Biotechnology Letters **26:** 1533–1539, 2004.

Segregation to non-dividing cells in recombinant *Escherichia coli* fed-batch fermentation processes

Heléne Sundström[1], Fredrik Wållberg[1], Erika Ledung[2], Bo Norrman[2], Christopher J. Hewitt[3] & Sven-Olof Enfors[1,*]

[1]*Department of Biotechnology, Royal Institute of Technology, Roslagstullsbacken 21, S-10691 Stockholm, Sweden*
[2]*Department of Biology and Chemical Engineering, Mälardalen University, P.O. Box 325 S-631 05 Eskilstuna, Sweden*
[3]*Department of Chemical Engineering (Biochemical Engineering), The University of Birmingham, Edgbaston, B15 2TT, UK*
[*]*Author for correspondence (Fax: +46 8 5537 8323; E-mail: enfors@biotech.kth.se)*

Received 18 June 2004; Revisions requested 1 July 2004; Revisions received 29 July 2004; Accepted 30 July 2004

Key words: bacterial cell division, *Escherichia coli*, fed-batch fermentation, flow cytometry, viability

Abstract

In *Escherichia coli* fermentation processes, a drastic drop in viable cell count as measured by the number of colony forming units per ml (c.f.u. ml^{-1}) is often observed. This phenomenon was investigated in a process for the production of the recombinant fusion protein, promegapoietin (PMP). After induction, the number of c.f.u. ml^{-1} dropped to ~10% of its maximum though the biomass concentration continued to increase. Flow cytometric analysis of viability and intracellular concentration of PMP showed that almost all cells were alive and contributed to the production. Thus, the drop in the number of c.f.u. ml^{-1} probably reflects a loss of cell division capability rather than cell death.

Introduction

In fed-batch processes for the production of recombinant proteins with *Escherichia coli* a drastic drop in viable cell counts, as measured by the number of colony forming units per ml growing on an agar surface (c.f.u. ml^{-1}), is often interpreted as cell death (Andersson *et al.* 1994). Glucose limitation in fed-batch processes has been discussed as a cause of this decline (Strandberg *et al.* 1994) and a drop in c.f.u. ml^{-1} was encountered when the specific glucose consumption rate (qS) approached a low level (0.04 g g^{-1} h^{-1}, Andersson *et al.* 1994). Further, a drop in c.f.u. ml^{-1} occurred at the same time as the specific growth rate (μ) fell below a critical value (0.02 g g^{-1} h^{-1}) in high cell density cultures (Andersson *et al.* 1996).

Colonies derived from single cells have been examined since Koch developed the technique more than a century ago. However, the strength of this technique, its high amplification factor (10^{9-12}), is also its weakness since it depends on post-sampling growth and its success is limited by our ability to grow bacteria in an artificial environment (Hewitt & Nebe-Von-Caron 2004). Classical microbiology states that a cell is only viable when it has been shown to reproduce and a demonstration of this has become the microbiologists 'gold standard' for proof of cell viability. However, the inability of a cell to divide is not the same as cell death and this argument is, and has been, subject to extensive discussions and investigations (Hewitt & Nebe-Von-Caron 2004, Nebe-Von-Caron *et al.* 2000). The occurrence of such viable but non-culturable cells (VBNC) is thought to be a survival strategy adopted by non-sporeforming bacteria when exposed to environmental stress (Colwell 2000). The *rpoS* gene and guanosine 5′-diphosphate 3′-diphosphate (ppGpp) have been implicated in the activation of the VBNC state (Boaretti *et al.* 2003). The expression of *rpoS*, is modulated by the growth rate and a large number of genes involved in the response to severe stress are

regulated by the RpoS protein (Booth 1999). ppGpp functions as an alarmone in the starvation activated stringent response which is coupled with a concomitant reduction in growth rate, ribosomal synthesis and cell size (Lengeler & Postma 1999).

