Application of flow cytometry analysis to elucidate the impact of scale-down conditions in Escherichia coli *cultivations*

P. Gil Salvador 2013 Award in Bioengineering category. (November 22, 2013 in the Annual General Assembly of the AIQS)

Anna M Marba-Ardebol[*1,2], Xavier Turon[1],Peter Neubauer[2], Stefan Junne[2]

[1]Bioengineering Department, Institut Químic de Sarrià, Universitat Ramon Llull, Barcelona, Barcelona [2]Chair of Bioprocess Engineering, Department of Biotechnology, Technische Universität Berlin, Berlin

Aplicación de la citometría de flujo para elucidar el impacto de las condiciones de scale-down en cultivos de Escherichia coli

Ganador del Premio P. Salvador Gil 2013 categoría de Bioingeniería (El pasado 22 de noviembre de 2013 durante la Asamblea General Ordinaria de la AIQS)

*Aplicació de la citometria de flux per elucidar l'impacte de les condicions d'scale-down en cultius d'*Escherichia coli

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Recibido: 26 de mayo de 2014; revisado: 3 de agosto de 2014; aceptado: 28 de octubre de 2015

RESUMEN

En bioprocesos de escala industrial aparecen gradientes; especialmente cuando no se alcanza una distribución homogénea ni de la alimentación ni del oxígeno. Para estudiar las consecuencias de estas condiciones heterogéneas sobre el cultivo, se han diseñado experimentos que imitan las condiciones de gran escala en el laboratorio (experimentos de scale-down). Estos estudios han puesto de manifiesto que las células expuestas a condiciones oscilatorias se ven afectadas en varios niveles celulares de regulación. No obstante, se han realizado pocas investigaciones en base a la observación del comportamiento de *Escherichia coli* a nivel unicelular. Por este motivo, fue la citometría de flujo (FCM) la técnica elegida para estudiar la viabilidad celular en experimentos de scale-down. Estos se llevaron a cabo en sistemas comparimentados de dos compartimentos (Two-CR), así como en el recién establecido de tres compartimentos (Three-CR). Debido a que los métodos de tinción de la citometría de flujo para la aplicación en bacterias aún no están establecidos, fue necesaria su optimización para ser utilizados en estos experimentos.

Palabras clave: Scale-down; citometría de flujo; fed-batch; heterogeneidades; tinción.

SUMMARY

Inhomogeneities appear in large-scale fed-batch bioprocesses; especially when the distribution of the feeding solution and of the oxygen is uneven. In order to study the consequences of these heterogenic conditions on the culture, scale-down bioreactor experiments have been designed. These studies have revealed that cells exposed to oscillatory conditions are affected on various cellular levels of regulation. However, not many of these studies have been performed on the observation of the behaviour of the single cell level of *Escherichia coli.* Therefore, flow cytometry (FCM) is chosen as analytical tool to study the cellular viability in the scale-down approaches of a two compartment reactor (Two-CR) and a newly established three compartment reactor (Three-CR). An optimization of different staining methods applied in these experiments is also performed, since staining procedures for flow cytometry studies of bacterial populations are still not well-established yet.

Key words: Scale-down; flow cytometry; fed-batch; heterogeneity; staining.

RESUM

En bioprocessos d'escala industrial apareixen gradients; especialment quan no s'assoleix una distribució homogènia ni de l'alimentació ni de l'oxigen. Per a estudiar les conseqüències d'aquestes condicions heterogènies sobre el cultiu, s'han dissenyat experiments que imiten les condicions de gran escala al laboratori (scale-down experiments). Aquests estudis han posat de manifest que les

*Corresponding author: annamarbaa@iqs.url.edu

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Paraules clau: Scale-down; citometria de flux; fed-batch; heterogeneïtats; tinció.

INTRODUCTION

Inhomogeneities in large-scale nutrient-limited fed-batch cultivations.

Gradients appear along the height of industrial scale bioreactors at nutrient-limited high-cell density processes (Lara, Galindo et al. 2006). The first drawback that faces scale-up from lab scale is the limitation in the power of mixing, and consequently the limitations in mass transport. The mixing times in large-scale bioreactors are usually in the range of several minutes (Junker 2004). The dissolved oxygen supply is not sufficient in zones of excess substrate, which appear near the inlet of the highly concentrated feed (Neubauer and Junne 2010). Thus, oxygen limitation occurs in these zones from a certain cell density onwards during the course of the cultivation. The fed-batch method is appropriate to achieve high cell densities, avoiding effects like metabolic overflow in most of the reactor zones. However, concentrations of the ingredients of the feed solution are higher near the feeding port, causing an alternation of the environment of the fast moving cells in the bioreactor. Thus, a pulse-wise increase of intracellular fluxes in zones of high substrate concentration leads to unwanted by products and usually decreased biomass and product yields. In zones far away from the feed port, cells are opposed to substrate limitation. Due to the stirring, cells are continuously exposed to alternating micro-environmental conditions between substrate excess and starvation (Junne, Klingner et al. 2011). These changes are not only found for the dissolved oxygen and substrate concentration, but also for the pH-value and dissolved carbon dioxide concentration. These changes lead to additional cell stress, which might influence the vitality and viability of cells and finally the process performance.

