Direct and Indirect Use of GFP Whole Cell Biosensors for the Assessment of Bioprocess Performances: Design of Milliliter Scale-Down Bioreactors

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Substrate limitation responsive biosensors have been used for the development of a mini-bioreactor platform that can be used as a scale-down tool. Three green fluorescent protein (GFP) transcriptional reporters have been chosen in Escherichia coli*, i.e.,* uspA::gfp*,* csiE::gfp*, and* yciG::gfp*. Our previous studies have shown that these kinds of promoters are induced in response to substrate limitation and are significantly repressed when cultures are carried out in heterogeneous bioreactors. This sensitivity to substrate limitation has been confirmed in the case of the* csiE *and* yciG *biosensors. A mini-scale-down platform is proposed as a high throughput tool to rapidly investigate the usefulness of a given microbial biosensor. This platform is composed of shake flasks able to operate in fed-batch mode either using the slow release or the intermittent feeding principle. Local heterogeneities were reproduced at the level of these mini-bioreactors (operating under the intermittent feeding principle) and caused a decrease in GFP expression as in conventional scale-down reactors. The presence of GFP in supernatants was also noted and seems to be correlated with the substrate limitation signal for the three cultivation systems considered in this work (i.e., chemostat, conventional and mini-bioreactors) and with membrane permeability.* © 2012 American Institute of Chemical Engineers *Biotechnol. Prog.,* 29: 48–59, 2013
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Introduction

Recent developments in bioreactor technologies follow the scaling-out principle, i.e., carrying out several cultures in parallel with controlled conditions for screening purposes. Several mini-bioreactor concepts, i.e., bioreactors with working volumes of 1 to 100 mL with controlling devices, have been developed following this principle.[1,2] In general, chemical engineering similarities between conventional stirred bioreactors and their miniature equivalent are well characterized.[3–5] However, the actual scaling-up rules are not able to cope with the complexity of the microbial stress response. Indeed, the microbial stress response still remains incompletely understood with respect to the process perturbations and environmental fluctuations accompanying the scaling-up to industrial bioreactors.[6–8] At this time, this kind of response can only be experimentally predicted by using scale-down bioreactors, i.e. lab-scale bioreactors designed in order to reproduce mixing imperfections that have to be expected at large-scale. However, the use of such an approach is time consuming and requires highly qualified personnel to elaborate the scaling-down protocols. This work

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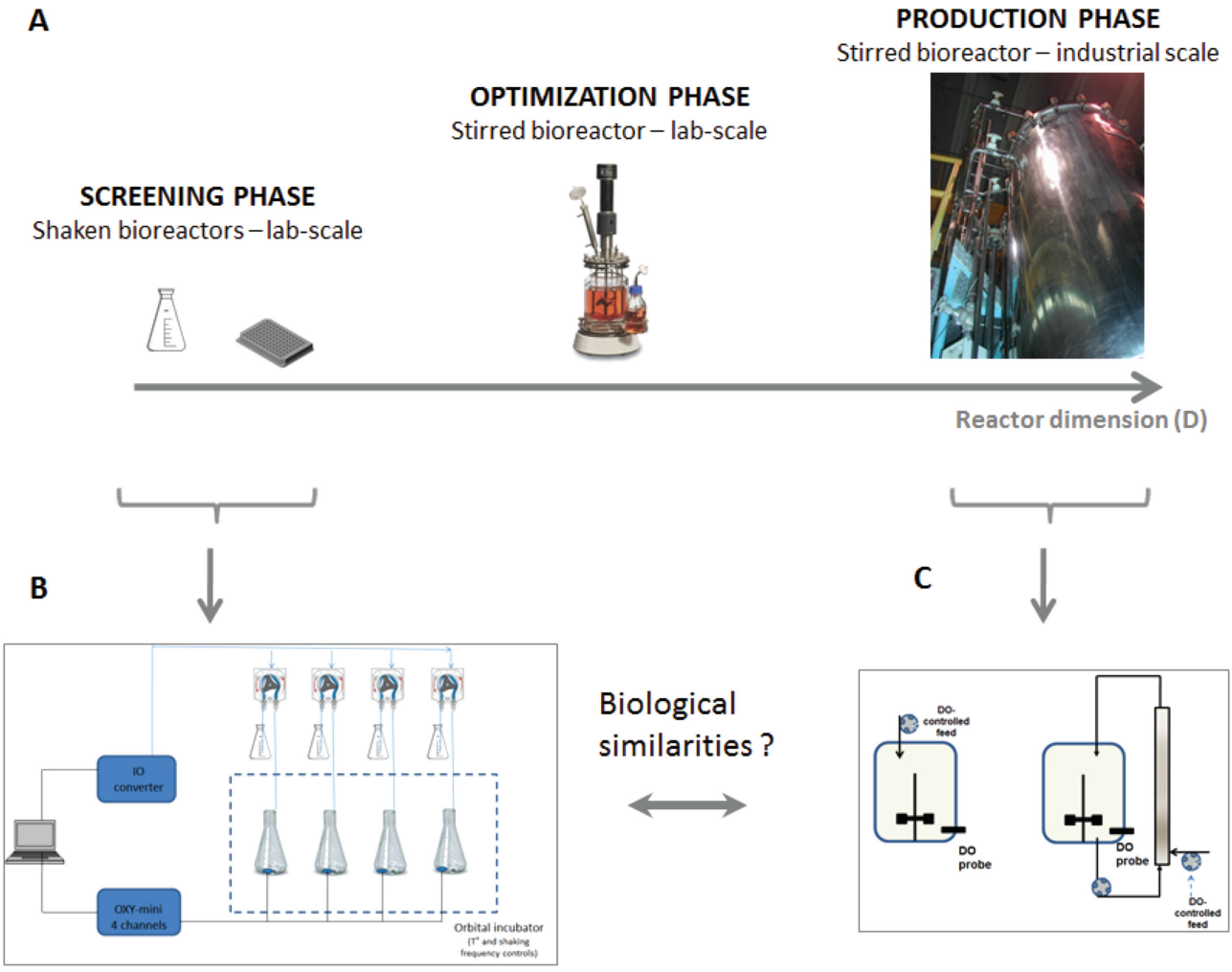

Figure 1. Summary of the scientific strategy used in this work.

A: bioprocess development involves three main steps, i.e., screening, optimization and production phases, in which microbial cells are exposed to very different environmental conditions between the screening phase (homogeneous conditions) and the production phase (environmental fluctuations). The aim of this work is to evaluate similarities between a mini-scale-down platform (B) and scale-down bioreactors mimicking industrial conditions (C).

aims to determine to what extent milliliter-scale bioreactors are similar to conventional bioreactors, and how these mini-bioreactors can be used as a scale-down tool. Indeed, bioprocess development involves several steps, which cannot necessarily be linked with each other considering the different cultivation equipment required (Figure 1A).