The advantages of multi-parameter flow cytometry over the more conventional micro-biological techniques such as dilution plating (c.f.u. ml^{-1}) are well documented (Hewitt *et al.* 1999, Nebe-Von Caron & Badley 1995) but, briefly, it is possible to resolve the physiological state of an individual microbial cell beyond culturability, based on the presence or absence of an intact polarised cytoplasmic membrane and the transport mechanisms across it, enabling assessment of population heterogeneity. This technique has successfully been applied to the study of high cell density fed-batch fermentations of *E. coli* where a similar, but less extensive, drop in cell viability was observed (Enfors *et al.* 2001, Hewitt *et al.* 2000).

Clearly, the problem of the presence of VBNC bacterial cells in fermentation processes remains unresolved. Further, since the presence of a high proportion of dormant, dying or dead cells at any time during a fermentation process will have an obvious detrimental effect on the synthesis of any desired product it was proposed, in this study, to compare the more traditional techniques of viable cell count (c.f.u. ml^{-1}) with the viability as measured by multi-parameter flow cytometry during an *E. coli* fed-batch fermentation process to produce the recombinant fusion protein promegapoietin (PMP). In this way we hoped to highlight the dangers of relying on measurement of the numbers of c.f.u. ml^{-1} alone to monitor biomass viability in fermentation processes.

Materials and methods

Organism and growth conditions

Escherichia coli MON105 was used. This is a prototrophic strain derived from *E. coli* K-12 strain W3110 containing rpoH358 as a chromosomal mutation (Obukowicz *et al.* 1992). The production of promegapoietin is controlled by a production plasmid and induced by nalidixic acid. The strain was kindly provided by Pharmacia AB (Sweden).

The culture medium was composed of (per litre distilled water): $Na_2PO_4 \cdot 2H_2O$ 8.71 g; KH_2PO_4 3 g; NaCl 0.5 g; NH_4Cl 1 g; yeast extract 20 g; Breox polyalkylene glycol (antifoam) 0.5 ml; 1 M $MgSO_4$ 2.5 ml; 0.1 M $CaCl_2$ 3.5 ml; trace elements solution 3 ml; glucose 50% (w/w), 8 g in shake flask and 10 g initially in the bioreactor. The trace element solution was composed of (per litre distilled water): thiamine · HCl 0.833 g; $FeCl_3 \cdot 6H_2O$ 12.6 g; $ZnSO_4 \cdot 7H_2O$ 0.133 g; $CoCl_2 \cdot 6H_2O$ 0.233 g; $NaMoO_4 \cdot 2H_2O$ 0.233 g; $CuSO_4 \cdot 5H_2O$ 0.267 g; H_3BO_3 66.7 mg; $MnSO_4 \cdot H_2O$ 0.167 g; conc. H_2SO_4 1.13 ml. All chemicals used were of analytical or pharmaceutical grade.

E. coli MON105 was inoculated into the shake flask medium and cultivated at 37 °C until the dry cell wt, DCW (g l^{-1}), was between 2–6. Fed-batch cultivations were performed in a 10 l lab-scale bio-reactor (Belach, Sweden) with an initial volume of 5 l and an inoculum volume of 100 ml. NH_4OH (25% v/v) was added on demand to maintain the pH at 7 and temperature was controlled at 37 °C. A feed of 50% (w/w) glucose solution, was started when the initial concentration of glucose was consumed as indicated by a rise in the DOT and pH signals. A low constant feed of glucose, 3–4 g l^{-1} h^{-1}, was used to ensure carbon limitation so that the DOT was maintained above the 30% saturation level. At a DCW $\sim$ 5 g l^{-1}, the recombinant protein was induced with nalidixic acid (0.05 g l^{-1}) and cells were harvested $\sim$15 h after induction, when the dry cell weight was $\sim$ 30 g l^{-1}.

Analyses

Cell biomass was measured turbidimetrically by optical density at 550 nm in a double beam spectrophotometer, calibrated against dry cell weight (DCW g l^{-1}) at 105 °C to constant weight. The concentrations of glucose and acetic acid were measured enzymatically with test kits from Boeringer Mannheim GmbH (Mannheim, Germany). Samples for glucose analysis were quenched with perchloric acid and then immediately frozen (−20 °C) until required (Larsson & Törnkvist 1996). The filtered supernatant from the DCW samples was used for analysis of acetic acid and total protein. Analysis of total protein in the medium was performed according to the Bradford procedure. For each measurement the average value of triplicate representative analyses was used.

