higher concentrations of antifoam agents the k_La rises. They concluded that this could be due to the detrimental effects of bubble coalescence and reduced a reaching a limit and contacting bubbles coalescing suppressing surface motility and decreasing surface tension. This then leads to decreasing bubble size and k_La rises again. Secondly it is possible that antifoams accumulate oxygen from rising bubbles as they have good oxygen solubility, and release it to the aqueous phase. Bubbles bursting at the surface also disperse small drops of the antifoam causing more oxygen to be released[58, 59]. In the case of oils which have a greater oxygen solubility than water, oil droplets may increase oxygen permeability in the

water boundary layer of the gaseous dispersion[202]. Koide suggested that the ability of antifoams to reduce k_L is less for bubble swarms than for a single bubble[97]. Yagi *et al* suggested that surfactants can lead to rippling or eddying which influences the $k_L a$. They found that k_L was not greatly affected by antifoam agents, and their main effect was upon a [57]. This could explain why a change in the trends of $k_L a$ were observed between the shake flasks and bioreactors, as there is a significant difference in the surface area of the cultures.

In the shake flask investigations there was no correlation between the yield of GFP and the $k_L a$. There also does not appear to be any trend between the $k_L a$ and protein yield in bioreactors. It is therefore possible that the $k_L a$ is already providing sufficient DO for the cells to grow and produce protein and is not a limiting factor, suggesting a combination of factors is responsible for the increases in GFP yield observed. As mentioned previously, in bioreactor cultures of *P. pastoris* producing GFP, a switch in the concentration effects of the antifoams was observed. A major difference between shake flasks and bioreactors is the oxygenation. However, investigations into the effect of the antifoams upon the $k_L a$ and the actual DO concentrations did not suggest that the antifoams had improved the yield by influencing these parameters. The precise reason for the switching in concentration effect of the antifoams upon scale up could not be explained from these investigations. However, a combination of factors is responsible for the way the cells grow and produce recombinant protein, which varies with each individual bioprocess. Therefore changing conditions changes the effects and interactions of these parameters, and when the culture conditions are scaled up, different factors to those in the shake flasks would affect the cultures resulting in a different antifoam effect. For these reasons the optimum conditions, including antifoam addition, should be evaluated for each bioprocess and system.

6.3 The effects of antifoam upon protein purification

GFP was successfully purified using nickel affinity columns and in two cases, with J673A and SB2121, appeared to improve the amount of GFP purified. This could be explained by the antifoams aiding the binding of the protein to the resin beads, as there also appeared to

be less GFP present in the flow through of these samples. For this protein and purification method, high concentrations of antifoam are not an issue.

6.4 Conclusion and future work

The aim of this thesis has been to investigate the range of effects that antifoams could influence in bioprocesses to highlight the importance of careful selection of antifoam type and concentration for each individual process as well as to provide information regarding their mode of action. The main focus of this work has been to determine the effects of high concentrations of antifoam addition to cultures, as low concentrations are more frequently used due to concerns that high concentrations affect purification. This study has not only shown antifoams added at high concentrations in shake flasks can increase protein yields, but also that they do not have a detrimental effect upon purification of the protein.

The biological effects of antifoams are poorly understood and this is in part due to the range of types available and the lack of information regarding their compositions being available from the manufacturers. Antifoams have commonly been added to bioprocesses without considering their possible effects, but as an additive, these effects should be assessed. This work has demonstrated that each antifoam not only destroys foam with a range of effectiveness, but may also affect the cells and the proteins themselves.

From these studies, it has been demonstrated that the concentration of antifoams added to the process can vastly influence the yield of protein, to both positive and negative effect. The type of antifoam, too is important, as some antifoams, such as J673A seem to have an overall less detrimental effect than others, for example Antifoam C. The system to which the agents are added can also completely reverse the effects observed, namely when scaling up from a shake flask into a bioreactor. Finally, the protein that is to be produced is also an important factor; certain antifoams and concentrations, while beneficial to the production of one protein could prove to be detrimental to the production of another.

This study found that antifoams can increase the yield of GFP, by enhancing its secretion, improving growth rates or increasing biomass. While the antifoams were found to increase and decrease k_La both in shake flasks and bioreactors, it did not appear to be sufficient to cause any change to the productivity of the cells. Their effect upon the DO in shake flasks was found to be minimal. This suggests that their influence upon these factors is not as important as the many articles detailing their effects suggest. Instead their effects upon the cells themselves, such as effects upon the permeability of the membrane leading to an increase in nutrient or methanol uptake rate, seems to be of more consequence.

Some of the mechanisms investigated in this study which may explain the improvements in GFP yield is summarized below for each antifoam:

- Antifoam A: Improvements to protein secretion
- Antifoam C: Improvements to protein secretion
- J673A: Increased specific growth rate (and possibly higher nutrient uptake) and improvements to protein secretion
- P2000: Increased specific growth rate (and possibly higher nutrient uptake) and increased biomass
- SB2121: Increased specific growth rate (and possibly higher nutrient uptake) and increased biomass

Of the antifoams tested, J673A produced the most beneficial effects overall. It was the most effective antifoaming agent at foam destruction, and produced some of the biggest increases to protein yield in both shake flask and bioreactor GFP cultures. In shake flask cultures of EPO and A_{2a}R at the concentrations tested, it had the least detrimental effect and produced similar yields to the control cultures. Cultures in shake flasks containing 1% J673A also had higher growth rates than with the other antifoams. A summary of the effects of the antifoams is shown in Table 6.1.

Table 6.1: Summary of the effect of antifoams upon recombinant GFP production by *P. pastoris* in both shake flasks and bioreactors

Antifoam	Defoaming time	Maximum GFP yield [‡]		Optimum concentration		Affects growth of cells		Growth rate	Affects secretion	Metabolized at 5% v/v ^{‡‡}
		Shake flask	Bioreactor	Shake flask	Bioreactor	Shake flask	Bioreactor			
Antifoam A	12 min	358.06 µg	13.5 mg	0.6%	0.6%	No	No	0.12 h ⁻¹	Yes	Yes
Antifoam C	8 min	347.89 µg	21.68 mg	0.8%	0.1%	No	Yes	0.09 h ⁻¹	Yes	No
J673A	0.5 min	393.55 µg	43.02 mg	1%	0.1%	No	No	0.19 h ⁻¹	Yes	No
P2000	9 min	421.89 µg	47.17 mg	1%	0.1%	Yes	Yes	0.15 h ⁻¹	No	No
SB2121	0.5 min	395.51 µg	58.66 mg	1%	0.1%	Yes	No	0.14 h ⁻¹	No	No

[‡] Maximum GFP yield at the end of the fermentation, approximately 100 h post-inoculation for all cultures except 0.6% Antifoam A (51 h post-inoculation).

^{‡‡} At 5% v/v in the absence of glycerol or methanol

In summary, the investigations conducted in this thesis have illustrated that antifoams, could increase the productivity of a process or hinder it. The benefits of investigating the optimum type of antifoam and concentration were demonstrated by experiments in both shake flask and bioreactor cultures of *P. pastoris* producing GFP where significant improvements in yield were observed. The importance of selecting the most appropriate concentration was also highlighted by investigations with antifoams upon EPO and A_{2a}R yield. It is not likely that the precise mechanisms of antifoams action will be easily understood, especially as has been shown by this work, that a combination of factors may have led to the effects upon protein yields. For these reasons, it is important to thoroughly evaluate the effects of antifoam addition to fermentation cultures on both a small and large scale.

As the antifoams affect several factors, there is still useful research that could be conducted in this area. Future work could include testing the antifoams upon EPO and A_{2a}R at a wider range of concentrations to determine whether antifoams could be used to boost the productivity of these proteins as well as GFP. The effects of different concentrations of antifoam upon the binding affinity of A_{2a}R and its ligands could also be informative if the presence of antifoams could potentially improve the affinity. Further concentrations could also be tested in bioreactors with GFP cultures at lower concentrations to determine whether 0.1% is actually the highest yielding concentration. TAM data suggested that there may be a difference in metabolism of the cells in the presence of J673A. This coupled with an increase in nutrient or substrate uptake due to a change in the permeability of the cells could explain its mechanism of action. This theory could be further explained by measuring the rate of methanol uptake using a methanol probe in the presence of different antifoams and concentrations. Polysome profiling could also reveal an insight into the effects of antifoams upon translation within the cell which in turn could provide more information about how the cells are growing in the presence of these agents.

Previously, antifoams have been added to bioprocesses with little regard to their possible influence. This study has demonstrated their effect upon cells and recombinant protein production, and emphasizes the benefit of their optimization.

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8. APPENDICES

8.1. RCP-TEV-GFP sequence alignment

Alignment of the plasmid DNA sequence with the expected construct sequence using T-Coffee software version 8.99.

		* BAD AVG GOOD			
RCP-TEV-GFPsequ	:	100			
RCP-TEV-GFP	:	100			
cons	:	100			
RCP-TEV-GFPsequ	1	ATTCGCAGCATCCTCCGCATTAGCTGCTCCAGT		33	
RCP-TEV-GFP	1	ATTCGCAGCATCCTCCGCATTAGCTGCTCCAGT		33	
cons	1	*****		33	
RCP-TEV-GFPsequ	34	CAACACTACAACAGAAGATGAAACGGCACAAT		66	
RCP-TEV-GFP	34	CAACACTACAACAGAAGATGAAACGGCACAAT		66	
cons	34	*****		66	
RCP-TEV-GFPsequ	67	TCCGGCTGAAGCTGTCATCGGTTACTCAGATTT		99	
RCP-TEV-GFP	67	TCCGGCTGAAGCTGTCATCGGTTACTCAGATTT		99	
cons	67	*****		99	
RCP-TEV-GFPsequ	100	AGAAGGGGATTTTCGATGTTGCTGTTTTGCCATT		132	
RCP-TEV-GFP	100	AGAAGGGGATTTTCGATGTTGCTGTTTTGCCATT		132	
RCP-TEV-GFPsequ	133	TTCCAACAGCACAATAACGGGTTATTGTTTAT		165	
RCP-TEV-GFP	133	TTCCAACAGCACAATAACGGGTTATTGTTTAT		165	
cons	133	*****		165	
RCP-TEV-GFPsequ	166	AAATACTACTATTGCCAGCATTGCTGCTAAAGA		198	
RCP-TEV-GFP	166	AAATACTACTATTGCCAGCATTGCTGCTAAAGA		198	
cons	166	*****		198	
RCP-TEV-GFPsequ	199	AGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGA		231	
RCP-TEV-GFP	199	AGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGA		231	
cons	199	*****		231	
RCP-TEV-GFPsequ	232	AGCTGCAGGAATTCTTATGGAAGTGAAGGATGC		264	
RCP-TEV-GFP	232	AGCTGCAGGAATTCTTATGGAAGTGAAGGATGC		264	
cons	232	*****		264	

