Physiological studies on bacterial fermentations using multi-parameter flow cytometry

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Abstract

Two staining protocols were formulated that enabled the detection of cellular stress at the single-cell level for *Bacillus cereus*. Both $DiOC_6(3)$ and RedoxSensor Green™ can be employed to detect perturbations in the energetic status of the cell at concentrations of 0.30 $\mu g.mL^{-1}$ and 3.0 µM respectively. These methods can be employed for sensitive analysis of bacteria of both industrial and clinical interest. Flow cytometry was used throughout this work in order to assess the quality of recombinant *Escherichia coli* populations present within an agitated bioreactor. It was demonstrated in shake-flask culture that the cells could be grown to moderate cell densities ($OD_{600nm} \approx 25$) whilst producing measurable levels of antibody fragment. Despite being described in a patent which claims invention of a 100 % effective repression system (Hodgson et al., 2006), there was extensive evidence of promoter leakiness. Fab production was usually synonymous with cellular breakdown, however, a strategy based on simultaneous feeding and induction, before the exhaustion of the primary carbon source, yielded the highest concentration of Fab, 105 $mg.L^{-1}$, with more than 50 $mg.L^{-1}$ successfully targeted to the extracellular environment. Unlike all the previous cultures, this attainment also preceded the breakdown in the cellular structure.

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List of Abbreviations (ordered by first use)

DNA – Dexoyribose Nucleic Acid

SCID – Sever Combined Immunodeficiency

rDNA – Recombinant DNA

pDNA – Plasmid DNA

Ig – Immunoglobulin

Fc – Fragment crystallisable

ELISA – Enzyme-Linked Immunosorbent Assay

Fab – Fragment antigen binding

LPS – Lipopolysaccharide

CHO – Chinese Hamster Ovary

lac operon – DNA sequence controlling the expression of genes associated with the transport and metabolism of lactose in *E. coli*.

RNA – Ribose nucleic acid

σ – RNA polymerase subunit responsible for targeting the core enzyme to specific DNA sequences.

HSP – Heat shock protein

ppGpp - Guanosine tetraphosphate

GSP – General secretory pathway

TAT – Twin arginine translocase

ATP – Adenosine triphosphate

EDTA – Ethylene diamine tetraacetic acid

λ P_R & P_L – promoters found in the bacteriophage lambda

cI – Repressor molecule that controls expression from lambda controlled genes.

phoA – Promoter controlling expression of genes for growth and survival in low phosphate environments.

GTP – Guanosine triphosphate

trp operon – DNA sequence controlling the expression of gene products when tryptophan is limiting.

cAMP – Cyclic adenosine monophosphate

CRP – cAMP receptor protein

*lac*UV5 – Mutated form of the *lac* operon

tac operon – Combination of upstream elements of the *lac* operon and *trp* operon.

IPTG – Isopropyl-β-D-1-thiogalactopyranoside

TMG – Methyl-β-D-1-thiogalactoside

lacYZA – Shorthand term for the lac operon

mRNA – Messenger RNA

OD_{XXXnm} – Optical density, where XXX nm indicates the wavelength of light used for analysis.

$CFU.mL^{-1}$ – Colony forming units per millilitre

DCW – Dry cell weight

PBS – Phosphate buffered saline

GFP – Green fluorescent protein

PI – Propidium iodide

$DiBAC_4(3)$; BOX; bis-oxonol – Bis-(1,3-dibutylbarbituric acid)trimethine oxonol

$DiOC_6(3)$ – 3,3′-Dihexyloxacarbocyanine iodide

NADH – Nicotinamide adenine dinucleotide

NADPH – Nicotinamide adenine dinucleotide phosphate

FADH – Flavin adenine dinucleotide

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

HPLC – High-pressure liquid chromatography

SEC – Size-exclusion chromatography

qPCR – Quantitative polymerase chain reaction

LB – Lysogeny broth

PPG – Polypropylene glycol

DO – Dissolved oxygen

FACS – Flow-activated cell-sorting

OS1 – Osmotic shock solution 1; designed to remove the outer membrane from Gram negative cells, liberating the contents of the periplasm

OS2 – Osmotic shock solution 2; designed to disrupt the inner membrane of Gram negative cells (following treatment with OS1), liberating the soluble contents of the cytoplasm

BCA – Bicinchoninic acid

FS Log – Forward scatter logarithmic axis

PMT1 Log – Photomultiplier tube 1 logarithmic axis

SD – Standard Deviation

DMSO – Dimethyl sulfoxide

GMO – Genetically modified organism

tRNA – Transfer RNA

RT-PCR – Reverse transcriptase PCR

1. Introduction

Biopharmaceuticals, elaborate biological molecules used in the treatment of a variety of illnesses, are a large and ever-growing market. It has been estimated that the value of the biologics market could be as much as US$26 billion by 2010 (Pharmaceutical Business Review, 2006). Due to the innate complexity of these products the most viable current method for their manufacture is the use of biological organisms as "cell factories". *Escherichia coli* is the most widely used cell-type for this purpose, due to the wealth of information and resources available, although other cells are increasing in prominence, such as *Bacillus subtilis*, and *Bacillus licheniformis* (Law *et al* 2003; Lopes-Silva *et al* 2005). From both a cost-effectiveness and demand-driven perspective, it is desirable to develop processes which can generate as much product, in the shortest time as possible. It is estimated that approximately 80 % of the total cost of manufacturing a biopharmaceutical can be attributed to the downstream purification (separating the required molecule from its "factory" before purifying sufficiently for use as a therapeutic compound) (Dwyer, 1984). In order to mitigate this cost factor, the downstream process is considered when the choices of vector and expression system are being made with preference given to more easily recoverable products. In the context of an *E. coli* expression system, the low level of secretion into the extracellular environment makes periplasmic targeting an attractive compromise.

The production of a foreign molecule by a cell exerts a toll on its metabolism, the larger and more plentiful the foreign molecule, the greater this toll becomes. In certain cases, the metabolic load experienced by the cell is too great to bear, leading to cessation of growth, and even widespread cell death and lysis (Glick, 1995).

It is an unfortunate contradiction that periplasmic expression of foreign proteins often increases the drain on cellular resources still further by the activation of numerous stress response pathways (Balagurunathan and Jayaraman, 2008). In order to more fully understand the impact of these subcellular events on the organisms in particular, and the process in general, information from a variety of different analytical procedures must be collated. In order to maximise the value and quantity of information about cell cultures, it is becoming increasingly necessary to combine traditional, population-averaged, techniques with more modern single-cell analyses.

2. Literature Review

2.1. Biopharmaceutical Industry

The pharmaceutical industry, in one form or another has been around since the middle ages, where the medicinal properties of plants and naturally occurring compounds were used to treat diseases and ailments. Originating in the middle-east, these pharmacies spread across Europe, however, it wasn't until the mid-nineteenth century that a pharmaceutical industry, in the modern sense, was born.

By the mid-20th century, increasing understanding of human biology and its maladies lead rapidly to increasingly complex medical treatments.
The development of the pharmaceutical industry must also be considered alongside its current sibling, the biotechnology industry. For the purposes of this review, biotechnology will be considered shorthand for unicellular biotechnology, to exclude animal husbandry methods.

Biotechnology, the co-optation of biological processes for the benefit of mankind, is far older than the pharmaceutical industry, preceding it, in fact, by a few thousand years. The first accounts of the use of biotechnology describe alcoholic fermentation in ancient Egypt and Mesopotamia. This was followed by the use of fermentative yeasts for the production of leavened bread. These early processes were likely arrived at through stochastic means,

with no real understanding of the underlying causes of the macroscopic changes being exploited.

The first hurdle in the path to a broadly applicable discipline for biotechnologists was surmounted by Louis Pasteur in 1857 with his demonstration that fermentation processes are caused by the growth of microorganisms. This was the first proof that the established fermentative processes were reliant on a microbial population. Three major events combined to form the first modern-day biotechnology process; discovery of penicillin (Fleming, 1929), demonstration of its therapeutic potential (Chain *et al.*, 1940) and the development of a fermentation process for its large-scale production by Margaret Hutchinson-Rousseau. The elucidation of the structure of DNA (Watson and Crick, 1953) and subsequent discoveries of the nature of heritable units allowed the manipulation of DNA beyond anything previously performed by selective breeding. This recombinant age marks another significant step in the march towards the biotechnology used today. There are a number of subclasses within the field of biotechnology, briefly:

Blue – Describes the use and application of biotechnology within the marine environment such as degradation of marine oil spills by recombinant bacteria (Rheinwald *et al.*, 1973).

Green – Biotechnology as applied to agricultural processes and materials e.g. the Flavr Savr™ tomato generated by Calgene (Hiatt *et al.*, 1987).

Red – Medical applications, such as antibiotic production, and the emerging tissue therapies (Asano *et al.*, 2002).

White – Industrial usage, typically high volume, low value. Used in a variety of industries from solvent production, to paper milling (Jaeger and Eggert, 2004).

It should be noted that in all the different types of biotechnology listed above, there are both native and recombinant organisms involved, although the latter are increasingly the majority. Following the discovery of the structure of DNA came an ever-expanding body of knowledge surrounding the genetic structure of living organisms and, with that knowledge, the ability to manipulate this most fundamental of features.

The founding of Genentech™, the first company which was completely dependent on recombinant microbial technology, was a landmark in the modern biotechnological age, the first proof that these new tools could be applied in an industrial context for profitable ends. The first product in this new age was recombinant human insulin, which began manufacture in 1982 (Chance and Hoffmann, 1983). The new "genetic era" opened up a wide array of possibilities in the development and manufacture of more sophisticated treatments for diseases with the new-found freedom to manipulate biology to greater extent.

Insulin is an example of a peptide hormone, consisting of a short sequence of amino acids. The simplicity of this molecule was a major factor in its

development as the first biopharmaceutical, manufactured using *Escherichia coli* (Keen *et al.*, 1980).

Broadly speaking, biopharmaceuticals can be divided into a three distinct classes:

- Cell therapies
- rDNA (recombinant DNA)
- rProtein (recombinant Protein)

2.1.1. Cells

The first instance of the use of, specific, whole cells as medical therapies is vaccination. The most common early use of vaccination on record is against the smallpox virus, with the use of weakly virulent strains of smallpox as the vaccinating agent. Edward Jenner, among others, showed that exposure to cow pox, a relative of smallpox, could confer resistance to the latter. Louis Pasteur further developed Jenner's work using organisms subjected to treatments which decreased or removed their virulence.

Beyond the use of attenuated viruses and bacteria as immunizing agents, the use of whole cells as therapeutics has been the overwhelmingly minor part of the industry. Recent elucidation of techniques for the *in vitro* cultivation of human embryonic stem cells (Thomson *et al.*, 1998) has re-invigorated this part of the biotechnology industry. These pluripotent cells, and their progeny,

are currently being investigated for use in *in vivo* cell-replacement therapies, as well as *in vitro* platforms for animal-free drug-screening. Some work has also shown the benefits of mesenchymal stem cells as an alternative to surgery for osteoarthritic knee cartilage damage (Centeno *et al.*, 2008).

The other major use of cells as clinical therapies was the oft-highlighted and sporadically effective use of retroviruses in the treatment of SCID (Severe combined immunodeficiency). In that case, an rDNA copy of a gene absent in patients with the disease, is put into a retrovirus which are then used to "infect" a sample of the patients own cells. These cells are then transplanted back into the patient whereupon they express the previously missing proteins, curing the illness. The reason that this technique was not applied beyond phase I clinical trials was due to the development of leukaemia in 40 % of the 17 patients treatmented (Gansbacher, 2003).

2.1.2. rDNA

In conjunction with the production of insulin, and also intricately associated with the abortive SCID therapy, rDNA is an integral tool in modern biotechnology. The application of extrachromosomal plasmid DNA (pDNA) is predominantly used to import industrially useful characteristics into a desired organism from expression of recombinant products (Ladisch and Kohlmann, 1992; Yee and Blanch, 1993), co-expression of chaperones to assist product formation (Schäffner *et al.*, 2001), co-expression of cell permeation factors (van der Wal *et al.*, 1995; van der Wal *et al.*, 1998) to

addition of exotic enzymes able to carry out unusual chemical conversions (Di Gennaro *et al.*, 1997) among others.

Despite these primary uses of rDNA in biopharmaceutical processes there has also been a significant quantity of research into the use of naked DNA as a vaccine against a variety of ailments such as cancer (Johnson *et al.*, 2006), bird-flu (Laddy *et al.*, 2007) and multiple sclerosis (Stüve *et al.*, 2007). A stimulation of the immune response as a result of the presence of the naked pDNA has also been observed, which will only serve to increase the efficacy of these medicines (Krieg *et al.*, 1995; Jakob *et al.*, 1998).

2.1.3. rProtein

There are a number of different polypeptide compounds of interest in medical and related fields, with the main difference between them being the final functional state of the molecule. Many of these different types of proteinaceous compounds, described below, have both *in vivo* and *ex vivo* uses.

2.1.3.1. Small Peptides

These are the simplest amino acid polymers and usually consist of between 10 and 50 amino acids in length. The most well known of this group is insulin, mentioned earlier (Ladisch and Kohlmann, 1992), but also includes

somatostatin (Brazeau *et al.*, 1973) and atrial natriuretic peptide (de Bold, 1985).

The second major category of small peptide molecules is that of the antimicrobial peptide, such as; d-lysin, Magainin 2 and melittin (Almeida and Pokorny, 2009). These molecules are near-ubiquitous in nature and are capable of exerting a lethal effect on a wide variety of different organisms such as: bacteria (Gram positive and negative), viruses and fungi among others. Their modes of action vary depending on the specific sequence but are capable of both membrane permeablisation and interference with intracellular targets (Papagianni, 2003).

2.1.3.2. Enzymes

Enzymes are a naturally occurring biological catalyst constituting a folded polypeptide chain whose function is defined by the three-dimensional shape formed. Enzymes are a potent and efficient tool able to perform a wide variety of reactions under an equally wide range of conditions. When being considered from a bioprocess perspective, enzymes can be viewed as either an end product (Prakash *et al.*, 2009), or as a process ingredient, where the enzyme is first manufactured then subsequently used to catalyse a desired reaction (Goldberg *et al.*, 2008). The main reason for the need for the use of enzymes (whose manufacture can be a complex process) is the unparalleled degree of specificity achieved in some enzyme catalysed reactions. In

manufacturing processes where a specific optical isomer is required, enzymes are often the most cost-effective way of achieving this goal.

2.1.3.3. Antibodies

Antibodies, or Immunoglobulins (Ig), are an integral part of the mammalian immune system, consisting of two pairs of polypeptide chains linked by disulfide bonds.

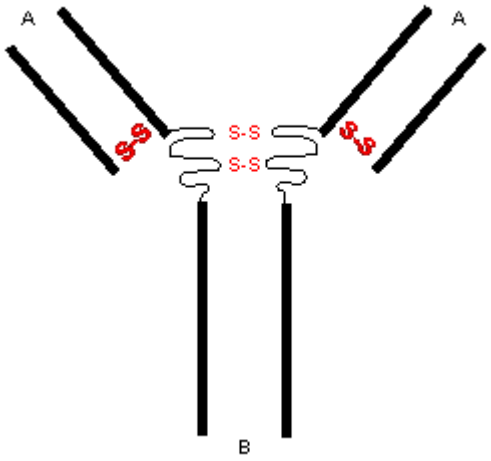

Figure. 2.1. An IgG molecule. A denotes the antigen-binding portion of the molecule B indicates the Fc region which modulates the immune system response. The flexible hinge regions (the thinner lines) of the heavy chains are covalently linked. The light chains are connected to one heavy chain each by a single disulfide bond. (Parham, 2000b))

In vivo, naturally occurring, antibodies are produced in a number of different isotypes, which perform distinct physiological functions.

Table 2.1. Major antibody isotypes (Parham, 2000c)		
Name	Description	Appearance
IgA	Secreted to the external surfaces of the body	Dimeric
IgD	Antigen receptor	Monomeric
IgE	Involved in the allergic response	Monomeric
IgG	Perform the bulk of antibody-based immunity against invading pathogens	Monomeric
IgM	Active against pathogens before sufficient IgG has been produced	Pentameric

For use as a therapeutic or diagnostic tool, the most suitable class of antibody is the IgG, as a result of its initial method of production (injection of antigen into animals, and subsequent recovery of the raised antibodies) and the ease with which functional fragments can be generated by enzymatic digestion of IgG (Figure 2.2. p. 15).

One of the greatest difficulties in obtaining antibodies by purification from an animal is the process of recovering a specific antibody from the mixture present. The work of Köhler and Milstein (1975) revolutionised the process of antibody production with the first production of an immortalised cell capable of producing a known, specific, antibody (a monoclonal antibody). The downsides to this method of production are the high cost and sensitivity to process conditions not experienced with more traditional biopharmaceutical production organisms, such as *Escherichia coli*.

The majority of therapeutic antibodies are still made using mammalian cells as a production system, despite the drawbacks mentioned above. The reason

for this is that bacteria are unable to perform post-translational glycosylation of the antibodies in question. Chiba and Jigami (2007) describe the current state of the art regarding chemical and molecular methods for adding glycans to recombinant proteins either following, or during expression from bacterial and yeast-based systems. It is possible to achieve titres of 2 $g.L^{-1}$ in bacterial systems (Carter *et al.*, 1992), however, the yield of that kind of process would necessarily be impacted by the following steps where glycosylation is required (or the increased doses, reflecting decreased binding efficiency *in vivo*).

The high degree of binding specificity exhibited by antibodies can be exploited to a number of different ends, both as *in vivo* therapeutics as well as *in vitro* diagnostic testing.

2.1.3.3.1. *In Vivo* Therapeutic

There are three major avenues being explored in order to realise the treatment of diseases with antibody therapy. The most prevalent is that of immune system targeting. Antibodies are generated which recognise specific molecules associated with disease (such as proteins expressed by cancerous cells) and can then recruit other important components of the immune system enabling the body to raise an immune response (Parham, 2000d).

Antibodies can also be utilised in ameliorating an immune overreaction such as allergic responses to the environment or autoimmune diseases. Antibody fragments without the Fc region are more useful here (2.1.3.3.3. Antibody

Fragments) due to the fact that, in this case, a reduced immune response is required (Cochet *et al.*, 1998; Pelat *et al.*, 2007; Depetris *et al.*, 2008).

Some research has also been performed with a view to conjugating xenobiotics to antibody fragments, in an attempt to reduce the effects of cytotoxicity of chemotherapeutic agents on healthy cells (Pietersz and Mackenzie, 1992).

2.1.3.3.2. *In Vitro* Diagnostic Testing

Antibodies form the basis of a variety of diagnostic tests, such as ELISA analysis, radioactive assay for myasthenia gravis (Oger *et al.*, 1987) and septicaemia (Eisenhardt *et al.*, 2007).

2.1.3.3.3. Antibody Fragments

A single molecule of IgG has a molecular weight of approximately 150 kDa, with only a very small portion of this dedicated to performing its specific binding function. When manufactured by purifying antibodies from an animal, a significant portion of the final IgG is not only unnecessary, but due to being from a non-human source, can be recognised by the immune system as foreign and acted upon as such. One of the solutions to this issue was enzymatic digestion of full-length antibodies with papain or pepsin creating Fab and $F(ab')_2$ respectively (Figure 2.2.).

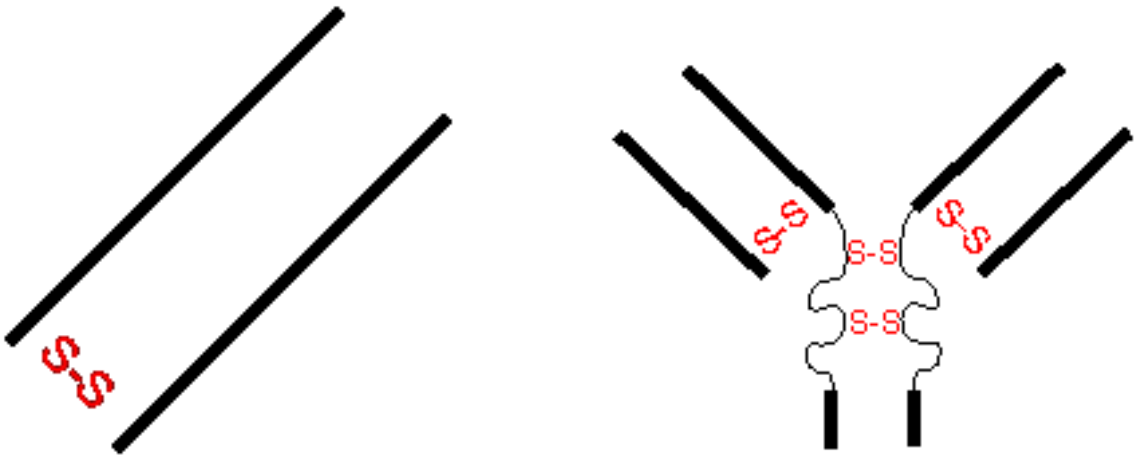

Figure. 2.2. Left Fab fragment; Right F(ab')$_2$ fragment. Originally created by enzymatic digestion of the whole IgG molecule from Fig. 2.1. (Parham, 2000a)

Both of these fragments have far less surplus sequence which is immunogenic in nature making them more suitable for therapeutic use in that sense. This reduction in size also had an additional unwanted effect however, with an observed reduction in circulation time in the body. This problem has been overcome by the addition of a tail of polyethylene glycol to antibodies to increase their size and mass preventing them being filtered out by the kidneys before they can have a therapeutic effect (Veronese and Pasut, 2005; DeFrees *et al.*, 2006). There are also many other types of antibody fragment available with a wide variety of functionalities, Holliger and Hudson (2005) have written a comprehensive review including a list of antibodies currently in clinical use and development.

One of the most attractive features of the availability of antibody fragments is their ease of production, relative to full-length monoclonal antibodies. The smaller size and decreased reliance on disulfide bonds means that the production options are much more varied than they would be otherwise.

Simmons *et al* (2002) have shown that the expression of full-length IgG molecules is possible in *Escherichia coli* however this is still very much the exception rather than the rule.

2.1.4. Cells as Factories

The use of biological cells as factories is the only viable method for the manufacture of complex biological molecules as has been previously described here. There are a number of factors involving the choice of host organism which are all interlinked such as: nature of product, its location, process operation methods, etc.

2.1.4.1. Bacteria

Bacteria are one of the most popular choices for large-scale biotechnological processes. This is a consequence of their rapid growth rate, ease of manipulation (both physically and genetically) and potential to achieve very high cell densities. As with all of the different production methods, there are downsides too, such as the increased load of lipopolysaccharide (LPS, the pyrogenic component of the outer membrane) when utilising Gram negative organisms and rare, protein-specific, post-translational modifications such as glycosylation (Sherlock *et al.*, 2006), which are necessary for the function of certain products (Bolt et al., 2005). It is also the case that larger and more complex proteins often cannot be produced in bacterial systems due to the limits of the cellular expression machinery.

2.1.4.1.1. *Escherichia coli*

First described in 1885 and named for its discoverer, Theodor Escherich, *E. coli* is a Gram negative bacterium and a normal commensal organism of the human gastrointestinal tract. Individual cells consist of rod-shapes approximately 2 μm in length by 0.5 μm in diameter, although specific mutations in membrane proteins have formed "filaments" (de Pedro *et al.*, 2002), and spherical particles (Markiewicz *et al.*, 1982; Zaritsky and Woldringh, 2003). These microorganisms also have the capability of metabolising a wide variety of carbon sources in both the presence and absence of oxygen. *E. coli* can be further subclassified into strains, differentiated by mutations in the genome, which often elicit phenotypic variations. In most cases, the differences between one strain and another have very little impact on the ability of the organisms to grow and reproduce in an ideal environment. However, if these organisms are challenged with a non-ideal environment, the different phenotypes displayed as a result of strain variations can enable an array of responses with the possibility of increasing survival rate of the genotype within a mixed population of cells.

Escherichia coli has been closely studied for many years, used as a model organism by research groups around the world for understanding bacterial physiology and was the first organism in which the process of horizontal gene transfer, conjugation, was studied (Lederberg and Tatum, 1946). In 1997 the complete genome of *E. coli* MG1655 was sequenced and published (Blattner

et al., 1997), although this was as a result of its prominence as an organism of choice for so many rather than a precursor. Most of the strains in use in laboratories today (including MG1655, above) are descended from the K12 strain of *E. coli*. These laboratory strains are distinct from the wild type organisms by their inability to colonise the gut and, in some cases, to form biofilms (Vidal *et al.*, 1998).

As a result of its *de facto* status as a model organism, there is a great deal of knowledge available surrounding the physiology of E. coli. In addition, the array of cloning vectors and expression systems that have been developed for use with E. coli mean that it is often the simplest and quickest route to manufacture of a complex biological molecule.

2.1.4.1.2. *Bacillus* spp.

Routinely found in soil, *B. subtilis* is a Gram positive, rod-shaped bacterium used by some as an organism for the manufacture of recombinant products (Law *et al.*, 2003; Takesue *et al.*, 2007). Gram positive organisms, such as B. subtilis, do not contaminate the final product with LPS which must be removed from Gram negative-produced recombinant materials. B. subtilis also possesses a much more efficient secretion system than *E. coli*, due to the absence of a periplasm (Han *et al.*, 2003) which can ease the purification of a biopharmaceutical. It also holds Generally Recognized As Safe (GRAS) status due to the prevalence in the environment and exceedingly rare incidence of associated food poisoning (Westers *et al.*, 2004).

Bacillus cereus is a closely related member of the genus, which is capable of causing food poisoning but otherwise has similar characteristics in terms of Gram staining and sporulation under stress. *Bacillus cereus* was chosen for this work due to the fact that it is an organism of clinical relevance for which understanding of stress tolerance is of interest. It also serves as a model for Gram positive behaviour in general, ideally situating it as a platform for the development of new techniques.

2.1.4.1.3. *Streptomyces* spp

The Streptomycetes are an important source of antibiotics; the natural source of, among others: erythromycin, tetracycline and chloramphenicol. As a result, this family was particularly important in the pre-genomic era, allowing large scale manufacture of clinical quantities of antibiotics. *Streptomyces* spp. are still being used today in modern biotechnological processes with the advantages of an efficient secretion system and naturally occurring glycosylation of protein products (Cowlishaw and Smith, 2001).

2.1.4.2. Mammalian Cells

Typically the term “mammalian cells” excludes human cells, and is usually used to describe cells from sources such as rat, mouse and monkey.

One of the most widely utilised mammalian cell types in this context is the Chinese Hamster Ovary (CHO) cell, first cultured in 1958 (Tjio and Puck, 1958). This cell type can be cultivated in attachment dependent and suspension cultures (following suitable adaptation), making it a very versatile cell type for biotechnological use. Furthermore, CHO cells are capable of applying glycosylation to manufactured proteins which is similar (but not identical) to that of native human proteins (Sheeley et al., 1997; Bolt et al., 2005). The other common expressions system of choice in this arena is the NS0 cell line derived from mouse myeloma described by Barnes *et al* (2000). Both of these cell types have similar advantages and disadvantages, and two of the major disadvantages are the slow growth rate and high cost of growth medium. The latter has been partly diminished by the development of synthetic growth media and additives that circumvent the prior necessity for more expensive, naturally occurring additives (e.g. foetal bovine serum). Mammalian cells also present a number of challenges from a bioprocess perspective, distinct from bacterial cells. Their more complex physiology makes the design of media and feeds more difficult, in conjunction with a reduced tolerance for mechanical stresses, often found within bioreactors, which combine to prescribe narrower engineering tolerance limits for operating parameters.

2.1.4.3. Human Cells

This particular area of research is expanding rapidly; however, the uses of these cell types are, generally, not as factories to manufacture a recombinant

product. The cost of manufacture and youth of the technology mean that most of the current work is aimed towards using these cells either as replacement therapies in cases of insufficient or poorly functional cells in a patient or as *in vitro* drug-screening platforms which will be vital if animal testing is to be discontinued.

2.1.4.4. Yeast/Fungi

As mentioned earlier, yeasts are some of the oldest biotechnological organisms, used in the creation of raised bread products and alcohol by *Saccharomyces cerevisiae*. Additional genetic material has been added to *S. cerevisiae* in order to broaden its range of substrates for the production of ethanol from a larger variety of sources (Ho *et al.*, 1998). Another kind of fungus was responsible for changing the landscape of modern medicine entirely. Discovered in 1928, penicillin, so named as a product of the *Penicillium* genus of fungus, was the first proven antibiotic compound (Fleming, 1929) (prior to this plants were often used, but the source of the antibacterial effect was unknown).

In more recent times, *Pichia pastoris* is gathering prominence as an effective production organism (Cregg *et al.*, 2000), offering a compromise between bacterial and mammalian systems. *P. pastoris* is a methylotrophic yeast capable of performing glycosylation and disulfide bonding on proteins, rendering it an attractive choice for recombinant protein manufacture. The most commonly used expression system is the AOX promoter, which is

repressed in the presence of glucose and active in the presence of methanol, a similar system to the *lac* operon in *E. coli* (Charoenrat *et al.*, 2005; Charoenrat *et al.*, 2006). This enables the growth and production phases to be discrete in a manufacturing process.

It has also been shown that glycosylation by *Pichia pastoris* can increase the efficiency of binding of certain molecules (Medzihradszky *et al.*, 2004). In addition to this, secretion by this yeast is much more easily achieved, and reliably performed than in the case of poor secretors such as *E. coli* with far higher biomass achievable (Cregg *et al.*, 2000).

Bacterial production systems still dominate the biopharmaceutical manufacturing mien. The primary reasons for this are ease of regulatory approval for the process, stability of production following strain selection and rapid generation time, which impacts both development cycle-time and production space-time efficiency. The combination of these is ample justification for using a process based on bacterial expression systems, in preference to the other options mention above.

2.1.5. Stress

Cells of all types can be subjected to stresses from a variety of sources, that those cells have persisted means they have evolved mechanisms to enable them to tolerate, and even grow in the presence of, those stresses.

To properly understand stress, it must first be defined. It is also vital that the effect of the stress on the cell is characterised, at the molecular level, which will inform the nature and extent of the responses enacted by the cell.

2.1.5.1. Stress – A Definition

"Stress is any change in the genome, proteome or environment that imposes either reduced growth or survival potential. Such changes lead to attempts by a cell to restore a pattern of metabolism that either fits it for survival or for faster growth." (Booth, 2002).

This definition encompasses stresses which are intrinsic to the normal operation of the cell (such as DNA mutations through random copying errors) as well as extrinsic factors, more normally associated with raising cellular stress responses (*e.g.* heat, heavy metals, antibiotics, *etc.*), and as such provides a comprehensive and satisfactory definition of stress, as experienced at the single-cell level.

The most well-studied stress, relating to single-cells, is the effect of a sudden increase in temperature, eliciting the heat shock response. First discovered in *Drosophila melanogaster* (Ritossa, 1963), and later found to be present in almost all living organisms, the heat shock response enables cells to survive in the presence of a sudden increase in temperature to unusually high levels. Other stressors include: heavy metals (Sharma *et al.*, 2006), oxygen (both too much and not enough) (Kalmar and Greensmith, 2009), antibiotics (Barcina

et al., 1995), starvation (Cuny *et al.*, 2005), mechanical stresses (Sukharev *et al.*, 1999) and recombinant protein production (Dürrschmid *et al.*, 2008).

2.1.5.2. Stress Effects

There are three major classes of molecule whose integrity and function must be preserved by the cell in order to remain viable under stress

- Membrane
- DNA
- Protein

The cytoplasmic membrane is responsible for separating the cell from its environment; it suffices to say that if a cell is unable to maintain the integrity of its membrane the internal contents of the cell will be quickly diluted in the surrounding medium. In addition, the membrane is also required to maintain electrolyte gradients for the generation of energy and transport of nutrients. This electrolyte imbalance is described as the membrane potential of the cell. The resting membrane potential of the cell is approximately 100-200 mV negative inside with respect to the outside (Novo et al., 1999). When the cells are subjected to stresses, their ability to maintain this membrane potential could be impaired due to the diversion of energy and resources away from these processes to cellular repair and stress tolerance mechanisms (Hewitt et al., 1999; Lewis et al., 2004). An extreme example of this is the ability of *E. coli* to accumulate recombinant protein up to 80 % of dry cell weight (Demain

and Vaishnav, 2005). Production in this quantity represents a significant drain on cellular resources away from normal housekeeping functions.

The DNA comprising the cellular genome contains all of the heritable information required for continuing survival and proliferation. Fidelity of copying the information is important, and all living cells have a vast array of repair mechanisms operating continuously to establish this. Even under ideal conditions, the rate of mutation is non-zero, allowing the occurrence of occasional changes in the genome, however under the pressure of mutagens (chemical or radiative) the level of expression of the repair functions is increased (Bridges *et al.*, 1987).

Protein is the last major class of molecule affected by stresses to the cell. Protein molecules are the workhorses of the cells, providing the means by which the information in the DNA sequences can be controlled, transcribed, translated and exported. They enable metabolites to be imported and processed providing the energy for all cellular operations, as well as controlling the flow of ions across the membrane to establish the gradients mentioned above.

2.1.5.2.1. Metabolic Load

Metabolic load, or burden, is the term given to an often observed phenomenon when using living organisms to manufacture complex biopharmaceuticals. The presence of foreign DNA within the cell (Ow *et al.*,

2006), as well as the manufacture of proteins from it (Kane, 1995; Bonomo and Gill, 2005) cause a decrease in the ability of the cell to carry out its normal metabolism. In the case of Kane's (1995) and Bonomo & Gill's (2005) work the net effect of the recombinant protein expression is the induction of the starvation response as a result of rare codons/amino acids found in eukaryotic proteins. The response to this increase in the requirement for resources diverted away cellular maintenance and proliferation usually entails a decrease in growth rate (Glick, 1995). The responses of the cell to the effects of metabolic load are carried out through responses to the particular effects detailed below (2.1.5.3. Stress Responses).

2.1.5.3. Stress Responses

E. coli regulates a significant amount of its gene expression at the transcriptional level. The genetic information is transcribed, by RNA polymerase into messenger RNA, before being translated into a primary peptide sequence where it can perform the function of that specific protein. The RNA polymerase enzyme is a quaternary structure featuring a number of subunits which form the "core" enzyme and a more easily dissociated subunit, designated the σ subunit. The σ subunit is responsible for targeting the core RNA polymerase to a specific DNA sequence. The most commonly utilised σ subunit is the σ^{70} (70 denotes the molecular weight – 70 kDa) which targets the core enzyme to the "housekeeping" genes, those whose transcription is necessary to maintain basic cellular function. A number of other σ factors exist, which serve to recruit the RNA polymerase core enzyme

to genes associated with stress response proteins, which are designated by molecular weight, in the case of a number, or by function (e.g. E = envelope stress).

The onset of a significant upward change in temperature alters the conformation of σ^{32} transcription factor. The σ^{32} subunit preferentially binds to the promoters of the so-called heat shock proteins (HSP), and recruits RNA polymerase, effecting their transcription. Many HSPs have been demonstrated to be chaperones that assist correct folding of proteins into their functional conformation (Gaitanaris *et al.*, 1990). It has been shown that although the HSPs are expressed following a rise in temperature their expression is necessary, but not sufficient, to achieve thermotolerance (VanBogelen et al., 1989). The variety of possible stressors encountered by bacteria is very wide, and so it seems unlikely that they would be able to utilise specialised stress response mechanisms. A number of other stimuli have elicited expression of HSPs, as well as providing varying measures of thermotolerance. The heat shock response, and the associated thermotolerance, has been raised by the exposure of cells to ethanol, $CdCl_2$, antibiotics and H_2O_2 (VanBogelen et al., 1989; López-Sánchez et al., 1997). Most of the evidence suggests that non-heat derived induction of the heat shock response may be due to accumulation of improperly folded proteins (Chaudhuri *et al.*, 2006; Díaz-Acosta *et al.*, 2006).

An additional alternative sigma factor, σ^S, is responsible for modulating the general bacterial stress response. Particularly implicated as part of the

bacterial response to starvation (Lange and Hengge-Aronis, 1991), σ^S is increased as the cells transition from exponential-phase growth to stationary-phase (Jishage and Ishihama, 1995). As the name implies, the general stress response is also induced by stimuli other than the starvation and reduction in growth rate associated with transition into stationary phase (Notley and Ferenci, 1996), such as high osmolarity (Muffler *et al.*, 1996) and low pH (Bearson *et al.*, 1996).

A third alternative σ subunit, σ^E, is responsible for modulating the response to envelope stresses. During bacterial growth the proper functioning of the membrane, and its associated proteins, are impaired by events such as mechanical stress, antibiotics and misfolding of membrane proteins (Ami et al., 2009). These effects are transduced through the membrane allowing the expression of proteases and chaperones that assist the correct folding of outer membrane proteins, membrane biosynthesis proteins and genes with predicted periplasmic localisation (Dartigalongue *et al.*, 2001). Envelope stress is particularly important in a biotechnological context due to the possibility that accumulation of recombinant proteins in the periplasm can trigger the same stress response due to both the appearance of misfolded proteins in the compartment (Hayden and Ades, 2008), as well as the increased physical pressure exerted on the membrane by the contents of the periplasm (Narayanan *et al.*, 2008).

It is common to have one or more of these alternative factors present in a cell simultaneously in addition to the housekeeping σ^{70}. The σ^{32} mediated

response exemplifies this, as described above, with heavy metals and oxidative stress eliciting the heat-shock response. This has the effect of reducing the number of RNA polymerase core enzyme complexes available to transcribe constitutive cellular proteins. For this reason, a secondary effect of the stress responses is the lowering of the growth rate, which in turn, reduces the quantity of basal metabolism needed (Want *et al.*, 2009).

One of the features exhibited in cells entering stationary-phase growth is the stringent control of ribosome production (Sands and Roberts, 1952). The molecule responsible for modulating this effect is the unusual nucleotide guanosine tetraphosphate, ppGpp (Cashel and Gallant, 1969), which exerts its effect through binding to the β and β' subunits of the RNA polymerase core enzyme (Chatterji et al., 1998). It has been established that ppGpp increases the affinity of the RNA polymerase core enzyme for alternative sigma factors, such as σ^S (Jishage *et al.*, 2002) thus inducing the general stress response in addition to restriction of ribosome production. This sequence of events increases transcription from stress response genes, whilst limiting the production of ribosomes to increase the competition between transcripts, leading to increased translation of stress response gene products. Costanzo (2006) found that ppGpp also impacts on σ^E targeted genes although it is, as yet, unknown if this is caused by increased affinity of RNA polymerase for σ^E.

2.2. Culture Strategy

2.2.1. Expression Strategy

When expressing a protein in a Gram negative bacterium, there are four possible options when considering where the protein might be targeted:

- Extracellular environment (secreted)
- Intracellular
  - Soluble
  - Insoluble (inclusion bodies)
- Periplasmic space

Each of these options can be employed with a variety of proteins and their suitability is determined by the nature of the product (size, number of disulfide bonds, etc.).

2.2.1.1. Secretion

The overwhelming majority of bacteria are capable of secreting proteins into the extracellular environment. The principal benefit of this in environmental samples of bacteria is the manipulation of the extracellular milieu, to its own advantage. There are a variety of functions performed by these secreted proteins (Fernández and Berenguer, 2000); from enzymes able to digest surrounding macromolecules (Voigt *et al.*, 2006), to pathogenicity factors

(Brown *et al.*, 2004), even combinations of the two where the enzyme function increases potency of the pathogenic activities (Egea *et al.*, 2007). Secretion of proteins from Gram negative bacteria is a more involved process than is the case for Gram positive organisms. In order to be liberated into the extracellular medium from a Gram negative cell, the protein must pass through two lipid membranes, a highly energetically unfavourable process given the highly hydrophilic nature of the majority of soluble proteins. In order to achieve the export of proteins, bacteria are able to utilise one of a number of different pathways; type I, II and the twin arginine translocase pathways being the most prominent.

2.2.1.1.1. Type I Secretion

Type I secretion, also referred to as signal-sequence independent (although the modern consensus is that there is, actually, a signalling sequence at the C-terminal end of proteins destined for this kind of export (Sandkvist and Bagdasarian, 1996)), is a single-step process where a protein is passed through both membranes at once and is present in a variety of genera including *Escherichia coli* (Kenny *et al.*, 1991; Palacios *et al.*, 2001). Type I secretion is very rapidly saturated with proteins if translation rates are not properly controlled (Shokri et al., 2003) further, the signal sequence is not cloven from the protein during export. The combination of these two factors ensures that type II secretion is the most widely used system of this type in industrial processes.

2.2.1.1.2. Type II Secretion

Type II secretion, also known as the general secretory pathway (GSP), is a two-stage process where the excreted protein is first passed into the periplasmic space, and then secreted through the outer membrane as a fully folded protein. Following translation, the protein destined for export binds to elements of the secretory machinery by interaction with an N-terminal signal sequence usually between 10 and 30 amino acids in length. This signal sequence is removed from the protein as it is passed through the inner membrane into the periplasm. It has been established that secreted proteins pass through the outer membrane after interacting with a large number of protein factors (Pugsley, 1993) although the exact means of this interaction has yet to be elucidated, indications are that it may be dependent on the three-dimensional structure of the protein and additionally, may vary from protein to protein (Sandkvist and Bagdasarian, 1996).

Bacterial strains derived from the K12 strain may have been stripped of their pathogenic activity, however, the secretion machinery utilised by the organism in those processes remain intact. *Escherichia coli*, generally, secretes very low quantities of protein into the extracellular medium (Sandkvist and Bagdasarian, 1996).

This low level of secretion of endogeneous proteins makes *E. coli* an ideal candidate for use as a production organism; due to the low levels of host-cell proteins contaminating the downstream purification. Secretion of

recombinant protein product also obviates the need for a cell disruption step, ensuring that contamination with host cell DNA and proteins can be avoided. Other benefits of this type of production methodology are; increased stability (Talmadge and Gilbert, 1982) and reduction in immunogenicity of the final product, as a result of decreased LPS contamination (Mergulhão *et al.*, 2000).

Due to the constitutively low levels of secretion by *E. coli*, the pathways which enable newly produced proteins to be passed through the two lipid membranes are ill-equipped, both physically and energetically, to translocate protein in the quantities, and at the rates of production, usually achieved with recombinant expression (Simmons and Yansura, 1996). Incomplete translocation across the inner membrane can result in blockage of the membrane-bound complex responsible for transferring protein from the cytoplasm to the periplasm (Baneyx, 1999) and concomitant accumulation of heterologous protein in inclusion bodies within the cell (Mergulhão and Monteiro, 2004; Mergulhão *et al.*, 2004). These can be caused by protein size (Koster *et al.*, 2000), amino acid composition of the leader peptide, and the sequence of the expressed protein (Kajava *et al.*, 2000), especially in the region of the export signal sequence.

2.2.1.1.3. Twin Arginine Translocase (TAT) Pathway

Discovered in *E. coli*, and independent of the GSP, the TAT is so called due to a pair of consecutive, conserved arginine residues within the N-terminal signal sequence. The TAT pathway is unusual due to its ability to transport

fully folded proteins (DeLisa *et al.*, 2003) across the inner membrane (Stanley *et al.*, 2000) in the absence of ATP (Yahr and Wickner, 2001). The transport is instead driven by the proton motive force generated by the bacteria (de Leeuw et al., 2002). In spite of this, export is performed at very slow rates when compared to the type II system (DeLisa *et al.*, 2004).

2.2.1.2. Intracellular Protein Expression

The potential utility of secretion as a means of achieving ease of purification from *E. coli* cultures means that research in this area is being pursued as a high priority; however, bacterial systems are currently unable to attain the yields required for therapeutic uses.

The most obvious alternative is the expression of the protein within the cells, leading to extraction and subsequent purification. Proteins can occur in one of two possible forms inside the cell, soluble and insoluble.

2.2.1.2.1. Soluble

In bacterial cells, this is the default expression option for the overwhelming majority of proteins. It is, therefore, the easiest method on offer, but in some ways the most problematic. Many proteins that one might wish to express in *Escherichia coli* are able to fold correctly within the cytoplasm, however, those requiring the presence of disulfide bonds in order to achieve their correct 3-dimensional structure cannot achieve this in the highly reducing

bacterial cytosol (Bessette *et al.*, 1999). Any proteinaceous material present within the cell also presents a prime target for intracellular proteases requiring the use of protease deficient bacterial strains to reduce product degradation. However, this would also compromise the ability of the organism to deal with stress and could further complicate the fermentation. At high intracellular concentrations of protein, insoluble inclusion bodies can be formed (Section 2.2.1.2.2. Insoluble) and there is not yet any reliable means for predicting whether an individual protein will be sequestered, or at what concentration.

2.2.1.2.2. Insoluble

In many cases insoluble intracellular expression is not an unwanted by-product of overexpression, but a desired feature of the process. It was originally considered that inclusion bodies were formed by the aggregation of misfolded proteins in instances where their numbers overwhelmed the chaperone-assisted protein folding (Kane and Hartley, 1988). The whole process of inclusion body formation is now recognised as being more complex than first thought, with significant quantities of correctly folded protein within bacterial inclusion bodies (Ventura and Villaverde, 2006; Martínez-Alonso *et al.*, 2009). Inclusion bodies can easily be separated from the bulk of a disrupted bacterial cell, due to their increased density relative to other intracellular components. They also exhibit excellent purity and shield the desired protein from proteolysis (Singh and Panda, 2005). Following this separation, the extremely costly and low yield refolding process takes place

using high concentrations of chemicals such as urea or guanidine hydrochloride (Datar *et al.*, 1993). Driven by the extremely poor recovery and economics of these refolding processes, a great deal of work has been carried out concerning the extent of refolding necessary from a variety of inclusion bodies, with far milder conditions than conventionally used for a number of different proteins (Tsumoto *et al.*, 2003; 2004; Umetsu *et al.*, 2005).

2.2.1.3. Periplasmic Expression

Periplasmic expression represents a compromise between the absence of additional engineering required for intracellular expression, and the ease of purification allowed by true secretion. As described earlier, the first step in secretion of a protein from Gram negative organisms is translocation across the inner membrane, and periplasmic expression obviates the need for the additional cellular resources required to pass the protein through the outer membrane. Furthermore, the oxidizing environment of the periplasm ensures that disulfide bonds can be formed where necessary (Missiakas *et al.*, 1995). The periplasm contains approximately 4 % of the total cell protein of wild type *E. coli* cells allowing simplified purification of product if the outer membrane of the cell can be selectively removed or otherwise negated (Nossal and Heppel, 1966; Makrides, 1996).

Protein can be liberated from the cell by “partial” lysis – methods which remove, or render passive, the outer membrane of the cell

- Osmotic shock (lysozyme/EDTA/sucrose)
- L-form cells

Osmotic shock is the most well established method for disrupting cells, requiring inexpensive chemicals and minimal processing. This method uses ethylene diamine tetraacetate (EDTA) to weaken the membrane by chelation of magnesium ions that stabilise the teichoic acids in the membrane, along with sucrose which diffuses into the periplasm. The cells are then exchanged into a solution free of sucrose and the resulting osmotic gradient causes influx of water into the periplasm, causing it to swell and then burst (Heppel, 1967). Chen *et al* (2004) have performed experiments indicating that pre-treatment of the cells with divalent cations can increase the efficiency of the osmotic shock technique when recovering recombinant proteins from *E. coli*.

The derivation of Gram negative bacteria that are missing their cell wall (L-form cells) has allowed another potential avenue for an alternative type of expression. These bacteria possess a stable mutation which prevents them from correctly forming the outer cell membrane (Rippmann *et al.*, 1998). This effect has been utilised as a recombinant expression system in a number of species including *Proteus mirabilis* and *Escherichia coli*. Due to the absence of the outer membrane and cell wall, any protein which is targeted for release to the periplasm will instead be secreted into the extracellular milieu. These cells have an absolute requirement for complex medium sources, which introduce an extra layer of variability into the process, rendering them less suitable for biotechnological exploitation (Gumpert and Hoischen, 1998).

2.3. Expression Systems

2.3.1. Constitutive

Many proteins found within cells are required at specific levels at all times in order to carry out basal functions. In order to facilitate this, these proteins are expressed at low levels at all times.

On the face of it, constitutive protein expression appears to be an attractive means of producing heterologous proteins with continuous expression throughout the length of the culture. This kind of expression system effectively links biomass accumulation to increases in product, with an easily administered process.

In practice, constitutive expression is almost never used for the production of recombinant products due to a number of factors. Most significantly, the manufacture of a recombinant product exerts a toll on the organism in question in terms of both energy and resources. The magnitude of this toll is amplified by the scarcity of these components during rapid cell growth. Continuous production of recombinant proteins also increases the risk of proteolytic degradation due to the higher residence time of the proteins within the cell.

2.3.2. Induced

By far the more common means of expressing recombinant proteins is that of induction. A variety of methods are available whereby the cells are first grown, and then induced to manufacture the desired compound. The greatest advantage of this approach is maintaining a separation of cellular growth from product formation. The classical paradigm of this type of process is growth of cells up to the maximum possible cell density, followed by induction. Approaching the process in this manner enables the use of much stronger promoters than are suitable for constitutive expression meaning that, in some cases, far greater quantities of protein can be produced in a relatively short induction period. Induced systems also provide greater scope for process optimisation in comparison to constitutive ones.

There are a variety of different methods for achieving induction of protein expression, and they generally achieve better results than constitutive methods with improved process control. There is also evidence that some induction regimes can be harmful to cells, by expressing recombinant protein too fast for the existing cellular infrastructure causing a breakdown in the expression systems that impacts on the production of constitutively required components (Section 2.1.5.2.1. Metabolic Load). Furthermore, some of the induction conditions are not easily scaled due to either physical restrictions, such as heat and mass transfer, or cost-effectiveness.

2.3.2.1. Inducers

2.3.2.1.1. Heat

The most common means for control of protein expression in *Escherichia coli* by temperature change is the bacteriophage λ P_L and P_R promoter system. Manufacture of proteins which are controlled by this system are held under repression by the cI repressor at temperatures less than 42 °C. Jechlinger *et al* (1999) have shown that a mutant repressor (cI857) exhibits an increased sensitivity to temperature, with induction of the system at temperatures exceeding 30 °C. Cells utilising temperature control are usually grown in the range of 30-37 °C, however it has been shown that temperatures prior to, during and following induction, as well as the duration of the induction, can vary the extent of both the expression and its effect on the organism. Cultivation at repressive temperatures (30 °C) followed by an upward shift and brief hold (39 °C) before returning to a non-repressive condition with increased cellular activity (37 °C) can yield particularly good results in terms of measured protein activity (Chao *et al.*, 2002). The model described here is the standard means of operating this kind of process (although the specific temperatures vary from protein to protein). There are two major factors which prevent permanent upshift in temperature; the observation that protracted derepression of P_R and P_L controlled genes can lead to segregational instability and reduction in the number of plasmid-bearing cells within the bioreactor (Siegel and Ryu, 1985). High temperatures also carry the risk of triggering the bacterial heat shock response, necessarily

entailing diversion of resources away from recombinant protein production, as well as increased protease activity.

Temperature-controlled induction does not lend itself well to large-scale cultures due to the limitations on the speed of heat transfer encountered. While these effects can be partially mitigated by process changes such as utilisation of a second bioreactor (Hortacsu and Ryu, 1990), this impacts significantly on the cost-effectiveness of the process.

It has also been shown that reduction in temperature can increase the expression from a number of promoters, including the λ P_L promoter (Giladi *et al.*, 1995) and the cold-shock response proteins (Phadtare *et al.*, 1999) although these effects are not generally exploited for recombinant protein expression.

2.3.2.1.2. Nutrient Limitation

2.3.2.1.2.1. Phosphate

Alkaline phosphatase, transcribed from the *phoA* gene, is responsible for performing hydrolysis of phosphate containing substrates (Schwartz and Lipmann, 1961). When the availability of inorganic phosphate falls, the *phoA* gene is derepressed. The *phoA* promoter can be used in conjunction with a recombinant DNA sequence, in order to link phosphate depletion to product formation. This type of control of recombinant protein expression is

particularly elegant, ensuring an automatic disconnection between growth and product formation, particularly due to the lack of a requirement for exogenous material. However, the phosphate limitation can have negative effects on the cell's metabolism (Lübke et al., 1995) which diminish its ability to manufacture the protein of interest. The effects of the phosphate limitation on metabolism can be mitigated by optimisation of the feeding strategy and batch medium (Lübke et al., 1995; Wang et al., 2005) though the presence of phosphate in some of the most significant biomolecules (nucleotides and ATP/GTP). As a result of this, Lübke *et al* (1995) observed plasmid loss during the induction phase (likely resulting from the cell having insufficient phosphate to maintain the plasmid within the cell), but were able to compensate by increasing the level of inoculum. This action also managed to increase the productivity of the fermentation with the most recombinant protein produced in tandem with the highest level of inoculum (10 % compared with 1 %). Wang *et al* (2005) employed a pH-stat fed-batch strategy and achieved a ten-fold increase in recombinant protein production as a result, using a similar phosphate limitation method. This recombinant protein production was further increased by supplementing amino acids into the simple glucose feed, although this effect was less significant.

Fine control of this kind of expression is very difficult to achieve using complex media (Section 2.4.2. Nitrogen source) due to the variability of components within it, therefore use of phosphate-limited induction places constraints on the possible choices of growth medium.

2.3.2.1.2.2. Tryptophan

The *trp* operon encodes proteins which are responsible for synthesising tryptophan from other amino acids when it is not present within the bacterial environment. Genes under the control of the *trp* operon are repressed in the presence of tryptophan, and derepressed by either its absence or the presence of the gratuitous inducer 3-β-indole acrylic acid. Repression of this type, however, has been found to be incomplete (Kane and Hartley, 1988) which could be problematic in the case of production of a toxic product. There are processing techniques available that can completely repress the *trp* operon though, such as the addition of yeast extract (whose effect was shown to be distinct from that of tryptophan added alone) or the use of monosaccharides such as glucose to enable a catabolite repressive effect (Yoon *et al.*, 1996). Care must be taken with this system though, as it may be unsuitable for the expression of proteins which have large quantities of tryptophan, or other aromatic amino acids.

2.3.2.1.3. Chemical Induction

2.3.2.1.3.1. *lac* Operon

The *lac* operon is present in many *E. coli* strains and controls the expression of proteins responsible for the import and metabolism of, the disaccharide, lactose. The *lac* operon encodes three proteins, of which two are necessary for lactose metabolism: β-galactactoside permease (responsible for import of

lactose and its analogues) and β-galactosidase (capable of catalyzing hydrolysis of lactose to galactose and glucose, and transgalactosylation of lactose to form allolactose). In the absence of lactose, the expression of these two proteins is repressed, although often not completely, allowing small quantities of the *lac* operon products to be made (Elowitz *et al.*, 2002). This is particularly important, due to the fact that without β-galactoside permease none of the lactose can enter the cell. More significantly, the natural inducer of the *lac* operon is actually allolactose, which means that both the permease and galactosidase enzymes are required in order to fully induce expression. It has also been shown that the products of β-galactactoside reactions (galactose, glucose and allolactose) are all excreted from the cell in large quantities. It is suggested this may have the dual effect of preserving the allolactose from hydrolysis to act on uninduced cells, and the connection between proton-linked efflux of glucose and the proton-linked influx of lactose and allolactose (Huber *et al.*, 1980).