Our study was more particularly focused on the comparison of a mini-bioreactor platform with conventional stirred bioreactors, and more specifically between mini-bioreactors operating under heterogeneous conditions and scale-down reactors mimicking industrial conditions (Figures 1B,C). The rationale behind this approach relies on the generation of relevant biological kinetics data directly during the screening phase for an appropriate microbial strain for a target application. Indeed, there is still an important gap between the screening phase and the scaling-up phase, since in small reaction volumes environmental conditions are relatively homogeneous compared to those experienced in large-scale bioreactors. Spatial substrate heterogeneities are the main source of process perturbations during scaling-up procedures[9,10] and it is thus of primary importance to characterize the impact of such heterogeneities at the biological level. A recent review demonstrates the potential of milliliter-scale bioreactors in order to investigate microbial cell population heterogeneity.[11]

The proposed mini-bioreactor platform was thus investigated in this context. The mini-reactors considered here are simply based on baffled shake flasks as cultivation vessels. These flasks are equipped with dissolved oxygen probes and can be operated in fed-batch mode following different control strategies. The first feeding mode involves the use of a glucose slow release system commercialized under the name Enbase Flo.[12] As this system is based on a slow release of glucose from a polymer by a specific enzymatic reaction, substrate fluctuations are completely avoided in the reaction medium (homogeneous conditions). The second feeding strategy is based on intermittent feeding of glucose by a peristaltic pump. This strategy is recognized as generating strong substrate fluctuations.[13] The combination and parallelization of these two feeding modes can be the basis of a high-throughput scale-down system. These investigations were carried out by using microbial GFP biosensors as physiological tracers for the environmental fluctuations met in industrial heterogeneous bioreactors.[14,15] Whole cell microbial biosensors responsive to carbon limitation are very useful for the detection of spatial substrate heterogeneities, as demonstrated in our previous works:[15,16] the behavior of a *csiE* biosensor has already been studied in conventional stirred reactors. In the case of these specific reporter systems, GFP is actively synthesized under homogeneous conditions (environmental conditions met in the case of

a lab-scale reactor) and is deactivated when the biosensor is exposed to glucose heterogeneities in a scale-down reactor able to reproduce industrial conditions.[15] Another aspect that will be investigated in this work involves indirect use of the biosensors by analyzing the amount of extracellular GFP coming either from permeabilized membranes (protein leakage) or from lysed cells. Indeed, a significant release of GFP in the extracellular medium can be observed in relation to the mixing performances of bioreactors with the *csiE* biosensor.[15,17] This works aims to determine to what extent miniaturized bioreactors are similar to conventional bioreactors based on biological parameters (GFP induction and GFP release from biosensors). The above-mentioned results were also extended to other biosensors responsive to carbon limitation.

Material and Methods

Strains and Medium Composition

E. coli K12 MG1655 bearing a pMS201 (4,260 bp) plasmid with a stress promoter (*uspA*, *csiE*, or *yciG* gene) followed by the *gfpmut2* gene and a kanamycin resistance gene was used as the main chassis for the construction of the GFP reporter systems used throughout this work (BamH1 and Xhol were used as restriction sites, rnnB T1 as terminator). All the strains came from a cloning vector library at the Weizmann Institute of Science[18] and are maintained at −80°C in working seeds vials (2 mL) in solution with LB media and 40% glycerol. Pre-cultures and cultures were performed in a defined mineral salt medium containing (in g/L): K_2HPO_4 14.6, $NaH_2PO_4.2H_2O$ 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, and kanamycin 0.1. Thiamin and kanamycin were sterilized by filtration (0.2 μm). The medium was supplemented with 3 mL/L of trace mineral solution, 3 mL/L of a $FeCl_3.6H_2O$ 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 solution contained (in g/L): $CoCl_2.H_2O$ 0.74, $ZnSO_4.7H_2O$ 0.18, $MnSO_4.H_2O$ 0.1, $CuSO_4.5H_2O$ 0.1, and $CoSO_4.7H_2O$ 0.21. Before operating in mini- or classical stirred bioreactors, a pre-cultivation step was performed in 100 mL of the above-mentioned medium in a baffled shake flask at 37°C under orbital shaking at 160 rpm. Cell growth was monitored by optical density (OD) at a wavelength of 600 nm. Cell dry weight was determined on the basis of filtered samples (0.45 μm) dried for 24 h at 105°C. Glucose concentration was monitored by an electro-enzymatic system YSI (Yellow Spring Instrument). Samples were injected in a room filled with a buffer solution and the sample was diffused through a polycarbonate membrane that limited the reaction rate. Membrane-immobilized glucose oxidase produced hydrogen peroxide that diffused through a cellulose acetate membrane and was oxidized by a palatine electrode. The signal was proportional to the glucose concentration up to 2.5 g/L. More concentrated samples were diluted.[19] The instantaneous growth rate was computed from the microbial growth curve using the following equations. Over relatively short time intervals, the differential term dX/dt can be approximated by the finite difference term $\Delta X/\Delta t$:

$$r_x(t,i) = \frac{x_{i-1} - x_{i+1}}{t_{i-1} - t_{i+1}} \quad (1)$$

$$\mu(t) = \frac{r_x(t)}{X(t)} \quad (2)$$

Table 1. Glucose Concentrations Used for the Different Experiments

Types of Reactor Mode	Glucose Concentrations in the Feeding Bottle
Fed-batch mode	400 g/L
Chemostat mode	5 g/L

Stirred Bioreactor Experiments: Fed-Batch and Chemostat Experiments

Lab-scale stirred bioreactors (Biostat B-Twin, Sartorius with a total volume of 3 L; initial working volume of 1 L; final working volume of 1.5 L; mixing provided by a standard RTD6 rushton turbine) were used for chemostat and fed-batch experiments. The bioreactor platform comprised two cultivation vessels in parallel monitored and controlled by the same control unit (remote control by MFCS/win 3.0 software). For fed-batch experiments and for each reporter strain, experiments were conducted in parallel comparing a culture performed in the classical stirred vessel and another conducted with the stirred vessel connected to a recycling loop. This latter apparatus corresponded to a scale-down strategy allowing reproduction of the heterogeneities expected in large-scale bioreactors.[10,20] The scale-down reactor arrangement was based on the previously described stirred bioreactor connected to a recycle loop (silicon pipe; internal diameter 0.005 m; length 6 m). Residence time distribution determined by tracer experiments (data not shown) indicated a mean residence of 36 seconds and a standard deviation of 10 s. This value is similar to those measured in other studies and corresponds to a typical value for circulation time encountered in large-scale bioreactors.[21] Continuous recirculation of the broth between the stirred reactor and the recycle loop was ensured by a peristaltic pump (Watson Marlow 323), with the glucose feed solution added at the inlet of the recycle loop in order to generate a concentration gradient. During the experiments, pH was maintained at 6.9 (regulation by ammonia and phosphoric acid) and temperature at 37°C. The stirrer rate was maintained at 1,000 rpm with a RDT6 impeller and the air flow rate was set to 1 L/min at the beginning of the culture. Once the fed-batch was started, agitation rate and air flow rate were progressively raised to 1,300 rpm and 2 L/min, respectively. The culture was fed with 500 mL of a solution containing 400 g/L glucose diluted in mineral medium (see above for composition). The glucose feed profile was performed on the basis of a DO-stat control with a set point of 30% dissolved oxygen from saturation. For chemostat mode, after a classic batch phase, different dilution rates were imposed on the cell by different flow rates of the feeding pump in a classical stirred vessel. The glucose solution always contained 5 g/L of glucose diluted in mineral medium. To keep the same total volume in the reactor, excess medium was eliminated (Table 1).

Mini-Bioreactor Platform

A slow release and an intermittent feed system were used as a scale-down platform in order to assess the responsiveness of several GFP microbial whole-cell biosensors. The two feeding systems were implemented at the level of baffled shaker flasks (1 L, working volume: 200 mL of mineral medium described previously) equipped with dissolved oxygen optical mini-sensors (oxy-mini, purchased from Presens company). Ruthenium oxide was used as the sensitive element in the DO spot sensor (excitation wavelength: 505 nm; emission wavelength: 650 nm). This patch was adhered

inside the flasks and immersed in the cultures. The measurement is based on the attenuation of the emitted light by oxygen: the relationship between dissolved oxygen and fluorescence intensity is nonlinear and can be expressed by the Stern-Volmer equation.[22,23] Sensitivity is optimal at low concentrations, which is the relevant range for fermentations. The cultures were performed in an orbital incubator (160 rpm, 37°C). The slow release system was based on the liquid version of the EnBase enzymatically controlled glucose release system. Formerly sold in a gel version,[24] the new liquid version allows on-line monitoring of the dissolved oxygen inside the flask by using newly developed optical sensors.[25] The composition of EnBase Flo is based on a mineral salt medium similar to those previously described. The kit was used without adding the booster medium (containing a complex cocktail of amino acids).[12] Glucose is released by the action of a glucoamylase on a polymer (composition not provided) incorporated in the liquid medium. The enzyme was added to the medium in order to reach an activity of 1.2 U/L. This enzyme activity allows the release of 2.4 g h^{-1} L^{-1} of glucose. The same glucose release rate was used in the case of the intermittent feed for comparison. The intermittent fed-batch mode was performed by using peristaltic pumps (Watson Marlow 101 U/R, silicone tubing with external/internal diameter of 6/2 mm) working at a basic flow rate of 0.06 mL/s. The flow rate was stopped intermittently, following a switching profile controlled by a dedicated MatLab code. The feeding solution comprised 200 mL of the above-mentioned mineral medium containing 30 g/L of glucose. Samples were taken as infrequently as possible in order to limit process disturbances generated by the arrest of the orbital shaker. These samples were used for off-line monitoring of optical density, glucose concentration, pH and GFP intensity.