Analysis of promegapoietin (PMP)

Bacterial cell samples were disintegrated using a French press (internal working pressure ≈90 MPa). To dissolve the inclusion bodies, samples were treated

with equal volumes of solubilizing solution containing: SDS (2% v/v), dithiothreitol (0.2 M) and Tris (0.4 M). The solubilizing reaction took place at room temperature for 30 min. Cell debris was then removed by centrifugation at 10 600 *g* for 2 min. The supernatant was used for quantification of the PMP by reversed phase HPLC using a VydacTM Protein C4 analysis column (Grace Vydac) coupled with a UV detector (214 nm) and an acetonitrile gradient with trifluoroacetic acid. The percentage (v/v) of acetonitrile was gradually increased from 42% to 70% over a period of 14 min, after which it was gradually decreased from 70% to 42% over a further 11 min.

Labelling inclusion bodies in permeabilised cells

Samples taken from the bioreactor were stored at −20 °C until analysed. Cells were thawed at room temperature and diluted to a DCW of ∼1. One ml of the diluted sample was centrifuged at 10 600 *g* for 2 min. Pellets were re-suspended in 1 ml DMSO and then washed twice in PBS by centrifugation at 10 600 *g* for 2 min. Pellets were re-suspended in 1 ml of PBS and incubated with lysozyme at 1 mg ml^{-1} for 15 min at room temperature. To stop the lysozyme reaction, samples were washed as above. Five μl of the primary antibody (anti-PMP) solution was added and the samples were incubated for 30 min at room temperature. After incubation, samples were washed as above and 5 μl of a secondary antibody conjugated to Alexa Fluor 488 (Molecular Probes) was added and incubated for 30 min at room temperature. Samples were washed once more as above before being analysed on the flow cytometer.

Measurement of viability by number of c.f.u. ml^{-1}

Samples were diluted in a saline solution (0.9% NaCl) and 100 μl was spread-plated on to Luria–Bertani agar plates, in triplicate, for each dilution. Plates were incubated at 37 °C for 24 h and the c.f.u. ml^{-1} calculated.

Multi-parameter flow cytometric measurements

All flow cytometric measurements were made using a Partec PAS flow cytometer (Partec GmbH, Münster, Germany) with 488 nm excitation from an argon-ion laser at 20 mW. Samples taken from the fermenter were immediately diluted with PBS (0.16 M NaCl, 0.003 M KCl, 0.008 M Na_2HPO_4 and 0.001 M KH_2PO_4, pH 7.3) and stained with a mixture of two fluorescent dyes, propidium iodide (PI, Sigma) and bis-(1,3-dibutylbarbituric acid) trimethine oxonol (BOX, Molecular Probes). Stock solutions of each dye were prepared as follows; PI was made up at 200 μg ml^{-1} in distilled water. BOX was made up at 10 mg ml^{-1} in DMSO. DMSO stock solution was maintained at −20 °C and the distilled water stock solution was maintained at 4 °C. The working concentrations of PI and BOX were 5 μg ml^{-1} and 10 μg ml^{-1} respectively in PBS. In order to facilitate staining with BOX, 4 mM EDTA was added to the working solution. Ten μl of the working solution was then added to approx. 1 ml of the appropriately diluted cell sample suspension for staining. All solutions were passed through a 0.2 μm filter immediately prior to use to remove particulate contamination. Additionally, software discriminators were set on the right angle light scatter (RALS) signal to further reduce electronic and small particle noise. The optical filters were set up as standard for the Partec PAS flow cytometer so PI fluorescence was measured above 630 nm whilst both BOX and Alexa Fluor 488 fluorescence were measured at ∼520 nm. Since there is a spectral overlap between BOX and PI-emitted fluorescence, the software compensation was set up in such a way that BOX-emitted fluorescence was eliminated from the PI-emitted fluorescence detector and *vice versa*. Samples were analysed at a data rate of ∼1500 counts sec^{-1} and ∼50 000 cells were analysed (error <0.1%, Shapiro 2003) for each measurement in triplicate. For determination of total cell number the Partec true volumetric absolute counting function was used (www.partec.de/products/PartecPAS.pdf, 2004). All measurements were calibrated using 3 μm diam. fluorescent beads (Standard 05-4008, Partec GmbH). Data analysis was made by Partec FloMax software (version 2.4b) and MATLAB.