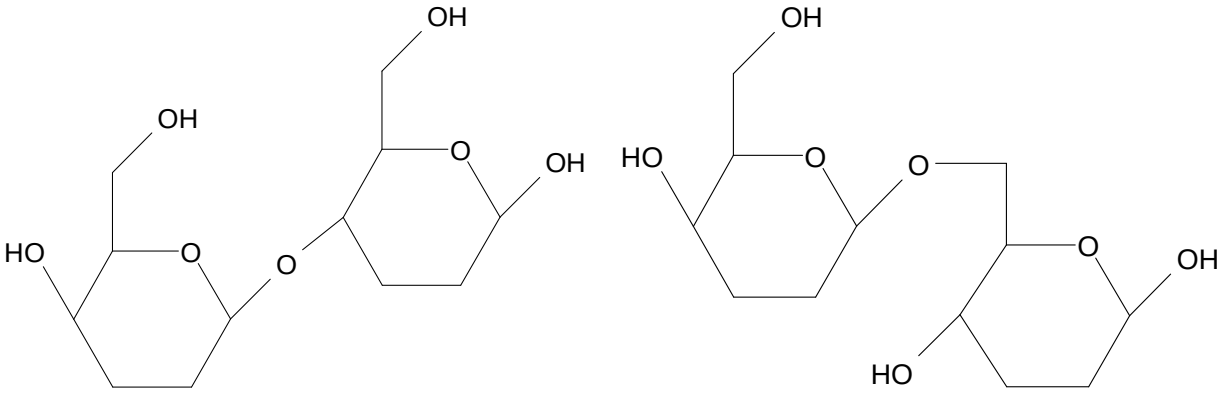

Figure. 2.3. Lactose (left) naturally occurring disaccharide and Allolactose (right) its enzymatically altered (*lac* operon inducing) isomer.

The *lac* repressor molecule is a tetrameric protein complex which obstructs the transcription initiation site, preventing access by RNA polymerase. Each

subunit of the *lac* repressor can bind a single allolactose molecule, requiring 4 molecules for each repressor to achieve complete derepression (Lewis et al., 1996).

Escherichia coli also uses an ancillary method for control of the *lac* operon, whose effect was first described by Jacques Monod in 1942. The observed effect was that of diauxie (preferential use of one carbon source over another) later expounded upon by Monod himself (1949) as well as specifically relating to the glucose/lactose dyad (Loomis and Magasanik, 1967). When both glucose and lactose are present in the environment, *E. coli* will utilise glucose as a carbon source whilst the *lac* operon remains repressed. This is an effect termed "catabolite repression" which classical scientific orthodoxy indicates is caused by high levels of intracellular cAMP, in turn binding to cAMP receptor protein (CRP) which regulates the transcription in the absence of glucose (Ullmann and Monod, 1968). The established model states that growth using glucose suppresses cAMP levels within the cell, a suppression which is lifted when metabolism is changed to alternative carbon sources, such as lactose. Inada *et al* (1996) demonstrated that the apparent catabolite repression is actually an inducer exclusion effect of the phosphotransferase system (responsible for the import of simple sugars such as glucose) on the β-galactosidase permease.

2.3.2.1.3.2. *lac*UV5 Operon

The *lac*UV5 operon is a variation of the *lac* operon that is not susceptible to the apparent catabolite repression discussed in 2.3.2.1.3.1. If the carbon source of choice is likely to be glucose, the use of the *lac*UV5 operator ensures that the cells can be adequately fed during induction.

2.3.2.1.3.3. *tac* Operon

The *tac* operon is a hybrid of the -20 to -35 region of the *trp* promoter, attached to the -20 to -1 section of the *lac*UV5 operon. This hybrid has been shown to increase transcription by 11 times relative to the *lac*UV5 sequence and 3-fold in comparison to the *trp* operon (de Boer *et al.*, 1983).

2.3.2.1.3.4. *lac* Operon Inducers

The natural inducer of this system, allolactose (derived from lactose), has already been mentioned above (Section 2.3.2.1.3.1. *lac* operon). There is, however, another type of inducer that is relevant because of its prolific use within academic research, the so-called gratuitous inducers. These compounds are non-hydrolysable analogues of lactose which bind to the *lac* repressor causing expression of foreign proteins under control of the *lac* operon. The most widely used of these chemicals is isopropyl-β-D-1-thiogalactopyranoside (IPTG).

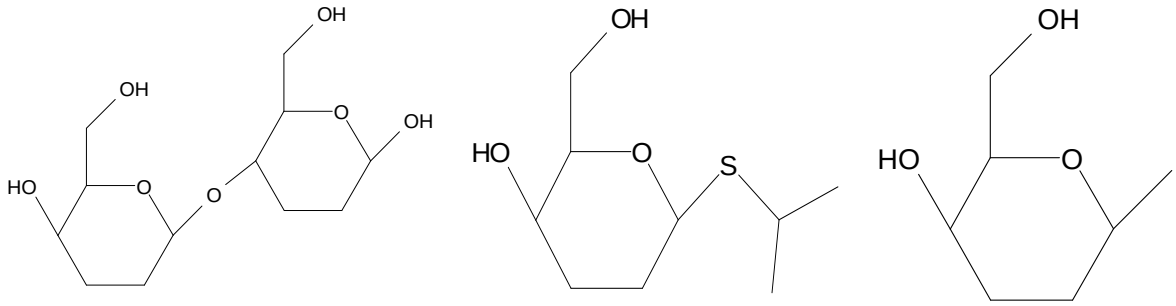

Figure. 2.4. Lactose (left) compared with gratuitous inducers of the *lac* operon; IPTG(middle) TMG (right).

These synthetic chemicals exhibit a number of other features which distinguish them from their natural alternative. Methyl-β-D-1-thiogalactoside (TMG) concentrates 100-fold within cells when supplied at 10 μM. β-galactoside permease has approx a 100-fold higher affinity for IPTG than for lactose (Sistrom, 1958), although it is also well established that lac permease is not a significant factor in the influx of IPTG into cells (Fried, 1977). IPTG is becoming less widely used due to its unsuitability for industrial application resulting from high cost, potential toxicity to humans (Makrides, 1996) and an effect similar to the bacterial heat shock response although with no significant change in growth rate observed (Kosinski et al., 1992).

2.3.2.2. Induction Conditions

As mentioned previously, the use of induction to trigger the manufacture of the protein of interest provides additional opportunities and scope for development and optimisation. The variety of options available is also dependent on the promoter system in use:

2.3.2.2.1. Temperature

A repressive growth temperature must first be established, which should be lower than 37 °C with 100 % repression achieved at 30 °C or below (Tabandeh et al., 2004) although the exact optimal repressive and inductive temperatures vary depending on the product. This is consistent with all expression systems, where the specific recombinant sequence to be induced will change the metabolic profile of the cell, and hence, the optimum conditions for expression. Following this, a temperature to which the culture is raised must be determined, based on desired culture parameters, such as; product yield/quality and cell viability. In many cases, the temperature is downshifted subsequently to create an environment more conducive to minimizing bacterial stress responses (Section 2.1.5.3. Stress Responses).

In tandem with the discovery of optimal temperatures for initial induction and post-induction growth and manufacture, the length of time of induction must also be considered, with particular attention to the scalability of the protocol (Caulcott and Rhodes, 1986; Tabandeh et al., 2004; Soares et al., 2008).

2.3.2.2.2. Nutrient Limitation

This method of induction allows far less control than is the case with the other, more frequently applied, methods. The only real measure of control available is adjusting the time at which cells are induced through the use of

feeds containing the limited component. This kind of induction also has the greatest potential for creating a mosaic effect within the culture due to the existence of microenvironments with distinct nutrient limitations or excesses causing patches of differential gene expression within the reactor. As volumes increase towards production-scale, these effects will become even more pronounced as mixing efficiency falls.

2.3.2.2.3. Chemical Induction

2.3.2.2.3.1. Inducer concentration

This is one of the primary factors controlling the level of expression of recombinant genes. The relative amounts of inducing agent and lac repressor molecules determine to what extent the gene is expressed in a population of cells. Furthermore, the induction regime is usually extremely variable based on the choice of inducer. In the case of the *lac* operon, IPTG is usually the inducer of choice, although this is often because host strains which are *lacYZA*$^{-}$ are often used for initial cloning procedures. *E. coli* strains which are *lacYZA*$^{+}$ allow for the use of lactose as either inducer, or both carbon source and inducer enabling a greater level of flexibility within the process.

If lactose is being used as a sole inducer in a *lacYZA*$^{+}$ strain of *E. coli*, the metabolism of lactose will cause the concentration to fall within the culture as it proceeds. This metabolism, combined with the growth of the cells, causes the concentration of lactose (or, more specifically allolactose) to fall

both in absolute terms and in proportion to the number of cells. This problem can be circumvented by the use of a lactose feed (Gombert and Kilikian, 1998; Kilikian et al., 2000), however, the strains utilising the *lac*UV5 operon should enable employment of lactose solely as an inducer, with minimal degradation.

The other solution to the problem of falling inducer levels is the use of a gratuitous inducer, such as IPTG. The cell's inability to metabolically process IPTG means that the absolute concentration is maintained throughout the culture. This could still present the problem of post-induction increases in biomass, however, its passage through the cell membrane is not significantly restricted even in the absence of β-galactoside permease (Fried, 1977). Despite this, Hansen *et al* (1998), have indicated that the presence of *lac* permease can, in fact, be beneficial for proteins synthesised from *lac* operon controlled genes. Wood & Peretti (1991) showed that protein expression from the *lac* operon increased linearly up to 1 mM IPTG, after which there was no further increase in β-galactosidase, a result of mRNA degradation rather than a lack of induction. The optimal concentration of IPTG (the concentration which achieves the highest yield of functional protein) to be used is affected by a number of factors but is heavily influenced by the subcellular target (intracellular/periplasmic/secreted). Intracellular proteins are often best induced using approximately 1 mM IPTG and extracytoplasmic proteins usually achieve best results with 0.1 mM or less (Chalmers et al., 1990; Shibui and Nagahari, 1992; Laffend and Shuler, 1994).

The choice between using a natural physiological inducer (such as lactose) and a gratuitous inducer (e.g. IPTG) presents more scope for process optimisation in addition to the potential for variation of concentration.

2.3.2.2.3.2. Induction Point

Not only is the concentration of inducer important, so is the bacterial growth phase at the point of addition. The optimal point for addition of the inducing agent is influenced by manifold and various factors including; pre-induction growth rate (Curless *et al.*, 1990), size and type of product, cellular localisation of product (Ramirez *et al.*, 1994) and metabolic changes brought about by transitions between energy sources (Want *et al.*, 2009).

2.4. Medium

2.4.1. Carbon Source

2.4.1.1. Glucose

Glucose is one of the most widely used carbon sources for microbial cultures. Its transport, and metabolism, by the cell is well understood, and it provides high yields of biomass (Castan *et al.*, 2002; Soini *et al.*, 2008).

When glucose is used as a carbon source, care must be taken to maintain a low concentration at all times. High glucose concentrations have been shown

to inhibit growth (Luli and Strohl, 1990) and recombinant product formation (Jensen and Carlsen, 1990) from production of acetate through anaerobic metabolism.

2.4.1.2. Glycerol

Glycerol is one of the more common alternative carbon sources to glucose, with its recent drop in price from being a by-product of biodiesel manufacture being a significant factor in this (Yazdani and Gonzalez, 2007). Cells metabolising glycerol typically exhibit lower growth rates and a lower biomass yield on substrate than similar cultures fed with glucose (García-Arrazola et al., 2005). This is because each glucose molecule produces twice as much pyruvate as each glycerol molecule.

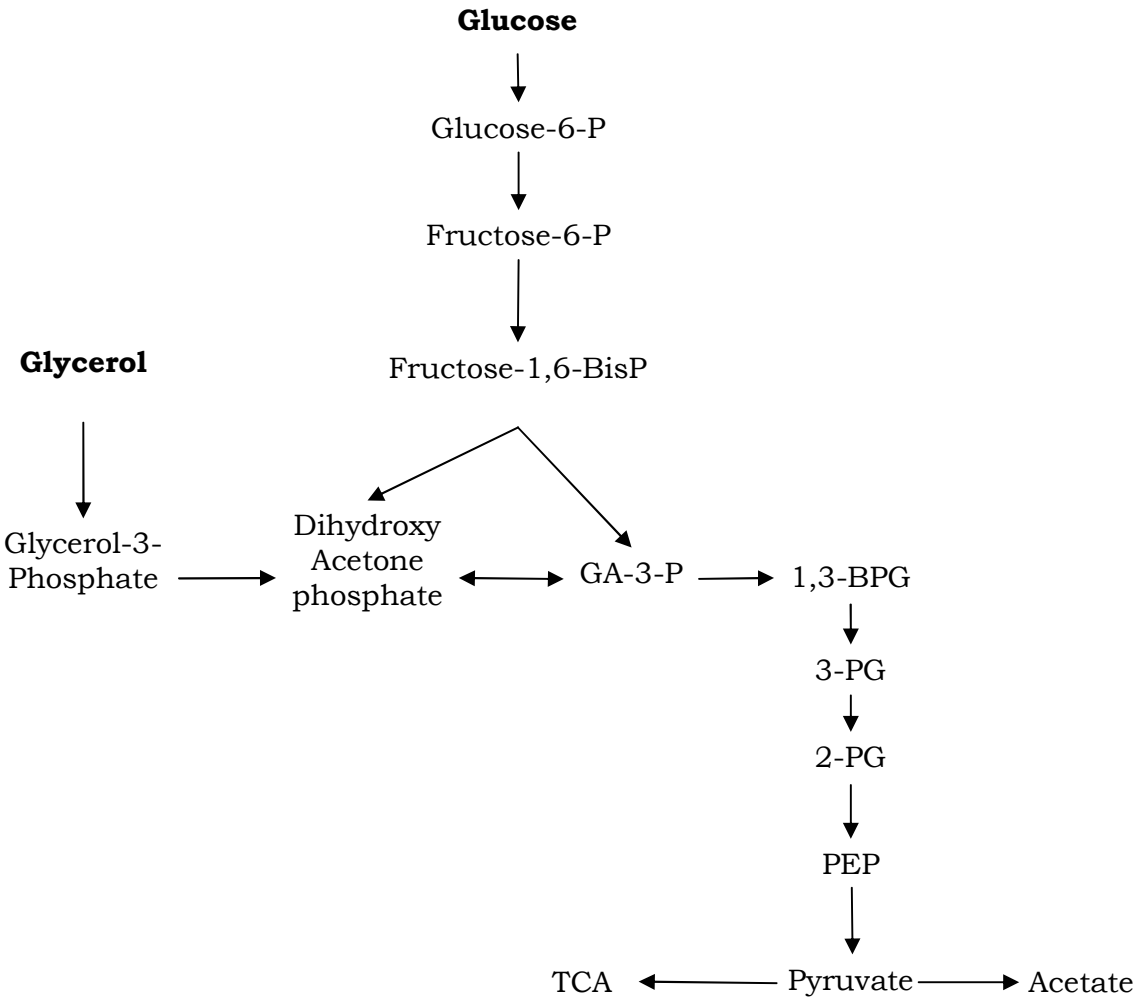

Figure 2.5. Central carbon metabolism showing entry of glucose and glycerol. GA-3-P (glyceraldehydes-3-phosphate); 1,3-BPG (1,3-bisphosphoglycerate); 2-PG (2-phosphoglycerate); PEP (Phosphoenolpyruvate); TCA (tricarboxylic acid cycle) (Stryer, 1995) and (Luli and Strohl, 1990)

Cells growing on glycerol are not subjected to overflow metabolism in the same way as glucose-grown cells. It is a commonly stated opinion that *E. coli* utilising glycerol as the primary carbon source do not produce acetate (Shiloach and Fass, 2005). Where acetate has been shown to coincide with glycerol use (García-Arrazola et al., 2005; Guebel et al., 2009), levels are far below those shown to inhibit growth and product formation. The use of glycerol does present problems from a material handling perspective due to

the high viscosity, although it is soluble at higher concentrations than glucose.

2.4.1.3. Lactose

Lactose is similar to glycerol in that glucose is preferentially metabolised when both are present in the environment (Fraser and Yamazaki, 1983), which could be due, in this case, to the extra metabolism required of lactose prior to entry into glycolysis. Lactose is also not suitable as a primary carbon source in recombinant strains utilising the *lac* operon for induction of the product.

2.4.1.4. Fructose and Others

It has been shown that cultivating *E. coli* on fructose reduces the level of acetate, relative to growth on glucose, whilst simultaneously increasing the quantity of overexpressed β-galactosidase (Aristidou *et al.*, 1999). Xylose and arabinose have also been used both individually and as a mixture with glucose to increase levels of 3-Dehydroshikimic acid produced by recombinant *E. coli* (Li and Frost, 1999). It seems unlikely that glucose and glycerol will be deposed as the automatic first and second choices for bacterial fermentation in the foreseeable future due to their abundance and high biomass yield in fermentation processes.

2.4.2. Nitrogen Source

The nitrogen required by growing cell cultures is usually supplied in one of two ways; either a so-called "complex" source (e.g. yeast extract, tryptone/peptone, etc.) or via a "defined" inorganic means such as an ammonium salt.

Using complex medium can increase the amount of both cell mass and specific product formation within a bioreactor (Hoffmann *et al.*, 2004). In addition, Hoffmann *et al* showed that yeast extract can prevent accumulation of insoluble protein, relative to a defined nitrogen source. It should be noted, however, that these effects were only observed at very low cell densities. This result was observed using two different products; one under heat-induction and the other under IPTG induction of T7 RNA polymerase so it is possible that these effects are correlated rather than causative. Tsai *et al* (1987) have also shown that the use of an organic nitrogen source can increase the productivity of a recombinant fermentation, however, they also found that this increase was concomitant with an increase in the amount of insoluble target protein.

Comparison between complex and defined nitrogen sources have also been made and a third category included, that of semi-defined medium, a compromise between the two extremes (Tabandeh et al., 2004). This work showed that in terms of overall growth potential, the semi-defined medium ranks, unsurprisingly, between complex (the highest) and defined (the

lowest). There was no data presented for the recombinant protein productivity of the semi-defined medium, however, more than twice the amount of human growth hormone was produced in complex medium compared with the defined medium.

2.5. Cultivation Techniques

2.5.1. Batch

The simplest of the available cultivation techniques, comprising a one-step process, where all nutrients are supplied to a single inoculum of cells. The standard microbiological shake-flask approach uses a batch method, and, whilst suitable for achieving growth this method is unable to achieve the high-cell densities which are required for biotechnological purposes.

2.5.2. Fed-Batch

Built on the principal of the batch process, the fed-batch begins with an initial batch phase. At a pre-defined point in the process, a feed is pumped into the fermenter, in order to provide additional nutrients to the cells. Fed-batch operation allows the attainment of much higher cell-densities than are possible with batch processes, which in turn enable higher productivities. On top of this, fed-batch processes can control growth rate, limiting the accumulation of growth, or product, inhibitory metabolites (Luli and Strohl, 1990) as a consequence of overflow metabolism. Large arrays of possible

feeding strategies also entail greater potential for process optimisation than is the case with batch processes (Yee and Blanch, 1992; Korz *et al.*, 1995; Belo and Mota, 1998; Kim *et al.*, 2004). The increases in production capacity usually outweigh the increase in process time considerably, such that batch methods are rarely used in preference.

2.5.3. Continuous

Continuous culture is the third possible option for bacterial cultivations. Continuous cultures are operated in such a fashion that a specific parameter is maintained at a constant value. This is achieved, first, by growing the cells to high density, then using a pump to continually remove the contents of the fermenter with replenishment of the same volume of fresh medium. The rate of this replenishment controls the growth rate of the organisms (Jung, 2006). This method has the benefit of constant removal of growth/product limiting metabolites, as well as the ongoing addition of nutrients. This method is most commonly used in research, to provide a steady-state reactor where the deviations from this state can be attributed to experimental perturbations (Villaverde *et al.*, 1993; Aucoin *et al.*, 2006). From a bioprocess standpoint, these reactions also enable the continual removal of product from the reactor, which can avoid detrimental effects associated with product accumulation, such as proteolysis or aggregation. This approach is rarely used in industry due to the long process times, where increasing process time concomitantly increases the risk of failure, with the associated cost.

2.6. Culture Monitoring

2.6.1. Growth

2.6.1.1. OD

Optical density (OD) uses monochromatic light, usually from a white light source and filtered to the desired wavelength, shone through a sample, with the emerging light detected. Measurements can be taken in either transmission or absorbance mode, and for the purposes of biomass estimation, are equivalent and reciprocal. This method assumes a linear correlation between the absorbance of a sample and the number of cells present. This assumption holds as long as the cells in question maintain a constant size throughout the culture.

2.6.1.2. $CFU.mL^{-1}$

Analysis of colony forming units can be used to monitor the growth of a bacterial population. Samples are diluted, spread on agar plates and incubated

for at least 24 hours. From these samples, the number of reproductively viable cells per unit volume can be determined. The requirement for post-sampling proliferation from a single-cell to a visible colony places certain time restrictions on this type of analysis. In order to form a visible colony, a single cell must be incubated for 18-24 hours with the result that

information gathered from this analysis is not immediately available. Its greater utility, therefore, is in the analysis of bacterial propagation under stressful conditions (Section 2.1.5. Stress). This technique must be considered alongside the fact that it is estimated that only 2 % of all known microorganisms can be grown in an artificial environment (Kaeberlein et al., 2002). This would suggest that there would have to be relatively little change in the physiological state of a cell in order to make it non-resuscitatable whilst still being viable as measured by cellular integrity and energy generation.

2.6.1.3. DCW

Dry cell weight measures the quantity of cell mass within a defined volume by sampling, separating cells from broth, and then drying the cells at high temperature (usually between 70 and 100 °C). This method can achieve high levels of accuracy, although accuracy falls with falling numbers of cells and decreasing sample size.

2.6.1.4. Cell Counting

Direct microscopic visualisation of cells, within a defined volume, allows determination of quantity of cells per unit volume. Usually this would need to be repeated 3-4 times to increase data reliability, therefore this method is both labour-intensive and time-consuming. This is the only method for

determining microbial growth listed here which relies on the analysis of single-cells rather than analysis of an averaged sample.

2.6.1.5. Microscopy

2.6.1.5.1. White Light Microscopy

Conventional light microscopy can be allied with specific stains, such as methylene blue to give an indication of the ability of the cells to exclude the dye as a crude measure of cellular metabolism. This method is often carried out in conjunction with manual cell counting, which increases the quantity of information that can be obtained from cell counting although the method is still laborious and time-consuming.

2.6.1.5.2. Fluorescence Microscopy

Fluorescence microscopy obeys many of the same principles of white light microscopy, however the illuminating source is usually filtered to allow only a specific wavelength (or group of wavelengths) to strike the cells. If the cells in the sample are pre-mixed with one or more fluorescent dyes, respondent to the filtered light wavelength, extra information can be gathered relating to the metabolic status of the cell (covered in more detail in Section 2.6.1.6. Multi-Parameter Flow Cytometry). The statistical resolution of this assay is extremely poor with samples measured in the hundreds, however, when used

in conjunction with flow cytometry the cells which are visualised are taken from a subpopulation with similar fluorescence characteristics.

2.6.2. Cell Quality

2.6.2.1. Multi-Parameter Flow Cytometry

Flow cytometry: literally the measuring of cells suspended in a moving fluid. Cells are first mono-dispersed in an isotonic liquid (usually phosphate buffered saline (PBS)) and then forced to flow through a measurement chamber (flow-cell). This stream of cells is hydrodynamically focussed within an outer stream of similar fluid such that the stream of cells forms a single strand within a laminar flow regime. This moving column of single-cells is then intersected with a monochromatic light source (usually a laser, although more than one may be used) at right angles to the direction of flow. As the light strikes the cells it is scattered, with no change in wavelength (elastic scattering), in all directions (Kerker, 1983). This scattered light is then focussed and collected using lenses and optical-electronic interfaces in two different directions:

- Forward Angle Light Scatter
- Right Angle Light Scatter

The combination of these two parameters is characteristic for specific organisms/particles and deviations imply a change in the cells (such as a

change in size/formation of inclusion bodies) (Vives-Rego et al., 2000; Lewis et al., 2004).

In order to analyse parameters of cells in addition to size and intracellular composition, fluorescent dyes can be added to the cell suspension. These chemicals absorb energy from a light source within a defined range of wavelengths, resulting in the promotion of an electron to a higher energy level. As the electron loses energy and returns to its ground-state, it emits light at a wavelength longer than that which excited it, with the difference between these two wavelengths known as Stokes' shift. The fluorescent dyes are chosen according to their excitation and emission peak wavelengths, with the former as close to that of the laser source as possible, whilst the latter should be distinct from all the other dyes used in the mixture.

Most uses of flow cytometry employ some kind of binary live/dead stain, often propidium bromide (ethidium bromide is also useful here, although is partly permeable through the membrane, whereas propidium is not). Propidium is excited, most typically, by a 488 nm (blue-green) laser and emits light around 615 nm (red) (Rault et al., 2007). There is an array of different fluorescent molecules (chemicals, fluorescent antibodies, reporter proteins such as GFP) available for monitoring characteristics such as; intracellular pH, membrane potential and intracellular protein content (Tracy et al., 2010). The fluorescence characteristics can also be used to select specific cells and populations for further study via fluorescence activated cell sorting. By sorting into microwell plates or directly onto solid agar, cells

which have exhibited a specific fluorescence profile can be further cultivated in either solid or liquid medium.

The ability of flow cytometry to analyse very large quantities of cells (up to 10,000 cells per second in some cases) provides for a high degree of statistical reliability on the data obtained from it. If, in addition, sorting is employed then some degree of spatial resolution can also be obtained.

Throughout this thesis, four different fluorescent dyes have been employed for the elucidation of heterogeneous populations:

2.6.2.1.1. Propidium Iodide

Propidium is a membrane impermeant; positively charged DNA chelator. It binds to DNA with a stoichiometry of 1: 4-5 base pairs. Fluorescence is considerably enhanced by binding to DNA, relative to free PI.

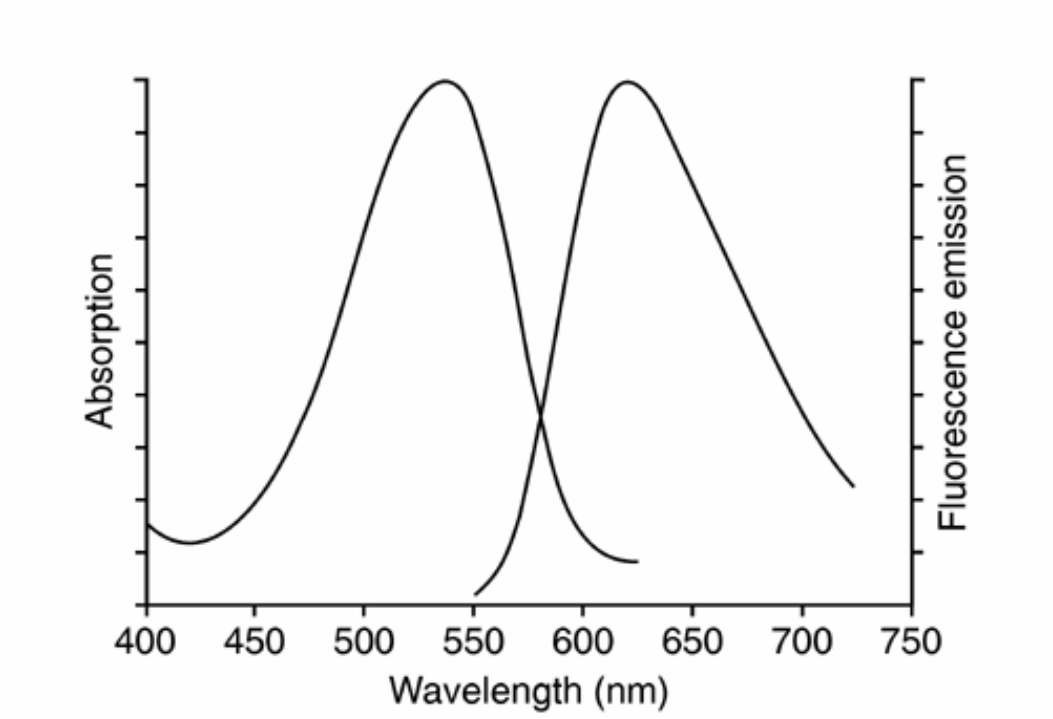

Figure 2.6. Absorption/emission spectrum for propidium iodide from www.probes.com.

2.6.2.1.2. $DiBAC_4(3)$; BOX

Bis-(1,3-dibutylbarbituric acid)trimethine oxonol is a lipophilic, anionic dye, excluded from cells which have a “normal” membrane potential. It freely enters cells as the membrane potential approaches zero.

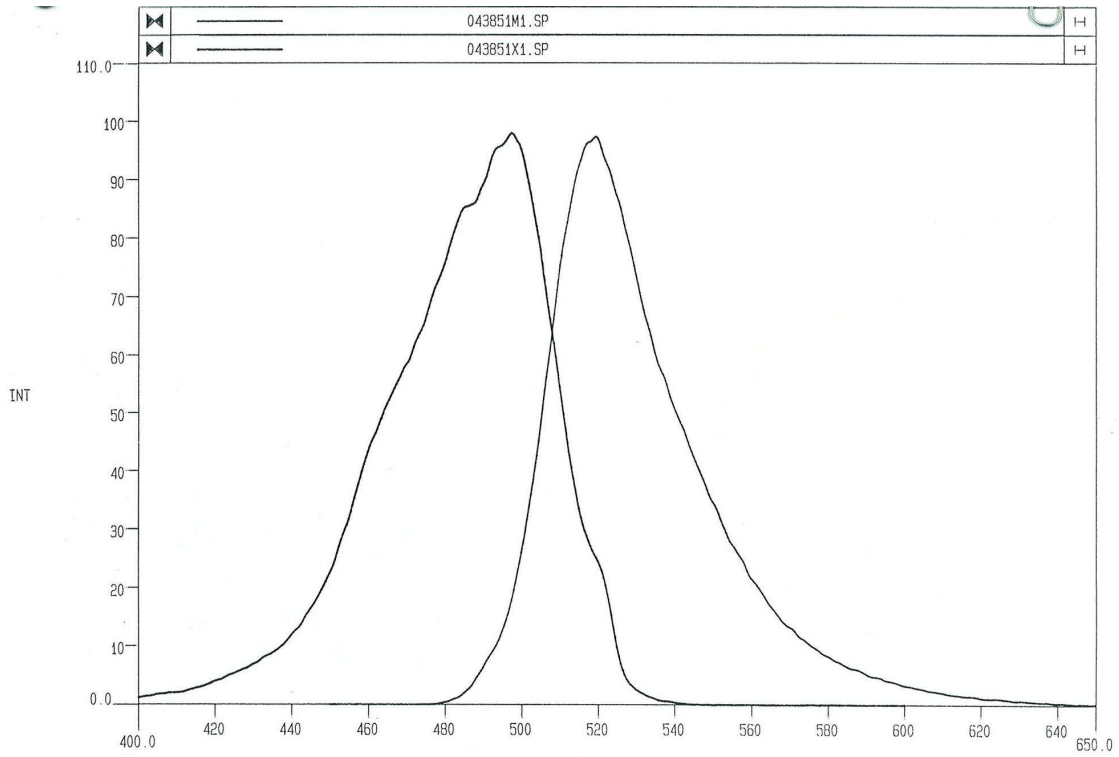

Figure 2.7. Absorption/emission spectrum for $DiBAC_4(3)$ supplied by Molecular Probes (Invitrogen).

2.6.2.1.3. $\underline{DiOC_6(3)}$

3,3′-Dihexyloxacarbocyanine iodide is a lipophilic cationic dye and follows a Nernstian distribution pattern. The ratio of the internal and external concentrations of a membrane permeant, cationic molecule is determined by the resting potential across that membrane.

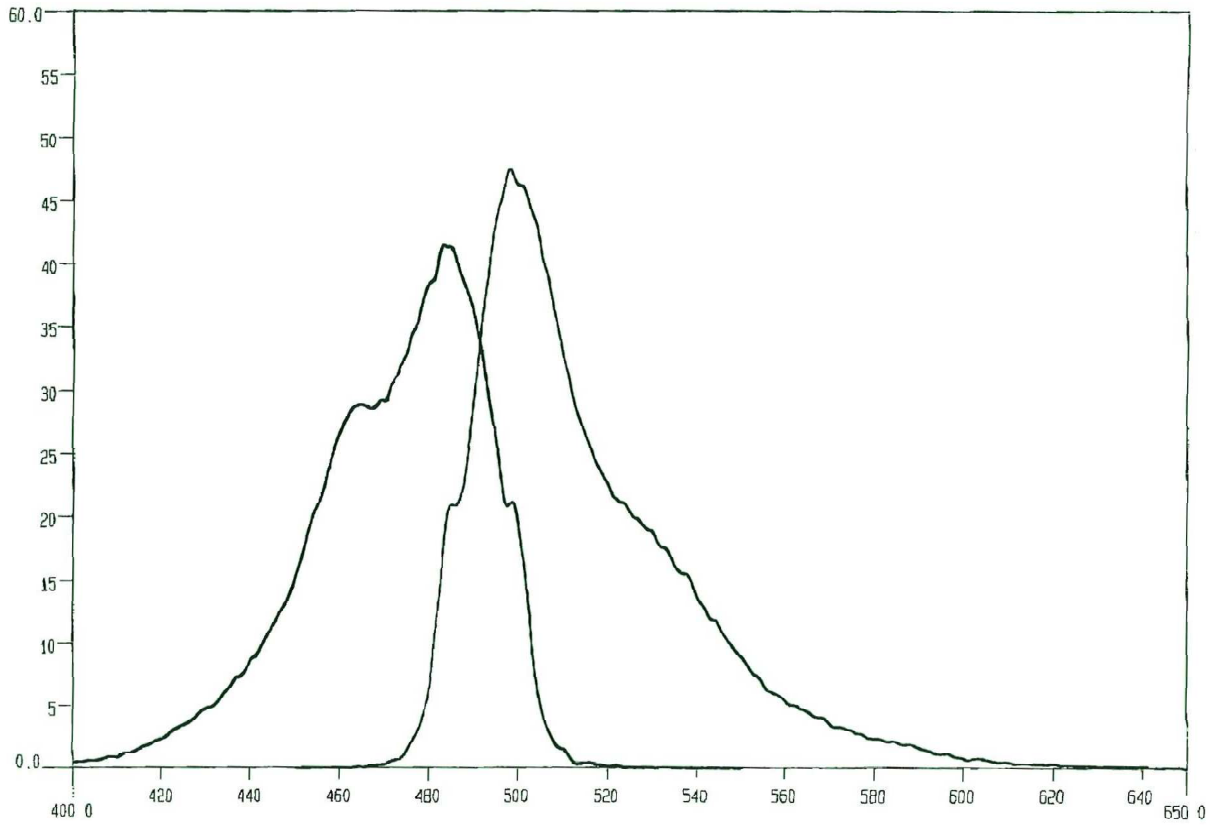

Figure 2.8. Absorption/emission spectrum for $DiOC_6(3)$ supplied by Molecular Probes (Invitrogen)

2.6.2.1.4. RedoxSensor Green™

Supplied by molecular probes, the RedoxSensor Green™ stain exists in two forms: an oxidised, afluorescent molecule and a reduced, fluorescent one. If the dye enters a cell, with a functioning reduction machinery (one capable of generating reducing power in the form of NADH/NADPH/FADH, as well as functional reductase enzymes) the fluorescent form of the molecule is created. The ability of a cell to carry out this process is, in turn, linked to the capacity of a cell to perform central carbon metabolism (Gray *et al.*, 2005). In

its reduced state, the molecule absorbs light in the 488 nm region and emits light at approximately 525 nm.

2.6.2.2. Productivity

2.6.2.2.1. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis is a technique which uses charge, as a surrogate for size, to separate protein molecules. First described by Laemmli (1970), it entails coating of the protein in a detergent, which linearises the peptide chain. This linearization allows the detergent to coat the chain along its length, with the resulting charge of the molecule being congruent with the number of amino acids. This method is limited by the sensitivity of the detection technique, where a non-specific protein stain (Coomassie blue or silver) binds to the proteins in the gel, however it is the standard technique for laboratory detection and quantification of protein species. The correlation of charge with size only holds when there is an approximately even distribution of the different amino acids within the molecule. If a protein with a preponderance of low molecular weight (e.g. glycine) or high molecular weight (e.g. Tyrosine) amino acids is analysed, then the correlation could be unreliable.

2.6.2.2.2. Size Exclusion Chromatography

Size exclusion chromatography is a high pressure liquid chromatography (HPLC) method whereby a protein sample is passed through a porous matrix, where larger molecules will be transmitted sooner than smaller ones, in a manner consistent with differing molecular weights. Unlike SDS-PAGE, a more direct measure of protein mass can be determined with this technique, although the 3D structure of the proteins being analysed can also have a significant effect on this procedure, where it is assumed that the size of the molecule in 3D is strongly correlated with its molecular weight. SEC must, however, be used in conjunction with a purified sample due to a necessity for low loading volumes (around 10 % of the column volume at any one time) and so is not as immediately applicable method as SDS-PAGE.

2.6.2.2.3. Western Blotting

Western blotting is a method for increasing the sensitivity of SDS-PAGE. An antibody, specific for the protein of interest is conjugated to an enzyme capable of catalysing an easily detectable reaction (usually conversion of a coloured substrate to a different colour). The protein is transferred from the SDS-PAGE gel to a nitrocellulose membrane, again driven by charge. This membrane is then treated with the conjugated antibody, following which, the enzyme substrate is added for a defined incubation period. After this period has elapsed, the reaction is stopped and a visible band can be seen where the protein of interest lies. The weakness of this technique is that it requires

a number of processes prior to the end measurement, whose efficiency will all impact the final reading.

2.6.2.2.4. Enzyme-Linked Immunosorbent Assay (ELISA)

An ELISA uses the same principle as the Western Blot, detailed above, but is usually carried out in a microwell plate format, using serial dilutions of samples. This difference in technique allows for more accurate quantitation of the amount of product present than is capable with SDS-PAGE, when compared with a known concentration of reference material. The major weakness of the technique is its reliance on serial dilutions, which are prone to error

2.6.2.2.5. Quantitative Polymerase Chain Reaction (qPCR)

Quantitative polymerase chain reaction, also encompassing quantitative reverse transcriptase polymerase chain reaction is a method by which the quantity of DNA in a sample can be determined by the use of a DNA binding fluorophore (Bustin, 2000).

When qPCR and reverse transcriptase are used in tandem, it is possible to quantify specific mRNA sequences of interest, recombinant products, for example. As a technique, qPCR is generally more commonly applied to mammalian and higher cell analysis (Lattenmayer *et al.*, 2007) with this author only identifying one investigation targeting bacterial fermentation

processes, and this was focussed on determination of plasmid copy number (Skulj *et al.*, 2008).

Employment of qPCR for the analysis of transcription rates under different fermentation conditions could give significant insight into any disjunct between expression of the completed recombinant product and the transcription of the genes prior to and following induction.

2.7. Aims and Objectives

Bacteria are still the default choice for the production of recombinant biomolecules, despite the wealth of alternatives, due to their rapid growth rate, and low-cost process. It is clear that *E. coli*-based manufacturing methods are currently more suitable for some products than others. In order to improve the processes by which biologics are made, a deeper understanding of the product and its effect on the organism must be obtained. This can be achieved by the application of niche analysis techniques, such as flow cytometry, which allow rapid, statistically-reliable, detection of bacterial stress as a consequence of protein overproduction.

The purpose of this work is as follows:

- To increase the variety of staining procedures available for determination of stress on bacterial cells by flow cytometry.

- To employ extant, and newly-developed, staining protocols for the analysis of recombinant *E. coli* fermentations to produce antibody fragments; a poorly understood industrial process.
- Use the information gained, regarding the ability of cells to withstand the production environment, to make alterations to the protocol in order to maximise productivity.
- Monitor the new process using flow cytometry, to detect early signs of stress, which precede cellular disruption, resulting from the foreign protein expression.

3. Materials and Methods

3.1. Chemicals

Table 3.1 Chemicals List	
Chemical	Supplier
Nutrient Agar	Oxoid, Hampshire, UK
Nutrient Broth	Oxoid, Hampshire, UK
Peptone	Difco (BD, NJ, USA)
Yeast Extract	Difco (BD, NJ, USA)
NaCl	Sigma-Aldrich, MO, USA
$(NH_4)_2SO_4$	Sigma-Aldrich, MO, USA
Glycerol	Sigma-Aldrich, MO, USA
KH_2PO_4	Sigma-Aldrich, MO, USA
K_2HPO_4	Sigma-Aldrich, MO, USA
Citric Acid	Sigma-Aldrich, MO, USA
$MgSO_4$	Sigma-Aldrich, MO, USA
$CaCl_2$	Sigma-Aldrich, MO, USA
Tetracycline	Sigma-Aldrich, MO, USA
Polypropylene Glycol 2000 (PPG 2000)	Sigma-Aldrich, MO, USA
$FeSO_4.7H_2O$	Sigma-Aldrich, MO, USA
$ZnSO_4.7H_2O$	Sigma-Aldrich, MO, USA
$MnSo4.H_2O$	Sigma-Aldrich, MO, USA
$Na_2MoO_4.2H_2O$	Sigma-Aldrich, MO, USA
$CuSO_4.5H_2O$	Sigma-Aldrich, MO, USA
H_3BO_3	Sigma-Aldrich, MO, USA
Conc. H_3PO_4	Fisher Scientific, MA, USA
NaOH	Sigma-Aldrich, MO, USA
NH_4OH	Fisher Scientific, MA, USA
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Melford Laboratories, Suffolk, UK
Phosphate Buffered Saline (PBS)	Oxoid, Hampshire, UK
Coulter Clenz™	Beckman Coulter, CA, USA
Isoton II	Beckman Coulter, CA, USA
Propidium Iodide	Invitrogen, CA, USA
Bis-(1,3-dibarbituric acid)-trimethine oxanol ($DiBAC_4(3)$)	Invitrogen, CA, USA
3,3'-Dihexyloxacarbocyanine iodide ($DiOC_6(3)$)	Invitrogen, CA, USA
RedoxSensor Green™	Invitrogen, CA, USA
TrisHCl	Sigma-Aldrich, MO, USA
Trizma	Sigma-Aldrich, MO, USA
Tetrasodium ethylenediaminetetra-acetate (EDTA)	Sigma-Aldrich, MO, USA

Sucrose	Sigma-Aldrich, MO, USA
Na_2CO_3	Sigma-Aldrich, MO, USA
$NaHCO_3$	Sigma-Aldrich, MO, USA
Lysozyme	Sigma-Aldrich, MO, USA
Bovine Serum Albumin (BSA)	Sigma-Aldrich, MO, USA
Goat anti-human Fab peroxidase conjugate	Sigma-Aldrich, MO, USA
KPL peroxidase substrate	KPL, MD, USA
pH 4 standard solution	Sigma-Aldrich, MO, USA
pH 7 standard solution	Sigma-Aldrich, MO, USA
Glutaraldehyde	Sigma-Aldrich, MO, USA
PBS	Sigma-Aldrich, MO, USA
Osmium Tetroxide	Sigma-Aldrich, MO, USA
Ethanol	Fisher Scientific, MA, USA
Propylene oxide	Sigma-Aldrich, MO, USA
Uranyl Acetate	Sigma-Aldrich, MO, USA
Reynold's Lead citrate	Sigma-Aldrich, MO, USA

3.2. Consumables

Table 3.2. Consumables

Item	Supplier
Microcentrifuge tube (1.5 mL)	Triple Red, Bucks, UK
Centrifuge tube (50 mL)	Nunc, NY, USA
Microwell plate (96 well)	Sarstedt,Nürnbrecht, Germany
Constricted Cuvette (2 mL)	Sartorius, Göttingen, Germany
Minisart (0.2 µm)	Sartorius, Göttingen, Germany
Nitrocellulose Filter (0.2 µm)	Sartorius, Göttingen, Germany
Midisart filter (0.2 µm)	Sartorius, Göttingen, Germany
Sartobran-PH 0.2 µm	Sartorius, Göttingen, Germany

3.3. Equipment

Table 3.3 Equipment List

Equipment	Manufacturer
Shaker Incubator	Gallenkamp, Germany
FerMac 310/60 Fermentation Controller	Electrolab, Gloucestershire, UK
Electrolab Fermentation Manager Lite software	Electrolab, Gloucestershire, UK
Dissolved Oxygen (DO) probe	Broadley Technologies, CA, USA
pH probe	Broadley Technologies, CA, USA

Uvikon 922 Spectrophotometer	Kontron Instruments, Eching, Germany
5415D Microcentrifuge	Eppendorf, Hamburg, Germany
C422 Centrifuge	Jouan, France
pH meter	Hanna Instruments, RI, USA
EPICS ELITE Flow Cytometer	Beckman Coulter, CA, USA
Coulter Electronics Elite software	Beckman Coulter, CA, USA
Windows Multiple Document Interface (WinMDI) v. 2.8	Available from http://facs.scripps.edu
Microwell plate shaker/incubator	Infors-HT, Bottmingen, Switzerland
Multiskan MS plate reader	Thermo Fisher Scientific, MA, USA
Ortholux II	Leitz, Wetzlar, Germany
Camera	Nikon, Japan
Ultracut E microtome	Reichert-Jung, NY, USA
1200 EX Electron Microscope	Jeol, Tokyo, Japan

All water used was filtered by a Millipore ultrafiltration unit (Millipore, MA, USA). All materials, for which it was suitable, were sterilised by autoclaving at 121 °C for 30 minutes. Materials unable to be autoclaved were filtered through 0.20 μm Minisart filter as indicated in 3.2. Consumables. All chemical concentrations expressed are final values in the relevant solution. All flask cultures were grown in an orbital shaker incubator with a 2.5 x 5 cm throw.

3.4. Culture Techniques

3.4.1. Organisms

3.4.1.1. *Bacillus cereus*

Bacillus cereus NCTC 11143 was obtained from the National Collection of Type Cultures (NCTC, London, UK) (Part of the United Kingdom National Culture Collection).

3.4.1.2. *Escherichia coli*

E. coli W3110 (F^-; λ^-; IN(rrnD-rrnE)1; rph^{-1}) was obtained from the American Type Culture Collection (ATCC, Virginia, USA).

E. coli W3110::pAVE046 was obtained from Avecia Biologics Ltd. pAVE046 was derived from pZT7#2.0 (Kara *et al.*, 2003) and described as *E. coli* CLD048 in WO/2007/088371 (Hodgson et al., 2006).

3.4.2. Medium

Nutrient agar was used as the non-selective solid medium for the cultivation of both *B. cereus* and *E. coli*.

3.4.2.1. *Bacillus cereus*

B. cereus was grown in nutrient broth as liquid medium, for both inoculum and experimental cultures.

3.4.2.2. *Escherichia coli*

E. coli starter cultures were grown using modified lysogeny broth (LB) (Bertani, 1951) (5 % peptone; 10 % yeast extract; 10 % NaCl, all (w/v)).

Stirred-tank reactor cultures of *E. coli* were grown in the medium shown in Table 3.4.

Table 3.4. Fermentation medium composition	
Medium Component	Concentration
$(NH4)_2SO_4$	14 $g.L^{-1}$
Glycerol	35 $g.L^{-1}$
Yeast Extract	20 $g.L^{-1}$
KH_2PO_4	2 $g.L^{-1}$
K_2HPO_4	16.5 $g.L^{-1}$
Citric Acid	7.5 $g.L^{-1}$
1 M $MgSO_4$	10 $mL.L^{-1}$
1 M $CaCl_2$	2 $mL.L^{-1}$
Trace Element Solution	34 $mL.L^{-1}$
Tetracycline	15 $\mu g.mL^{-1}$

The shaded portion of Table 3.4. indicates where solutions were added, aseptically, after sterilisation and cooling of the medium. For stirred-tank reactor fermentations 0.66 $mL.L^{-1}$ of Polypropylene glycol 2000 (PPG) was added to the medium, as antifoam, prior to sterilization. In order to return the pH to 7 prior to fermentation, ammonium hydroxide (35 % w/v) was added.

Table 3.5. Trace element solution composition	
Trace Element Component	Concentration
$FeSO_4.7H_2O$	3.36 $g.L^{-1}$
$ZnSO_4.7H_2O$	0.84 $g.L^{-1}$
$MnSO_4.H_2O$	0.51 $g.L^{-1}$
$Na_2MoO_4.2H_2O$	0.25 $g.L^{-1}$
$CuSO_4.5H_2O$	0.12 $g.L^{-1}$
H_3BO_3	0.36 $g.L^{-1}$
Conc. H_3PO_4	48 $mL.L^{-1}$

3.4.3. Shake Flask

3.4.3.1. *Bacillus cereus*

Cultures were performed using 500 mL Ehrlenmeyer flasks, each with four baffles placed at 90° intervals. A liquid volume of 50 mL nutrient broth was sterilised inside each of the flasks, with 2 flasks for the inoculum and 24 for the experimental culture. An agar plate was prepared with *B. cereus* seeded from a glycerol stock, using a sterile loop, and incubated overnight (16 ± 4 h at 37 ± 1 °C). Single colonies were then picked, again with a sterile loop, and used to inoculate two flasks which were incubated overnight (14 ± 2 h; 37 ± 1 °C; 200 RPM). The contents of the flasks were visually assessed for growth, and the contents of one flask were used to inoculate the 24 experimental flasks (2 % v/v). The contents of the second flask were assayed for optical density. The experimental flasks were placed into a shaking incubator (37 ± 1 °C; 200 RPM). Sampling was performed every 1-2 hours via the sacrifice of two flasks per time point. Each flask was analysed as described in Section 3.5. Methods.

3.4.3.2. *Escherichia coli*

Cultures were performed using 500 mL Ehrlenmeyer flasks, each with four baffles placed at 90° intervals. A liquid volume of 50 mL LB was sterilised inside two flasks for the inoculum. After cooling, and immediately prior to commencement of the cultivation, 15 $\mu g.mL^{-1}$ tetracycline was added to each

flask. The 24 experimental culture flasks had 50 mL of the medium described in Table 3.4, sterilised *in situ*, with the post sterilisation additions (Table 3.4 and Table 3.5.) made, aseptically, after cooling of the medium. For the preparation of the inoculum, 0.1 % (v/v) of a glycerol stock was thawed and added aseptically, and incubated overnight (14 ± 0.5 h at 37 ±1 °C; 200 RPM). The contents of the flasks were visually assessed for growth, and the contents of one flask were used to inoculate the 24 experimental flasks (2 % v/v). The contents of the second flask were assayed for optical density. The experimental flasks were placed into a shaking incubator (37 ± 1 °C; 200 RPM). Sampling was performed every 1-2 hours via the sacrifice of two flasks per time point. Antibody production was induced using 0.1 mM IPTG. Each flask was analysed as described in Section 3.5. Analysis Techniques.

3.4.4. Stirred-tank reactor

Fermentations were carried out in a 5 L cylindrical bioreactor, with dimensions as indicated in Figure 3.1.

Table 3.6. Fermentation parameters

Parameter	Set Point
Agitation (RPM)	1000 ± 50
Temperature (°C)	37.0 ± 0.2
pH	7.00 ± 0.1
Air flow rate (vvm)	1.0 ± 0.1

The fermenter was equipped with two 6-bladed, paddle-type, impellers. Four baffles were positioned around the edge of the reactor. The aerated liquid height was, at all times below the level of the baffles. Oxygenation of the

medium was performed using compressed air through a 0.2 μm filter (Midisart) and sparged beneath the lower impeller. Water vapour in the off-gas was condensed, and the air filtered with a Sartobran capsule. Temperature was maintained at 37 °C throughout each of the fermentations. The fermenter was filled with 3 L of culture medium (Table 3.4.) and autoclaved. Immediately prior to the fermentation, the post-sterilisation additions (Table 3.4. and Table 3.5.) were filter-sterilised (Minisart) and added to the fermenter. The pH of the medium was adjusted to 7.0 using 35 % (w/v) ammonium hydroxide. Antibody production was induced using 0.1 mM IPTG.

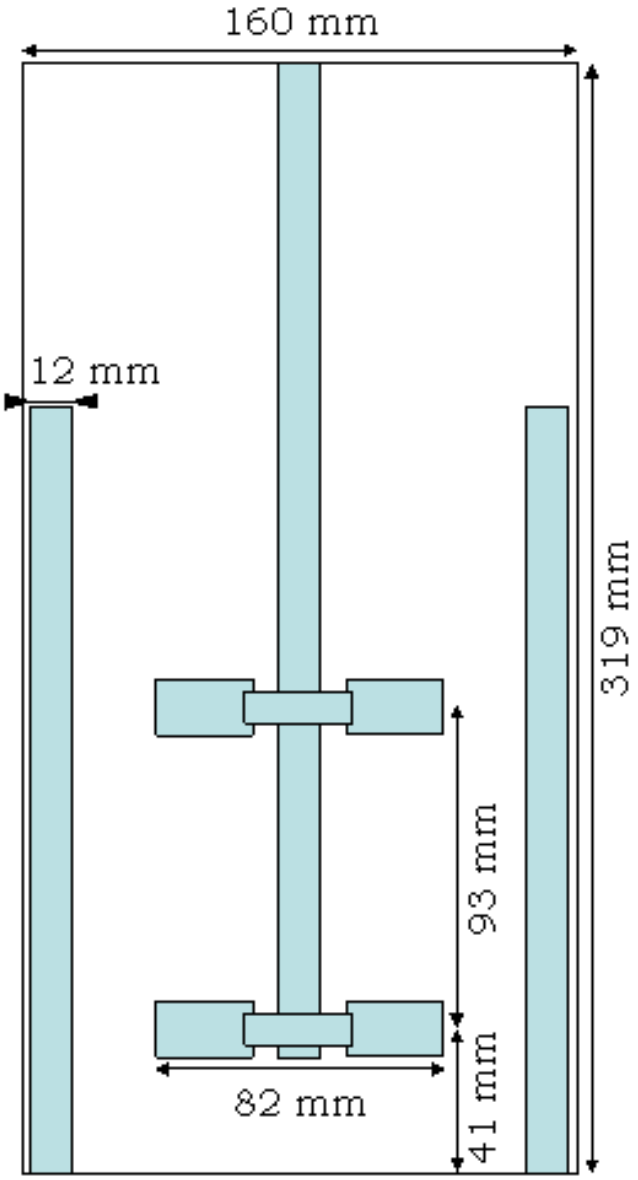

Figure 3.1 Fermenter Geometry. Scale 1:2

3.4.4.1. Dissolved Oxygen & pH

Dissolved oxygen (DO) was monitored by means of a polarographic probe. The probe was cleaned, sterilised and calibrated prior to use. pH was measured using a suitable probe and adjusted by adding 35 % (w/v) ammonium hydroxide via a computer controlled pump.

3.4.4.2. Inoculum

A LB medium (120 mL) was sterilised within a baffled 500 mL Ehrlenmeyer flask, with a sealed side arm port for connection to the fermenter. For *E. coli* CLD048 cultivations, 15 $\mu g.mL^{-1}$ tetracycline was added after cooling, before addition of 100 µL of glycerol stock. For cultivations of *E. coli* W3110 antibiotic was not added. The inoculum was then incubated overnight (14 ± 0.5 h; 37 ± 1 °C; 200 RPM) before being added to the fermenter.

3.4.4.3. Batch Fermentation

Batch fermentations were carried out as described with 30-40 mL samples being taken, aseptically, at 1-2 hour intervals and submitted to appropriate testing for growth, product formation and cell viability.

