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**Induction studies with *Escherichia coli* expressing recombinant interleukin-13 using multi-parameter flow cytometry.**

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## **ABSTRACT**

The expression of interleukin -13 following induction with IPTG in *E. coli* results in metabolic changes as indicated by multi-parameter flow cytometry and traditional methods of fermentation profiling (O<sub>2</sub> uptake rate, CO<sub>2</sub> evolution rate and optical density measurements). Induction early in the rapid growth phase was optimal although this led to lower overall biomass concentrations and lower maximum specific growth rates. In contrast, induction in the mid-rapid growth phase was the most detrimental to cell quality as measured by cytoplasmic membrane depolarisation.

## **INTRODUCTION**

The over-expression of heterologous recombinant proteins in *Escherichia coli* often results in severe growth inhibition of the host cells, accompanied by metabolic changes. These adverse effects, often characterised by an increased metabolic burden, are mainly attributed to the presence of plasmid replication systems, rDNA transcription and plasmid-encoded mRNA translation, to which cellular resources are redirected at the expense of normal host cell metabolic processes (Bentley *et al.* 1990, Anderson *et al.* 1996, Soriano *et al.* 2002). These changes may therefore manifest themselves as a decrease in growth rate, enhanced production of heat-shock proteins and inclusion body formation (Kurland & Dong 1996). This growth and metabolic process inhibition usually becomes apparent following transcriptional induction of the foreign gene but is difficult to measure in real-time.

Over the last 10 years there have been many advances in the use of multi-parameter flow cytometry coupled with fluorescent staining techniques for the analysis of bacterial physiological state at the single cell level (Nebe-von-Caron *et al.* 2000,

Hewitt & Nebe-von-caron 2004). Since the analyses used in this study were essentially the same as in our earlier work (Hewitt *et al.*, 2007), only a brief discussion will be included here. The advantages of multi-parameter flow cytometry over the more conventional microbiological techniques such as dilution plating (c.f.u. per ml) are well documented (Hewitt & Nebe-von-Caron 2004, Nebe-von-Caron *et al.* 2001) but, briefly, by using various mixtures of fluorescent dyes [e.g. propidium iodide, bis-(1,3-dibutylbarbituric acid) trimethine oxonol], it is possible to resolve an individual microbial cells' physiological state beyond culturability based on the presence or absence of an intact polarised cytoplasmic membrane and the transport mechanisms across it, in real- time, enabling assessment of population heterogeneity.

The study described here seeks to build on our earlier work (Lewis *et al.* 2004, Sundstrum *et al.* 2004), where the physiological consequences of the production of recombinant protein by *E. coli* using various expression strategies was studied. The production of a model recombinant protein (AP50), as an insoluble inclusion body accumulating in the cytosol, correlates very well with any detrimental change in cell physiological state as measured by multi-parameter flow cytometry. Here we describe the use of both conventional fermentation analyses and multi-parameter flow cytometry to study the physiological effects of IPTG-induction on *Escherichia coli* BL21 expressing the foreign protein, interleukin-13, at different times during the cell growth phase. Interleukin-13 is an important cytokine secreted by T helper type 2 (Th2) cells implicated in the mediation of allergic inflammation in humans and a potential therapeutic target. The aim here was to determine the point during growth when induction would have the least detrimental effect on cell physiology, metabolism and protein expression leading to real process improvements.

## MATERIALS AND METHODS

### *Organisms and growth conditions*

The strain used, *Escherichia coli* IL 13, was a modified *BL21* (DE3) strain containing a pET11a vector (Novagen, Madison, WI, USA) with the interleukin-13 gene under the control of an IPTG-inducible promoter. *E.coli* IL-13 was cultured in Luria Bertani (LB) medium at 25 g l<sup>-1</sup> with ampicillin at 100 mg l<sup>-1</sup>. All medium components except ampicillin were sterilized *in-situ* in the fermenter by heating to 121 °C at 1 bar pressure for 30 min. Polypropylene glycol P 2000 (0.15 ml l<sup>-1</sup>) was added to the bioreactor prior to media sterilization as an antifoam. Ampicillin was filter-sterilized and aseptically added to the media immediately prior to inoculation.