RCP-TEV-GFPsequ	265	CAATTCTGCGCTTCTCAGTAACTACGAGGTATT	297
RCP-TEV-GFP	265	CAATTCTGCGCTTCTCAGTAACTACGAGGTATT	297
cons	265	*****	297
RCP-TEV-GFPsequ	298	TCAGTTACTAACTGATCTGAAAGAGCAGCGTAA	330
RCP-TEV-GFP	298	TCAGTTACTAACTGATCTGAAAGAGCAGCGTAA	330
cons	298	*****	330
RCP-TEV-GFPsequ	331	AGAAAGTGGAAAGAATAAACACAGCTCTGGGCA	363
RCP-TEV-GFP	331	AGAAAGTGGAAAGAATAAACACAGCTCTGGGCA	363
cons	331	*****	363
RCP-TEV-GFPsequ	364	ACAGAACTTGAACACTATCACCTATGAAACGTT	396
RCP-TEV-GFP	364	ACAGAACTTGAACACTATCACCTATGAAACGTT	396
cons	364	*****	396
RCP-TEV-GFPsequ	397	AAAATACATATCAAAAACACCATGCAGGCACCA	429
RCP-TEV-GFP	397	AAAATACATATCAAAAACACCATGCAGGCACCA	429
cons	397	*****	429
RCP-TEV-GFPsequ	430	GAGTCCTGAAATTGTCAGAGAATTTCTCACAGC	462
RCP-TEV-GFP	430	GAGTCCTGAAATTGTCAGAGAATTTCTCACAGC	462
cons	430	*****	462
RCP-TEV-GFPsequ	463	ATTGAAAAGCCACAAGTTGACCAAAGCTGAGAA	495
RCP-TEV-GFP	463	ATTGAAAAGCCACAAGTTGACCAAAGCTGAGAA	495
cons	463	*****	495
RCP-TEV-GFPsequ	496	GCTCCAGCTGCTGAACCACCGGCCTGTGACTGC	528
RCP-TEV-GFP	496	GCTCCAGCTGCTGAACCACCGGCCTGTGACTGC	528
cons	496	*****	528
RCP-TEV-GFPsequ	529	TGTGGAGATCCAGCTGATGGTGGAAAGAGAGTGA	561
RCP-TEV-GFP	529	TGTGGAGATCCAGCTGATGGTGGAAAGAGAGTGA	561
cons	529	*****	561
RCP-TEV-GFPsequ	562	AGAGCGGCTCACGGAGGAGCAGATTGAAGCTCT	594
RCP-TEV-GFP	562	AGAGCGGCTCACGGAGGAGCAGATTGAAGCTCT	594
cons	562	*****	594

RCP-TEV-GFPsequ	595	TCTCCACACCGTCACCAGCATTCTGCCTGCAGA	627
RCP-TEV-GFP	595	TCTCCACACCGTCACCAGCATTCTGCCTGCAGA	627
cons	595	*****	627
RCP-TEV-GFPsequ	628	GCCAGAGGCTGAGCAGAAGAAGAATACAAACAG	660
RCP-TEV-GFP	628	GCCAGAGGCTGAGCAGAAGAAGAATACAAACAG	660
cons	628	*****	660
RCP-TEV-GFPsequ	661	CAATGTGGCAATGGACGAAGAGGACCCAGCAGC	693
RCP-TEV-GFP	661	CAATGTGGCAATGGACGAAGAGGACCCAGCAGC	693
cons	661	*****	693
RCP-TEV-GFPsequ	694	GGCCAGCCGGCCGTCTCGGATCGGTACCTCGA	726
RCP-TEV-GFP	694	GGCCAGCCGGCCGTCTCGGATCGGTACCTCGA	726
cons	694	*****	726
RCP-TEV-GFPsequ	727	AAATCTTTATTTTCAAGGTCCGCGGC66CCGCT	759
RCP-TEV-GFP	727	AAATCTTTATTTTCAAGGTCCGCGGC66CCGCT	759
cons	727	*****	759
RCP-TEV-GFPsequ	760	TATGAGTAAAGGAGAAGAAGACTTTTCACTGGAGT	792
RCP-TEV-GFP	760	TATGAGTAAAGGAGAAGAAGACTTTTCACTGGAGT	792
cons	760	*****	792
RCP-TEV-GFPsequ	793	TGTCCCAATTCTTGTTGAATTAGATGGTGATGT	825
RCP-TEV-GFP	793	TGTCCCAATTCTTGTTGAATTAGATGGTGATGT	825
cons	793	*****	825
RCP-TEV-GFPsequ	826	TAATGGGCACAAATTTTCTGTCAAGTGGAGAGGG	858
RCP-TEV-GFP	826	TAATGGGCACAAATTTTCTGTCAAGTGGAGAGGG	858
cons	826	*****	858
RCP-TEV-GFPsequ	859	TGAAGGTGATGCAACATACGGAAAACCTTACCCT	891
RCP-TEV-GFP	859	TGAAGGTGATGCAACATACGGAAAACCTTACCCT	891
cons	859	*****	891
RCP-TEV-GFPsequ	892	TAAATTTATTTGCACTACTGGAAAACCTACCTGT	924
RCP-TEV-GFP	892	TAAATTTATTTGCACTACTGGAAAACCTACCTGT	924
cons	892	*****	924

RCP-TEV-GFPsequ	925	TCCATGGCCAACACTTGTCACTACTTTCTCTTA	957
RCP-TEV-GFP	925	TCCATGGCCAACACTTGTCACTACTTTCTCTTA	957
cons	925	██	957
RCP-TEV-GFPsequ	958	TGGTGTTC AATGCTTTTCCCGTTATCCGGATCA	990
RCP-TEV-GFP	958	TGGTGTTC AATGCTTTTCCCGTTATCCGGATCA	990
cons	958	██	990
RCP-TEV-GFPsequ	991	TATGAAACGGCATGACTTTTTCAAGAGTGCCAT	1023
RCP-TEV-GFP	991	TATGAAACGGCATGACTTTTTCAAGAGTGCCAT	1023
cons	991	██	1023
RCP-TEV-GFPsequ	1024	GCCCGAAGGTTATGTACAGGAACGCACTATATC	1056
RCP-TEV-GFP	1024	GCCCGAAGGTTATGTACAGGAACGCACTATATC	1056
cons	1024	██	1056
RCP-TEV-GFPsequ	1057	TTTCAAAGATGACGGGAAC TACAAGACGCGTG	1089
RCP-TEV-GFP	1057	TTTCAAAGATGACGGGAAC TACAAGACGCGTG	1089
cons	1057	██	1089
RCP-TEV-GFPsequ	1090	TGAAGTCAAGTTTGAAGGTGATACCOCTTGTTAA	1122
RCP-TEV-GFP	1090	TGAAGTCAAGTTTGAAGGTGATACCOCTTGTTAA	1122
cons	1090	██	1122
RCP-TEV-GFPsequ	1123	TCGTATCGAGTTAAAAGGTATTGATTTTAAAGA	1155
RCP-TEV-GFP	1123	TCGTATCGAGTTAAAAGGTATTGATTTTAAAGA	1155
cons	1123	██	1155
RCP-TEV-GFPsequ	1156	AGATGGAAACATTCTCGGACACAAACTCGAGTA	1188
RCP-TEV-GFP	1156	AGATGGAAACATTCTCGGACACAAACTCGAGTA	1188
cons	1156	██	1188
RCP-TEV-GFPsequ	1189	CAACTATAACTCACACAATGTATACATCACGGC	1221
RCP-TEV-GFP	1189	CAACTATAACTCACACAATGTATACATCACGGC	1221
cons	1189	██	1221
RCP-TEV-GFPsequ	1222	AGACAAACAAAAGAATGGAATCAAAGCTAACTT	1254
RCP-TEV-GFP	1222	AGACAAACAAAAGAATGGAATCAAAGCTAACTT	1254
cons	1222	██	1254
RCP-TEV-GFPsequ	1255	CAAAATTGCCACAACATTGAAGATGGATCCGT	1287
RCP-TEV-GFP	1255	CAAAATTGCCACAACATTGAAGATGGATCCGT	1287
cons	1255	██	1287

RCP - TEV - GFPsequ	1288	TCAACTAGCAGACCATTATCAACAAAATACTCC	1320
RCP - TEV - GFP	1288	TCAACTAGCAGACCATTATCAACAAAATACTCC	1320
cons	1288	*****	1320
RCP - TEV - GFPsequ	1321	AATTGGCGATGGCCCTGTCCTTTTACCAGACAA	1353
RCP - TEV - GFP	1321	AATTGGCGATGGCCCTGTCCTTTTACCAGACAA	1353
cons	1321	*****	1353
RCP - TEV - GFPsequ	1354	CCATTACCTGTCGACACAATCTGCCCTTTCGAA	1386
RCP - TEV - GFP	1354	CCATTACCTGTCGACACAATCTGCCCTTTCGAA	1386
cons	1354	*****	1386
RCP - TEV - GFPsequ	1387	AGATCCCAACGAAAAGCGTGACCACATGGTCCT	1419
RCP - TEV - GFP	1387	AGATCCCAACGAAAAGCGTGACCACATGGTCCT	1419
cons	1387	*****	1419
RCP - TEV - GFPsequ	1420	TCTTGAGTTTGTAACTGCTGCTGGGATTACACA	1452
RCP - TEV - GFP	1420	TCTTGAGTTTGTAACTGCTGCTGGGATTACACA	1452
cons	1420	*****	1452
RCP - TEV - GFPsequ	1453	TGGCATGGATGAGCTCTACAAAATTCTAGAACA	1485
RCP - TEV - GFP	1453	TGGCATGGATGAGCTCTACAAAATTCTAGAACA	1485
cons	1453	*****	1485
RCP - TEV - GFPsequ	1486	AAAACATCATCTCAGAAGAGGATCTGAATAGCGC	1518
RCP - TEV - GFP	1486	AAAACATCATCTCAGAAGAGGATCTGAATAGCGC	1518
RCP - TEV - GFPsequ	1519	CGTCGACCATCATCATCATCAT	1543
RCP - TEV - GFP	1519	CGTCGACCATCATCATCATCAT	1543
cons	1519	*****	1543

Translated:

LFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDF
DVAVL PFSNSTNNGLLFINTTIASIAAKEEGVSLEKRE
AEAAGILMetEVKDANSALLSNYEVFQLLTDLKEQRKE
SGKNKHSSGQQNLNTITYETLKYISKTPCRHQSP EIVR
EFLTALKSHKLTKA EKLQLLNHRPVTAVEIQLMetVEE
SEERLTEEQIEALLHTVTSILPAEPEAEQKKNTNSNVA
MetDEEDPAAAQPAVSDRYLENLYFQGPRRPLMetSKGE
ELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKL
TLKFICTTGKLPVPWPTLVTTFSYGVQCFSRYPDHMetK
RHDFFKSAMetPEGYVQERTISFKDDGNYKTRAEVKFE
GDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIT
ADKQKNGIKANFKIRHNIEDGSVQLADHYQQNTPIGD
GPVLLPDNHYLSTQSALSKDPNEKRDHMetVLLEFVTA
AGITHGMetDELYKILEQKLISEEDLNSAVDHHHHH

8.2 Human growth hormone sequence alignment

Alignment of the plasmid DNA sequence with the expected construct sequence using T-Coffee software version 8.99.