3.4.4.4. Fed-Batch Fermentations

Fed-batch fermentations were carried out using the same process as the batch fermentations in the first instance, with samples taken every two hours. As the dissolved oxygen measured in the fermenter began to rise, the feedstock was added at a rate of 45 $mL.h^{-1}$. The feed was supplied via a manually activated pump, external to the bioreactor controller, operated according to Avecia's standard protocol at 11 $g(glycerol).L^{-1}.min^{-1}$ with

approximately constant volume over the course of the fermentation, due to sample taking, and evaporation.

Table 3.7. Feed Composition	
Component	Concentration ($g.L^{-1}$)
Glycerol	714
Magnesium Sulfate (1 M)	30 $mL.L^{-1}$

3.5. Analysis Techniques

3.5.1. Optical Density (OD)

Measurements were taken at 580 nm and 600 nm for *B. cereus* and *E. coli* respectively. All samples were measured in the range 0-0.6, using serial 1:10 dilutions with PBS where necessary, with actual OD values determined by calculation.

3.5.2. Dry Cell Weight (DCW)

3.5.2.1. Shake Flask

3.5.2.1.1. *Bacillus cereus*

First, a filtration method was used to determine DCW, using pre-weighed 0.2 μm filters (Nitrocellulose) inserted into a capsule. A defined volume was syringed through the filter and the filters were dried out in an oven at 100 ± 5 °C overnight until a constant weight was achieved. Initially, the

applied volume was used to determine DCW, however, this was changed to use of the permeate volume due to leakage of liquid following filter blocking at high cell-densities. The latter was more accurate (as can be seen from a comparison of the error bars in Figures 4.3. and 4.15.); however, subsequently a simpler, alternative method was found. This method was then used the *E. coli* analysis and is described below in; 3.5.2.1.2 *Escherichia coli.* More realistic correlations between optical density and dry cell weight were yielded, based on comparisons with literature values.

3.5.2.1.2. *Escherichia coli*

Between six and eight 1 mL samples were taken from the flask and each transferred to a pre-dried, pre-weighed microcentrifuge tube. The samples were then spun down (16,100 g; 5 minutes; ambient temperature), after which the supernatant was discarded and the tubes placed in an oven at 100 ± 5 °C overnight until they achieved a constant weight. Concurrently, 8 empty tubes were also placed in the oven for the same time period and the average weight-loss from these tubes determined. This value was then used to correct for any weight loss in the experimental tubes.

3.5.2.2. Stirred-tank Reactor

From the sample removed from the fermenter, 20 mL was taken and divided equally between two pre-weighed 50 mL centrifuge tubes. These tubes were then centrifuged (3,260 g; 10 minutes; ambient temperature), the

supernatant discarded and the tubes dried in the oven at 100 ± 5 °C overnight until constant weight was achieved. In addition, 8 empty, pre-weighed, tubes were oven-dried over the same time period, and the average weight-loss determined. This value was then used to compensate for the weight-loss experienced by the experimental tubes.

3.5.3. pH measurement

pH was measured off-line for shake-flask cultures and online for fermenters.

3.5.4. Colony Forming Units (CFU.mL^{-1})

Samples were taken and diluted into PBS using 10-fold serial dilutions, 100 µL of three consecutive dilutions were spread onto nutrient agar plates using a sterile spreader. Each dilution was analysed in duplicate, and placed into a 25 °C incubator for 72 h or 37 °C overnight. All colonies were counted on plates containing fewer than 400 colonies; plates with more than 400 colonies were discarded.

3.5.5. Multi-Parameter Flow Cytometry

3.5.5.1. Analysis

Prior to analysis, samples were diluted to at least 1:2000 in PBS, such that the flow cytometer was operating in the region of 1-1500 cells.s^{-1} and a total

of 25,000 cells were counted from the gated region of the forward scatter/side scatter plot. Dyes were used at the working concentrations in table 3.8.

Table 3.8. Fluorescent dyes used and final concentrations

Dye	Conc. *B. cereus* ($\mu g.mL^{-1}$)	Conc. *E. coli* ($\mu g.mL^{-1}$)
Propidium Iodide (PI)	0.30	3.03
Bis-(1,3-dibarbituric acid)-trimethine oxanol ($DiBAC_4(3)$; BOX; bis-oxonol)	0.30	0.61
3,3'-Dihexyloxacarbocyanine, iodide ($DiOC_6(3)$)	0.30	3.03
RedoxSensor Green™	3.03 µM	N/A

The working concentrations for PI and BOX were arrived at based on previous experience in the laboratory with these fluorophores (Lewis et al., 2004; Lopes-Silva et al., 2005). The other dyes were applied to the cellular systems to be tested at varying concentrations and incubation periods until the results achieved were in agreement with the established PI/BOX system for both healthy and heat-treated control cells.

Cells were analysed immediately following addition of PI/$DiBAC_4$ and PI/RedoxSensor Green™. *B. cereus* cultures were also analysed immediately following addition of PI/$DiOC_6(3)$, however, *E. coli* cultures were incubated for 30 seconds prior to analysis. Propidium iodide was used in all dye staining protocols in conjunction with one of the other dyes.

Where appropriate, samples were subjected to flow-activated cell sorting (FACS) based on their fluorescence characteristics, and the resulting samples

were further analysed either by spotting onto agar plates and incubation (25 °C; 24 h) or by visualization by fluorescence microscopy (Section 3.5.8. Fluorescence Microscopy).

3.5.6. Osmotic Shock Procedure

Table 3.9. Composition of Osmotic Shock Solutions	
Solution Name	Composition
Osmotic Shock Solution 1 (OS1)	TrisHCl 0.39 $g.L^{-1}$
	Trizma 2.64 $g.L^{-1}$
	Tetrasodium EDTA 1.04 $g.L^{-1}$
	Sucrose 200 $g.L^{-1}$
Osmotic Shock Solution 2 (OS2)	TrisHCl 0.39 $g.L^{-1}$
	Trizma 2.64 $g.L^{-1}$
	Tetrasodium EDTA 1.04 $g.L^{-1}$

Samples were diluted to $OD_{600nm} \approx 5$, then centrifuged (16,100 g; 2 minutes; ambient temperature). The supernatant was transferred to -20 °C and the pellet resuspended in 1 mL of OS1. The resulting suspension was incubated (4 °C; 10 minutes), before being centrifuged (16,100 g; 2 minutes; ambient temperature). The OS1 supernatant was stored at -20 °C and the pellet resuspended in OS2 and incubated at 4 °C for 10 minutes. The OS2 suspension was centrifuged (16,100 g; 2 minutes; ambient temperature), and the supernatant and pellet frozen separately at -20 °C.

3.5.7. ELISA

Table 3.10. Composition of ELISA Solutions	
Solution	Components
Coating buffer	Na_2CO_3 1.59 $g.L^{-1}$
	$NaHCO_3$ 2.93 $g.L^{-1}$
	pH 9.6
Coating with Lysozyme	Coating buffer
	Lysozyme 1 $g.L^{-1}$
Blocking buffer	BSA (Bovine Serum Albumin) 0.1 $g.L^{-1}$ in PBS
Washing buffer	Tween 20 1 $mL.L^{-1}$ in PBS
Detection antibody	Goat anti-human Fab peroxidase conjugate (Sigma A0293) 2 µL
	20 mL blocking buffer
Substrate	Peroxidase substrate kit as per manufacturer's instructions
Phosphoric Acid	1 M Phosphoric acid

Coating buffer with lysozyme was added to a microwell plate (120 µL per well) and incubated overnight at 4 °C. Coating buffer with lysozyme was replaced with 200 µL blocking buffer and incubated with shaking (37 °C; 500 RPM; 1 h).

A dilution plate with 180 µL of neat sample in each well in one row, and 120 µL of blocking buffer added to the remaining wells. Serial dilutions were performed, transferring 60 µL per well down the plate.

After the incubation, the first plate was washed with 3 x 300 µL washing buffer. The diluted samples were then transferred from the dilution plate to the coated plate, 100 µL per well.

The coated plate was incubated (37 °C; 500 RPM; 1 h) then washed with 3 x 300 µL washing buffer. 100 µL detection antibody was added to each well and incubated (37 °C; 500 RPM; 1 h).

The coated plate was washed once more (3 x 300 µL washing buffer) and 100 µL peroxidase substrate solution added to each well, incubated at room temperature for 10 minutes.

The reaction was stopped by the addition of 100 µL of phosphoric acid and the plate analysed by absorbance at 450 nm. Antibody concentrations were determined following construction of a standard curve based on a known concentration of the antibody (Figure 3.2.), purified from fed-batch fermentation by protein G chromatography and ultrafiltration, concentration established by BCA assay.

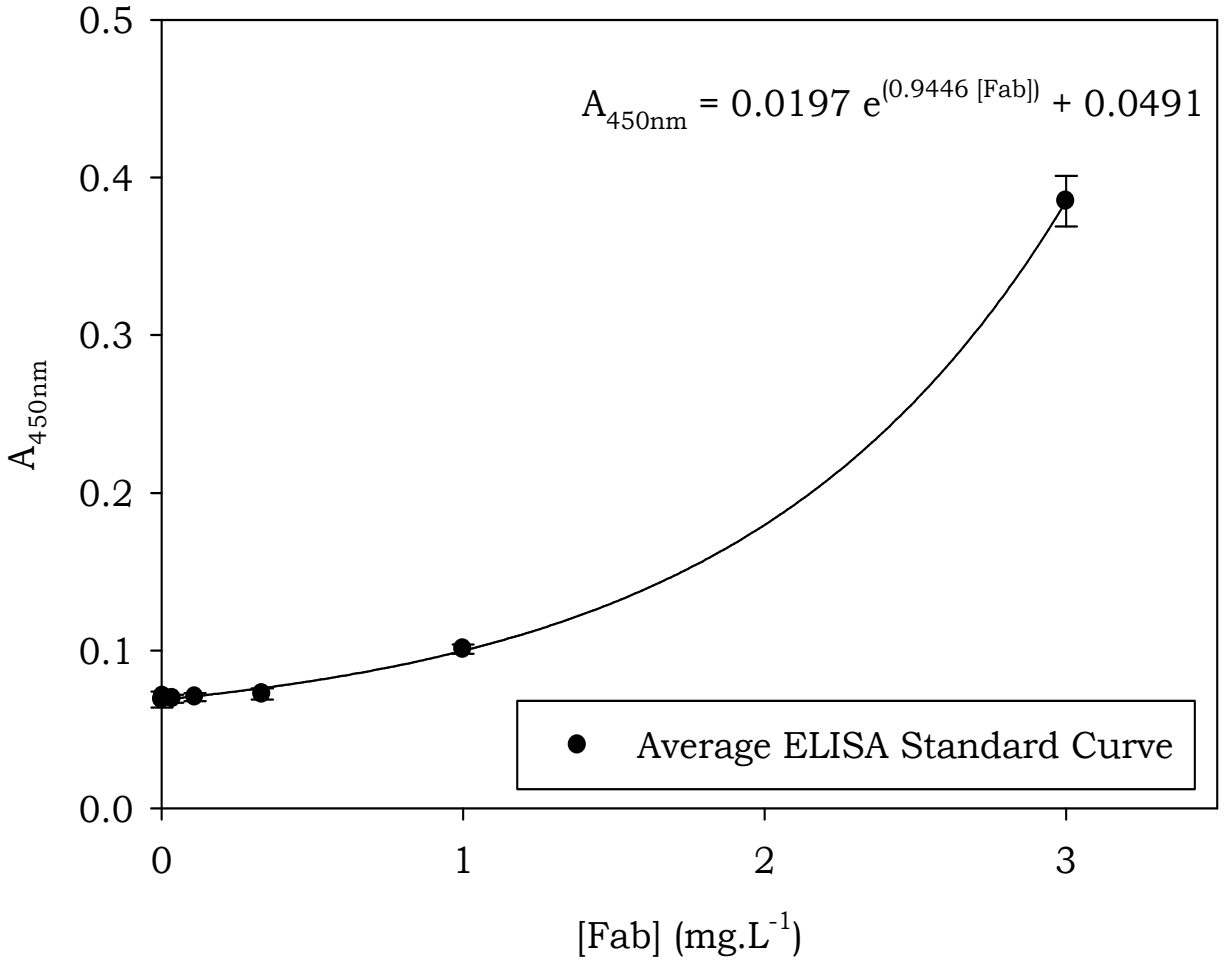

Figure 3.2. ELISA calibration curve. The points plotted here are average values taken from two separately performed ELISAs. The curve was fitted using Sigmaplot v. 10.0.

3.5.8. Fluorescence Microscopy

Two thousand cells were sorted, according to specific fluorescence parameters, onto a filter membrane. The cells were then covered with aluminium foil prior to observation, where they were illuminated with a mercury lamp, and the wavelength filtered to 488 nm. Images were recorded through a 40x objective lens.

3.5.9. Transmission Electron Microscopy

Samples were taken and spun in a microcentrifuge (16,100 g; 2 minutes; ambient temperature), after which the supernatant was aspirated and discarded. The cells were then resuspended in 2.5 % glutaraldehyde in 0.1 PBS pH 7.2, and placed into the fridge for a maximum of 2 days. They were then passed onto the University of Birmingham Centre for Electron Microscopy where they were dehydrated using ethanol. The sample was then embedded in a 1:1 mixture of polypropylene oxide and araldite resin and left to polymerise. Ultra-thin sections (70 nm) were cut using a Reichart-Jung Ultracut E microtome and stained with uranyl acetate and Reynold's lead citrate.

4. Results & Discussion

4.1. *B. cereus* Shake-Flask Fermentation

Shake flask cultures are used in a wide variety of biological research environments, and are the most basic tool to control and monitor the growth and behaviour of microorganisms. These experiments are usually performed as a sacrificial culture where a large number of flasks are initially seeded and at each time point where analysis is required, one or, usually, more flasks are removed and subjected to testing. The assumptions governing this type of experiment are that the flasks are homogeneous, relative to each other, at the beginning of the experiment, and that they are given the same conditions throughout. At such a small scale, the assumption of equal treatment of each flask holds due to rapid mass and heat transfer within, and without, the culture entailing low flask-flask variation (Solomons, 1969).

The ultimate aim of the work presented here is to arrive at a greater understanding of an *E. coli* fermentation process. The first step to approach this was an evaluation of new flow cytometric staining protocols compatible with the analysis of Gram positive organisms in a model system. For this reason, a number of shake-flask cultures were carried out using *Bacillus cereus*. This particular strain of *B. cereus* is non-sporulating, and it was originally recovered from a patient's emesis. As a result of this, its behaviour under stress is of particular interest, as well as the potential for exploitation

as a model for the development of flow cytometric dye combinations for use with other Gram positive organisms.

In the course of this work, reference will be made to three broad cell states informed by the measurement of fluorescence with the various dyes used. A complete explanation of the mechanism of action of the dyes used in this thesis is included in 2.6.1.6.1-4. The first condition of note is that of "healthy" cells, these are generally stained green with $DiOC_6(3)$ and unstained with $DiBAC_4(3)$ (also referred to as bis-oxonol, or BOX) due to their ability to maintain their membrane potential, and lacking red fluorescence indicating their ability to exclude PI. The measurement of membrane potential as an indicator of viability of bacteria is well established and has been used extensively (Nebe-von-Caron *et al.*, 2000; Shapiro, 2001). The converse condition is that of "unhealthy" cells, which have for whatever reason lost the ability to maintain sufficient membrane potential to exclude $DiBAC_4(3)$ (or include $DiOC_6(3)$). The flow cytometer can measure the presence or absence respectively of these cells and inferences can be made about the overall condition of the cells. The final major cell-state of interest is the dead, intact cell where the contents of the cell are still contained within the membrane, but the cell has lost all ability to segregate the internal environment from the external. If there is a hole in the membrane big enough for PI to pass through, but small enough to still contain the genome, PI will penetrate the cell (by diffusion), intercalate into the DNA and fluoresce red.

The first experiment that was performed was a brief evaluation of the difference between using baffled and unbaffled flasks. Baffled flasks provide the vessels with increased capacity for mass transfer, which should increase the growth rate of the cells within (Gupta and Rao, 2003; Galindo *et al.*, 2004). This increased growth rate may accentuate the effect of any stresses present, which would amplify the signal. These results (Figure 4.1.) show a superficial analysis comparing the optical density and pH of the growing cultures.

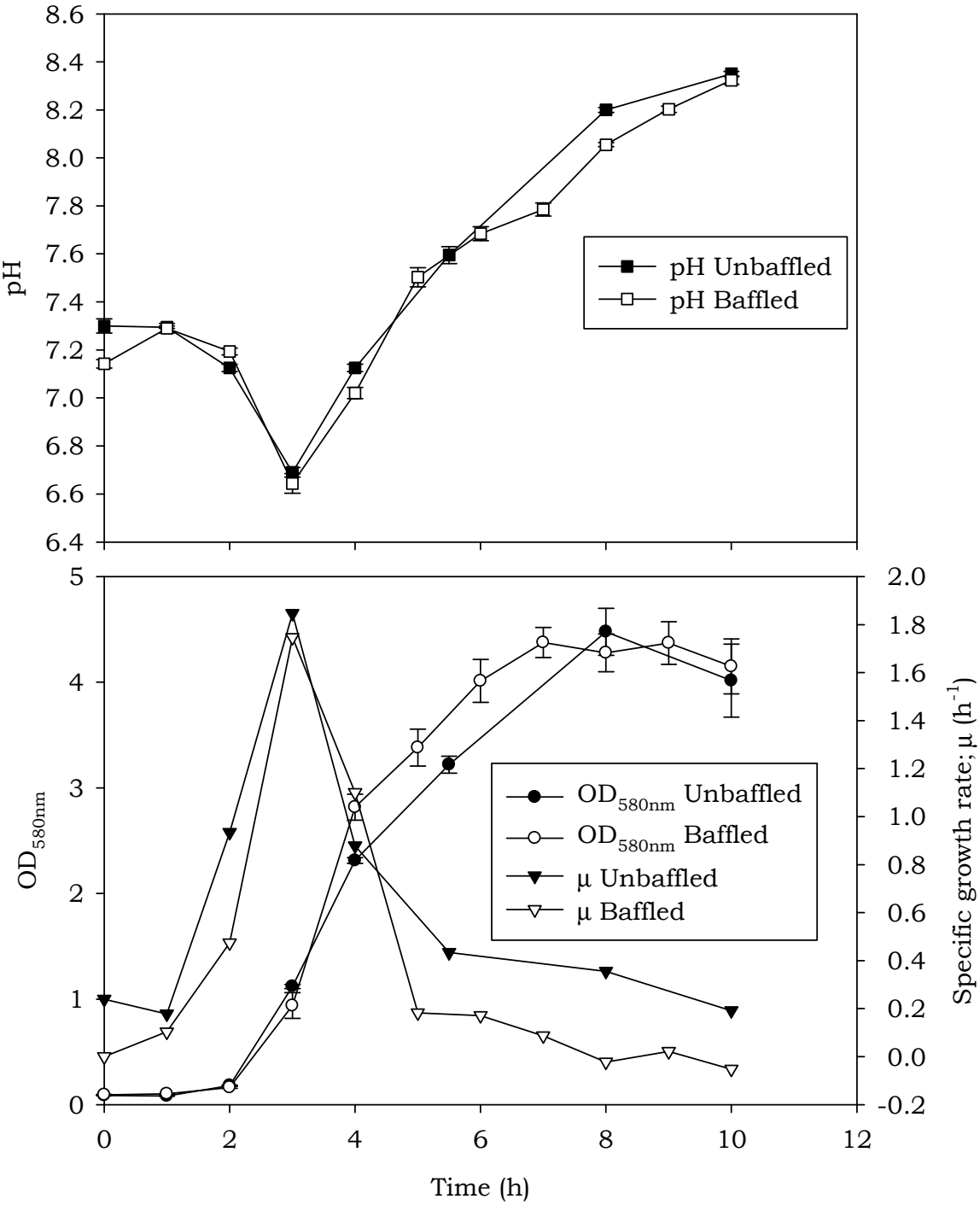

Figure 4.1. Comparison of growth of *B. cereus* in baffled and unbaffled shake-flask fermentations (n = 1). All points plotted are mean values of 2-4 replicates from a single pair of experiments. Error bars show the standard deviation.

The data in Figure 4.1. seems to indicate that, in this particular case, the baffles have provided no discernible advantage in terms of growth rate or final achieved turbidity. The variation in pH appears consistent when

comparing the baffled and unbaffled flasks, reflecting a similar rate of nutrient consumption; with both cultures experiencing a switchover to gluconeogenic metabolism at the same time. Interestingly, the growth rate of the baffled flask was lower than that of the unbaffled for all but one of the time points. Between 3 and 4 hours, in the mid-exponential phase of growth, the baffled flasks achieved a higher growth rate than the unbaffled (1.0 h^{-1} vs 0.8 h^{-1}). This is just after the peak of the growth rates, and suggests that there may be a minimal effect whereby the specific growth rate can be maintained at a higher level for longer as a result of the baffles. The limited observation of this effect might be attributable to the nutritive quality of the medium. Even if oxygen transfer was increased by the baffles, a metabolic bottleneck could have developed in the processes which supply material to the electron transport chain, limiting the potential growth rate of the culture.

Figure 4.2. shows typical growth characteristics associated with cultivation of bacteria in shake-flask culture; a short lag phase, followed by a rapid period of growth indicated by both the OD_{580nm} and $CFU.mL^{-1}$ curves. The OD_{580nm} curve is then marked by a deceleration, plateau and slight decline after 24 hours in culture (reaching the peak optical density between 7 and 10 hours after inoculation). The lack of a high energy-providing component to the medium, such as glucose or glycerol, significantly limits the productivity of the fermentation, achieving a maximum OD_{580nm} of < 5. In concert with this, the pH drops from approximately 7 to 6.5, probably as a result of build up of acidic by-products of metabolism of an undefined carbon source in the medium, before rising again after only 3 hours. The exception to this is

shake-flask culture 3, indicated by the closed square, where the minimum pH is achieved after 4 h in culture. This reinforces the reliability of this simple method as an indirect measure of metabolism, because it coincides with a protracted lag phase, as measured by optical density.

The progression of pH variation is likely caused, firstly, by utilisation of the aforementioned acidic by-products to supply energy once whatever preferred sources had been consumed. Secondly, catabolism of proteinaceous compounds to yield ATP-generating compounds results in the release of ammonium, which would significantly raise the pH (Morita, 1957). This explanation seems reasonable given the make-up of the medium in which the cells are growing consisting of three different kinds of complex media (peptone, yeast extract and “Lab Lemco” powder) with no single, defined, carbon source and an abundance of hydrolysed proteins.

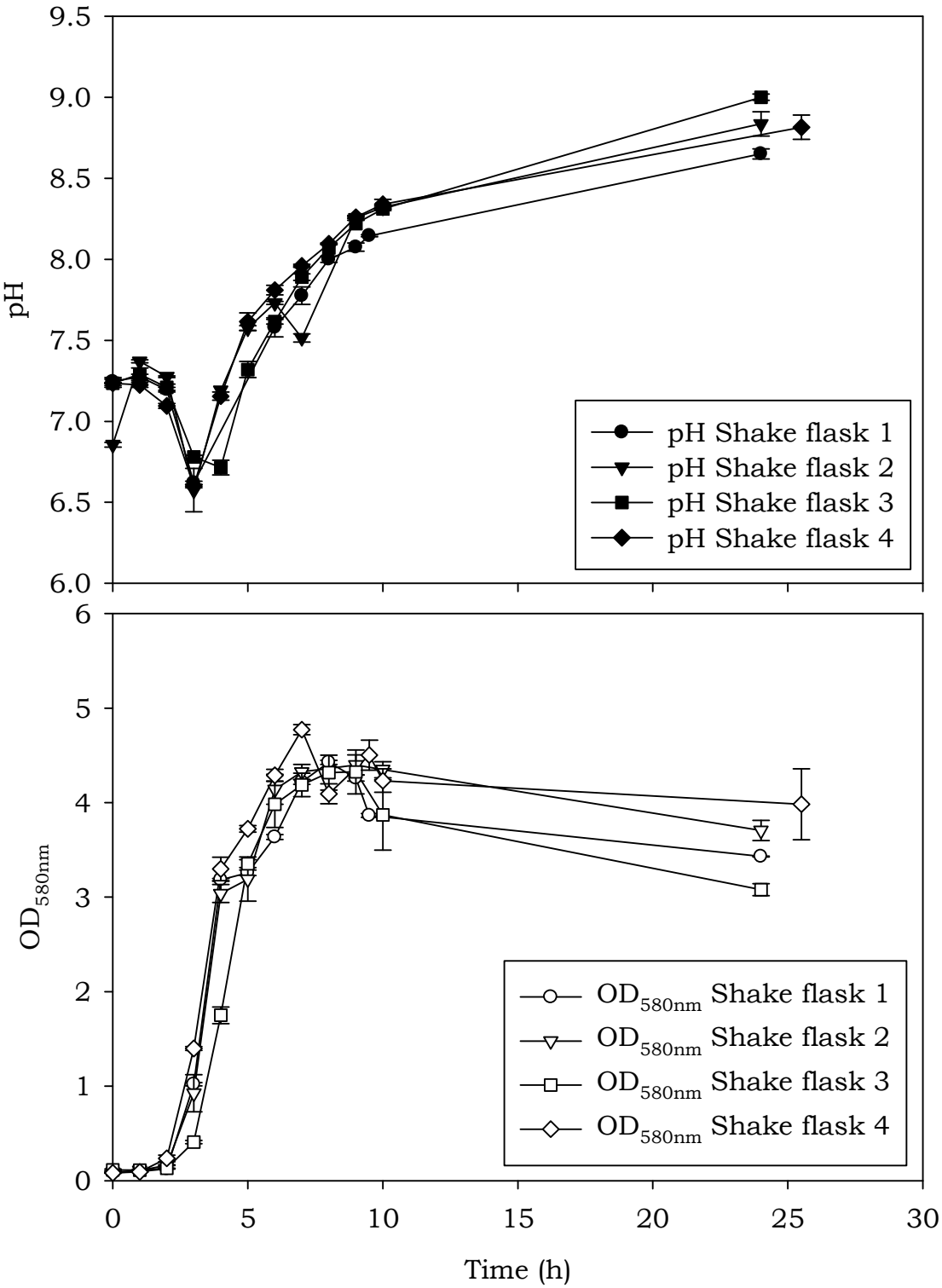

Figure 4.2. *B. cereus* shake-flask fermentation (n = 4). All points plotted are mean values from replicates within each experiment. The error bars show the standard deviation.

The comparison of OD_{580nm} and dry cell weight in Figure 4.3. below shows that the correlations established here are consistent with others in the

literature, although are on the low-end of the scale (Tännler et al., 2008; Balagurunathan and Jayaraman, 2008) with values in the range of 0.3-0.6 more common. The two fitted curves in Figure 4.3. correspond with two different methods of dry cell weight analysis. The first method, signified by the open circles used a filtration-based method which was prone to overloading of the filter with sample, leading to blockage before all of the sample could be passed. The second method, indicated by the open, inverted, triangle was more consistent, as measured by the higher r^2 value. This new method employed centrifugation of samples, which enabled the analysis of more replicates per sample, giving less variation among the samples from any given time point. The combination of these factors lead to the discarding of the filtration method in favour of the centrifugation for the remainder of this thesis.

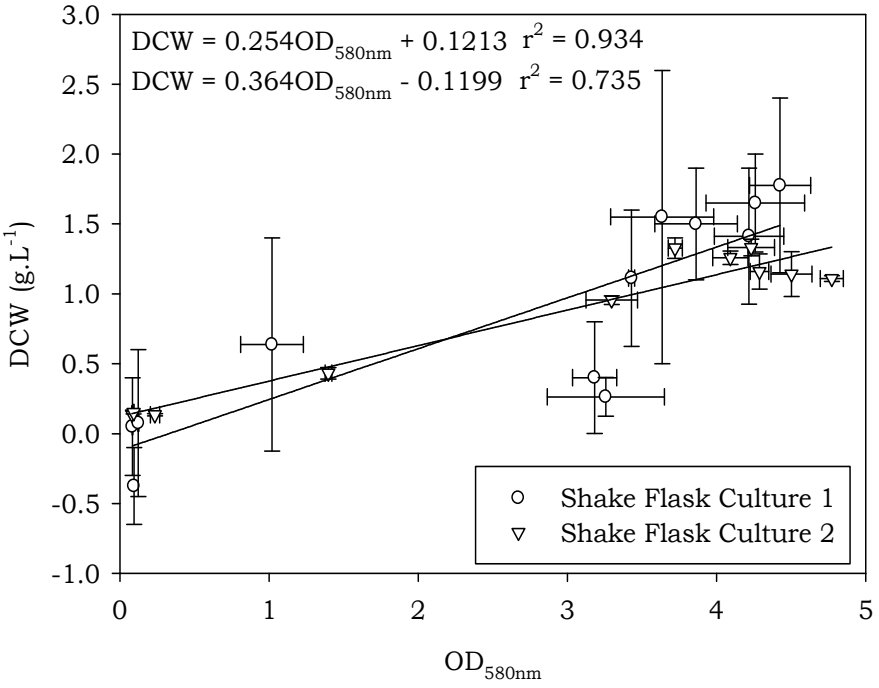

Figure 4.3. OD_{580nm} vs DCW with r^2 value for *B. cereus* shake-flask fermentation (n = 2). The data plotted mean values of 2-4 replicates within each experiment. The error bars indicate the standard deviation.

Figure 4.4 shows the variation of colony forming units with time for the *B. cereus* shake-flask cultures. It can be seen that the same generally increasing trend is repeated here, as with the optical densitometry; the corollary being that the increase in turbidity and mass are as a result of the increase in number of cells. The CFU analysis shows an increase in cells from approximately 10^6 to 10^8, a 100-fold increase, which appears inconsistent with the magnitude of the observed changes in turbidity and cell mass. Assuming that the total observed dry mass is distributed amongst the constituent cells equally, and that there is no significant change in cell size during the shake-flask culture, one might expect the linear relationship between OD and DCW to extend to the CFU analysis. It is estimated that the mass of a bacterial cell is approximately 1×10^{-12} g (Davis, 1973), which would mean that, for the range of optical densities recorded here, $CFU.mL^{-1}$ could be expected to vary from 10^8 - 5×10^8. The apparent underestimation of cell numbers by this technique, at least, in the early stages of the culture could reflect the difficulty of cultivating any bacterium *in vitro* (Kaeberlein et al., 2002). This apparent viable but non-culturable state (Oliver, 2005) may not be the only reason for this disparity, for instance there could be a wide distribution of cell sizes or masses, although neither of these is particularly likely given the lack of available evidence that either of these parameters varies significantly during a bacterial growth cycle over such a short period of culture.

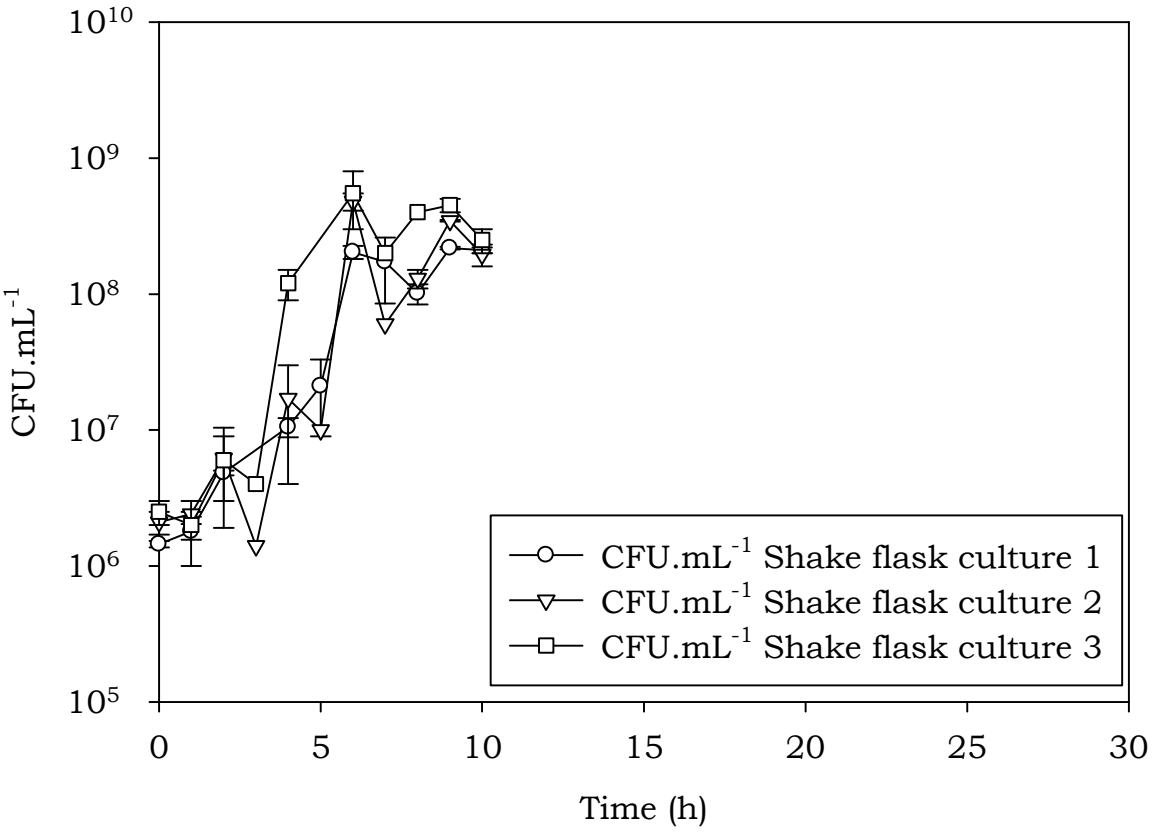

Figure 4.4. B. cereus CFU analysis. The data plotted show 3 experiments and each point is a mean value of 4-6 replicates. The error bars indicate the standard deviation of the data.

The flow cytometer is capable of detecting subtle changes in cell size and intracellular composition by detecting the incident light scattering characteristics as seen in Figure 4.5. In this case, it seems that there is no significant change in cell size over the course of the culture, from mid-exponential culture (3-4 h), peak optical density (7-8 h) through to the declined 24 h sample. The consistency of the position of these populations throughout the culture indicates that, along with consistent size (as demonstrated by forward scatter (FS Log)), there is no detectable change in the intracellular composition, as would be detected by right-angle scatter on PMT1 Log.

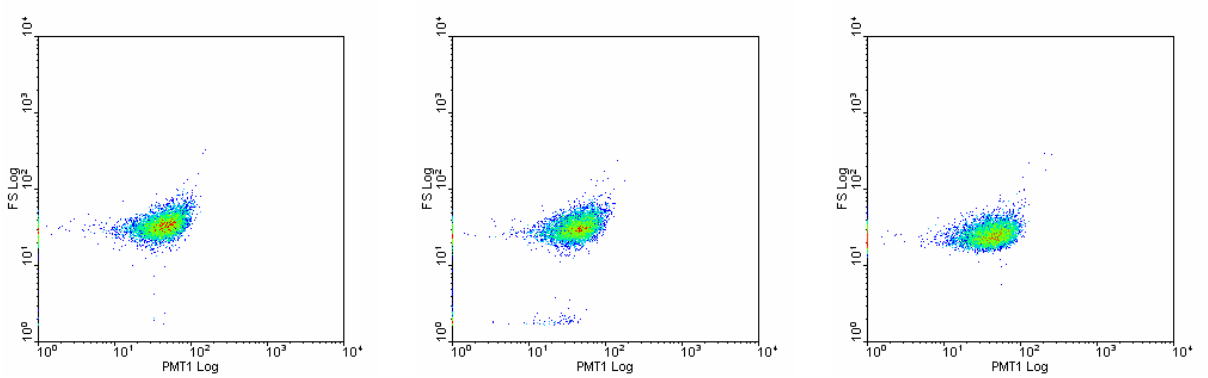

Figure 4.5. Flow cytometric analysis of forward scatter (FS Log) and side scatter (PMT1 Log) for *B.* cereus at 3; 7 and 24 h post-inoculation

It appears that the most likely explanation for this mismatch between the different methods' assessments of cell number may be the existence of viable but non-culturable cells. Normally, when this term is applied, it is used in reference to a drop in observed viability (Oliver, 2005), rather it appears that in this instance there is a large underestimate of the number of cells in the early stages of the culture. During the early stages of growth, the cells adapt their metabolism to grow rapidly in the liquid medium environment; it may be that the different demands of metabolism for growth on solid agar prevent the cells from forming observable colonies.

The use of PI/$DiBAC_4(3)$ for differentially staining healthy (unstained), unhealthy ($DiBAC_4(3)$ positive; Green fluorescent) and dead (PI and $DiBAC_4(3)$ positive; concurrent green and red fluorescence) cells is well established for use with Gram negative organisms, such as *E. coli* (Hewitt et al., 1999; Onyeaka et al., 2003; Lewis et al., 2004). Density plots, of the kind in Figure 4.4. are the simplest method of representing multiparameter flow cytometric data of the kind generated in this work. The plot indicates the fluorescence parameters of a particular cell with, in this case, a single blue dot whose X

value is red fluorescence and Y value is green fluorescence. The dot plot shows the accumulation of 25,000 data points, and, where multiple cells are detected with coincident fluorescence characteristics the colours transition from green > yellow > red for each additional cell. The gating method is designed to bisect specific populations such that they can be quantified. The plot is divided into quadrants, the divisions of which are drawn such that the lines are found at the lowest point of fluorescence intensity between two populations.

Overall, a highly heterogeneous starting material is indicated by Figure 4.4. exhibiting significant green fluorescence (almost 75% of the cells), indicating an abnormal membrane potential.

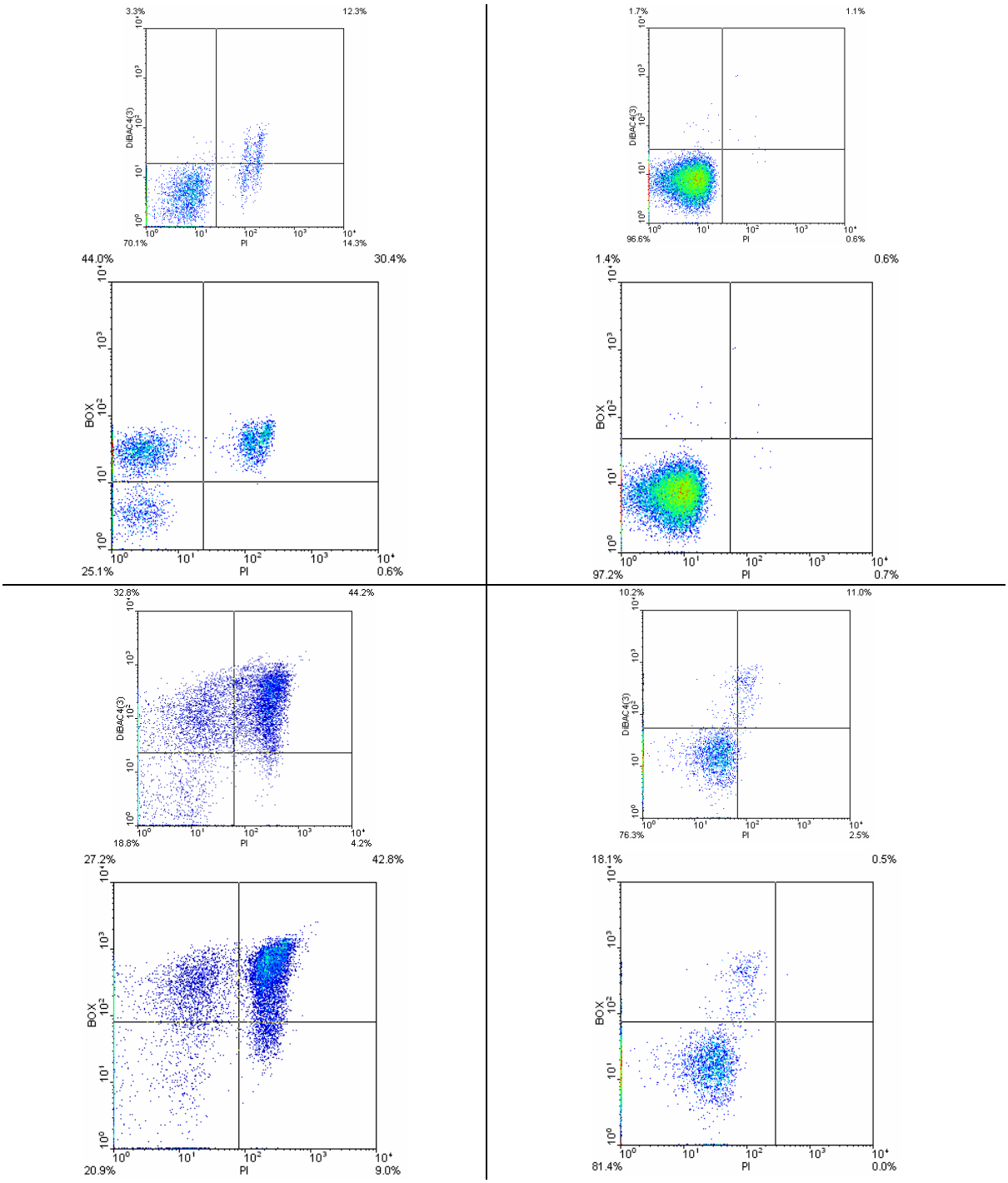

Figure 4.6. Flow cytometric analysis of the *B. cereus* shake-flask culture in Figure 4.2. stained with PI & BOX. Each quadrant shows plots from two different experiments. Clockwise, from top-left: Inoculum, 4 hours, 9 hours and 24 hours.

Between two and four hours following inoculation however, the proportion of cells able to exclude the two dyes is > 95 %, evidence that the previously

observed fluorescent cells have either lysed, become diluted out, or recovered, the latter only in the case of solely green fluorescent cells.

Sampling of the cells in stationary phase, around 9 hours after inoculation, shows the number of green fluorescent cells has increased again to approximately 18 %. Given the changes in metabolism undergone during this time, and the previously demonstrated widespread fluorescence after 16 hours in culture, it was expected that the cells would transition towards a higher level of fluorescence (both green, and eventually red/green).

After 24 hours it can be seen that there are non-fluorescent (21 %), green and red/green fluorescent cells but distinguishing discrete populations of fluorescent cells has become an entirely arbitrary process. All that can be concretely determined is that around 52 % of the cells are PI positive, where there is still good resolution, but the same cannot be said for $DiBAC_4(3)$ positive populations.

Thus, for most of the bacterial culture described here, the PI/$DiBAC_4(3)$ protocol can be used to analyse Gram positive organisms, but it is likely that a more robust staining procedure can be found which is able to achieve clear discrimination under all growth conditions.

With this in mind, it was decided to evaluate the possibility of using $DiOC_6(3)$ as an alternative. The use of $DiOC_6(3)$ is already well established in the measurement of mitochondrial membrane potential in eukaryotic organisms

(Ko *et al.*, 2007; Maftoum-Costa *et al.*, 2008), but its use in the analysis of bacterial cells is far more limited to only a handful of investigations (Ratinaud and Revidon, 1996; Lopes-Silva et al., 2005; Reis et al., 2005).

The same cultures as those analysed in Figure 4.6. were also stained with PI and $DiOC_6(3)$ with the data shown in Figure 4.7.

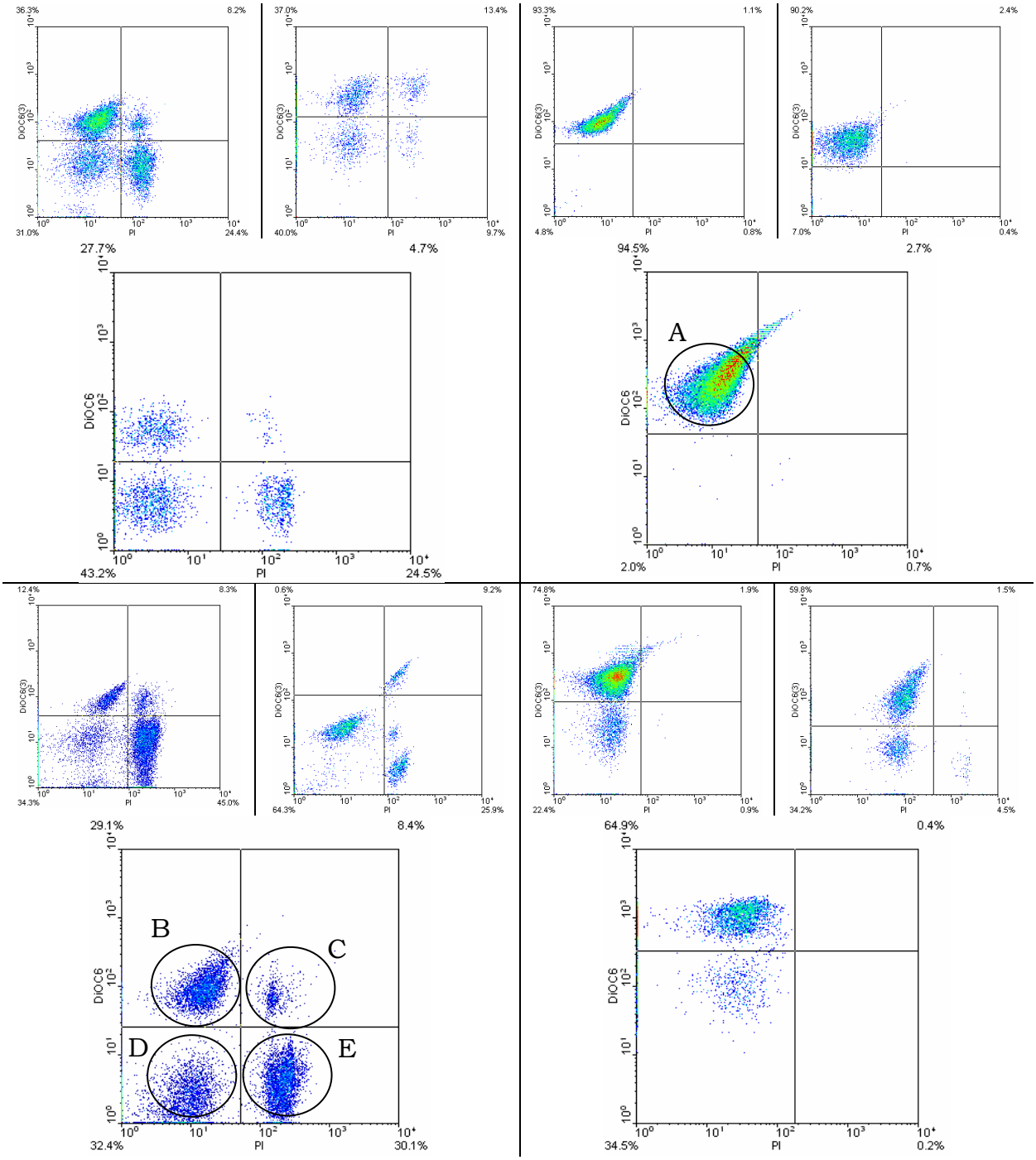

Figure 4.7. Flow cytometric analysis of the *B. cereus* shake-flask culture in Figure 4.2. stained with PI & $DiOC_6(3)$. Each quadrant shows plots from two different cultures. Clockwise, from top-left: Inoculum, 4 h, 9 h and 24 h.. Populations A-E were subjected to cell sorting as shown in Figure 4.8.

When the population sizes of the $DiBAC_4(3)$ and $DiOC_6(3)$ are compared as in Table 4.1. it can be seen that as far as the non-PI positive populations there is good agreement between the two methods:

Table 4.1. Amalgamation of the flow cytometry plots from the inoculum of Figures 4.4. and 4.5 (upper left quadrant, largest plot). The proportion of cells from each quadrant in those figures is listed below as a percentage of the total. The background colours have been chosen to demonstrate the colour of that specific population as recorded by the fluorescence detectors in the flow cytometer.

(%)	PI		Total
$DiBAC_4(3)$	44.0	30.4	100
	25.1	0.5*	
$DiOC_6(3)$	27.7	4.6	100
	43.2	24.5	

The two green fluorescent dyes react to the cells in different ways; $DiBAC_4(3)$ is unable to penetrate the membrane of a healthy cell, therefore cells with a normal membrane potential do not exhibit any green fluorescence. $DiOC_6(3)$ works in the opposite manner, enabling detection of green fluorescence from cells with a "normal" membrane potential. Table 4.1. briefly shows a comparison of the cultures used for inocula prior to experimentation with the

displayed percentages of the whole population attributed to particular staining characteristics. The non-PI stained populations are in broad agreement over the proportion of healthy and unhealthy cells (unhealthy being those cells unable to generate a normal physiological membrane potential (around -100 to -200 mV)) (Shapiro, 2000) with the amounts being within approximately 1.5 %. When this is compared with the PI population, however, there is a disparity between the measurements of nearly 3 times this amount, and, in fact when the respective quadrants of the dot plot are compared, there is no visible evidence of a single population for $DiBAC_4(3)$ in this region. The flow cytometry data indicates that 0.5 % of the cells are solely PI positive (indicated by a * in Table 4.1.), however on closer inspection of the relevant quadrant, there is no visible population suggesting that this may be experimental error, attributable to electronic noise. Given this fact, it is probable that all of the flow cytometry data presented here is subject to this same margin of error. In order to properly mitigate this, two populations would only be considered significantly different if there was more than 10% deviation between them.

The remainder of the plots in Figure 4.7. show a similar progression as seen in Figure 4.6., from an initially heterogeneous culture, through homogeneity (in terms of membrane potential) and back to a highly heterogeneous culture after 24 hours. The proportion of cells which exhibit both red and green fluorescence simultaneously at the 24 hour time point is greater than in the inoculum, but only by a few percent. This apparent limitation on the size of this population may be indicative of a short lifetime of the particular

phenomenon generating this dual fluorescence. This population is of particular interest due to the known action of $DiOC_6(3)$ and PI, whereby PI stains cells whose membrane integrity has failed whilst $DiOC_6(3)$ fluorescence is usually only associated with cells capable of generating a normal membrane potential. This unusual population was also discovered during previous work with *B. licheniformis* (Reis et al., 2005; Lopes-Silva et al., 2005) however, they were unable to demonstrate why these cells were simultaneously absorbing both stains. For this reason, these populations were subjected to fluorescence activated cell sorting onto agar plates for further determination of post-sampling growth potential of specific populations and visualisation using a fluorescence microscope.

The top-most image in Figure 4.8. (U) shows cells sorted from mid-exponential growth phase, in order to demonstrate that the sorting process itself has no impact on the ability of the cells to grow on the agar.

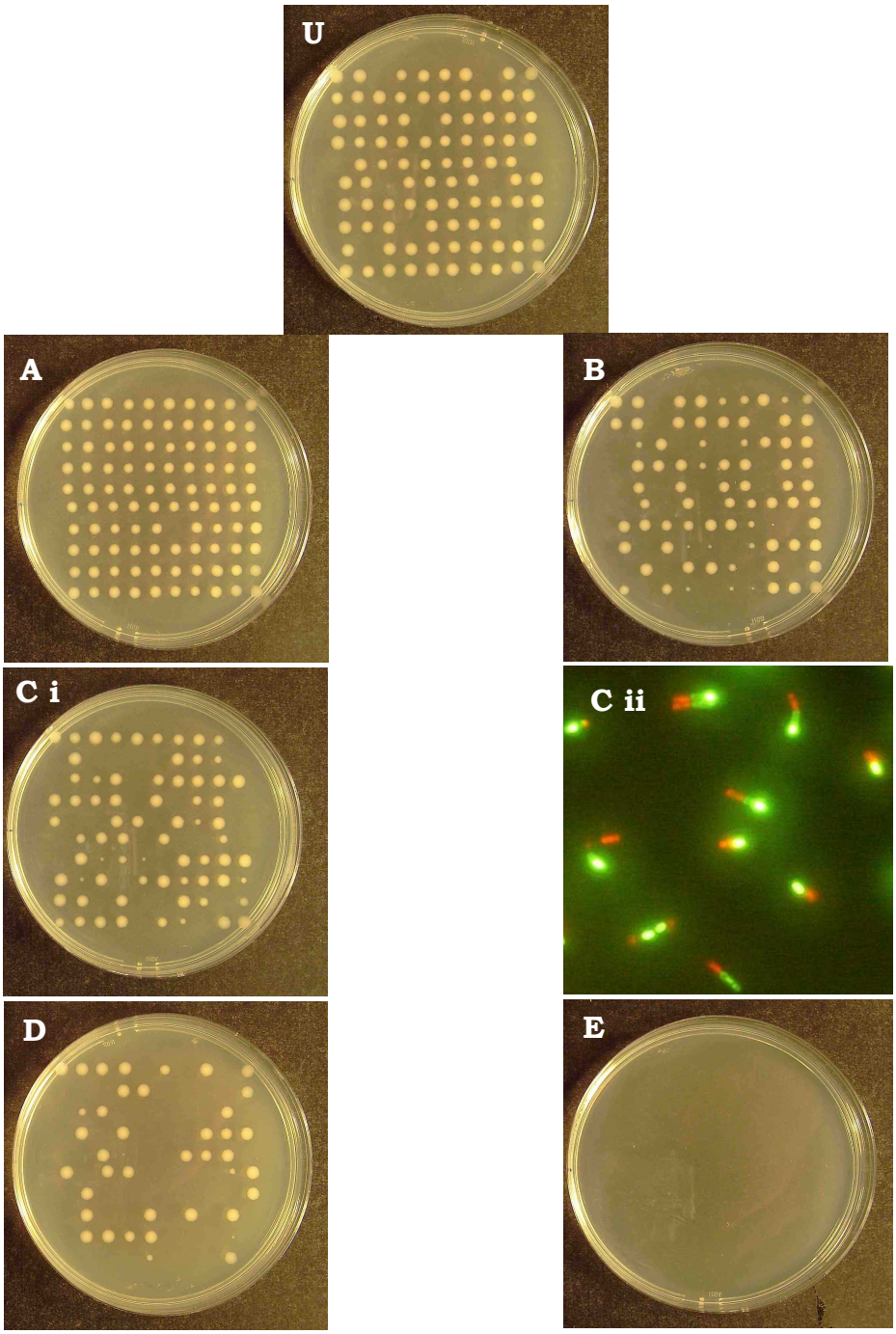

Figure 4.8. Sorted *B. cereus* cells analysed by cultivation on nutrient agar and fluorescence microscopy. Labelled as follows U. Unstained cells. A. Healthy (mid-exponential phase), B, C i, D and E (24 hours after inoculation) $DiOC_6(3)$ & PI stained cells from the populations indicated in Figure 4.7. C ii: Fluorescence microscopy of cells sorted from population C on Figure 4.7. The agar plates shown here are representative of 4-6 for each sample (full breakdown of plate counts shown in Table 4.2.).

Plate A features cells from the same population, with PI and $DiOC_6(3)$ applied at the working concentrations used in the protocol. Plates B, C, D and E correspond to the populations highlighted in Figure 4.5.

Table 4.2. Results of counting colonies on sorted plates from Figure 4.6. after 24 h incubation at 25 °C. The bottom two rows show the mean and standard deviation (SD) of the values.

	U	A	B	C	D
	90	94	72	73	42
	94	97	76	74	40
	90	99	78	70	30
	98	98	68	74	37
	97	100	78	74	39
					39
Mean	93.8	97.6	74.4	73.0	37.8
SD	3.8	2.3	4.3	1.7	4.2

Table 4.3. Results of unpaired student's t-tests on colony number (%) from sorted plates shown in Figure 4.6 and Table 4.2. T-tests performed using Sigmaplot v. 10. If the P value for the data is greater than the threshold, this indicates that the means are significantly different.

