FERMENTATION, CELL CULTURE AND BIOENGINEERING

Real-time monitoring of cell viability and cell density on the basis of a three dimensional optical reflectance method (3D-ORM): investigation of the effect of sub-lethal and lethal injuries

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Abstract Cell density and cell viability have been followed on-line by using a three-dimensional optical reflectance method (3D-ORM) probe. This method has allowed to highlight the differences between a well-mixed and a scaledown bioreactor configured in order to reproduce mixing deficiencies during a fed-batch culture of *Escherichia coli*. These differences have been observed both for the obscuration factor (OBF) and the coincidence probability delivered by the probe. These parameters are correlated to flow cytometry measurement based on the PI-uptake test and cell density based on optical density measurement. This first set of results has pointed out the fact that the 3D-ORM probe is sensitive to sub-lethal injuries encountered by microbial cells in process-related conditions. The effect of lethal injuries has been further investigated on the basis of additional experiments involving heat stress and a sharp increase of the OBF has been observed indicating that cells are effectively injured by the increase of temperature. However, further improvement of the probe are needed in order to give access to single-cell measurements.

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Abbreviations

COP	Coincidence probability
FBRM	Focused beam reflectance method
FSC	Forward scatter
GFP	Green fluorescent protein
MSCA	Multi scattering analysis
OBF	Obscuration factor
ORM	Optical reflectance method
PAT	Process analytical technology
PI	Propidium iodide
TOF	Time of flight

Introduction

Cell viability is among the most important parameters to be followed during bioreactor operations. In general, determination cell viability is based on off-line and time consuming methods such as plate counting. More recently, flow cytometry has been proposed as a very promising alternative for cell viability determination in process-related conditions [12]. This method is culture-independent, i.e., do not require cultivation steps, and gives information at the single cell level. By this way, microbial cells can be analyzed in approximately 20 min after sampling, depending on the staining protocol used. Indeed, a large range of fluorescents stains is available in order to highlight cytological parameters linked with cell viability, i.e., membrane integrity and intracellular enzyme activity [30]. Automated flow cytometry has been proposed as a method for microbial cell analysis in bioreactors [1, 18]. This technique has been used mainly for the determination of membrane permeability and the synthesis of recombinant protein based on the GFP technology [5]. However, this method has not been extensively applied considering the

complexity for interfacing flow cytometer to bioreactors. Beside this very promising fundamental technique, several process-oriented in situ probing systems have also been used in order to monitor cell viability based on the permittivity of the cells [3]. However, these techniques are not valid in the case of high cell densities, such as those encountered in fed-batch bioreactors. Under the increasing pressure made at the level of process validation by the regulation agencies, the process analytical technology initiative (PAT) has been established in order to increase the robustness of bioprocesses. This initiative implies notably the design of new online sensors in relation to important process variables, such as cell density and viability. A lot of efforts have been initially made for the development of a capacitance probe [32], but at this time, most of the researches are focused on two technologies, i.e., optical analysis [20, 26, 28] and spectroscopy [24]. Since some spectroscopy-based methods allows for the determination of biomass concentration, these techniques are mainly focused on the excretion of metabolites in the extracellular medium and do not take into account physiology-related parameters such as cell viability. On the other hand, significant advances have been performed at the level of the development of the optical-based method in order to increase both the throughput (i.e., number of events analyzed per second) and the representativeness of these methods (i.e., possibility for the simultaneous measurement of cell concentration and viability). Among these optical methods, the focused beam reflectance method (FBRM) allows for the analysis of a large amount of particles within a second [20]. This method is based on the detection of backscattered laser light and the time of flight (TOF) of the beam. However, only elongated particles can be efficiently detected by this way and particles like spherical-shaped bacteria cannot be analyzed. In this work, we have investigated the use of a three-dimensional optical reflectance method (3D-ORM). Unlike FBRM, the excitation beam can be moved in three dimensions, giving access to the analysis of spherical particles. This method has been integrated in a sterilizable probe that can be directly inserted in a bioreactor port in order to give real-time measurement. In addition, both cell density and cell viability can be analyzed simultaneously. The simultaneous measurement of these two parameters can also be performed by using a dark-field in situ microscopy probe, but this apparatus cannot be heat sterilized [31]. The efficiency of the 3D-ORM probe will be tested for the measurement of cell viability and density during a fed-batch culture of *Escherichia coli*. Our attention will be particularly focused on the modulation of the viability parameters. Indeed, cultivation-independent methods lead to a binary vision of the concept of viability, i.e., cells are either cultivable or not. A lot of researches have been done in order to

