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Research Article

Green fluorescent protein (GFP) leakage from microbial biosensors provides useful information for the evaluation of the scale-down effect

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Mixing deficiencies can be potentially detected by the use of a dedicated whole cell microbial biosensor. In this work, a *csiE* promoter induced under carbon-limited conditions was involved in the elaboration of such biosensor. The *cisE* biosensor exhibited interesting response after up and down-shift of the dilution rate in chemostat mode. Glucose limitation was accompanied by green fluorescent protein (GFP) leakage to the extracellular medium. In order to test the responsiveness of microbial biosensors to substrate fluctuations in large-scale, a scale-down reactor (SDR) experiment was performed. The glucose fluctuations were characterized at the single cell level and tend to decrease the induction of GFP. Simulations run on the basis of a stochastic hydrodynamic model have shown the variability and the frequencies at which biosensors are exposed to glucose gradient in the SDR. GFP leakage was observed to a great extent in the case of a culture operated in well-mixed fed-batch mode, by comparison with those operated in SDR. GFP leakage seems to be correlated to a higher membrane permeability, confirming previous studies highlighting a better cell viability in cultures operated in a fluctuating environment. Our results suggest that GFP leakage could be used in parallel to the normal GFP biosensor function in order to assess microbial viability in process conditions.

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1 Introduction

In previous works, the dynamics of GFP synthesis from several microbial biosensors has been evaluated in different scale-down bioreactor configurations [1, 2]. These studies involved the use of stress responsive promoters coupled to the GFP coding sequence in order to allow an easy detection of the fluorescent signal. In this context, we have used semi-specific whole cell microbial biosensors involving stress promoters responsive to carbon limitation. The term semi-specific means that the promoter can be induced by a set of environmental conditions [3], i.e. in our case such conditions leading to the induction of a stress regulon involving several interconnected genes. The induction of such stress regulon can have severe final consequences at the level of the bioprocess, such as a significant reduction of the growth rate. Since these

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Abbreviations: FSC, forward scatter channel of flow cytometer; **GFP,** green fluorescent protein; **PI,** propidium iodide; **RTD,** residence time distribution; **SDR,** scale-down reactor; **SSC,** side scatter channel of flow cytometer

conditions are usually strongly interrelated and met in fed-batch bioreactor, GFP is actively synthesized in normal cultivation conditions and is deactivated when biosensors are exposed to substrate heterogeneities. In this work, we used the carbon starvation-induced *csiE* promoter, which exhibits a significantly higher level of GFP expression than the previously used *rpoS* promoter [1]. This *csiE* promoter is subjected to the control of the *rpoS* sigma factor and is induced when cells encounter carbon limitation. Considering its location far below the global regulatory level (on the opposite of the *rpoS* promoter which is a global regulator located at the top of the transcriptional network [4]), we expect more specificity from this kind of promoter. This principle can thus be used to detect bioreactor mixing imperfections at the single cell level. However, during cultivations, significant release of GFP to the extracellular medium can be observed [5]. This phenomenon is not well documented in the literature in relation with bioprocess performances. It is known that about 30% of the proteome of Gram negative bacteria is exported to the extracellular medium, this fraction being described as the secretome [6]. The secretome fraction of *Escherichia coli* comes from the specific leakage (and not from membrane lysis) of proteins which are initially located in the outer membrane compartment [7]. Protein leakage can be correlated to the occurrence of stress and/or changes in growth conditions. There is indeed a strong correlation between growth rate, membrane stress and protein leakage [8—10]. More recently, a series of cultivation tests carried out in intensive fed-batch bioreactor has allowed the validation of the periplasmic origin of the secretome of *E. coli* and the fact that this secretome is modulated in function of cell density and growth rate [11]. The purpose of this work is to determine if this release can be correlated to the extracellular fluctuations met in the scale-down bioreactors. At this level, relationship between GFP leakage and membrane permeability would be a very interesting parameter to investigate, considering the fact that membrane integrity is one of the most used parameter to assess microbial viability [12]. If the relationship with GFP leakage can be established, this information could be potentially used to expand to the use of GFP microbial biosensors for viability assessment. As stated before, the proteome of *E. coli*, as well as its secretome fraction has been relatively well characterized in the literature [13], but only a few studies deal with the release of protein in process-related conditions. Only a single study involving the characterization of the *E. coli* secretome in high cell density culture with control of the main environmental variables (i.e., dissolved oxygen, glucose level, pH and temperature) has been performed so far [11]. However, this study has been conducted in a lab-scale, well-mixed bioreactor. In industrial bioreactors, considering the drop at the level of the mixing efficiency, microbial cells are exposed to fluctuations at the level of the main environmental variables [14, 15]. It could be thus interesting to characterize the secretome fraction affected by these fluctuations since some studies suggest a strong modulation of the secretome in function of the environmental conditions [16]. In this work, we have followed the leakage of GFP when microbial biosensors are exposed to changes in environmental conditions in chemostat and in fed-batch bioreactors operated in normal mode as well as in scale-down mode.

