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Image analysis as a tool for viability and recombinant protein production assessment during *E. coli* fermentations

S. Carneiro[1], A. C. A. Veloso[1,2], A. L. Amaral[1,3], I. Rocha[1] and E. C. Ferreira[1*].

[1]Centro de Engenharia Biológica, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal
[2]Escola Superior Agrária de Bragança, Campus de Santa Apolónia, Apartado 172, 5301-855 Bragança, Portugal
[3]Escola Superior de Tecnologia e de Gestão, Campus de Santa Apolónia, Apartado 172, 5301-855 Bragança, Portugal

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Abstract

The development of monitoring methods for physiological state assessment during recombinant fermentation processes has been encouraged by the need to evaluate the influence of processing conditions in recombinant protein production.

In this work, microscopy and image analysis techniques were used for the quantification of viability and protein production in two recombinant *E. coli* batch fermentations. Images obtained from light microscopy with phase contrast were used to assess the total number of cells in a given sample and, from epifluorescence microscopy, both producing and dead cells were counted using two different filters.

This methodology allowed the extraction of information related to cell viability and recombinant protein production. This information, combined with standard fermentation data, allowed the derivation of interesting hypothesis that can be used afterwards for experimental design and further validation.

Additionally, the ratios calculated in this work can be complemented with other parameters that can be extracted from image analysis.

1 Introduction

The ability to cultivate microbial strains expressing heterologous recombinant proteins is an increasingly important technique throughout the field of biotechnology. Among the many systems available for heterologous protein production, the Gram-negative bacterium *Escherichia coli* remains one of the most attractive because of its ability to grow rapidly and at high densities on inexpensive substrates, its well-characterized genetics and the availability of a large number of cloning vectors and mutant host strains (Jana and Deb, 2005).

However, the production of recombinant proteins can significantly influence cell metabolism by channelling bacterial resources towards the production of the target protein, thereby imposing a metabolic burden and stress to the host cell. In particular, the strong synthesis of a recombinant protein can induce a number of different stress signals connected to cellular regulons such as a heat-shock-like or unfolded protein response and the stringent response (Lin et al., 2004).

* Corresponding author. Tel +351 253 604 407. E-mail: ecferreira@deb.uminho.pt

Additionally, during fermentation processes, recombinant cells can be submitted to other stress conditions, including nutrient starvation, oxidative stress, and excessive carbon dioxide levels which can decrease growth rates and stimulate acetate formation (Enfors et al., 2001).

These phenomena can lead to a significant loss of protein production during the fermentation process, due to alternative metabolic pathway to cell maintenance rather than cell reproduction and protein production.

It is therefore essential to include in fermentation monitoring strategies the evaluation of the different physiological responses at the occurring culture conditions. These results can then be used for mathematical model construction and validation and for designing process control and optimization strategies.

Among the different physiological conditions, cellular viability assessment is one of the most relevant approaches because it is directly connected to process productivity and is relatively easy to evaluate.

However, traditional methods based on plate counting are unsuitable for assaying viability, because only culturable cells grow on plates, and depending on the situation, these may represent only a minor fraction of living cells (Sachidanandham et al., 2005).

Epifluorescent microscopy (EFM) has been described as a suitable method for a rapid and direct assessment of cell viability. The main advantage of direct methods based on fluorescent probes is the lack of incubation time. Fluorescent indicators of viability may be based on different approaches (Auty et al., 2001), from which membrane integrity is one of the most common. Membrane integrity analysis is based on the capacity of the cells to exclude fluorescent dye compounds, which when used at low concentrations do not normally cross intact membranes. Most of the membrane integrity assays use nucleic acid stains, due to the high concentrations of nucleic acids within the cells and the large fluorescence enhancement exhibited by nucleic acid stains upon binding, leading to a clear separation between intact and dead cells (Joux and Lebaron, 2000). A wide diversity of impermeant nucleic acid stains can be used, among which propidium iodide (PI). PI binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA.