Batch fermentations were carried out in a 30 l stirred tank reactor (STR, B. Braun Biotechnology, Allentown, PA, USA), with a working volume of 20 l. The STR was inoculated with a 2.5% (v/v) overnight (16h) culture. Culture pH was maintained at 7 ± 0.2 by the addition of 1 M NaOH on demand and temperature was maintained at 37°C. Dissolved O<sub>2</sub> tension (DOT) was measured using a polarographic probe (Ingold, Messtechnik, Urdorf, Switzerland) and maintained above the 30% saturation level by control of agitation speed (300-700 rpm) and aeration at 1 vvm. Vessel pressure was maintained at ≤ 0.5 bar. The culture was induced with 1 mM IPTG, dissolved in process water and filter-sterilized before being aseptically added to the fermentation vessel.

### *Analytical techniques*

Biomass concentration was measured as OD<sub>600</sub> and compared to the dry cell weight (DCW) of samples dried at 100 °C to constant weight. The CO<sub>2</sub> evolution rate (CER) and O<sub>2</sub> uptake rate (OUR) were measured and recorded via mass spectroscopic analysis of exit gases from the fermenter. DOT, CER and OUR data were collected using FIX software (GE Fanuc, Albany, NY, USA).

Flow cytometric analysis was carried out using a FACSCalibu dual laser flow, bench top analyser with 488 nm excitation from an argon-ion laser at 15 mW. Two fluorescent dyes were used, propidium iodide (PI) and bis- (1,3-dibutylbarbituric acid) trimethine oxonol (sometimes referred to as DiBAC<sub>4</sub> or BOX, Molecular Probes, Leiden, The Netherlands). Samples taken from the test culture were immediately diluted with Dulbecco's buffered saline (DBS), pH 7.2 and stained with a mixture of PI and BOX. Samples were held in the 'hot-spot' of a sonication bath for 3-4 s just prior to analysis in order to avoid problems associated with cell aggregation. Each dye was added from a stock solution: 200 µg PI per ml distilled water and 10 mg BOX per ml dimethyl sulphoxide (DMSO). The DMSO stock solution was held at -20°C and the distilled water stock solution was held at 4°C. The working concentrations of PI and BOX were 5 µg/ml and 10 µg/ml, respectively, in DBS. The optical filters were set up so that PI fluorescence was measured at 630 nm and BOX fluorescence was measured at 525 nm. Any interference caused by spectral overlap between the emitted fluorescence of the two stains was eliminated by the system's compensation software. Additionally, software discriminators were set on both the FALS signal and the RALS signal to further reduce electronic and small particle noise. Data analysis was

accomplished with the CellQuest Pro software (BD Biosciences, San Jose, CA, USA), which allows protocol definition and batch analysis.

The presence of interleukin-13 (IL-13) protein in *E.coli* IL-13 following induction with IPTG was determined via SDS-PAGE using the Laemmli protocol followed by silver staining to visualise the protein bands. Fermentation broth samples (10 ml) were harvested from the fermentation vessel prior to, and post-, induction. The cell mass was separated from the fermentation by centrifugation at 5000 *g* for 30 min. Samples were standardised to 1 OD unit before SDS-PAGE analysis. Protein was extracted from the cell pellet using the BugBuster Protein Extraction Reagent kit as directed by the manufacturer's protocol for protein extraction and inclusion body (IL-13 is present as inclusion bodies) purification via chemical lysis. Ten  $\mu$ l of a 1 in 10 dilution of the purified protein suspension was added to 10  $\mu$ l of Laemmli buffer and incubated at 90°C for 5 min to denature the protein, prior to being loaded onto a pre-cast 4-20%, 10-well Novex Tris/glycine/SDS gel (Invitrogen, Carlsbad, CA, USA). The gel was run in Novex Tris/glycine/SDS running buffer for 90 min at 150 V. Ten  $\mu$ l of protein size markers from 10 to 255 kDa in an equal volume of Laemmli buffer, was also loaded on to the gel to determine the size of the protein bands seen following silver staining of the SDS PAGE gels. Silver staining for protein visualisation was achieved using the SilverXpress Silver Staining kit (Invitrogen, Carlsbad, CA, USA).

## **RESULTS AND DISCUSSION**

A series of batch fermentations was carried out with the BL21 (DE3) strain where the point of induction of IL-13 by addition of 1mM IPTG was varied dependent on the turbidity of the culture in the range OD<sub>600</sub> 0.5 ± 0.05, 1 ± 0.2 and 3 ± 0.2, respectively.