2 Material and methods

2.1 Strains and medium

E. coli K12 MG1655 bearing a pMS201 (4260 bp) plasmid with a stress promoter and a kanamycin resistance gene. The strain is provided from a cloning vector library elaborated at the Weizmann Institute of Science [17]. The carbon starvation induced promoter *csiE* has been chosen as a biosensor of carbon limitation in our different bioreactor experiments. Microbial biosensors are maintained at –80°C in working seeds vials (2 mL) in solution with LB media and with 40% of glycerol. Precultures and cultures have been performed on a defined mineral salt medium containing (in g/L): K_2HPO_4 14.6, $NaH_2PO_4 \cdot 2H_2O$ 3.6; Na_2SO_4 2; $(NH_4)_2SO_4$ 2.47, NH_4Cl 0.5, $(NH_4)_2$-H-citrate 1, glucose 5, thiamine 0.01, kanamycin 0.1. Thiamin and kanamycin are sterilized by filtration (0.2 μm). The medium is supplemented with 3 mL/L of trace elements solution, 3 mL/L of a $FeCl_3 \cdot \cdot 6H_2O$ solution (16.7 g/L), 3 mL/L of an EDTA solution (20.1 g/L) and 2 mL/L of a $MgSO_4$ solution (120 g/L). The trace elements solution contains (in g/L): $CoCl_2 \cdot H_2O$ 0.74, $ZnSO_4 \cdot 7H_2O$ 0.18, $MnSO_4 \cdot H_2O$ 0.1, $CuSO_4 \cdot 5H_2O$, $CoSO_4 \cdot \cdot 7H_2O$. Before each bioreactor cultivation experiment, a precultivation step is performed in 100 mL of the above-mentioned medium in baffled shake flask at 37°C and under orbital shaking at 160 rounds per minute. Cell growth has been monitored by OD at a wavelength of 600 nm. Cell dry weight has been determined on the basis of filtered samples (0.45 μm) dried during 24 h at 105°C. Glucose concentration has been monitored by an electro-enzymatic system YSI.

Biotechnol. J. 2011, 6, 968–978

2.2 Bioreactor configurations: Fed-batch, SDR and continuous cultures

Microbial GFP biosensors was cultivated in a lab-scale stirred bioreactors (Biostat B-Twin, Sartorius) operated in fed-batch mode (total volume: 3 L; initial working volume: 1 L; final working volume: 1.5 L; mixing provided by a standard RTD6 rushton turbine). The bioreactor platform comprises two cultivation vessels in parallel monitored and controlled by the same control unit (remote control by the MFCS/win 3.0 software). For each reporter strains, experiments have been conducted in parallel by considering a culture performed in the classical stirred vessel and another one conducted with the stirred vessel connected to a recycle loop. This last apparatus correspond to a scale-down strategy allowing to reproduce heterogeneities expected in large-scale bioreactors [14, 18, 19]. The SDR arrangement is based on the previously described stirred bioreactor connected to a recycle loop (silicon pipe; diameter 0.005 m; length 12 m). A continuous recirculation of the broth between the stirred reactor and the recycle loop is ensured by a peristaltic pump (Watson Marlow 323) with the glucose feed solution being added at the inlet of the recycle loop in order to generate a concentration gradient. Fed-batch is controlled on the basis of dissolved oxygen (setpoint: 30% above saturation). The dissolved oxygen in the recycle loop is monitored by a set of sterilizable optical probes (Flow-through cell, Presens). The sensor spot inserted in the flow-through cell contains a fluorogenic compound that is excited at a wavelength of 540 nm. The emission signal can then be recorded at 640 nm. The dissolved oxygen measurement is based on the properties that molecular oxygen is able to absorb a part of the emission energy. The relationship between dissolved oxygen and fluorescence intensity is nonlinear and can be expressed by the Stern-Volmer equation [20]. The excitation and emission signals are generated/recorded at the level of a miniaturized set of excitation led and photomultiplier. The fluorescence signal coming from planar sensors is then processed and recorded at the level of an oxy-4 mini transmitter. During the experiments, pH was maintained at 6.9 (regulation by ammonia and phosphoric acid) and temperature at 37°C. Stirrer rate is maintained at 1000 rpm with a RDT6 impeller and air flow rate is set to 1 L/min at the beginning of the culture. Once fed-batch phase is started, the agitation rate and the air flow rate are progressively raised to 1300 rpm and 2 L/min, respectively. Culture is fed with 500 mL of a solution containing 400 g/L of glucose diluted in mineral medium (see above for composition). Continuous cultures have also been performed on the basis of the same stirred bioreactor with the same parameters, unless the working volume which is adjusted to 500 mL. In this case, culture medium is added continuously and spent medium is withdrawn in order to keep a constant volume. The feed rate of medium is modulated in order to reach dilution rate D within the range of 0.02 and 0.2 h^{-1}.

2.3 Flow cytometry

The analysis of the GFP expression level has been performed by fluorescence activated cell sorting (FACS) on a FACScan (Becton Dickinson) flow cytometer. Samples are taken directly from the reactor and are diluted in 900 µL of PBS and 100 µL of a chloramphenicol solution (50 µg/mL) in order to stop protein synthesis. For each measurement, 30 000 cells are analyzed. GFP is excited at 488 nm and emission signals are collected by using filters at 530 nm. The *gfpmut2* variant has been especially engineered to optimally match the excitation/emission range of the FACS instrument [21]. Considering that bacterial cells exhibit a high side scatter (SSC) signal [22], a threshold of 52 has been set up on the SSC channel in order to limit noise signal. The forward scatter channel of flow cytometer (FSC), FL1, FL2 and FL3 channels are logarithmically amplified with the following settings: FSC E00, FL1 620, FL2 420, FL3 420. The results have been analyzed by the FlowJo version 7.6.1 software. Flow cytometry has also been used in order to determine the residence time distribution inside the recycle loop of the SDRs and the membrane permeability of the cells (see above).