Scale-down reactors for the imitation of the large scale in lab scale.

In order to mimic the large scale in lab-scale experiments, several scale-down reactor design approaches have been evolved. For example, cyclic perturbations at the level of the sugar concentration and dissolved oxygen level have been generated by a feed control applying a DO sensor signal (Delvigne, Boxus et al. 2009).

Another option is using two reactors, each representing one distinct zone. Some of them comprise of a tubular section (most likely a plug flow reactor - PFR) and a conventional stirred tank reactor (STR). A system like this is usually called two compartment reactor (Two-CR). The variable location of the feeding and the sparger, as well as the profile of the feeding, enables to study the influence of various conditions at distinct residence times of the cells opposed to these gradients. One option is installing the feeding at the middle of the section of the plug flow reactor (PFR). In this case, the cells encounter firstly a strong starvation before they are opposed to glucose excess (Delvigne, Boxus et al 2009). Other studies are describing a bioreactor setup, where the feed addition was installed at the bottom of the PFR in order to generate a substrate concentration gradient along the height of the tubular reactor (Delvigne, Gorret et al. 2011), (Junne, Klingner et al. 2011).

A Two-CR system just reproduces two parts of the spatial segregation that the fluidic phase of a large-scale bioreactor cultivation typically expresses: (i) the homogeneous nutrient-limited bulk zone, and (ii) the feed zone. One zone is not represented, to which cells are usually exposed, too: oxygen excess and substrate limitation, which appears far away from the feed zone in areas with poor mixing conditions. In order to meet this specification, a second PFR without feeding port but of same dimensions can be added to the system, thus achieving the three zones in one system: high nutrient concentration and low oxygen availability around the feed zone, nutrient-limiting substrate availability and oxygen excess conditions in the bulk zone, and low nutrient and low oxygen availability in zones, which are poorly mixed (Lapin, Schmid and Reuss. 2006).

Flow cytometry for the investigation of the impact of stress on the cell population.

Traditional techniques (microscopic dye exclusion or cell-counting on plates) allow an overall study of the physiologic condition of the culture (viability, activity). Flow cytometry offers the analysis of large numbers of cells individually, which can provide an examination of cell viability and vitality among a representative amount of cells. Traditional techniques tend to underestimate the viability of the culture (Díaz, Herrero et al. 2010), just quantifying the dividing cells (CFU), but not all the living cells as flow cytometry does.

If the cell died or if the membrane ruptured or developed large holes, as it is often the case under exposure to heat, salinity, and other types of stress, the membrane potential is reduced and cells become (partially) depolarized or even permeabilized (Novo et al. 2000). Regarding this property, propidium iodide (PI) (Looser, Hammes et al. 2005), which stains permeabilized membrane cells and Bis-(1,3-dibutylarbituric acid)-trimethineoxonol (BOX) (Want, Thomas et al. 2009), which stains partially depolarized cells, can be applied. Moreover, some dyes interact with the metabolism of the cell, for instance 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), which is used for the characterization of the respiratory capacity of cells (Rodriguez 1992).

Fluorescent dyes can also interact with particles, which are not cells and, consequently, provide false results. Therefore, it is important to be able to discriminate between the target cells and debris or other particles, e.g. from media. SYTO13 can be used for this purpose, since it stains DNA (Khan, Pyle et al. 2010).

Hewitt and Nebe-von-Caron have applied FCM using several fluorescent dyes (rhodamine 123, propidium iodide, bis-oxonol and ethidium bromide) to study the differences between bacterial populations in batch, fed-batch and continuous fermentations in terms of physiological responses to environmental changes (Hewitt, Nebe-Von Caron et al. 1999); including the investigation in scale-down approaches (Hewitt and Nebe-Von-Caron 2001).

This paper will provide examples of bacterial cell staining for the suitable investigation of the physiological state of single bacterial cells in the Two-CR and Three-CR scale-down reactor concept in *E. coli* K12 W3110 cultivations. The suitability of different sample pre-treatment methods will be discussed and consequences for the experimental design will be described, as no standard protocols based on the examination of various procedures were established yet.

MATERIALS AND METHODS

Media preparation and strain cultivation

Escherichia coli K12 W3110 cells were cultivated at 10 L of working volume in a two-step procedure in a fed-batch mode as previously described (Xu, Jahic et al. 1999). Briefly, at first, the *in situ* enzymatic substrate release method EnBase®-Flo was applied until a biomass concentration of 5 gL^{-1} was reached. Then a mechanical feeding with a pump was started. This feed is exponentially increased, therefore cells are restricted to grow at a constant rate.The feed solution contained 440 gL^{-1} of glucose, macro and trace elements (Neubauer, Häggström et al. 1995)

Scale-down experiments

The applied Two-CR was used as described in Junne, Klingner et al. 2011, whereas the novel Three-CR was built by the connection of a second PFR module to the Two-CR system. The only difference between both PFRs is that the second one had no feed at the entrance. (Figure 1).