<div style="display: flex; align-items: center;"> + BAD AVG GOOD </div>					
hghsequencing	:	100			
hgh	:	100			
cons	:	100			
hghsequencing	1	ATGAGATTTGCTTCAATTTTTACTGCTGTTTTATTC	36		
hgh	1	ATGAGATTTGCTTCAATTTTTACTGCTGTTTTATTC	36		
cons	1	*****	36		
hghsequencing	37	GCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACT	72		
hgh	37	GCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACT	72		
cons	37	*****	72		
hghsequencing	73	ACAACAGAAGATGAAACGGCACAAAATTCGGGCTGAA	108		
hgh	73	ACAACAGAAGATGAAACGGCACAAAATTCGGGCTGAA	108		
cons	73	*****	108		
hghsequencing	109	GCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTTC	144		
hgh	109	GCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTTC	144		
cons	109	*****	144		
hghsequencing	145	GATGTTGCTGTTTTGCCATTTTCCAACAGCACAAAAT	180		
hgh	145	GATGTTGCTGTTTTGCCATTTTCCAACAGCACAAAAT	180		
cons	145	*****	180		
hghsequencing	181	AACGGGTTATTGTTTATAAATACTACTATTGCCAGC	216		
hgh	181	AACGGGTTATTGTTTATAAATACTACTATTGCCAGC	216		
cons	181	*****	216		
hghsequencing	217	ATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAA	252		
hgh	217	ATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAA	252		
cons	217	*****	252		
hghsequencing	253	AGAGAGGCTGAAGCTGAATTCACCATCACCATCAC	288		
hgh	253	AGAGAGGCTGAAGCTGAATTCACCATCACCATCAC	288		
cons	253	*****	288		

hghsequencing	289	CATATTGAAGGGAGATTCCCAACTATACCACTATCT	324
hgh	289	CATATTGAAGGGAGATTCCCAACTATACCACTATCT	324
cons	289	*****	324
hghsequencing	325	CGTCTATTCGATAACGCTATGCTTCGTGCTCATCGT	360
hgh	325	CGTCTATTCGATAACGCTATGCTTCGTGCTCATCGT	360
cons	325	*****	360
hghsequencing	361	CTTCATCAGCTGGCCTTTGACACCTACCAGGAGTTT	396
hgh	361	CTTCATCAGCTGGCCTTTGACACCTACCAGGAGTTT	396
cons	361	*****	396
hghsequencing	397	GAAGAAGCCTATATCCCAAAGGAACAGAAGTATTCA	432
hgh	397	GAAGAAGCCTATATCCCAAAGGAACAGAAGTATTCA	432
cons	397	*****	432
hghsequencing	433	TTCTGCAGAACCCCGAGACCTCCCTCTGTTTCTCA	468
hgh	433	TTCTGCAGAACCCCGAGACCTCCCTCTGTTTCTCA	468
cons	433	*****	468
hghsequencing	469	GAGTCTATTCCGACACCCTCCAACAGGGAGGAAACA	504
hgh	469	GAGTCTATTCCGACACCCTCCAACAGGGAGGAAACA	504
cons	469	*****	504
hghsequencing	505	CAACAGAAATCCAACCTAGAGCTGCTCCGCATCTCC	540
hgh	505	CAACAGAAATCCAACCTAGAGCTGCTCCGCATCTCC	540
cons	505	*****	540
hghsequencing	541	CTGCTGCTCATCCAGTCGTGGCTGGAGCCCGTGCAG	576
hgh	541	CTGCTGCTCATCCAGTCGTGGCTGGAGCCCGTGCAG	576
cons	541	*****	576
hghsequencing	577	TTCTCAGGAGTGTCTTCGCCAACAGCCTGGTGTAC	612
hgh	577	TTCTCAGGAGTGTCTTCGCCAACAGCCTGGTGTAC	612
cons	577	*****	612
hghsequencing	613	GGCGCCTCTGACAGCAACGTCTATGACCTCCTAAAG	648
hgh	613	GGCGCCTCTGACAGCAACGTCTATGACCTCCTAAAG	648
cons	613	*****	648
hghsequencing	649	GACCTAGAGGAAGGCATCCAAACGCTGATGGGGAGG	684
hgh	649	GACCTAGAGGAAGGCATCCAAACGCTGATGGGGAGG	684
cons	649	*****	684

hghsequencing	685	CTGGAAGATGGCAGCCCCGGACTGGGCAGATCTTC	720
hgh	685	CTGGAAGATGGCAGCCCCGGACTGGGCAGATCTTC	720
cons	685	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	720
hghsequencing	721	AAGCAGACCTACAGCAAGTTGACACAAACTCACAC	756
hgh	721	AAGCAGACCTACAGCAAGTTGACACAAACTCACAC	756
cons	721	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	756
hghsequencing	757	AACGATGACGCACTACTCAAGAACTACGGGCTGCTC	792
hgh	757	AACGATGACGCACTACTCAAGAACTACGGGCTGCTC	792
cons	757	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	792
hghsequencing	793	TACTGCTTCAGGAAGGACATGGACAAGGTCGAGACA	828
hgh	793	TACTGCTTCAGGAAGGACATGGACAAGGTCGAGACA	828
cons	793	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	828
hghsequencing	829	TTCTGCGCATCGTGCAAGTCCCGCTCTGTGGAGGGC	864
hgh	829	TTCTGCGCATCGTGCAAGTCCCGCTCTGTGGAGGGC	864
cons	829	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	864
hghsequencing	865	AGCTGTGGCTTCTAG	879
hgh	865	AGCTGTGGCTTCTAG	879
cons	865	XXXXXXXXXXXX	879

Translated:

Met R F P S I F T A V L F A A S S A L A A P V N T T T E D E T A Q I P A E A
V I G Y S D L E G D F D V A V L P F S N S T N N G L L F I N T T I A S I A A
K E E G V S L E K R E A E A E F H H H H H H I E G R F P T I P L S R L F D N
A Met L R A H R L H Q L A F D T Y Q E F E E A Y I P K E Q K Y S F L Q N P
Q T S L C F S E S I P T P S N R E E T Q Q K S N L E L L R I S L L L I Q S W L
E P V Q F L R S V F A N S L V Y G A S D S N V Y D L L K D L E E G I Q T L
Met G R L E D G S P R T G Q I F K Q T Y S K F D T N S H N D D A L L K N Y
G L L Y C F R K D Met D K V E T F L R I V Q C R S V E G S C G F Stop

8.3 Presentations and publications arising from this thesis

Presentation given at the IChemE Young Researchers Meeting 2010 and awarded first prize.



The effect of antifoam on recombinant protein yield

By Sarah Routledge

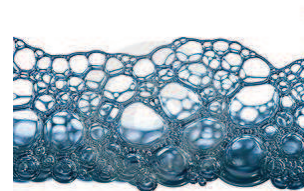
Fermentation

- ▶ Exploitation of microorganisms
 - ▶ Yeast used as expression systems
Pichia pastoris
- ▶ Proteins
 - ▶ Insulin
 - ▶ Human growth hormone
 - ▶ GFP
- ▶ Shake flasks
 - ▶ Initial screening for conditions
- ▶ Bioreactors
 - ▶ Culture thousands of litres

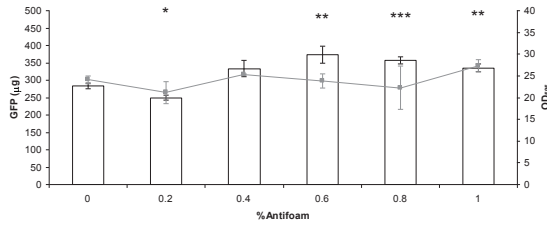


Foaming and Chemical Antifoams

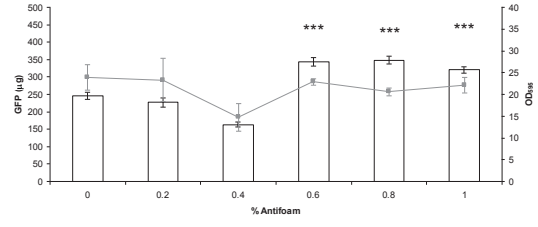
- ▶ Foaming reduces efficiency
 - ▶ Increased costs
 - ▶ Reduced productivity
 - ▶ Detrimental effects
- ▶ Both positive and negative effects observed
- ▶ Observation upon
 - ▶ Foam destruction
 - ▶ $k_L a$ – volumetric mass oxygen transfer coefficient
 - ▶ Concentration effect
 - ▶ Media
 - ▶ Growth of organisms
 - ▶ Protein production
- ▶ Usually added to a process without consideration of the effects