2.4 Determination of the residence time distribution inside the recycle loop of the SDRs and mathematical model for the characterization of the substrate profiles experienced at the single cell level

Fluorescent microspheres (Fluosphere 1 µm, density: 1060 g/L, molecular probes, Invitrogen) have been used as representative tracer for the determination of the residence time distribution of the microbial cells inside the recycle loop of the SDRs. Indeed, tracer test involving particulate dye instead of soluble dye has been recently recognized as more relevant to describe the transport of microbial cells [23]. We have also used this method previously for the characterization of the transport of fluorescently labeled microbial cells in SDRs [24]. Our methodology has been improved in the present work, mainly at the level of the method used to detect fluorescent particles. A tracer pulse of 1 mL

containing 10^9 fluorescent beads has been injected at the inlet of the recycle loop. Samples of 3 mL are taken at different time intervals at the outlet of the recycle loop. Samples are analyzed by flow cytometry. Beads are easily detected according to their high green fluorescent level (FL1 detection). The analysis is performed for 30 s and the number of events recorded during this period is used as a measure of the beads concentration. The number of events is gated on the basis of the FL1 parameter in order to make the distinction between fluorescent beads and background (software analysis performed on FlowJo 7.6.1.). The residence time distribution (RTD) curves are processed with MatLab to determine the following parameters:

$$t_R = \frac{\sum_i^{n-i} t_i C_i \Delta t_i}{\sum_i^{n-i} C_i \Delta t_i} \quad (1)$$

$$\sigma^2 = \frac{\sum_i^{n-i} t_i^2 C_i \Delta t_i}{\sum_i^{n-i} C_i \Delta t_i} - t_R^2 \quad (2)$$

With t_R being the mean residence time of the RTD (s); C_i the number of beads detected during the time interval t_i and σ^2 the variance of the RTD (s^2). In the case of our SDR, the following parameters have been determined: t_R = 79.8 s and σ^2 = 120.7 s^2.

These data have been exploited in order to elaborate a hydrodynamic model able to simulate the glucose gradient inside the SDR as well as the exposure of single microbial biosensor to these gradients. The most critical parameter that has to be taken into account in order to simulate efficiently glucose gradients generated in the SDR is the pulse frequency of the feed pump. In general, different categories of behavior can be depicted in function of the relative importance of the feed pump frequency and the mixing time of the SDR or the residence time inside the recycle loop [25]. For a time interval between two pulses (T_{pulse}) smaller than the global mixing time of the system, the mean concentrations experienced by the microorganisms are very low. This is due to the fact that, between two pulses, the concentration gradient at the level of the mixed part of the SDR disappears. For T_{pulse} approximatively equal or superior to the mixing time of the SDR, concentration gradient is maintained. This concentration gradient persistence leads to the increase of the mean concentration experienced by the microorganisms, as well as an increase of the variance of the frequency distribution. In order to take into account this very important source of glucose level variation, experimental data of the feed pump have been directly extracted from the bioreactor control unit (MFCS/win 3.0 software), processed with MatLab and added to the hydrodynamic model. Indeed, the glucose addition frequency is governed by a regulation loop involving the dissolved oxygen signal, i.e. when the DO signal raise above 30% from saturation, glucose is depleted and the feed pump is thus activated. On the opposite, when DO signal drops below 30% from saturation, glucose is accumulating in the broth and the oxygen transfer efficiency of the bioreactor is overwhelmed by the biological oxygen requirement, the feed pump is thus deactivated. At the level of the hydrodynamic model, the stirred part of the SDR is conceptualized as a single perfectly mixed reactor, whereas the recycle loop exhibits a strong plug flow behavior and must then be modeled as a series of n = 90 perfectly mixed reactor. The number n = 90 of perfectly mixed compartments has been adjusted on the basis of a RTD parameters measured previously (i.e., t_R and σ^2).

The compartimentalization of the SDR has been performed according to the following mathematical formulation. For the stirred part of the SDR

$$V_{\mathrm{M}} \frac{\mathrm{d}C_{\mathrm{M}}}{\mathrm{d}t} = qC_{\mathrm{T6}} - qC_{\mathrm{M}} \quad (3)$$

And for the recycle loop:

$$V_{\mathrm{T1}} \frac{\mathrm{d}C_{\mathrm{T1}}}{\mathrm{d}t} = qC_{\mathrm{M}} - qC_{\mathrm{T1}} \quad (4)$$

$$V_{\mathrm{T2}} \frac{\mathrm{d}C_{\mathrm{T2}}}{\mathrm{d}t} = qC_{\mathrm{T1}} - qC_{\mathrm{T2}} \quad (5)$$

$$V_{\mathrm{Tn}} \frac{\mathrm{d}C_{\mathrm{Tn}}}{\mathrm{d}t} = qC_{\mathrm{Tn-1}} - qC_{\mathrm{Tn}} \quad (6)$$

Leading to in matrix form:

$$\mathrm{d}\begin{bmatrix} C_M \\ C_{T1} \\ C_{T2} \\ . \\ . \\ . \\ C_{Tn} \end{bmatrix} / \mathrm{d}t =$$

$$\begin{bmatrix} -q/V_m & 0 & 0 & 0 & 0 & 0 & q/V_m \\ q/V_{T1} & -q/V_{T1} & 0 & 0 & 0 & 0 & \\ 0 & q/V_{T2} & -q/V_{T2} & 0 & 0 & 0 & \\ 0 & 0 & . & . & 0 & 0 & 0 \\ 0 & 0 & 0 & . & . & 0 & 0 \\ 0 & 0 & 0 & 0 & . & . & 0 \\ 0 & 0 & 0 & 0 & 0 & q/V_{Tn} & -q/V_{Tn} \end{bmatrix} . \begin{bmatrix} C_M \\ C_{T1} \\ C_{T2} \\ . \\ . \\ . \\ C_{Tn} \end{bmatrix} \quad (7)$$