Results and discussion

Two methods were used to measure the cell viability in recombinant fed-batch fermentation processes with *E. coli*: determination of the number of colony forming units (c.f.u. ml^{-1}) and determination of the number of cells with depolarised permeabilised cytoplasmic membranes (cells positively stained with propidium iodide) by multi-parameter flow cytometry. A series of duplicate fed-batch fermentations for the production of promegapoietin (PMP, as inclusion bodies) was carried out in order to gain a better understanding

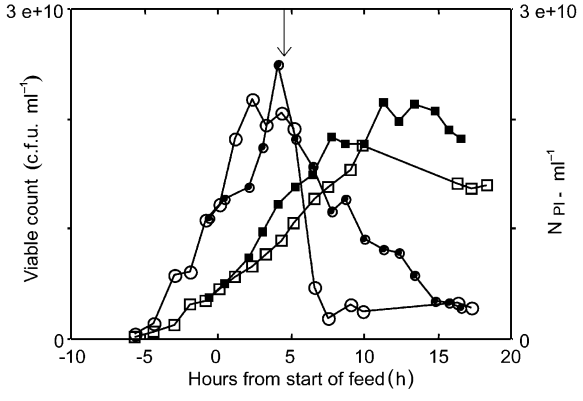

Fig. 1. Colony forming units (c.f.u. ml^{-1}, circles) and number of PI-negative cells (N_{PI}-ml^{-1}, squares) during two reproducible fed-batch processes. Open symbols show cultivation A. Filled symbols show cultivation B. The point of induction is signified by the arrow (4.5 h).

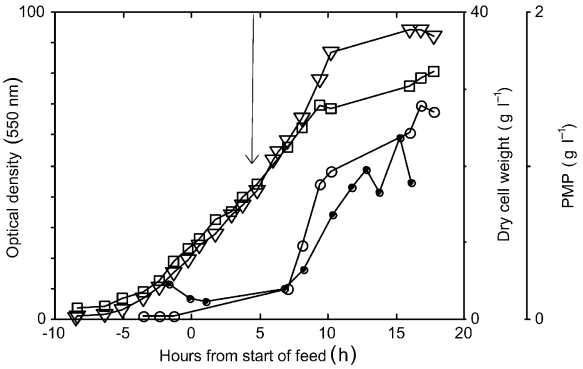

Fig. 2. Concentrations of PMP for the cultures shown in Figure 1 (g l^{-1}, circles). Dry cell weight (dcw g l^{-1}, squares) and OD_{550nm} (triangles) from one of the cultures (A) are also included for comparison. Corresponding dcw (g l^{-1}) and OD_{550nm} curves from cultivation B have been omitted for clarity. The point of induction is signified by the arrow (4.5 h).

of the apparent drastic drop in c.f.u. ml^{-1} observed in earlier work. The results from two representative cultivations are shown in Figure 1. A drastic drop in the number of the c.f.u. ml^{-1} was associated with the addition of the inducing agent and PMP expression at a cell dry weight of about 15–20 g l^{-1}.

However, while the c.f.u. ml^{-1} dropped by about 90% within 5–10 h, the DCW (g l^{-1}) continued to increase and the process was terminated when a DCW of 30 g l^{-1} was reached. PMP accumulated rapidly (Figure 2) during the period when the number of c.f.u. ml^{-1} was at its minimum post-induction (Figure 1) reaching a maximum of $\sim$1.5 g l^{-1}.