Flow Cytometry Analysis

Analysis of the GFP expression level was performed by flow cytometry on a FACScan (Becton Dickinson) instrument. Samples were taken directly from the reactor and were diluted in 900 μL of PBS and 100 μL of a cycloheximide solution (1 mg/mL) in order to stop protein synthesis. For each measurement, 30,000 cells were analyzed. GFP was excited at 488 nm and emission signals were collected using filters at 530 nm. The gfpmut2 variant has been especially engineered to optimally match the excitation/emission constraints when using a FACS instrument.[26] Considering that bacterial cells exhibit a high side scatter (SSC) signal,[27] a threshold of 52 was set on the SSC channel in order to limit the noise signal. The FSC (Forward SCatter), FL1, FL2, and FL3 channels were logarithmically amplified with the following settings: FSC E00, FL1 620, FL2 420, and FL3 420. The results were analyzed with CellQuest (Becton Dickinson) software and exported to FlowJo 7.6.1. Further visual observations of the biosensors were carried out on a fluorescence microscope (Zeiss, Axioscope). Propidium iodide (PI) was used to determine membrane permeability. After staining with PI, cells with compromised membranes were detected by flow cytometry using the following settings: FSC E00, FL1 620, FL2 420, FL3 520.

Data Treatment for Determination of the Different Biosensor GFP Classes

An important parameter to be considered in this work is the induction of the different biosensors on the basis of GFP expression in different bioreactor configurations. The classical range of variation in the GFP content of the cells, as measured on the FL1 channel of the flow cytometer with the settings presented in the previous section, is approximately one log of magnitude between the induced and non-induced states. This maximum range of variation has been confirmed previously for different biosensors belonging to the same library used in this work.[14] On the basis of this previous observation, FSC-FL1 flow cytograms are divided into three sub-classes GFP−, GFP+, and GFP++. In the case of the three biosensors used in this study, no events belonging to the GFP− subclasses were observed (Figure 2). The basal level of our three biosensors was located in the GFP+ quadrant. Accordingly, the results were expressed as the percentage of induced cells, those cells belonging to the GFP++ quadrant of the flow cytograms.

SDS-PAGE and Immunoblot Analysis

Samples from the different bioreactor configurations tested throughout this study were centrifuged at 13,500 rpm for 5 min and filtered on 0.2 μm cellulose membrane in order to remove cells. Proteins in the supernatant were separated (with a working volume of 20 μL) on 30% polyacrylamide gels (Biorad) by standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) procedures. After SDS-PAGE, proteins were transferred to a hybond hydrophobic polyvinylidene difluoride membrane (PVDF) membrane. Nonspecific binding sites were blocked by immersing the membrane in 5% non-fat dried milk with 0.1% Tween 20 in PBS for 1 h at room temperature on an orbital shaker. The membrane was washed twice with PBS, 0.1% Tween 20 and then incubated with a 1:10,000 dilution of the anti-goat IgG (sc-2768) secondary antibody in order to detect the band corresponding to GFP. The membrane was finally washed three times for 15 min with PBS, 0.1% Tween 20. Detection of the secondary antibody was performed using the enhanced chemiluminescence (ECL) plus detection system (Amersham) by exposure to X-ray film. In order to check for possible indications of cell lysis at the level of protein leakage, the total nucleic acid content of the supernatant was also analyzed (Hoescht method).

Extracellular Proteome (Secretome) Profiling

Cells were removed by centrifugation at 13,500 rpm for 5 min. The supernatant was passed through a 0.2 μm filter. Proteins were precipitated with trichloroacetic acid (TCA) 20% (v/v) for 15 min at 4°C, and washed with 300 μL of acetone to remove traces of TCA. The protein pellets were resolubilized in a thiourea/urea buffer and quantified with an RC/DC Protein Assay kit (Bio-Rad) with bovine serum albumin standards. Two hundred fifty micrograms of proteins were diluted in 450 μL of rehydration buffer (thiourea/urea buffer completed with 16 μL of ampholyte, 8 mg of dithiothreitol (DTT), and a trace of bromophenol blue by mL). Samples were incubated for 20 min in the dark before being loaded onto immobilized pH gradient (IPG) strips (Immobiline™ Dry Strip pH 3–11 NL, 24 cm). Isoelectric focusing (IEF) was run employing a Protean IEF Cell (Bio-Rad). First, the dry strip was re-hydrated for 9 h at 25°C. Then the following program was applied: 200 V for 2 h, then an increase from 200 V to 1,000 V over 4 h, followed by an increase of 1,000 to 10,000 V in 1 h, and electrofocalization at 10,000 V for 5 h 30 min. After IEF, proteins on the strip were first reduced for 15 min in 275 nm Tris (pH 8.8), 6 M