With C being the concentration of the species in the different compartments (g/L), index M referring to the mixed compartment and index T referring to the tubular recycle loop, V being the volume of the compartment (m^3) and q being the liquid flow rate between compartment (m^3/s). This system of equation (Eq. 7) can be resolved numerically in order to simulate, for example, the dynamics of homogenization of a substance in the SDR. In our case, we will use the model is order to simulate the glucose concentration gradient experienced by microbial cells during a fed-batch reaction performed in the SDR. In order to simulate this process, additional equations, representing the glucose addition and consumption and biomass formation must be considered. The displacement of microbial cells in the environment and in engineered systems such as a bioreactor can be represented roughly by convection and/or diffusion-based equations, according to the nature of the convoying medium. However, one of the biggest advantages of using whole cell biosensors is their ability to detect stress at the micrometer scale. If this kind of data has to be interpreted, the environmental fluctuations have to be described at the single cell level and the random component linked with the displacement of the cell has to be taken into account. In the case of bioreactor operations, such displacement can be described by a stochastic version of the hydrodynamic model presented previously. Roughly, the same equations are kept but the algorithm used to perform the simulation is quite different since the stochastic component of the process has to be added to the problem. The basic principle is to use the same model structure as used previously for the simulation of glucose gradient by integrating a random component. The matrix M containing the exchange flow rate (m^3/s) of the model will be assimilated as the generator matrix Q of the stochastic version of the model [26]:

$$M = Q = \begin{bmatrix} -q_{ii} & q_{ij} & 0 & 0 & 0 & 0 \\ q_{ij} & . & . & 0 & 0 & 0 \\ 0 & . & . & . & 0 & 0 \\ 0 & 0 & . & . & . & 0 \\ 0 & 0 & 0 & . & . & . \\ 0 & 0 & 0 & 0 & . & . \end{bmatrix} \quad (8)$$

Physically, Q contains the exchange rates (s^{-1}) between fluid zones. The main diagonal of Q contains the escape rate from the ith fluid zones and is described by the vector λ:

$$\lambda = \begin{bmatrix} q_{ii} \\ . \\ . \\ . \\ . \\ \\ \end{bmatrix} \quad (9)$$

Considering mass balance

$$q_{ii} = -\sum q_{ij} \quad (10)$$

This equation leads to the fact that the sum of each line of matrix Q (Eq. 8) equals zero. This property can be used to verify mass balance of the system. Value of vector λ (Eq. 9) can be used to compute the residence time distribution for a given fluid zone. This vector is then usually called sojourn time vector. For example, in the case of a fluid zone i:

$$P(x_i) = q_{ii} e^{-q_{ii} x_i} \quad (11)$$

The outlet flow rates λ_i will be used to define the occurrence of an event (i.e., in our case the displacement of a microbial biosensor from a fluid zone to another). The times at which events occur are determined from an exponential distribution:

$$T_i = -\frac{\ln(r)}{\lambda_i} \quad (12)$$

For which r is a random number determined from a uniform distribution on the interval [0 1]. When an event is determined, the displacement of the microbial biosensor, i.e. the coordinates of the incoming fluid zone, is determined from the generator matrix Q (Eq. 8):

$$P = \frac{Q}{\lambda} + \begin{bmatrix} 1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 & 0 \\ 0 & 0 & . & 0 & 0 & 0 \\ 0 & 0 & 0 & . & 0 & 0 \\ 0 & 0 & 0 & 0 & . & 0 \\ 0 & 0 & 0 & 0 & 0 & 1 \end{bmatrix} \quad (13)$$

In the algorithm, the occurrence of an event is determined by generating a random number and by comparing the obtained value with the probability value contained in the ith line of the P matrix (Eq. 13). The stochastic simulation can be then superimposed to the deterministic simulation of the glucose gradient field in the SDR, leading to the glucose concentration profile experienced at the level of a single biosensor cell.

2.5 Supernatant analysis: Fluorescence, SDS-page and western blot

Samples coming from bioreactor are centrifuged at 12 000 rpm for 3 min and filtered on 0.2 μm cellulose membrane in order to remove the cells. Fluorescence of the supernatant (samples of 200 μL on 96 wells black microtiter plate) is analyzed by spectrofluorimetry (Victor^3 V Wallac, Perkin Elmer). Proteins coming from the supernatant (20 μL) are separated on 30% polyacrylamide gels (Biorad). Immunoblot is performed in order to detect the band corresponding to GFP (ECL plus detection system, Amersham). Detailed procedures can be found in our previous work [5].

2.6 Membrane permeability

Samples taken from bioreactor are diluted in PBS in order to reach an OD of 4. Cells are stained by adding 10 μL of propidium iodide solution (PI) for 15 min at 37°C. After staining, cells are washed twice in order to remove excess PI by alternating centrifugation and resuspension in PBS. Samples are then analyzed by flow cytometry with the following settings: FSC E00, FL1 620, FL2 420, FL3 520. Heat stressed cells have been obtained by exposing 1 mL of cell suspension in PBS (at an OD of 4) to 60°C for 30 min. Full procedures for the analysis of PI stained cells by flow cytometry are detailed in the Supporting information online.

3 Results and discussion

3.1 Dynamics of *csiE* GFP microbial biosensor in chemostat conditions

In order to assess the responsiveness of the microbial biosensor, a culture in chemostat mode has been performed by considering a sharp variation at the level of the dilution rate. Indeed, the *csiE* biosensor is supposed to be induced upon carbon limitation, a condition that can be easily implemented in a glucose-limited chemostat (i.e., constant cell density and growth rate with constant environmental variables such as pH and dissolved oxygen). The first phase that has been followed corresponds to the transition of the batch phase to the continuous mode at a dilution rate $D = 0.02\ h^{-1}$ (Fig. 1). During the batch phase, *cisE* promoter is not active considering that cells are growing at their maximum rate, substrate being in large excess. Consequently, global GFP-related fluorescence remains at the basal level (Fig. 1A). Flow cytometry allows the measurement of GFP content at the single cell level. The specific fluorescence recorded by this way (i.e., the ratio of GFP fluorescence on the amount of microbial cells) tends to decrease with time, considering that no new GFP molecules are synthesized and that background GFP is diluted by cell division (Fig. 1B). As expected, *csiE* promoter is induced at the end of the batch phase according to the elevation of the GFP-relat-

