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Studies related to antibody fragment (Fab) production in *Escherichia coli* W3110 fed-batch fermentation processes using multi-parameter flow cytometry

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Running Title: Flow cytometric study of microbial fermentations.

Key terms: Bacteria; Membrane potential; Membrane integrity; Multi-parameter; Propidium iodide; bis-(1, 3-dibutylbarbituric acid) trimethine oxonol; Antibody fragment

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Background: Microbiology is important to industry therefore rapid and statistically representative measurements of cell physiological state, proliferation and viability are essential if informed decisions about fermentation bioprocess optimisation or control are to be made, since process performance will depend largely upon the number of metabolically active viable cells.

Methods: Samples of recombinant *Escherichia coli* W3110, containing the gene for the D1.3 anti-lysozyme Fab fragment under the control of the lac based expression system, were taken at various stages from fed-batch fermentation processes and stained with a mixture of bis-(1, 3-dibutylbarbituric acid) trimethine oxonol and propidium iodide (PI/BOX). Where appropriate, measurements of dissolved oxygen tension (DOT), OD_{600nm} and Fab concentration were made.

Results: Depending on time of induction the maximum amount of Fab accumulating in the supernatant varied quite markedly from 1 – 4 µgml⁻¹ as did subsequent cell physiological state with respect to PI/BOX staining with a concomitant drop in maximum biomass concentration.

Conclusion: Depending on point of induction a 4 fold increase in Fab production could be achieved accompanied by a ~50% drop in maximum biomass concentration but with a higher proportion of viable cells as measured by multi-parameter flow cytometry.

INTRODUCTION

The design of applications where a population of metabolising microbial cells interact within an artificially engineered environment (e.g. fermentation, bio-remediation, bio-transformation etc) is important for both human health and the economy, therefore many methods have been developed to count as well as identify micro-organisms during such bio-processes. Accurate measurements relating to cell physiological state, proliferation and viability are essential if informed decisions about process control are to be made, since process performance will depend largely upon the number of metabolically active cells [1]. In this way, desirable products can be harvested at optimal concentrations and inducible recombinant systems can be activated at the appropriate time so that high product yields are achieved. Such information is also important since a high number of dead, damaged or dormant cells present during any part of a process will have an obvious detrimental effect on the synthesis of any desired products or breakdown of waste materials [2].

Antibodies have emerged, in recent years, as important tools in the diagnosis and treatment of a variety of illnesses, with particular emphasis on cancer [3, 4]. As well as being able to exert a direct therapeutic effect, antibodies can also be utilised as delivery vehicles for other molecules and as blocking agents to shield antigens from the immune system. Additionally, antibody fragments can also be employed in *ex vivo* diagnostic devices (e.g. AIDS and pregnancy test kits). Where the availability of an Fc region (that which interacts with and recruits immune functions in the body) is unnecessary for the antibody's function, prokaryotic organisms can be used in order to manufacture the desired moiety in a cost-effective manner. *Escherichia coli*, whilst being poorly suited to whole antibody manufacture, is an ideal tool for the production of these simpler molecules [5] although heterologous proteins are rarely secreted into

the growth medium making subsequent downstream purification difficult [6]. From a bioprocessing perspective, the next best option to aid the purification of foreign proteins in *E. coli* other than secretion is targeting the protein to the periplasmic space. However, in the case of Fabs this has been shown to have a deleterious effect on the generation of fully functioning biomass [7] and it is this effect that must be managed when aiming to optimise the process. To date most of the work concerning optimisation of this type of process has been aimed at altering the genetics of the expression system to favour production [5, 8, 9]. However, Garcia-Arrazolla *et al* [10] have taken a more process-focussed approach by analysing the effect of carbon-limitation on the yield of Fabs. There has, however, been little development in the understanding of the effects of these changes on the physiology of the organism involved. Further, the ability to make informed process decisions designed to maximise the number of actively metabolising, and therefore productive, cells in the fermentation environment remains less well established.