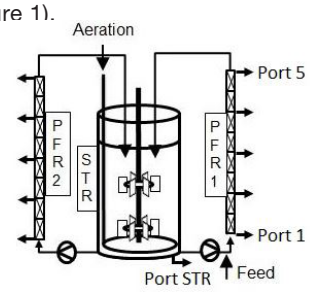

Figure 1: *Setup of the three-compartment scale down reactor (Three-CR) as applied in this study. It comprises of a stirred tank reactor (STR) with a working volume of 10 L and two identical plug flow reactor (PFR) modules of 1.8 L working volume (each). The modules are connected via peristaltic pumps at a flow rate of 1.8 Lmin-1, resulting in a mean residence time of approx. 1 min. At PFR module, the feed is supplied behind the peristaltic pump.*

Since this second PFR module is connected at another port of the STR module, the residence time within this second plug flow module differs in a range of a few seconds from the first one. However, since the mean residence time in the PFR is in the order of a minute, the small deviance in the residence time has likely no remarkable impact. Thus, in this scale-down approach, the EnBase background feeding supports the cells with nutrients (approximately 10 to 3% of the total nutrient supply, declining in the course of the cultivation), while cells are exposed to glucose excess for approx. 40 s in one of the PFR modules (when cells passed the feed addition).

Dry cell weight

For the dry cell weight analysis, 2 mL of culture were centrifuged in previously weighted 2 mL Eppendorf tubes (21500 g; 10 min; 4°C), the pellet was washed with 1 mL of 0.9 % w/w NaCl solution and centrifuged again. Then the Eppendorf tube was stored in a drying oven (75ºC) for at least 48h and weighted.

Flow cytometry

Flow cytometric measurements were performed using the MACSQuant® Analyzer (MiltenyiBiotec GmbH**,** BergischGladbach, GER). The excitation wavelength was set to 488 nm, which is generated by a blue argon-ion laser at 25 mW.

Four fluorescent dyes were used (alone, no double staining): propidium iodide (PI), $DiBAC_4(3)$(BOX or Bis-(1.3-dibutylbarbituricacid) trimethineoxonol)), 5-Cyano-2.3-di-(p-olyl)tetrazoliumchloride (CTC), which were purchased from Sigma-Aldrich (Munich, GER); and SYTO13, which was provided by Invitrogen. Filters were applied as follows: a band-passfilters: 488/10 for FSC/SSC, 525/50 for FL1 (SYTO13, BOX) and a long-passfilter: 655-730 for FL3 (PI, CTC).

A volume of 2 mL of the fed-batch samples was filtered with a vacuum filter using a filter pore size of 0.2 µm, in order to avoid undesired particles and background noises at the flow cytometric analysis. The cells retained by the filter were washed with 5 mL of 0.9 % w/w NaCl solution and dissolved with PBS to reach an OD_{600} between 0.02 and 0.03, which corresponds to a concentration between $2*10^5$ cells mL^{-1} and $5*10^5$ cells mL^{-1}. After that, depending on the applied dye, samples were stored at the appropriate temperature as described in the results section: 4ºC (ice), room temperature or 37ºC (thermoblock).

The final optimized concentrations for the dyes PI, SYTO13, CTC were 1 µg mL^{-1}, 3 µM and 13 mM, respectively. PI, SYTO13 and CTC were applied for 2 min in the

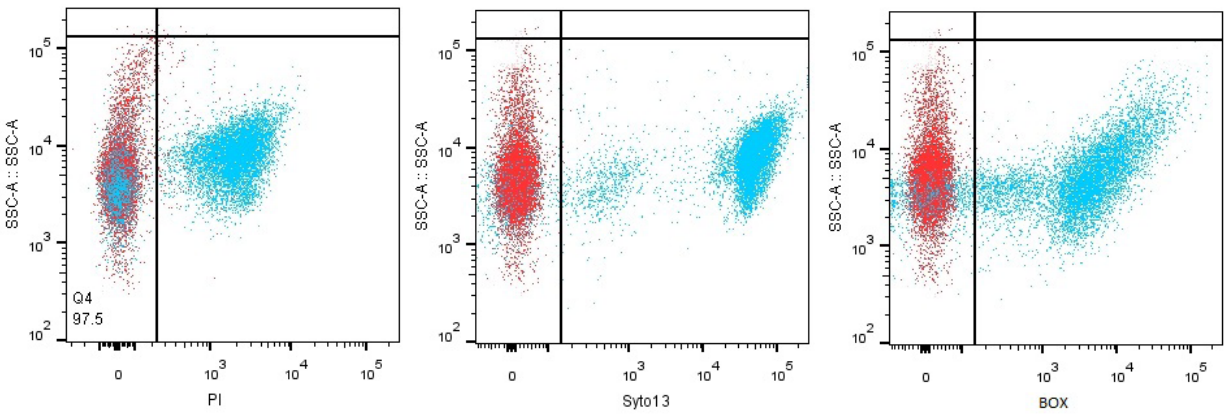

Figure 2*: Negative controls (left populations) and positive controls (right population) for PI, Syto13 and BOX.*

dark. PI and SYTO13 stainings were performed on ice (4ºC); meanwhile CTC staining was performed at 37ºC. These conditions were found through a screening using a design of experiments (DoE) approach. BOX staining was performed in the dark for 4 minutes at room temperature at a final concentration of 1 µM. BOX staining was applied based on previously published procedures (López-Amorós, Castel et al. 1997), (Amor, Breeuwer et al. 2002) and not in the focus of optimizations described in this paper.