In addition, control fermentations were carried out where IPTG was not added to a batch fermentation of the transformed host. Reproducible measurements of pH, DOT (% saturation), DCW g/L, OD<sub>600</sub>, CER (mmol/l.h) and OUR (mmol/l.h) were made (**Figure 1**, not all data shown). Additionally, from samples taken just prior to and 3 h after induction for the induced fermentations and at the end of the non-induced fermentation individual cell physiology was measured by multi-parameter flow cytometry (**Figures 2 - 5**). In all cases the batch fermentations were terminated when an OD<sub>600nm</sub> of between 2.5 and 4.5 had been achieved. In all cases, the point of induction has a clear effect on fermentation progression as measured by DOT (% saturation), OD<sub>600</sub>, CER and OUR (**Figure 1**). Without induction, a maximum OD<sub>600</sub> of 4.5 (= DCW 2.13 g/l) was reached at 5 h with a  $\mu_{\max}$  of 0.71 h<sup>-1</sup> and no protein was expressed. With induction at OD<sub>600</sub> of 0.5 ± 0.05, which is early in the rapid growth phase, a maximum OD<sub>600</sub> of 4 (= DCW 1.48 g/l) was then reached at 7 h which is lower and later than with the control. In this case, the  $\mu_{\max}$  before induction was 0.74 h<sup>-1</sup>, but after induction only reached 0.41 h<sup>-1</sup> and protein was expressed (**Figure 6**). This effect on growth was mirrored in the DOT, CER and OUR profiles.

When induction was carried out at an OD<sub>600</sub> of 1 ± 0.2, i.e. in the middle of the rapid growth phase, the maximum OD<sub>600</sub> of 2.5 (= DCW of 1.32 g/l) was achieved at 3 h without any further growth thereafter. Indeed, growth only continued for 1.5 h post-induction. The  $\mu_{\max}$  (recorded just post-induction) was 0.64 h<sup>-1</sup> and protein was expressed (**Figure 6**). This marked effect on growth is again mirrored in the DOT, CER and OUR profiles. When induction was carried out at an OD<sub>600</sub> of 3 ± 0.2, late in the growth phase, the maximum OD<sub>600</sub> of 4.5 (= DCW of 2.20 g/l) was achieved at 5 h with a  $\mu_{\max}$  of 0.72 h<sup>-1</sup>. This is similar to the control except that protein was expressed (**Figure 6**). The DOT, CER and OUR profiles were also similar to the control.



In all cases, 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 (Fouchet *et al.* 1994, Laverne-Mazeau *et al.* 1996, Lewis *et al.* 2004, Wittrup *et al.* 1988) 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 (data not shown). Why this is so remains unclear and further work will need to be done to investigate this phenomenon further since phase-bright inclusion bodies could be observed in the cytosol of cells *via* light microscopy. Analysis of the fluorescent staining data as measured by flow cytometry showed that in all cases, both induced and un-induced, there is a progressive detrimental change in cell physiological state that continues throughout the course of each fermentation and that the extent of this is dependent on whether the IL-13 was induced or not and at which OD the IPTG was added, as revealed by BOX and PI dual (PIX) staining. As described previously (Hewitt *et al.* 2001, 2004), in **Figure 2 – 5**, area A, no staining, represents healthy cells; area B, stained with BOX, shows cells with a reduced cytoplasmic membrane potential (stressed cells); and area C, stained with both PI and BOX (PIX), shows cells with a depolarised permeabilised cytoplasmic membrane, (dead cells). The greatest detrimental effect on cell physiological state was seen when the IL-13 was induced in the middle of the rapid growth phase.

Chemical induction is a common method of initiating the expression of foreign genes in recombinant bacterial systems (Kosinski *et al.* 1992, Andersson *et al.* 1996, Lewis *et al.* 2004). However when this occurs during the growth period, the conditions can significantly affect the final quantity and quality of recombinant protein product

Patkar *et al.* 2002) as well as the physical and metabolic status of the host cell. Foreign protein synthesis in the host cell has been found to enhance the maintenance coefficient and has been reported to impose a metabolic burden on the normal functioning of the host cell, thus inducing severe metabolic stress (Bentley *et al.* 1990, Fouchet *et al.* 1994, Soriano *et al.* 1999).