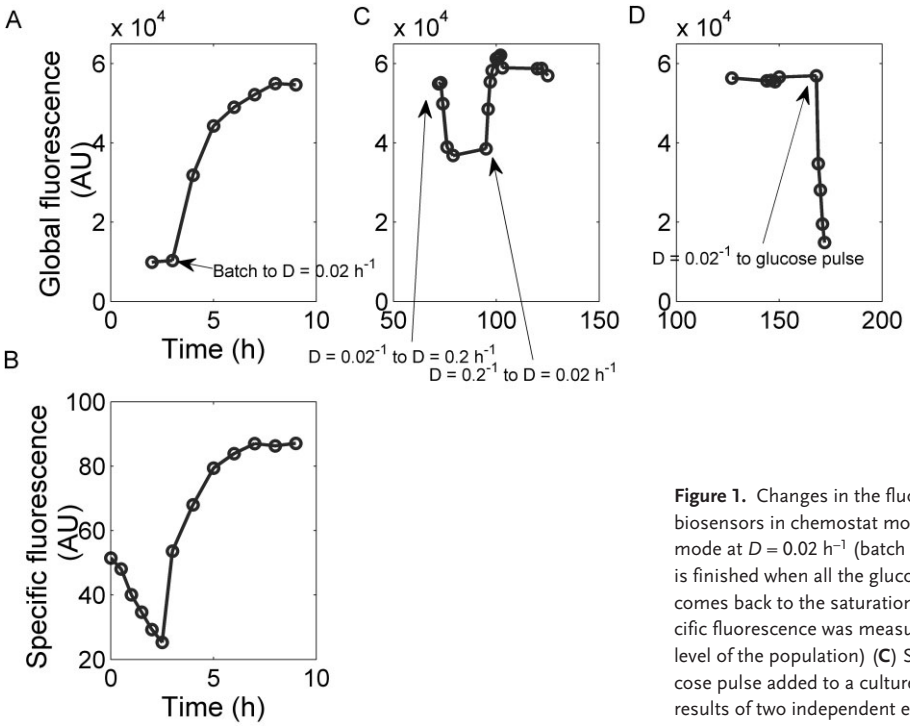

Figure 1. Changes in the fluorescence level during culture of the *csiE* biosensors in chemostat mode. (**A**) Shift from batch phase to chemostat mode at $D = 0.02\ h^{-1}$ (batch phase was started with 5 g/L of glucose and is finished when all the glucose is consumed and when dissolved oxygen comes back to the saturation level). (**B**) Same experiments as **A**, but specific fluorescence was measured by flow cytometry (mean fluorescence level of the population) (**C**) Shift from $D = 0.02$ to $D = 0.2\ h^{-1}$. (**D**): Glucose pulse added to a culture stabilized at $D = 0.02\ h^{-1}$ (representative results of two independent experiments).

ed fluorescence. Fluorescence reaches a constant level after 5 h of culture in chemostat at $D = 0.02\ h^{-1}$. In chemostat conditions, global GFP-related fluorescence and specific GFP are the same since cell density remains constant during the culture. The second phase that has been investigated is the evolution of fluorescence upon the shift of dilution rate from 0.02 to 0.2 h^{-1}. As expected, fluorescence intensity drops when D is modified. The fluorescence level reached at steady state is then lower than that obtained in the case of $D = 0.02\ h^{-1}$, but is significantly higher that the initial level, suggesting that the *csiE* promoter is always active, but to a lower extent, at $D = 0.2\ h^{-1}$. The microbial biosensor seems thus to exhibit a response proportional to the degree of substrate limitation in the reactor. The phenomenon is reversible when D is readjusted to 0.02 h^{-1}. The third phase (Fig. 1D) consists to follow the reaction of the biosensor after the injection of a glucose pulse to reach a final concentration of 5 g/L in the reactor. After the pulse, fluorescence drops rapidly to its initial level corresponding to a culture growing at its maximal growth rate in the presence of an excess of substrate. The *csiE* biosensor responds thus quite well to glucose limitation and could be used to detect local heterogeneities in large-scale or scale-down bioreactors. However, it must be taken into account that experiments have been carried out in constant environmental conditions with abrupt shift between dilution rates. In large-scale bioreactors, the frequency and the amplitude of the environmental fluctuations are by far higher [27] and the biosensor has thus to be tested in order to assess its performance in fluctuating environment. For this purpose, a SDR able to reproduce such environmental fluctuations will be used in a next section. Before this step, GFP leakage to the extracellular medium during upshift and down shift of dilution rates will be investigated.

3.2 Glucose limitation tends to increase GFP leakage when steady-state is reached

Western blot analyses of the supernatant taken at different time of the chemostat cultivation have been carried out (Fig. 2). A thin GFP band at 27 kDa has been detected at the end of the batch phase (3 h). When the bioprocess is shifted to chemostat mode at a dilution rate of 0.02 h^{-1}, this band disappears. However, when steady-state is reached GFP can be clearly detected in the supernatant. When dilution rate is shifted, GFP intensity in the supernatant tends to initially decrease when environmental conditions are perturbed. Once a new steady-state is established after the initial perturbations, GFP leakage can be detected again (Figs. 2B and C). These results suggest that GFP is excreted to the extracellular medium when microbial biosensors are exposed to glucose limitation, whereas leakage is stopped (or proteins are consumed) when they are exposed to environmental fluctuations. A second band at approximately 60 kDa has been detected by western blot. This band corresponds to a GFP dimer [28] and is only detected after prolonged cultivation at constant dilution rate. Membrane integrity has been determined in parallel on the basis of the permeability of PI. Flow cytometry reveals that microbial biosensors exhibit an intermediate permeability stage between healthy cells and heat damaged cells (figures showing flow cytograms are shown in the Supporting information online). This observation has been confirmed by observation by fluorescent microscopy where cells do not show any visible staining with PI, by comparison with the heat stressed standard. It seems also that membrane permeability of cell population is modulated and tends to be higher in the case of prolonged cultivation in chemostat mode, which correlated to the intensity of GFP leakage.