Multi-parameter flow cytometry has many advantages over conventional microbiological analyses such as dilution plating (c.f.u. per ml) and these have been extensively reviewed elsewhere [1, 2, 11], but briefly, using various mixtures of fluorescent dyes, it is possible to resolve an individual microbial cell's physiological state beyond culturability, in 'real-time' based on the presence or absence of an intact fully polarised cytoplasmic membrane and the transport mechanisms across it. The presence of both an intact polarised cytoplasmic membrane and the active transport systems across it are essential for a fully functional healthy cell. In this study we used propidium iodide (PI) which binds to nucleic acid but will not cross an intact cytoplasmic membrane (of *E. coli* cultured conventionally in industrial bioreactors) in combination with bis-(1, 3-dibutylbarbituric acid) trimethine oxonol (BOX) which

accumulates intracellularly when the cytoplasmic membrane potential is low. In this paper we use this fluorescent technique to gain a deeper understanding of the interaction of the *E. coli* cell with the fermentation engineering environment to produce Fabs leading to real process improvements.

MATERIALS AND METHODS

Organism and growth conditions.

Escherichia coli W3110 (ATCC 27325) transformed with a plasmid containing the gene for the D1.3 anti-lysozyme Fab fragment under the control of the lac based expression system was maintained at -80 °C in LB Medium supplemented with 20 % v/v glycerol. Starter cultures were grown by adding 100 µl of the glycerol stock to 120 ml LB without glucose, in a 500 ml baffled shake-flask at 37 °C and 200 rpm in an orbital shaker for ~14 h. The basal fermentation medium was made up containing: (g l⁻¹) (NH₄)₂SO₄ 14; glycerol 35; yeast extract 20; KH₂PO₄ 2; K₂HPO₄ 16.5; citric acid 7.5. Polypropylene glycol 2000 was added at 0.66 ml l⁻¹ to control foaming. After cooling, 4 separate solutions were filter sterilised and aseptically added to the fermentation medium (ml l⁻¹): 1M MgSO₄ 10; 1M CaCl₂ 2; tetracycline 1 (of a 15 mg ml⁻¹ stock) and trace element solution 34. The trace element solution contained (mg l⁻¹): FeSO₄·7H₂O 3.36; ZnSO₄·7H₂O 0.84; MnSO₄·H₂O 0.51; Na₂MoO₄·2H₂O 0.25; CuSO₄·5H₂O 0.12; H₃BO₃ 0.36; Conc. H₃PO₄ 48 ml.l⁻¹. Fermentations were carried out in the fed-batch mode with the feed made up as follows (g.l⁻¹): glycerol 714; MgSO₄ 3.6, and was added at a constant rate of 45 ml h⁻¹. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM in order to induce production of the Fabs at the designated time point. Laboratory scale fed-batch fermentations were carried out in a 5 l cylindrical glass bioreactor, (162 mm diameter x 300 mm total height), with an initial working volume of 3 l rising to ~ 4 l

at the end of the fermentation. The vessel was fitted with two 82 mm, six bladed radial flow paddle type impellers which were 80 mm apart with the lower impeller situated 80 mm above the bottom of the vessel. The vessel was also fitted with three equally spaced baffles, width 15 mm.

All fermentation vessels were equipped for the measurement of dissolved oxygen, pH, temperature and fermentation's were run at 37 °C. Impeller speeds and aeration rates were held constant (~1 vvm, adjusted for volume increases during feeding and 1000 rpm) so that the DOT was maintained above ~5%, the critical DOT level for *E. coli* [13]. pH was controlled above 7.0 using a 25% aqueous solution of ammonia.

Flow cytometry protocols.

Fluorescent measurements were made using a Coulter (High Wycombe, UK) EPICS ELITE flow cytometer with 488 nm excitation from an argon-ion laser at 15 mW. Samples taken from the culture were immediately diluted (at least 1:2000 v/v) with phosphate buffer solution (PBS, pH 7.0) and stained with a mixture of PI and BOX. Samples were kept in a sonication bath for 10s prior to analysis, in order to avoid problems associated with cell aggregation. Stock solutions of each dye were prepared as follows: BOX, was made up at 10 mg ml⁻¹ in DMSO and PI was made up at 2 mg ml⁻¹ in distilled water. The working concentrations of BOX and PI were 0.6 µg ml⁻¹ and 3 µg ml⁻¹, respectively in Dulbecco's buffered saline (pH 7.2, DBS). All solutions were passed through a 0.2 µm filter, immediately prior to use, to remove particulate contamination. Additionally, software discriminators were set on both the FALS and RALS signals to further reduce electronic and small particle noise. The optical filters were set up so that PI fluorescence was measured at 630 nm and BOX fluorescence was measured at 525 nm. Where there was some spectral overlap between the emitted

fluorescence of the fluorochromes used the systems compensation was set up to eliminate any interference.