Additionally to viability measurement, during recombinant protein production, it is important to measure not only the total amount of product obtained, but also to evaluate the fraction of producing and non-producing strains, mainly to evaluate parameters related to strain performance, like promoter strength and stability. For this purpose, fluorescent microscopy can also be used if the target protein exhibits fluorescent properties, like the model protein GFP (Green Fluorescent Protein) of the jellyfish *Aequorea victoria*. GFP needs no substrates or cofactors to fluoresce (Tsien, 1998, Lehtinen et al., 2003) and is an excellent choice for diverse cell research, having its applications increased widely in recent years (Banning et al., 2002; Lehtinen et al., 2004).

However, although fluorescence-based methods have remained very useful for a wide diversity of applications ranging from industrial to environmental microbiology, for many years, the counting of cells from microscopic images has been done manually. Recently, image analysis has become a valuable accessory for such quantification because it reduces subjectivity and allows automation. During the last years, the application of image analysis to cell technology has increased rapidly. In some fields, such as the fermentation of microorganisms, image analysis is now essential for characterizing the state of the culture, decreasing analysis costs are making microscopy a more practical technique.

The purpose of this study was to establish a rapid, simple, and accurate direct method to evaluate bacterial viability, physiological state and protein production during fermentation processes. A dual marker system based on the expression of GFP and PI staining has been used for the quantification of cell number, viability and protein production of *E. coli.*

2 Materials and methods

2.1. Cultivation conditions

A modified *E. coli* M15 (Qiagen - Germany) with the insertion of the EYFP (Enhannced Yellow Fluorescent Protein) gene (Clontech - USA) in a pREP4 plasmid (also from Qiagen) was cultivated in a minimal medium with composition described elsewhere (Rocha and Ferreira, 2002) with addition of 0.025 $g \cdot Kg^{-1}$ of kanamicin and 0.1 $g \cdot Kg^{-1}$ ampicilin. Fermentations were conducted in batch mode in a 5 L Biostat MD fermenter from B. Braun Biotech (Germany) equipped with pH, dissolved oxygen (DO) and temperature sensors connected to a digital control unit. The pH of the culture was maintained at 7.0 by automatic addition of 20 M H_3PO_4 or 3.5 M NH_3 with a pH controller. Temperature was kept at 33.5ºC, while the dissolved oxygen concentration was maintained above 27.5% air saturation. Induction was performed with 1.5 mM of IPTG (isopropyl-beta-D-thiogalactopyranoside).

Cell biomass was measured turbidimetrically by optical density at 600 nm in a Jasco (USA) V-560 spectrofotometer, calibrated against dry cell weight (DCW $g \cdot Kg^{-1}$) at 105 ºC to constant weight. The concentrations of glucose and acetate were measured by HPLC with a refractive index detector (Jasco) and a Chrompack organic acids column (Varian, USA) at 60ºC. Analysis of fluorescent protein was performed in a Jasco FP-6200 spectrofluorometer with excitation and emission wavelengths of 513 and 527 nm, respectively.

2.2. Bacterial staining

100 μL of undiluted biomass suspension was mixed with 20 μL of a 0.5 M PI solution and incubated in the dark for 10 min. A drop of the stained suspension was placed on a glass slide, covered with a cover slip, and sealed with a clear varnish to prevent drying of the sample during visualization with the microscope.

2.3. Acquisitions and analysis of epifluorescence images

The images were acquired in a Zeiss Axioscop microscope (Zeiss, Germany) coupled to a AxioCam (Zeiss) colour video camera with a size of 1300x1030 pixels in 24 bit (8 bits per channel). The public domain open source ImageJ 1.33u (NIH, USA) platform was used to develop the image processing and analysis programmes.