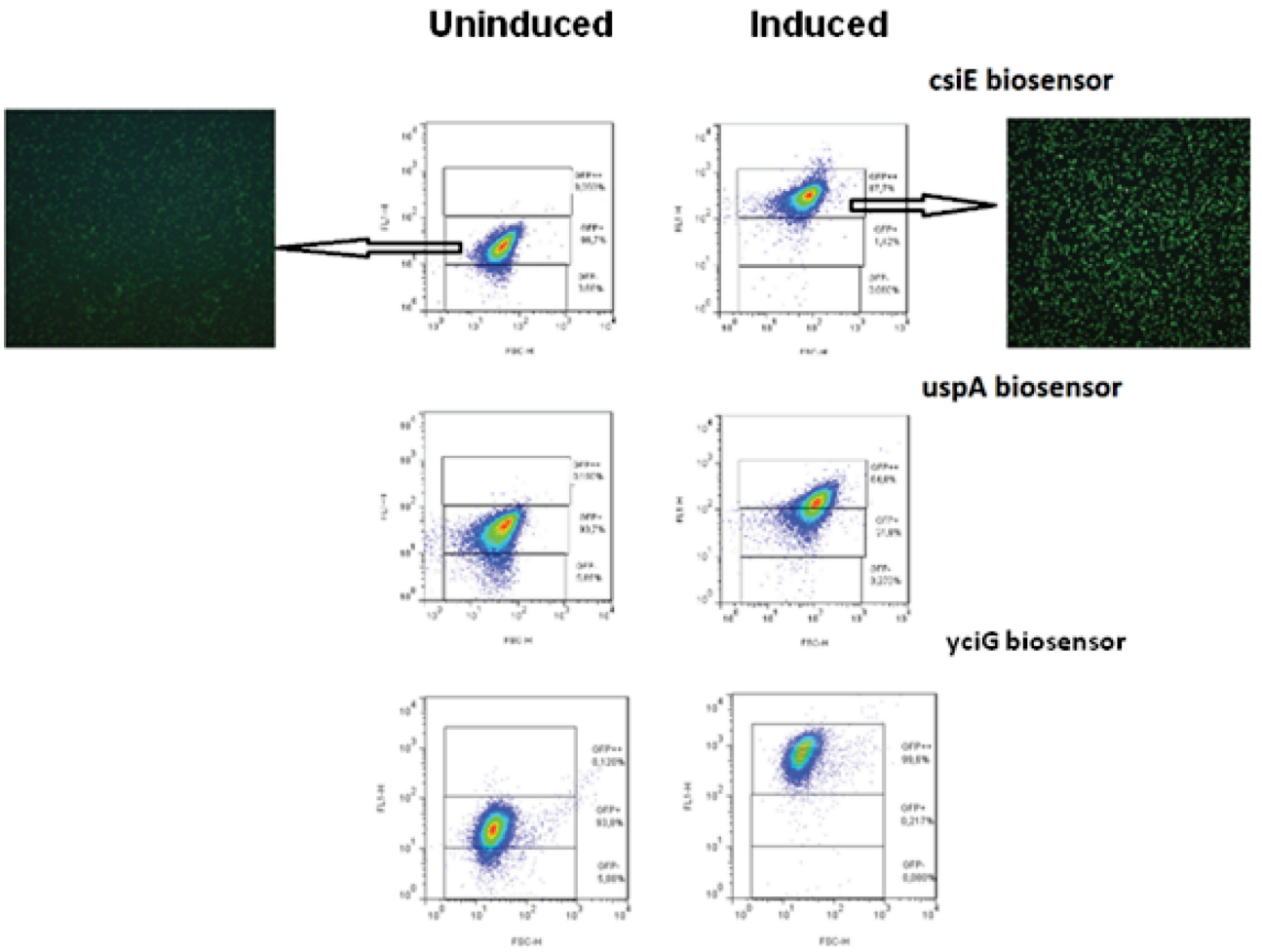

Figure 2. Flow cytometry analysis of the basal and maximal levels of GFP expression for the *yciG*, *cisE*, and *uspA* biosensors. The gating procedure enabling discrimination of the noninduced and induced states is shown for each biosensor.

urea, 20% v/v glycerol, 2% w/s and 130 mM DTT, and then alkylated for 15 min in 375 mM Tris (pH 8.8), 6 M urea, 20% v/v glycerol, 2% w/v SDS, and 135 mM iodioacetamide. The strip was charged on top of a 12% polyacrylamide gel. The SDS-PAGE was run at 100 W until the dye front reached the end of the gel in a Dodeca Cell tank (Bio-Rad). Proteins spots were visualized using a quick hot staining. Spots were manually excised and collected in 96-well plates designed for the automated digester (Proteineer dp, Bruker, Bremen, Germany). Briefly, samples were washed two times in a 50 mM NH_4HCO_3 buffer, and dehydrated with acetonitrile 100%. Trypsin digestion was performed with 3 μL/well of a 10 ng/μL solution (in ammonium hydrogen carbonate) for 4 h at 37°C. Peptides were extracted with 15 μL of trifluoroacetic acid (TFA) 1% for 30 min at 40°C. Protein digests (3 μL) were absorbed for 3 min on pre-spotted anchor chips using Proteinner dp automation (Bruker). Tryptic peptides were analyzed using an Ultraflex II MALDI mass spectrometer (Bruker) in positive reflectron mode with close calibration (peptide mass fingerprinting and tandem mass spectroscopy). The MASCOT search server and the National Center for Biotechnology Information protein database (available at: http://www.ncbi.nlm.nih.gov/) for bacteria were used for protein identification. The UniProt Knowledge database (on the ExPasy Proteomics Server website: http://kr.expasy.org/) was consulted to determine molecular function and subcellular localization of identified proteins.

Results and Discussion

Sensitivity of GFP Biosensors to Glucose Limitation: Chemostat Experiments

The first step in our experimental protocol was the determination of the effective sensitivity of the three biosensors when they were exposed to a substrate limitation signal. For this purpose, different degrees of substrate limitation can easily be generated in a chemostat bioreactor. Moreover, extracellular conditions are constants (i.e. constant cell density with constant environmental variables such as pH and dissolved oxygen), and the continuous mode imposes the elimination of old cells and renewal of the population, which enables an easier interpretation of the results compared to batch or fed-batch experiments. Chemostat reactors with four different dilution rates were set up for each biosensor. During all experiments, cell density remained constant during the continuous phase at 2.47 g/L (results not shown). The results are reported as the percentage of induced cells as a function of the dilution rate when the chemostat is stabilized (Figure 3).

Biosensors carrying the *yciG* and *csiE* promoters were modulated as a function of the dilution rate, whereas no difference in expression was noted in the case of the *uspA* biosensor. The *yciG* biosensor seems to have a lower operating range, since its fluorescence decreased rapidly when the dilution rate was increased. Only the *csiE* biosensor was modulated over the entire range of dilution rates considered.

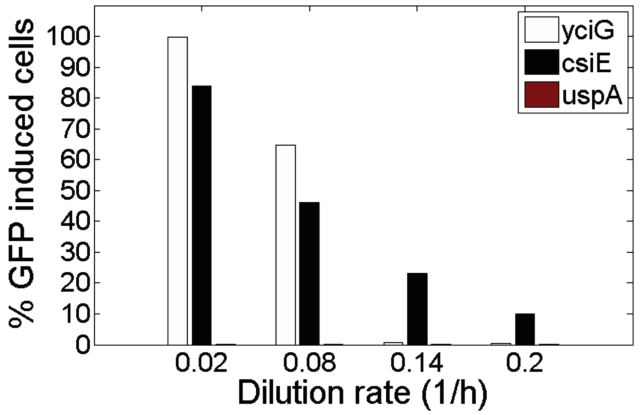

Figure 3. Percentage of induced cells as a function of dilution rate in chemostat reactors for the *yciG*, *csiE*, and *uspA* biosensors.

In the case of the *yciG* and the *csiE* biosensors, the variations between two independent experiments is less than 10%.

The responsiveness of the different biosensors can be linked to the specificity of the corresponding stress promoter. Indeed, synthesis of *uspA* (i.e. universal stress protein A) is induced in response to a variety of stimuli and stresses and is notably synthesized during growth inhibition caused by carbon starvation.[28] This kind of stress promoter is called "semi-specific" because the reporter gene is linked to a promoter responding to general conditions of stress.[29] The latter two genes, *csiE* and *yciG*, are part of the sigma S regulon and are more specific. In this context, a luxCDABE version of *yciG* has recently been used in order to detect spatial heterogeneities of glucose in bioreactors.[30] Knowing their range of variation in function of substrate limitation, these biosensors were used in the next section to compare classical stirred bioreactors with the mini-bioreactor platform.

Performance of the Mini Scale-Down Reactor Platform on the Basis of the Response of the GFP Biosensors

Several scale-down reactor configurations are available in order to reproduce the substrate heterogeneities encountered in large-scale bioreactors.[20,31] We have shown in our previous studies that biosensors sensitive to substrate limitation are activated when cultured in a well-mixed fed-batch bioreactor, demonstrating the progressive limitation appearing as cell density increases.[15,16] When cultivated in two-compartment scale-down reactors, the response of these biosensors is slowed down or decreases with time.

Several cultures were therefore set up in order to determine if the same features could be reproduced at the level of the mini-bioreactor platform. The cultures were then monitored on the basis of GFP expression for the three different biosensors (Figure 4A).

In mini-bioreactor configurations, the *yciG* and *csiE* biosensors were induced, whereas no GFP synthesis is observed in the case of the *uspA* biosensor. These results are in good accordance with the sensitivity analysis carried out in the chemostat (Figure 3). First, the two systems were compared: mini-bioreactors and conventional stirred bioreactors. Growth rate profiles showed a similar evolution for all the cultivation experiments, either in stirred or mini-bioreactors (Figure 4B). After an initial increase, the growth rate drops progressively during culture. However, the intensity of the growth rate was significantly different between the stirred and mini-bioreactors. Indeed, the growth rate values observed at the milliliter scale were lower than those observed in the case of the stirred bioreactors, considering the lower oxygen transfer performances of the mini-cultivation devices. Moreover, the growth rate decreased faster in the mini-bioreactors than in stirred bioreactors (Figure 4B). On this basis, it is possible to compare the two strains, i.e., *csiE* and *yciG*, effective sensitivities to glucose limitation, and understand the differences observed. Indeed, the *yciG* promoter is induced only at a very low dilution rate and the GFP signal is lost when D is raised to values above 0.08 h^{-1}.