Other analytical techniques.

Cell growth was monitored by optical density (600nm) using a double-beam spectrophotometer, with samples being diluted into the range 0-0.6 absorbance units prior to analysis. This was compared with the dry cell weight (DCW) of samples dried at 100 °C to constant weight.

The expression of D1.3 was monitored by ELISA using the following solutions (per litre); coating buffer: Na₂CO₃ 1.59 g; NaHCO₃ 2.93 g; pH 9.6; Lysozyme 1 g. Blocking buffer (per litre): 10 PBS tablets (Oxoid, Basingstoke, UK), bovine serum albumin 0.1 g. Washing buffer (per litre): 10 PBS tablets (Oxoid, Basingstoke, UK), Tween 20 1 ml. Detection antibody: goat anti-human Fab peroxidase conjugate (Sigma-Aldrich, Gillingham, UK) 2 µl in 20 ml blocking buffer. Peroxidase substrate (KPL, Gaithersburg, MD) used according to the manufacturers instructions. 1M Phosphoric acid. A 96 well (loading) plate with 120 µl of coating buffer with added lysozyme was incubated overnight at 4 °C. Following incubation, the coating buffer with lysozyme was removed and replaced with 200 µl blocking buffer and incubated at 37 °C at 500 rpm for 1 h. A dilution series was then created with 180 µl of neat samples 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. After the incubation was completed the plate was washed with 3 x 300 µl washing buffer. The diluted samples were then transferred from the dilution plate to the loading plate ~100 µl per well. The loading plate was then incubated at 37 °C for 1h at 500 rpm then washed with 3 x 300 µl washing buffer with 100 µl detection antibody added to each

well and further incubated at 37 °C and 500 rpm for 1 h. The loading plate was again washed (3 x 300 µl washing buffer) and 100 µl substrate added to each well and incubated at room temperature for 10 mins. The reaction was stopped by the addition of 100 µl of phosphoric acid and the plate analysed by measurement of absorbance at 450 nm. Antibody concentrations were determined following construction of a standard curve based on the absorbance of wells containing a known concentration of the recombinant antibody fragment.

RESULTS AND DISCUSSION

Duplicate fed-batch fermentations of *E. coli* W3110 where the point of induction of the D1.3 anti-lysozyme Fab (Fab) by the addition 0.1mM IPTG was varied, were carried out and reproducible measurements of DOT (% saturation), DCW (g l⁻¹), OD_{600nm} as well as µg Fab ml⁻¹ supernatant were obtained (Figure 1). In all cases µ_{max} was ~1.1 h⁻¹ but depending on time of induction the maximum amount of Fab accumulating in the supernatant varied quite markedly from ~1 – ~ 4 µg ml⁻¹ as did subsequent cell physiological state (Figure 2 - 4). Osmotic shock solutions were used to fractionate the total antibody fragment produced based on sub-cellular location, however, in all cases, unusually for this recombinant system, the overwhelming majority of the Fab (> 85%) was found in the extracellular milieu. Why this is, is unclear and will require further investigation since the protein was supposed to be targeted to the periplasmic space. However, since very little (~15% of the total) of the protein accumulated intracellularly it was not possible to follow protein accumulation based on changes in the intrinsic light scatter measurements of cells alone as has been reported previously [19, 20, 21, 22] and the characteristic bi-modal clustering with respect to forward and right angle light scatter associated with the rod-like shape of *E.*