IPTG-inducible systems are widely used in recombinant bacteria and are characterised by high product yields (Soriano *et al.* 2002). The reason that the OD at which the IPTG is added has such an effect on cell growth and physiology is usually related to the concentration of IPTG per cell at time of induction. Since the IPTG concentration is kept the same (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, the induction effect on the culture as a whole is stronger than at a higher OD. 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. Unfortunately, there is no reliable assay for IL-13 quantification so expression level data were not available however a correlation with the flow cytometry data can be conferred since the presence of a high number of dead or dormant cells at any time during the fermentation will have an obvious detrimental effect on total protein quantity. Why induction in the middle of the rapid growth phase has such an effect is more uncertain, however, any toxic effects of the IPTG can be largely discounted because in previous work (Lewis *et al.* 2004) the addition of IPTG to a non-recombinant fermentation of *E. coli* B21 had no

effect on fermentation progression. The effect may be due to a subtle metabolic switch since *E. coli* responds to various stress factors by the transcriptional induction of an alternative set of genes (Schweder *et al.* 1999) resulting in a protective effect on cells in or leaving the lag phase or in or entering the stationary phase that can lead to lower biomass concentrations and protein productivity overall.

## **CONCLUSIONS**

When compared to other more traditional microbiological techniques, it is recognised that the relative complexity and high cost of flow cytometry equipment probably prohibits it from becoming a routine analytical tool for use in most fermentation microbiology laboratories. Nevertheless, flow cytometry is proving to be a valuable research analytical technique for the study of microbial population dynamics within bioreactors, leading to informed improvements in process performance. Here, the point of induction of the IL-13 by 1 mM IPTG addition had a clear effect on fermentation progression and the physiological consequences of this can be followed by multi-parameter flow cytometry. Depending on point of induction a ~40% drop in maximum biomass concentration with respect to DCW g/l was achieved and subsequently growth rates were retarded.

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**Figure 1.** i) Profile of un-induced *E.coli* IL-13 batch fermentation. Early rapid growth phase, A; Mid rapid growth phase, B; Late rapid growth phase, C.1 ii-iv) Profiles for batch fermentations induced in early rapid growth phase (ii), mid rapid growth phase (iii) and late rapid growth phase (iv). Closed triangles, DOT; open squares, OUR; closed squares, CER and closed circles, OD.

**Figure 2.** Cell samples taken at 6 h (i) and 8 h ii) during the course of a batch fermentation where synthesis of IL-13 was not induced. Cells were stained with PIX, Three main sub-populations of cells can be distinguished, corresponding to healthy polarised cells (A), no staining, cells with no membrane potential (B), stained with BOX; and cells with permeabilised membranes (C), stained with both PI and BOX.

**Figure 3.** Cell samples taken (i) immediately before induction and (ii) ~ 3 h after induction during the course of a batch fermentation where synthesis of IL-13 was induced early on in the rapid growth phase (i.e. at point A Figure 1i). Cells were stained with PIX, three main sub-populations of cells can be distinguished, corresponding to healthy polarised cells (A), no staining, cells with no membrane potential (B), stained with BOX; and cells with permeabilised membranes (C), stained with both PI and BOX.

**Figure 4.** Cell samples taken (i) immediately before induction and (ii) ~ 3 h after induction during the course of a batch fermentation where synthesis of IL-13 was induced mid rapid growth phase (i.e. at point B Figure 1i). Cells were stained with PIX, three main sub-populations of cells can be distinguished, corresponding to healthy polarised cells (A), no staining, cells with no membrane potential (B), stained with BOX; and cells with permeabilised membranes (C), stained with both PI and BOX.

**Figure 5.** Cell samples taken (i) immediately before induction and (ii) ~ 3 h after induction during the course of a batch fermentation where synthesis of IL-13 was induced late on in the rapid growth phase (i.e. at point C Figure 1i). Cells were stained with PIX, three main sub-populations of cells can be distinguished, corresponding to healthy polarised cells (A), no staining, cells with no membrane potential (B), stained with BOX; and cells with permeabilised membranes (C), stained with both PI and BOX.



Figure 6. SDS PAGE gel of protein purified from non-induced and induced *E.coli* IL-13 fermentations. (SM) Protein size marker, (A) Non-induced samples, (B) Induced samples. Arrows point to the IL-13 band, which is 12.5kDa in size. Protein bands are visualised with silver staining.