The negative control for all dyes is considered to be the unstained sample. This sample is used to set up the threshold and detector sensitivity settings (influencing the display of the particles in the different channels).

The positive control is obtained with a heat shock (80ºC, 10 minutes), which results in the loss of cell viability. This control was stained with PI, BOX and SYTO13 (Figure 2).

Experimental design approach and data treatment

The DOE approach was performed in order to identify parameters of high sensitivity for the adjusted staining procedure. A multiple linear regression model was adjusted (MLR). Each experiment was defined with 3 factors (temperature, staining time and final dye concentration) at two levels, resulting in a set of 11 experiments with three replicates at the center point, in order to obtain statistic results. The DoE was analyzed with MODDE v. 9.1, MKS Umetrics AB. The effect of each factor was normalized from -1 to 1 (normalized coefficients), being 0 when the factor has no influence on the response. Besides, additional titration experiments were also performed in order to optimize the dye concentration.

RESULTS

Pre-preparation of *Escherichia coli* K12 W3110 for flow cytometric analysis

The impact on the pre-preparation method is tested, applying either vacuum filtration or centrifugation while washing cells. Although the portion of stained cells is similar in terms of SYTO13 and CTC dyes: vacuum filtration vs. centrifugation yielded for SYTO13 82.73% vs. 79.16 %, and for CTC 78.12% vs. 71.28%. Thus, more SYTO13 and CTC stained cells are obtained using vacuum filtration. In terms of the percentage of PI staining, the number of stained cells after washing with centrifugation is almost doubled than after washing with vacuum filtration (9.59% vs. 5.15%) (Table 1). Hence, a more viable culture is obtained after washing with vacuum filtration than when centrifugation is used, as usually proposed in many protocols. Also, results are more reproducible when cells are washed with vacuum filtration than with centrifugation (±1.7% vs. ± 4.8%).

Table 1: *Portion of E. coli cells stained with PI (1 µg mL^{-1}), SYTO13 (3 µM) or CTC (13 mM), after using vacuum filtration or centrifugation as cell washing method*

	%PI(+)	%SYTO13(+)	%CTC(+)	
Vacuum filtration	5.15	82.73	78.12	Average
	0.11	1.91	2.98	Std. Dev. (±)
Centrifugation	9.59	79.16	71.28	Average
	0.05	8.97	5.42	Std. Dev. (±)

Staining of *Escherichia coli* K12 W3110 with PI for viability analysis

At the normalized coefficients of the DoE analysis, it can be seen that the staining time has almost no influence on the results (0.04). The same holds true for the temperature (-0.24). In contrast, the concentration of the dye (PI) has a considerable impact (>1).

Apparently, the lower the PI concentration, the lower are the interferences with the viability of the culture (Figure 3) However, the working concentration should be sufficient in order to stain all non-viable cells of the culture. To accomplish this, a non-viable culture (positive control) was prepared and a titration procedure was performed. It was seen that a concentration of 0.2 µg mL^{-1} is not sufficient to stain all the non-viable cells,but 1 µg mL^{-1} (91.76% vs. 99.05%). Moreover, a small shift in the unstained population could be seen at PI concentrations above 2 µg mL^{-1}(data not shown), confirming that the concentration should not be higher than 1 µg mL^{-1} to avoid unspecific staining or high background noise or any other influence on the population.

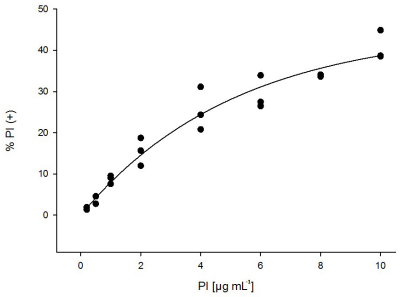

Figure 3: *Flow cytometry titration of PI stained E. coli samples.*

Staining of *Escherichia coli* K12 W3110 with SYTO13 for viability analysis

As revealed by a DoE analysis, no significant influences at the observed parameters could be identified for SYTO13 staining, but once again the staining time is a less important factor (normalized coefficients of the DoE analysis: SYTO13 concentration -0.50, temperature -0.68 and time 0.19). It came out that working at low temperature enhanced the performance of the dye, thus a larger percentage of cells are stained. So a titration was performed in order to identify the optimal concentration range. SYTO13 stained samples indicate no background staining. Above a concentration of 1 µM, no influence on the number of stained cells is observed (Figure 4). A culture of viable and non-viable cells (positive control) was prepared and stained with SYTO13 and PI following the optimized staining conditions. It is demonstrated that SYTO13 is able to stain all cells regardless their viability, although viable and non-viable cells lie in different intensity regions of the positive quadrant (the non-viable population have a higher intensity in comparison with the viable population). However, no quantitative (only qualitative) correlation between SYTO13 and PI is found, thus a direct correlation between them cannot be made on a mathematical basis. For that reason, only the viable population stained with SYTO13 was considered, meanwhile PI staining will be used for estimating the portion of non-viable cells.