coli remained unchanged throughout each process. In all cases after inoculation the OD_{600nm} began to rise with a concomitant fall in DOT and a characteristic double spike in DOT noted between 5-7 hrs, the first of which indicating carbon exhaustion upon which feeding commences. When induction occurs relatively late on (OD_{600nm} ~50 corresponding to a DCW of ~ 21 g l⁻¹) during the process a maximum OD_{600nm} of 66 (corresponding to a DCW of 25g l⁻¹) was achieved at 10 hrs whilst a maximum Fab concentration of 1 µgml⁻¹ was measured at 14 hrs when the fermentation was terminated. When induction occurred at the same time as the second DOT spike (OD_{600nm} of ~21 corresponding to a DCW of ~ 7 g l⁻¹) a lower maximum OD_{600nm} of 45 (corresponding to a DCW of ~ 20 g l⁻¹) was attained later at 12 hrs whilst a higher Fab concentration of 2.5 µg ml⁻¹ was measured earlier at 11hrs after which Fab concentration fell until termination presumably degraded by free protease in the fermentation broth. When induction occurred at the same time as feeding was started (essentially the same OD_{600nm} of ~21 as before but coinciding with the basal value after the first DOT spike) a much lower maximum OD_{600nm} of 29 (corresponding to a DCW of ~ 16 g l⁻¹) was attained at 9hrs with a much higher maximum Fab concentration of 4.1 µg ml⁻¹ measured at 11 hrs after which the fermentation was terminated. Analysis of the cell physiological state by multi-parameter flow cytometry throughout each process is very interesting (Figures 2 – 4). In the cases where Fab production was induced at an OD_{600nm} of ~50 and ~21 (at the basal point after the first DOT spike) there was a progressive but detrimental change in cell physiological state with respect to cytoplasmic membrane polarisation and permeability (Figure 2 and 4). Interestingly at the termination of both fermentations there was a high proportion of cells (~73% and ~40% respectively) being classified as having a low cytoplasmic membrane potential (i.e. positively stained with BOX only). In the case of the fermentation where the Fab production was induced at an OD_{600nm} ~21 (but at the

same time as the second DOT spike) cell physiological state remained largely the same throughout (Figure 3). The drop in final OD_{600nm} when higher maximum Fab concentrations are measured is not entirely unexpected [19], since energy is presumably being redirected away from cell growth towards protein synthesis in the higher producing cultures. The reason that the OD_{600nm} has such an effect on maximum Fab concentration is probably related to the concentration of IPTG per cell at time of induction. Since the IPTG concentration is relatively low and kept the same (0.1 mM) for each fermentation, we know that the concentration of IPTG per individual cell at the exact time of IPTG addition is very different. This means that as the OD gets higher fewer cells are fully induced (if at all) resulting in a heterogeneous culture of producing and non-producing cells. Therefore, at a low OD_{600nm}, the induction effect on the culture as a whole is stronger than at a higher OD_{600nm}. Provided that the IPTG is irreversibly bound to the repressor this will lead to a greater level of expression of the protein and a greater stress for the individual induced cell but not at all for cells un-induced. Why the point of induction on the first DOT spike had such an effect (on physiological state, max Fab concentration and max OD_{600nm}) compared to induction on the second DOT spike is more uncertain but is probably due to a subtle but rapid metabolic switch since *E. coli* is known to respond very quickly to changes in substrate concentration by the fast transcriptional induction of an alternative set of genes [23, 24]. Such 'stress genes', are rapidly transcribed, when a cell experiences changes in glucose concentration resulting in a protective effect on the cell but leading to lower protein productivity.

CONCLUSIONS

Here we show that it is not possible to follow soluble protein production using intrinsic light scattering when the majority (~85%) of the Fab is secreted into the fermentation medium. This is in contrast to the accumulation of insoluble inclusion bodies in the cytosol which can cause a change to the characteristic bi-modal clustering of *E. coli* with respect to forward and right angle light scatter. It is clear that the point of induction of the Fab by IPTG addition had a clear effect on fermentation progression and the physiological consequences of this can be followed by flow cytometry. Depending on point of induction a 4 fold increase in Fab production could be achieved accompanied by a ~50% drop in maximum biomass concentration but with a higher proportion of viable cells.

Over the past 15 years or so it is recognised that the relative complexity and high cost (cf traditional techniques) of flow cytometry equipment has prohibited it from becoming a routine analytical tool for use in most microbiology laboratories. Nevertheless flow cytometry is proving to be a valuable research measurement technique for the study of microbial population dynamics within bioreactors, leading to informed improvements in process performance.