Comparison	Mean (n)	P value	P value thresholds
U/A	93.8 (5)/97.6 (5)	0.091	> 0.05
A/B	97.6 (5)/74.4 (5)	0.0000056	< 0.001
A/C	97.6 (5)/73.0 (5)	0.000000058	< 0.001
A/D	97.6 (5)/37.8 (6)	0.00000000040	< 0.001
B/C	74.4 (5)/73.0 (5)	0.52	> 0.05
B/D	74.4 (5)/37.8 (6)	0.000000178	< 0.001

The null hypothesis under which these t-tests were performed was that there is no significant difference between the mean values of the compared populations, with the P value indicating the probability that discarding it would be incorrect. A P value of greater than 0.05 is the most widely used confidence limit and the comparisons of U/A and B/C are the only ones which can be demonstrated to be similar with a high degree of confidence. No

comparisons were made with plate E, which exhibited no growth as would be expected of cells which are unable to maintain their membrane integrity.

Populations A & D were demonstrated to be different, and this is no surprise when the plates and their respective means are analysed. Cells from population A showed almost 2.5-fold recovery in comparison with their counterparts from population D. Both of the populations seem to generate homogeneous colony sizes, although there probably aren't enough individual colonies analysed from population D to make a definitive statement in this regard.

The difference observed between A and B is a little more confounding, given that both of these populations exhibit similar levels of green fluorescence. It is clear from the disparity here that there is more going on than can be detected through this staining protocol and subsequent sorting. It is possible that this result is an artefact of the technique, where cells which have long-adapted for stationary phase culture and the metabolic demands associated with it. These cell's proteome may not transition sufficiently quickly to the alternative requirements for propagation on solid medium leading to underrepresentation of the viable cell number on the nutrient agar despite other signs of a functional metabolism, the so-called viable but non-culturable cell state *(*Xu *et al.*, 1982; Boaretti *et al.*, 2003; Oliver, 2005). The flow cytometric methods employed here only assay very small, specific, areas of cellular physiology. As a result of this analysis, all that can be definitively stated about these two populations is that they have similar membrane

potentials. Populations A and B, as a result of being from markedly different cell growth phases (exponential and stationary, respectively) likely have widely divergent internal biochemistries (Cohen *et al.*, 2006; Dreisbach *et al.*, 2008) which happen to produce a similar fluorescence, indicative of membrane potential.

Having established that there is a significant difference between populations A and B, it follows naturally that populations A and C are different also. This is shown by similar levels of green fluorescence along with congruent mean values of colony counts from plates B and C. A visual assessment of the plates yields yet more information, where it can be seen that plate C has more variation in colony size than plate B. However, a longer/higher temperature incubation period was not a viable option to try to increase the number of colonies, due to the overgrowth on the higher recovery plates.

Image C ii from Figure 4.8. shows the fluorescence micrograph of the sorted cells from population C in Figure 4.4. showing the source of the counterintuitive, dual-stained bacterial cell population to be two associated cells, one green fluorescent and one red. The consistent attachment of these cells along the longitudinal axis suggests that they were mid-way through cell division (Haeusser and Levin, 2008) when one of the cells died before the separation of the cells could be completed, but after one of the committed steps in the process. The appearance of cells conjoined in this way is interesting due to the rarity of the event. It has been demonstrated amply that cells with specific mutations in the pathway involving cell division can

form long filaments (Lau and Zgurskaya, 2005). No further analysis was performed here due to the fact that, even if a mutation was evident in this population, its effect was clearly fatal on one of the cells, suggesting that it would not persist in the culture long-term. Populations B & C were not shown to be statistically significantly different, in terms of the mean colony counts (Table 4.3.), however, there appears to be considerable variation in colony size. This is probably caused by the reasons suggested earlier concerning prolonged lag phase due to adaptation however might be due to an observed bacteriostatic effect of the flow cytometric dyes on bacteria (Browning *et al.*, 1924). The effect observed by Browning was recorded with dyes of the carbocyanine-type, making it likely that $DiOC_6(3)$, rather than PI, was responsible for any detriment to the growth of the bacterial colonies. As mentioned previously, the bacterial cell membrane is impervious to propidium (the main reason for its use in this assay), therefore, even though molecules of that type, specifically ethidium, have been shown to be bacteriostatic (Grant, 1969) it was not a factor here.

The comparison between the two control populations (U and A) would not be expected to be significantly different, indeed, one might expect that the stained cells may have a less robust recovery due to the presence of the dyes which might exert a bacteriocidal/bacteriostatic effect, limiting the growth rate sufficiently that colonies cannot be detected visually. This was not the case, in this instance, with the stained cells having a slightly higher mean colony count than the unstained cells, but the result of the unpaired

student's t-test ensures that this can be attributed to random variation in this instance.

Due to the small number of people using flow cytometry (and other fluorescence methods) to analyse bacterial cultures, it is important that the maximum utility is extracted from every resource possible when new dyes and reagents become available. For this reason, the RedoxSensor Green™ kit (Molecular Probes) was of particular interest with its ability to assay for enzymatic activity within living cells. The RedoxSensor Green™ is altered by reductase enzymes in the cell (Molecular Probes catalogue), which causes the generation of a fluorescent product that can be detected by e.g. flow cytometers.

Figure 4.9. shows similar trends to those observed with the previous two staining methods, an initially heterogeneous population, in this case, green, red and neither green nor red cells. On addition to fresh medium, there is a rapid transition towards a homogeneous, healthy population before degradation as the culture ages.

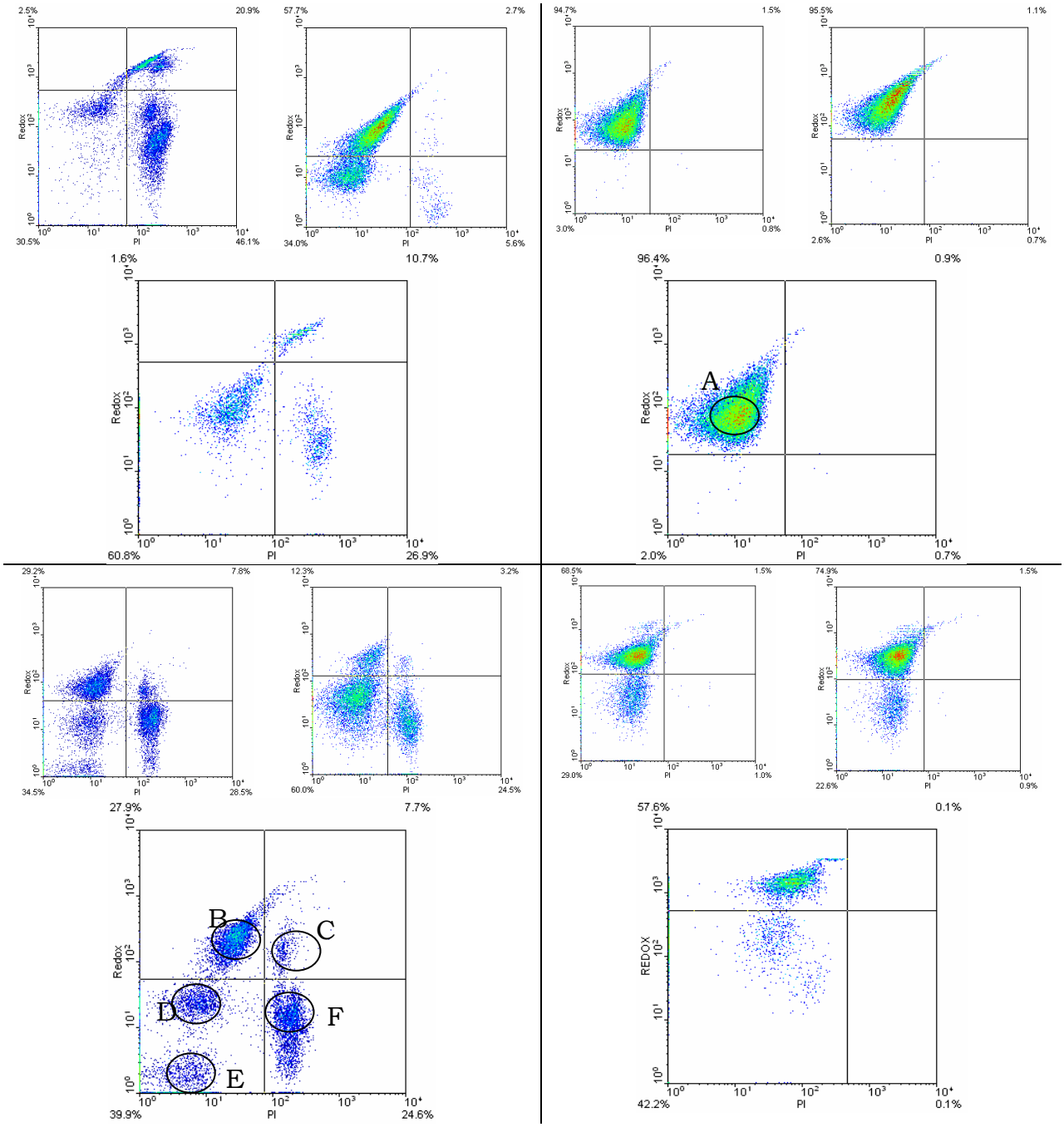

Figure 4.9. Flow cytometric analysis of the *B. cereus* shake-flask culture in Figures 4.2. stained with PI & RedoxSensor Green™. Clockwise, from top-left: Inoculum, 4 hours post, 9 hours and 24 hours. Analysis shows data from 3 fermentations. Populations A-F were sorted according to their fluorescence onto nutrient agar, and the results displayed in Figure 4.10.

Interestingly, after 24 hours, there are 5 clearly distinguishable populations (population F can, in fact, be seen as two populations however they were not sufficiently separated for individual analysis but it is likely that the

respective cells contain different quantities of DNA similar to Gasol *et al* (1999). In the work reported by Gasol *et al*, the differing DNA concentration was linked with an apparent increase in cellular volume at approximately constant density, as measured by centrifugation. The magnitude of the fluorescence measured by flow cytometry is linked to the cellular volume, which almost certainly made the difference between the populations greater than is the case in this work. The situation observed here is much more analogous to the work of Michelson *et al* (2010) who demonstrated, via flow cytometric DNA staining, that *L. lactis* exists as a diploid cell-line, exhibiting higher quantities of DNA, in this case in a similar cell volume. The difference observed here is far more minor, indicating that the quantity of DNA in the respective population's cells is much closer, probably a result of a well-aligned and sensitive system combined with multiple replication forks which appear within bacterial cells. The populations labelled B, C, D and F are the populations that appear to be analogous to those discovered using the other staining techniques, with the additional fifth population, E.

The most easily identified population stained with the PI/RedoxSensor Green™ couple is E. The detection of ghost cells (cells with no DNA/protein contents, but with sufficient intact membrane to be detected with distinct light scatter characteristics to intact cells) by flow cytometric methods is well understood (Lewis et al., 2004; Reis et al., 2005), and in some cases techniques have been optimised to increase the sensitivity to provide maximum discrimination between them (Haidinger *et al.*, 2003). These cells

were probably not detected during the other methods due to the voltages required on the photomultiplier tubes of the flow cytometer for those dyes.

Population B, shown in a similar position to population A in the upper right plot from Figure 4.7. indicates healthy, green fluorescent cells. D and F represent “stressed cells” where there is insufficient available energy to carry out the reduction of the fluorophore and red fluorescent, PI positive cells, with a compromised membrane, respectively.

Evidence, again, of a population which confounds expectations by fluorescing both red and green simultaneously which was further interrogated by fluorescence microscopy (Figure 4.10.; C ii).

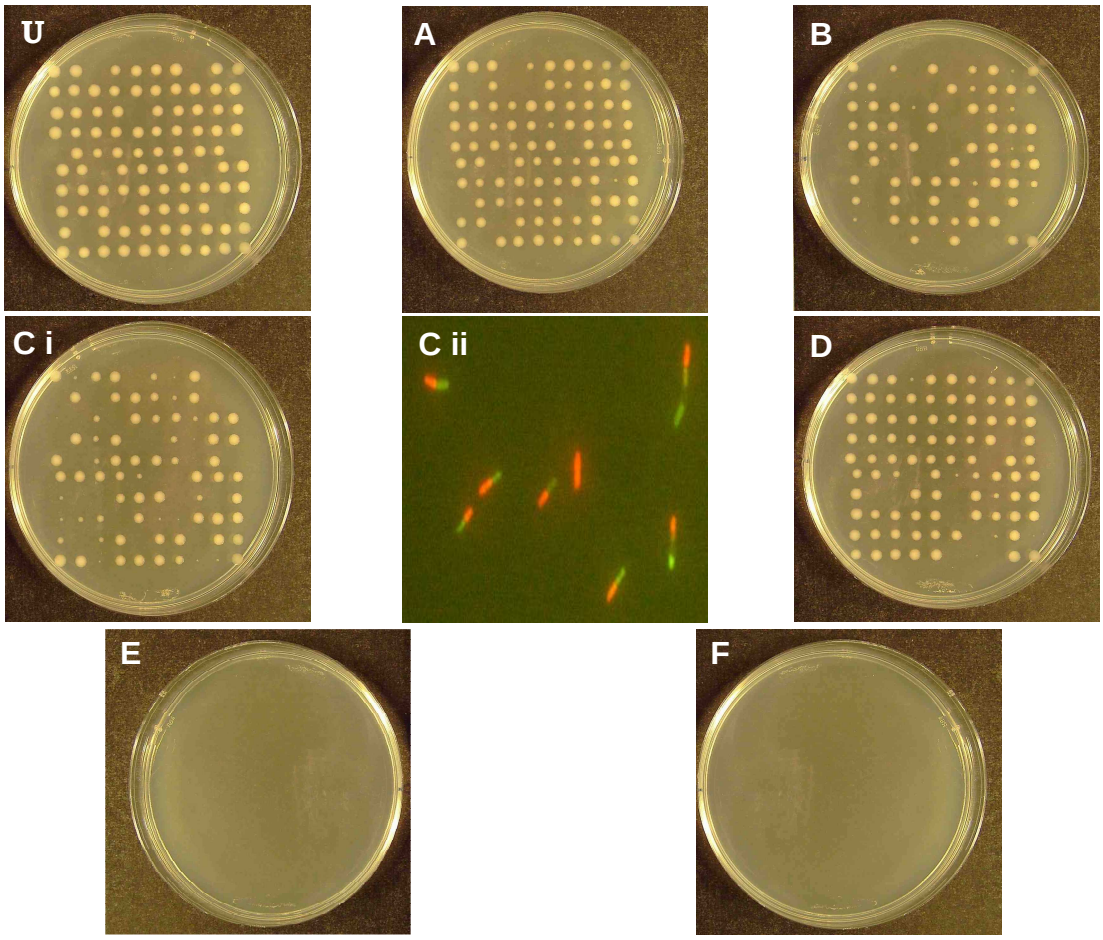

Figure 4.10. Sorted cells from *B. cereus* shake-flask fermentation analysed by cultivation on nutrient agar and fluorescence microscopy. Top-Left: Unstained cells. A. Healthy (mid-exponential phase), B, C i, D, E and F (24 hours after inoculation) RedoxSensor Green™ & PI stained cells from the populations indicated in Figure 4.9. C ii: Fluorescence microscopy of cells sorted from population C on Figure 4.7. The agar plates shown here are representative of 4-6 for each sample (full breakdown of plate counts shown in Table 4.4.).

Following sorting onto nutrient agar none of the cells from populations E and F, ghost cells and perforated membrane cells respectively, managed to produce colonies.

Table 4.4. shows the colony counts from the sorted agar plates following incubation, along with means and standard deviations.

Table 4.4. Colony counts from sorted cells, stained with PI/RedoxSensor™ Green and incubated on solid agar. The bottom two rows show the mean and standard deviation (SD) of the values.

	U	A	B	C	D
	90	89	60	74	89
	94	94	72	71	91
	90	90	83	75	90
	98	98	67	73	93
	97	98			
		96			
Mean	93.8	94.2	70.5	73.3	90.8
SD	3.8	3.9	9.7	1.7	1.7

Further, when subjected to the same unpaired student's t-test as the $DiOC_6(3)$ stained cells, the following data were revealed:

Table 4.5. Results of unpaired student's t-tests on colony number (%) from sorted plates shown in Figure 4.8. T-tests performed using Sigmaplot v. 10. If the P value for the data is greater than the threshold, this indicates that the means are significantly different.

Comparison	Mean (n)	P value	P value thresholds
U/A	93.8 (5)/94.2 (6)	0.88	>0.05
A/B	94.2 (6)/70.5 (4)	0.00059	<0.001
A/C	94.2 (6)/73.3 (4)	0.0000091	< 0.001
A/D	94.2 (6)/ 90.8 (4)	0.14	> 0.05
B/C	70.5 (4)/73.3 (4)	0.60	> 0.05

The results from the statistical tests in Table 4.5. indicate that there exists no significant difference between the number of colonies comparing unstained cells (U) and stained healthy cells (A) and non-fluorescent cells.

This test indicates, again, that population A is different from population B, this corroborates the same observation made with $DiOC_6(3)$ stained cells, and

the same explanations can be reasonably made in justification. The same is true of A/C where the comparison is between mid-exponential phase cells and stationary phase cells which have demonstrably lost some metabolic activity, being unable to perform the reactions necessary to be able to activate the fluorophore.

As before, the cells from populations B & C are shown to be similar, indicating that the cells in population C are able to produce sufficient energy to become green fluorescent despite existing in tandem with a dead cell as shown in Figure 4.8. C ii. Population C does, however, seem to have a greater heterogeneity than population B in terms of colony size, with a larger proportion of small colonies in contrast with the PI/$DiOC_6$(3) where there was no noticeable difference in colony size distribution. This effect is also noticeable when comparing plates U and A, with plate A exhibiting a few colonies that are visibly smaller than those on the unstained plate. It is possible that long-term exposure to the PI/RedoxSensor™ is detrimental to the health of the cells, the latter of which is supplied in DMSO which might enable penetration of PI into healthy cells which might disrupt the genome and alter the reproductive output of the cell on nutrient agar as shown with ethidium by Grant *et al* (1969).

4.1.1. Conclusions

Even at small-scale, the assumption of a truly homogeneous culture is optimistic at best. PI/$DiBAC_4$(3) can be used with Gram positive cells to

monitor cell viability, however there are flaws in the technique which lead to difficulties in interpretation of the data. PI/$DiOC_6$(3) gives a much clearer resolution of the populations of cells present within a "homogeneous environment" and has shown the presence of a fourth population consisting of cellular couplets of a single live and dead cell joined along the longitudinal axis. PI/RedoxSensor Green™ can be utilised as an alternative/adjunct to PI/$DiOC_6$(3) but care must be taken with length of exposure of the cells. This last point is especially important, because DiOC6(3) is a dye which stains cells in response to the energy status, whereas RedoxSensor Green is energy independent. In this example, the results given by the two systems are very similar.

4.2. *E. coli* CLD048 Shake-Flask Fermentation

Following completion of the work in the previous section with *B. cereus* in shake flasks, the work was repeated using *E. coli* CLD048, using the production of an anti-lysozyme antibody as a model for antibody fragment production in general. All experiments were carried out using the same baffled flasks as before, ensuring maximum oxygen transfer capacity within this batch culture, where it is expected that other factors will become limiting (e.g. soluble nutrients, such as glycerol and lack of pH control). The aim here was to obtain preliminary data, replicating the observation by Avecia Biologics Ltd. (the source of the recombinant *E. coli* strain, and funding for this research) of a large reduction in OD_{600nm} following induction of the recombinant Fab. These experiments were also used to determine the suitability, or otherwise, of the staining protocols developed for analysis of *B. cereus* cultures.

Figure 4.11. shows the resulting growth of shake-flask cultures when induced with 0.1 mM IPTG at $OD_{600nm} \approx 1$. The rapid curtailment of the growth of the cells, indicated by the OD_{600nm} curve, suggests that induction of the cells this early prevents their growth and productivity. This is confirmed by the pH plot, which shows that the cells had barely begun to metabolise the medium components in significant quantities, with cell death occurring prior to the onset of the pH drop in the control culture. This experiment, in combination with discussions with Avecia, lead to inductions henceforth being performed at a minimum $OD_{600nm} \approx 10$. Due to the nature of

the experimental protocol, this was within the range of 7.5 – 12.5 OD_{600nm} units each time. This experiment ably typifies the rapid reduction in culture viability following induction, reported by Avecia Biologics, which was the impetus for this research.

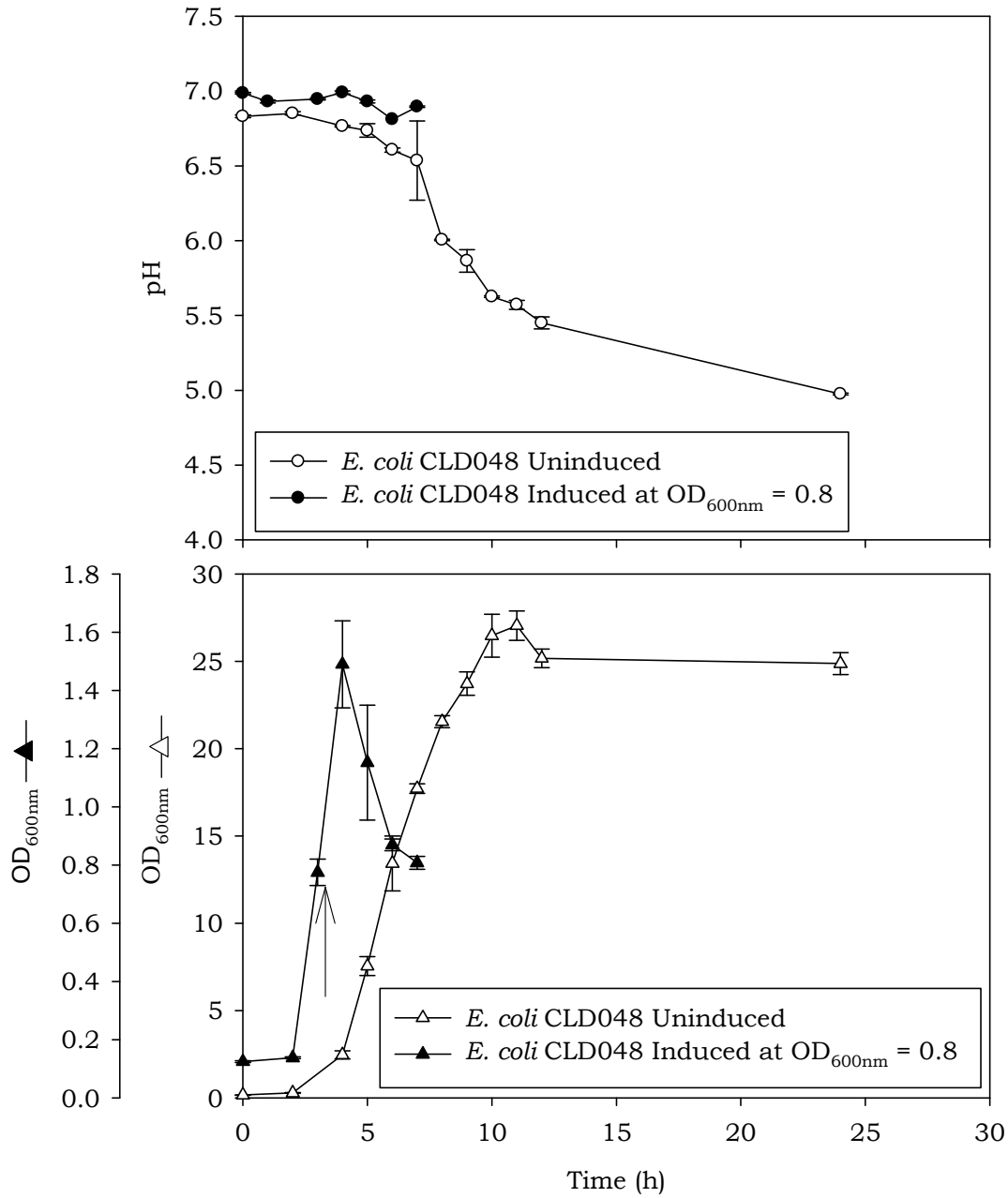

Figure 4.11. *E. coli* CLD048 shake-flask fermentation (n = 1). All points plotted are mean values from replicate analyses. The error bars show standard deviation of the data from which the mean was calculated. Induction carried out at OD_{600nm} ≈ 1 using 0.1 mM IPTG immediately following analysis of the sample indicated with the arrow.

As may have been expected, from the optical densitometry in Figure 4.11. the reproductive growth on agar, demonstrated in Figure 4.12. is also severely impacted by the induction of recombinant protein expression. Interestingly, where the peak optical densities of the induced and uninduced cultures differ by a factor of 15, the peak CFU.mL^{-1} also show a similar degree of separation. This suggests that this CFU.mL^{-1} method may be more reliable for the *E. coli* cultures than for the *Bacilli* where the method appeared to under-estimate the CFU.mL^{-1}, relative to the DCW and OD_{580nm}, quite considerably.

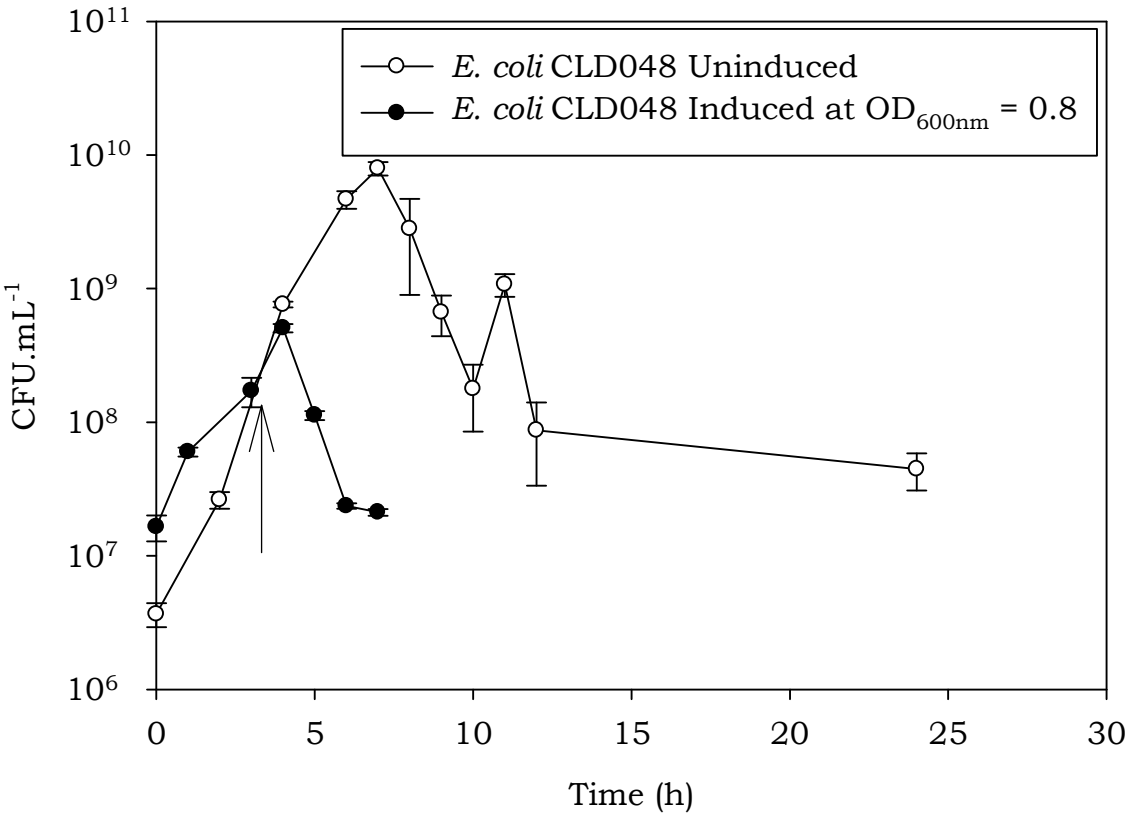

Figure 4.12. Colony forming units per millilitre for *E. coli* shake-flask culture. Each point displays a mean value from 4-6 dilution plates, with the error bars showing the standard deviation of those data. The point of induction (OD_{600nm} ≈ 1) is indicated by the arrow.

Figure 4.13. compares the recombinant *E. coli* cultures during induced and uninduced fermentations. The uninduced cultivation was performed once to demonstrate growth of the microorganism within the system. This was then compared with a similarly grown, induced culture, with IPTG added to 0.1 mM final concentration after 6 hours, indicated by the arrow on Figure 4.13.

Scrutiny of the OD_{600nm} curve, in Figure 4.13. shows that, following induction, the cells are limited to an $OD_{600nm} < 25$, more than 10 % lower than that achieved in the uninduced culture. It can also be seen that there is a significant different between the two induced cultures. The earlier of the two inductions (induction occurred at $OD_{600nm} \approx 7.5$) had a more profound effect on the final achievable optical density of the culture. This is characteristic of metabolic load, suppressing the growth potential of the cells as a result of diversion of resources to production of the recombinant product (Glick, 1995; Kilikian et al., 2000). It can be seen that as the strength of induction increases (from induction at 7.5-12.5; no induction) the metabolic toll on the cells also increases, as determined by optical density measurement. This observation is confirmed by the pH measurement, used here as an indirect measure of metabolism. The pH deviation observed was greatest for the uninduced culture, where the rates and onset of the deviations were approximately the same for all three cultures. This is reflective of the diversion of metabolic energy away from “normal” functions and into recombinant protein production. That the three cultures experience changes in pH to differing degrees, suggests that the contribution of recombinant protein production is minor in comparison with that of

vegetative growth. There is also a large downturn in the culture as it ages, with the 24 hour culture exhibiting an OD_{600nm} of less than 15 compared with 25 for the uninduced fermentation. This drop in the measured OD_{600nm} indicates probable cell lysis, which, in the context of an industrial fermentation, is undesirable causing increasing demands on the downstream purification (Dwyer, 1984). It has previously been mentioned (Sommer *et al.*, 2009) that one of the benefits of periplasmic expression as used with this strain, is the potential to avoid the need for whole cell lysis to liberate the product. It appears that there is significant lysis occurring at a late stage in the culture, and in fact it may be occurring earlier, masked by the rapid growth of the non-lysing cells. The analysis of pH for the two cultures is very similar, with both following the same pattern, out of step by the difference recorded at the start. Ordinarily, a culture that is observed to be lysing following stationary phase would be expected to increase in pH, as a result of gluconeogenesis resulting from scarcity of high-energy carbon sources. The fact that this culture, in fact, decreases in pH is suggestive that there is still sufficient carbon source in the medium to support further growth, suggesting that the stationary phase may have been induced by a lack of some other fundamental nutrient.

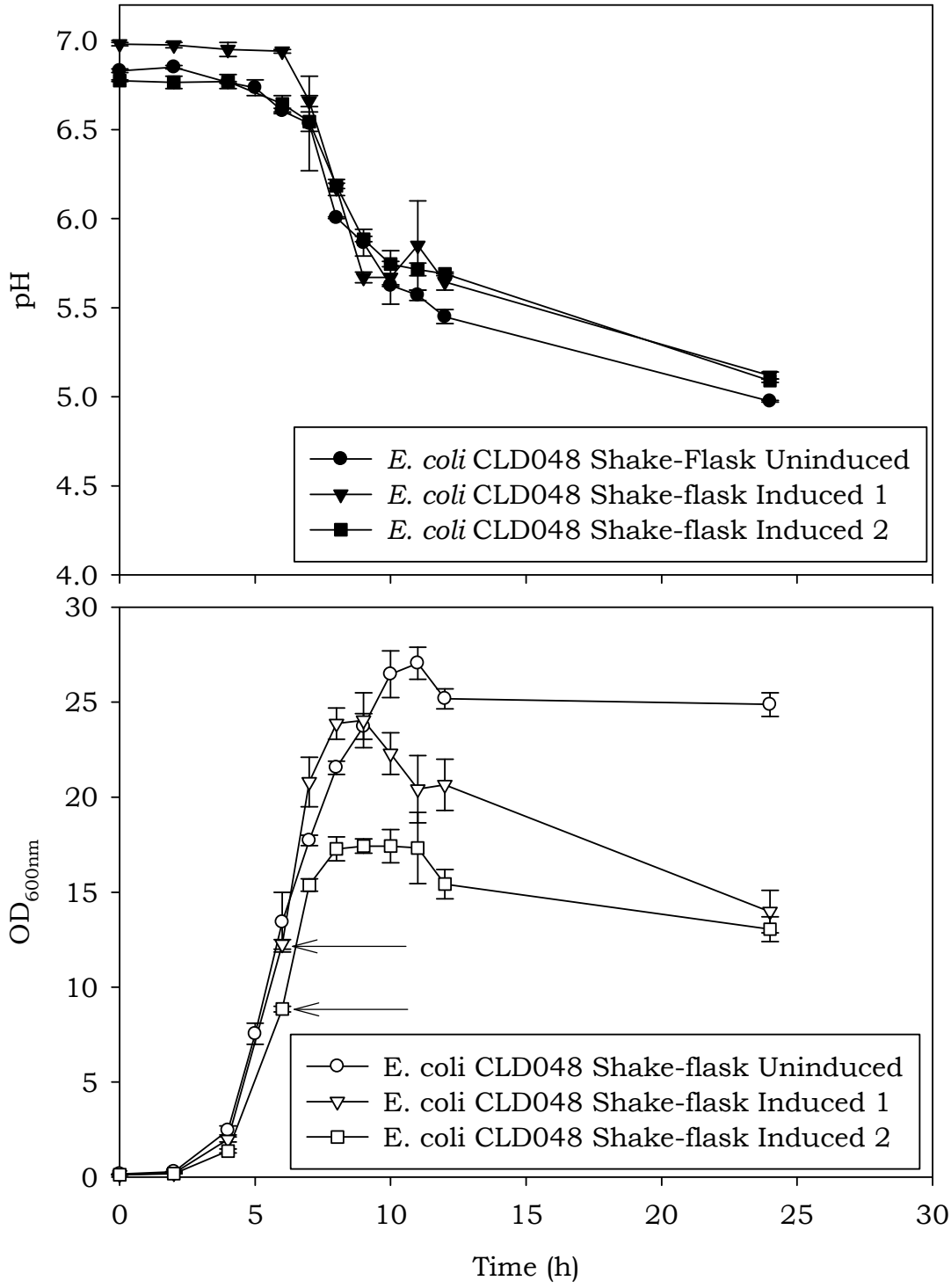

Figure 4.13. Comparison of shake-flask cultures of *E. coli* CLD048 with and without induction. All points plotted are mean values from replicate analyses. The error bars show the standard deviation of the data from which the mean was calculated. Induction carried out at OD_{600nm} ≈ 10 using 0.1 mM IPTG immediately following analysis of the sample indicated with the arrow.

Figure 4.14. shows the growth of the induced, recombinant, culture in terms of both turbidity and CFU.mL^{-1}. It can be seen that the CFU.mL^{-1} and

OD_{600nm} increase in a similar manner up to the point of induction, after which, there is a 10-fold drop in $CFU.mL^{-1}$ for little change in OD_{600nm}. The impact of induction on the cells is felt almost immediately afterwards, with a sharp decline in the number of colonies formed on nutrient agar falling by a factor of 100 from 1 to 3 hours post-induction.

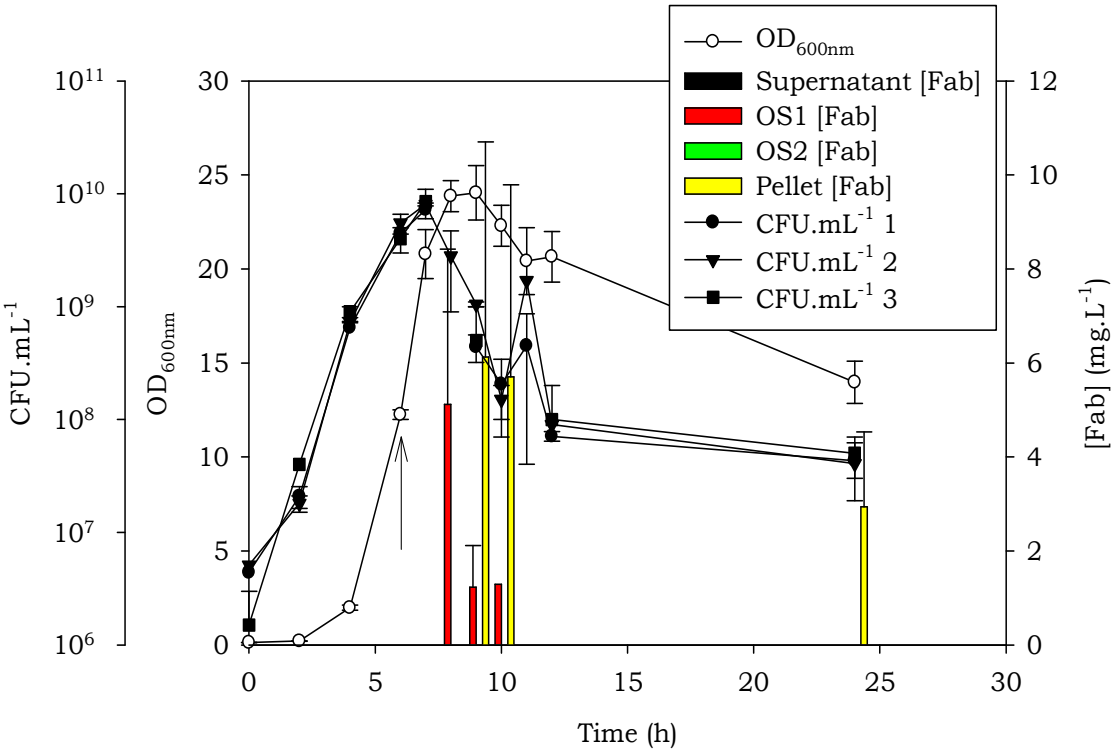

Figure 4.14. *E. coli* CLD048 shake-flask fermentation. All points plotted are mean values from replicate analyses within a single experiment. The error bars show the standard deviation of the data from which the mean was calculated. Induction carried out at OD_{600nm} ≈ 10 (indicated by the arrow) using 0.1 mM IPTG immediately following analysis of the sample indicated with the arrow. The Fab fragment samples shown are the only samples tested in this instance.

In common with the CFU analysis of the *Bacillus cereus*, the same parameter appears to have been underestimated for this *E. coli* culture as well, in the early stages at least. Above an optical density of 10, the CFU data appears to be far more congruent with what would be expected based on the estimation of bacterial cell mass in combination with the measured dry cell weight from

Figure 4.13. The recombinant Fab fragment was produced in small quantities over the course of this fermentation, with a maximum of 7 $mg.L^{-1}$ for any one sample. This production was also limited to only two of the intracellular regions under test; within the periplasm, and associated with the pellet. The first measurement detected Fab fragment within the periplasm (OS1) at a concentration of 5 $mg.L^{-1}$, with the concentration in this partition falling rapidly over time. This fall was in concert with an increase in the quantity of material associated with the cell pellet; implying that it was insoluble.

In comparison with the published information on this strain (Hodgson et al., 2006), the specific quantity of recombinant protein produced in this batch shake-flask culture is much lower; 0.29 $mg.L^{-1}.OD_{600nm}^{-1}$ compared with 4.5 $mg.L^{-1}.OD_{600nm}^{-1}$. This results from a much stronger induction in the process described in the patent (addition of 0.1 mM IPTG at $OD_{600nm} \approx 0.5$-0.7) however, there is no mention of any detrimental effect on the optical density of the culture. A similar effect on the post-induction growth would indicate that this production was achieved at very low optical density. The shake-flask fermentation described in Figure 4.11., then, was almost certainly more productive in terms of total Fab concentration than that used by Hodgson *et al* (2006).

After 24 hours in culture, the Fab concentration has dropped to a fraction of that observed at the peak of growth. This corroborates the inference that widespread cell lysis is responsible for the drop in OD_{600nm}, an ancillary effect

of which would be the liberation of intracellular proteases that would degrade the product.

The method of quantification of Fab, ELISA, can only detect Fab where the complimentarity determining regions are intact and able to bind their antigen as well as the epitope recognised by the secondary antibody. As such, the total amount of protein produced (including any that may be in insoluble forms) cannot be ascertained using this method. In order to determine the total quantity of protein, a more generic procedure could be utilised such as SDS-PAGE, however, the quantity of protein was insufficient to detect the purified protein.

Figure 4.15. displays the relationship between OD_{600nm} and DCW for this *E. coli* culture when the DCW is determined by centrifugation rather than filtration, followed by drying. In contrast with the previously used filtration method, this example displays an good r^2 value, nearly 0.95, with the slope of the curve ($DCW = 0.381OD_{600nm} + 0.110$) is well within the normal range found in the literature (Tännler et al., 2008; Balagurunathan and Jayaraman, 2008).

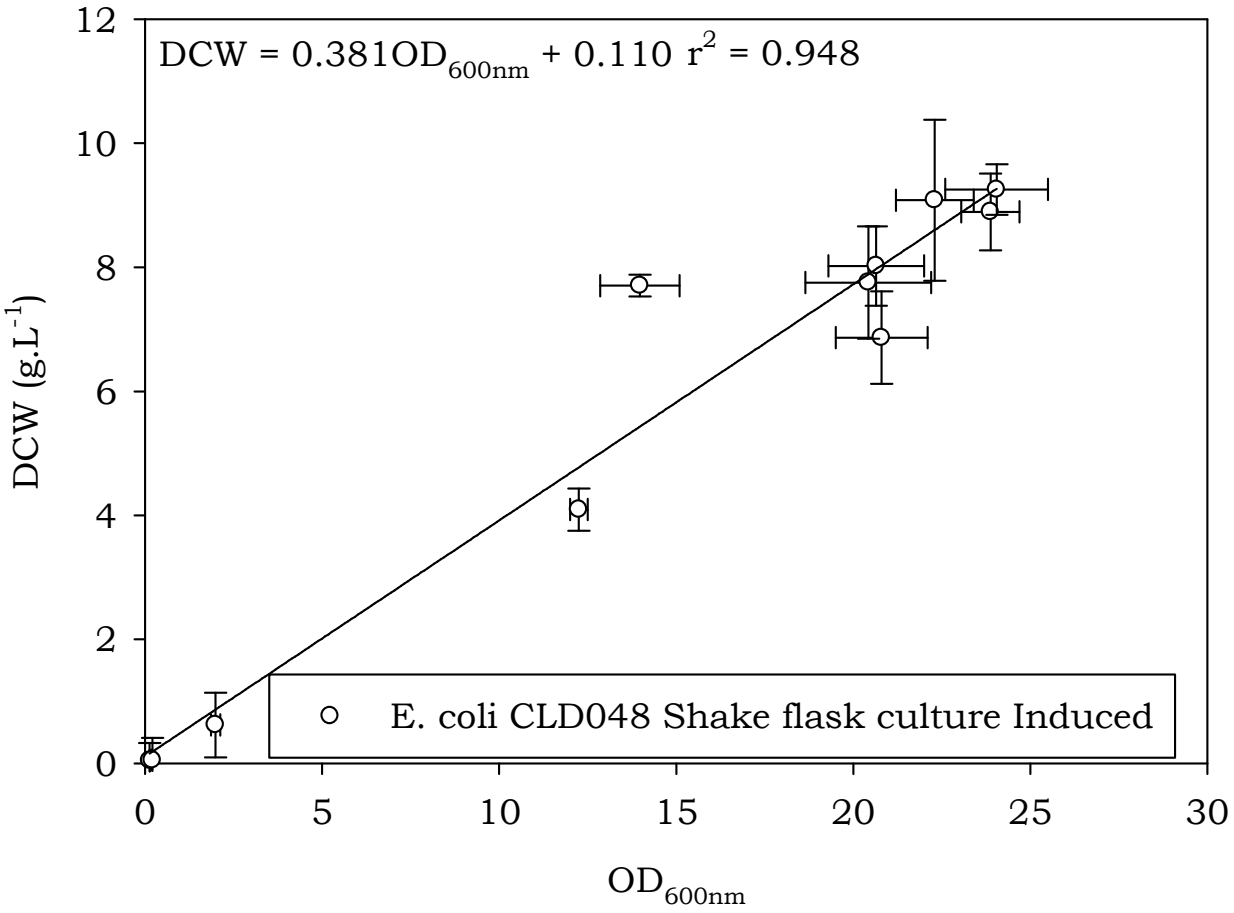

Figure 4.15. OD_{600nm} vs DCW for *E. coli* shake-flask fermentation. The data plotted are mean values of 2-4 replicates with the error bars indicating the standard deviation. Induction was carried out by addition of 0.1 mM IPTG at an $OD_{600nm} \approx 10$.

As seen in Figure 4.13., the OD_{600nm} reaches a plateau and then falls sharply following induction. Despite this observation, consideration of the specific growth rate in Figure 4.16. illustrates that, in fact, the cells had already passed beyond their maximum growth rate at the point of induction. The average specific growth rate (μ) for the length of the process, approximately 0.2 h^{-1} is a little misleading due to the long, slow decline in OD_{600nm} between 12 and 24 hours and may lead one to infer that, over this time period, growth was relatively slow. Growth-rate controlled cultures are usually maintained in the region of 0.1-0.3 h^{-1} to diminuate the build up of harmful by-products

and to ensure that the production of recombinant protein is not hampered by the stress associated with nutrient limitation at high growth rates (Åkesson *et al.*, 2001; Eiteman and Altman, 2006). Thus, the average specific growth rate between the beginning of the experiment and the peak OD_{600nm} is a more appropriate guide for the overall growth of the culture at 0.6 h^{-1}. It is likely that the combination of a rate of growth unrestrained by nutrient limitation and the recombinant protein production causes many of the cells to break down, resulting in the effects seen here.

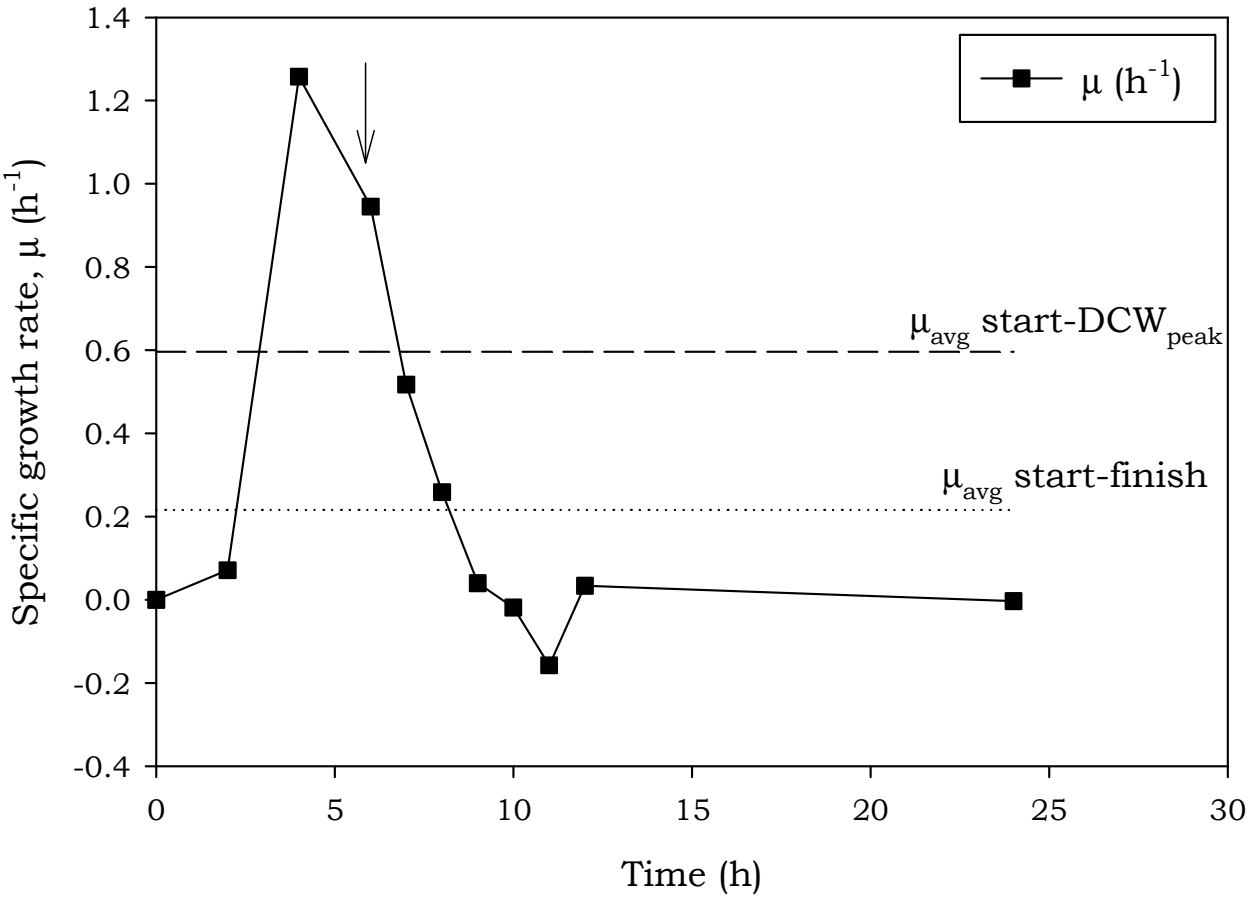

Figure 4.16. Variation of specific growth rate for *E. coli* shake-flask fermentation in Figure 4.13. The data are calculated from the mean DCW. Induction was carried out by addition of 0.1 mM IPTG at the point indicated by the arrow.

Following the initial overnight incubation, Figure 4.17. detects a significant proportion of the culture exhibiting green fluorescence with $DiBAC_4(3)$ (38.7 %). This is a repeat of the observation of the *Bacillus cereus* culture, also prior to inoculation in Figure 4.6, indicating that the initial incubation period may be too long from the perspective of an industrial process, causing a breakdown in the overall quality of the cells prior to the beginning of the fermentation. It is worth noting that the *B. cereus* culture recorded a far higher quantity of $DiBAC_4(3)$ positive cells, nearly 75 % with around 40 % of these having compromised membrane integrity. The magnitude of the impact of the initial culture conditions seems to be greater for the Gram positive organism. Barcina *et al* (1995) showed a similar effect when using ciprofloxacin to artificially arrest cell division, with Gram positive bacteria showing a lower recovery on solid medium than the *E. coli* strains tested. Interestingly, it appears that a rod-shaped morphology seems to minimise this effect with the worst recoveries being demonstrated by the cocci in Barcina *et al*'s work (1995). It's possible that, were this flow cytometric analysis to be performed on spherical Gram positive cells, such as *Streptococcus gordonii*, the extent of the unhealthy populations may be even greater than seen here.

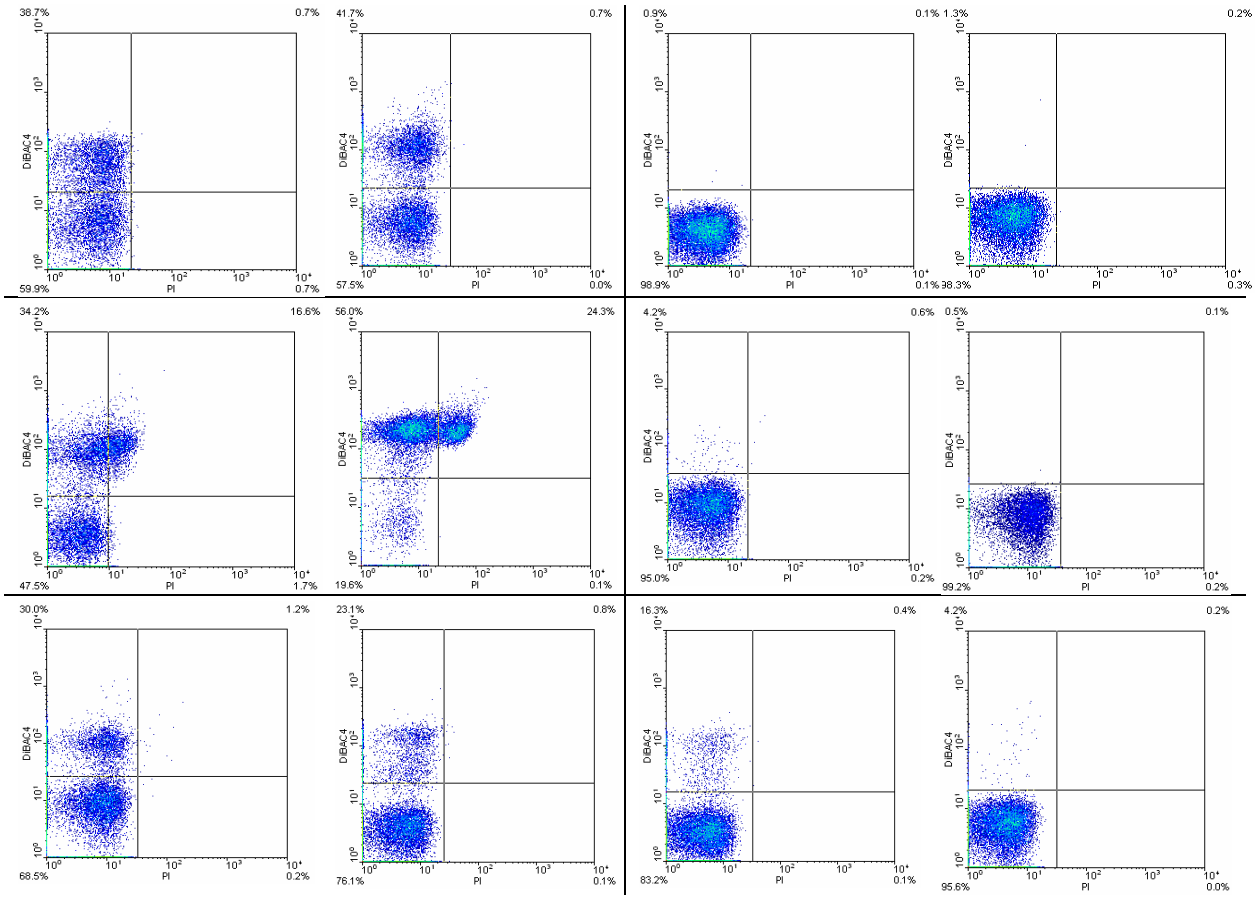

Figure 4.17. Flow cytometric analysis of *E. coli* shake-flask fermentation in Figure 4.13. stained with PI & $DiBAC_4(3)$. Each grid space shows similar time points for two different fermentations, clockwise from top left: Inoculum; 4; 7; 9; 11; 24 hours. Cultures were induced at OD_{600nm} ≈ 10 using 0.1 mM IPTG.

This large population of stressed cells impacts heavily on the remainder of the fermentation and in order to avoid this issue the cells would have to be grown over a far shorter time period. This approach is less amenable to a process executed by people, because the setup of the inoculum may fall outside the normal working day.