Moreover, in mini-bioreactors the slow release system provides a very smooth feeding profile without any perturbations or fluctuations, whereas intermittent feeding induces an oscillation in the level of substrate concentrations. These behaviors can be clearly seen on the basis of the dissolved oxygen profiles recorded for the mini-bioreactor systems (Figure 5).

According to the substrate fluctuations (intermittent feeding system), the substrate limitation signal is relieved and GFP synthesis is stopped or slowed down. This observation can be validated in the case of the *csiE* and the *yciG* biosensors exhibiting a lower GFP signal in the case of cultures performed with intermittent feeding compared with cultures using the slow release system. However, fewer differences were observed between the slow release and the intermittent feeding system in the case of the *yciG* biosensor, suggesting that this reporter system is less sensitive to environmental perturbations. This result is supported by the sensitivity analysis of the *yciG* promoter exposed to different dilution rates in the chemostat (Figure 3).

Finally, it is important to point out some other differences between conventional bioreactors and mini-bioreactors, which potentially explain the difference observed at the level of GFP synthesis between mini and conventional bioreactors. First, the cell density is about 10 times higher in the case of conventional bioreactors than in our mini-bioreactors (final dry weight for a well-mixed stirred bioreactor: 53.5 g/L; final dry weight for a mini-bioreactor using the slow release system: 4.4 g/L). Cell density is known to have a strong impact at the level of transcriptional regulation (i.e. quorum sensing related phenomena),[32] potentially explaining the differences noticed with the *uspA* reactors. Second, pH is not controlled in the mini-bioreactors,[33] potentially leading to the induction of some related regulation network linked with substrate starvation (i.e., general stress response pathway[34–37]). It must be noted that this technical issue could be corrected in the future by appropriate sensor-actuator systems at the milliliter scale.[23,38] However, the media are buffered, so the maximum decrease in pH is 0.5 units (data not shown).

In conclusion, this first set of results clearly shows that, on the basis of the cell response to environmental conditions, the milliliter scale cannot exactly match conditions experienced in conventional and scale-down bioreactors. However, the main features can be reproduced, i.e., progressive substrate limitation with a proportional response in the case of the most sensitive promoter, i.e., *cisE*., and decreases in GFP expression due to environmental fluctuations. So our mini-bioreactor platform is able to highlight differences for the biosensors between homogeneous and heterogeneous conditions at the level of GFP synthesis, exhibiting sensitivity to substrate limitation as confirmed in the chemostat (*csiE* and *yciG* promoters).

The following section illustrates a further insight regarding the usefulness of the mini-bioreactor in the selection of biosensors able to respond to process-related perturbations. Indeed, we have recently shown that GFP leakage occurs during cultivation

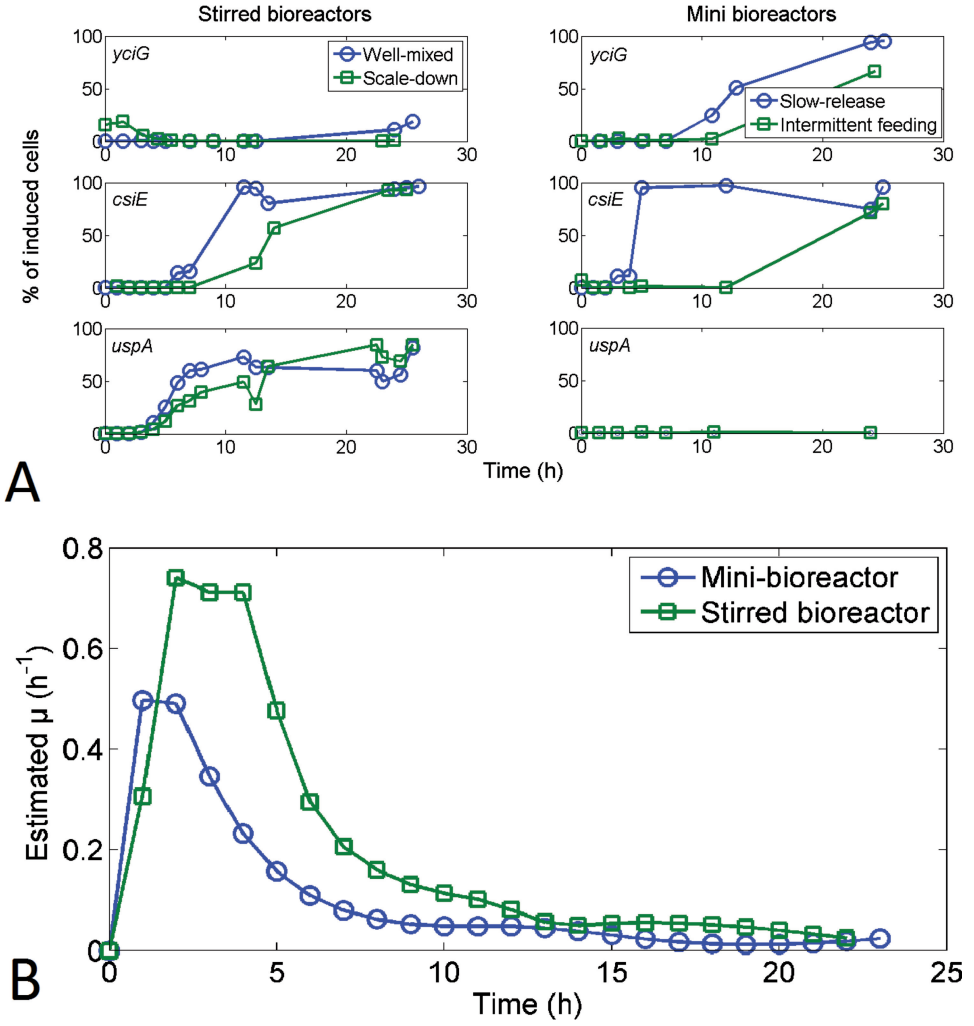

Figure 4. A: Percentage of induced cells for the *yciG*, *csiE*, and *uspA* biosensors during fed-batch experiments in well-mixed and scale-down bioreactors, and in the mini-scale-down platform with either a slow release (EnBase) or intermittent feeding.

B: Typical evolution of the growth rate computed for the well-mixed stirred bioreactor and the corresponding slow release mini-bioreactor (Eqs. 1 and 2 were used in order to compute these results). For the determination of the percentage of induced cells, the variation between two independent experiments is always less than 15%.