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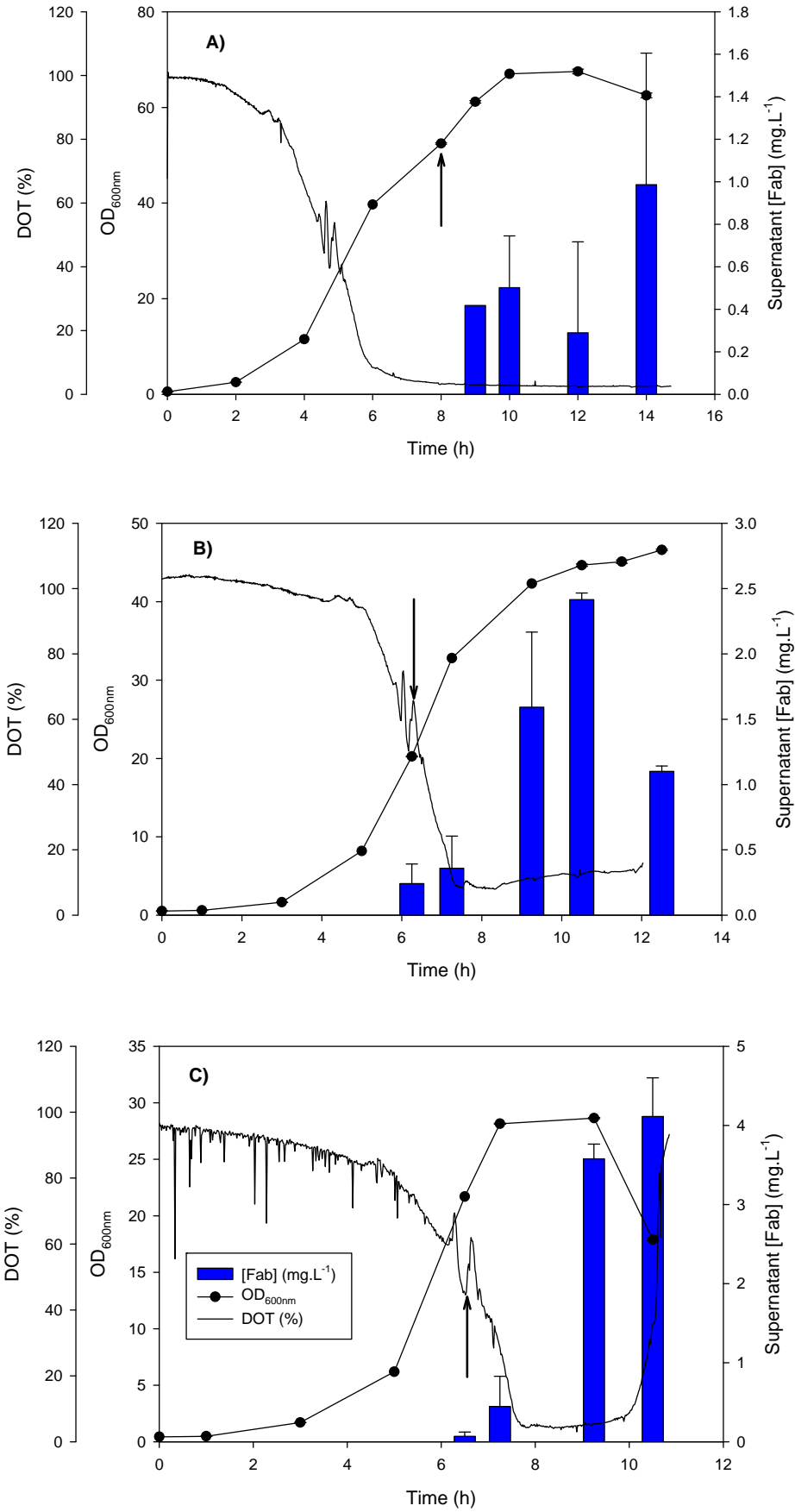