Flow cytometry was used to analyse the total cell number and the number of dead cells (those with de-

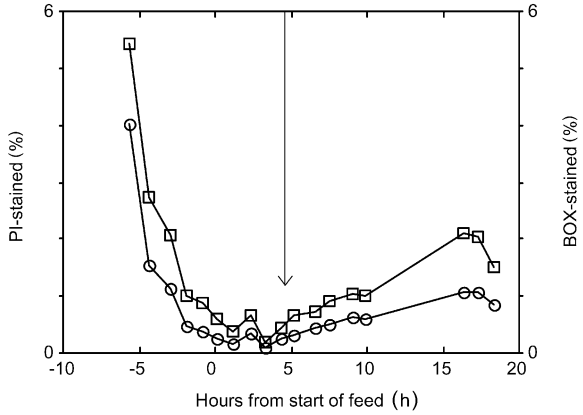

Fig. 3. Percentage of PI-stained (circles) and BOX-stained (squares) cells. The point of induction is signified by the arrow (4.5 h). Data presented is from cultivation A. Cultivation B resulted in similar data but is not presented.

polarised permeabilised cytoplasmic membranes, i.e. PI positively stained cells). The difference between total cell number and the number of PI positive cells was calculated as the total number of viable cells ml^{-1} (Figure 1).

The number of viable (PI-negative) cells followed the DCW (g l^{-1}) profile and the amount of dead (PI-positive) cells was less than 1% for the remainder of the process after the start of the glucose feed (t = 0) (Figure 3). As discussed earlier, PI does not penetrate and stain cells with intact cytoplasmic membranes but, when the cytoplasmic membrane is permeabilised, it becomes PI-permeable and the cells are stained. Since the cytoplasmic membrane is the way a cell communicates selectively with its environment and an intact cytoplasmic membrane is essential for a healthy viable cell, PI-positively stained cells are considered to be dead and PI-negative cells are considered to be alive. To further investigate the possibility that the PI-negative cells were just impermeable to PI, but metabolically inactive, an attempt was made to counter-stain the cells with BOX. BOX is anionic, lipophilic and only enters a cell if the cytoplasmic membrane potential has collapsed. Since the cytoplasmic membrane potential is driven by the passage of charged molecules across the membrane, and is therefore energy dependent, cells stained positively with BOX can be considered to be metabolically compromised (Hewitt *et al.* 1999, Nebe-Von Caron & Badley 1995). Figure 3 shows that the proportion of BOX-stained cells was only slightly larger than that of the PI stained cells, which indicates that almost all cells

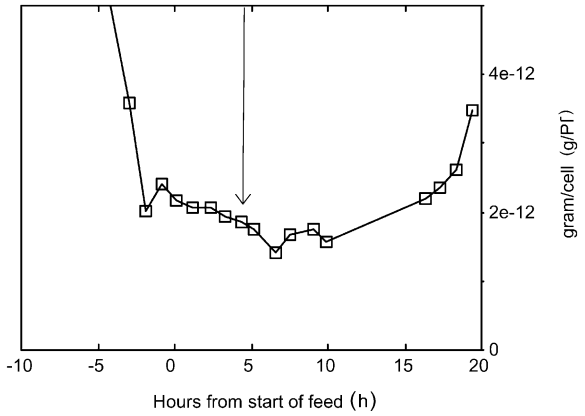

Fig. 4. Flow cytometric analysis with PI-staining during a representative fed-batch fermentation process. Mass (dcw) per cell in grams/PI-negative cell per ml. The point of induction is signified by the arrow (4.5 h). Data presented is from cultivation A. Cultivation B resulted in similar data but is not presented.

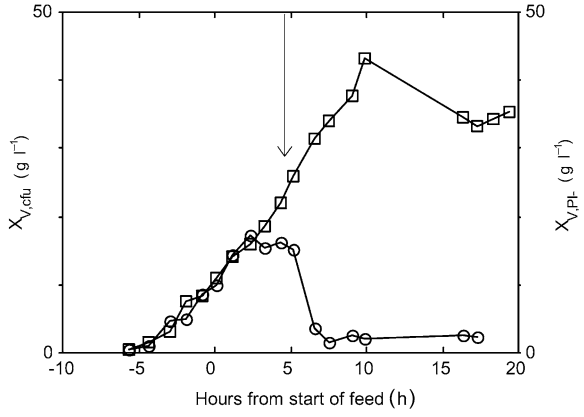

Fig. 5. Biomass profiles for viable cells based on the two viability measurements used in this study, c.f.u. ml^{-1} (circles) and $N_{PI-}ml^{-1}$ (squares). The point of induction is signified by the arrow (4.5 h). Data presented is from cultivation A. Cultivation B resulted in similar data but is not presented.