It is worth to mention that the stained samples spread to higher intensities in the SYTO13 plots within time, i.e. towards the non-viable intensity range. For this reason, sam-

ples should be treated as fast as possible in order to obtain reliable results.

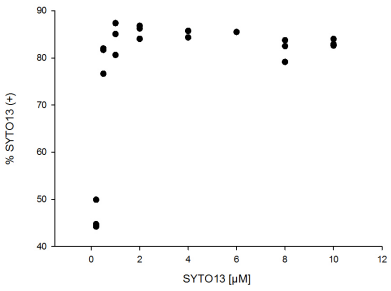

Figure 4: *Flow cytometry titration of SYTO13 stained E. coli samples.*

Staining of *Escherichia coli* K12 W3110 with CTC for the analysis of the respiratory activity

The analysis of the DoE reveals the temperature as the factor with a higher impact on the response (percentage of stained cells) than the dye concentration or time. (Normalized coefficients: temperature 0.96, CTC concentration 0.42 and time 0.08). As with the other optimization procedures, the time has almost no impact on the staining. This result is similar to the ones obtained in previous studies, where the portion of CTC-stained cells quickly reached a plateau, despite the fact that the fluorescence per cell continued to increase (Del Giorgio, Prairie et al. 1997), (Sieracki, Cucci et al. 1999).

At higher temperatures, the respiratory capacity of the cells increases, leading to a higher CTC reduction to formazan. From a biological point of view, the velocity of the metabolic transport and turnover rates of cells decreases at lower temperature. The temperature has also an impact on the deviation, which is reduced when working at higher temperatures (37ºC). The toxicity of CTC might be critical, since it interferes with the electron transport system, altering the bacterial metabolism. However, the toxic effects are posterior to the incorporation and reduction of CTC. Furthermore, it has been already proven by several authors that the intracellular reduction continues after the number of CTC stained cells remain stable (Sieracki, Cucci et al. 1999), (Gasol and Aristegui 2007). In order to verify this assumption, a titration was performed (Figure 5). A saturation point has been reached above a concentration of 14mM. The stained samples spread towards lower respiratory activity along with a prolonged sample treatment, i.e. so their respiratory capacity is reduced. Like SYTO13 staining, it is important to process samples as fast as possible. Thus, the staining time was set to 2 minutes.

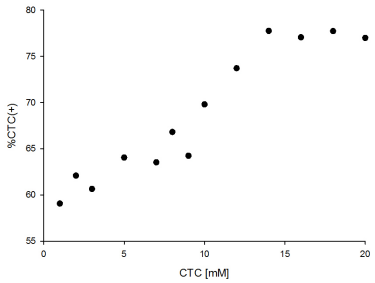

Figure 5: *Flow cytometry titration of CTC stained E. coli samples.*

Cultivation characteristics in *Escherichia coli* K12 W3110 scale-down cultivations (Two-CR and Three-CR)

Growth in the Two-CR is very similar to growth under homogeneous conditions in the STR (Figure 6). This is not a surprising result as this is seen often when cultivating *E. coli* strains under such conditions. Oscillating substrate availability can even have a stimulating effect on carbon conversion, as intracellular limitations that occur under steady nutrient limitation are resolved in the zone of excess substrate availability. In the STR cultivation, the substrate uptake capacity seems to be decreased, but what is actually occurring is that the oscillating conditions increase the substrate uptake capacity of the culture. In the Three-CR, the adaptation probably takes longer, thus an increased substrate uptake capacity is seen later. This phenomena has been already stated earlier (Sandoval-Basurto et al. 2005). When comparing the two scale-down approaches, the accumulated extracellular glucose is greater in the Two-CR. This is due to the lack of substrate in the second PFR, in which no feed is added. Hence, whenever cells are entering this PFR module, substrate depletion is occurring immediately. If this culture broth is re-entering the STR, only a limited amount of substrate is available.