As seen earlier in the analysis of *B. cereus* with this same combination of stains, the culture attains homogeneity at least according to the fluorescence of the cells with these two stains. The *E. coli* cells actually managed to reach

this homogeneously fluorescent population after only 2 hours in culture, whereas the *Bacilli* generally needed 4 hours to completely dilute out or otherwise eradicate the green fluorescent cells. This is likely attributable in part to the content of their respective media. The very high glycerol concentration (35 $g.L^{-1}$) probably enabled the healthy *E. coli* cells to grow at a much faster rate than the *Bacilli*, preventing detection of the green fluorescent cells even by such a sensitive technique as flow cytometry. Another explanation, given the lack of PI positive cells in the inoculum, is that, rather than being diluted out, the green fluorescent cells had sufficient nutrients to recover their ability to generate a normal physiological membrane potential. The image displayed for the four hour time point accurately depicts the condition of the cells from 2 hours post-inoculation through to 6 hours, immediately prior to induction.

One hour after induction, 7 hours post-inoculation, the green fluorescent population begins to re-appear, although only in very small quantities (4 %). The CFU analysis at this point (Figure 4.14.) indicates the peak in the number of colonies, prior to the fall that is observed immediately afterward. This increase in the number of cells unable to maintain their cytoplasmic membrane potential contributed to the observed drop in the expected recovery of the cells on nutrient agar, demonstrating that this flow cytometric method can yield signs of bacterial stress at-line, more sensitively than detectable by CFU analysis.

Three hours after induction, the proportion of green fluorescent cells has increased to 16 %, and it is in this period that the amount of Fab produced becomes detectable by ELISA. At the peak quantity of measured Fab, the culture is 30 % green fluorescent, indicating the onset of significant cellular stress, compared with the value of 38.7 % for the inoculum after an additional 5 hours of growth in a far less nutrient rich medium. This large number of cells that have become metabolically compromised can be extenuated by observation of the location of the Fab produced in this time. At 11 hours, most of the Fab is located within the pellet, and only very small quantities are associated with the periplasm. The Fab is passed from the cytoplasm to the periplasm utilising part of one of the Tat or Sec pathways, which are dependent on the proton motive force (de Leeuw et al., 2002) or nucleoside triphosphate hydrolysis (Pugsley, 1993) respectively. As such, this inability of the cells to maintain their cytoplasmic membrane potential could directly prevent passage of the Fab through the membranes as a result of a depressed proton-motive force (PMF), or it could be symptomatic of a general increase in the energy demands within the cell diverting resources away from recombinant protein translocation (Natale *et al.*, 2008).

After 24 hours cultivation, the culture further degrades in quality with 50 % of the cells exhibiting green fluorescence, of which, approximately one third are also red fluorescent. This is the first point where PI positive cells can be detected in significant quantities during this shake-flask culture. The dearth of red fluorescent cells observed prior to this may be caused by a number of factors, the first of which would be that the cells aren't dying. This doesn't

seem to make sense given the large falls in OD_{600nm}, $CFU.mL^{-1}$ and DCW in the same time frame. It has been well established that propidium is incapable of penetrating an intact cellular membrane (Phe *et al.*, 2007), therefore, only cells whose membrane structure has been compromised fluoresce red when exposed to propidium. Due to the action of propidium as an intercalator into DNA, it naturally follows that the perforations in the membrane must be sufficiently small to contain the genome. Once the holes in the membrane reach sufficient size for the DNA to leak out, PI is no longer an effective means of determining the number of dead cells. This would require a rapid breakdown in the cellular structures, such that it remains undetected by flow cytometric analysis. The cells under stress, resulting from the combination of nutrient limitation and recombinant protein production (specifically with periplasmic targeting, known to increase the burden on producing cells (Aldor *et al.*, 2005))) could have succumbed to programmed cell death (Engelberg-Kulka *et al.*, 2006). This process would bring about a rapid and complete breakdown in the cell integrity releasing the contents sufficiently quickly that an intermediate stage exhibiting PI fluorescence may not have been present long enough to be detected. The death and release of intracellular contents would by no means reduce the productive capacity of the culture, due to the release of IPTG along with the cell's contents. This IPTG would then be taken up by surrounding cells causing a stronger induction leading to a continuing increase in recombinant Fab, even with falling cell numbers.

When compared with the work performed by Hewitt *et al* (2007) the effect of performing periplasmic expression appears to have little impact on the viability of the cells as measured by flow cytometry. Both this investigation, and Hewitt *et al* (2007) used E. coli culture for production, however, there were significant differences also. Where Hewitt *et al* used a T7 promoter, controlling the expression of a product targeted to the bacterial cytoplasm, here a T7A3 promoter was used, which reduces the strength of expression, as well as eradicating the need for co-transformation with the viral RNA polymerase. The main reason for the divergence in subcellular target for expression was that the antibody fragment used requires an oxidising environment in order for disulfide bonds to form, whereas the AP50 protein produced by both sets of experiments show small levels of green fluorescence shortly after induction with higher levels observed with increasing temporal displacement. However, Lewis *et al* (2004) showed a large degree of culture heterogeneity and green fluorescence (>65%) within 3 hours of induction, when these cells are grown and analysed in stirred-tank reactors then more appropriate comparisons can be made.

Figure 4.18. shows that, whilst the $DiOC_6(3)$ stain seems to be efficient at staining healthy cells in mid-exponential phase of growth when the culture approaches stationary phase, the PI positive, $DiOC_6(3)$ negative population observed with *B. cereus* is not seen in these samples. The presence of the green/red fluorescent population later in the culture is anomalous, especially considering the lack of any, solely, PI positive cells. The apparently red/green fluorescent cells are an artefact of the high concentration of the dye,

confirmed by the absence of this population with the PI/DiBAC$_4$(3) stained cells (Figure 4.17.).

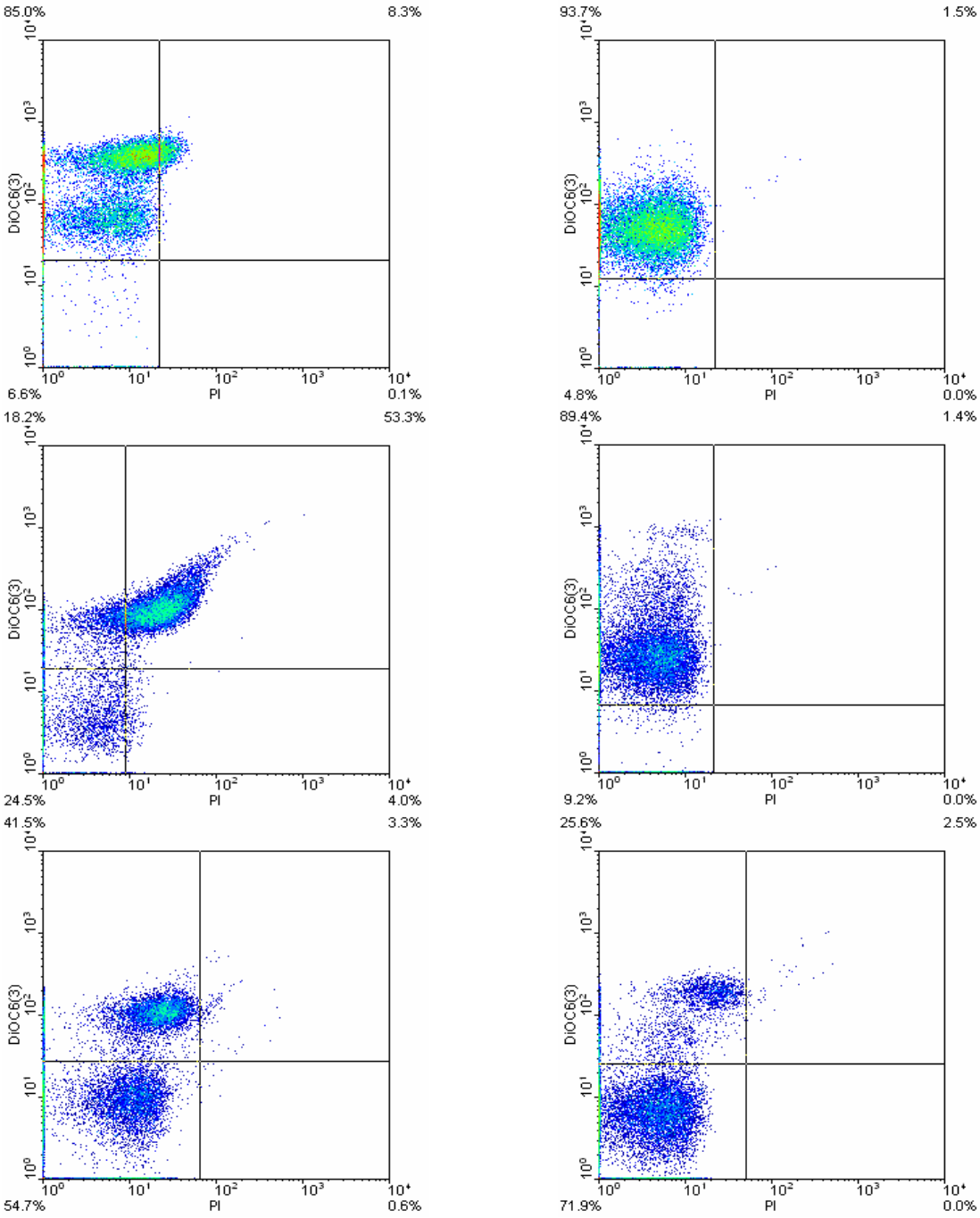

Figure 4.18. Flow cytometric analysis of *E. coli* shake-flask fermentation in Figure 4.13. stained with PI & DiOC$_6$(3). Cells were induced at OD$_{600nm}$ ≈ 10 using 0.1 mM IPTG. Clockwise from top left: Inoculum; 4; 7; 9; 11; 24 hours.

Following identification of the PI/$DiOC_6$(3) double-positive population in Figure 4.18. additional fluorescence parameters were investigated, displayed in Figure 4.19. During mid-exponential phase, where the cells were demonstrated to be at their most homogeneous and healthy by a lack of $DiBAC_4$(3) fluorescence, Figure 4.19. shows that the forward scatter (FS)/side scatter (SS) plot yields a similarly homogeneous culture to that observed for PI/$DiOC_6$(3) fluorescence of the same samples.

The 24 hour time point from Figure 4.19. shows a deviation away from the 6 hour samples, with an observed reduction in forward scatter. When the forward scatter is then plotted against $DiOC_6$(3) fluorescence, it becomes clear that the highly green fluorescent population is coincident with the reduced forward scatter population. This change in forward scatter is associated with a change in cell size (López-Amorós *et al.*, 1994), and, the population can be seen to encroach on the area where small particles and electronic noise are filtered out, indicating a significant reduction in size of the particles. If the pressure on the cells caused by the protein production is great enough to lyse the cells (as suggested by the fall in OD_{600nm}) this might account for the reduction in observed particle size. The high level of green fluorescence of these small, probably fragmented, cell particles could be attributed to the hydrophilic nature of the dye allowing it to remain associated with the cellular fragments following lysis.

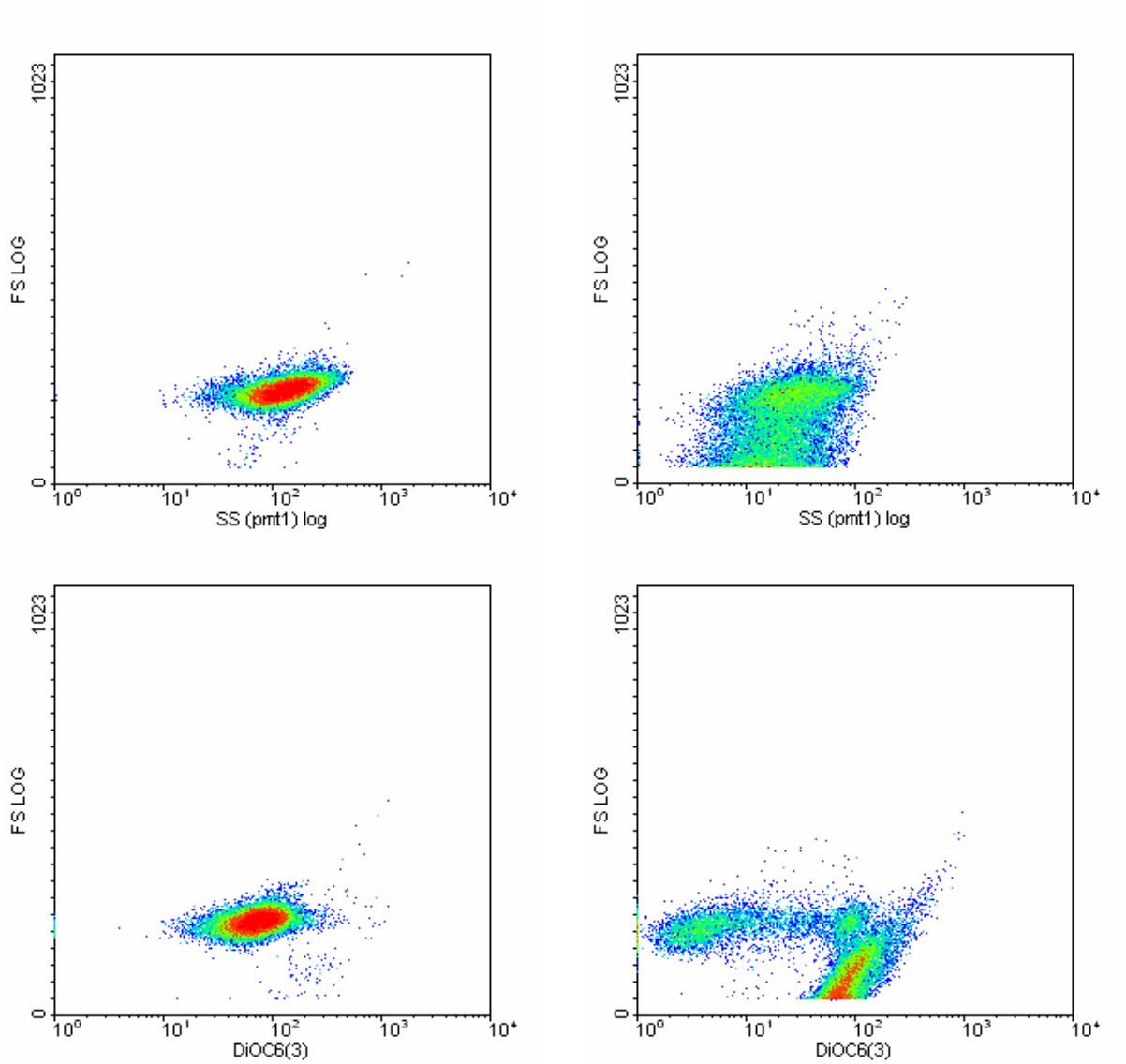

Figure 4.19. Flow cytometric analysis of *E. coli* shake-flask fermentation in Figure 4.13. stained with PI & $DiOC_6(3)$. Cells were induced at OD_{600nm} ≈ 10 using 0.1 mM IPTG. Left: 6 hours post-inoculation; Right: 24 hours post-inoculation
(18 hours post-induction).

4.2.1. Conclusions

Flow cytometry can be used in conjunction with PI/$DiBAC_4(3)$ to reliably detect the effect of recombinant protein production on these cells. The expression of the Fab fragment inhibits cell growth, as compared with uninduced control samples, and, additionally, causes cell lysis later in the fermentation. This lysis is undesirable from a process perspective, and is one of the conditions which this method was employed to avoid. Relatively little recombinant protein is actually produced in this example, less than 7 $mg.L^{-1}$ (and most of that associated with the cells rather than free in the periplasmic space). $DiOC_6(3)$ is unsuitable as a dye for use with *E. coli*. Whilst it appears able to demonstrate the presence of homogeneous, viable cultures, the lack of resolution of different populations in heterogeneous cultures produces inconsistent results.

4.3. *E. coli* Stirred-Tank Reactor Batch Fermentations

The process that is central to this thesis entails cultivation of the bacteria within a conventional stirred-tank reactor, followed by induction with IPTG to enable recombinant protein expression. Following demonstration within the scaled-down shake-flask model that the deleterious effect of the expression of this Fab fragment can be duplicated in our laboratories, the next step was to perform the same experiments in stirred-tank reactors. The standard protocol provided by Avecia Biologics Ltd. describes a fed-batch process, designed to achieve the optimum productivity, with the same widespread cell disruption. The first analysis performed was the determination of the conditions within a batch culture to provide a baseline for the process at this scale from which the fed-batch might be better understood.

Figure 4.20. shows the data from batch stirred-tank reactor fermentations, with data shown from two runs each of the induced GMO, uninduced GMO and induced wild type. It is clear from this plot that the induction of the recombinant gene is having a significant, and deleterious, effect on the growth of the culture. Interestingly, it appears that the uninduced GMO cells indicated by the open shapes, have an advantage in terms of their growth over the wild type organism. There are two factors at work in this instance which might impact on the growth of these cells; the uninduced GMO cell (*E. coli* CLD048) culture has a metabolic burden, not experienced by the wild type organism, that of maintenance of the recombinant plasmid (diverting resources away from genome replication) (Sato and Kuramitsu, 1998). In

addition, these cells also have to manufacture antibiotic resistance components (efflux pumps, in this case (Chopra, 2006)) enabling the cells to grow in the presence of the selecting antibiotic. In contrast with this, the wild type organism has been exposed to a xenobiotic compound, IPTG, that has been shown to affect the proteome in *E. coli* (Kosinski et al., 1992). In Kosinski *et al*'s work, however, it was not sufficiently demonstrated that this alteration in expression profile (affecting only a handful of genes) impacted the growth of the cells significantly. Analysis of the genotype of the W3110 strain indicates that the organism is *lac*$^{+}$, which would lead to gratuitous induction of the lac operon and would likely cause a significant metabolic load in itself. It appears from these results that the metabolic toll taken by the gratuitous inducer is of a greater magnitude than the combined requirement of plasmid maintenance and antibiotic resistance (although these two are linked, the requirements of the cell to fulfil the respective tasks comprise different spheres of metabolism, and so their effect can and should be separated). Addition of the IPTG to *E. coli* CLD048 leads to a drastic decline in the optical density of the cultures. Prior to induction, all of the cultures appear to be growing at approximately the same rate (bar the closed square, which experienced a prolonged lag phase), with the only exception, matching the other cultures after exiting the initial lag period.

When compared with the shake-flask culture it can be observed that the productive capacity (the extent to which the bioreactor can create biomass) is twice as great in the stirred-tank. This is due to the increased mass transfer, as well as the control of factors such as pH that can deleteriously affect the

metabolism of the constituent organisms (Glass *et al.*, 1992). This may also increase the magnitude of any detrimental effect of the induction conditions on the cells, with a greater quantity of resources diverted to cellular growth, fewer are available to respond to the stresses associated with the recombinant protein production (Jishage and Ishihama, 1995; Chatterji et al., 1998; Graves et al., 2008; Rosano and Ceccarelli, 2009).

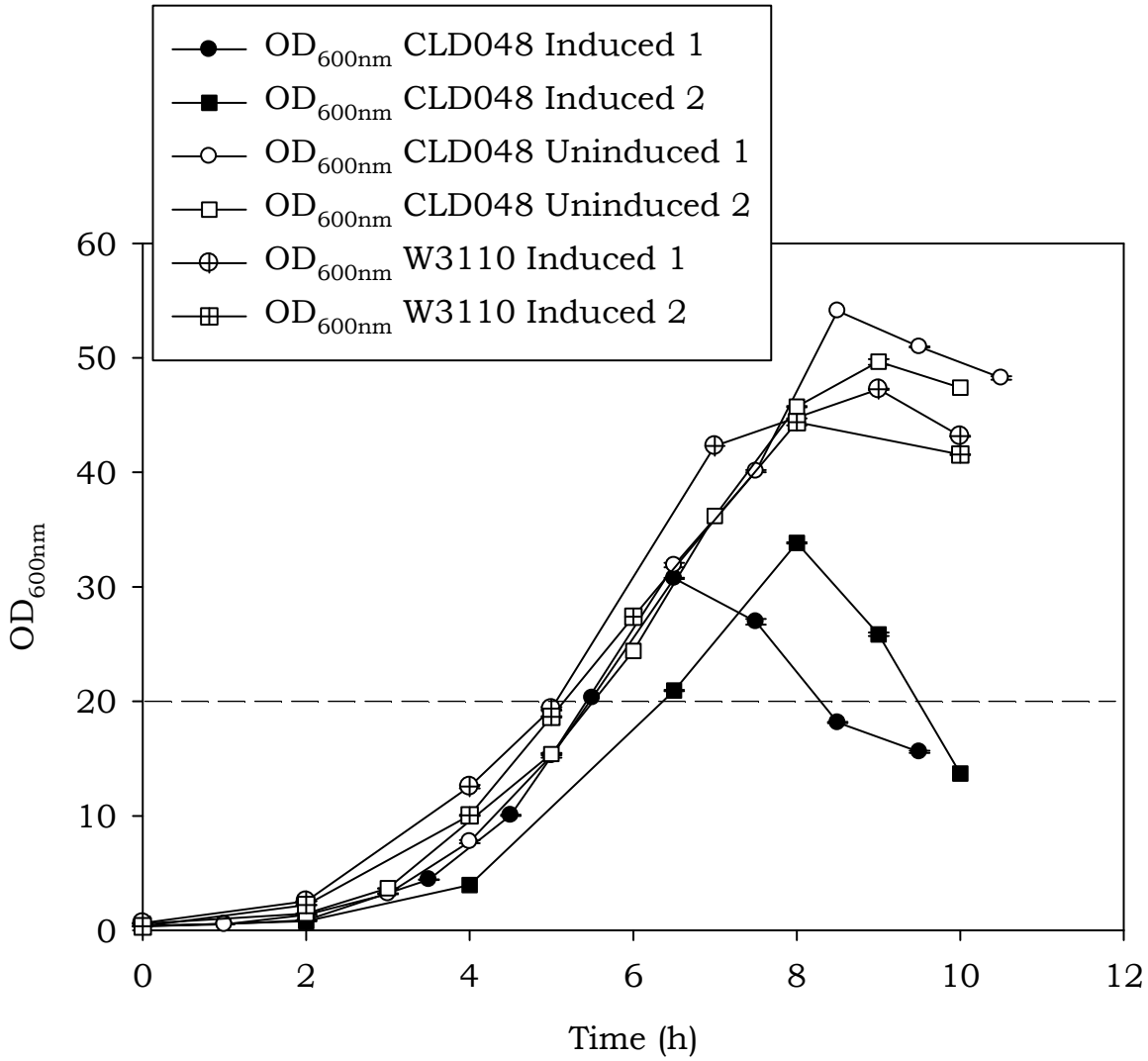

Figure 4.20. *E. coli* batch stirred-tank fermentation; CLD048 is the code for the recombinant organism, W3110 is the identity of the wild type organism from which the recombinant organism is derived. All points plotted are mean values from replicate analyses. The error bars show the standard deviation of the data. Induction was carried out at OD_{600nm} ≈ 20 using 0.1 mM IPTG immediately following analysis of the sample closest to the dashed horizontal line in the induced samples.

The first feature of Figure 4.21. which draws attention is the presence of measurable Fab production without the addition of any inducing agent. During the shake-flask culture, it was assumed that non-induced production of the recombinant product was nil, or negligible due to the use of a putative leak-free promoter system (Hodgson et al., 2006). The production of antibody fragment in the non-induced case was easily in excess of that achieved for the induced shake-flask fermentation but without the associated collapse in the turbidity. This is likely due to a large number of cells producing small quantities of Fab, consistent with promoter "leakiness", rather than the converse case of a smaller number of highly active operons under the influence of an inducing agent. It has, however, also been demonstrated that yeast extract can often contain small quantities of lactose (Nair *et al.*, 2009) which would undertake a natural induction of the expression system without the addition of IPTG.

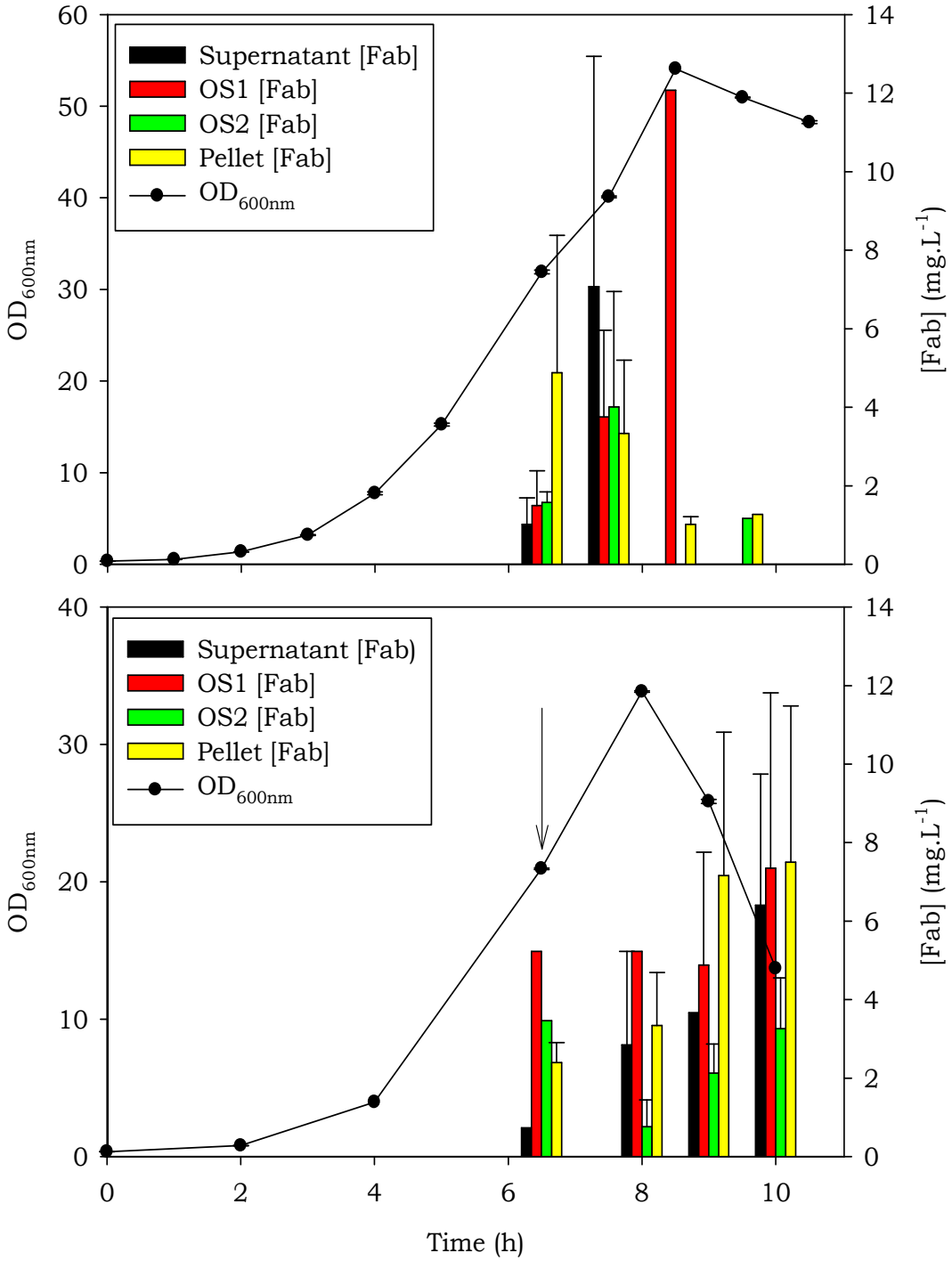

Figure 4.21. Batch fermentation of E. coli CLD048 (top; uninduced, bottom; induced). The arrow shows where 0.1 mM IPTG was added as an inducing agent.

It can also be seen in Figure 4.21. that the expression of the Fab fragment in the induced system has resulted in a more even distribution of the protein among the various intracellular compartments. This is evidenced by the

increased quantity of total protein in the induced culture, relative to the uninduced (a maximum of 27 $mg.L^{-1}$ and 18 $mg.L^{-1}$, respectively). It should also be noted, that, the specific productivity of the induced fermentation is approximately 2 $mg.L^{-1}.OD_{600nm}^{-1}$, considerably greater than the comparable shake-flask fermentation, although still lower than recorded by Hodgson *et al* (2006). There is no evidence of preferential partitioning of the Fab in the periplasmic space (as would be illustrated by a high proportion of Fab in osmotic shock solution 1). It is unlikely that the extent of collapse observed in the optical density observed here can be attributed to the production of the quantity of recombinant protein seen here. Even if a combined effect of gratuitous induction of the native lac operon and pre-induction derepression of the operon by lactose in the yeast extract were taken into account, this impact is anomalous. The best explanation for this data is that there is some portion of recombinant protein unobserved by the assay.

An ELISA is only capable of quantifying and antigen where the specific epitope is available and conformationally similar to that in which the initial antibody-antigen interaction took place. If protein is being produced at a sufficient rate to overwhelm the protein translocation system, this can lead to an accumulation of improperly folded or truncated recombinant proteins that sequester chaperone functions within the cell for their disposal, rendering the organism less able to respond to other stresses it might encounter, such as endogenous protein misfolding at high growth rates (Gasser *et al.*, 2008; Johansson *et al.*, 2008).

It should also be noted that the induction was carried out in the stirred-tank at twice the optical density of that in the shake-flask culture. This value was arrived at in an attempt to utilise approximately the same proportion of the growth curve for induction in each experiment; inducing at OD_{600nm} ≈ 10 for shake-flasks with a maximal potential OD_{600nm} ≈ 25, compared with induction at OD_{600nm} ≈ 20 for stirred-tanks with a maximal potential OD_{600nm} ≈ 50. This would entail a necessarily stronger induction (with more IPTG molecules per cell) in the shake-flask. Using the correlation mentioned earlier (Section 4.1. p. 108), which delivers a value of 1 OD unit ≈ 10^8 cells, it can be shown that the 0.1 mM solution of IPTG ensures that there are approximately 3 x 10^7 molecules per cell under stirred-tank induction and 6 x 10^7 molecules per cell for shake-flask induction. This quantity is likely enough to fully titrate all available binding sites on all copies of the *lac* repressor molecule even after expansion of the culture to the peak OD_{600nm} (10 molecules per cell, 4 binding sites per molecule (Lewis et al., 1996)).

The protein production within the bioreactor fermentation demonstrated that not only can greater quantities of protein be made than in shake-flask, but that there is a difference in the subcellular distribution of the recombinant product. The Fab found within the shake-flask culture was limited to the periplasm and latterly, the pellet. When produced by the stirred-tank reactor, recombinant Fab was first detected associated with the pellet, and a lesser quantity in the cytoplasm. This first detection of the Fab is an interesting departure from the observations made with the shake-flask culture, where there was no protein detected prior to induction (the time point where

induction is indicated was taken first, measured and the remaining culture subsequently induced). This is the first indication that there is some "leakiness" of the promoter allowing expression of the protein in the absence of the inducing agent. The patent which this cell line forms a part of specifically concerns the development of a non-leaky promoter system (Hodgson et al., 2006) making this result all the more intriguing.

Figure 4.22. correlates OD_{600nm} and DCW for the bioreactor culture and is much less convincing than in the shake-flask with an r^2 value of only 0.752 (compared with 0.948 in shake-flasks) described by the solid line. The uninduced fermentation of *E. coli* CLD048 and the wild type *E. coli* W3110 with IPTG (dashed and dotted lines respectively) both display significantly better correlations (1.000 and 0.999), suggesting that the deviation observed in the *E. coli* CLD048 induced fermentation is a result of the fall in optical density, and, further, that that fall is caused by the expression of the recombinant product. A qualitative observation was made during performance of the experiment; following induction there was difficulty compacting the pellet prior to dry cell weight analysis with *E. coli* CLD048 after induction with IPTG. The uncompacted mass was poured away with the supernatant, which should have caused the DCW to be underestimated, relative to previous cultures. In this case, the DCW is overestimated, in comparison with the highly-correlated cultures. This inability to form a compact pellet (along with an unclarified supernatant) was probably not related to the differences in the relationship between OD_{600nm} and DCW. The most probable explanation for this observation is cells in the bioreactor have

become more dense than those in the shake-flask culture with the unsedimented particles being cellular debris, where the centrifugal force was not sufficient to sediment them along with the cells.

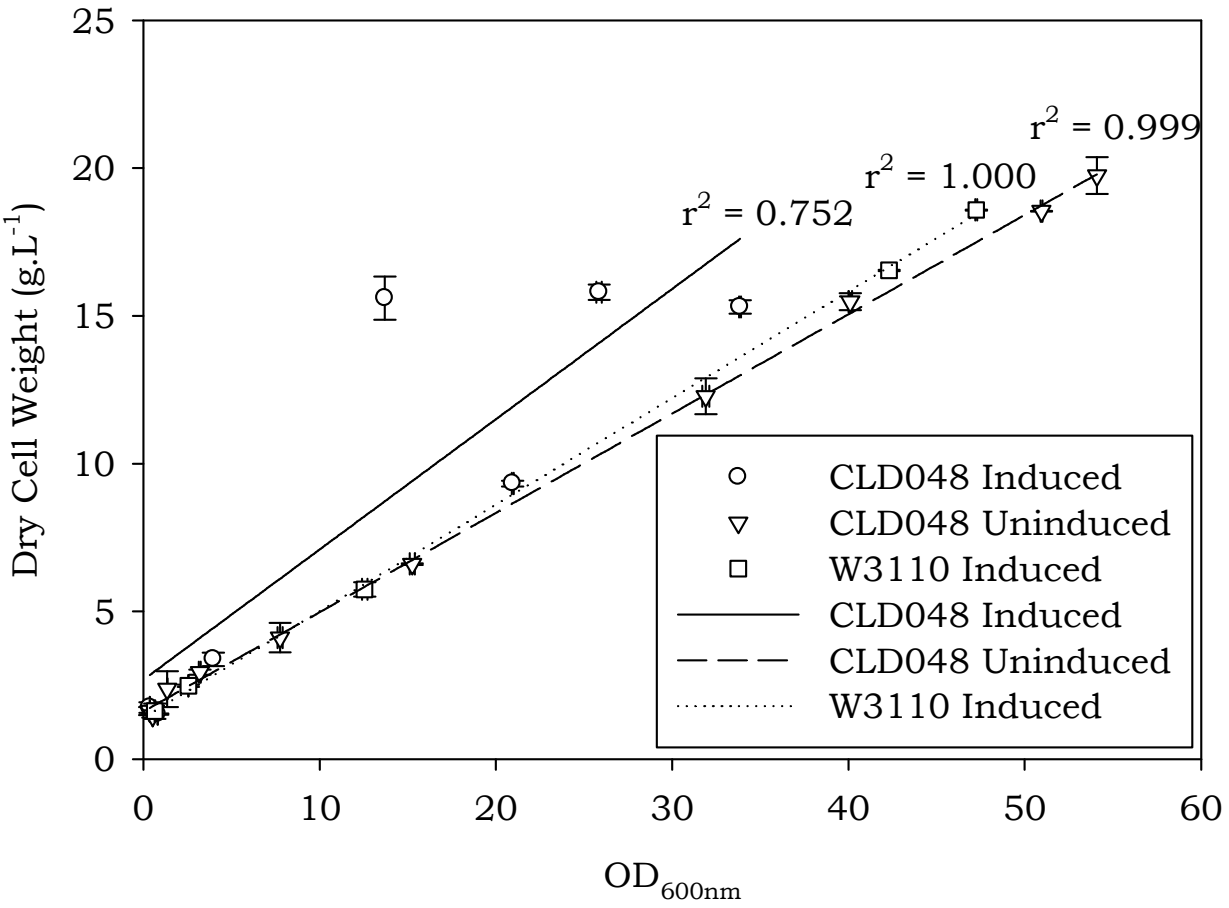

Figure 4.22. OD_{600nm} vs DCW with r^2 value for *E. coli* batch stirred-tank fermentation. The data plotted are mean values of 4-6 replicates from single experiments. The error bars indicate the standard deviation of the data from. Induction was carried out by addition of 0.1 mM IPTG at an $OD_{600nm} \approx 20$.

Akerlund *et al* (1995) demonstrated that the medium and growth conditions experienced by a cell can significantly affect the size of the cell, without a concomitant effect on the turbidity. It is, therefore, a reasonable postulation that other growth parameters (such as DCW) may become disconnected from spectrophotometric determination of cell proliferation. It has also been

established that for thy$^-$ phenotypes, the average cell mass increases with decreasing thymine availability in the medium (Zaritsky and Pritchard, 1973; Begg and Donachie, 1978) however, the complexity of the medium used here makes it impossible to determine whether there might be some component in this medium causing a similar phenomenon. The development of several subpopulations of cells differing in their buoyant density was discovered by differential gradient centrifugation of *Escherichia coli* cells following transition from exponential to stationary phase (Makinoshima *et al.*, 2002), lending further weight to the interpretation that the growth conditions the cells find themselves in can strongly affect simple bioprocess metrics such as dry cell weight. Whilst it cannot be definitively stated that any of the above mechanisms are at work in the batch fermentation, it, at least, demonstrates that these kinds of effects can be explained in terms of cellular physiology/biochemistry, though such an investigation is beyond the scope of this work.

A much lower specific growth rate was achieved in the stirred-tank reactor (Figure 4.23.) than in the shake-flask (Figure 4.16.). The maximum specific growth rate achieved in the shake-flask culture was 1.2 h^{-1}, compared with 0.4 h^{-1} for the bioreactor. Both of these measurements were taken for their respective cultures between 2 and 4 hours, although the OD_{600nm} was 4 and 2 for the bioreactor and shake-flask respectively. Robinson *et al* (2001) showed a marked effect of inoculum size (in terms of total cell number) on length of lag phase. The shake-flask and stirred-tank fermentations in this study used 4 and 2 % inocula respectively, which could have been

responsible for the unexpected differences in growth rate. An increase in the percentage inoculum may negatively impact growth rate by increasing competition for resources, although there are no studies which have investigated this. Interestingly, the variation in specific growth rate appears to be consistent across each of the fermentations, whereas the optical densities are significantly different. It is probable that, without the disjunct between optical density and dry cell weight, observed in the induced, GMO, fermentation there would have been a concomitant downturn in the specific growth rate

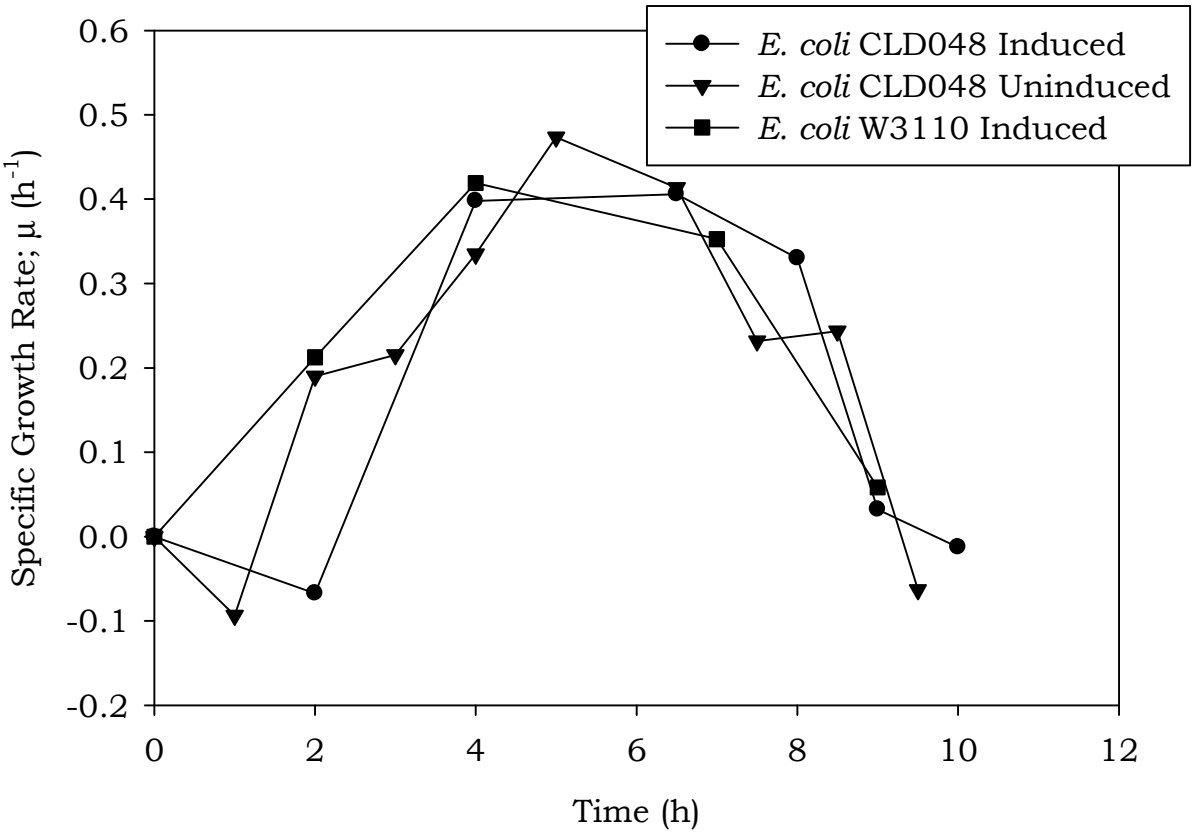

Figure 4.23. Variation of specific growth rate for *E. coli* batch stirred-tank fermentation in Figure 4.17. The data plotted are calculated from the mean DCW of 6-8 replicates per time point. Induction was carried out by addition of 0.1 mM IPTG at an OD ≈ 20.

Figure 4.24. shows the variation of specific growth rate as calculated as a function of optical density, rather than dry cell weight or cell number. What is much more clearly shown, in this example is that, despite a similar picture overall, the end of the fermentation is clearly differentiated by dropping much farther below 0 than the two control experiments.

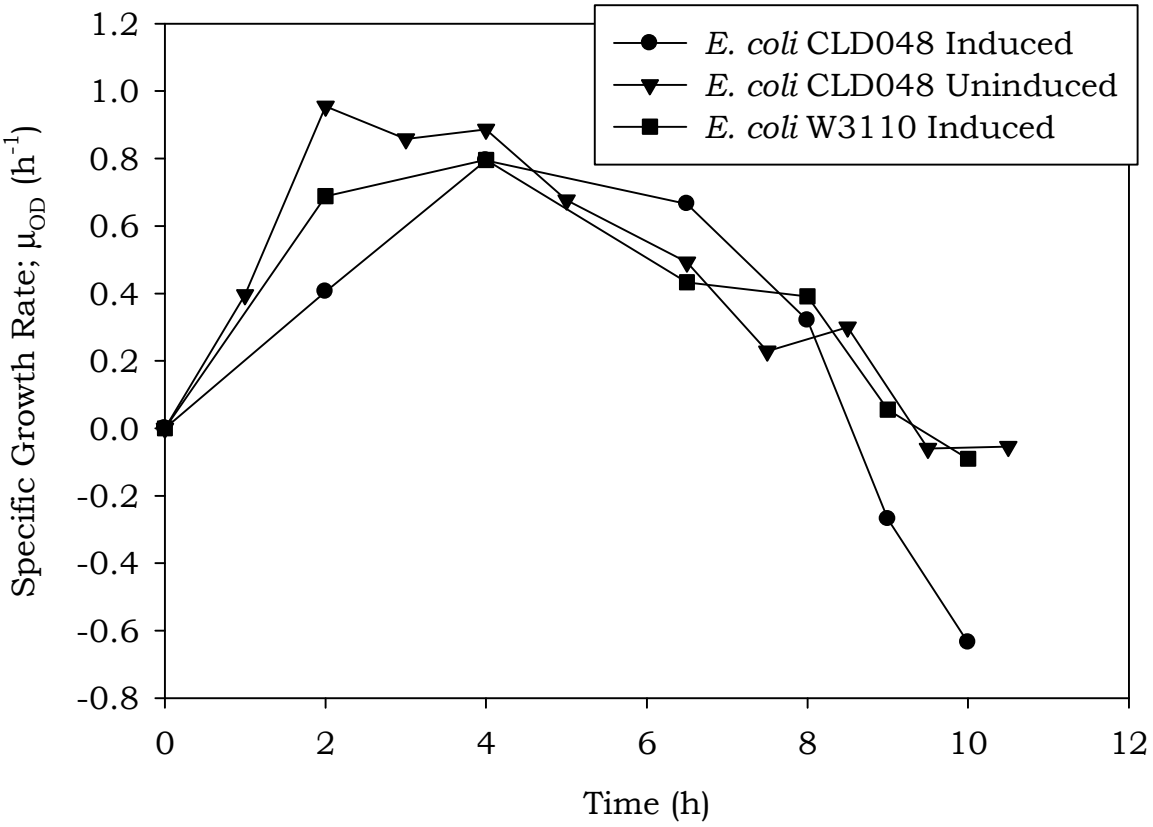

Figure 4.24. Variation of specific growth rate, as calculated using optical density from the same dataset shown in Figure 4.23.

The induction of the bioreactor fermentation exerts a similar effect, over a similar timescale, as in the shake-flask culture, in spite of the fact that the bioreactor was induced at double the OD_{600nm} of the shake-flasks. This may have resulted from the shake-flasks remaining unagitated during induction with an associated confinement of the added IPTG to a subpopulation of cells

within the locus of the addition. However, the stirred-tank reactor was kept operational whilst the inducing agent was added, distributing the compound more thoroughly among the cells enabling induction of a greater number of them, causing the steeper decline in the culture.

When analysed by flow cytometry, the inoculum culture consistently yielded similar results showing a large proportion of the cells to be green fluorescent, indicating a compromised cytoplasmic membrane potential. As can be seen in Figure 4.25. below, this, much like the shake-flask culture transitions to a healthier state, exhibiting negligible green fluorescence. The sample immediately following induction (8 h) already shows signs of an increase in green fluorescent cells, 6.5 %; comparable with a similar stage in the shake-flask fermentation. After only 2 more hours, however, the proportion of green fluorescent cells has increased to more than 65 % (including 11 % fluorescing red and green simultaneously). In contrast, the shake-flask culture achieves a maximum of just over 50 % green fluorescent cells, further evidence of the additional deleterious effect of the bioreactor on the cells relative to the shake-flask experiments. Images from control experiments can be found in appendix (8.1. and 8.2.)

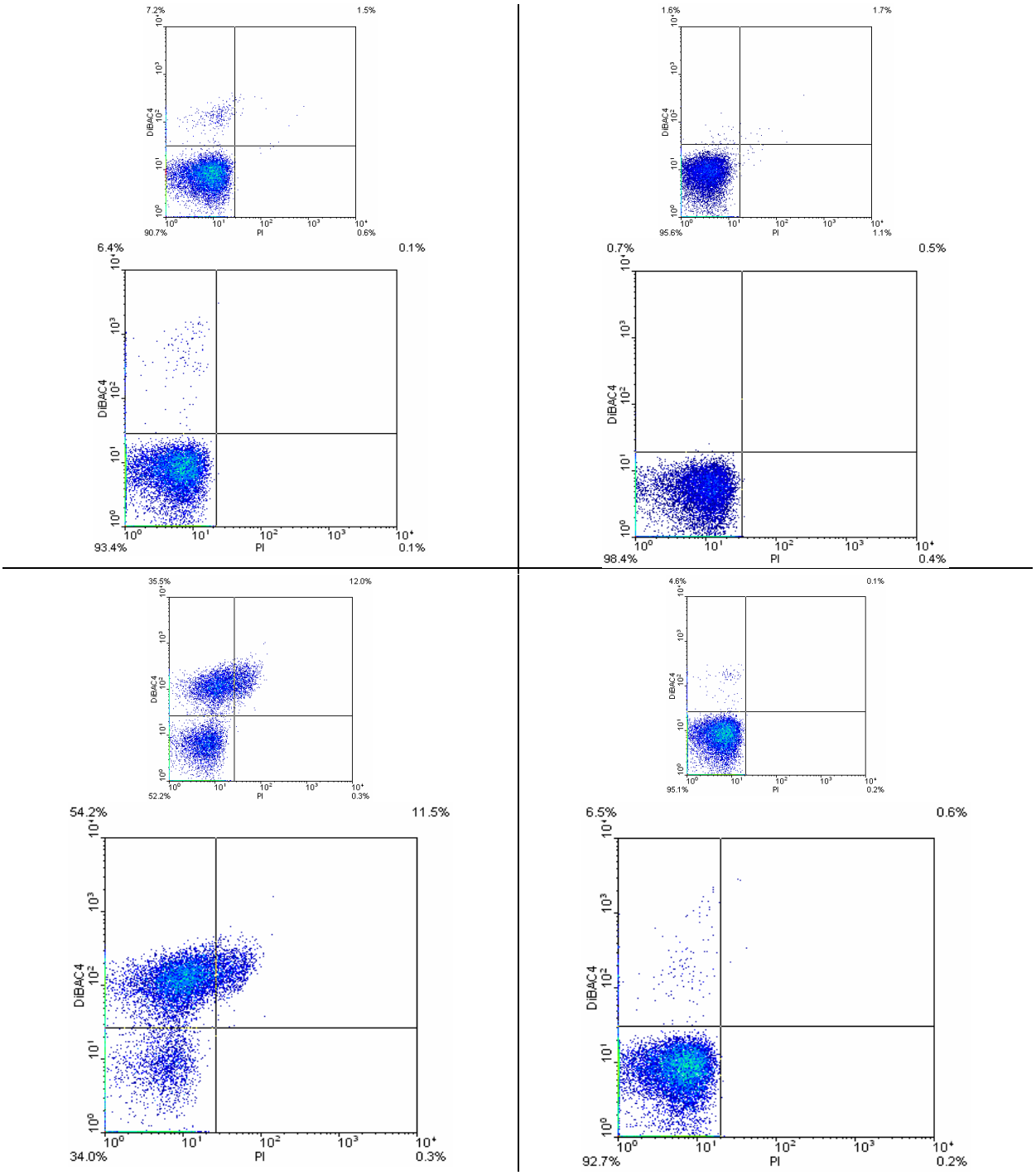

Figure 4.25. Flow cytometric analysis of *E. coli* batch stirred-tank fermentation stained with PI & $DiBAC_4(3)$. Cells were induced at OD_{600nm} ≈ 20 using 0.1 mM IPTG. Clockwise from top left: Inoculum; 6.5; 8(8.5) and 10(9.5) hours. Figures in parentheses show the time points from the alternative fermentations. Each quadrant shows similar time points from different batch fermentations Data from equivalent control experiments can be found in Appendix (8.1. & 8.2.)

Following the repeat of the analysis performed on the shake-flask fermentation, transmission electron microscopy was employed in order to attempt to discern any visible effect on the cell as a result of induction. The resulting electron micrographs (Figure 4.26.) show that prior to induction the periplasm is clearly visible between the two constituent membranes, representing a very small portion of the cell. Images from the uninduced *E. coli* CLD048 batch fermentation are included in the appendix (8.1.1. and 8.2.1.).

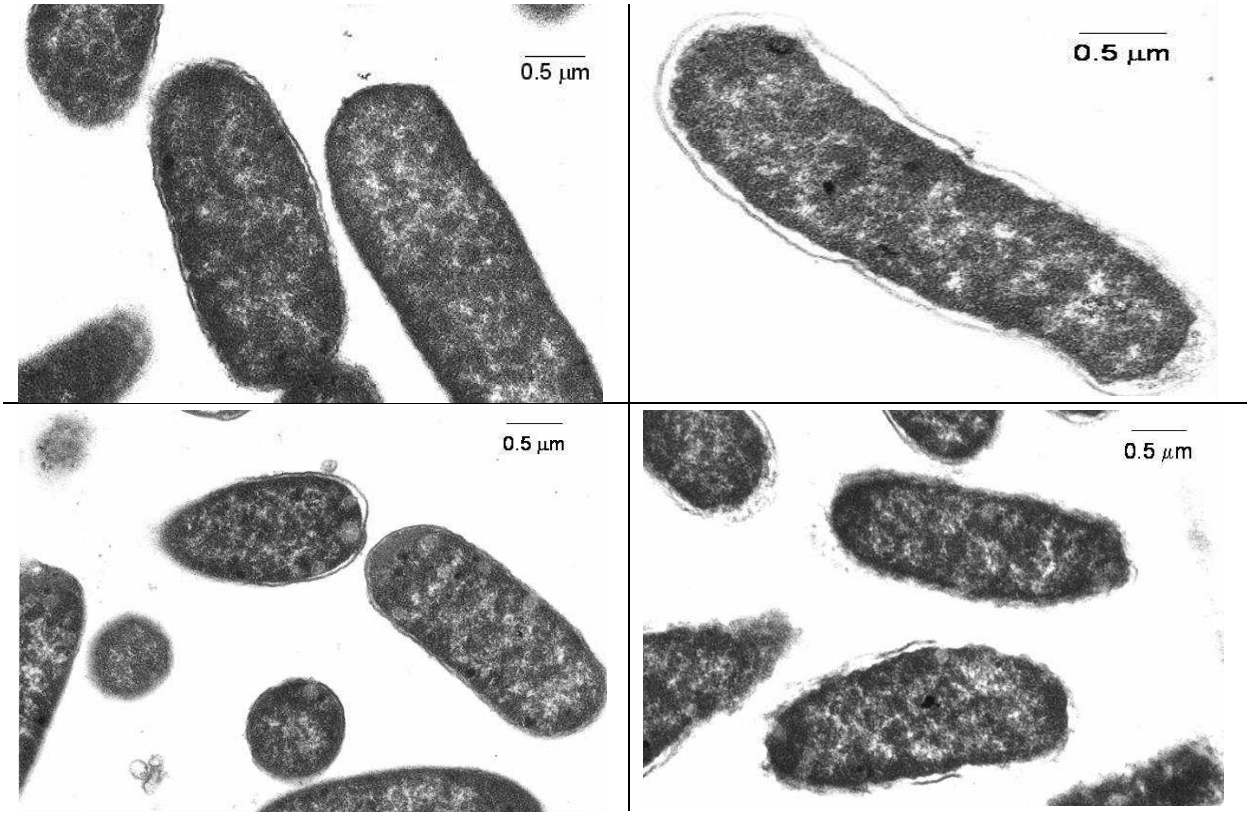

Figure 4.26. Transmission Electron Micrographs of *E. coli* CLD048 induced, batch stirred-tank fermentation in Figure 4.17. Cells were induced at OD_{600nm} ≈ 20 using 0.1 mM IPTG. Clockwise from top left: 6; 8, 9 and 10 hours. Analysis was performed on samples from a single fermentation. Data from equivalent control experiments can be found in Appendix (8.1. & 8.2.)

Only 1.5 hours after induction an obvious increase in the breadth of the periplasm can be observed, even when the increased magnification is taken

into account. At this stage, the levels of Fab detected by ELISA were similar to that of the uninduced control (where this breakdown of the periplasmic membrane was not observed; Figure 8.1.1 and 8.2.1.).

At this stage in the culture, there was no Fab detectable in the periplasmic fraction (OS1) however; the presence of additional material in the periplasm seems the most plausible explanation for this effect. Ami *et al* (2009) demonstrated that protein misfolding and aggregation adversely affected bacterial membrane structure, and these events would also explain the lack of protein detected by the ELISA.

When the same analysis is performed after 9 and 10 hours, the breakdown of the periplasm can be seen directly, especially after 9 hours where intact sections of membrane can be seen interspersed with gaps. These images confirm the proposed mechanism of release of recombinant protein into the extracellular space, with only a partial periplasmic membrane. The ideal goal, from a complete bioprocess perspective would be to manage this membranous breakdown such that the periplasmic membrane is removed, whilst maintaining the integrity of the individual cells.

The transmission electron microscopy failed to detect any partially disrupted or ghost cells, however, one of the major weaknesses in this technique is the low level of statistical resolution. Only a handful of cells can be captured in any one image, and these images are, as far as possible, representative of those observed in a wider observation of 30-40 microbes per analysis point.