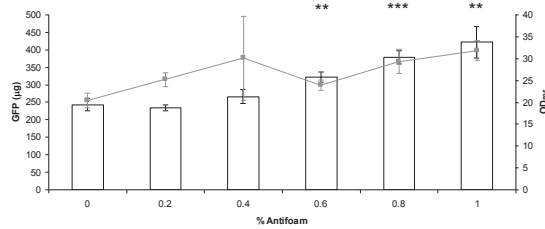
Antifoam A



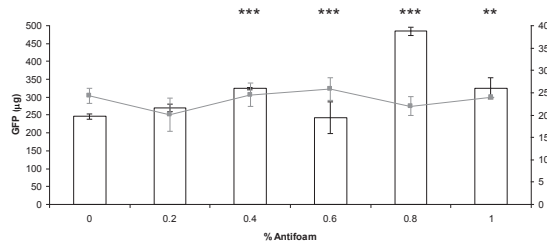
Antifoam C



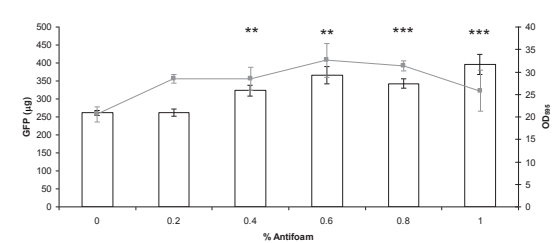
P2000



J673A



SB2121



Antifoams Affect Total Protein Yield in Shake Flasks

- ▶ All antifoams increased total protein yield at higher concentrations than those normally used
- ▶ Antifoams A and C show significant effect upon yield of GFP above 0.6%
- ▶ Antifoam J673A shows the best effect of the antifoams tested at 0.8%
- ▶ The effects of antifoam addition are not due to changes in growth rate of the cultures, except for P2000 and SB2121

Influence of Antifoams Upon Oxygen Availability

- ▶ Volumetric mass oxygen transfer coefficient

- ▶ $k_L a (t_2 - t_1) = \ln \left(\frac{c_{1, \infty} - c_{1, t_1}}{c_{1, \infty} - c_{1, t_2}} \right)$

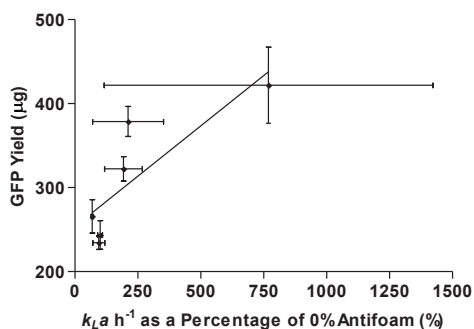
- ▶ Antifoams reported to reduce $k_L a$ at low concentrations
- ▶ Certain antifoams may increase $k_L a$ at higher concentrations



Correlation of $k_L a$ and Protein Yield

- ▶ Antifoams had a positive effect upon the $k_L a$ for each of the antifoams except SB2121.
- ▶ The only antifoam that increased the $k_L a$ at each concentration was Antifoam C.
- ▶ $k_L a$ at increasing concentrations of P2000 is correlated with the yield of GFP obtained in shake flasks at the same concentrations

- ▶ R^2 of 0.7
- ▶ P value > 0.05



- ▶ The rest of the antifoam $k_L a$ measurements and yields of GFP were not found to have any correlation, suggesting that changes in $k_L a$ are not the mechanism leading to increased protein yield for these antifoams.

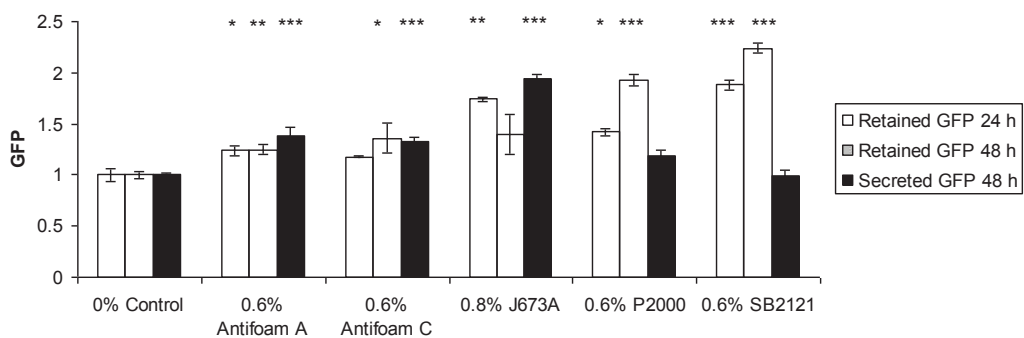


Flow Cytometry

- ▶ Determine whether more GFP was retained or secreted under the influence of antifoams
- ▶ Flow cytometry data used to determine retained GFP
- ▶ Total GFP yield used to determine secreted GFP
- ▶ Assume control data = 1 to compare between antifoam effects



Effect of Antifoams on GFP Retained and Secreted by *P. pastoris* X33



Antifoam A - 52%

Antifoam C - 51%

J673A - 58%

P2000 - 38%

SB2121 - 31%



Summary

- ▶ Antifoams can increase recombinant protein yield at higher concentrations than normally used in shake flasks
- ▶ They affect the $k_L a$ in bioreactors both positively and negatively depending upon the antifoam and the concentration
- ▶ Increased $k_L a$ was correlated to increased yield for P2000
- ▶ They can alter the ability of the cell to secrete GFP
- ▶ Antifoams tested can be split into two different groups by their mechanism of action

- ▶ Changes to growth
 - ▶ P2000
 - ▶ SB2121

- ▶ Improved secretion:
 - ▶ Antifoam A
 - ▶ Antifoam C
 - ▶ J673A

RESEARCH

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Antifoam addition to shake flask cultures of recombinant *Pichia pastoris* increases yield

Sarah J Routledge¹, Christopher J Hewitt², Nagamani Bora¹ and Roslyn M Bill^{1*}

Abstract

Background: *Pichia pastoris* is a widely-used host for recombinant protein production. Initial screening for both suitable clones and optimum culture conditions is typically carried out in multi-well plates. This is followed by up-scaling either to shake-flasks or continuously stirred tank bioreactors. A particular problem in these formats is foaming, which is commonly prevented by the addition of chemical antifoaming agents. Intriguingly, antifoams are often added without prior consideration of their effect on the yeast cells, the protein product or the influence on downstream processes such as protein purification. In this study we characterised, for the first time, the effects of five commonly-used antifoaming agents on the total amount of recombinant green fluorescent protein (GFP) secreted from shake-flask cultures of this industrially-relevant yeast.

Results: Addition of defined concentrations of Antifoam A (Sigma), Antifoam C (Sigma), J673A (Struktol), P2000 (Fluka) or SB2121 (Struktol) to shake-flask cultures of *P. pastoris* increased the total amount of recombinant GFP in the culture medium (the total yield) and in the case of P2000, SB2121 and J673A almost doubled it. When normalized to the culture density, the GFP specific yield ($\mu\text{g OD}_{595}^{-1}$) was only increased for Antifoam A, Antifoam C and J673A. Whilst none of the antifoams affected the growth rate of the cells, addition of P2000 or SB2121 was found to increase culture density. There was no correlation between total yield, specific yield or specific growth rate and the volumetric oxygen mass transfer coefficient ($k_L a$) in the presence of antifoam. Moreover, the antifoams did not affect the dissolved oxygen concentration of the cultures. A comparison of the amount of GFP retained in the cell by flow cytometry with that in the culture medium by fluorimetry suggested that addition of Antifoam A, Antifoam C or J673A increased the specific yield of GFP by increasing the proportion secreted into the medium.

Conclusions: We show that addition of a range of antifoaming agents to shake flask cultures of *P. pastoris* increases the total yield of the recombinant protein being produced. This is not only a simple method to increase the amount of protein in the culture, but our study also provides insight into how antifoams interact with microbial cell factories. Two mechanisms are apparent: one group of antifoams (Antifoam A, Antifoam C and J673A) increases the specific yield of GFP by increasing the total amount of protein produced and secreted per cell, whilst the second (P2000 or SB2121) increases the total yield by increasing the density of the culture.

Background

The laboratory-scale production of recombinant proteins using *P. pastoris* requires that cells are cultured either in large shake flasks or in continuously stirred tank bioreactors. In these vessels, the formation of foam is an issue that requires intervention. This is in contrast to the situation in the small vessels typically used in the

initial stages of protein production experiments where foaming is minimal [1].

Foaming can lead to reduced yields since bursting bubbles can damage proteins [2] and can also result in a loss of sterility if the foam escapes [3]. In bioreactors, foaming can lead to over-pressure if a foam-out blocks an exit filter. To prevent the formation of foam, mechanical foam breakers, ultrasound or, most often, the addition of chemical antifoaming agents (or “antifoams”) [3] are routinely employed.

There is a well-established literature on antifoams [3]. One useful classification categorizes them as either

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hydrophobic solids dispersed in carrier oil, aqueous suspensions/emulsions, liquid single components or solids [4-6]. Several mechanisms of action for these agents have been suggested which include bridging-dewetting, spreading fluid entrainment and bridging-stretching [7]. Many are commercially-available, with 19 being sold by Sigma-Aldrich alone. While little information is routinely given about their composition, their specific antifoam properties have been thoroughly investigated. These include their effects on foam height with time, their influence on the volumetric oxygen mass transfer coefficient (k_{La}) of the system, their gas hold-up characteristics and their globule size and distribution in relation to their action upon foams [3,5,7-11]. Such studies have been performed in various growth media in both the absence and presence of cultures of prokaryotic and eukaryotic microbes.

In contrast, literature on the biological effects of antifoams on recombinant protein yields from microbial cell factories is more limited. Additional file 1: Table S1 shows an analysis of representative examples of this body of work including previous studies on four bacterial hosts and one yeast species. In some cases, the additives tested are not antifoams *sensu stricto*. It is also noteworthy that the yeast, *Schizosaccharomyces pombe*, is not widely used in biotechnology applications and that there have been no prior studies on the biological effects of antifoam addition to recombinant *P. pastoris* cultures. A recent review stated that in the last 15 years, 80% of all recombinant genes reported in the literature were expressed in either *Escherichia coli* or *P. pastoris* [12]. In this study, we therefore examined five antifoams that are widely used in controlling the foaming of recombinant *P. pastoris* cultures [13-16] in order to analyze effects over and above that of their de-foaming action. We looked at polypropylene glycol (PPG) P2000 that is analogous to previously-examined liquid single components of the PPG-type [11] as well as examples from other categories such as Antifoam A and Antifoam C, which are silicone polymers, SB2121, which is a polyalkylene glycol, and J673A, which is an alkoxyated fatty acid ester on a vegetable base and has not previously been documented in this context: for all antifoams examined, this was the first report of their effect on the yield of recombinant GFP secreted from shake-flask cultures of *P. pastoris*.