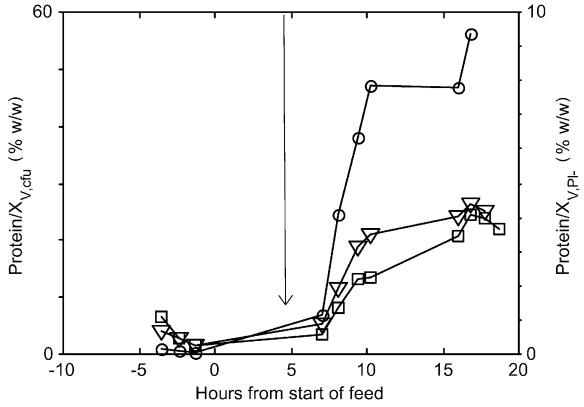

Fig. 6. Promegapoietin concentration per cell mass (% w/w). Cell mass according to: c.f.u. ml^{-1} analysis (PMP/Xv,c.f.u. circles), PI-staining (PMP/Xv,PI^- squares) and cell dry weight (PMP/dcw triangles). The point of induction is signified by the arrow (4.5 h). Data presented is from cultivation A. Cultivation B resulted in similar data but is not presented. Note the different scales.

retained their cytoplasmic membrane potential and hence their energy dependent metabolic activity.

PMP was induced by the addition of nalidixic acid, which is known to inhibit DNA synthesis and cell division, resulting in cell elongation (Goss 1965, Keller *et al.* 1990). However, in this case the induction of PMP did not inhibit cell division after induction at the concentration used, since the total number of cells (as measured by multi-parameter flow cytometry) and DCW g l^{-1} increased (Figures 1 and 2). Furthermore, based on the flow cytometry data, the cell size was relatively constant, at 0.2×10^{-11} g $cell^{-1}$, except for the period before the glucose feed was started and at the very end of the process, where for both cases an increase in cell size was measured (Figure 4).

From the acquired viability data, the dry weight of 'viable' cells (X_V), according to the two viability analytical methods, was calculated (Figure 5). Calculations were based on an average of the DCW per 'viable' cell at the time before the start of the carbon feed ($9 \cdot 10^{-13}$ g/c.f.u. and $2 \cdot 10^{-12}$ g/N_{PI-}).

Almost all of the PMP was produced during the period (7–16 h after the start of the carbon feed), when the viable cell concentration, according to the number of c.f.u. ml^{-1}, was only 10–15% of the concentration at the time of induction (Figures 1 and 2). If we assume that the protein was produced only by 'viable' cells according to the two methods of viability analysis, the protein concentration was 3–4% of the total viable mass when related to the total number of PI negative cells, but 40–55% when related to the number of c.f.u. ml^{-1}. The latter is an unrealistically high value (Figure 6).

In order to investigate the possibility of extensive cell lysis (which should naturally follow a drastic drop in cell viability) during the fermentation process, the concentration of extracellular protein in the medium was measured. Since the medium contained yeast extract the basal protein concentration was about 0.08 g l^{-1} before inoculation and this concentration only increased to ~0.17 g l^{-1} during the entire process. The resulting amount, when the background had been deducted, was then used to estimate the extent of cell lysis during the process, assuming that a cell