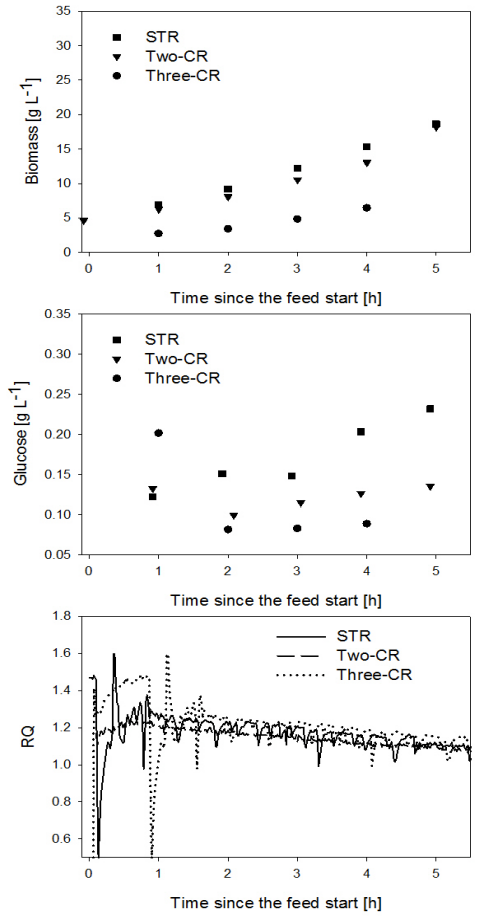

Figure 6: *Process parameters of the STR, Two-CR and Three-CR cultivations: biomass and glucose concentration and respiratory coefficient.*

Flow cytometry analysis of *Escherichia coli* K12 W3110 scale-down cultivations (Two-CR and Three-CR)

Firstly, an increase of viability is seen after feed start. This response can be observed either with PI or SYTO13

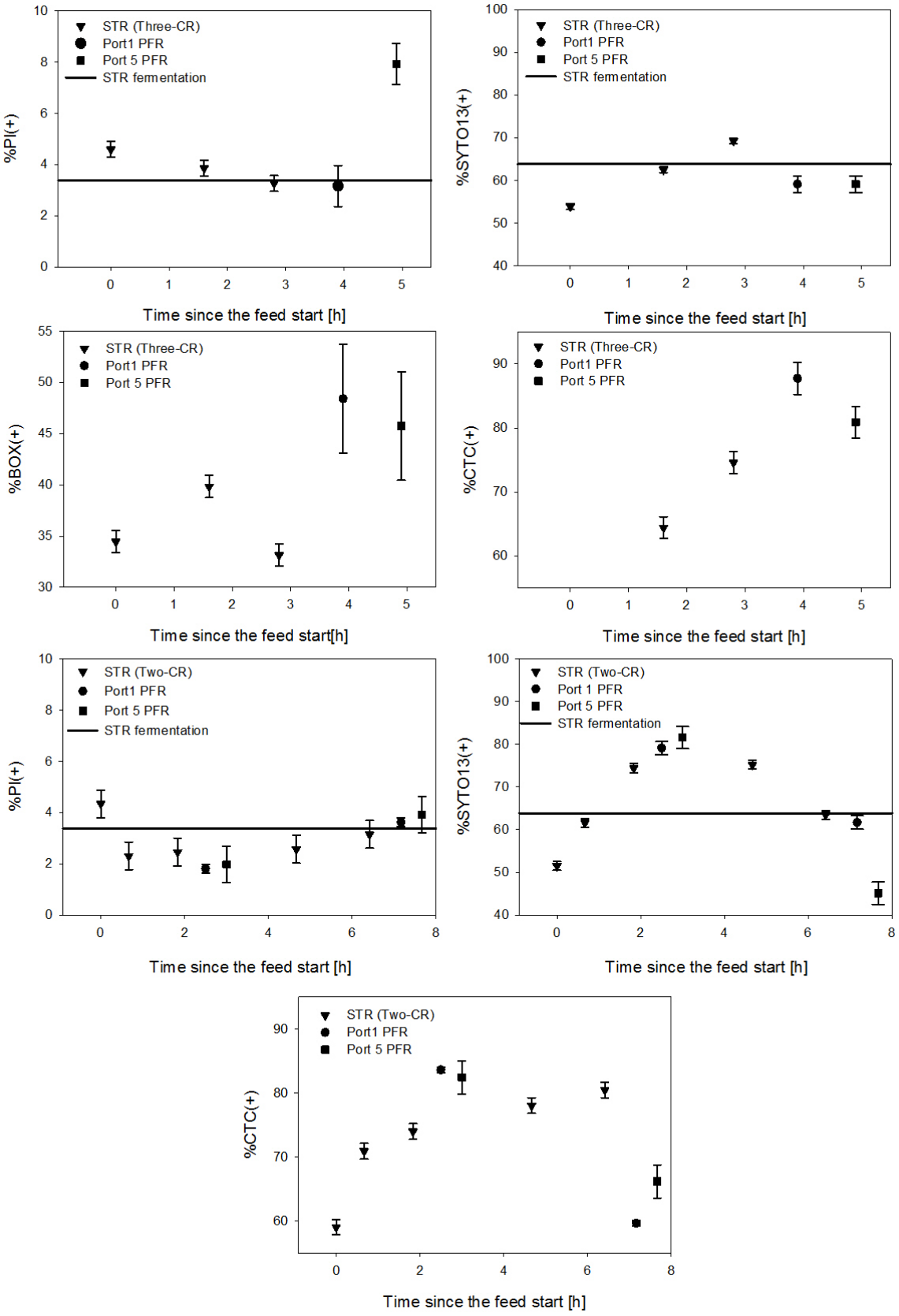

***Figure 7**: Evolution of the portion of PI, SYTO13 and CTC stained E. coli cells along the Two-CR and Three-CR cultivations.*

staining, in both scale-down cultivations. However, in the Three-CR the adaptation is slower than in the Two-CR, in which the viability is even higher than in the STR cultivation. This could indicate that the conditions at the Three-CR are more stressful for the cells (Figure 7). Nevertheless, it should be differentiated between the adaptation due to kinetic regulation and due to changed enzyme expression. The first one is present when the environment of cells suddenly changes and fluxes represent a response to altered metabolite concentrations, whereas the second one is a (long term) adaption of cells.