Results

We wanted to establish whether antifoams affect recombinant protein yield in *P. pastoris* X33 cultures, and if so to investigate the underlying mechanisms. To examine this we chose an experimental system, under the control of the methanol-inducible *AOX1* promoter, comprising GFP secreted from 20 mL cultures in shake

flasks in the presence of five different antifoams at a range of concentrations from 0-1% v/v. These concentrations are higher than the 0.1% routinely used for de-foaming purposes. The total amount of GFP in these 20 mL cultures (the total yield) was measured by fluorimetry 48 h post-induction.

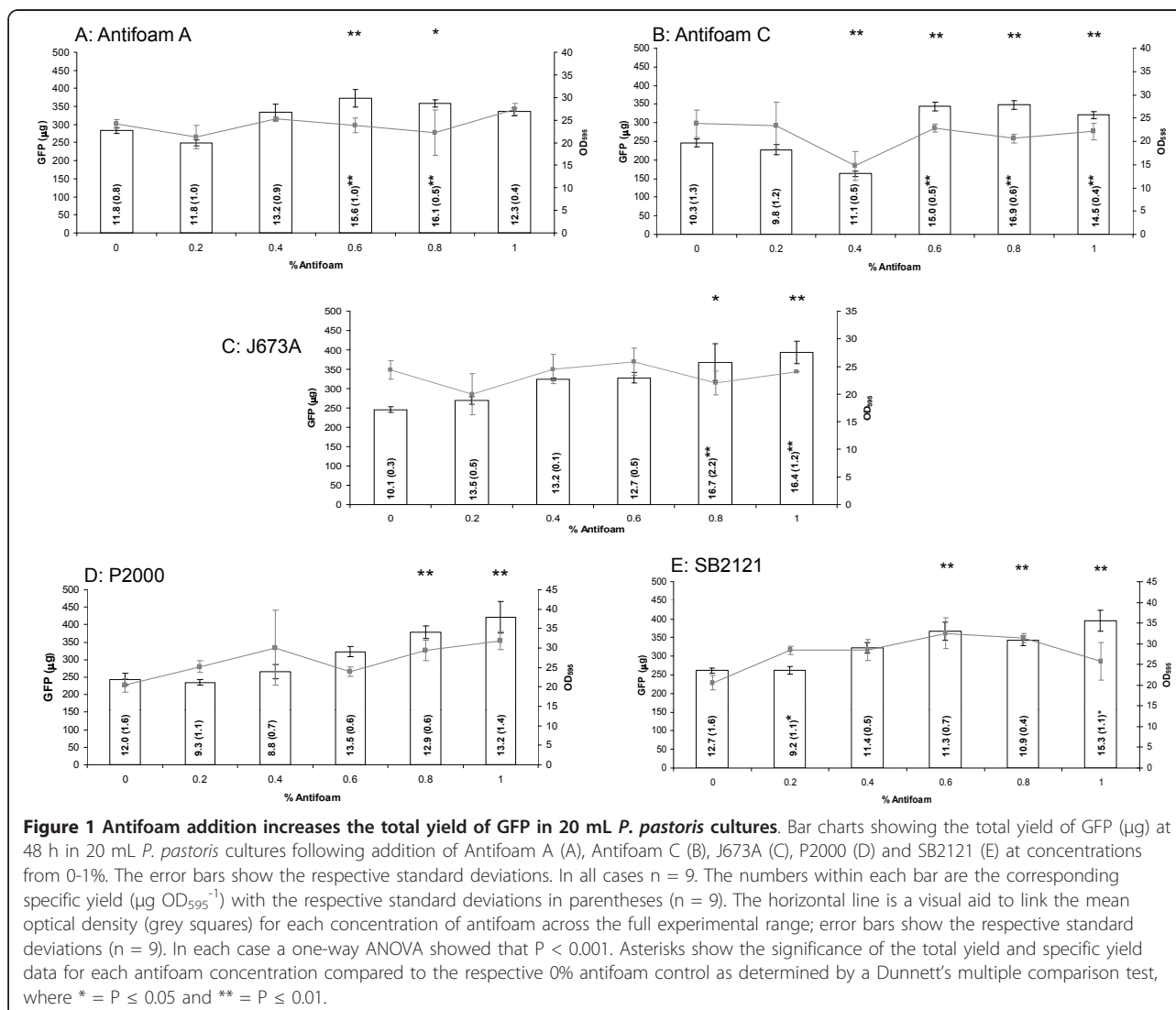
Antifoam addition affects total GFP yield in shake flasks

The total yield of GFP as a function of Antifoam A addition rose significantly at concentrations of 0.6% and above (Figure 1A) with no further increases above 1% (data not shown). A similar but more pronounced trend was observed for Antifoam C (Figure 1B), which is unsurprising since Antifoam C is a 30% emulsion of the same antifoam concentrate as Antifoam A, but with different non-ionic emulsifiers [17]. Figure 1C shows that addition of 1% J673A almost doubled the total yield of GFP compared to the control without antifoam, representing one of the largest effects of the antifoams evaluated. At concentrations above 1%, the total yield of GFP decreased (data not shown). Addition of P2000 (Figure 1D) also resulted in a significant increase in total yield at or above 0.6%, while addition of SB2121 (Figure 1E) increased total yield at concentrations above 0.4%. In both cases the largest improvement was obtained on addition of 1% of the antifoam, again almost doubling the yield. Overall, the five antifoams tested all increased the total yield of GFP at concentrations in the range of 0.4-1% v/v. The highest yield was achieved by adding 1% P2000 (422 μ g GFP) followed by 1% SB2121 (396 μ g GFP), 1% J673A (394 μ g GFP), 0.6% Antifoam A (373 μ g GFP) and 0.8% Antifoam C (348 μ g GFP). All five yields were significantly higher than the corresponding yields from the 0% control, as shown in Figure 1.

The effects of antifoam addition are due to changes in culture density for P2000 and SB2121

To account for any changes in the growth characteristics of the cells on addition of the antifoams, we normalized the total yield to the optical density of the cultures to obtain the specific yield (μ g OD₅₉₅⁻¹). OD₅₉₅ was demonstrated to be a reliable measure of cell density in these experiments by comparing the number of cells at a given OD₅₉₅ in the absence and presence of a range of concentrations of the different antifoams used in our study: there was no statistically significant difference in cell number between cells harvested at a given OD₅₉₅ in the absence or presence of all antifoam concentrations tested. Typical values were 4.8×10^7 cells/mL at an OD₅₉₅ of 20.5 in the absence and presence of 0.5% SB2121.

For Antifoam A, Antifoam C and J673A, the specific yield data were similar in trend to the total yield data

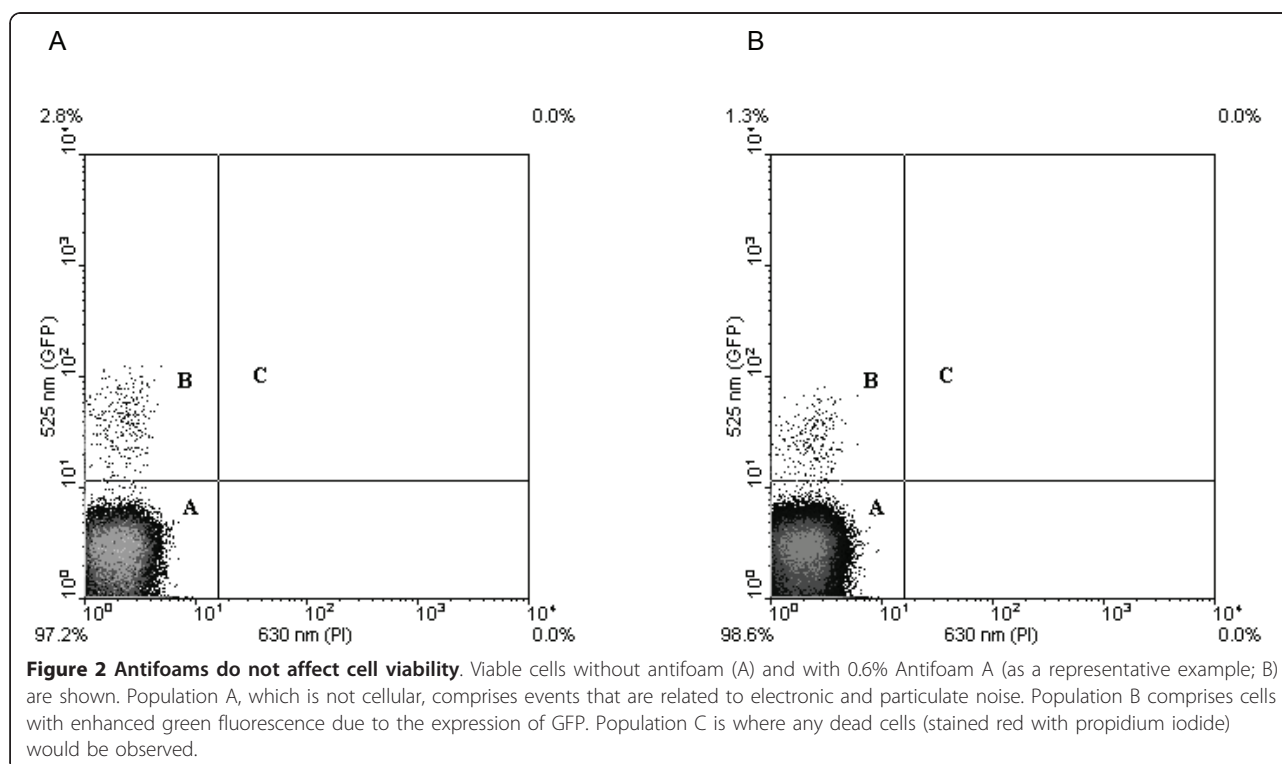


(Figure 1A-C): addition of these antifoams in the range 0.6-1% v/v caused a significant increase in specific yield compared to the control cultures with no antifoam. For cultures containing P2000 or SB2121, however, there was no statistically significant difference in the specific yield at each antifoam concentration compared with the control except for 1% SB2121 where P < 0.05 (Figure 1D-E). This suggested that the enhancements in total yield due to P2000 or SB2121 addition might be attributable to changed growth characteristics of the cells. The specific growth rates (μ) for cultures containing either 1% P2000 or 1% SB2121 were 0.17 h^{-1} and 0.18 h^{-1} respectively compared with 0.17 h^{-1} for the control samples (0% antifoam) indicating that the growth characteristics during the log phase were not affected by the presence of the antifoams. However, we noted an increase in OD₅₉₅ (at both 24 and 48 h) with increasing antifoam concentration for both antifoams (Figure 1D-E; 48 h data), which was

less pronounced for Antifoam A, Antifoam C and J673A (Figure 1A-C). We concluded, therefore, that there was more than one mechanism of antifoam action: one due to changed culture density (P2000, SB2121) and a second due to increased cellular production levels of recombinant GFP (Antifoam A, Antifoam C, J673A).