Both dead and protein producing cells ratios was obtained by means of an image processing and analysis methodology. For that purpose, three sets of images were taken for each sample after staining with PI. The first set constitutes bright field *E. coli* images intended to determine the total number of cells in a given image. The second set of images was obtained with a blue filter (wavelength of 450-490 nm) in order to determine the number of EYFP producing cells (yellow-green cells). Finally the third set of images was obtained with a green filter (wavelength of 546/12 nm) to establish the number of PI stained dead cells (red cells). The percentage of dead cells and protein producing cells was thus determined by the ratio between the dead cells or protein producing cells, respectively, and the total number of cells. The acquisition methodology relied on taking consecutively the images using the blue filter, green filter and bright field images at each given field of the sample.

In order to determine the total number of cells the developed programme consisted on four stages: a pre-processing initial step for enhancing structures with a width bellow 10 pixels by the use of a bandpass filter of size 10, followed by a background correction algorithm; an image segmentation step by the automatic Otsu thresholding algorithm; a debris removal step based on morphological parameters in order to eliminate small debris and ultimately the image registration. Regarding the determination of the dead cells the developed programme consisted on three stages: pre-processing by choosing the red channel of the 24 bit colour image and masking with the binary total *E. coli* image; determination of the dead cells by the red channel mean cell value and finally the results and image registration. With respect to the protein producing cells the programme consisted on three stages: the pre-processing step in order to obtain the enhanced images of the protein producing cells (green

channel) and the dead cells (red channel) subsequently masked with the binary total *E. coli* image; a second step regarding the determination of the protein producing cells by the enhanced green channel mean cell value on one hand and the determination of the dead cells by the enhanced red channel mean cell value on the other; a third final step for the results and images registration.

3. Results and Discussion

Bacterial viability analysis by EFM, performed by single staining of EYFP producing *E. coli* cells with PI, revealed the existence of three different cell populations, particularly, dead (PI stained), EYFP producing and live EYFP non-producing cells.. All the cells non-stained by PI were considered to be in active state, either EYFP producing or non-producing cells.

The described methodology was applied to two batch fermentation runs, where all conditions were the same, except for the time of induction: while in fermentation 1 the induction was performed at the beginning of exponential phase, in fermentation 2 cells were already in the middle of exponential phase when 1.5 mM of IPTG was added. During the course of the fermentations, cell physiology was evaluated by statistical analysis of the different populations defined previously. Figs. 1 and 2 show the progress of these fermentations. Biomass, carbon source (glucose) and main by-product (acetate) concentrations are shown in the left charts, together with arbitrary fluorescent units (AFU) measured with the spectrofluorometer, considered to be proportional to recombinant protein concentration. On the right figures, the fractions of both dead and recombinant protein producing cells are represented.

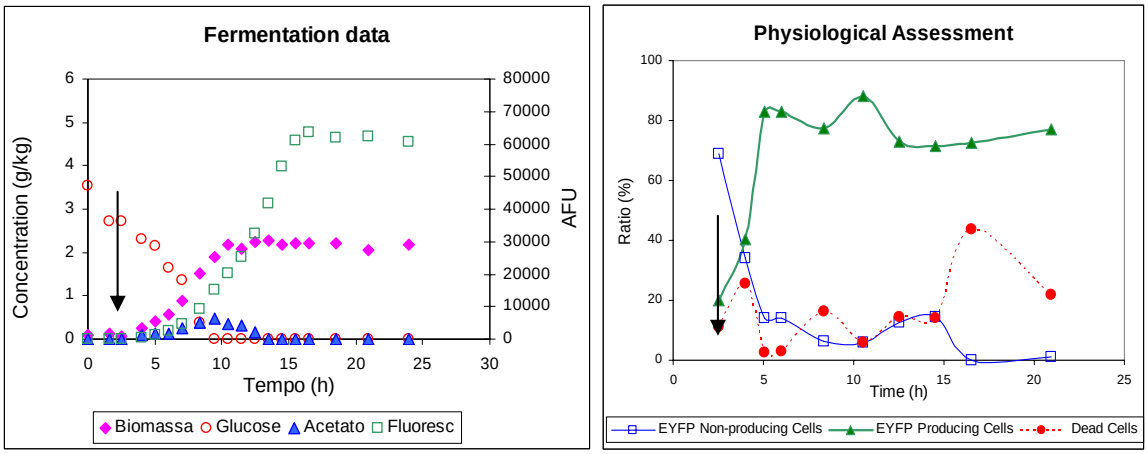

Figure 1 – Batch fermentation process of *E. coli* induced with IPTG at 2.5 hours of fermentation as indicated by the arrow (fermentation 1).