highlight the frontier between a live microbial cell and a dead one and until now, no parameters are available in order to decipher these states [2, 8]. There is, thus, a need for new approaches in order to parameterize the different stages involved in the passage of microbial cells from viability to death. The 3D-ORM probe will be used in this context and the results will be compared to flow cytometry.

Methods

Strain and bioreactor operating conditions

Escherichia coli K12 MG1655 bearing a reporter plasmid with a *fis* promoter followed by the *gfpAAV* gene and a chloramphenicol resistance gene was throughout this work. This strain was maintained at −80 °C in working seeds vials (2 mL) in solution with LB media and with 40 % of glycerol. Precultures and cultures were performed on a defined mineral salt medium containing (in g/L): K_2HPO_4 14.6, $NaH_2PO_4\cdot2H_2O$ 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, chloramphenicol 0.05. Thiamin and kanamycin were sterilized by filtration (0.2 μm). The medium was supplemented with 3 mL/L of trace solution, 3 mL/L of a $FeCl_3\cdot6H2O$ 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 contains (in g/L): $CoCl_2\cdot H_2O$ 0.74, $ZnSO_4\cdot7H_2O$ 0.18, $MnSO_4.H_2O$ 0.1, $CuSO_4\cdot5H_2O$ 0.1, $CoSO_4\cdot7H_2O$ 0.21. Before operating in a bioreactor, a precultivation step was performed in 100 mL of the above mentioned medium in a baffled shake flask at 37 °C and under orbital shaking at 160 rounds per minute. Fed-batch cultures were performed inlab-scale stirred bioreactors (Biostat B-Twin, Sartorius with a total volume of 3 L; initial working volume of 1L; final working volume of 1.5 L; mixing provided by a standard RTD6 rushton turbine). The bioreactor platform comprises two cultivation vessels in parallel monitored and regulated by the same control unit (remote control by the MFCS/win 3.0 software). Experiments were conducted in parallel by considering a culture performed in the classical stirred vessel (well-mixed condition) and another one conducted with the stirred vessel connected to a recycle loop (scale-down condition). This last version was used in order to reproduce heterogeneities expected in large-scale bioreactors. The scale-down reactor arrangement is 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 was determined by tracer experiments (data not shown) indicating a mean residence of 36 s and a standard deviation of 10 s. This value is similar to those measured in other studies and corresponds

to typical value of circulation time encountered in large-scale bioreactors. 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. 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 1,000 rpm with a RDT6 impeller and air flow rate is set to 1 L/min at the beginning of the culture. Once fed-batch is started, agitation rate and air flow rate are progressively raised to 1,300 rpm and 2 L/min, respectively. Culture was fed with 500 mL of a solution containing 400 g/L of glucose diluted in mineral medium (see above for composition). The glucose feed profile was set-up on the basis of a DO-stat control with a set point of 30 % of dissolved oxygen from saturation.

Flow cytometry analysis

Cell permeability was determined on the basis of the propidium iodide (PI) uptake test. The PI penetrates into microbial cells exhibiting either a damaged or permeabilized membrane and gives a red fluorescence upon binding to DNA. Cells were stained with 10 μl of PI solution (1 mg/L) during 15 min at 37 °C. The PI uptake was followed on the FL3 channel of the flow cytometer (C6Flow Accury Cytometer). An amount of 30,000 events were collected for each analysis (a threshold of 80,000 was set-up on the FSC channel in order to exclude the background). Flow cytograms FL3/FL1 were analyzed by the CFlowPlus Analysis Software. The GFP signal was recorded on the FL1 channel (excitation wavelength: 488 nm; emission wavelength: 530 nm).