Antifoam addition does not affect cell viability

We investigated the influence of antifoams on cell viability by propidium iodide exclusion and flow cytometry. In this assay, dead cells are stained red [18] and appear in population C (Figure 2) while live cells fluoresce green due to GFP production and appear in population B. The data shown in Figure 2A suggest that there are no dead cells present in cultures containing 0% antifoam. Figure 2B shows that the same result was obtained in the presence of 0.6% Antifoam A. This result was seen for all antifoams tested.



The foam destruction capacity of an antifoam is related to its ability to improve GFP yield

We wanted to understand how the five antifoams increase total yield and hence began by evaluating their foam destruction properties. Simple methods of determining the ability of antifoams to reduce foam are the Bartsch shaking test [19] and the Ross-Miles pouring test [20]. A Bartsch shaking test was conducted (Figure 3) and demonstrated that in the absence of an antifoaming agent, initial foam destruction was quick until a stable foam was formed. Foam height reduced slowly and in the 15 min testing time did not reach zero (Figure 3A). The most effective agent for foam reduction was J673A, where less foam was formed after initial shaking, and destruction was rapid. Antifoam C had the least activity of the agents tested. All antifoams were effective at foam destruction and most foam was destroyed within one minute (Figure 3B). Although there was no statistical correlation between foam destruction capacity and either total or specific yield, J673A was the most effective at foam destruction and one of the best at increasing GFP yield, whilst Antifoam C was the least effective.

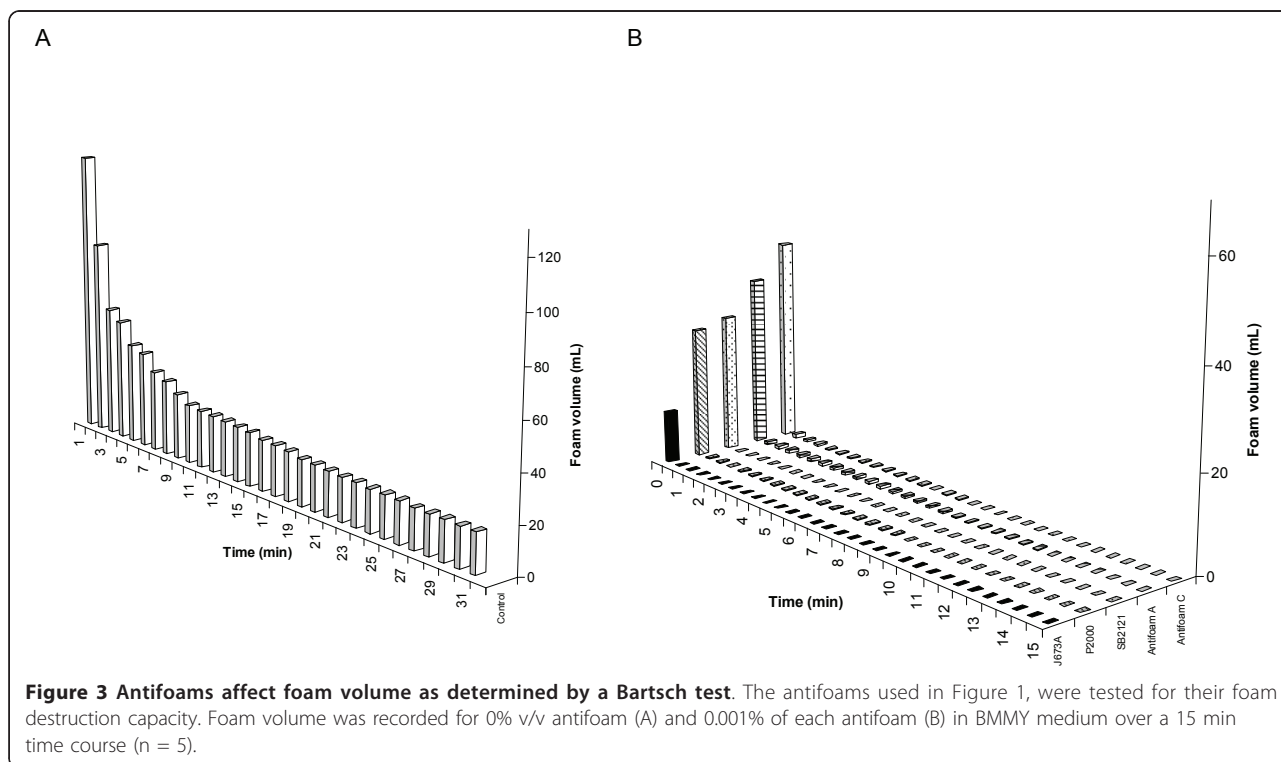
Improved yields cannot be explained by antifoam-induced changes in GFP fluorescence

In order to determine whether any of the antifoams affected the fluorescence of GFP, 1% antifoam was incubated in BMMY for 48 h to mimic the experimental set-up. This was then spiked with a similar concentration of

recombinant GFP standard to that obtained in Figure 1. There was no significant difference between the fluorescence of GFP in the presence and absence of any of the antifoams suggesting that they did not influence the sample readings. The fluorescence values of the antifoams themselves were also measured at 1% and found to be minimal, similar to the buffer control readings.

The $k_L a$ characteristics of antifoam-containing cultures are not correlated with improvements in GFP yield

As P2000 and SB2121 affected the density of the cultures, we investigated the possibility that the oxygen availability in the system was affected by antifoam addition and that this could explain increased GFP yields. The $k_L a$ was therefore measured in shake flasks in the presence of 0-1% v/v of these antifoams. Addition of 0.4% or 0.6% Antifoam A caused a large increase in $k_L a$ compared to the control (Figure 4A), whereas addition of the related antifoam, Antifoam C, led to an initial reduction in $k_L a$, which increased on addition of antifoam up to 0.8% and then returned to control levels at 1%. After an initial decrease in $k_L a$ was caused by J673A addition up to 0.4%, it remained relatively constant up to 1%. Addition of P2000 at all concentrations tested caused relatively minor changes to the $k_L a$. SB2121 addition did not substantially increase the $k_L a$ at any of the concentrations tested. Overall, there was no correlation between $k_L a$ and total yield for any of the conditions tested, or with the density of the cultures,



suggesting that changes in $k_L a$ may not directly lead to increased protein yield for these antifoams.

DO in shake flasks is not affected by the presence of antifoams P2000 and SB2121

In addition to measuring $k_L a$, we looked at the dissolved oxygen (DO) content of the cultures. The effect of 1% P2000 or 1% SB2121 addition on DO was assessed in shake flask cultures with PreSens DO patches and online monitoring. Figure 5 shows that there was no difference in DO in the flasks in the presence or absence of antifoam: after approximately 12 h for each culture condition the DO in the flasks became limiting. Since functional GFP can be expressed by anaerobic bacteria and in media containing 0.1 ppm dissolved oxygen [21], there was no concern that this would influence our data. DO decreased as the cells metabolized the methanol present in the medium and rose once they had consumed it. DO remained high until additional methanol was added at which point the DO immediately decreased and utilization continued. Methanol concentrations were confirmed by gas chromatographic analysis (data not shown). Overall, there was no difference in the DO content of cultures containing antifoam and those without.

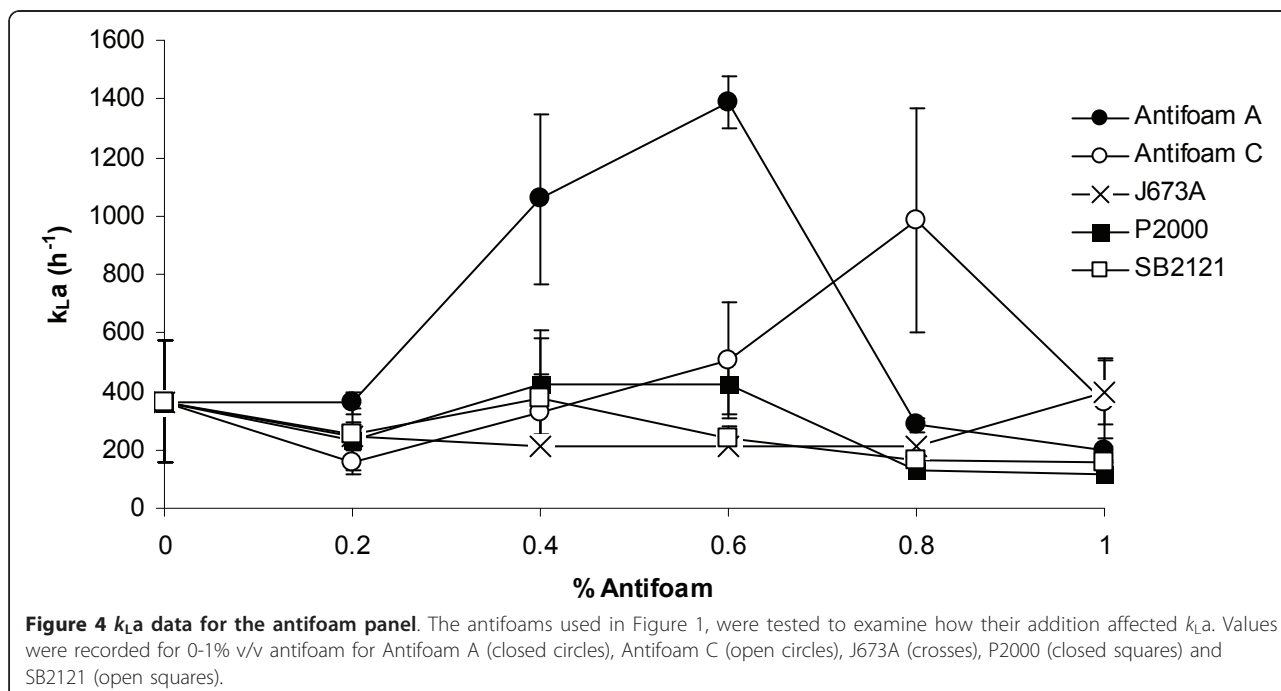
Addition of Antifoam A, Antifoam C or J673A affects the total yield of GFP secreted into the medium

We next investigated whether antifoam addition might have a physical influence on the cells. We therefore

measured the amount of GFP retained in the cell (by flow cytometry) and that in the culture medium (by fluorimetry). Figure 6 shows that addition of Antifoam A, Antifoam C and J673A caused a statistically significant increase ($P < 0.01$) in the amount of GFP secreted into the medium compared with the 0% antifoam control. The amount of protein retained in the cells was also greater suggesting that antifoam addition enhanced the ability of the cells to produce recombinant GFP. For P2000 however, more GFP was retained inside the cells compared with the 0% antifoam control. This is consistent with the growth of the cells being affected by P2000 addition rather than resulting in improved secretion efficiency, and also suggests that there has been some metabolic change to the cells compared to the control. Data for SB2121 was similar to that for P2000. We also noted that addition of antifoam did not cause any change in the total concentration of all proteins in the supernatant (measured using a bicinchoninic acid (BCA) assay) for cultures containing antifoams at representative concentrations of 0%, 0.5% and 1%, except for 0.5% Antifoam C ($P < 0.05$) and 1% SB2121 ($P < 0.01$). In the presence of these 2 antifoam concentrations, a decrease of 13-14% was observed in the total protein concentration of the supernatant compared to 0% antifoam-containing control cultures.