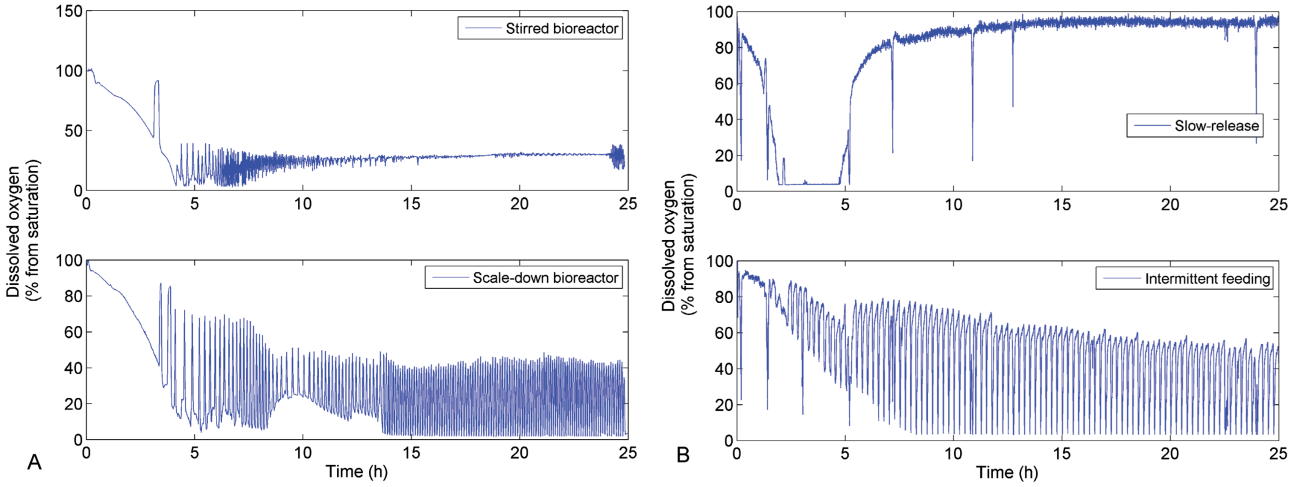

Figure 5. A: Typical dissolved oxygen profiles observed in the case of stirred bioreactors (well-mixed and scale-down bioreactors).

B: Typical dissolved oxygen profiles observed in the case of mini-bioreactors (slow release and intermittent feeding systems). Sudden decreases at the level of the dissolved oxygen profile correspond in this case to arrest of the orbital shaking when sampling the devices.

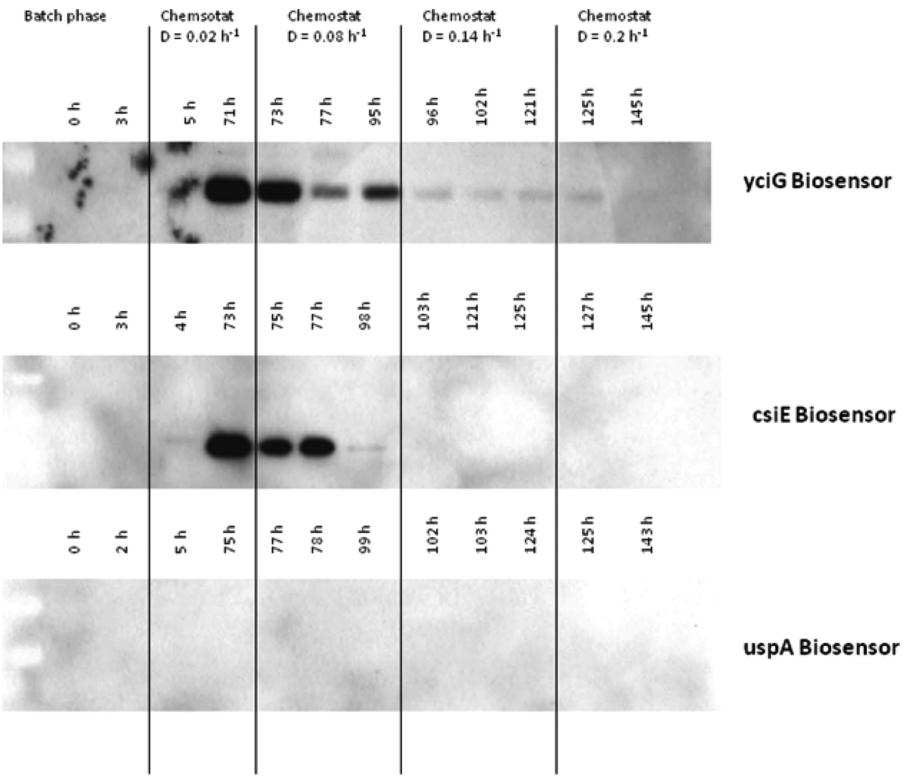

Figure 6. Western blot analysis of supernatants from chemostat reactors. GFP is observed at a mass corresponding to 27 kDa.

of the biosensors and that this GFP leakage is influenced by spatial substrate perturbations in bioreactors.[17]

Indirect Use of GFP Biosensors: Evaluation of Protein Leakage and Cell Lysis Between the Different Scales

Another important feature for comparison between the different cultivation scales is cell viability. Several studies involving the scale-down principle have highlighted membrane integrity as a key parameter affected by extracellular conditions.[39,40] A clear link has also been established between the permeability of the cellular membrane and the release of proteins. This phenomenon is named protein leakage and is related to the release of outer-membrane and periplasmic components into the extracellular medium. The major fraction of the proteins released in this way have a periplasmic origin, as previously observed by two-dimensional gel electrophoresis in supernatants extracted from high-density cell cultures.[41] It is quite difficult to make the distinction between proteins released from permeabilized cells and from lysed cells. During our experiments, no DNA was detected in the culture supernatant. However, it has been shown that DNA released from lysed cells can be adsorbed on cell membranes.[42]

In order to assess the origin of the proteins released into the extracellular medium, i.e., whether by leakage or cell lysis, a two-dimensional gel electrophoresis analysis was performed on a culture supernatant collected from a stirred bioreactor running in fed-batch mode. MALDI-TOF analysis was performed in order to identify extracellular proteins (mass fingerprinting and MS-MS). The conditions were chosen so that the supernatant collected exhibited a high protein content. All the results are presented in the Supporting Information. Analysis of the extracellular proteome revealed that the most abundant proteins have a periplasmic origin (64% of this extracellular proteome is composed of periplasmic and outer membrane proteins). This observation is in accordance with the results of Funke et al. (2011)[38] who found, respectively, 65% of periplasmic proteins and 56% of outer membrane proteins with the W3110 *Escherichia coli* strain. However, some major cytoplasmic proteins have been identified, such as chaperonin *GroE*, chaperone *DnaK*, and elongation factor Tu. It has been reported that *E.coli* are able to release some cytoplasmic proteins, including *DnaK* and EF-Tu, by an unknown mechanism. The hypothesis in these previous studies was simple leakage or shedding of outer membrane components with little or no cell lysis, as reported previously for *E. coli* cultures. In our case, we postulate principally cell leakage with a possible limited occurrence of cell lysis. Investigation of the secretome has been limited at this stage in the context of this work and this aspect will be more thoroughly investigated in further work.

The following investigation was mainly based on the extracellular GFP, which was considered an indirect tracer for protein leakage under process conditions. The supernatants from chemostat experiments were analyzed by western blot. GFP leakage from *csiE* and *yciG* biosensors was observed in the chemostat reactors under the most limiting conditions, i.e., $D = 0.02\ h^{-1}$ and $0.14\ h^{-1}$ (Figure 6).