Three-dimensional optical reflectance method (3D-ORM)

The probe used in this work in order to monitor the viability and cell density is based on an optical reflectance method (Fig. 1). This method has been previously applied in order to characterized emulsions and crystals in different industrial processes. The 3D-ORM probe is equipped with a highly efficient focus allowing for scanning precisely microbial cells entering the multiple focal planes surrounding the probe. The 3D ORM SMF system comprises a laser beam with the intensity inferior to 5 mW. The inlet of the laser beam into the probe is accomplished by a 12.8 μm single-mode fibre. The separation of incoming and outgoing signals is realized with a fibre optic coupler. As a result of the rotation of the optical system and the formation of the dynamic focal point, the
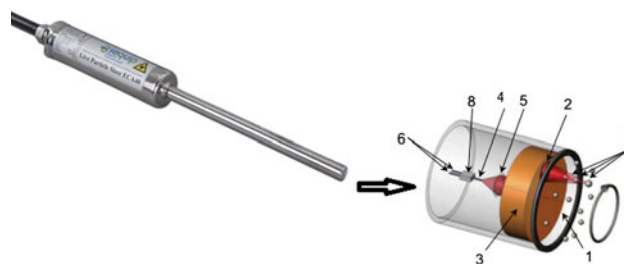

Fig. 1 Schematic representation of the 3D ORM probe: *1* = sapphire window, *2* = focusing lens, *3* = rotating optical system, *4* = fiber *5* = coupling in lens, *6* = single mode fibre, *7* = dynamic focus *8* = fibre optic coupler

imaging of a spiral path with the diameter of 8.5 mm takes place. The coupling out of the reflected signals from the probe is realized with the incoming single-mode fibre. The small cross section of such fibres ensures that only particles directly or in close proximity of the focal point are detected. The reflected signals are perceived as pulses per time in the following photomultiplier of the particular measurement systems. On the basis of reflected light, the OBF and COP parameters are determined. The probe can be mounted directly on a DN25 bioreactor port and is heat sterilizable (internal parts containing electronics can be removed for this purpose). More information can be gained from the constructor [21].

Immunoblot analysis of culture supernatants

Samples coming from well-mixed and scale-down bioreactors were centrifuged at 13,500 rpm for 5 min and filtered on 0.2 μm cellulose membrane in order to remove cells. Proteins coming from the supernatant were separated (with a working volume of 20 μl) on 30 % polyacrilamide gels (Biorad) by standard SDS-PAGE procedures. After SDS-PAGE, proteins have been transferred to a hybond PVDF membrane. Non-specific binding sites were avoided by immersing the membrane in 5 % non-fat dried milk, 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 1:10,000 dilution of secondary antibody antigoat IgG (sc-2,768) in order to detect the band corresponding to GFP. The membrane was finally washed 3 times for 15 min with PBS, 0.1 % Tween 20. Detection of the secondary antibody was performed using ECL plus detection system (Amersham) by exposition to an X-ray film. In order to check for possible implication of cell lysis at the level of protein leakage, total nucleic acid content of the supernatant was analyzed (Hoescht method). The total protein concentration of these samples was measured by the Folin method.