Figure 1

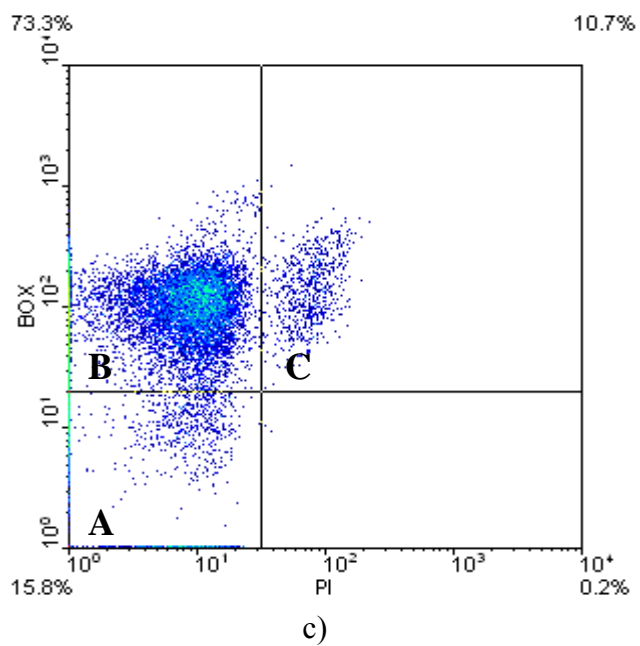
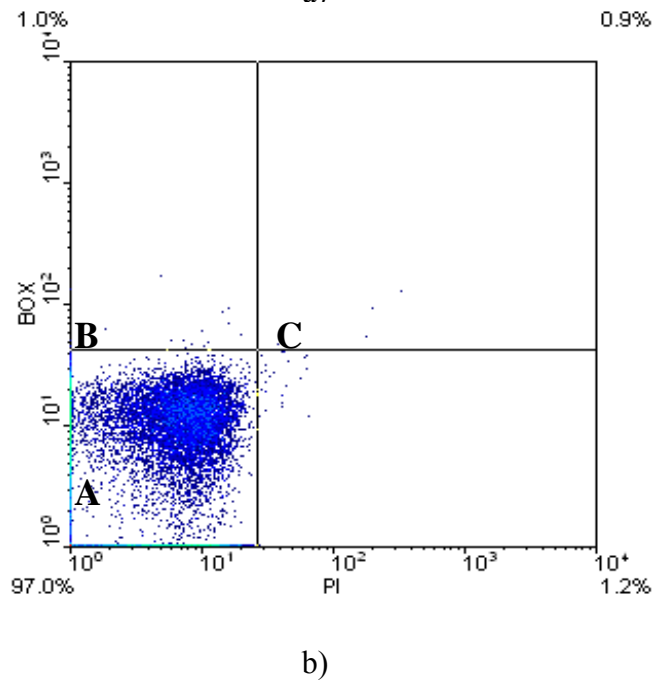
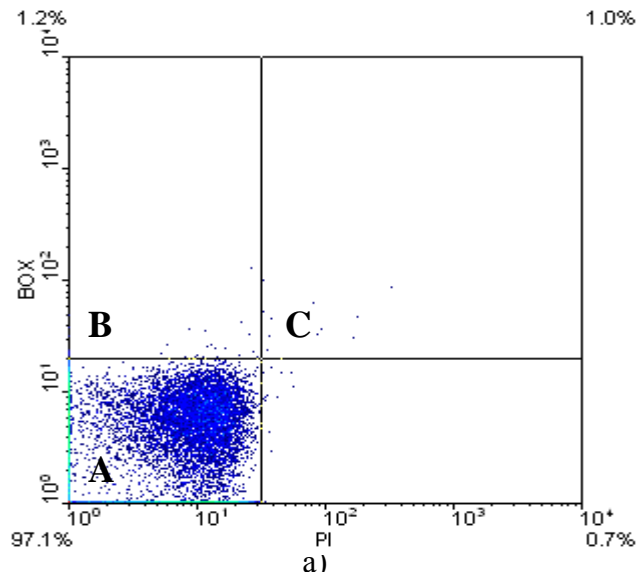