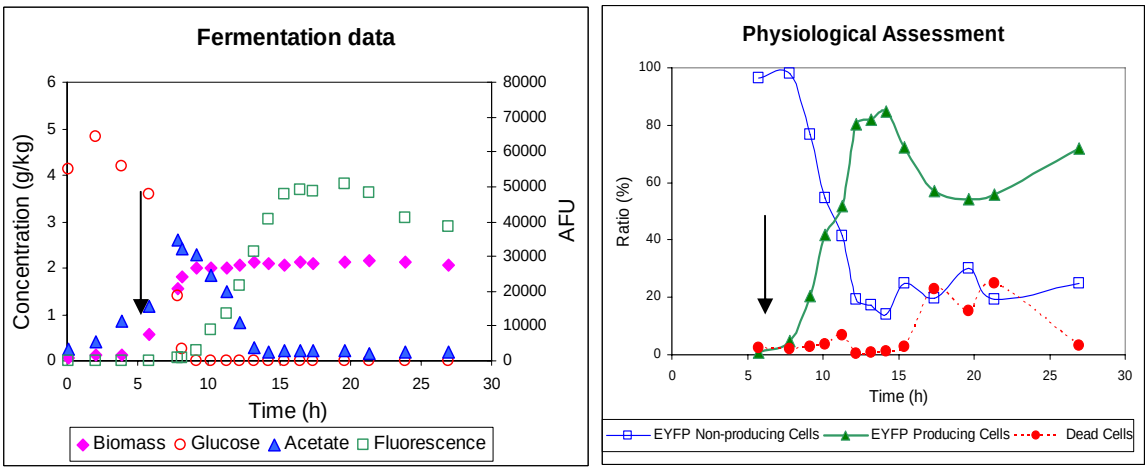

Figure 2 – Batch fermentation process of *E. coli* induced with IPTG at 6 hours of fermentation as indicated by the arrow (fermentation 2).

It can be seen from figures 1 and 2 that the time of induction had a clear effect on fermentation progression. Regarding recombinant protein production, it is perceptible a higher maximum value for AFU at the earlier induced batch fermentation. It is however clear that AFU values *per se* do not give a broad picture of the production patterns of the culture, as those differences can be due to either an increase in the productivity of individual cells or to an increase in the number of producing cells, or even a combination of both. In this case, image analysis results indicate that the fraction of producing cells is also higher.

Those facts could be explained by differences in the physiological state of the two cultures at the moment of induction, indicating that in a younger culture the number of cells able to produce recombinant proteins is higher. However, that phenomenon is observed together with lower acetate levels for the earlier induced culture, explained by the deviation of the carbon supply to protein production, altering the levels of the flux entering the TCA cycle. As it has been described (for example by (Farmer and Liao, 1997), acetate presence can decrease recombinant protein production levels, a fact that could also be a reasonable explanation for the differences observed in terms of protein production. On the other hand, the mechanisms by which acetate inhibits recombinant protein production are not yet clear and, if the hypothesis raised here is proved to be true, then acetate would act by totally inhibiting protein production of a fraction of cell population.

Regarding viability, the ratio of dead cells begins to increase earlier and to higher levels for the earlier induced culture, although the relationship with ITPG addition is not clear. There is, however, an obvious increase in the number of dead cells when both glucose and acetate are exhausted, as a consequence of nutrient starvation. The final ratio of dead cells is between 20 and 40%, a value that should not be neglected for process modelling purposes.

Additionally, for the latter induced culture, there is a pronounced decrease in AFU values after 15-20 hours of fermentation, that is again coherent with results obtained from image analysis, a fact that is probably related with increased cell death.