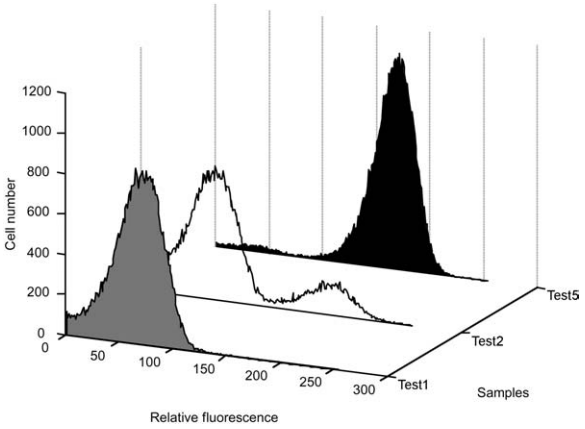

Fig. 7. Flow cytometric analysis of intracellular PMP inclusion bodies stained with fluorescent antibodies. Sample 1: negative control of cells lacking PMP; sample 2: mixture of cells containing PMP and negative control cells; sample 3: cells from the end of the process.

is made up of 50% of its total weight as protein. The total amount of extracellular total protein in the medium ranged from about 0.5–1.5% of the total protein present at the end of the cultivation, indicating that only about 1% of the total population had undergone cell lysis.

If the drop in c.f.u. ml^{-1} (Figure 1) were to truly represent cell death, then a large population should have no, or very low, concentrations of PMP since most of the cells would have died (Figure 1) before PMP production was initiated at ∼7.5 h (Figure 2). Most of the PMP would then be synthesised only by 'viable' and growing cells after this time. This possibility was investigated by analysis of the distribution of PMP in the population by means of intracellular staining with PMP specific fluorescent antibodies followed by flow cytometric analysis. If this scenario was correct, then ∼50% of the cells present before the drop in the number of c.f.u. ml^{-1} and induction (7 h after the start of the carbon feed, Figures 1 and 2) should have no or very low amounts of PMP.

The results of such an analysis (Figure 7) show that the negative control, with cells lacking PMP, produces a weakly fluorescent peak, while the cells analysed at the end of the process contains mainly one strongly fluorescent peak, with only about 3% of the total population corresponding to those cells with a fluorescent intensity similar to the negative control. As a further control, and to prove that a mixed population of cells could be resolved by the labelling technique used in this study, a mixture of producing and non-producing cells were analysed. In this case two distinct peaks were obtained corresponding to those cells containing PMP and those not. Thus, at the end of the process, almost all of the cells (∼97%) contained PMP and almost no cells had lysed though the number of c.f.u. ml^{-1} indicated that the majority of the cells had lost their capacity to divide on the LB agar plates used in this study. Why this should be is not clear. However, it is known that conditions need to be absolutely perfect for any cell to divide (Hewitt & Nebe-von-Caron 2004) and the environmental conditions on the surface of a solid LB agar plate are very different to those experienced by a cell when submerged in a liquid medium in a bioreactor. Therefore it is possible that whilst cells will not divide on the LB agar plate they may be capable of division in the bioreactor or other growth media (solid or otherwise).

Conclusions

In this study we have shown that recombinant promegapoietin was produced as inclusion bodies at a high rate in *E. coli* glucose-limited, fed-batch culture and that there was a drastic drop (80%–90%) in the number of c.f.u. ml^{-1} whilst viability with respect to cytoplasmic membrane integrity as measured by multi-parameter flow cytometry did not decline significantly. All of the evidence presented suggests that this drop in the number of c.f.u. ml^{-1} probably represents a loss in cell dividing capacity rather than cell death. These results highlight the problem of solely using the number of c.f.u. ml^{-1} in recombinant protein processes to follow metabolically active protein producing cells. Further, it demonstrates the power of multi-parameter flow cytometry for providing important physiological information at the individual cell level about process efficiency that is difficult to obtain in any other way.

Acknowledgements

The project was supported by VINNOVA (project number P14369-1) and by a consortium of industrial and academic partners, among which the contribution of Pfizer AB, Strängnäs, Sweden is especially acknowledged.

References

Andersson L, Strandberg L, Enfors S-O (1996) Cell segregation and lysis have profound effects on the growth of *Escherichia coli* in high cell density fed-batch cultures. *Biotechnol. Prog.* **12**: 190–195.

Andersson L, Strandberg L, Häggström L, Enfors S-O (1994) Modelling of high cell density fed-batch cultures. *FEMS Microbiol. Rev.* **14**: 39–44.