The substrate limitation is not so severe anymore after the mechanical feed start (see glucose concentration in Figure 6). Not only the glucose provision increases (by a factor of ten), but also mineral salts are provided (mineral salt medium, trace elements and thiamine). This shift, also beneficial for the nutrient supply of the cells, causes cell stress, which leads to a spread in the population. Cells try to get adapted to these hostile conditions evolving to one culture. Those stressful conditions are translated also into a lower biomass achieved with the Three-CR cultivation in comparison with the STR and Two-CR cultivation (Figure 6). The feeding at the inlet of the PFR module results in a substrate gradient along the height of it. In parallel, this substrate heterogeneity causes also a gradient of the oxygen concentration. Dissolved oxygen is nearly depleted along the height of the PFR. However, cells adapt also to these conditions in the Two-CR system. Hence, the viability of the culture increases (see in Figure 7 the STR results), even the culture seems well adapted to the oscillating conditions induced by the PFR, as proven at similar portions of PI and SYTO13 stained cells in the PFR as in the STR reference cultivation. When considering the results of PI and SYTO13 staining, it seems that weak cells die at the beginning of the feed start. The increase of viability as revealed by PI staining occurs thereafter. In contrast, other cells undergo a process of adaptation during the fermentation. This adaptation can be observed with SYTO13 staining, while the percentage of stained cells is increasing throughout the cultivation.

In the Three-CR cultivation, BOX staining indicates no significant change of the membrane polarity after the feed start; instead the depolarization level remains constant along the fermentation. However, an increase of the portion of BOX-stained cells is observed at substrate excess in the PFR module (Figure 8).

The respiratory capacity is also a key factor related to the vitality of the cells, as examined with CTC staining. As it has been observed with applying PI and SYTO13, a change after the feed start can be noticed in both cultivations. After this perturbation, the percentage of CTC stained cells increases slightly until the end. The culture exhibits a higher respiratory capacity when passing through the PFR at the Two-CR cultivation. This effect is not seen any more towards the end of the cultivation. In comparison to the observation in the Two-CR cultivation, the portion of CTC stained cells in the Three-CR is lower by 10%. This difference is a consequence of the greater time cells are exposed to gradients, leading to unfavorable cultivation conditions. The greater stress conditions are leading to a reduced growth, as values of 14.0 and 13.5 $gcellL^{-1}$ are obtained in the STR and Two-CR cultivations in the first 4 hours, while 6.5 $gcellL^{-1}$ are achieved in the Three-CR in the same time.

DISCUSSION

A defined standard procedure for FCM analysis has been evaluated and optimized for the purposes of this study. Starting with the pre-preparation of the sample, centrifugation is the method of choice for cell harvest before staining (Looser, Hammes et al. 2005, Lopez-Amoros), (Castel et al. 1997, Herrera, Martinez et al. 2002). The authors have used different procedures concerning repetitions, speed and time. However, not always a washing step is performed (Hewitt and Nebe-Von-Caron 2004). As it has been already pointed out for the CTC and SYTO13 staining procedures, the lower the time for treatment, the higher will be the viability of the culture. Hence, washing is assumed to be necessary to obtain sufficiently reliable results. In this attempt, the vacuum filtration procedure possesses advantages not only in terms of viability and vitality, but also in terms of reproducibility. PI can only enter cells where the membrane integrity (permeability) is lost. In previous studies, only 4% of the *E.coli* cells stained with PI were able to grow, proving that cells stained with PI are non-viable cells (Khan, Pyle et al. 2010). Meanwhile the cells, which were not stained with the dye, possess a growth capacity of 96%, meaning that 96% of the cells can be cultivated thereafter. However, in order to get specific staining, the minimal necessary concentration of the dye has to be used.