Discussion

Antifoams have previously been suggested to alter the growth of cells and influence protein yield in bioprocesses

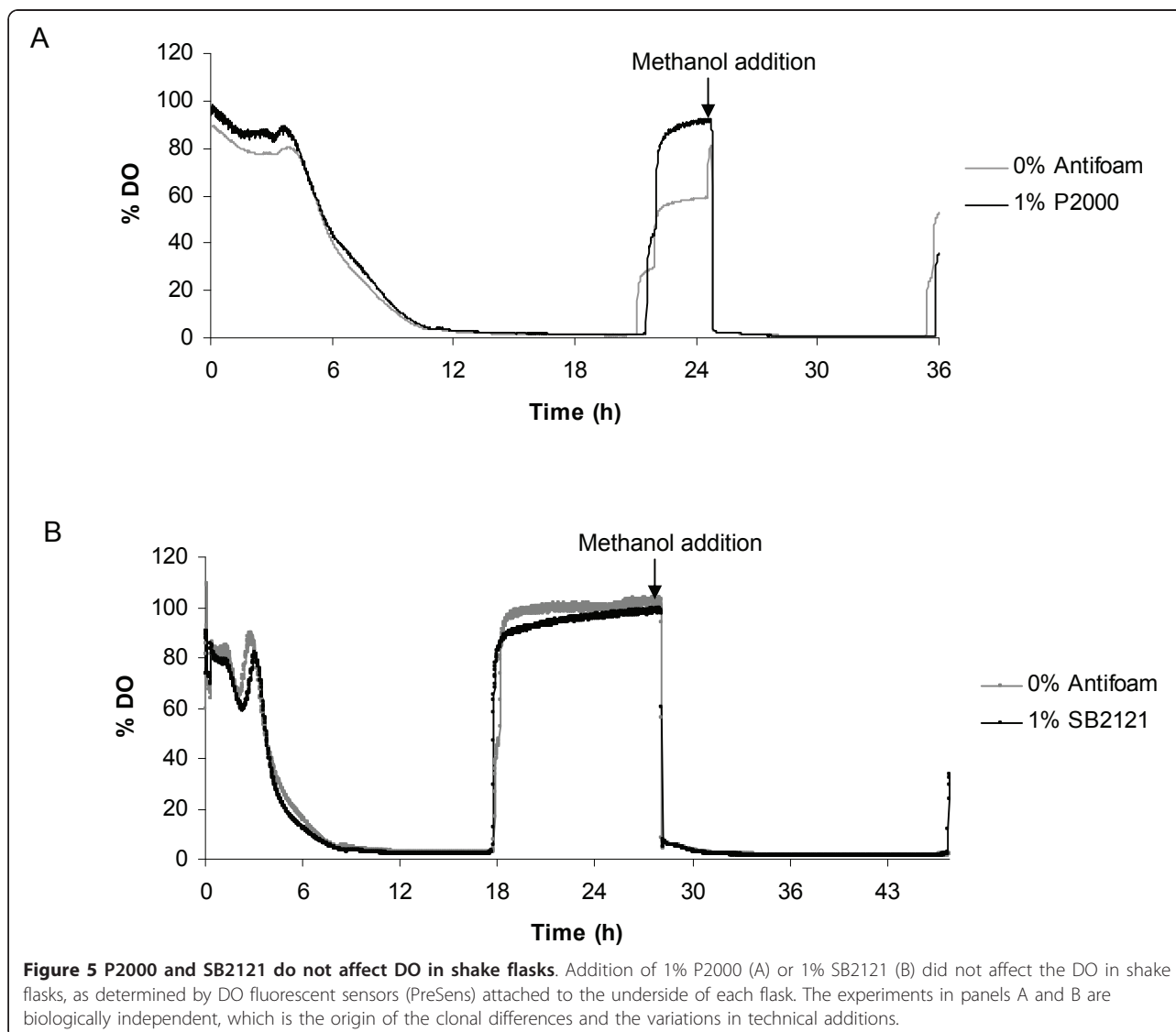


[2,11], but their addition to *P. pastoris* cultures has never been examined systematically. In this study, addition of the five antifoams tested increased the total yield of secreted recombinant GFP produced by 20 mL *P. pastoris* cultures. Generally, the total GFP yield secreted into the culture medium was increased when antifoam was added at concentrations of at least 0.4% v/v compared with the 0% antifoam control. Addition of 1% J673A to a 20 mL culture yielded 394 μ g GFP compared with 246 μ g GFP when there was no antifoam present. This is notable as J673A is approved for industrial use.

Antifoams can be split into two categories of fast and slow antifoams, depending on their mechanism of foam destruction. Slow antifoams are often oils which destroy foam over a longer period of time. Fast antifoams, as examined in this study, are generally mixed agents which enter the foam film and destroy it by a bridging-stretching mechanism [7]. It has also been observed that the most effective agents at destroying foam are those with the most efficient oil film spreading characteristics [22]. The least effective de-foaming agents in this study were Antifoams A and C, which are 30% aqueous emulsions of Antifoam A concentrate [17]. Their reduced de-foaming capability was accompanied by the weakest ability to increase the yield of protein. In contrast, J673A addition produced one of the best results, almost doubling the yield. This antifoam is an alkoxyated fatty acid ester on a vegetable oil base and it is known that vegetable oils can alter the structure of foams by increasing bubble size and reducing the stability of the foam [23]. While vegetable oils may be metabolized as a

carbon source [23], which might explain why J673A addition enhanced the yield of GFP, our data show that J673A did not influence the growth of the cells (Figure 1C), but rather enhanced the amount secreted into the medium (Figure 6). J673A was additionally found to be the most effective de-foamer of the panel of five antifoams that we assayed (Figure 3).

Antifoams are also known to affect the k_{La} of a system, which can be influenced by several factors such as medium viscosity, the presence of organisms and their by-products. These variables affect both k_L (ms^{-1}) and a (specific surface area m^{-1}) [24,25]. For example, antifoam addition is known to have an effect [26] by enhancing bubble coalescence and increasing bubble size which leads to a reduction in the specific surface area thereby lowering k_{La} [3,8,24,25,27]. However, it has also been previously observed [25,28] that at higher concentrations of antifoam the k_{La} rises possibly due to the detrimental effects of bubble coalescence. Consequently the reduced specific surface area (a) reaches a limit and bubbles coalesce suppressing surface motility and decreasing surface tension. This then leads to decreasing bubble size and k_{La} rises again. Additionally it is possible that antifoams accumulate oxygen from rising bubbles, as they have good oxygen solubility, and then release it to the aqueous phase. Bubbles bursting at the surface disperse small drops of antifoam causing more oxygen to be released [25,28]. In the case of oils which have a greater oxygen solubility than water, oil droplets may increase oxygen permeability in the water boundary layer of the gaseous dispersion [23]. Yagi and colleagues suggested that



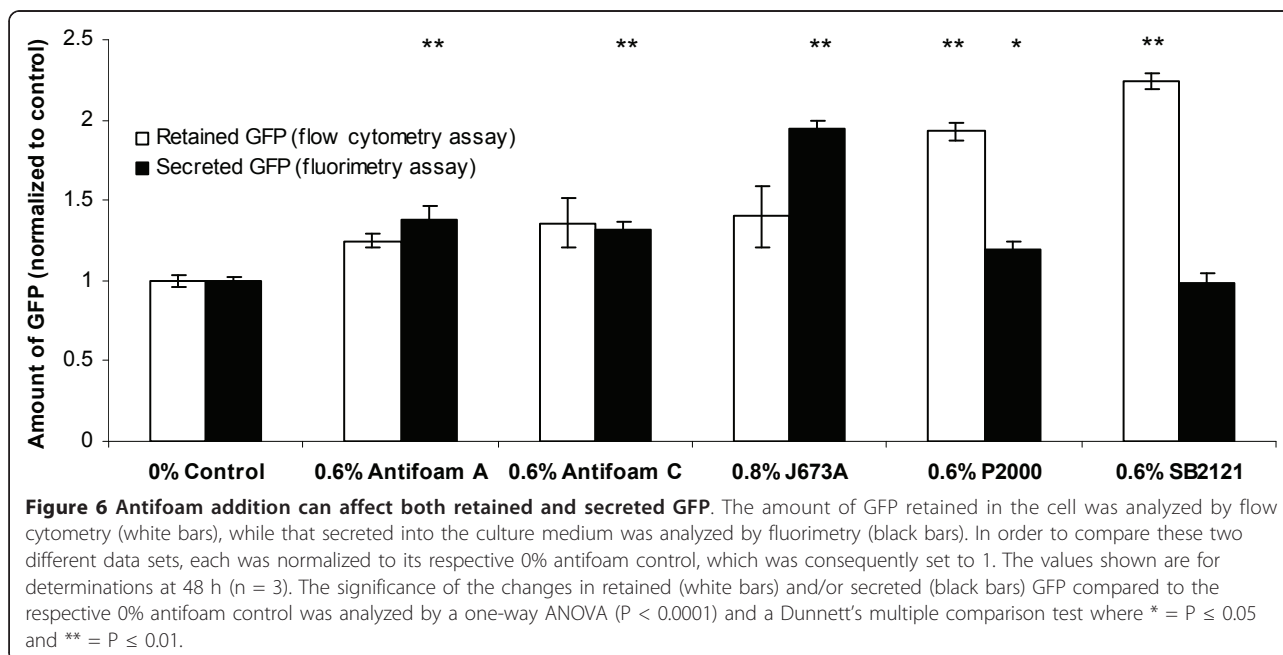
surfactants can cause rippling or eddying which influences the k_{La} [26]. They also found that k_L was not greatly affected by antifoams, but that their main effect was on a [26]. Koide subsequently suggested that the ability of antifoams to reduce k_L is less for bubble swarms than for a single bubble [29].