With the analysis of this batch fermentation, we appear to have conflicting data, with some indications of widespread cell death (OD_{600nm}, $CFU.mL^{-1}$, DCW) and some techniques in which the effects of this are conspicuously absent (flow cytometry, transmission electron microscopy).

4.3.1. Conclusions

There is considerable evidence that the repression system used in this organism is "leaky" with the induced culture producing 27 $mg.L^{-1}$ compared with 18 $mg.L^{-1}$ for the uninduced culture. The increased Fab expression is associated with a sudden and rapid decline in both total and viable cell number, moreso than shown in shake-flasks. Despite the expression system being designed for periplasmic expression, there is no preference for this compartment in terms of accumulation of conformationally active Fab (as would be demonstrated by increased Fab concentrations in OS1). In fact, During the decline phase of the culture the quantity associated with the insoluble fraction is dominant comprising 30-40 % of the total. The leakiness, combined with the increasing proportion of Fab present in cellular compartments other than the periplasm show that this system, at least in batch mode, is poorly optimised for the intended purpose.

4.4. *E. coli* CLD048 Fed-Batch 1 Stirred-Tank Reactor Fermentation

Following on from the batch fermentation, a fed-batch protocol was obtained from Avecia. The protocol consists of an initial batch phase, followed by a feed, enabling the bacteria to achieve a higher optical density than that in the original batch experiment. The fed-batch culture was induced at an OD_{600nm} of approximately 50. The onset of feeding was triggered by an increase in the dissolved oxygen within the reactor, at which point it was assumed that the level of glycerol in the reactor had been exhausted. With no reliable, at-line method in use during this process to measure glycerol, the dissolved oxygen in the medium was used as a surrogate. As the *E. coli* culture switches from metabolising glycerol to a secondary energy source (in this medium likely to be some constituent of the yeast extract) a brief pause in the consumption of oxygen would be expected as the cells upregulate the enzymes necessary to carry this out.

The point at which to add the feedstock was determined by monitoring of the dissolved oxygen. Figure 4.27. shows the variation of dissolved oxygen for duplicate runs of *E. coli* CLD048 induced, uninduced and *E. coli* W3110 induced. The onset of feeding was triggered by manual activation of a pre-calibrated pump, when the dissolved oxygen began to rise. Each of the cultures shows that, from an initially saturated state, the oxygen in the medium is consumed at a rate faster than it can be supplied by the growing organisms. Midway through this descent, there is a distinct, and conserved, double-peak where the dissolved oxygen rises, before continuing its fall. It is

believed that this rise is attributable to an alteration in the cells' primary source of energy, due to exhaustion. Attempts to quantify the glycerol in this culture were unsuccessful, however, alteration in growth as a result of adaptation to an alternative energy source is well-understood (Loomis and Magasanik, 1967). In this case, there is no observable change in the growth rate, however, it is plausible that a shift in the intracellular biochemistry required to make a change of this type would likely alter the usage of oxygen. The fact that this lapse in oxygen usage is so short also explains the apparent lack of an effect on the growth of the bacteria. Hofmann *et al* (2010) showed that it is possible to detect exactly the change in metabolism that has been proposed here using an online probe, whilst Losen *et al* (2004) showed that the depletion of glycerol in a rich medium caused the oxygen transfer rate to fall, which would have the effect of increasing dissolved oxygen as observed in Figure 4.23.

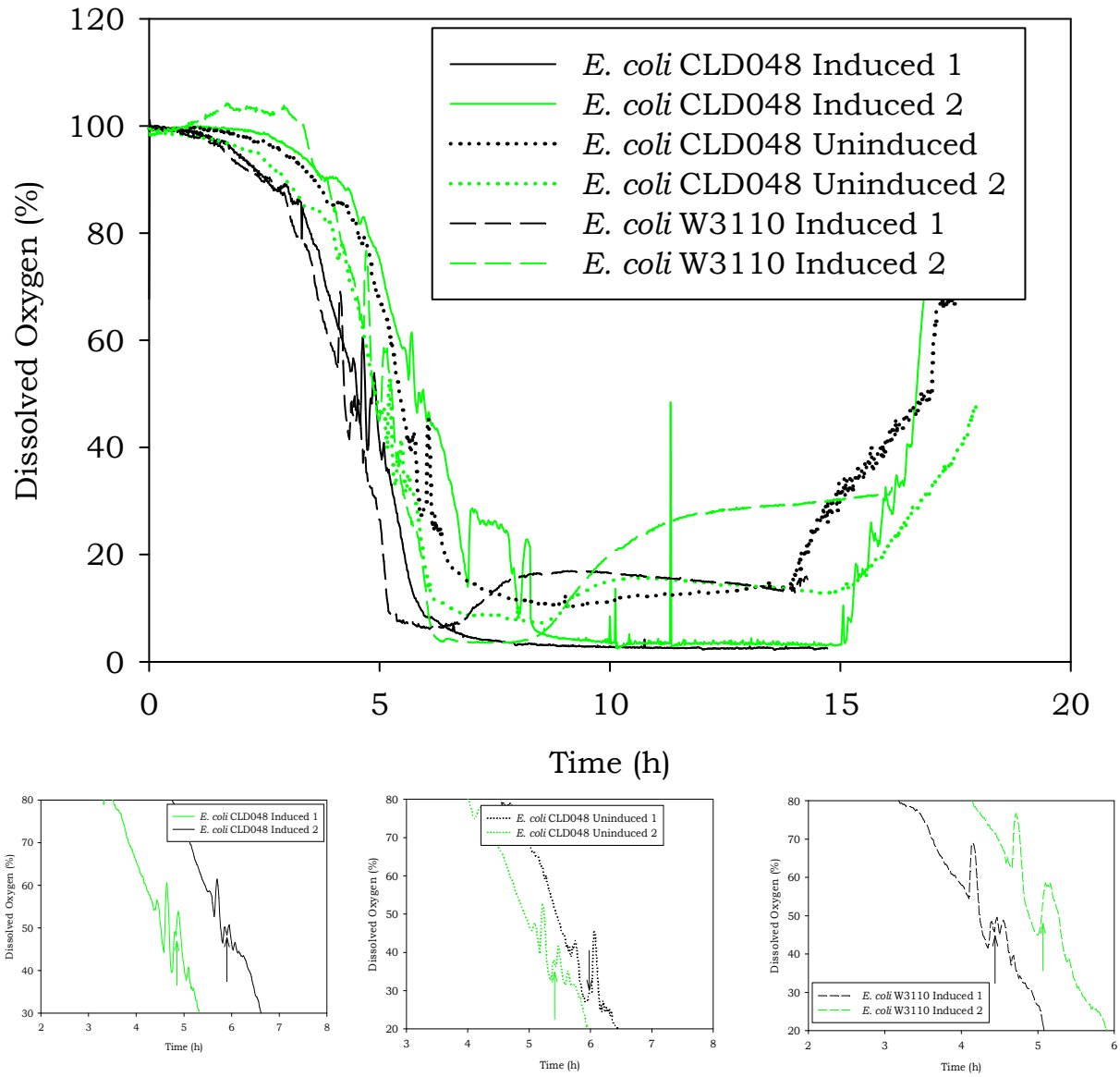

Figure 4.27. Top: Plot of the dissolved oxygen measured in the first fed-batch fermentation protocol. Bottom Left-Right: Plots showing close-up views of the point of feeding for *E. coli* CLD048 induced and and uninduced and E. coli W3110 induced, respectively. Arrows indicate the point of onset of feeding for each fermentation

The lower plot in Figure 4.28. shows the variation of optical density during the fed-batch process. The top plot indicates the detail of when the cultures were fed, using the time points from Figure 4.27. What is evident from the top plot here is that each of the cultures was fed at approximately the same optical density (20-24). That this fluctuation in dissolved oxygen occurs consistently across a specific, small, range of optical density values, provides

additional support for the suggestion that said fluctuation is related to a change in internal metabolism.

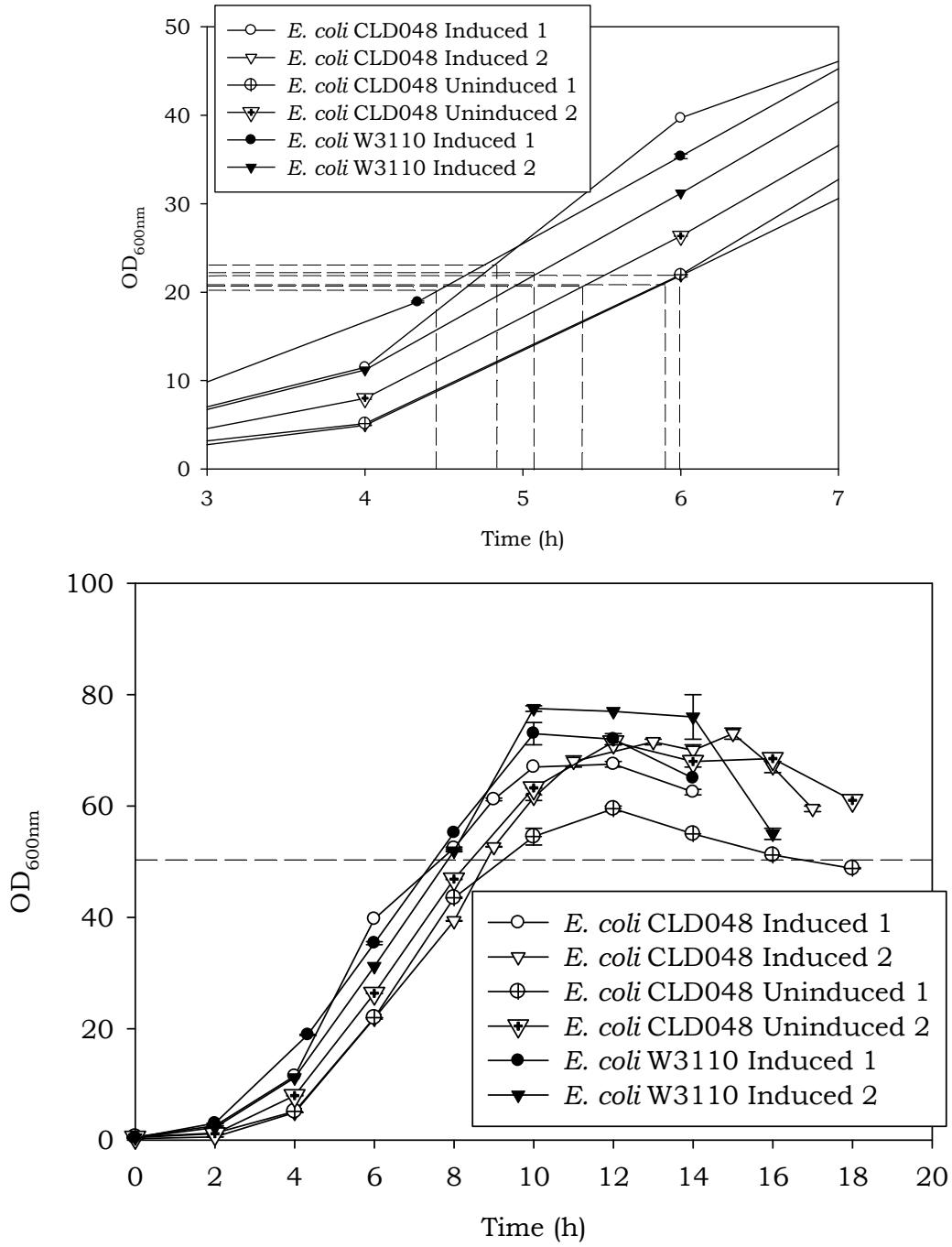

Figure 4.28. *E. coli* CLD048 fed-batch stirred-tank fermentation. All points plotted are mean values from 4 replicate analyses. The error bars show the standard deviation. Induction was carried out at OD_{600nm} ≈ 50, the point on the curve nearest to the dashed line, using 0.1 mM IPTG, immediately following analysis of the sample. The top plot indicates the point of feeding onset with droplines.

When the antibody productivity for the induced and uninduced fermentations is compared (Figure 4.29.) it is clear that the promoter leakiness observed during the batch culture is still evident. The level of expression from the uninduced system is lower than seen in the equivalent batch fermentations however shows a similar trend of being unconcentrated into any particular subcellular compartment, as well as being approximately constant over the range of samples analysed. However, the induced culture produced far more antibody, based on peak values, than either the uninduced or batch cultures with a maximum of 35 $mg.L^{-1}$. This value was achieved a very short time following induction, with the titres in samples following it markedly lower (although still achieving a total of 11 $mg.L^{-1}$). The sudden drop in optical density, following induction, that was previously observed in the batch experiment was not found here. This might be attributable to a number of factors; firstly, the induction of the fed-batch was carried out at a much higher optical density, necessarily meaning a lower quantity of inducer per cell than in the batch fermentation, although the inducing agent is still likely in excess, relative to *lac* repressor binding sites (p.150).

The fed-batch was also successful in preventing the large drop in optical density, post-induction. Due to the large excess of inducer, it is unlikely that the increased cell numbers at induction is responsible. However, the continual supply of glycerol has probably enabled the cells to mount a better response to the stress of heterologous protein production.

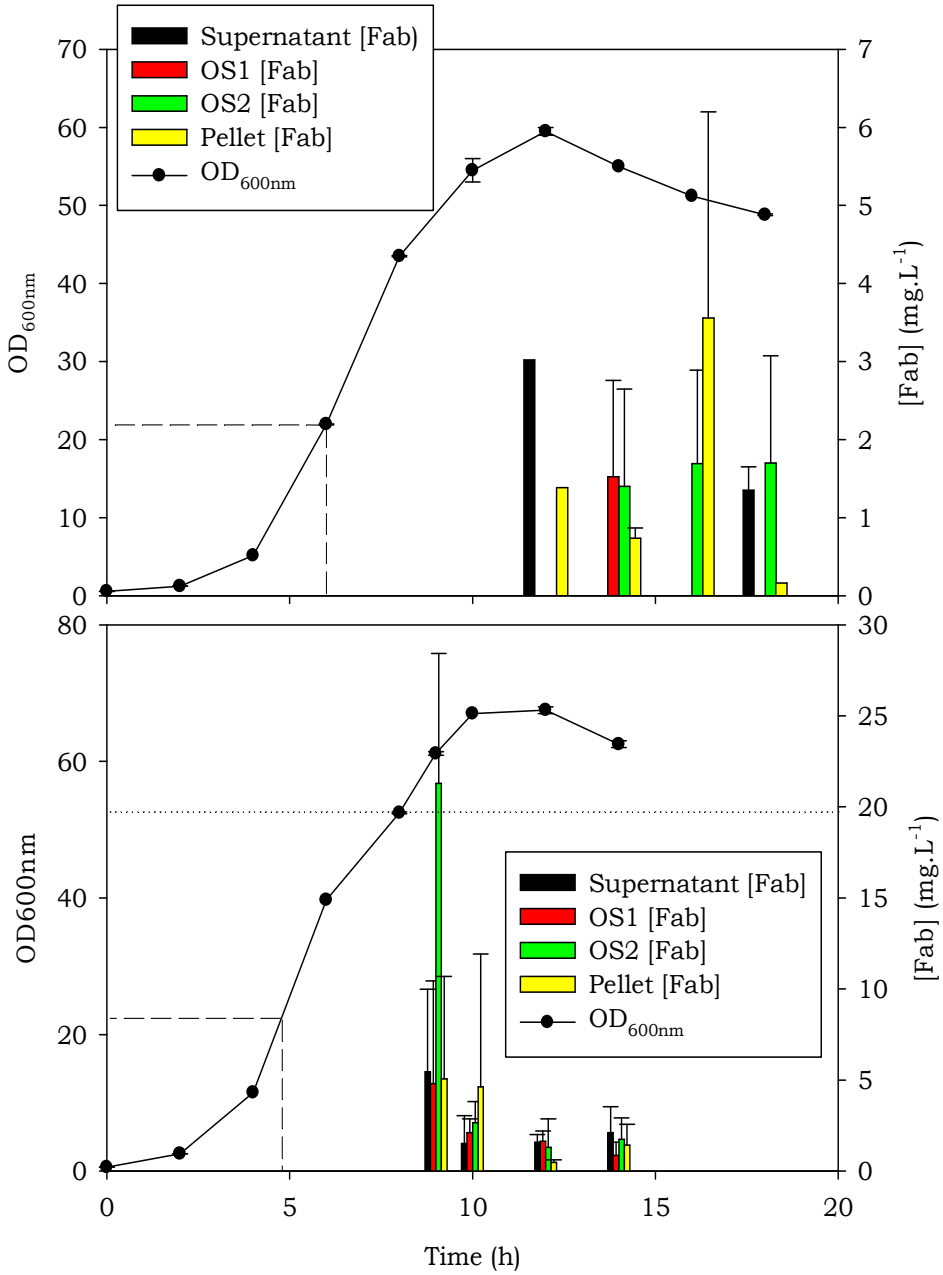

Figure 4.29. Fed-batch fermentation 1 of E. coli CLD048 (top; uninduced, bottom; induced). The dashed lines indicate the point where feeding was initiated, in response to a change in dissolved oxygen (Figure 4.27.). The dotted line shows where 0.1 mM IPTG was added as an inducing agent.

This feeding strategy is markedly dissimilar to others found in the literature, which usually rely on using the feed to control the concentration of a growth limiting substrate (Ramírez and Bentley, 1995; García-Arrazola et al., 2005). It is overly simplistic to suggest that merely the combination of feed strategy and feed composition was sufficient to limit the productivity of the

fermentation. Garcia-Arrazola *et al* (2005) used a similar level of glycerol in both the primary medium and the feed in order to obtain significantly higher yields of a periplasmic Fab, in the region of 0.1 $g.L^{-1}$. This increased yield relative to the fed-batch protocol described here is just that available in the periplasmic space, so it is likely that additional optimisation or recovery from additional subcellular fractions could increase this yield still further. There are a number of factors whose effect probably accumulated to exert this positive effect on productivity. The most important of these would be;

- Temperature: Garcia-Arrazola controlled at 30 °C before reducing to 27 °C after induction. The stated aim of this was to reduce the level of misfolding of protein.
- Induction with lactose: lactose is a less potent inducer than IPTG, and is also actively metabolised in the cells which it induces, reducing its effectiveness with time. This prevents the cellular protein production apparatus from being overwhelmed by recombinant transcripts.
- Gas blending: Maintaining constant impeller speed at a relatively low leve, and combining this with gas-blending with oxygen, enabled the creation of an oxygen unlimited environment within the bioreactor, without the necessity of harsh agitation conditions.

The rationale for using glycerol as the primary carbon source was, at least in part, a result of the commonly held belief that *E. coli* is unable to produce acetate when utilising glycerol as a primary carbon source (Koh *et al*, 1992; Han *et al*, 2003 Shiloach and Fass, 2005). An extension of this opinion is

that there is no requirement for feed rate restriction when using a glycerol feed, as compared to glucose-based cultures capable of producing acetate concentrations sufficient to inhibit growth and recombinant protein production (Luli and Strohl, 1990). However, both Garcia-Arrazola *et al* (2005) and Guebel *et al* (2009) have both shown that acetate can be produced by *Escherichia coli* growing on glycerol. The latter used principal component analysis to determine that the acetate was not a by-product of overflow metabolism caused by oversupply of glycerol. Both studies also failed to produce enough acetate to reach 2 $g.L^{-1}$, the level known to cause product and growth inhibition (Luli and Strohl, 1990). The source of acetate suggested by Guebel *et al* (2009); ornithine, was likely not present in significant quantities in the defined medium of Garcia-Arrazola, hence their conflicting conclusion that overflow metabolism was the cause of the acetate accumulation in that case.

Figure 4.30. illustrates that the linear correlation between OD_{600nm} and dry cell weight is maintained throughout the fed-batch process. This trend is consistent across both induced and uninduced GMO cultures, as well as induced wild type fermentations. This is in contrast with the batch processes, where a significant drop in the optical density was recorded following induction. It is clear from the control experiments that this breakdown is not attributable to an effect of the inducer on the cell, in the absence of the inducer, nor to the presence of the recombinant material and associated selection pressure.

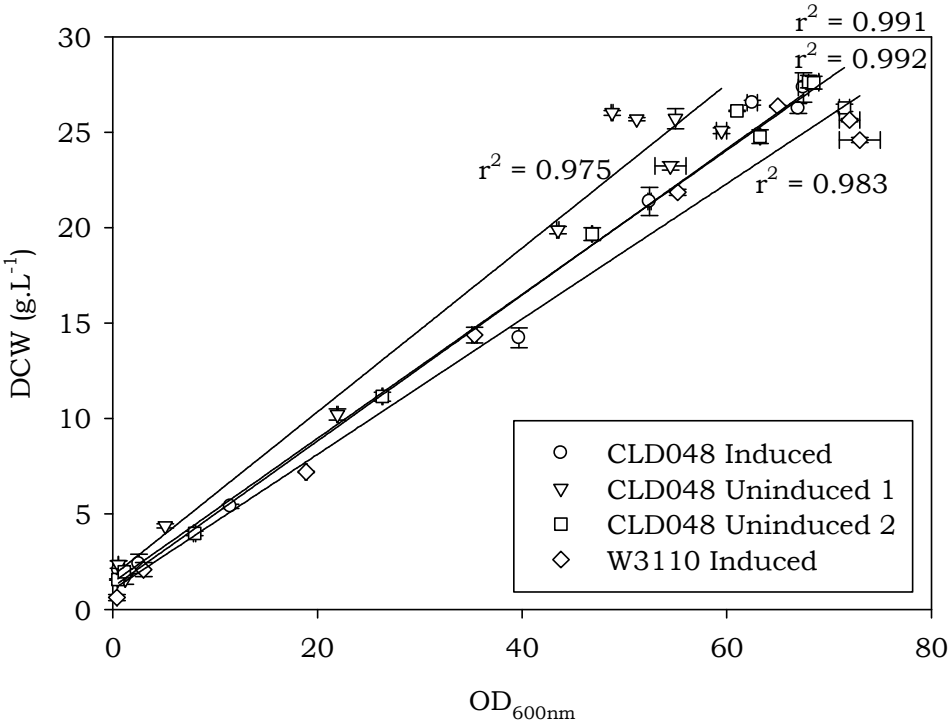

Figure 4.30. *E. coli* fed-batch stirred-tank fermentation. All points plotted are mean values from replicate analyses within single experiments. The error bars show the range of the data from which the mean was calculated. Induction carried out at OD_{600nm} ≈ 50 with 0.1 mM IPTG. The included correlation coefficients refer to the closest fitted curves.

The variation of specific growth rate for the fed-batch culture (Figure 4.31.) was very similar to the batch fermentation for the first 4 hours, unremarkable given that this is the "batch" phase of the fed-batch process. Feeding had an immediate impact on the cells, with the fed-batch culture reaching a peak of 0.5 h^{-1} compared with 0.4 h^{-1} for the batch. Following this peak, the cells in the fed-batch experience a gradual descent in their specific growth rate, with their deceleration into stationary phase. Conversely, the batch culture experienced a precipitous decline in specific growth rate, further showing the lesser impact of the induction on the cells in the fed-batch culture. Ihsen & Egli (2004) have shown that reduction of specific growth rate can increase the general stress response in *E. coli*. The fed-batch process experiences a protracted decline in μ as the fermentation proceeds;

this could lead to increases in concentration of intracellular proteases yielding one explanation for the reduced product yield in this fed-batch, compared with the batch protocol.

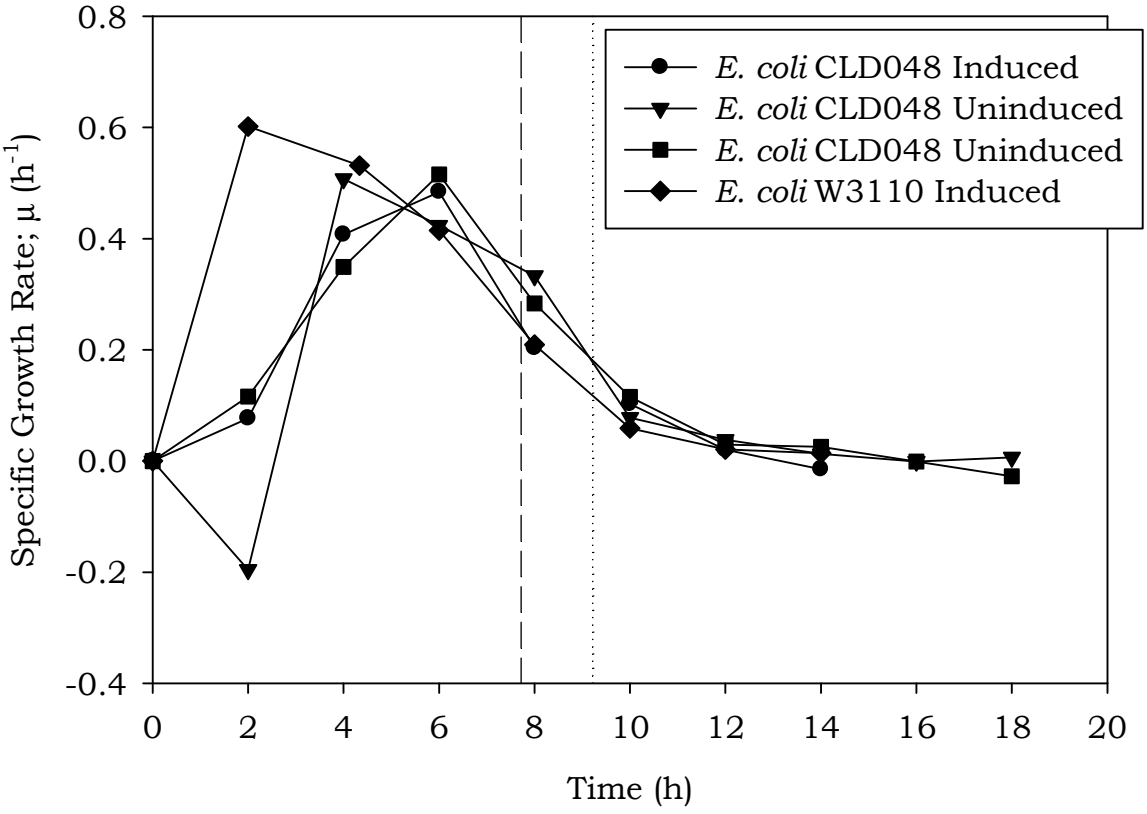

Figure 4.31. Variation of specific growth rate for fed-batch stirred-tank fermentations in Figure 4.24. The data plotted are calculated from the mean DCW. Induction was carried out by addition of 0.1 mM IPTG at an OD_{600nm} ≈ 50 (CLD048, dashed line; W3110, dotted line).

Multiparameter flow cytometry using PI and $DiBAC_4(3)$ (Figure 4.26.) shows a dearth of either green or red fluorescent cells, concurring with the maintenance of high optical density and dry cell weight, even after induction. Given this large proportion of healthy cells (> 90 % throughout the fermentation), it is apparent that the induction of this fed-batch culture is having a far less detrimental effect on the survival and proliferation of the cells.

The lack of deviation from the physiological norm in the cytoplasmic membrane potential is supported by the absence of OD_{600nm} decrease following induction. Despite the higher level of total protein manufactured by the fed-batch fermentation, the reason for the lack of an observable effect on the culture may be illuminated by the specific productivity. The batch fermentation Fab concentration peaked at 3.5 $mg.L^{-1}.g_{DCW}^{-1}$, however, this was measured in the period of culture decline after the optical density had halved from the highest value. The importance of this is the impact of this point in the culture on the future processing of the product, increasing the quantity of contaminating materials (host cell protein, host cell DNA, etc.) which complicates the purification. In comparison, the fed-batch fermentation achieved a specific productivity of 1.4 $mg.L^{-1}.g_{DCW}^{-1}$ at the peak total Fab concentration. This difference explains why the levels of stress observed, by flow cytometry, in the batch fermentation were greater than in the fed-batch method.

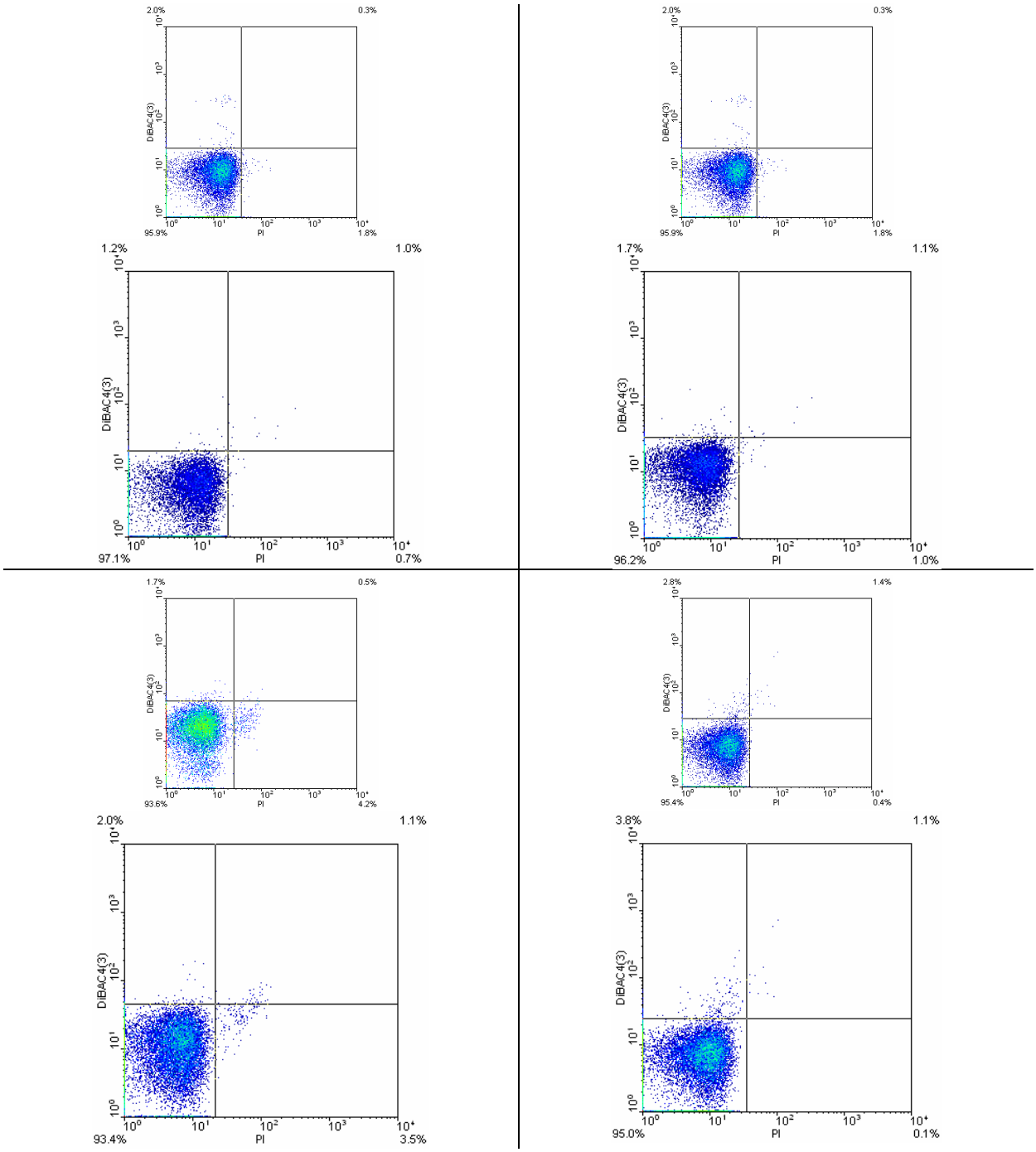

Figure 4.32. Flow cytometric analysis of *E. coli* CLD048 fed-batch stirred-tank fermentation in Figure 4.28. stained with PI & $DiBAC_4(3)$. Cells were induced at OD_{600nm} ≈ 50 using 0.1 mM IPTG. Quadrants show similar time points from two different fermentations; clockwise from top left: 4; 8; 10 and 14 hours. Data from equivalent control experiments can be found in Appendix (8.1. & 8.2.)

Images of the cells in Figure 4.33. show that prior to induction the periplasm is intact, as would be expected for healthy cells and demonstrated earlier

with the batch fermentation. Following induction and at the latter stages of the culture, the two membranes (cytoplasmic and periplasmic) are less well defined. This could be the result of a similar breakdown that was more clearly observed during the batch fermentation (Figure 4.26.) precipitated by the passage of large quantities of protein through the cytoplasmic membrane into the periplasm. Damage to the cell membrane integrity is a well-studied phenomenon resulting from periplasmic overexpression of recombinant proteins (Shokri et al., 2003; Balagurunathan and Jayaraman, 2008). Shokri *et al* (2004) showed that changes in membrane structure during fed-batch processes at low specific growth rate can increase the robustness of the organism to mechanical and other stresses, which offers a reason for the maintenance of cell membrane integrity here.

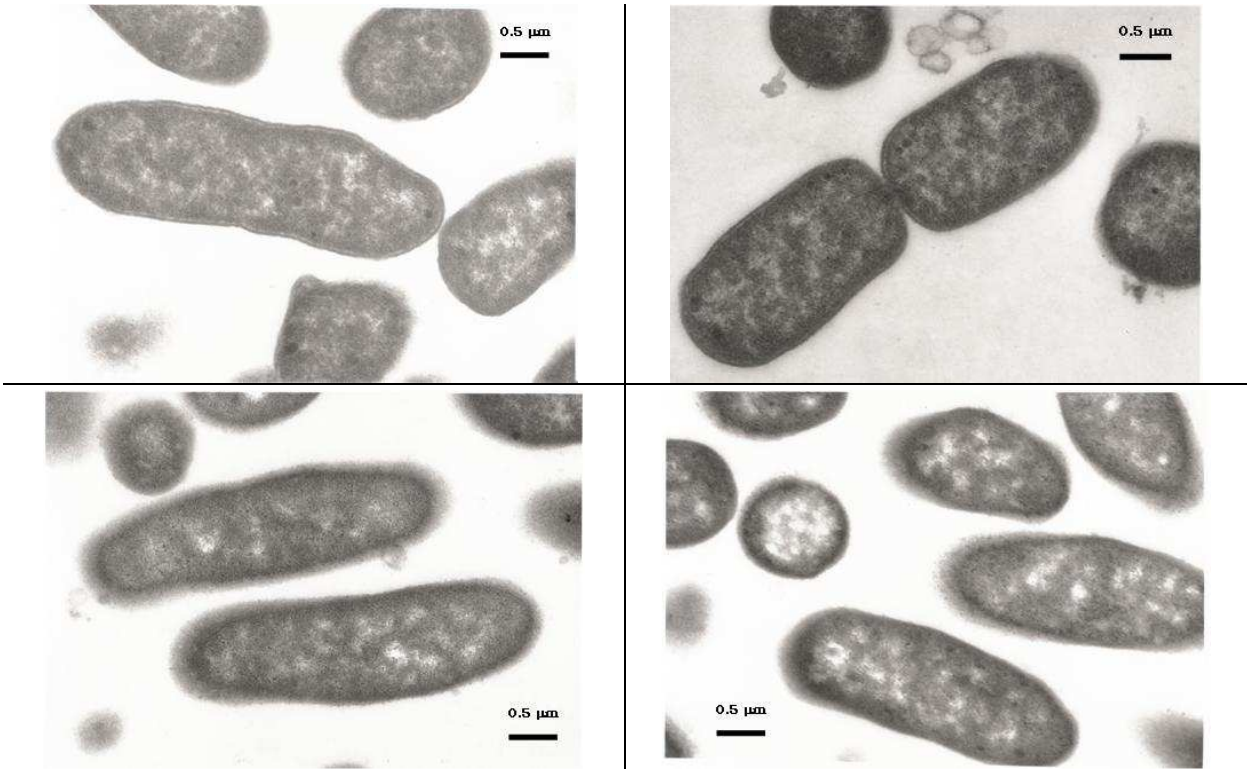

Figure 4.33. Transmission electron micrographs of *E. coli* fed-batch stirred-tank fermentation in Figure 4.28. Cells were induced at OD_{600nm} ≈ 50 using 0.1 mM IPTG. Clockwise from top left: 6; 8, 12 and 14 hours. Analysis was performed on samples from a single fermentation. Data from equivalent control experiments can be found in Appendix (8.1. & 8.2.)

4.4.1. Conclusions

A simple feeding strategy using a high-glycerol feedstock can achieve significant increases in culture turbidity over the batch process. 1.4 $mg.L^{-1}g_{DCW}^{-1}$ recombinant Fab can be expressed during the fed-batch process, which, whilst less than achieved in batch mode (3.5 $mg.L^{-1}.g_{DCW}^{-1}$), is attained without the associated decline in the culture. There is no apparent partitioning of the product into the periplasmic space (as would be indicated by more of the Fab being present in OS1 following osmotic shock). This fed-batch process is a viable method for production of this Fab, without the associated breakdown in cell integrity observed in the batch process. Employment of a method of this type would decrease the load on subsequent downstream purification, but the low titres observed here indicate that there is considerable room for optimisation.

4.5. *E. coli* CLD048 Fed-Batch 2 Stirred-Tank Reactor Fermentation

Having demonstrated that recombinant Fab could be manufactured by the cells with both a batch and fed-batch operating mode, it was decided to alter the protocol in an attempt to improve product yield. It was surmised that, rather than separating the induction phase from the feeding phase, Fab yield might be improved through feeding and inducing at the same time, with the feed triggered by the same rise in DO as in the previous protocol. The concentration of IPTG per cell is greater at the point where feeding is commenced ($OD_{600nm} \approx 20$) than in the earlier fed-batch method ($OD_{600nm} \approx 50$), resulting in a stronger induction. In concert, the addition of a highly concentrated glycerol feed may serve to mitigate the impact of this induction on the cells by increasing the available energy resources.

Figure 4.34. shows the variation of dissolved oxygen with time for fermentations of *E. coli* CLD048 (with control experiments performed with uninduced CLD048 and *E. coli* W3110). It can clearly be seen that the same fluctuation in dissolved oxygen is apparent in this culture, with the second of these perturbations signifying the point of addition of feed (and inducing agent where relevant).

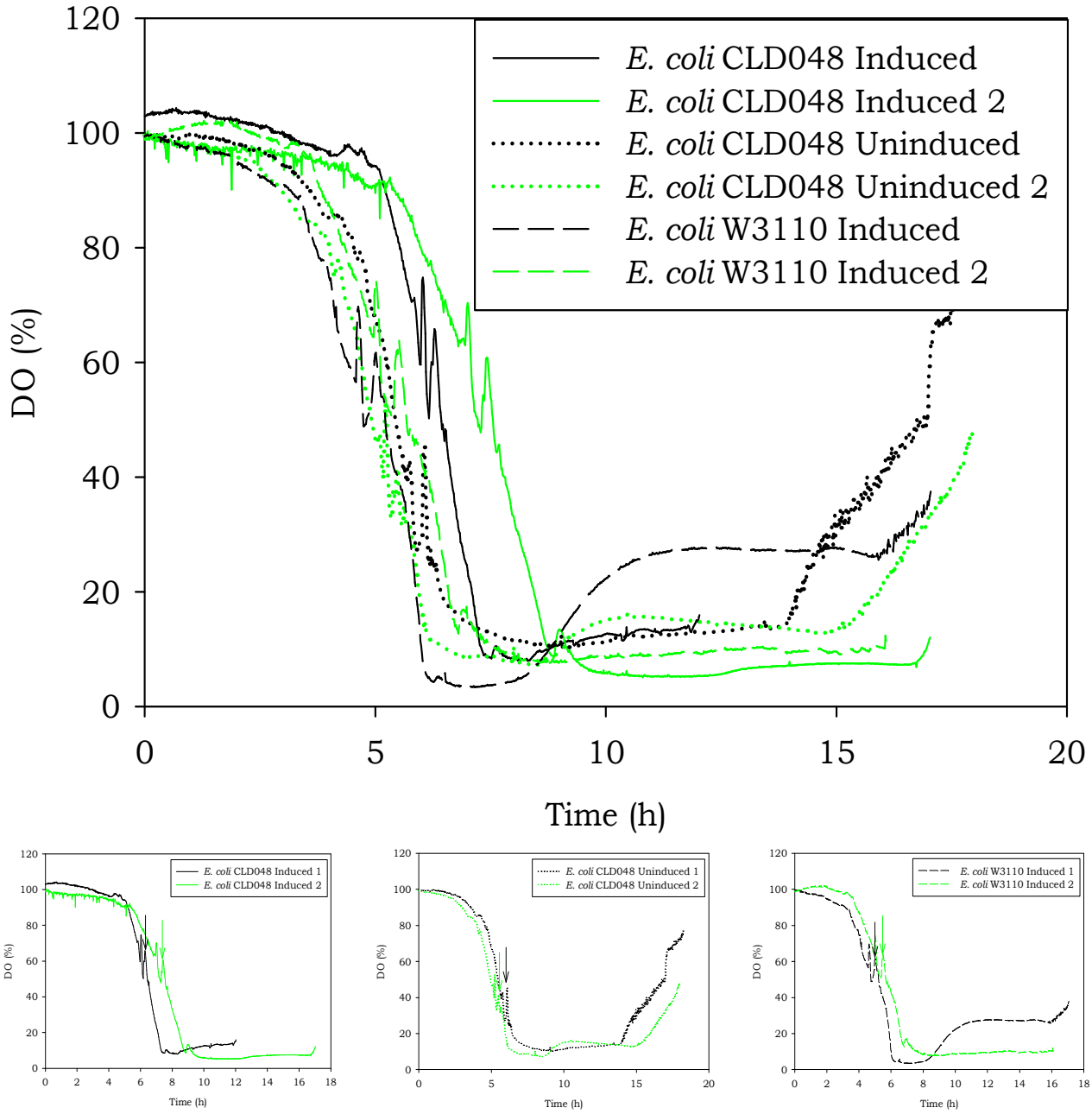

Figure 4.34. Top: Plot of the dissolved oxygen measured in the second fed-batch fermentation protocol. Bottom Left-Right: Plots showing close-up views of the point of feeding for *E. coli* CLD048 induced and and uninduced and E. coli W3110 induced, respectively. Arrows indicate the point of feeding, and induction where applicable, for each fermentation.

When the point of feeding (and induction) is transposed onto the optical density curves in Figure 4.35. it can be seen, as in the prior fed-batch fermentation all of the feed additions are commenced at an OD_{600nm} of approximately 20. What is clear from the optical densitometry is that there is

a stark difference in the growth capacity of induced GMO cultures and the control experiments. The maximum OD_{600nm} achieved by the induced recombinant fermentation was 50, a lower level compared to that of the first fed-batch protocol, whilst the control experiments reached the same, or higher, levels as previously observed (Figure 4.28.). This suggests that the effect of the simultaneous feeding and induction on the organism lies between the two extremes already shown (batch and first fed-batch protocol).

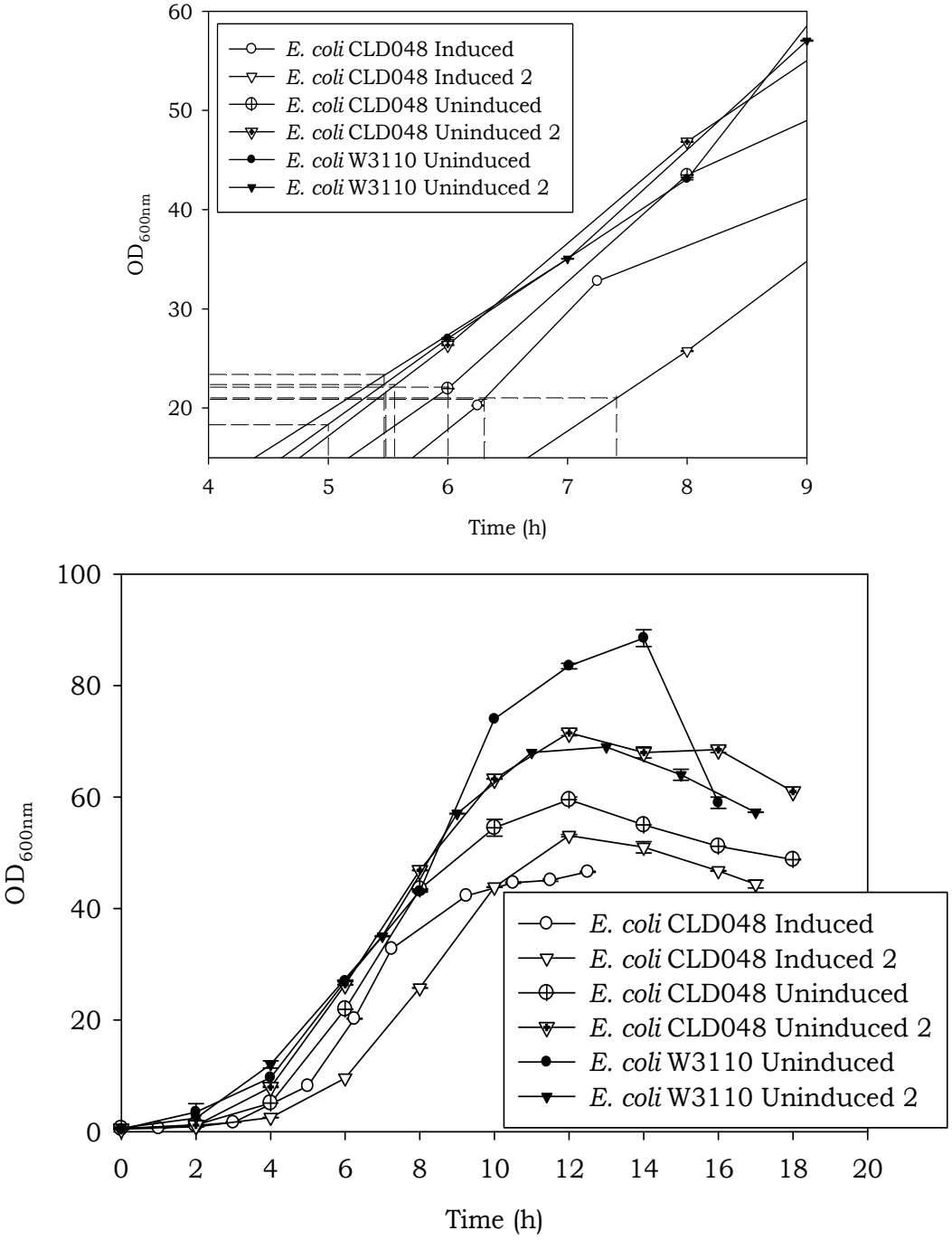

Figure 4.35. *E. coli* CLD048 fed-batch stirred-tank fermentation 2. All points plotted are mean values from replicate analyses. The error bars show the standard deviation. Induction using 0.1 mM IPTG, and concurrent feeding was carried out at the points indicated by the droplines on the top plot.

Reported earlier, there is also evidence here of expression of the Fab fragment before addition of the inducing agent (IPTG) (Figure 4.36.), further

suggesting incomplete repression. This is supported by the uninduced control, which also shows measurable quantities of recombinant protein, although at low levels. This “leakiness” could be caused by the single, perfect-palindrome sequence (Hodgson et al., 2006) not binding the LacI repressor molecule strongly enough, but may also result from stochastic effects from use with the artificial induction system (van Hoek and Hogeweg, 2007). The productivity of this system is more than both the batch and prior fed-batch method achieved in terms of total yield, with this protocol attaining a peak Fab titre of 40 $mg.L^{-1}$ compared with 27 $mg.L^{-1}$ (batch) and 35 $mg.L^{-1}$ (fed-batch 1). The predominant location of the Fab in this fed-batch method is also distinct, with the majority of the Fab associated with the insoluble, pellet fraction (previously there was no obvious partitioning in the batch culture and a majority for cytoplasmic expression in the first fed-batch method). The specific productivity using this fed-batch protocol is mid-way between the batch and first fed-batch method, approximately 1 $mg.L^{-1}.OD_{600nm}^{-1}$ (2 $mg.L^{-1}.g_{DCW}^{-1}$). This is the first indication that Fab productivity can be disconnected from the collapse in cellular numbers seen in the previous batch cultures (cultures in the first fed-batch culture remained at peak OD_{600nm} levels, but with very low productivity, relatively speaking).

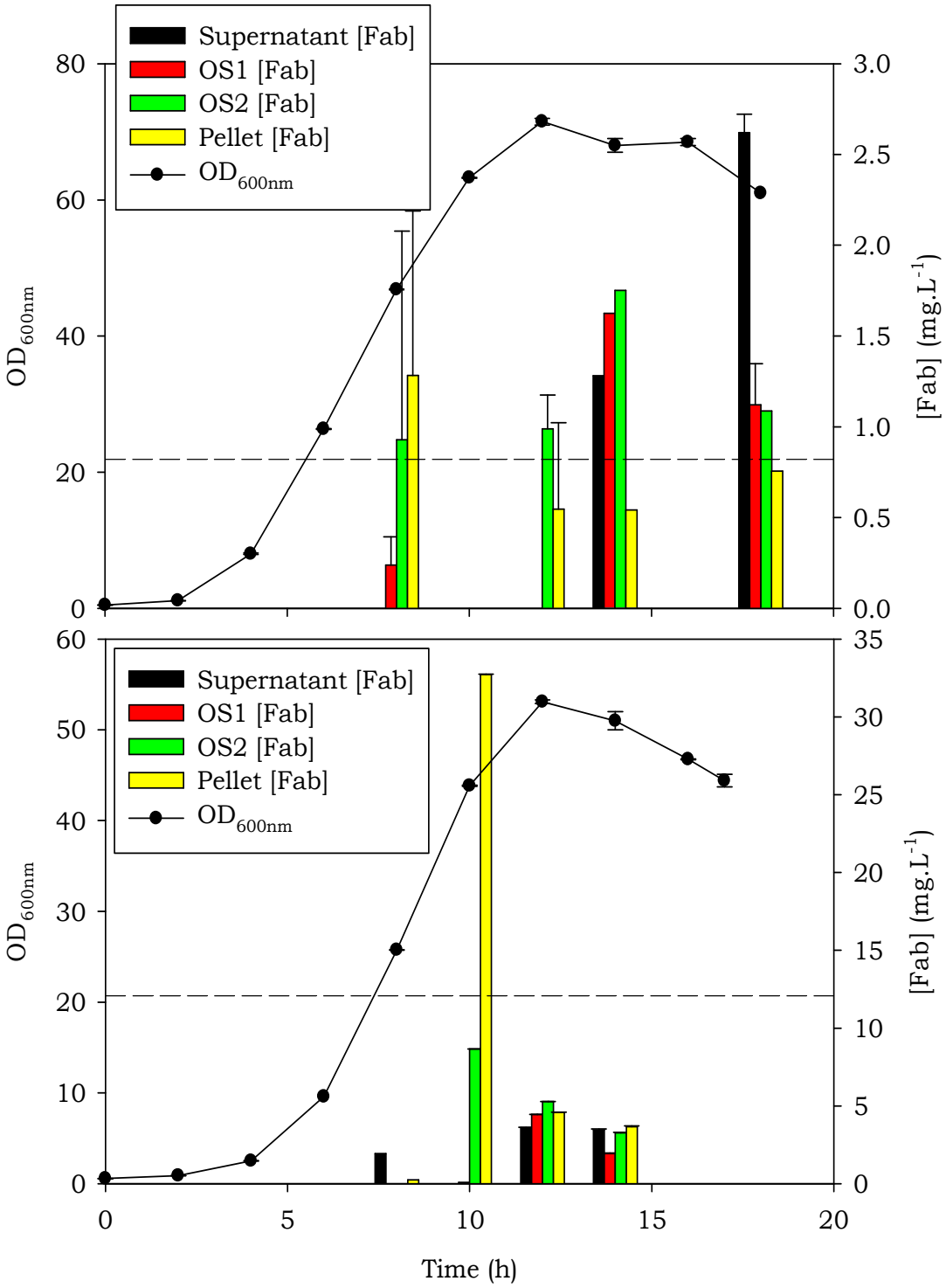

Figure 4.36. Fed-batch fermentation 2 of *E. coli* CLD048 (top; uninduced, bottom; induced). The dashed lines indicate the point where feeding was initiated (and 0.1 mM IPTG added: bottom), in response to a change in dissolved oxygen (Figure 4.30.).

Figure 4.37. shows that, whilst there appears to be a tendency for the induced culture to produce correlations between OD_{600nm} and DCW with

larger gradients, all of the cultures manifest similar correlation coefficients (> 0.95). A demonstration that, unlike the batch fermentation, the linear relationship between turbidity and dry cell weight is consistent even after induction for this fed-batch culture. When the gradients are compared for the CLD048 and W3110 induced cultures (0.515 and 0.380, respectively) it is clear that caution should be taken in using optical density as a proxy for dry cell weight, but that a correlation factor of 0.4 seems reasonable, taking into account all of the stirred-tank reactor data, with $r^2 > 0.95$, presented previously.

Table 4.6. Correlation coefficients (r^2 values) for all of the stirred-tank reactor fermentations performed in this work. Supporting data can be found in Figures 4.22., 4.30., 4.37. and 4.44.

	CLD048i	CLD048ui	CLD048ui	W3110i
Batch	0.752	0.999		1.000
FB1	0.975	0.991	0.992	0.983
FB2	0.973	0.975	0.992	0.988
FB3	0.920			

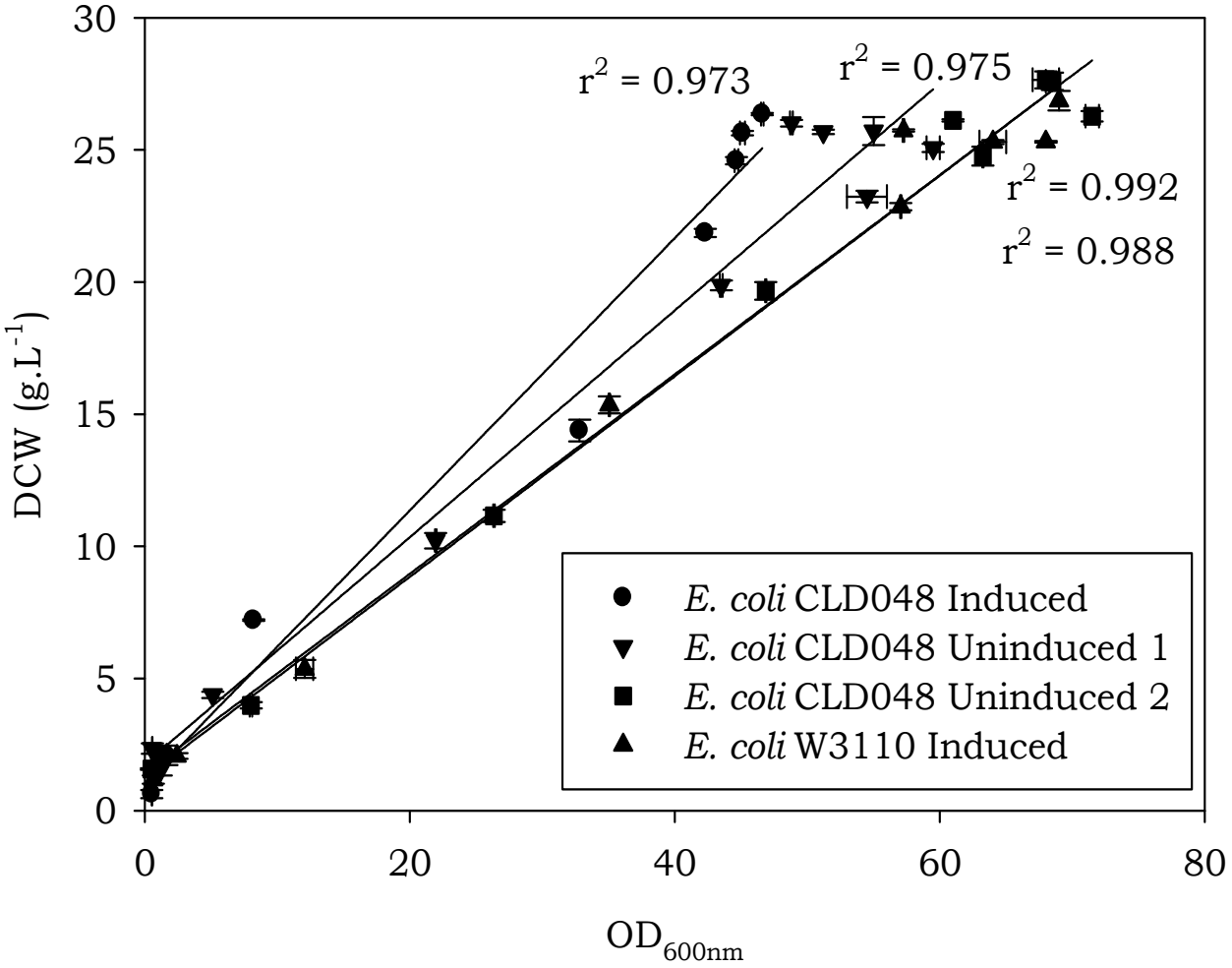

Figure 4.37. *E. coli* CLD048 fed-batch stirred-tank fermentation 2 in Figure 4.35. All points plotted are mean values from replicate analyses. The error bars show the standard deviation from which the mean was calculated. Induction and feeding were carried out at OD_{600nm} ≈ 20 with 0.1 mM IPTG and glycerol feedstock.