Figure 2

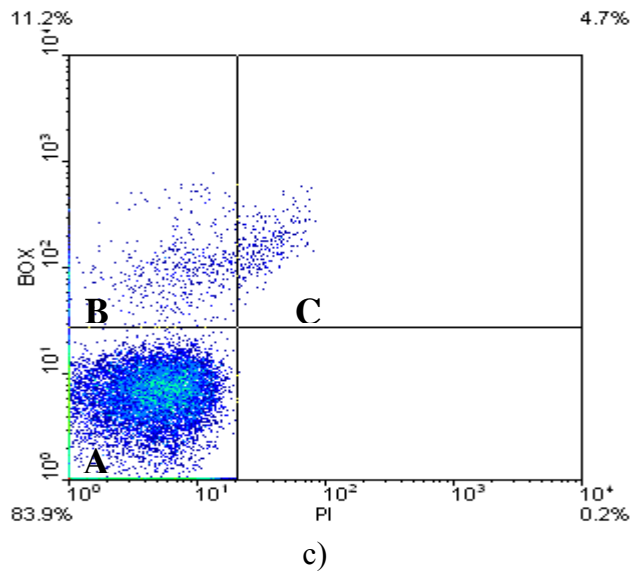
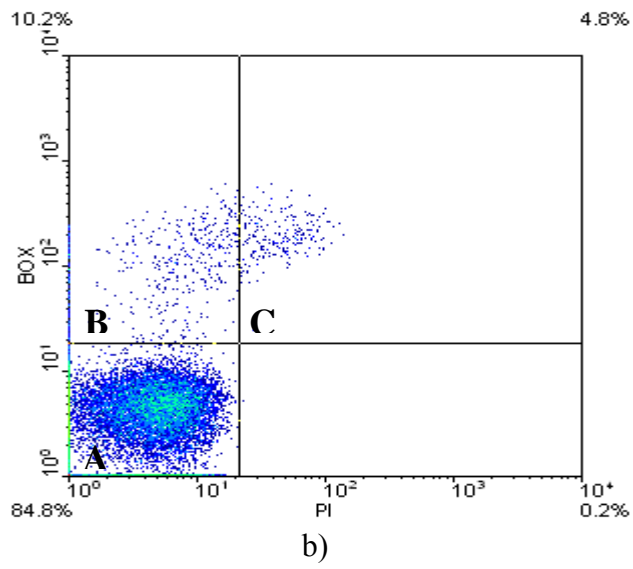
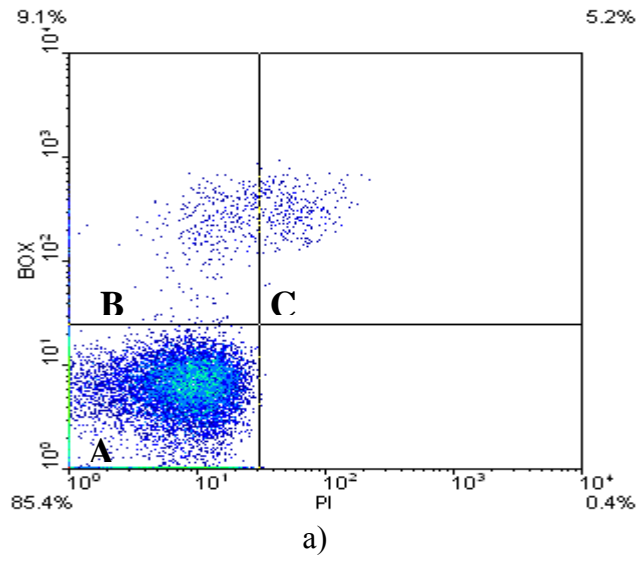