These experiments confirm the fact that GFP leakage is correlated with the intensity of glucose limitation, since no GFP is noticed in the supernatants for the higher dilution rates. Indeed, GFP leakage seems to occur when cells are exposed to prolonged substrate limitation, as reported by other recent works.[43] Obviously no GFP band was found for the chemostat carried out with the *uspA* biosensor because GFP has to be induced and synthesized before being

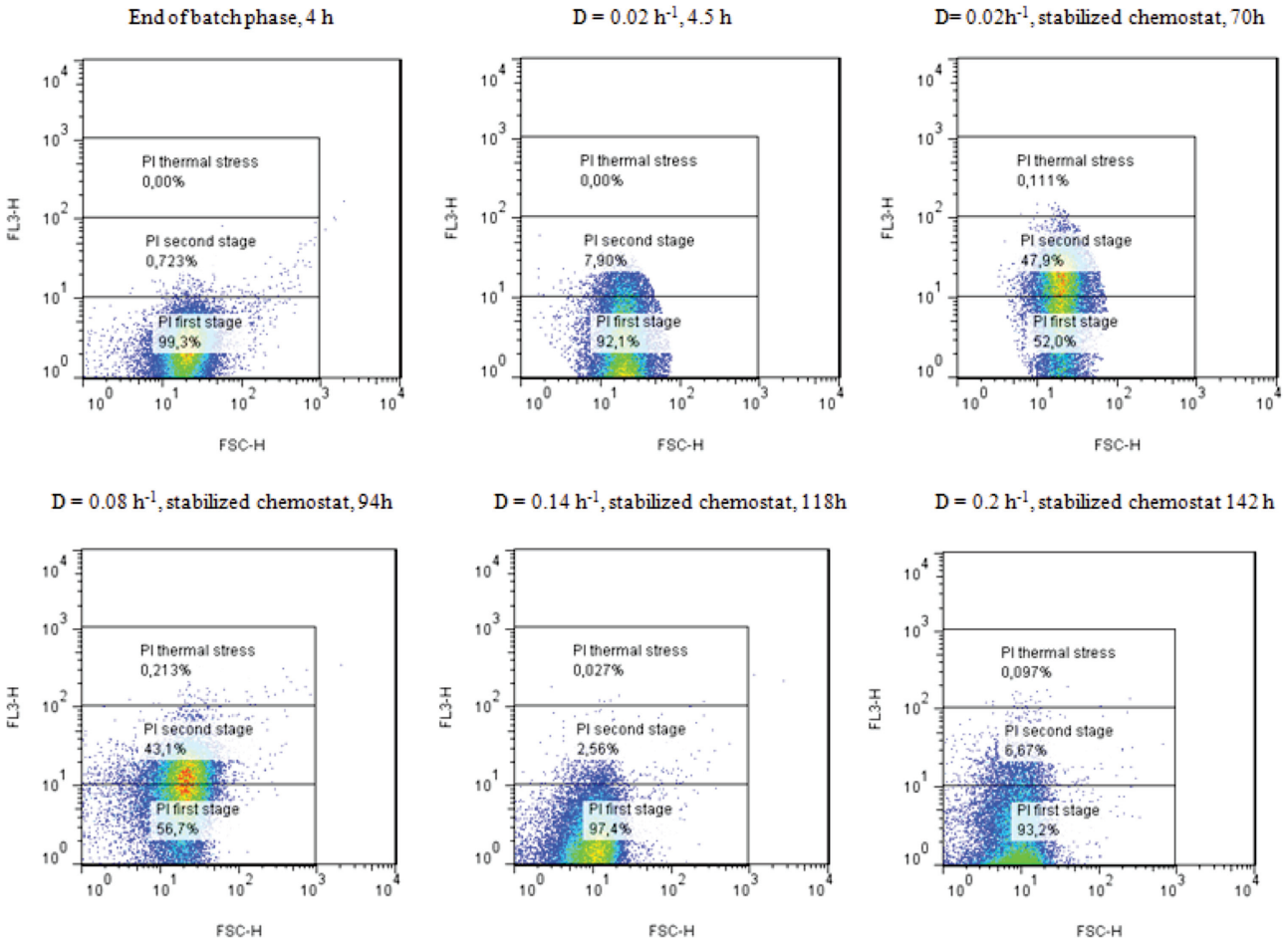

Figure 7. Flow cytometry diagrams showing the evolution of membrane permeability according to propidium iodide tests during chemostat experiments.

Propidium iodide accumulates in membrane-compromised cells and is measured on the FL3 channel, whereas cell size is measured on the FSC channel. Representative results of three independent experiments with the three different biosensors are shown.

excreted. In parallel, membrane integrity was determined on the basis of PI staining (Figure 7).

Flow cytometry diagrams show an intermediate population between the quadrant corresponding to healthy cells and that corresponding to heat stressed cells. It is very interesting to note that this intermediate population exhibits a strong modulation as a function of the dilution rate. Indeed, judging by this population, cell permeability decreases when the dilution rate is increased, supporting the fact that leakage is influenced by the degree of substrate limitation.

These analyses were repeated in order to detect GFP leakage in conventional and mini-bioreactors (Figure 8).

In conventional reactors, GFP leakage is significantly attenuated under scale-down conditions for the two biosensors tested. Once again, this observation is in alignment with the degree of cell permeability, as depicted by flow cytometry analysis (Figure 9). This observation is also in accordance with previous results showing a higher cell viability and a lower permeability in the case of cultures carried out in heterogeneous bioreactors where cells are exposed to spatial substrate fluctuations (scale-down reactors).[10] This phenomenon is further supported by several works focusing on changes in membrane composition during fed-batch processes, most notably on the levels of the pools of saturated and unsaturated fatty acids recognized to have a profound impact on membrane fluidity.[44,45]

The same phenomenon was observed for the cultures carried out in mini-bioreactors operating with intermittent feeding (heterogeneous conditions), for which no GFP leakage was observed (Figure 8). On the other hand, GFP leakage was observed in the case of mini-bioreactors operating under the slow release regime (homogeneous conditions). However, leakage was less intensive in comparison with cultures carried out in conventional reactors, considering the lower cell density. It must also be pointed out that membrane permeability was very low for cells cultivated in the mini-bioreactor platform, explaining the reduced leakage observed under these conditions (Figure 10).

In conclusion, GFP leakage seems to be correlated with the intensity of glucose limitation and glucose fluctuations observed for the different bioreactor operating conditions considered in this work. GFP leakage is more pronounced in the case of cultures growing under homogeneous conditions by comparison with those experiencing substrate fluctuations. Interestingly, this observation is valid both in the case of conventional stirred reactors (well-mixed and scale-down reactors) and in mini-bioreactors (slow release system and intermittent feeding).

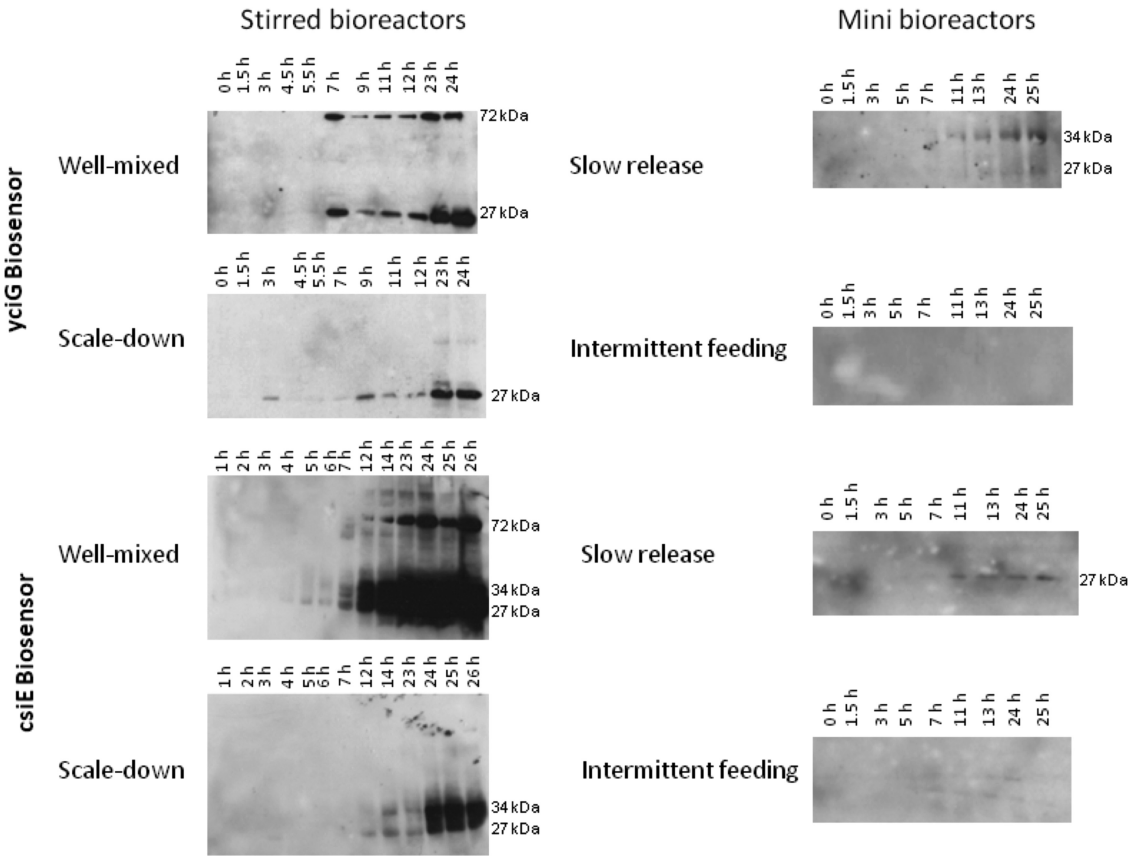

Figure 8. Western blot analysis of supernatants from stirred and mini-bioreactors for *yciG* and *csiE* biosensors.