Boaretti M, Lleò MM, Bonato B, Signoretto C, Canepari P (2003) Involvement of *rpoS* in the survival of *Escherichia coli* in the viable but nonculturable state. *Environ. Microbiol.* **5**: 986–996.

Booth IR (1999) Adaptation to extreme environments. In: Lengeler JW, Drews G, Schlegel HG, eds. *Biology of the Prokaryotes*, Stuttgart Germany: Thieme, pp. 652–672.

Colwell RR (2000) Viable but nonculturable bacteria: a survival strategy. *J. Infect. Chem.* **6**: 121–125.

Enfors SO, Jahic M, Rozkov A, Xu B, Hecker M, Jurgen B, Kruger E, Schweder T, Hamer G, O'Beirne D, Noisommit-Rizzi N, Reuss M, Boon L, Hewitt C, McFarlane C, Nienow A, Fuchs L, Kovacs T, Revstedt J, Tragardh C, Friberg PC, Hjertager B, Blomsten G, Skogman H, Hjort S, Hoeks F, Lin HY, Neubauer P, van der Lans R, Luyben K, Vrabel P, Manelius A (2001) Physiological responses to mixing in large scale bioreactors. *J. Biotechnol.* **85**: 175–185.

Goss WA (1965) Mechanism of action of nalidixic acid on *Escherichia coli. J. Bacteriol.* **80**: 1068–1074.

Hewitt CJ, Nebe-Von-Caron G (2004) The application of multi-parameter flow cytometry to monitor individual microbial cell physiological state. In: Enfors S-O, ed. *Advances in Biochemical Engineering/Biotechnology – Physiological stress responses in bioprocesses*. Berlin, Germany: Springer-Verlag, pp. 197–223.

Hewitt CJ, Nebe-Von-Caron G, Axelsson B, McFarlane CM, Nienow AW (2000) Studies related to the scale-up of high cell density *Escherichia coli* fed-batch fermentations using multi-parameter flow cytometry: effect of changing microenvironment with respect to glucose and dissolved oxygen concentration. *Biotechnol. Bioeng.* **70**: 381–390.

Hewitt CJ, Nebe-Von-Caron G, Nienow AW, McFarlane CM (1999) Use of multi-staining flow cytometry to characterise the physiological state of *Escherichia coli* W3110 in high cell density fed-batch cultures. *Biotechnol. Bioeng.* **63**: 705–711.

Keller J, Gerber R, Del Tito Jr B, Vannicolo D, Arcuri E (1990) Effects of mutations altering SOS regulation on a nalidixic acid-inducible system for the production of heterologous proteins in *Escherichia coli. J. Ind. Microbiol.* **6**: 199–206.

Larsson G, Törnkvist M (1996) Rapid sampling, cell inactivation and evaluation of low extracellular glucose concentrations during fed-batch cultivation. *J. Biotechnol.* **49**: 69–82.

Lengeler JW, Postma PW (1999) Global regulatory networks and signal transduction pathways. In: Lengeler JW, Drews G, Schlegel HG, eds. *Biology of the Prokaryotes*. Stuttgart, Germany: Thieme, pp. 491–523.

Nebe-Von Caron G, Badley RA (1995) Viability assessment of bacteria in mixed populations using flow cytometry. *J. Microsc.* **179**: 55–66.

Nebe-Von-Caron G, Stephens PJ, Hewitt CJ, Powell JR, Badley RA (2000) Analysis of bacterial function by multi-colour and single cell sorting. *J. Microbiol. Meth.* **42**: 97–114.

Obukowicz MG, Staten NR, Krivi GG (1992) Enhanced heterologous gene expression in novel rpoH mutants of *Escherichia coli. Appl. Environ. Microb.* **58**: 1511–1523.

Shapiro HM (2003) *Practical Flow Cytometry*, 3rd edn. New York: Alan R. Liss Inc.

Strandberg L, Andersson L, Enfors S-O (1994) The use of fed-batch cultivation for achieveing high cell densities in the production of a recombinant protein in *Escherichia coli. FEMS Microbiol. Rev.* **14**: 53–56.