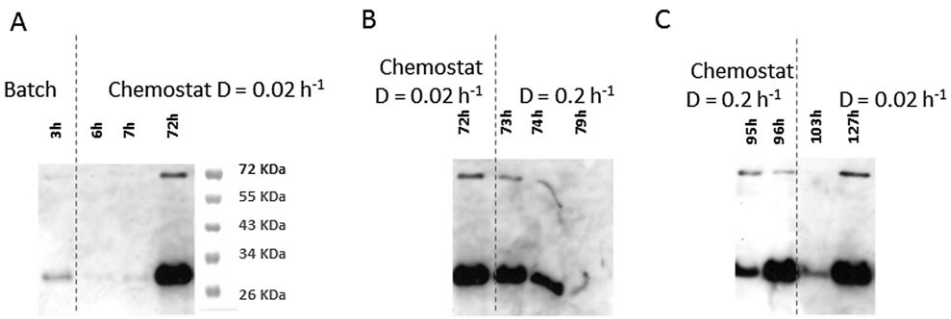

Figure 2. Immunoblot analysis of the supernatant for denatured GFP during cultivation of the csiE biosensor in batch and chemostat mode. (**A**) shift from batch phase to chemostat mode at $D = 0.02\ h^{-1}$ (batch phase is started with 5 g/L of glucose and is finished when all the glucose is consumed and when dissolved oxygen comes back to the saturation level). (**B**) Shift from $D = 0.02$ to $D = 0.2\ h^{-1}$. (**C**) Shift from $D = 0.2$ to $D = 0.02\ h^{-1}$. Denatured GFP can be easily visualized at a band corresponding to 27 kDa (representative results of two independent experiments).

3.3 Effect of scale-down conditions on the *csiE* promoter activity

The extracellular fluctuations are more complex in the case of cultures operated in fed-batch mode and in scaled-down conditions. Indeed, the extracellular environment of the biosensor depends on the global mixing efficiency of the system as well as the residence time in the recycle loop. In order to get a better insight at this level, a stochastic hydrodynamic model has been used to simulate glucose concentration profiles experienced at the single cell level (Fig. 3). This model has been validated by RTD measurement performed on the basis of 1 μm fluorescent beads and by incorporating the experimental pulse frequency of the glucose feed pump (for additional informations, see material and methods section). Simulations show that, considering our operating conditions, microbial biosensors are exposed to strong glucose fluctuations when crossing the recycle loop of the SDR. Electroenzymatic analysis performed at the different locations of the SDR and at different cultivation times highlights a glucose variation ranging between 0.01 and 0.25 g/L (results not shown) which is in good accordance with the simulated glucose concentration profile perceived at the single cell level. These results are of importance because the simulated glucose levels are sufficient to induce overflow metabolism resulting in lower biomass yield [29]. However, this simulation (Fig. 3) is only representative of the glucose profile experienced by a single microbial biosensor. According to the fact that microbial biosensors displacement is stochastic and depends on the respective residence time inside the stirred part and the recycle loop of the SDR, each biosensor exhibits its own glucose concentration profiles. Simulation shows that the passage frequency of microbial biosensor varies in the range of one to several hundred seconds. Considering that oxygen is required for GFP maturation [30], dissolved oxygen has been measured at two points of the recycle loop during the culture by a set of optical probes (results not shown). Oxygen limitation is observed at the outlet of the recycle loop for the whole duration of the culture. However, considering the fact that GFP maturation time (about 4 min. in the case of the *gfpmut2* variant [21]) is significantly higher than the mean residence time in the recycle loop, oxygen limitation is expected to have no impact on GFP folding. This process seems to have not impact at the level of the maximum GFP expression level, as shown by the flow cytometry diagrams recorded for the different cultivation tests (Fig. 4A). However, the maximal level for GFP induction has been noticed after 14 h of culture in normal mode, whereas the same process take 23 h in the case of the SDR, suggesting that the exposure of microbial biosensors to glucose excess in the recycle loop tends to diminish *csiE* promoter activation. Another mechanisms possibly involved are the leakage of GFP to the extracellular medium. This hypothesis

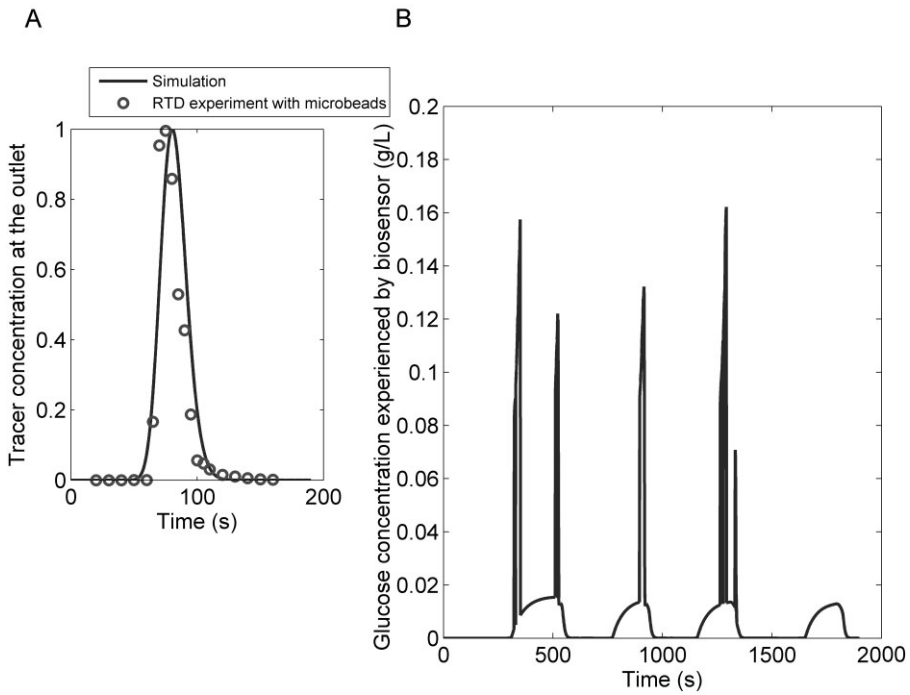

Figure 3. Simulation of the SDR hydrodynamics. (**A**) RTD inside the recycle loop. (**B**) Glucose concentration profile experienced at the single cell level using a stochastic formulation of the model.