Results and discussion

Global bioprocess trends and OBF/COP profiles: impact of sub-lethal injuries

A set of cultures has been performed in well-mixed, as well as in scale-down conditions. It is generally recognized that large-scale bioreactors are strongly heterogeneous. The impact of these environmental heterogeneities on microbial physiology has been thoroughly investigated [13, 23], but the biological response of a cell facing environmental fluctuations is still not well understood. More particularly, the dynamics of these stress responses is not known due to the lack of appropriate on-line sensors [25]. The 3D-ORM probe will, thus, be used in this context since the follow-up of the cultivation test carried out in well-mixed and scale-down conditions exhibits different trends at the level of the parameters recorded by this system (Fig. 2). Cell growth is very similar for the two operating conditions during the first 5 h. After this period, cell concentration is higher in the well-mixed bioreactor than in the scale-down bioreactor. This phenomena can be linked to the heterogeneities met by cells in the scale-down reactor resulting in the production of by-products (e.g., acetate and formate) to the detriment of biomass yield [11, 13]. These trends are relatively well represented by the COP profile. Indeed, COP seems to be effectively correlated to the evolution of cell density inside the bioreactors, with a COP value more elevated in the case of the well-mixed bioreactor, which is in accordance with the reported off-line values for cell dry weight (in our case, the COP signal has a maximal value corresponding to approximately 50 g/L of cell dry weight, but the ORM probe can be parameterized in order to take into account higher cell densities). The OBF signal follows the same trend, i.e., is more elevated in the case of the well-mixed bioreactor than in the case of the scale-down one. It means, thus, that cell viability is higher in the scale-down conditions since light reflective capacities of healthy cells are lower than those of dead cells. This result is in accordance with the general trends observed in scale-down bioreactors. Indeed, even if biomass yield is affected by the scale-down conditions, previous studies have reported a higher cell viability in heterogeneous bioreactors [13, 19]. During high cell density cultures, microbial cells are progressively exposed to increasing substrate limitation conditions. E. coli has developed several nutritional strategies in order to cope with nutrient limitations [29]. Among these strategies, E. coli is able to modulate the amount of porins and cell membrane transporter in order to increase its affinity for substrate [14, 15]. The side effect of this strategy is a global increase of cell membrane permeability leading to cells more sensitive to stress factors. In scale-down conditions, microbial cells are sequentially exposed
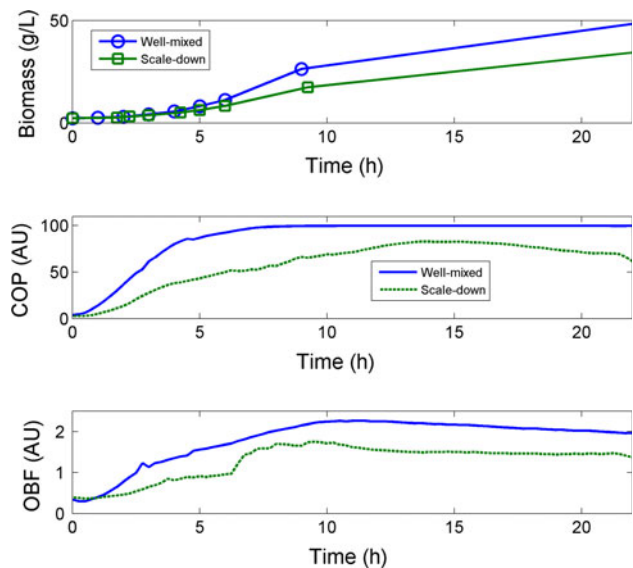

Fig. 2 Evolution of cell density, COP and OB signals during a fed-batch culture of *E. coli* carried out either in well-mixed or scale-down reactor. COP and OBF signals are expressed in arbitrary units (AU)

to substrate excess. It can be supposed that these localized substrate excess are enough to relieve the nutrient limitation signal leading to cells exhibiting a lower cell membrane permeability and thus able to better cope with stressors. This hypothesis has been partially validated in our previous works [6, 9, 10]. These observations highlight the need for appropriate methods in order to estimate cell viability in process-related conditions. However, as stated in the introduction section, the concept of cell viability has not been quantified so far [8] and new methods are needed for this quantification. In the next section, the OBF signal will be compared with flow cytometry results.