In addition to these observations, physiological evaluation using microscopy can be very useful to illustrate some events that cannot be detected for example, with flow cytometry, like the occurrence of cell lysis and cell aggregation. Cell lysis was observed in both cultures with some significance when a constant EYFP production was achieved, together with other interesting features, like the DNA packaging involving other cell components (EYFP protein) forming huge aggregates.

4. Conclusions

In this work a simple application of image analysis was developed that allow the extraction of information related to cell viability and recombinant protein production. This information, combined with standard fermentation data allowed the derivation of interesting hypothesis that can be used afterwards for experimental design and further validation.

Additionally, the ratios calculated in this work can be complemented with other parameters that can be extracted from image analysis.

References

Auty,M.A.E., Gardiner,G.E., McBrearty,S.J., O'Sullivan,E.O., Mulvihill,D.M., Collins,J.K., Fitzgerald,G.F., Stanton,C., and Ross,R.P. (2001) Direct in situ viability assessment of bacteria in probiotic dairy products using viability staining in conjunction with confocal scanning laser microscopy. *Applied and Environmental Microbiology* **67** (1), 420-425.

Banning,N., Toze,S., and Mee,B.J. (2002) Escherichia coli survival in groundwater and effluent measured using a combination of propidium iodide and the green fluorescent protein. *Journal of Applied Microbiology* **93** (1), 69-76.

Enfors,S.O., Jahic,M., Rozkov,A., Xu,B., Hecker,M., Jurgen,B., Kruger,E., Schweder,T., Hamer,G., O'Beirne,D., Noisommit-Rizzi,N., Reuss,M., Boone,L., Hewitt,C., McFarlane,C., Nienow,A.,

Kovacs,T., Tragardh,C., Fuchs,L., Revstedt,J., Friberg,P.C., Hjertager,B., Blomsten,G., Skogman,H., Hjort,S., Hoeks,F., Lin,H.Y., Neubauer,P., van der Lans,R., Luyben,K., Vrabel,P., and Manelius,A. (2001) Physiological responses to mixing in large scale bioreactors. *Journal of Biotechnology* **85** (2), 175-185.

Farmer,W.R. and Liao,J.C. (1997) Reduction of aerobic acetate production by *Escherichia coli*. *Appl.Environ.Microbiol.* **63** (8), 3205-3210.

Jana,S. and Deb,J.K. (2005) Strategies for efficient production of heterologous proteins in Escherichia coli. *Applied Microbiology and Biotechnology* **67** (3), 289-298.

Joux,F. and Lebaron,P. (2000) Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. *Microbes and Infection* **2** (12), 1523-1535.

Lehtinen,J., Nuutila,J., and Lilius,E.M. (2004) Green fluorescent protein-propidium iodide (GFP-PI) based assay for flow cytometric measurement of bacterial viability. *Cytometry Part A* **60A** (2), 165-172.

Lehtinen,J., Virta,M., and Lilius,E.M. (2003) Fluoro-luminometric real-time measurement of bacterial viability and killing. *Journal of Microbiological Methods* **55** (1), 173-186.

Lin,H.Y., Hoffmann,F., Rozkov,A., Enfors,S.O., Rinas,U., and Neubauer,P. (2004) Change of extracellular cAMP concentration is a sensitive reporter for bacterial fitness in high-cell-density cultures of Escherichia coli. *Biotechnology and Bioengineering* **87** (5), 602-613.

Rocha,I. and Ferreira,E.C. (2002) On-line simultaneous monitoring of glucose and acetate with FIA during high cell density fermentation of recombinant *E. coli*. *Analytica Chimica Acta* **462** (2), 293-304.

Sachidanandham,R., Gin,K.Y.H., and Poh,C.L. (2005) Monitoring of active but non-culturable bacterial cells by flow cytometry. *Biotechnology and Bioengineering* **89** (1), 24-31.

Tsien,R.Y. (1998) The green fluorescent protein. *Annual Review of Biochemistry* **67** 509-544.