The specific growth rate, again, shows a steep increase during the logarithmic portion of the growth curve, Figure 4.38., toward the maximum for each culture (0.5-0.6 h^{-1}). This peak is similar to both previous fermentation methods (batch and first fed-batch) before the addition of the inducer, indicating that the initial gradient of decline is part of the natural deceleration associated with depletion of nutrients. Both of the induced fed-batch methods exhibit a more prolonged descent of specific growth rate than the batch culture and this is closely associated with the lower Fab titres. It

appears that this breakdown in cellular integrity is, if not required, then, at least strongly correlated with production of large amounts of antibody. It is unclear whether this is a discrete or continuous process, but the data gathered here so far is suggestive of the latter, with no sign of a threshold of productivity which limits the ability of the culture to proliferate.

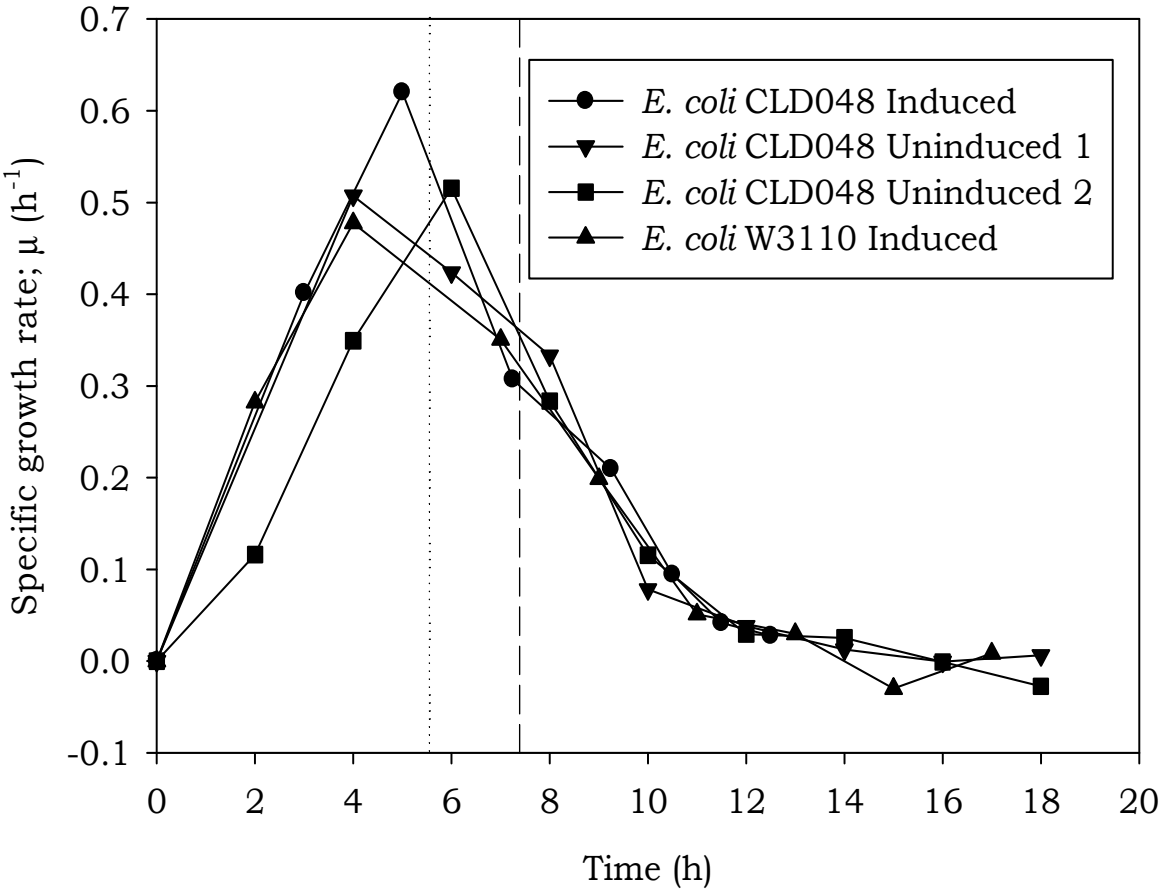

Figure 4.38. *E. coli* fed-batch stirred-tank fermentation 2 in Figure 4.35. The data plotted are calculated from the mean DCW. Induction and feeding were carried out by addition of 0.1 mM IPTG and glycerol feedstock at an OD_{600nm} ≈ 20. CLD048 induced at dashed line; W3110 induced at dotted line.

The behaviours of these cells have been well-characterised as showing an almost uniform population, in terms of cytoplasmic membrane potential, up to the point of induction with the prior analysis of the batch fermentation. The data in Figure 4.39. shows the effect on the cell's membrane potential

following induction in this second fed-batch method. As the experiment proceeded, the proportion of cells in each of the quadrants representing non-fluorescent, green fluorescent and green/red fluorescent remains consistent at approximately 85, 10 and 5 % respectively. This effect was observed for both fermentations run according to this protocol. As described earlier, this fed-batch method uses a stronger induction than the primary one, and the elevated level of green fluorescence, relative to that same fermentation is entirely expected. The impact of the expression of the recombinant product on these organisms is sufficient to retard the growth potential as evidenced by the limitation on the optical density and dry cell weight analyses. The cells, however, do not experience the same widespread breakdown in cytoplasmic membrane integrity, mediated by the supplementation of the glycerol in the feed providing a much-needed source of energy.

The $MgSO_4$ in the feed might additionally influence the condition of the cells by bolstering the membrane, providing additional interactions with LPS (Neidhardt, 1996). The effect of feed components exerting a protective effect on the producing cells was not considered for the first fed-batch protocol due to the lower level of induction used for that process. If present, however, this protective effect would have been greater in the first fed-batch, due to the feed being initiated in advance of the induction, rather than along with it.

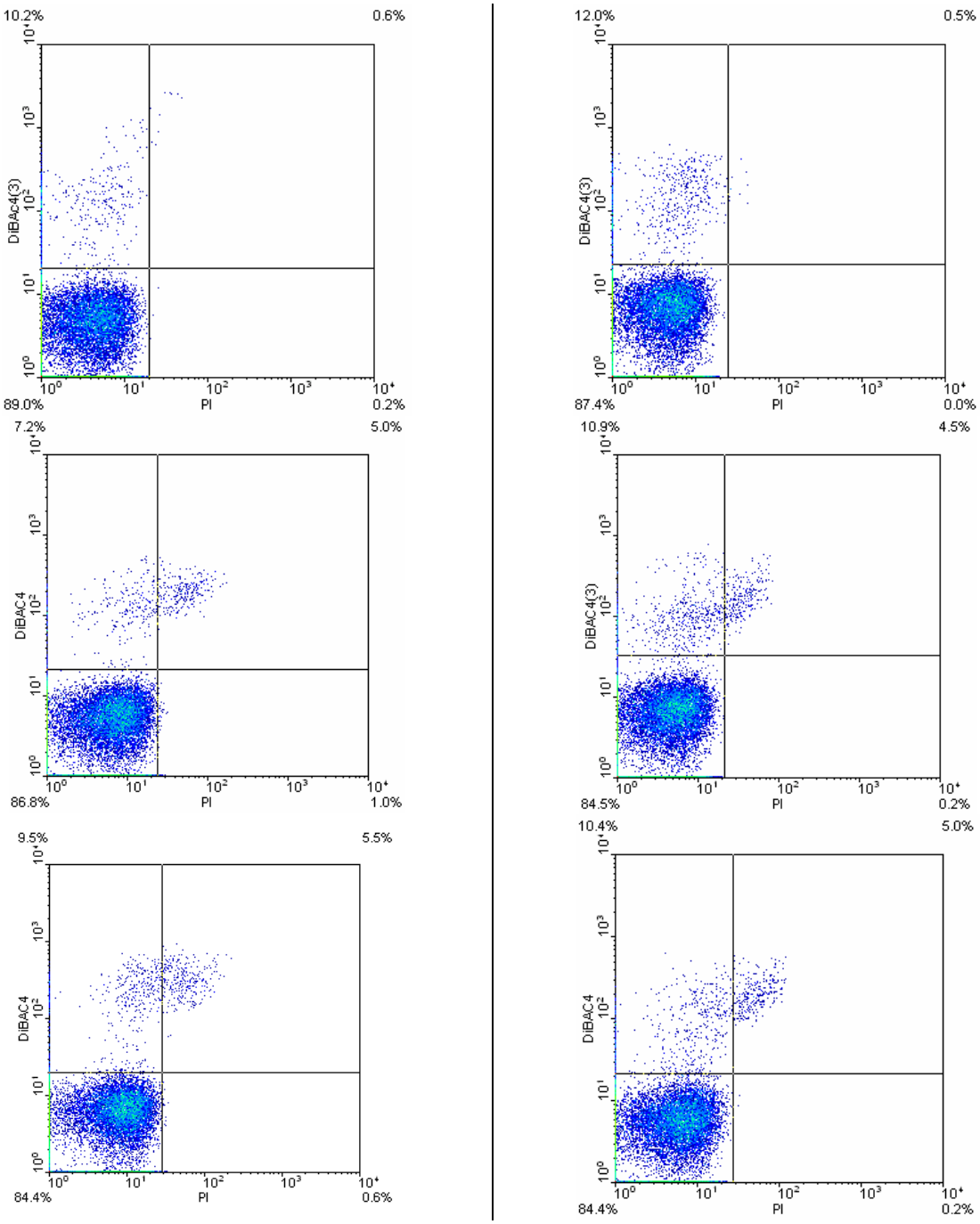

Figure 4.39. Flow cytometric analysis of *E. coli* fed-batch stirred-tank fermentation in Figure 4.35. stained with PI & $DiBAC_4(3)$. Cells were fed and induced at OD_{600nm} ≈ 20 using 0.1 mM IPTG. From top: 7.25(10); 10.5(12) and 12.5(16) hours with fermentation 1 on the left and fermentation 2 on the right. Figures in parentheses are time points for fermentation 2. Data from equivalent control experiments can be found in Appendix (8.1. & 8.2.)

When analysed by transmission electron microscopy (Figure 4.40), the data gathered share a great deal in common with the first fed-batch method: A

small, almost undiscernible periplasm just after induction, a reflection of the time required for the newly formed polypeptide chain of the recombinant product to make its way through the secretory system. After 9.5 h, a swelling of the periplasm can clearly be seen on the organism for which there is a longitudinal view, a feature also seen during the batch fermentation (Figure 4.26.). This appearance of an enlarged periplasm does not feature in the first fed-batch method (Figure 4.33.), suggesting that the increased strength of induction is exerting a pressure on the membrane. This swelling of the periplasm was not observed during any of the control experiments (see Appendix 8.1.1., 8.1.2. & 8.1.3.)

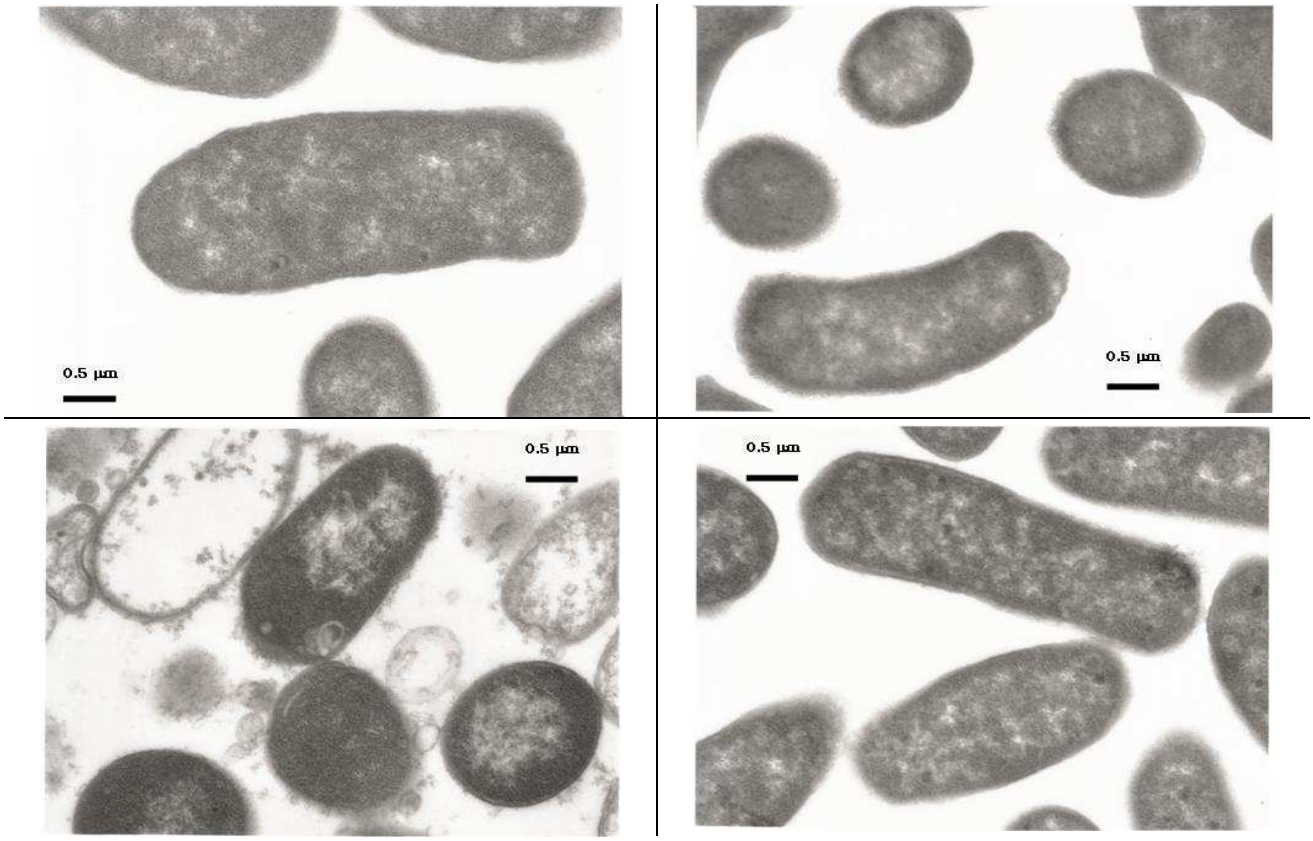

Figure 4.40. Transmission electron micrographs of *E. coli* CLD048 fed-batch stirred-tank fermentation 2 in Figure 4.35. Cells were induced at OD_{600nm} ≈ 20 using 0.1 mM IPTG. Clockwise from top left: 7.25; 9.5, 11.5 and 12.5 hours. Analysis was performed on samples from a single fermentation. Data from equivalent control experiments can be found in Appendix (8.1. & 8.2.)

As the fermentation proceeds, the same difficulty in resolving the periplasmic space becomes apparent although, as with the first fed-batch method, this seems to have had little impact on the ability of the cell to maintain its cytoplasmic membrane potential in this instance.

Figure 4.40. also shows the presence of "ghost" cells, cells which have become emptied of their contents with retention of the cytoplasmic membrane and structure (Lubitz et al., 1999; Reis et al., 2005), unseen with this organism in the prior fermentations. This appearance might be a function of the low statistical resolution of electron microscopy as a technique, mentioned previously. If this culture is indeed the only one of the three fermentation methods followed so far to actually contain ghost cells, then it's likely that these were not present in the other fed-batch due to the weaker induction. The cellular breakdown may be too rapid in the batch culture for ghost cells to exist in any more than a transient state making them almost impossible to detect.

4.5.1. Conclusions

Feeding the cells along with inducing agent of similar concentration to the batch method provides protection to the cells against widespread disruption. Volumetric productivity of Fab for this method is the lowest so far, however, specific productivity is higher here than in the previous fed-batch. At one of the time points (9.5 h) the whole of the measured Fab concentration (35

mg.L^{-1}) is located within the extracellular medium, a higher extracytoplasmic titre than has been achieved in either of the two other methods. This fed-batch method was the first instance where ghost cells were detected in culture following induction. These cells, emptied of their contents but maintaining the cellular structure, are dead cells which exhibit no PI fluorescence due to the lack of a genome. They have only been identified in a single TEM image, and maybe be a result of the low statistical resolution of the technique, because they would probably have been detected by flow cytometry (by a change in the side scatter) before this point.

4.6. *E. coli* CLD048 Fed-Batch 3 Stirred-Tank Reactor Fermentation

This fed-batch method was discovered serendipitously, on attempting to repeat the second fed-batch protocol (Figure 4.35.). A very slight, relative, difference in induction/feeding time between the two runs caused a very large impact on the behaviour of the cells, with a much more "batch-like" rapid decline in OD_{600nm}.

Figure 4.41. illustrates the difference in induction/feeding times between fed-batch 2 and 3 with the relative difference (i.e. the separation of the distinct characteristic points on their respective curves) being approximately 6 minutes.

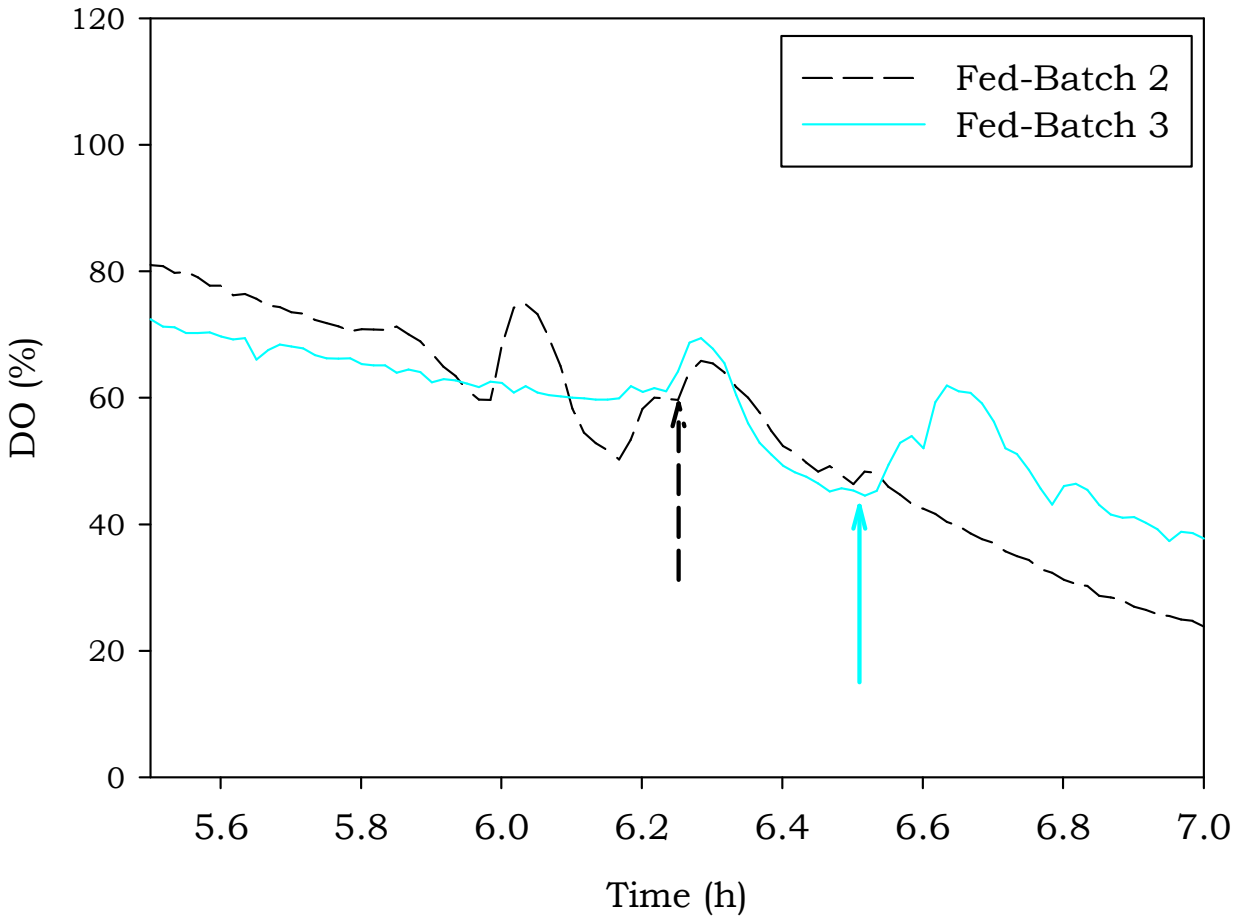

Figure 4.41. DO of a single run each of fed-batch 2 and 3, with the arrows indicating the addition of 0.1 mM IPTG and feeding of glycerol feedstock. Each curve is representative of 2 separate experiments, with the DO measurements taken online, every minute.

This unusual double-peak that occurs as the DO drops from its initial value of 100 % is seen consistently across all of the cultures performed where online DO measurement was performed and action taken on it (Figures 4.23. and 4.30.). This fluctuation in the ability of the culture to remove oxygen from the environment has been discussed in the previous chapters as likely due to a change in the metabolism of the cells, brought about by transition to dependence on a new metabolite.

As displayed previously, for the other fed-batch methods used in this thesis, Figure 4.38. shows the variation of dissolved oxygen for the cultures utilising this method. Due to issues of scale, it is not possible to identify the difference in the feed/induction time points, hence why an example has been expanded to form Figure 4.37. The DO plots for the induced GMO fermentation, both cases, show a sudden rise in dissolved oxygen after 2-2.5 h of plateau. This was caused by a large quantity of foam formation during the culture, which was counteracted by a bolus of polypropylene glycol antifoam (1 mL) in order to continue the culture.

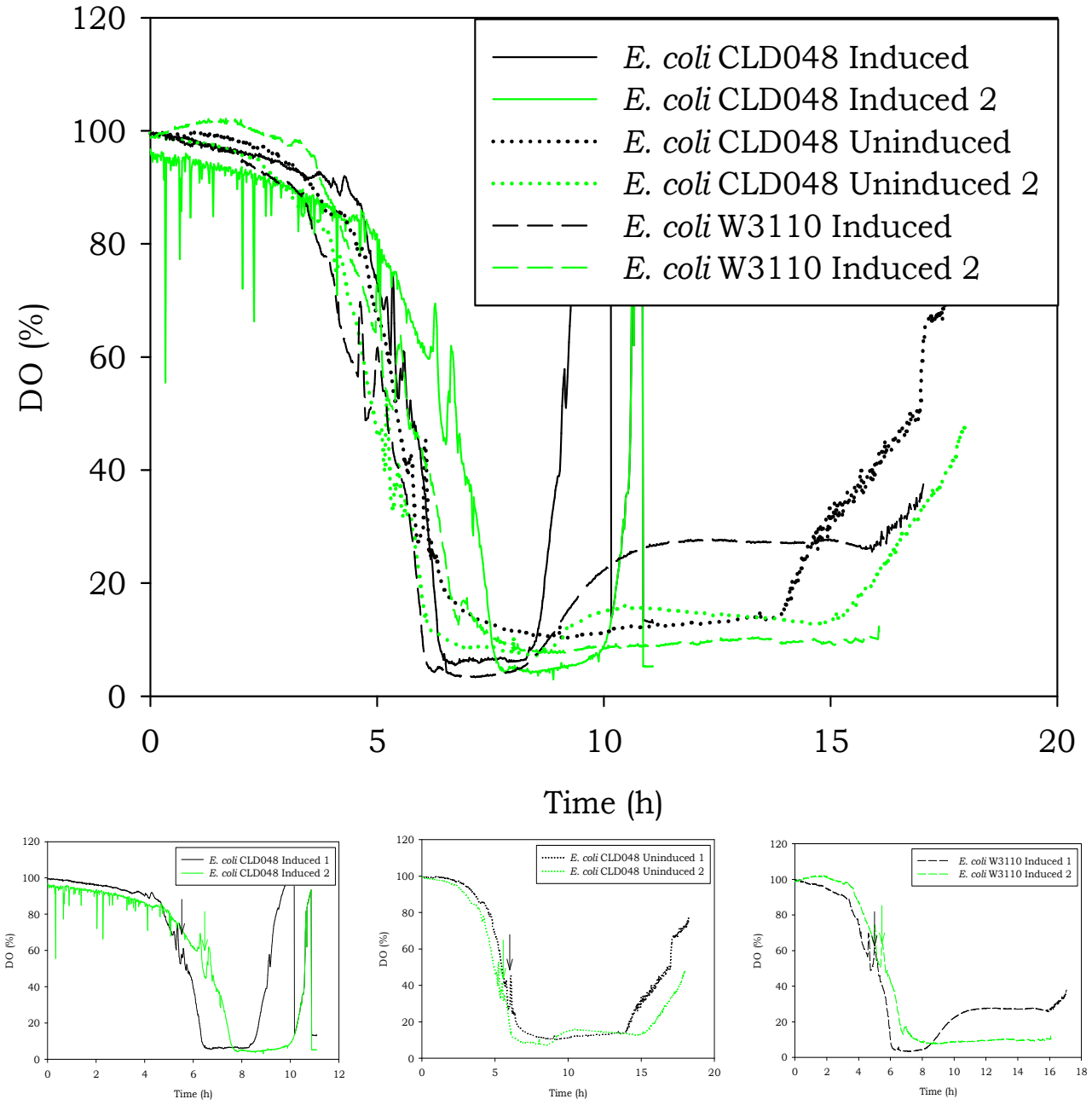

Figure 4.42. Top: Plot of the dissolved oxygen measured in the third fed-batch fermentation protocol. Bottom Left-Right: Plots showing close-up views of the point of feeding for *E. coli* CLD048 induced and and uninduced and *E. coli* W3110 induced, respectively. Arrows indicate the point of feeding, and induction where applicable, for each fermentation

Figure 4.43. illustrates the significance of the relative difference in feeding/induction points for fed-batch 3, compared with both uninduced GMO and induced wild type fermentations. This shows that the combination

of the metabolic switch that is presumed to be occurring at this point, and the powerful derepression of the recombinant gene causes a much more “batch-like” profile, in terms of optical density achieved and length of process.

What is apparent, more obviously from the lower plot in Figure 4.43., is the plateau in optical density after induction. Where the equivalent batch fermentation peaked and fell into a steep decline between 1 and 2 h after induction it seems as though this fed-batch method does not. The spacing of the samples (2 h separation) means that this may not be a real effect, where the middle hour of the plateaux could, in fact be a peak. However, this would still mean that the cultures continued to increase turbidity for at least 1.5 h post-induction; an improvement on the batch fermentation. It is likely that this improvement is a result in the addition of the feedstock at the early part of the DO fluctuation, where the cells have not undergone the metabolic changes hypothesised earlier.

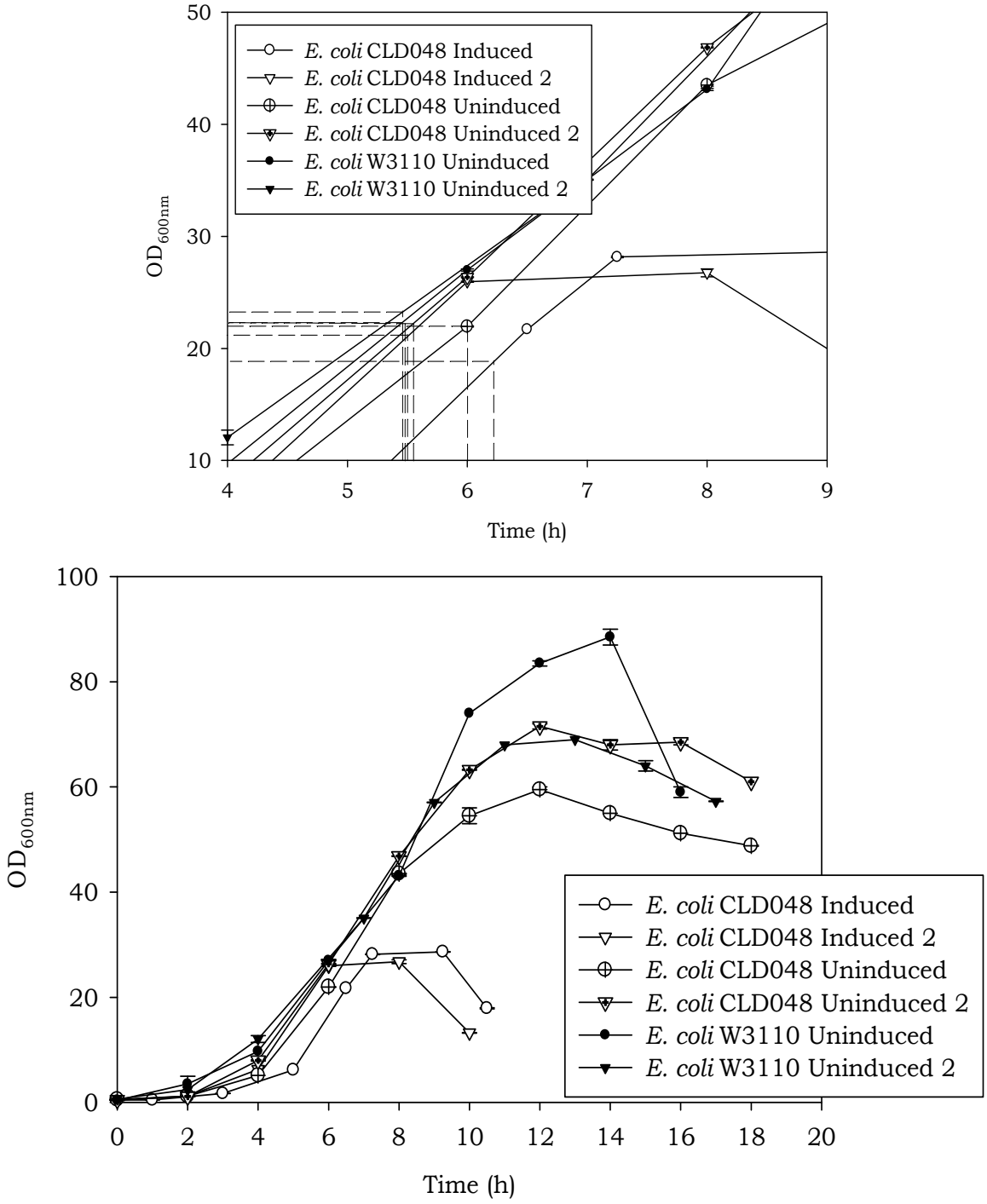

Figure 4.43. *E. coli* fed-batch stirred-tank fermentation 3. All points plotted are mean values from replicate analyses. The error bars show the standard deviation. Induction using 0.1 mM IPTG, and concurrent feeding was carried out at the points indicated by the droplines on the top plot.

The effect of this altered induction regime can be seen in Figure 4.44. where the induced GMO is compared with the uninduced equivalent fermentation (where only the feed was added to the fermenter). As in the previous cultures,

the non-induced GMO fermentation exhibited very little Fab production peaking at approximately 5.5 $mg.L^{-1}$. Due to the maintenance of optical density at, or near to, peak values, this translates to a very small specific productivity (less than 0.1 $mg.L^{-1}.OD_{600nm}^{-1}$).

In comparison, the induced *E. coli* CLD048 outstrips the productivity of all previous fermentations so far in this thesis with a peak productivity of 105 $mg.L^{-1}$. More importantly, when considering a holistic perspective is that most of that Fab was located in the periplasm; the originally intended target for subcellular location. In addition, this high level of Fab production preceded the reduction in optical density, indicating that the protein production and cellular breakdown can be temporally distinct, although not significantly, giving a small window in which the cells might be harvested and the product recovered prior to the release of contaminating intracellular materials.

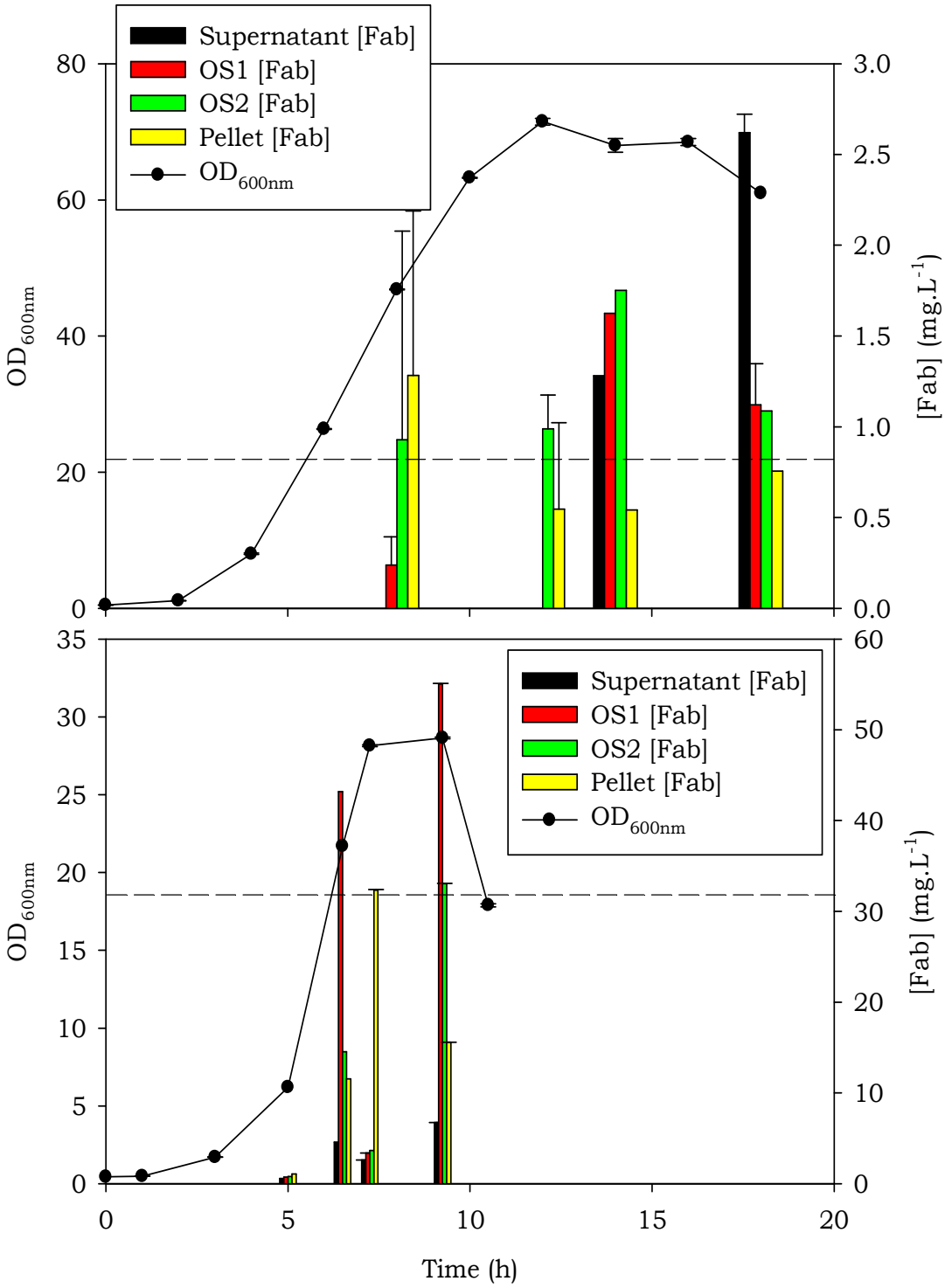

Figure 4.44. Fed-batch fermentation 3 of *E. coli* CLD048 (top; uninduced, bottom; induced). The dashed lines indicate the point where feeding was initiated (and 0.1 mM IPTG added: bottom), in response to a change in dissolved oxygen (Figure 4.42.).

The correlation between OD_{600nm} and DCW is shown in Figure 4.45. and displays the excellent linear fit for the control experiments, as demonstrated

earlier (Figures 4.22, 4.30 & 4.37.). The same cannot be said for the induced GMO fermentation, whilst still displaying a coefficient greater than 0.90, showing a much less robust relationship. This is an improvement on the batch fermentation r^2 value, indeed, the reason for this drop can likely be attributed to the outlier on Figure 4.45. (DCW ≈ 15; OD_{600nm} ≈ 12), with the rest of the points clustered around the fitted curve.

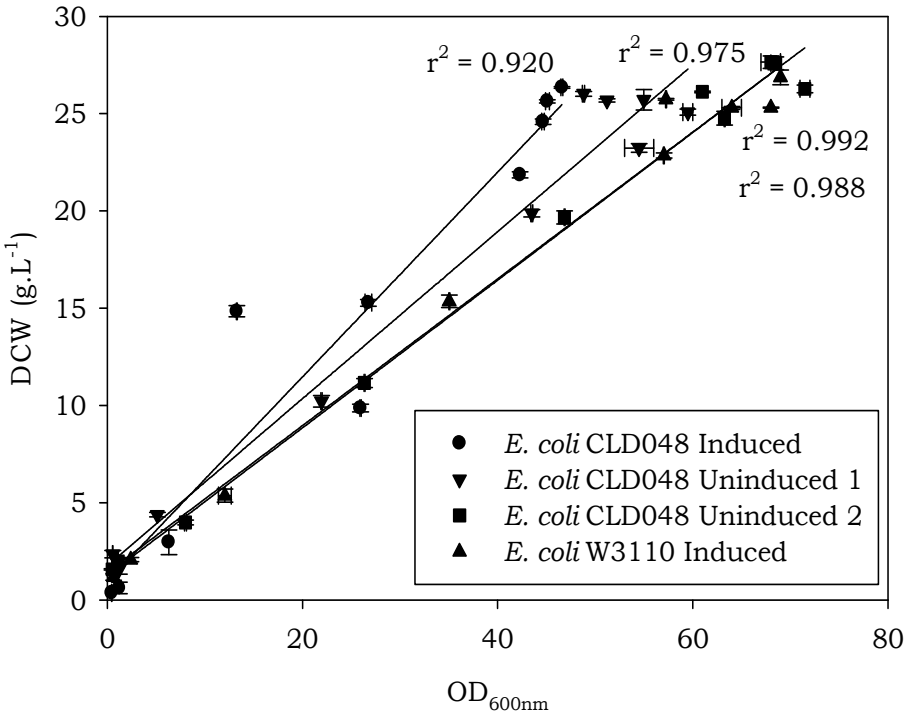

Figure 4.45. *E. coli* CLD048 fed-batch stirred-tank fermentation 3 in Figure 4.42. All points plotted are mean values from replicate analyses. The error bars show the standard deviation. Induction and feeding were carried out at OD_{600nm} ≈ 20 with 0.1 mM IPTG and glycerol feedstock. Correlation coefficients apply to the closest curve to each one.

Following the peak specific growth rate (Figure 4.46.), the descent observed for the third fed-batch fermentation is less steep than the batch process but a steadier decline than the other fed-batch methods. The first and second fed-batch processes follow a "first-order" logarithmic fall in μ, which results in a faster decline whose rate falls as it approaches zero. This difference

results from the entry into stationary phase experienced by the other cultures, whereas the third fed-batch is marked by a steep decline in OD_{600nm} after induction.

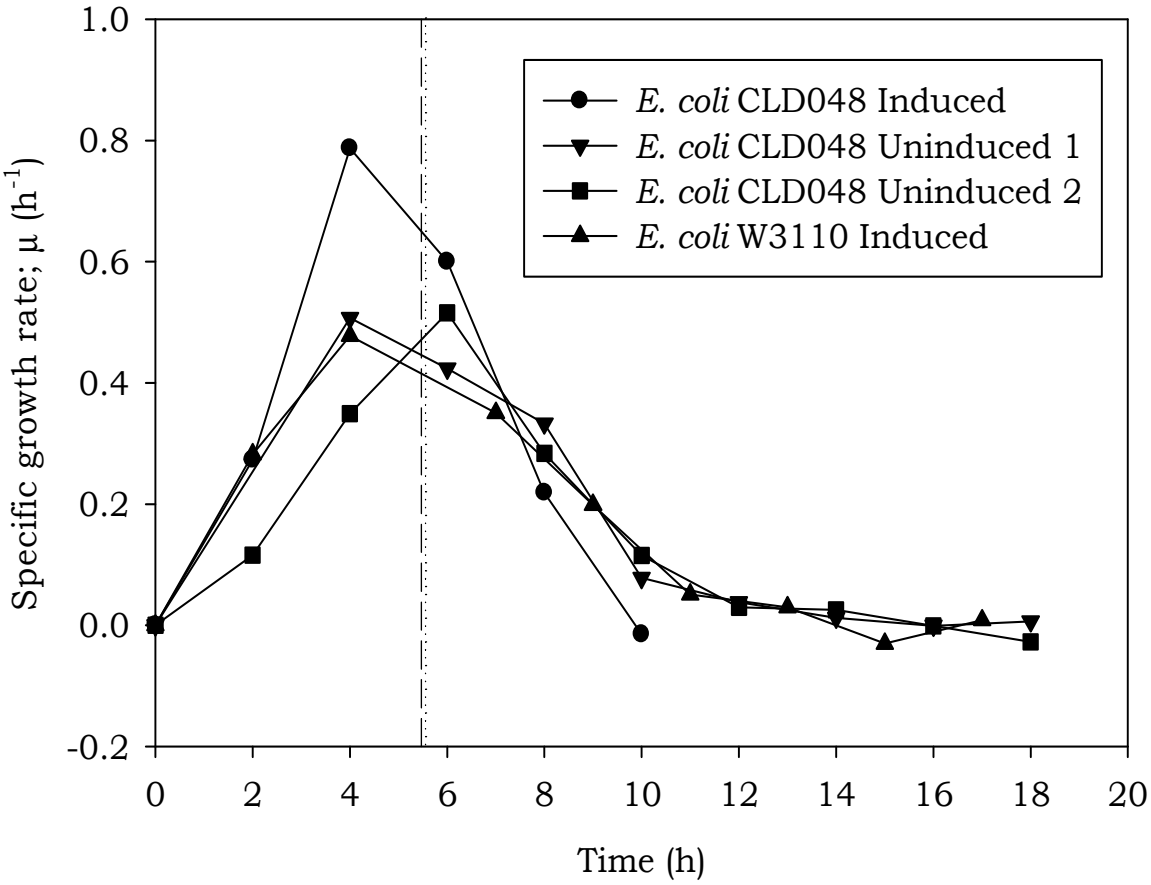

Figure 4.46. *E. coli* fed-batch stirred-tank fermentation 3 in Figure 4.42. The data plotted are calculated from the mean DCW. Induction and feeding were carried out by addition of 0.1 mM IPTG and glycerol feedstock at an OD_{600nm} ≈ 20. CLD048 induced at dashed line; W3110 induced at dotted line.

Figure 4.46. displays the flow cytometry data for this third fed-batch method with distinct similarities with the batch process, ending with significant green and red fluorescence. As with the batch fermentation, the point of maximum productivity doesn't actually coincide with the point of maximum stress as measured by this method. Instead, the highest proportion of green

and red cells, relative to the non-fluorescent, healthy, population is highest at the point where cell breakdown is the greatest, at the end of the cultures.

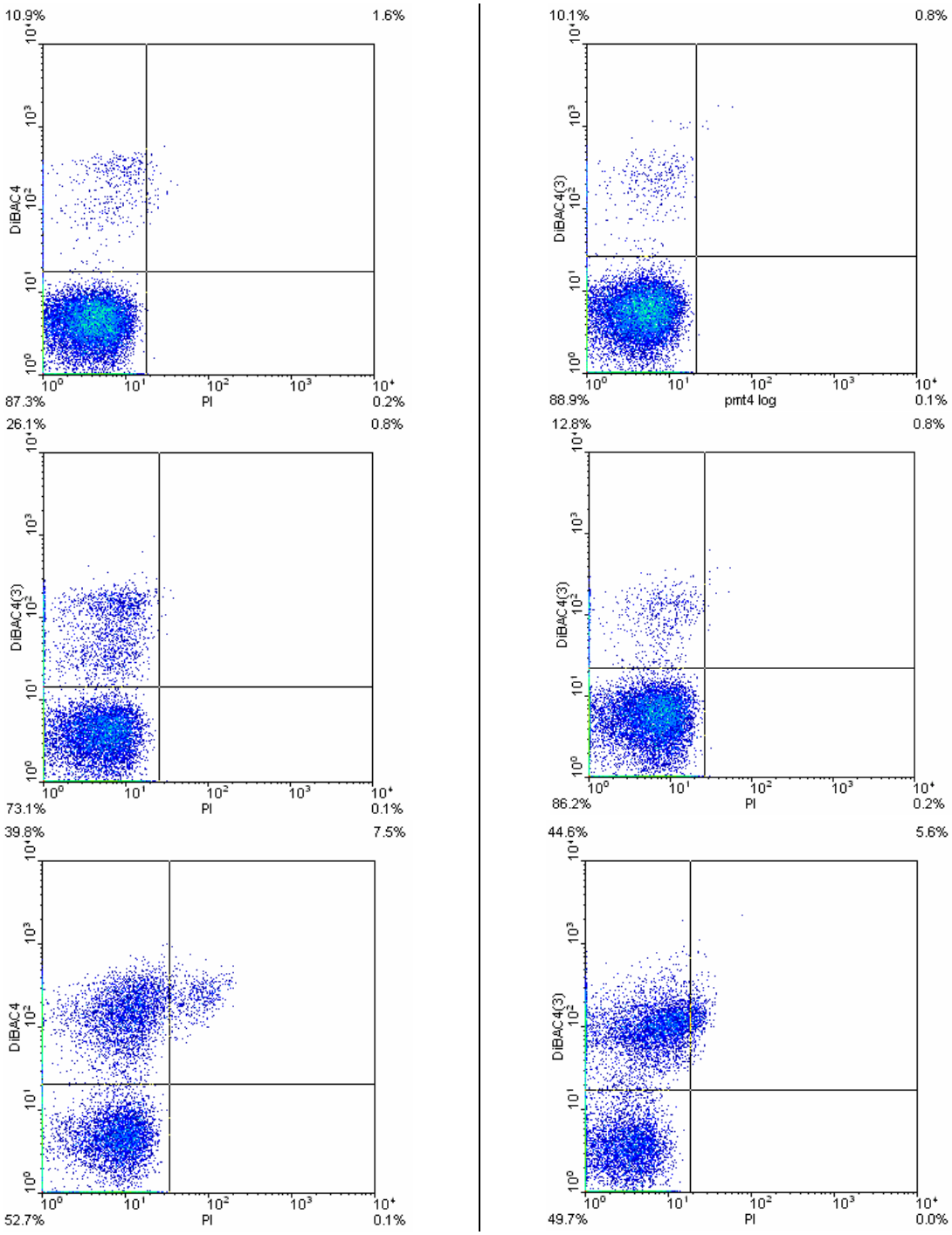

Figure 4.47. Flow cytometric analysis of *E. coli* CLD048 fed-batch stirred-tank fermentation 3 in Figure 4.42. stained with PI & $DiBAC_4(3)$. Cells were induced at OD_{600nm} ≈ 20 using 0.1 mM IPTG. Data gathered from: 6; 8 and 10 hours with fermentation 1 on the left and 2 on the right. Data from equivalent control experiments can be found in Appendix (8.1. & 8.2.)

The electron microscopy of fed-batch 3 shows two major differences with the majority of the other cultures. First, the ghost cells appear much earlier in this culture than detected in the prior fed-batch (Figure 4.40.). Second, at the culmination of the experiment, there were far more ghost cells present than were found at any stage in the other three stirred-tank reactor protocols.

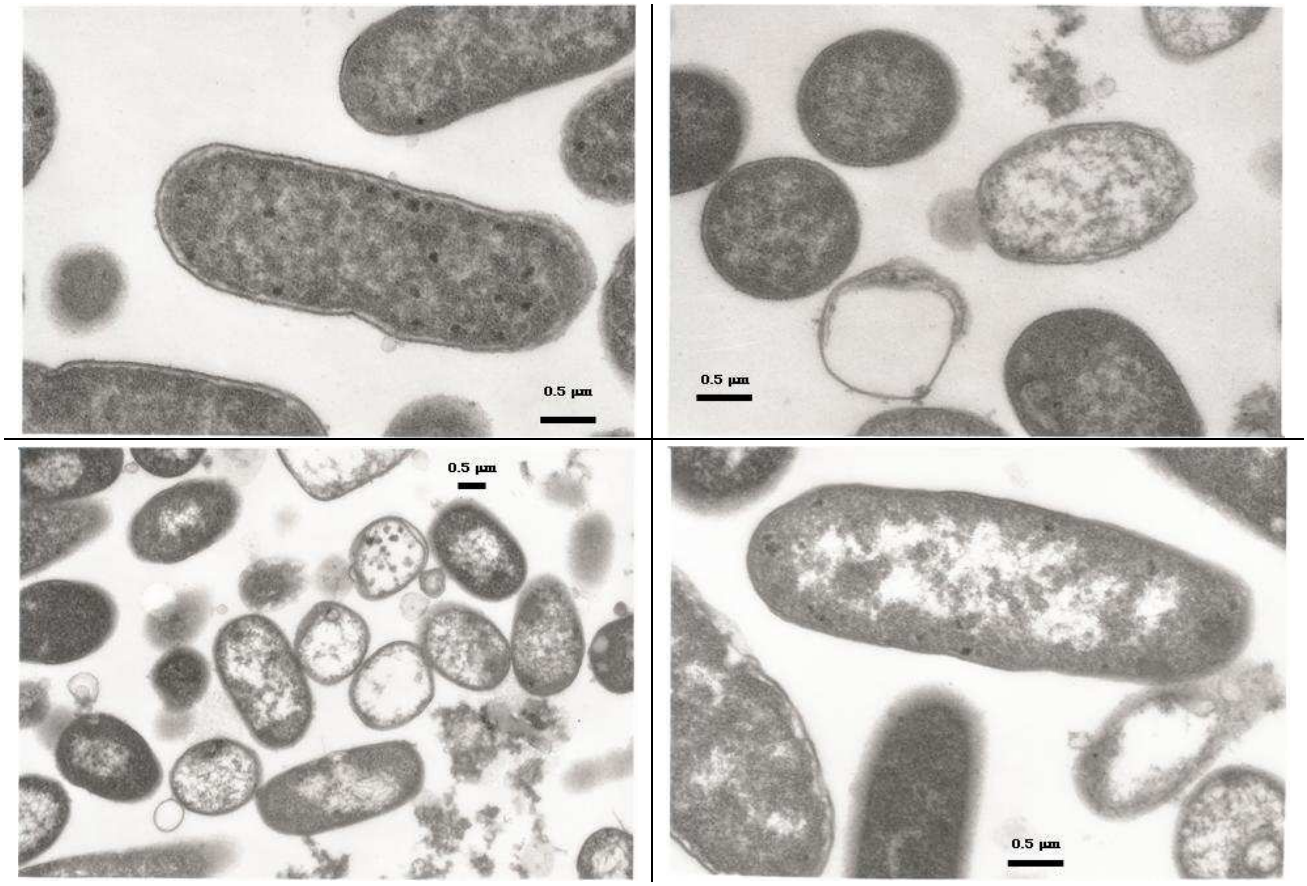

Figure 4.48. Transmission electron micrographs of *E. coli* fed-batch stirred-tank fermentation 3 in Figure 4.42. Cells were induced at OD_{600nm} ≈ 20 using 0.1 mM IPTG. Clockwise from top left: 5, 7; 8 and 10 hours. Analysis was performed on samples from a single fermentation. Data from equivalent control experiments can be found in Appendix (8.1. & 8.2.)

The appearance of these cells might be a result of the statistical resolution of the technique, mentioned earlier, where they may have been present in the cultures for which they were undetected but too few cells were analysed to

provide a fully representative sample. However, the limitation on growth observed in this culture is greater than in any of the previous experiments also, therefore the abundance of ghost cells in this case might be caused by this alternative method. If this increase in ghost cells is not an artefact, or result of the sampling errors mentioned prior, this would have a significant impact on the downstream purification of the Fab. An abundance of ghost cells would entail large quantites of host cell protein and host cell DNA being released into the liquid medium, increasing the challenges associated with purification.

4.6.1. Conclusions

This third fed-batch produced the greatest peak Fab yield of any of the fed-batch processes, 105 $mg.L^{-1}$. This protocol was also successful in achieving far higher periplasmic expression than any of the other methods, more than 50 $mg.L^{-1}$ of Fab being present in the periplasm at peak productivity. Whilst the same total cell breakdown was observed, in common with the batch method, this was significantly asynchronous with the cellular breakdown, suggesting that there may be scope for this to be avoided by alternative processing.

The large difference in the number of detected ghost cells during this culture, suggests that the contamination of the process fluid with host cell molecules has become a significant factor. It does seem like this is even more likely to be an artefact, given that the first appearance of ghost cells is apparent in a culture which appears healthy by all other measurements.