Monitoring of PI uptake by flow cytometry

Samples coming from the different bioreactor configurations have been analyzed by flow cytometry. As stated in the introduction section, flow cytometry is becoming widely used as a cultivation-independent technique for cell viability determination [17]. Traditional, cultivation-based, microbiological methods based on plate counting leads only to an estimation of cell viability on a binary basis, i.e., cells are defined to be either cultivable or non-cultivable. Flow cytometry is able to quantify the intensity of cell injuries and is then able to deliver a modulated estimation of cell viability. In our case, this estimation has been based on propidium iodide (PI) uptake. According to this method, when the cell membrane is compromised, PI enters and binds to DNA. Cell viability can thus be estimated by quantifying the red fluorescence related to PI. Cell membrane is indeed a good indicator of viability, even if the frontier between live and dead has not been parameterized
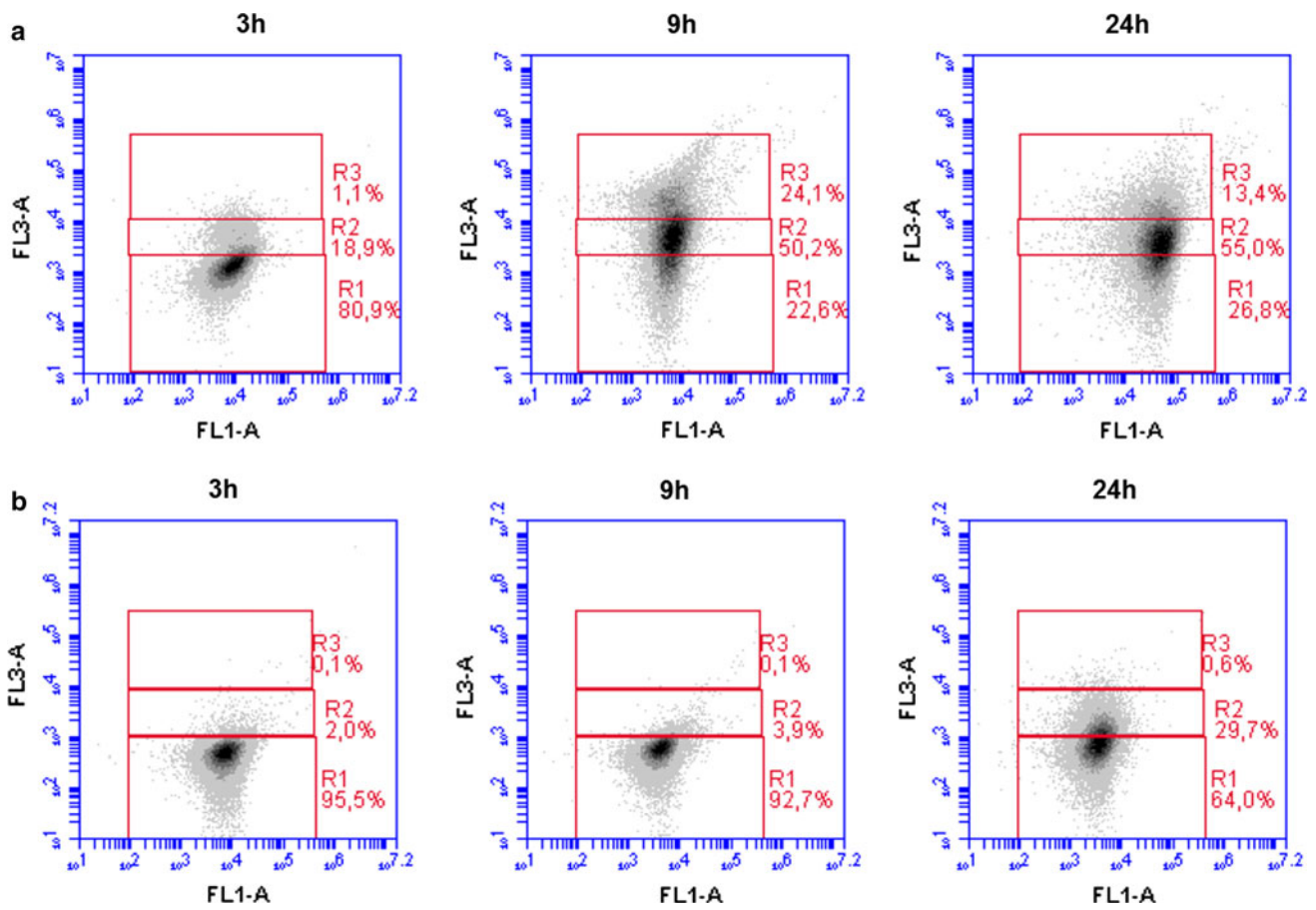
Fig. 3 Evolution of PI-uptake during culture carried out either in **a** well-mixed bioreactor **b** scale-down reactor. Representative results of two independent experiments