We found that in shake flasks, the k_{La} was higher at mid-range values and decreased with increasing concentration, but that there was no statistically significant correlation between increased k_{La} and total yield. It is therefore possible that the k_{La} is already sufficient for the cells to grow and produce protein and is not a limiting factor, or that a combination of factors is responsible for the increases in total yield that we observed. This is supported by the DO shake flask data which suggest there is no difference between the DO in flasks without antifoam

and those with either P2000 or SB2121 added. Combining flow cytometry and fluorimetry data showed that the antifoams can influence the amount of GFP retained inside the cell as well as the amount secreted into the medium. Antifoam A, Antifoam C and J673A enhanced the GFP secreted compared to 0% antifoam suggesting that the increase in total yield observed could be due to this secretion effect. This is consistent with an earlier study which suggested that antifoams can affect cell permeability in yeast by perturbing sterol biosynthesis which then alters the permeability of the membrane [30]. This is currently under investigation.

Conclusions

We show that when Antifoam A, Antifoam C, J673A, P2000 or SB2121 are added at concentrations higher



than those routinely used for de-foaming purposes, they all increase the total yield of shake-flask cultures. Two effects are apparent: one group of antifoams (Antifoam A, Antifoam C and J673A) increases the specific yield of GFP by increasing the total amount of protein produced and secreted per cell, whilst the second (P2000 or SB2121) increases the total yield by increasing the density of the culture. Addition of commonly-used antifoaming agents to shake flask cultures of yeast is therefore an effective way to increase the total yield of the recombinant protein being produced; any necessary changes to downstream steps such as protein purification are therefore likely to be worthwhile. Furthermore, this study provides insight into the manner in which antifoams interact with microbial cell factories: any data contributing to a fuller understanding of the specific effects of an antifoam on the growth and yield characteristics of such cultures, in addition to its primary action as a de-foamer, will be essential in bioprocess optimisation. These findings should provide an impetus to increase productivity in shake flask cultures of *P. pastoris*.

Methods

Yeast strains and culturing conditions

Pichia pastoris strain X33 transformed with pPICZ α A-GFPuv (designated X33GFPuv) [1] was used in all experimental procedures. Cells were cultured in shake flasks in medium buffered to pH 6.0 with 1 M potassium phosphate buffer and at 30 °C and 220 rpm. BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 4×10^{-5} % biotin, 1% glycerol

[31]) was used for the initial biomass accumulation stage before transferring to the induction medium, BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 4×10^{-5} % biotin, 0.5% methanol [31]), to induce production of GFP.

Shake flask cultures

Cells were cultured in 50 mL BMGY in 250 mL baffled shake flasks to accumulate biomass. 20 mL BMMY was then inoculated to a final OD₅₉₅ of 1.0 and transferred to a 100 mL non-baffled shake flasks for antifoam evaluations. Each evaluation was done in triplicate, with each flask containing the desired concentration of antifoam (0%, 0.2%, 0.4%, 0.6%, 0.8% or 1.0% (v/v)) with incubation at 30 °C and 220 rpm. After 24 h, 100% sterile methanol was added to 1% v/v to maintain production of GFP [1]. All optical density measurements were blanked against the relevant antifoam-containing medium. Since the antifoams themselves might influence OD₅₉₅, we analyzed the relationship between OD₅₉₅ readings in the absence and presence of a range of concentrations of different antifoams. In all cases the pair wise relationship was linear (R^2 was 0.91-0.99). We further verified that OD₅₉₅ was a reliable measure of cell density by comparing the number of cells at a given OD₅₉₅ in the absence and presence of a range of concentrations of different antifoams. There was no statistically significant difference in cell number between cells harvested at a given OD₅₉₅ in the absence or presence of any of these antifoam concentrations, suggesting that OD₅₉₅ is indeed a robust measurement of cell density.

Antifoam agents

The antifoams tested in this study were Schill and Schelinger's Struktol SB2121 (a polyalkylene glycol), Schill and Schelinger's Struktol J673A (an alkoxyated fatty acid ester on a vegetable base), Fluka P2000 (a polypropylene glycol), Sigma Antifoam A (a 30% emulsion of silicone polymer) and Sigma Antifoam C (a 30% emulsion of silicone polymer). All antifoams were autoclaved prior to use and each shake flask experiment was performed in triplicate, with the undiluted antifoam being added directly to the medium.

Fluorescence measurements

Culture supernatants (100 μ L) were assayed at 24 h and 48 h post-induction for GFP fluorescence using a Spectramax Gemini XS plate reader with an excitation wavelength (λ_{exe}) of 397 nm, and emission wavelength (λ_{em}) of 506 nm. Triplicate determinations were performed for each independent sample. All samples and blanks were buffered to pH >7.0 using 50 μ L 1 M potassium phosphate pH 7.5. Data were collected at 25 °C. To determine the concentration of GFP in each of the samples, a recombinant GFP standard (Vector Laboratories Ltd) was used to construct a standard curve relating RFU to protein concentration, as previously described [1]. All data were analyzed using a one-way ANOVA to test for a significant difference between any of the means. In all cases $P < 0.001$ indicating a high degree of significance. A Dunnett's multiple comparison test was then performed to compare each treatment mean (addition of various antifoam concentrations) and the control mean (0% antifoam).

Total protein analysis

The total protein content of culture supernatants (2 μ L) at 48 h post-induction was analyzed by bicinchoninic acid (BCA) assay. Cultures were examined in the presence of representative concentrations of 0%, 0.5% and 1% antifoam. 4.9 mL of proprietary BCA solution (B9643, Sigma) was mixed with 100 μ L 4% mM copper (II) sulfate solution (C2284, Sigma). 200 μ L of this solution was used to assay each independent supernatant sample in duplicate using a plate reader (BioTek Instruments) at 570 nm. To determine the concentration of protein in the samples, a bovine serum albumin standard (Sigma) was used to plot a standard curve. The data were analyzed using a one-way ANOVA ($P < 0.0001$) and a Dunnett's multiple comparison test.

Bartsch antifoam test

Bartsch tests were conducted following a protocol adapted from that outlined by Denkov and colleagues [32]. A 500 mL graduated glass cylinder was filled with 166 mL BMMY medium and in all cases except for the

control, antifoam was added to 0.01% v/v. The cylinder was sealed with parafilm and shaken ten times at ambient temperature. The height of the foam was recorded using the graduations on the cylinder every 30 s for 15 min. Determinations were performed in quintuplet for each antifoam. The activity of a given antifoam was reported as a volume [32], obtained by subtracting the volume of medium from the total volume (foam plus medium) in the cylinder.

k_La determination

The influence of each antifoam on the volumetric mass oxygen transfer coefficient (k_La) in 125 mL plastic non-baffled shake flasks with DO fluorescent sensors (PreSens; the closest available size to our previous experimental set-up using the same total:working volume ratio of 5:1) was measured using a dynamic method adapted from that of Bandyopadhyay and Humphrey [33]. A working volume of 25 mL BMMY was used for each determination, with each antifoam being added in a step-wise manner to a final concentration of 0%, 0.2%, 0.4%, 0.6%, 0.8% and finally 1.0% (v/v). Shake flasks were sealed with foam bungs and incubated at 220 rpm, 30°C. The medium was saturated with 1.5 L min⁻¹ compressed air and flushed with N₂. Determination of the k_La was carried out in triplicate by adding the required volume of antifoam at 100% DO, flushing with N₂ until the DO was 0% and then allowing the DO to return to 100%. The data were logged every second using SFR software (PreSens). The data logged during the increase in DO from 0% to 100% were used to calculate the k_La with the following formula, where t_1 and t_2 are consecutive time points, c_{1,t_1} is the oxygen concentration at time t_1 and $c_{1,\infty}$ is the oxygen saturation concentration.

$$k_La(t_2 - t_1) = \ln \left(\frac{c_{1,\infty} - c_{1,t_1}}{c_{1,\infty} - c_{1,t_2}} \right)$$

Dissolved oxygen measurements

Dissolved oxygen was measured in 125 mL non-baffled shake flasks with DO fluorescent sensors (PreSens) attached to the underside of each flask. The flasks were placed on a shake flask reader which excites the dyes in the sensors and allows the DO data to be logged over 48 h with SFR software (Presens).

Flow cytometry

Shake flask cultures of *P. pastoris*, as described above, were used to generate samples for flow cytometry analysis. The antifoams used were Antifoam A at 0.6%, Antifoam C at 0.6%, J673A at 0.8%, P2000 at 0.6% and SB2121 at 0.6% (v/v). Triplicate flasks were used for each antifoam. 48 h samples were diluted 1:1000 in phosphate buffered saline to a final concentration of

10^6 - 10^7 cells mL⁻¹, as determined using a haemocytometer. Fluorescent measurements were made using a Beckman Coulter (High Wycombe, UK) flow cytometer with $\lambda_{\text{exc}} = 488$ nm from an argon-ion laser at 15 mW. Diluted samples were additionally stained with 10 μ L propidium iodide (PI; 1 mgmL⁻¹ in water). All solutions were passed through a 0.2 μ m filter, immediately prior to use, to remove particulate contamination. The optical filters were set up so that PI fluorescence was measured at 630 nm and GFP fluorescence was measured at 525 nm. The data were analyzed using a one-way ANOVA ($P < 0.0001$) and a Dunnett's multiple comparison test.

Additional material

Additional file 1: Table S1: Summary of the biological effects of antifoam addition to microbial cell factories.

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Authors' contributions

SR was involved in all aspects of the experimental design, data collection, data analysis and interpretation and was supported by NB. CH oversaw the experimental design and data analysis for the flow cytometry component of the study. RB directed the study, coordinated the data analysis and interpretation and, together with SR, drafted the manuscript. All authors contributed to, read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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