5. Conclusions

Even at small-scale, the assumption of a truly homogeneous culture is optimistic at best. PI/$DiBAC_4(3)$ can be used with Gram positive cells, such as *B. cereus*, to monitor cell viability, however there are flaws in the technique which lead to difficulties in interpretation of the data. PI/$DiOC_6(3)$ gives a much clearer resolution of the populations of cells present within a "homogeneous environment" and has shown the presence of a fourth population consisting of cellular couplets of a single live and dead cell joined along the longitudinal axis. PI/RedoxSensor Green™ can be utilised as an alternative/adjunct to PI/$DiOC_6(3)$ but care must be taken with length of exposure of the cells. Flow cytometry can be used in conjunction with PI/$DiBAC_4(3)$ to reliably detect the effect of recombinant protein production in *Escherichia coli*. The expression of the Fab fragment inhibits cell growth, as compared with uninduced control samples, and, additionally, causes cell lysis later in the fermentation. This lysis is undesirable from a process perspective, and is one of the conditions which this method was employed to avoid. Relatively little recombinant protein is actually produced in this example, less than 7 $mg.L^{-1}$ (and most of that associated with the cells rather than free in the periplasmic space). $DiOC_6(3)$ is unsuitable as a dye for use with *E. coli*. Whilst it appears able to demonstrate the presence of homogeneous, viable cultures, the lack of resolution of different populations in heterogeneous cultures produces inconsistent results. There is considerable evidence that the repression system used in this organism is "leaky" with the induced culture producing 27 $mg.L^{-1}$ compared with 18

mg.L^{-1} for the uninduced culture. The increased Fab expression is associated with a sudden and rapid decline in both total and viable cell number, moreso than shown in shake-flasks. Despite the expression system being designed for periplasmic expression, there is no preference for this compartment in terms of accumulation of conformationally active Fab (as would be demonstrated by increased Fab concentrations in OS1). In fact, During the decline phase of the culture the quantity associated with the insoluble fraction is dominant comprising 30-40 % of the total. The leakiness, combined with the increasing proportion of Fab present in cellular compartments other than the periplasm show that this system, at least in batch mode, is poorly optimised for the intended purpose. A simple feeding strategy using a high-glycerol feedstock can achieve significant increases in culture turbidity over the batch process. 1.4 mg.$L^{-1}g_{DCW}^{-1}$ recombinant Fab can be expressed during the fed-batch process, which, whilst less than achieved in batch mode (3.5 mg.L^{-1}.g_{DCW}^{-1}), is attained without the associated decline in the culture. There is no apparent partitioning of the product into the periplasmic space (as would be indicated by more of the Fab being present in OS1 following osmotic shock). This fed-batch process is a viable method for production of this Fab, without the associated breakdown in cell integrity observed in the batch process. Employment of a method of this type would decrease the load on subsequent downstream purification, but the low titres observed here indicate that there is considerable room for optimisation. Feeding the cells along with inducing agent of similar concentration to the batch method provides protection to the cells against widespread disruption. Volumetric productivity of Fab for this method is the lowest so far, however,

specific productivity is higher here than in the previous fed-batch. At one of the time points (9.5 h) the whole of the measured Fab concentration (35 $mg.L^{-1}$) is located within the extracellular medium, a higher extracytoplasmic titre than has been achieved in either of the two other methods. This third fed-batch produced the greatest peak Fab yield of any of the fed-batch processes, 105 $mg.L^{-1}$. This protocol was also successful in achieving far higher periplasmic expression than any of the other methods, more than 50 $mg.L^{-1}$ of Fab being present in the periplasm at peak productivity. Whilst the same total cell breakdown was observed, in common with the batch method, this was significantly unsynchronised with the cellular breakdown, suggesting that there may be scope for this to be avoided by alternative processing.

6. Further work

The most widespread method for the manufacture of low-volume high-value products usually begins with a fermentation phase, optimised in isolation to maximise product formation. In an environment where 80 % of the total operating cost of the final bioprocess is estimated to be accounted for by the downstream purification, there is an ever-increasing need to formulate a holistic optimisation approach whereby the impact of the output from the production phase is more fully accounted for. The aim of the work in this project was to begin this process, by attempting to express a protein product to the periplasm, thereby making it more easily recoverable during downstream purification. The challenge when exploiting this route of manufacture is to create sufficient quantities of product whilst preserving the integrity of the cells, as demonstrated in the batch cultures (shake-flask and stirred-tank) displayed in Chapter 4: Results & Discussion.

There is a wealth of available methods that can be used to make changes to this process beyond the simple alterations of induction conditions investigated here. One widely used method is the use of a defined medium (García-Arrazola et al., 2005; Hewitt et al., 2007; Bäcklund et al., 2008), from which the effect of changes to the process can be more precisely measured in the absence of complex, undefined media components. Moreover, the variability in the growth rates and final achievable biomass should also become more consistent than in this case where, whilst general trends were replicable, the exact values varied in both value and timescale. There is also

strong evidence that control of the specific growth rate at low values (less than 0.3 h^{-1}) can have beneficial effects on the recombinant protein production, even in glycerol-fed cultures, where acetate accumulation is not a primary concern (García-Arrazola et al., 2005). A variety of molecular methods have been employed to improve productivity such as altering the protease profile of the cell (Chen et al., 2004), altering codon usage in the expressed product (Angov *et al.*, 2008) or adjusting the amino acid content of the primary sequence to more closely reflect the tRNA pool of the host (Bonomo and Gill, 2005).

A variety of strategies are available to accomplish expression of a recombinant protein via the *lac* operon, with inducer concentration and time of addition being the most obvious. The most commonly used gratuitous inducer, IPTG, was used in this work however, this chemical is not ideal for use at large scales due to toxicity problems associated with disposal and exposure of operating personnel (Makrides, 1996). The absolute concentration here (0.1 mM) was not changed because some research has shown that concentrations in this range are most often conducive to periplasmic expression of recombinant products in Escherichia coli (Shibui and Nagahari, 1992). Additional studies have investigated the use of lactose, both as a carbon source for a fed-batch method, as well as a tool to induce the lac operon (García-Arrazola et al., 2005) demonstrating a viable alternative to use of IPTG. In addition to modification of the induction conditions, it has also been shown that lowering the temperature of the

reactor following addition of the inducer can also improve the quality and quantity of manufactured product (Marx et al., 2007; Ruiz et al., 2009).

The basic analysis methods, which have a long history of use with microorganisms, such as optical densitometry and dry cell weight analysis yielded an acceptable overview of the cultures. The flow cytometric dying protocols employed were limited, using only 4 dyes in total, with only two for E. coli. The number of dyes available for use with bacteria is relatively limited, with flow cytometry mainly associated with analysis of mammalian cells. It would, therefore, be prudent to extend the range of dyes used to encompass others which have been used successfully with bacteria, such as carboxyfluorescein diacetate succinimidyl ester, for the measurement of intracellular pH (Hoefel *et al.*, 2003) or alternative redox dyes e.g. cyano-2,3-ditolyl tetrazolium chloride (CTC) (Fiksdal and Tryland, 1999). During the course of this research, there were no cell counts performed due to the labour-intensity of the process, and in consideration of the volume of other analyses that were also being utilised.

HPLC analysis was performed for quantification of organic acids and glycerol in the E. coli fermentations, however, the data was inconsistent at best and not reliable enough to draw useful conclusions. This method has been used for analysis of bacterial cultures (Ruiz et al., 2009) previously, so it is likely that the system and method in use at Birmingham needs optimisation. A more robust, but also more limited, method for metabolic analysis is provided by bioanalysers, which utilise a combination of enzyme-based

assays and ion selective electrodes, so this might be a further option although the cost of these items is significant (minimum £14,000 at time of writing).

Analysis of product formation by ELISA was used as a benchmark, with insufficient quantities of protein produced to be detectable by routine SDS-PAGE. Further information regarding the levels of production detectable by ELISA (as mentioned earlier, ELISA can only detect functional antibody fragments, able to bind to their antigen) could be gained by using size exclusion chromatography. This method would not give the same, functional, data gained from the ELISA (the ELISA could still be employed in tandem) however, it would detect antibody fragment fragments or dimers and other multimers. RT-PCR of the cell lysate, following RNA isolation, can show the levels of transcription to assess the strength of induction from the promoter.

Given this plethora of possible opportunities, this work could best be furthered by utilisation of lactose in the feed, combined with a control of oxygen by agitation speed around a set point between 30 and 50 %. This would enable better control of the specific growth rate both before induction and during the fed phase also. RT-PCR and a more robust HPLC method could also add significant knowledge regarding the pre-translation productivity from the recombinant gene and a more comprehensive metabolic profile than the use of inference based on indirect measurements such as the DO. The process-based changes are much more easily implanted than the more fundamental molecular alterations listed above, which would be more

suitable at the beginning of the project or if the organism itself was deemed to be irretrievably insufficient for the required purposes after extensive attempts at process optimisation.

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8. Appendix

8.1. Uninduced *E. coli* CLD048 Culture

8.1.1. Batch: Flow Cytometry and TEM data

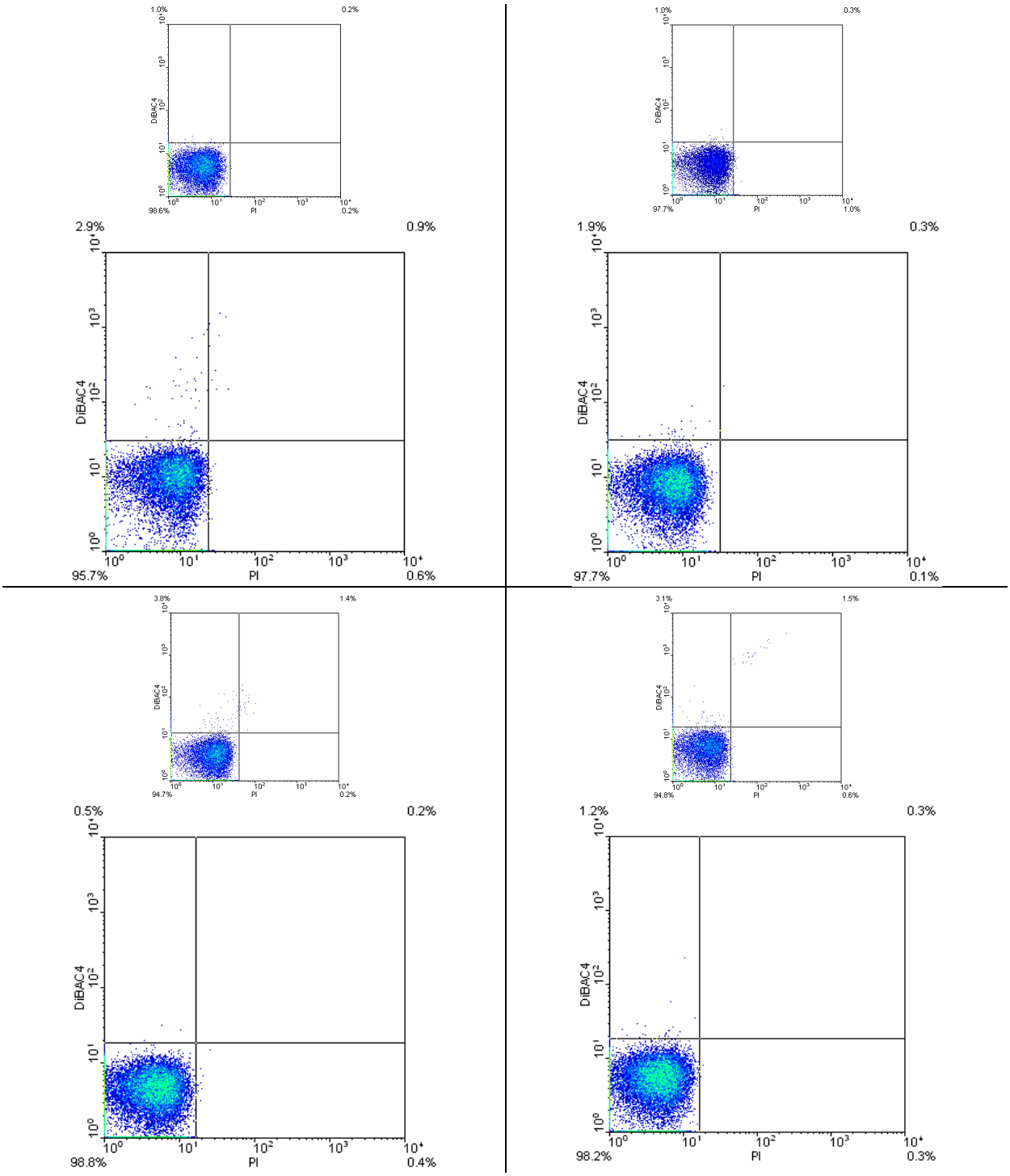

Figure 8.1. Flow cytometric analysis of *E. coli* CLD048 uninduced, batch stirred-tank fermentation in Figure 4.21. stained with PI & $DiBAC_4(3)$. Quadrants show plots from two different fermentations, clockwise from top left: 4; 6.5; 9.5 and 10.5 hours.

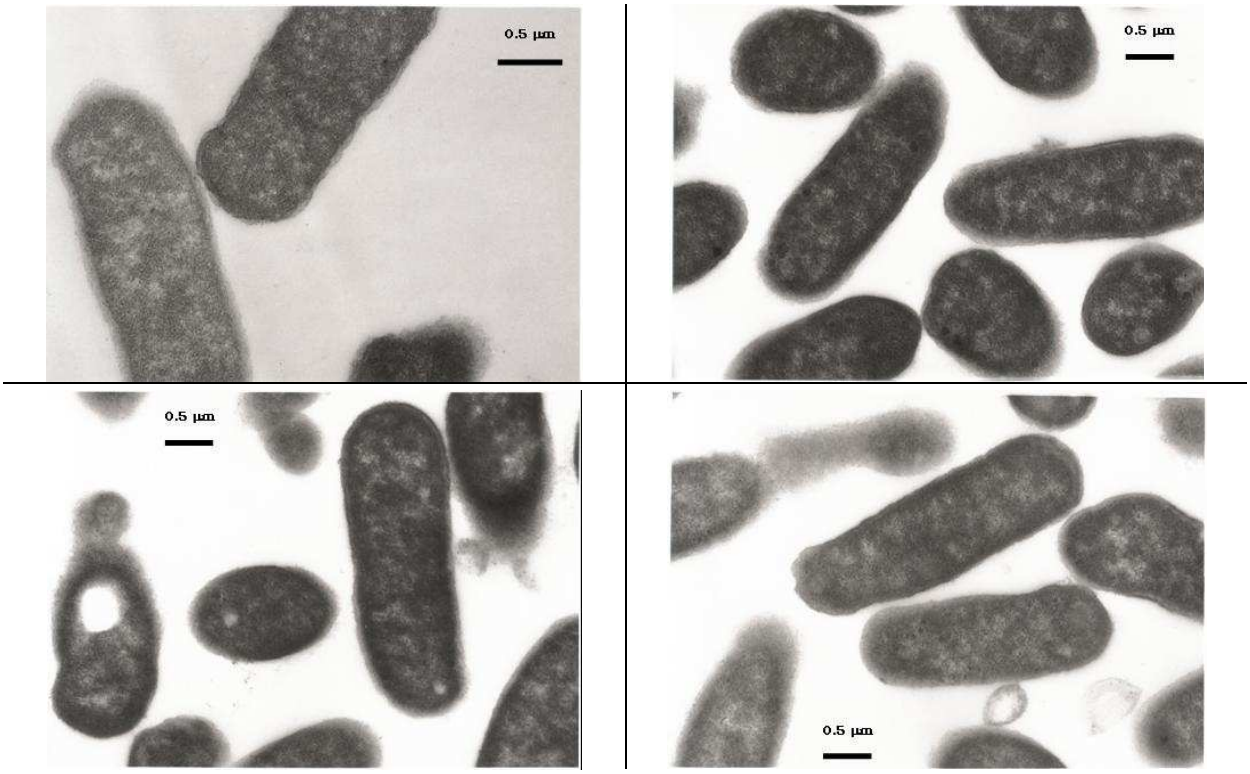

Figure 8.2. Transmission Electron Micrographs of *E. coli* CLD048 batch stirred-tank fermentation in Figure 4.21. Cells were induced at OD_{600nm} ≈ 20 using 0.1 mM IPTG. Clockwise from top left: 6; 8, 9 and 10 hours. Analysis was performed on a single fermentation, which was similar to the others performed in the other measured characteristics.

8.1.2. Early Fed-Batch: Flow Cytometry and TEM Data

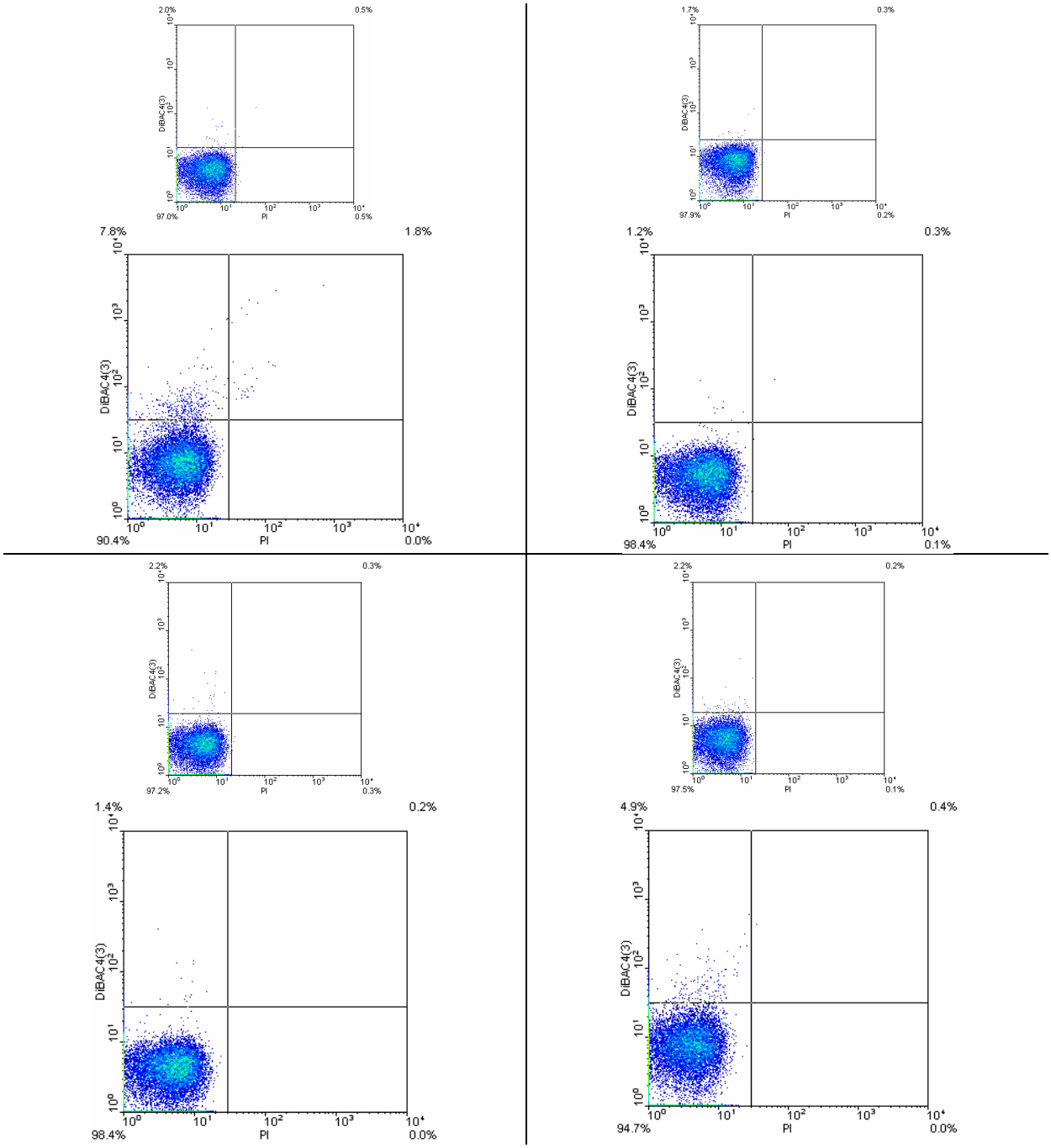

Figure 8.3. Flow cytometric analysis of *E. coli* CLD048 uninduced, fed-batch stirred-tank fermentation in Figure 4.29. stained with PI & $DiBAC_4(3)$. Clockwise from top left: 6; 10; 14 and 18 hours. Each quadrant shows equivalent data from two different fermentations

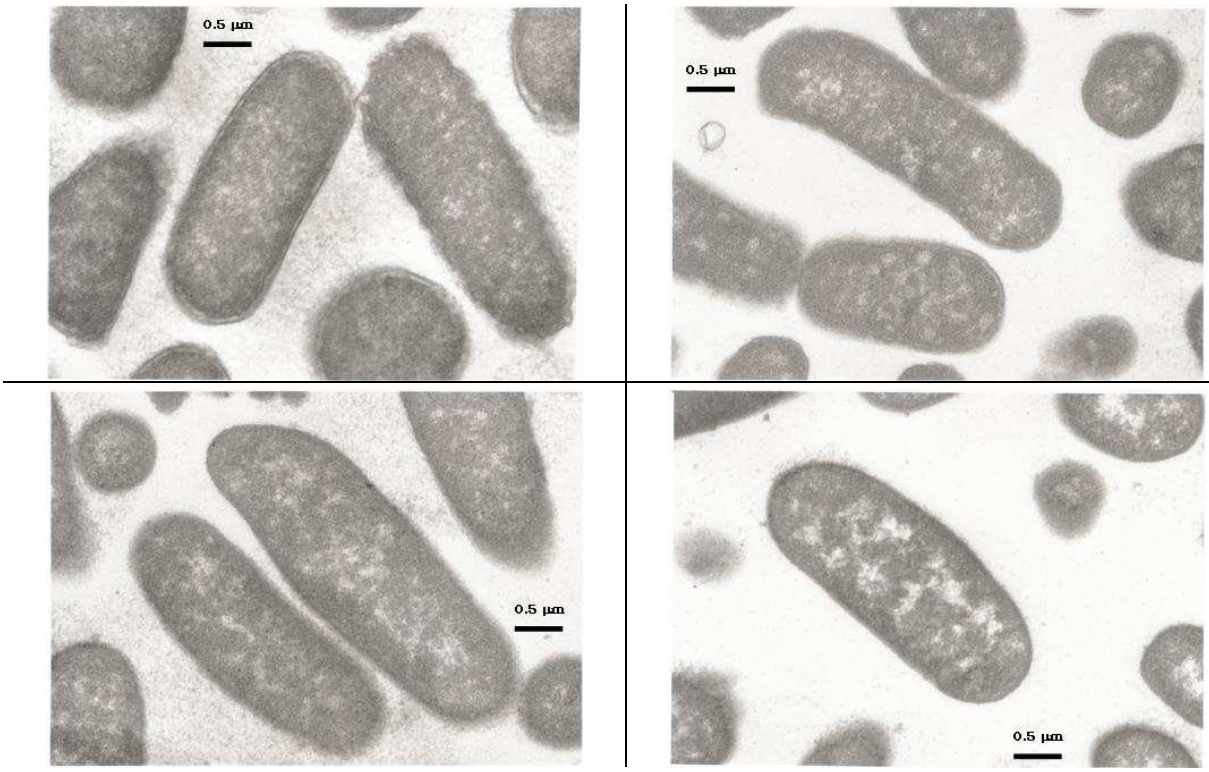

Figure 8.4. Transmission electron micrographs of *E. coli* CLD048 fed-batch stirred-tank fermentation in Figure 4.29. Clockwise from top left: 6; 10, 14 and 18 hours. Analysis was performed on samples from a single fermentation.

8.1.3. Late Fed-Batch: Flow Cytometry and TEM Data

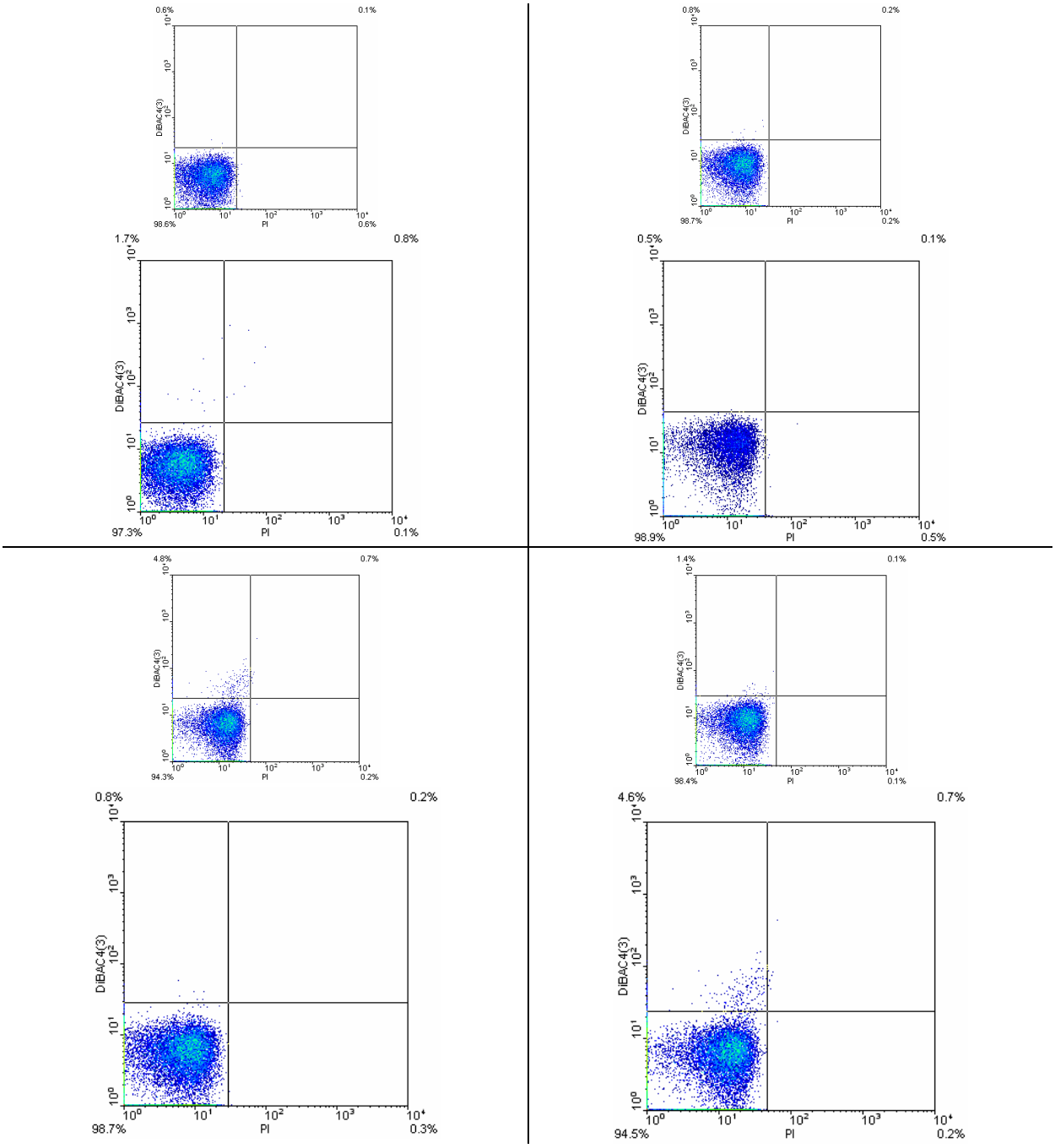

Figure 8.5. Flow cytometric analysis of *E. coli* fed-batch stirred-tank fermentation in Figure 4.29. stained with PI & $DiBAC_4(3)$. Clockwise from top left: 6; 10; 14 and 18 hours. Each quadrant shows equivalent data from two fermentations.

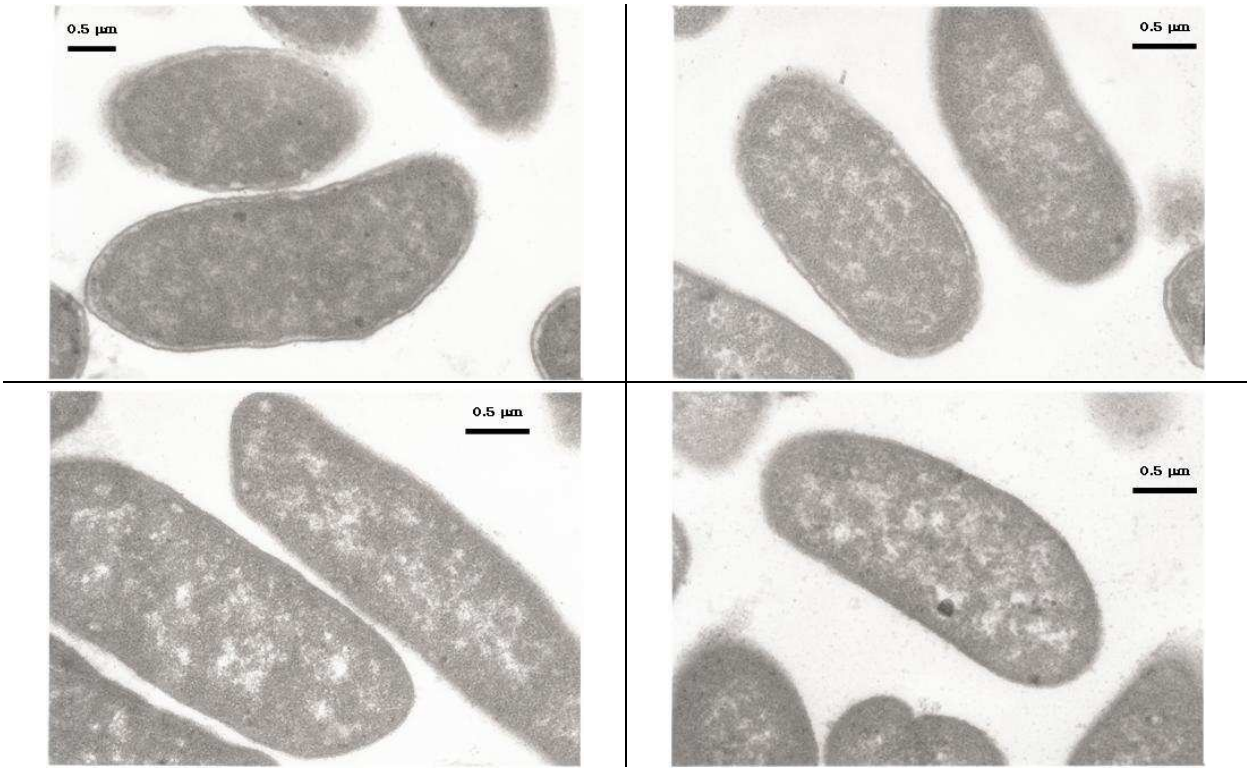

Figure 8.6. Transmission electron micrographs of *E. coli* fed-batch stirred-tank fermentation in Figure 4.29. Clockwise from top left: 6; 10, 14 and 18 hours. Analysis was performed on samples from a single fermentation.

8.2. Induced *E.* coli W3110 Stirred-Tank Reactor Culture

8.2.1. Batch: Flow Cytometry Data

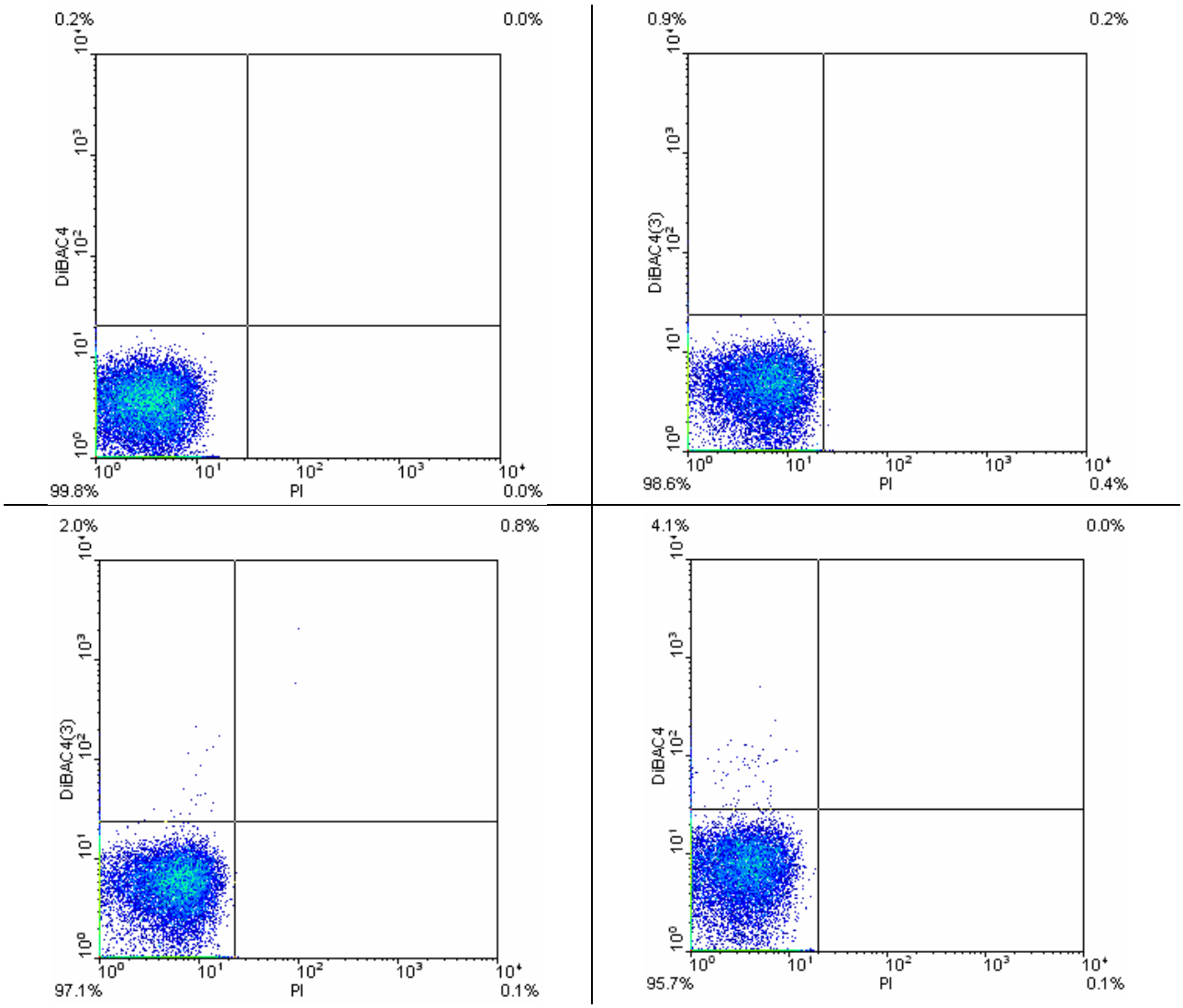

Figure 8.7. Flow cytometric analysis of *E. coli* batch stirred-tank fermentation in Figure 4.21. stained with PI & $DiBAC_4(3)$. Cells were induced at OD_{600nm} ≈ 20 using 0.1 mM IPTG. Data shows cells analysed at 7 and 10 hours.

8.2.2. Fed-Batch 1: Flow Cytometry Data

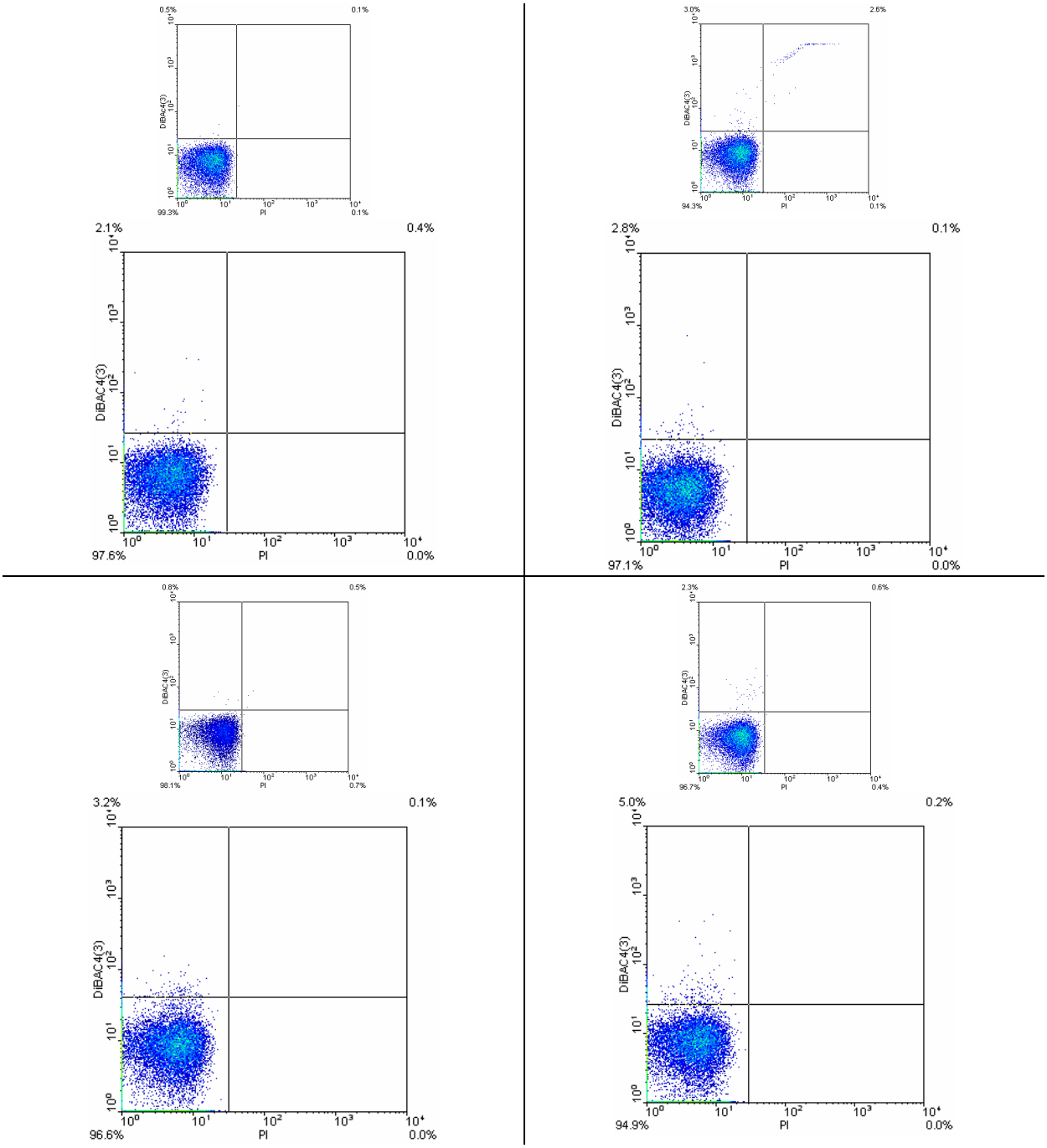

Figure 8.8. Flow cytometric analysis of *E. coli* fed-batch stirred-tank fermentation 1 in Figure 4.29. stained with PI & $DiBAC_4(3)$. Cells were induced at OD_{600nm} ≈ 50 using 0.1 mM IPTG. Each quadrant shows equivalent data from two fermentations; clockwise from top left: 6; 10; 12 and 14 hours..

8.2.3. Fed-Batch 2: Flow Cytometry Data

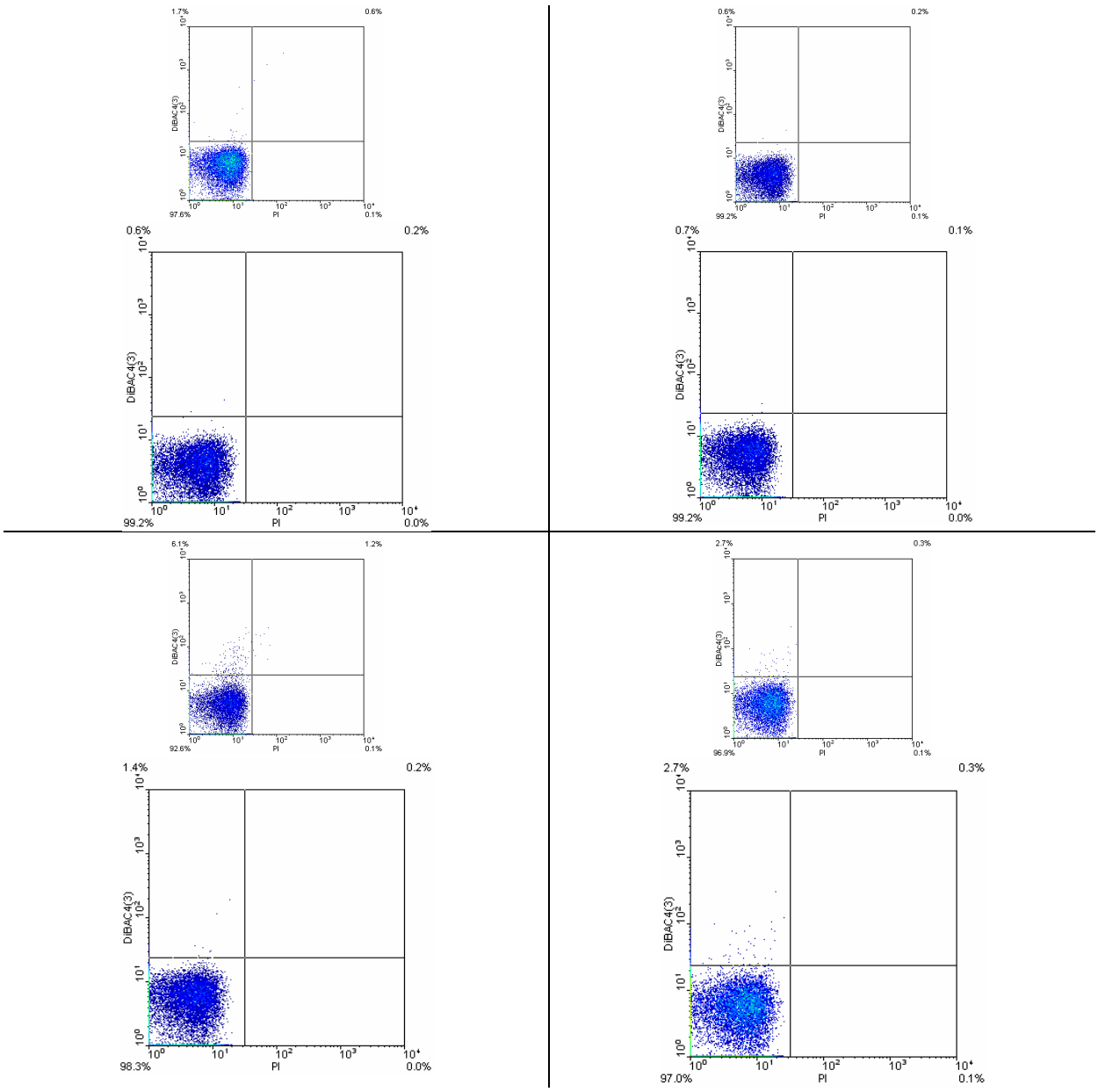

Figure 8.9. Flow cytometric analysis of *E. coli* fed-batch stirred-tank fermentation in Figure 4.35. stained with PI & $DiBAC_4(3)$. Cells were induced at OD_{600nm} ≈ 20 using 0.1 mM IPTG. Each quadrant shows equivalent data from two fermentations; clockwise from top left: 7; 9; 13 and 17 hours.

8.2.4. Fed-Batch 3: Flow Cytometry Data

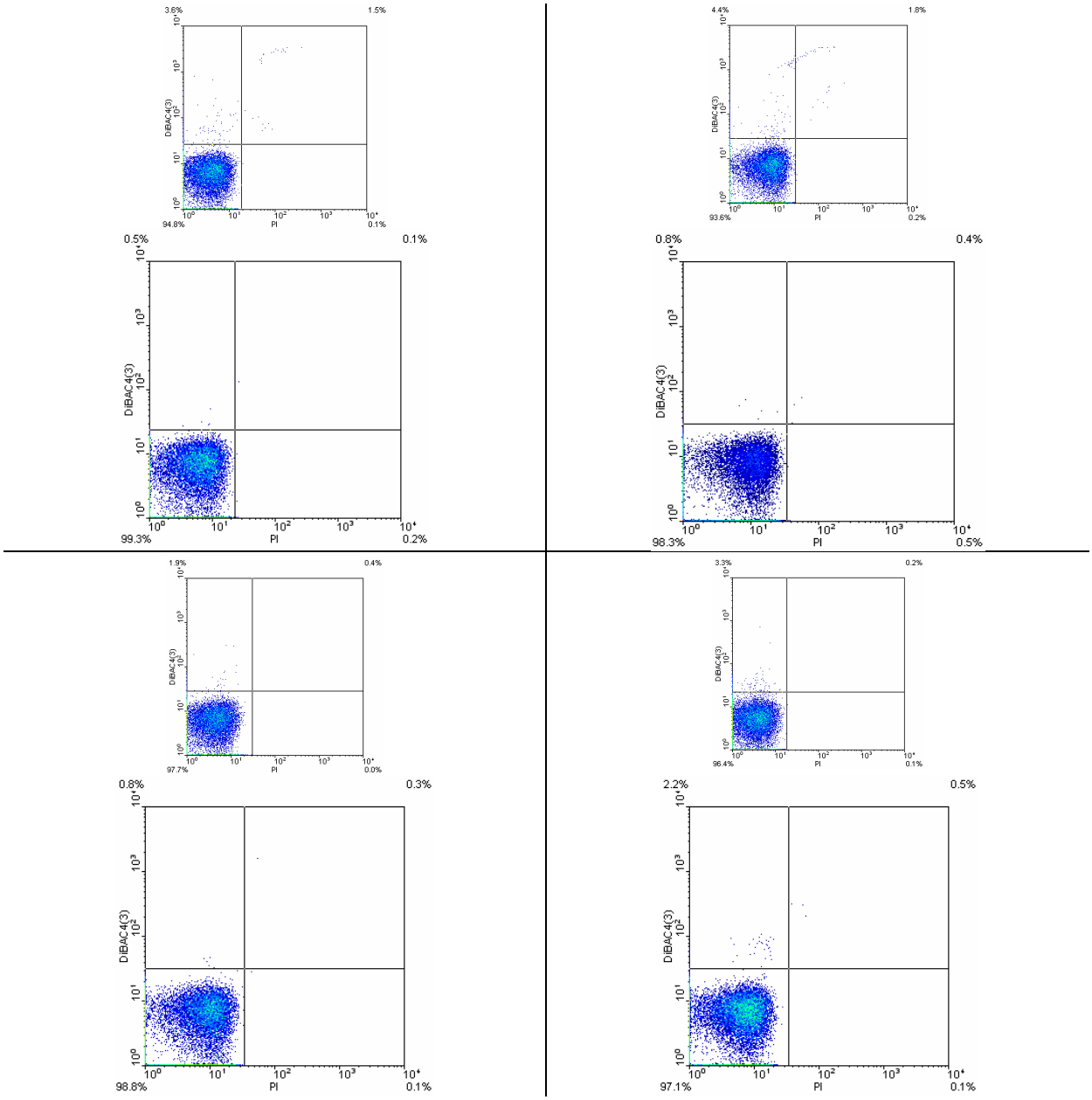

Figure 8.10. Flow cytometric analysis of *E. coli* W3110 fed-batch stirred-tank fermentation in Figure 4.43. stained with PI & $DiBAC_4(3)$. Cells were induced at OD_{600nm} ≈ 20 using 0.1 mM IPTG. Each quadrant shows equivalent data from two fermentations; clockwise from top left: 7; 9; 13 and 15 hours.

8.3. Publication

8.3.1. Conferences

8.3.1.1. Poster

Society for General Microbiology 158th Meeting: Warwick University, UK; 3-6th April 2006.

Individuals behave differently – multi-parameter flow cytometry for monitoring *Bacillus cereus* batch fermentation processes

Microbiology is important to both human health and industry, therefore many methods have been developed to count microorganisms in the process environment. Accurate measurements relating to cell proliferation and viability are essential if informed decisions about a process are to be made, since process performance will depend largely upon cell number and individual cell
physiological state. The development of multi-parameter flow cytometric techniques in our laboratories has led to a functional classification of the physiological state of single celled microorganisms. This classification is based on the presence or absence of an intact fully polarized cytoplasmic membrane and the transport systems across it. Using these techniques it is possible to resolve a cell's physiological state, beyond culturability to include metabolic
activity enabling assessment of population heterogeneity. Importantly results are available in real-time, 1-2 minutes after a sample is taken enabling informed decisions to be taken about a process.

http://www.sgm.ac.uk/meetings/pdfabstracts/warwick2006abs.pdf
Accessed: 7th July 2010

Society for General Microbiology Spring Meeting: Edinburgh, UK. 31st March-3rd April.

Analysis of recombinant protein production in *Escherichia coli* and its effects on the organism, during batch and fed-batch fermentation

Due to the overwhelming body of knowledge surrounding *E. coli*, it is a natural choice for use as a vector for the manufacture of recombinant biological products. The aim of this work was to attempt to improve the fate of the micro-organisms and gain some measure of control over the breakdown of said organisms by adjusting fermentation parameters or other extraneous factors. We have utilized flow cytometry alongside more traditional techniques, to better understand the effect of protein overexpression on the *E. coli* cell using differing modes of operation and

inducer concentration. We have seen that the effort expended in directing the product in question to the periplasm has been misspent, with widespread cell fragmentation and product aggregation resulting from induction of protein expression.

http://www.sgm.ac.uk/meetings/pdfabstracts/edinburgh2008abs.pdf
Accessed 7th July 2010

8.3.1.2. Oral

European Symposium on Biochemical Engineering Science 6th Meeting: Salzburg, Austria; 27-30th August 2006

Individuals behave differently - multi-parameter flow cytometry for monitoring bacterial batch fermentation processes.

Microbiology is important to both human health and industry, therefore many methods have been developed to count micro-organisms in the process environment. Accurate measurements relating to cell proliferation and viability are essential if informed decisions about a process are to be made, since process performance will depend largely upon cell number and individual cell physiological state. The advantages of using cytometric techniques over the more traditional microbiological analyses are well-documented and the development of multi-parameter flow cytometric techniques in laboratories around the world has led to the functional classification of the physiological state of single-celled micro-organisms. This classification is often based on either 1) the presence or absence of an intact, fully polarised cytoplasmic membrane and the transport systems across it or 2) energy dependent/independent intracellular enzyme activities. Using all of these techniques it is possible to resolve an individual microbial cell's physiological state, beyond culturability (the latter usually based on the measurement of number of c.f.u./mL) to include metabolic activity enabling assessment of population heterogeneity and dynamics. In this work we compare three well-known flow cytometric techniques for measuring cell physiological state, namely PI/$DiBAC_4(3)$, PI/$DiOC_6(3)$ and the RedoxSensor Green™ kit (Molecular Probes/Invitrogen) on batch cultures of *Bacillus cereus*, *Bacillus Licheniformis* and *Escherichia coli*. All three methods were found to work well with comparable results but the RedoxSensor Green™ kit (Molecular Probes/Invitrogen) may have advantages when endospores as well as vegetative cells are present in a culture.

http://www.esbesweb.org/esbesweb_media/Downloads/ESBES+6+Salzburg/esbes_final_programme.pdf
Accessed: 7th July 2010

Analysis of microbial cells at the single cell level 3rd Meeting: Bad Schandau, Germany. 22-25th May 2008

Measuring the Effect of Recombinant Antibody Fragment (Fab) Production in *Escherichia coli* at the Single-Cell Level Using Multiparameter Flow Cytometry

Despite a long history of successful exploitation of the *E. coli* cell for mass production of medicines, there is still much more information that can be gathered. In this study, we have been able to identify and follow cellular changes during batch and fed-batch manufacture of Fab fragments. We have observed that onset of induction during batch culture has had an immediate and deleterious effect on the ability of the cells to grow and reproduce. Flow cytometric analysis using $DiOC_6(3)$, a carbocyanine and $DiBAC_4(3)$, an oxanol dye both in conjunction with propidium iodide, has shown us that this negative effect can be monitored at the single-cell level giving us at-line information regarding the level of metabolic stress being exacted on the cell by the process conditions. Subsequent analysis has also shown that the cells have also been unable to manufacture significant quantities of the desired product in batch culture. Analysis of fed-batch cultures has shown that the cells have produced significant quantities of Fab enabled by their ability to withstand the demands placed on them by the process with the flow cytometry indicating a slower transition from a healthy state to that of a metabolically compromised organism.

http://www.organobalance.de/EFB/
Accessed: 7th July 2010

Society for General Microbiology Spring Meeting: Edinburgh, UK. 31st March-3rd April.

Analysis of recombinant protein production in *Escherichia coli* and its effects on the organism, during batch and fed-batch fermentation

Due to the overwhelming body of knowledge surrounding *E. coli*, it is a natural choice for use as a vector for the manufacture of recombinant biological products. The aim of this work was to attempt to improve the fate of the micro-organisms and gain some measure of control over the breakdown of said organisms by adjusting fermentation parameters or other extraneous factors. We have utilized flow cytometry alongside more traditional techniques, to better
understand the effect of protein overexpression on the *E. coli* cell using differing modes of operation and inducer concentration. We have seen that the effort expended in directing the product in question to the periplasm has been misspent, with widespread cell fragmentation and product aggregation resulting from induction of protein expression.

http://www.sgm.ac.uk/meetings/pdfabstracts/edinburgh2008abs.pdf
Accessed 7th July 2010 – Note; this material was presented in both oral and poster form, due to a last moment replacement of a speaker who failed to attend the conference. As a result, this abstract is not mentioned in the oral presentation section of the conference details linked above.

European Symposium on Biochemical Engineering Science 7th Meeting: Faro, Portugal. 7-10th September 2008

Flow cytometric analysis of recombinant antibody fragment production in *Escherichia coli* under Batch and Fed-Batch cultures

Micro-organisms are in widespread use throughout the biopharmaceutical industry. It is their rapid growth, and simple, robust physiology that makes them the ideal microscopic "factories" for the manufacture of complex biological medicines. In this study our research concerns the production of a recombinant antibody fragment (Fab), targeted to the periplasm, in *E. coli*. Flow cytometry was used to gain a more complete understanding of the effects of cultivation and Fab production on the cells and their population heterogeneity within the bioreactor. Production was performed in both batch and fed-batch modes of operation, to examine the effect of different, industrially-significant processes on cell vitality. During batch manufacture of Fab, significant stress effects were observed on the cells following addition of the inducing agent which were not due to a toxic effect of the inducer, and the level of Fab expression (measured by ELISA and SDS-PAGE) was very low. In contrast, much higher Fab expression and longer cell survival post induction was observed in fed-batch operation mode. Taken collectively these findings imply that together protein production and depletion of resources combine to exhibit a strongly deleterious effect on *E. coli* cell physiology. When the cells are supplied with additional nutrients, they can survive much longer, consequently giving rise to increased yields of target protein.

http://www.esbesweb.org/esbesweb_media/Downloads/ESBES_7__Faro-p-90/Programm_ESBES_2008.pdf
Accessed: 7th July 2010.

Society for General Microbiology Autumn Meeting: Trinity College, Dublin, Ireland. 8-11th September 2008

Analysis of recombinant protein production in *Escherichia coli* and its effects on the organism, during batch and fed-batch fermentation

Due to the overwhelming body of knowledge surrounding *E. coli*, it is a natural choice for use as a vector for the manufacture of recombinant biological products. The aim of this work was to attempt to improve the fate of the micro-organisms and gain some measure of control over the breakdown of said organisms by adjusting fermentation parameters or other extraneous factors. We have utilized flow cytometry alongside more traditional techniques, to better
understand the effect of protein overexpression on the *E. coli* cell using differing modes of operation and inducer concentration. We have seen that the effort expended in directing the product in question to the periplasm has been misspent, with widespread cell fragmentation and product aggregation resulting from induction of protein expression.

http://www.sgm.ac.uk/meetings/pdfabstracts/dublin2008abs.pdf
Accessed 7th July 2010
Note; this abstract is the same content as that presented at the SGM conference in Edinburgh. As a result of that poster presentation, I was invited to give a presentation at the Dublin meeting as a contestant in the Young Microbiologist of the Year Award.

8.3.2. Peer-Reviewed Literature

Following this page are copies of one publication in the journal Cytometry Part A, as well as a manuscript in the final stages of preparation for submission to the Journal of Microbiological Methods.