Figure 3

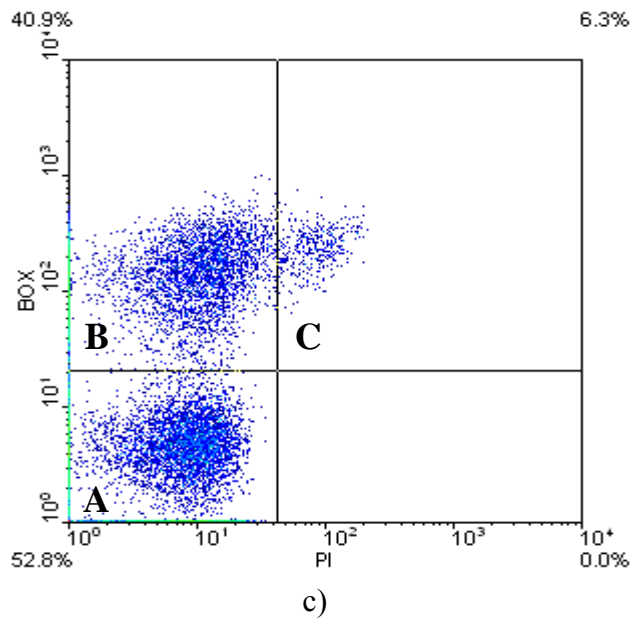
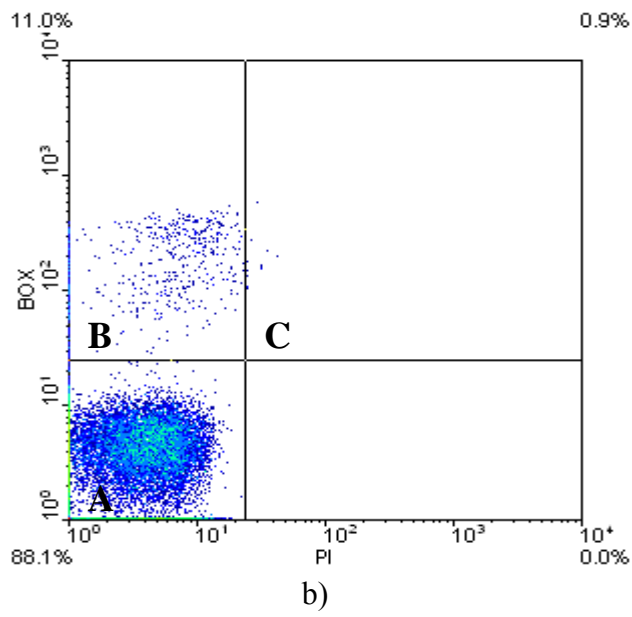
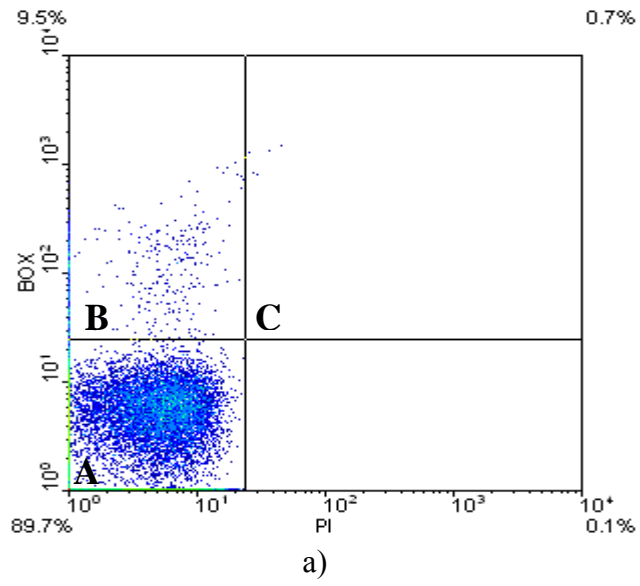


Figure 4

Figure 1. Optical density (OD_{600nm}) and DOT (% sat) Fab ($ng.ml^{-1}$ supernatant) profiles for a series of fed-batch fermentations where the point of induction of Fab synthesis was varied by addition of 0.1 mM IPTG at different optical densities and time points. A) Induction occurred at an OD_{600nm} of 50. B) Induction occurred at an $OD_{600nm} \sim 21$ (but at the same time as the second DOT spike). C) Induction occurred at an $OD_{600nm} \sim 21$ (at the basal point after the first DOT spike).

Figure 2. Cell samples taken at (a) 0hrs immediately after inoculation (b) 4hrs and (c) 14hrs during the course of a batch fermentation where synthesis of Fab was induced as in Figure 2A. Cells were stained with PI and BOX, up to three main sub-populations of cells can be distinguished, corresponding to healthy polarised cells (A), no staining, cells with a low cytoplasmic membrane potential (B), stained with BOX; and cells with depolarised permeabilised cytoplasmic membranes (C), stained with both PI and BOX.

Figure 3. Cell samples taken at (a) 0hrs immediately after inoculation (b) 3hrs and (c) 11hrs during the course of a batch fermentation where synthesis of Fab was induced as in Figure 2B. Cells were stained with PI/BOX, up to three main sub-populations of cells can be distinguished, corresponding to healthy polarised cells (A), no staining, cells with a low cytoplasmic membrane potential (B), stained with BOX; and cells with depolarised permeabilised cytoplasmic membranes (C), stained with both PI and BOX.

Figure 4. Cell samples taken at (a) 0hrs immediately after inoculation (b) 3hrs and (c) 13hrs during the course of a batch fermentation where synthesis of Fab was induced as in Figure 2C. Cells were stained with PI/BOX, up to three main sub-populations of

cells can be distinguished, corresponding to healthy polarised cells (A), no staining, cells with a low cytoplasmic membrane potential (B), stained with BOX; and cells with depolarised permeabilised cytoplasmic membranes (C), stained with both PI and BOX.