Biotechnol. J. 2011, 6, 968–978

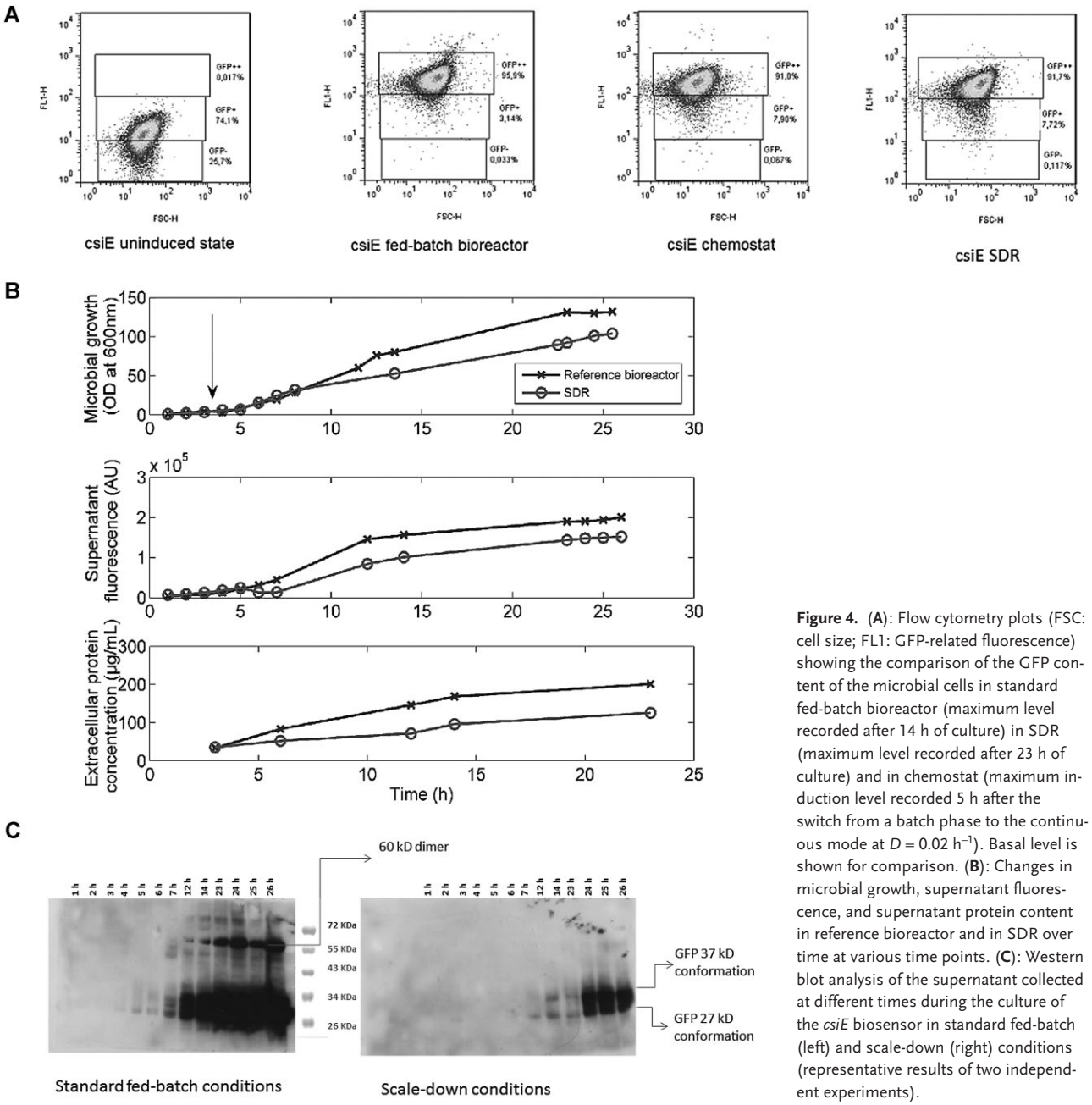

Figure 4. (**A**): Flow cytometry plots (FSC: cell size; FL1: GFP-related fluorescence) showing the comparison of the GFP content of the microbial cells in standard fed-batch bioreactor (maximum level recorded after 14 h of culture) in SDR (maximum level recorded after 23 h of culture) and in chemostat (maximum induction level recorded 5 h after the switch from a batch phase to the continuous mode at $D = 0.02\ h^{-1}$). Basal level is shown for comparison. (**B**): Changes in microbial growth, supernatant fluorescence, and supernatant protein content in reference bioreactor and in SDR over time at various time points. (**C**): Western blot analysis of the supernatant collected at different times during the culture of the *csiE* biosensor in standard fed-batch (left) and scale-down (right) conditions (representative results of two independent experiments).

is not valid since the amount of protein excreted is significantly higher in the case of the culture operated under the normal fed batch mode (Fig. 4B). Immunoblot analysis confirms this observation for the GFP (Fig. 4C). In the case of the classical bioreactor, GFP is detected earlier after only 7 h of culture. GFP is only detected after 14 h of culture in the SDR and the amount excreted are significantly lower than in the case of the classical fed-batch bioreactor. A 37 kDa band corresponding to GFP conformation exhibiting fluorescence and a 60 kDa band, probably corresponding to a GFP dimer have been detected in great amount only in the case of the culture operated in normal mode. The presence of such form indicates the leakage of a large amount of GFP in the medium, with a fraction being not affected by the denaturing procedure preceding SDS–PAGE analysis. GFP leakage seems also to be correlated with membrane permeability, as shown by PI staining experiments (Fig. 5).