SYTO13 is a cell permeable dye and thus, it is used for the purpose of staining the overall cell population or the viable cells. In terms of SYTO13 staining, a correlation between the counts obtained with SYTO13 and with DAPI staining,

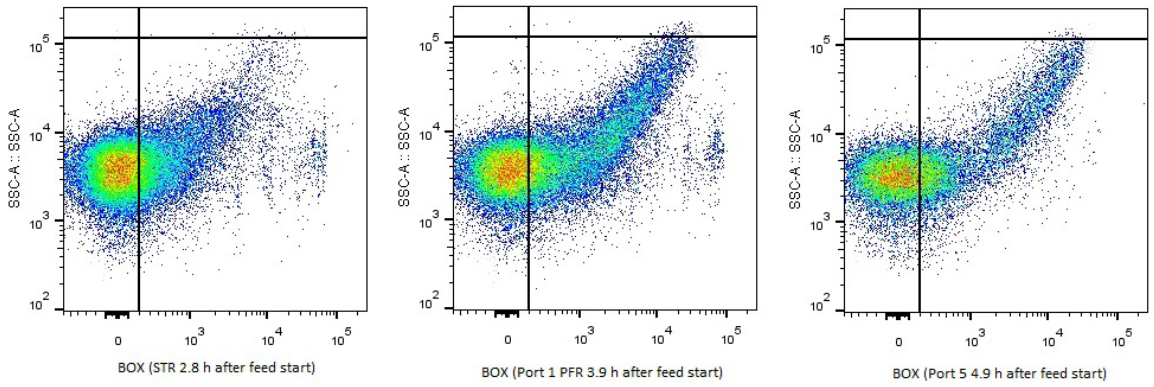

Figure 8: *Flow cytometry analysis of BOX stained E.coli samples from the STR and PFR (Port 1 and 5) during the Three-CR cultivation.*

was found (Troussellier, Courties et al. 1999). However, it has to be pointed out that, although several authors have used SYTO13 in combination with PI in order to subtract the number of PI-stained cells from the SYTO13-stained cells (Comas and Vives-Rego 1997), (Khan, Pyle et al. 2010), no quantitative correlation between PI and SYTO13 can be found in our experiments. There was only a qualitative relation. Thus, SYTO13 staining has been used to determine the percentage of viable cells within a culture. The non-viable cells show a more intensive staining of SYTO13 than the viable ones, so it seems that the membrane structure represents a barrier for the dye to diffuse into the cell. SYTO13 passes the cell through passive diffusion, so a more damaged membrane offer less resistance to this dye.

By staining cells with CTC, the respiratory capacity of the cells is estimated since CTC becomes fluorescent when it is reduced via the electron transport activity. Several authors have considered that CTC is toxic for cells, since it interferes with the electron transport system (Ullrich, Karrasch et al. 1996), (Lebaron, Servais et al. 2001). However, during the staining optimization with titration, no toxicity has been observed as other studies also have evidenced (Gasol and Aristegui 2007), (Del Giorgio, Prairie et al. 1997), (Sieracki, Cucci et al. 1999).

At all applied methods, the internal deviation of staining (PI ±0.3%, SYTO13 ±1%, BOX 1.3% and CTC 1.8%) is lower than the differences observed at samples taken during the course of the cultivation (Figure 7), thereby providing results that indicate relevant changes of the physiology.

Several studies have used scale-down bioreactors similar to the Two-CR used in this study in order to mimic the poorly mixed conditions found in a large-scale bioreactor (Hewitt and Nebe-Von-Caron 2001; Onyeaka, Nienow et al. 2003). This literature is in accordance with the results obtained in this study. Although slightly lower growth rates are obtained in the Two-CR, higher cell viability is observed than at homogeneous conditions in the STR cultivation.

In contrast to the Two-CR scale-down approach, in the newly designed Three-CR scale-down approach, the viability measured was lower than in the STR cultivation. It has been observed that starvation and other stresses induce cells to change to non-culturable states, as it is the case for *Lactococcuslactis* (Ganesan, Stuart et al. 2007) or *E.coli* (Dinu and Bach 2011). However, this state does not necessarily represent a loss of cell viability, sometimes they can even increase the resistance to other stresses like to low salinity inactivation(Wong and Wang 2004).

The change of the design of the scale-down approach leads to a considerable change in the viability of the cells. The FCM analysis provides a proof that the extent, to which cells are confronted with oscillatory conditions, matters. This leads to the necessity that the scale-down conditions have to reflect the conditions in an industrial scale reactor very precisely. This reflects a demanding challenge, since the design of industrial bioreactors varies and data are not easy to collect from industrial operators. However, the currently applied scale-down approaches are probably too broad to cover main aspects. Therefore, the consideration of population heterogeneity, and in general viability and vitality, should be considered as scale-down criterion, since a similar development of these parameters might guarantee a good transfer of conditions from one scale to the other.

CONCLUSION

A flow cytometry protocol has been optimized for staining a bacterial population in order to study the viability and vitality of *E .coli* cells. The most suitable staining conditions and sample pre-preparation methods were identified. Shifts of viability and vitality have been observed at scale-down conditions: an increased viability of cells was measured in the Two-CR cultivation in comparison to the STR cultivation (homogeneous conditions). Results from FCM show that there is a higher portion of affected cells, and a longer phase of adaptation in the Three-CR in comparison to the Two-CR cultivation. Further studies are ongoing to elucidate the regulatory response to the increased gradients. The next step for an improved understanding is the online single-cell analysis, achieving data about vitality and viability of the culture in real time. This information might be used as a mean of increasing the overall process efficiency and scale-down accuracy.

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