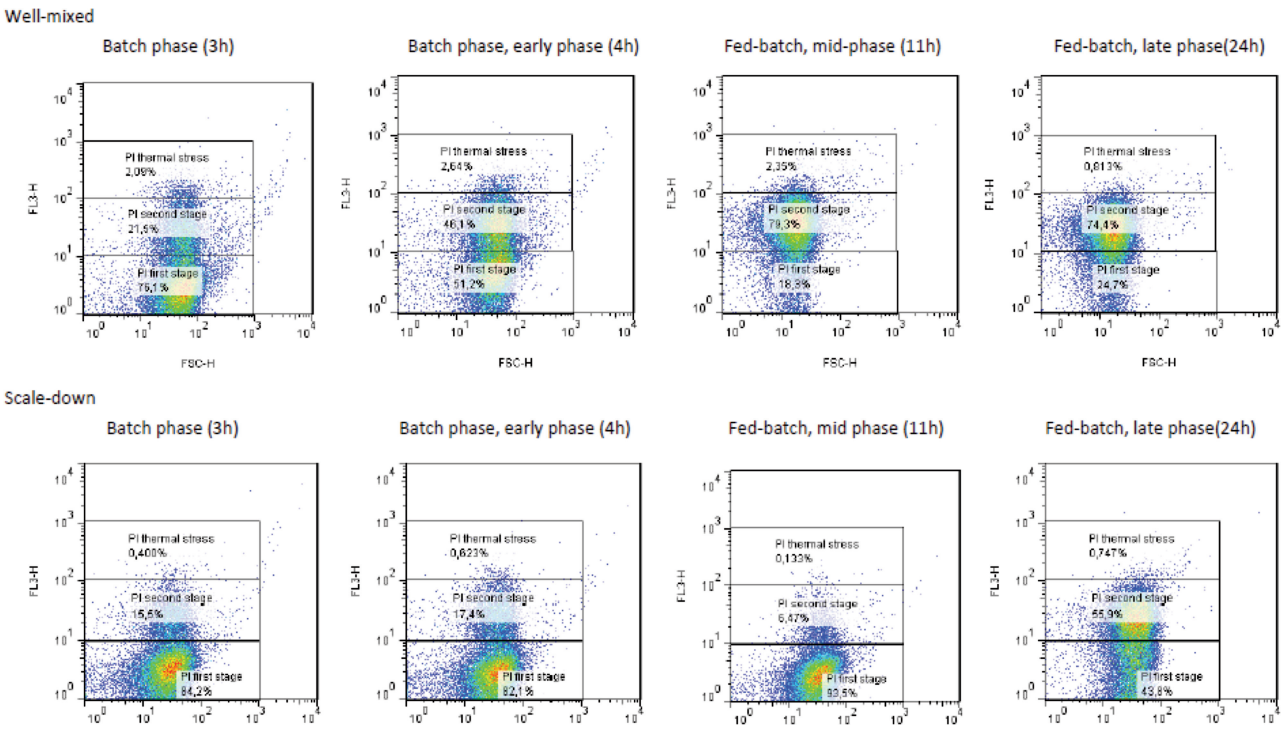

Figure 9. Membrane permeability according to propidium iodide tests in stirred bioreactors (well-mixed and scale-down bioreactors).

Conclusion

We have shown that scale-down data can be acquired in relatively simple set-ups in which high-throughput analysis can be performed. Three biosensors were used for the development of the mini-bioreactor platform. In order to validate the feasibility of our system, observations were based on intracellular GFP accumulation as well as on GFP leakage to the extracellular medium. Valid comparisons between

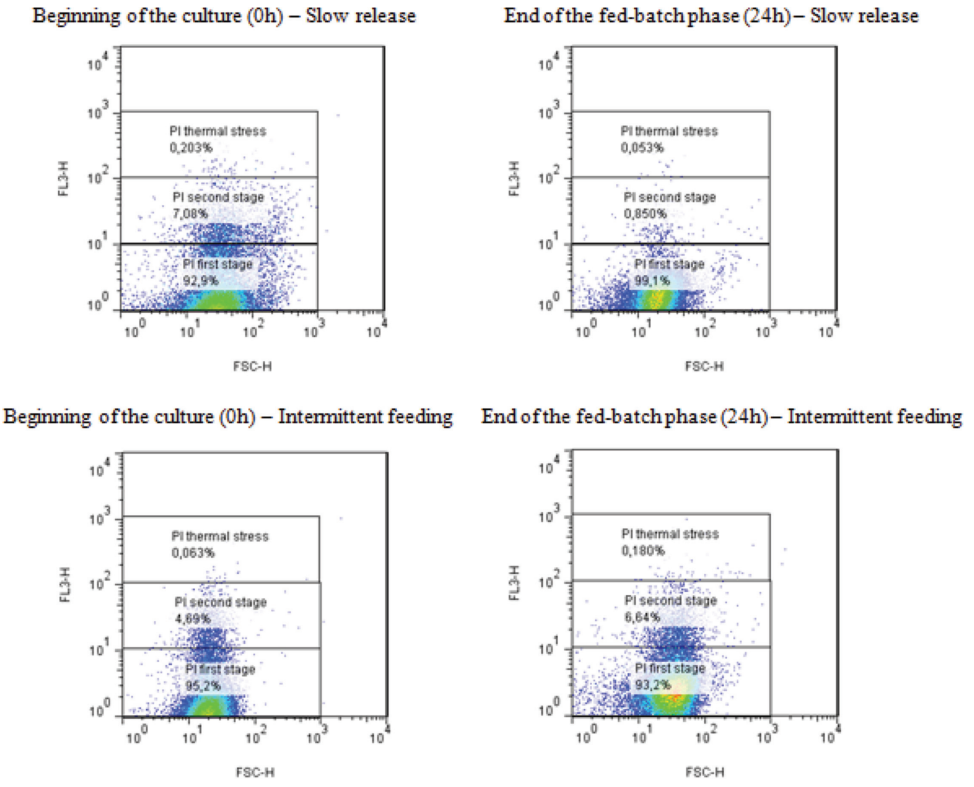

Figure 10. Membrane permeability according to propidium iodide tests in mini-bioreactors (intermittent feeding and slow-release systems).

conventional bioreactors and their miniaturized equivalents can be made using GFP biosensors carrying a promoter sensitive to substrate limitation (*csiE* and *yciG* promoters). Local heterogeneities generated in the mini-bioreactor caused decreases in GFP expression similar to those observed in the scale-down reactor. Moreover, the same phenomenon of GFP leakage into the extracellular medium was observed in mini-bioreactors and in stirred bioreactors. The mini-bioreactors could thus be proposed as a scale-down tool to rapidly investigate the usefulness of a given microbial biosensor. However, the main limitation of this mini-bioreactor platform is the effect of cell density; thus reporters associated with cell-density dependent processes, such as quorum sensing, cannot be efficiently tested in this way.

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