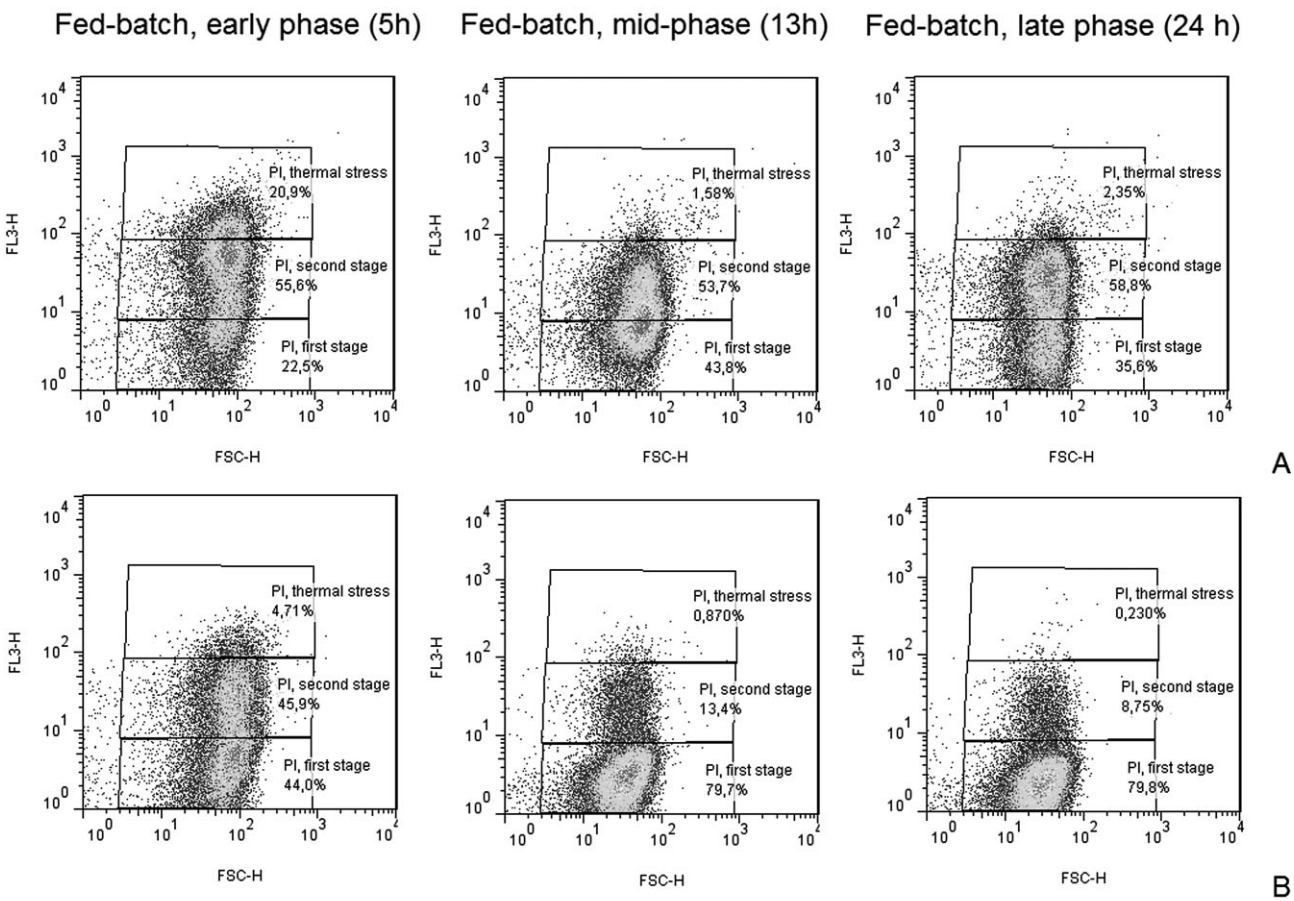

Figure 5. Changes in the membrane permeability of microbial biosensors according to permeability to PI. Red intensity related to the accumulation of PI inside cells is detected at the level of the FL3 channel. Cell size is determined on the basis of the FSC channel. (**A**) Culture operated in normal mode, (**B**) culture operated in SDR. (representative results of two independent experiments).

4 Conclusions

Detection of GFP in the extracellular medium is correlated with bioreactor operating mode. Our results suggest that the amount, but also the forms (mono or oligomeric) of the GFP in the extracellular medium are indicative of the stress encountered by microbial cells in a given bioreactor. Interestingly, membrane state seems to be better in the case of the SDR than in the case of the classical fed-batch bioreactor. This result is in good accordance with those obtained previously by Hewitt and coworkers [31] using approximately the same techniques, suggesting that in SDR biomass yield is lower but cell viability tends to be better. This statement can be confirmed by our results showing that the secretome exhibited by the *csiE* GFP biosensor exhibits a significantly lower amount of GFP, probably due to a lower membrane permeability. In the case of the classical bioreactor, GFP is detected earlier during the fed-batch phase and tends to be excreted in great amount in the medium. Our results suggest that GFP leakage could be used in parallel to the normal GFP biosensor function in order to assess microbial viability in process conditions. In addition, the results highlighted in this work are also of practical importance in the case of recombinant protein expression since our observations suggest that protein excretion is lower in scale-down conditions.

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