so far [8]. In our case, flow cytometry analyses based on PI-uptake show very different profile for cells extracted from well-mixed and scale-down bioreactor (Fig. 3). Indeed, three sub-populations have been delimited, i.e., $R1$ corresponding to healthy cells, $R2$ corresponding to intermediate cells and $R3$ corresponding to damaged cells. The subpopulation $R3$ has not been observed during the cultures carried out in scale-down bioreactor, suggesting that cell viability is higher in bioreactors exhibiting mixing defects. Anew, it is quite surprising since bioreactor heterogeneities are intuitively linked with a decrease of the bioprocess performances. This observation is very similar to those made previously at the level of the OBF parameter and has been previously reported by another research team on the basis of experiments carried out in well-mixed, scale-down as well as in a 20 m^3 industrial bioreactor [13, 19]. For the scale-down and industrial bioreactors, cell viability remained close to 100 %, whereas for the well-mixed lab-scale bioreactor, viability recovery dropped below 85 %. These observations are very interesting, since PI-uptake dynamics is correlated to the OBF signals. Indeed, the OBF signal is always more elevated in the case of the well-

mixed than for the scale-down bioreactor (Fig. 2). This effect is has been confirmed by the PI-uptake test with a sub-population $R2$ being present during the whole culture carried out in well-mixed conditions. After 9 h of cultivation, the OBF signal is maximal and is correlated with the observation of a $R3$ subpopulation in the well-mixed bioreactor corresponding to damaged cells. For the scale-down bioreactor, the OBF signal remains lower during the culture, this observation being linked with the absence of either a $R2$ or a $R3$ subpopulations for these operating conditions, except for the last stage of the culture for which the $R2$ subpopulation becomes visible. This observation leads to the conclusion that the OBF signal seems to be affected differently according to the type of cell injuries. Indeed, the R2 subpopulation corresponds to permeabilized cells keeping their ability to grow. This fact has been confirmed by cell sorting experiments of the cells located in $R2$ and their cultivation on a petri dish. After incubation, these cells exhibit almost 100 % of recovery confirming that they have kept their ability to divide (the same observation has been done for the $R1$ region). On the other hand, cells located in the $R3$ region exhibit damaged

membranes and have lost their ability to grow on agar medium. It seems, thus, that the OBF signal is less sensitive to the appearance of the $R2$ subpopulation than the $R3$ one. The intensity of the OBF signal is thus modulated in the function of the sub-lethal or lethal nature of the stresses encountered by the microbial cells. It is important to point out that mainly sub-lethal stresses have been considered so far, i.e., no significant loss of cell viability have been observed during the cultures carried out either in well-mixed or in scale-down conditions according to the proportion of cells located in $R3$. The impact of lethal injuries will be more thoroughly investigated in a next section.

Effect of lethal injuries

At this stage, only sub-lethal injuries have been investigated, i.e., injuries coming from intensive process conditions. These injuries affect cell vitality (protein synthesis rate, intracellular enzymatic activities,…) but not necessarily cell viability. In the context of this study, it will be interesting to test if the 3D-ORM probe can efficiently detect the effect of lethal injuries, such as an increase of temperature, on microbial cells. A new fed-batch culture has been performed in well-mixed conditions and the temperature has been progressively raised at the end of the process (Fig. 4). Temperature has been progressively raised according to the following profile: 10 min at 50 °C followed by 10 min at 60 °C followed by maintained the culture at 70 °C for the remaining of the culture. It can be seen that the OBF parameter rises in response to heat stress. After a short period when temperature is raised, the COP parameter drops, indicating that cell density decreases in front of heat stress. Flow cytometry has been also
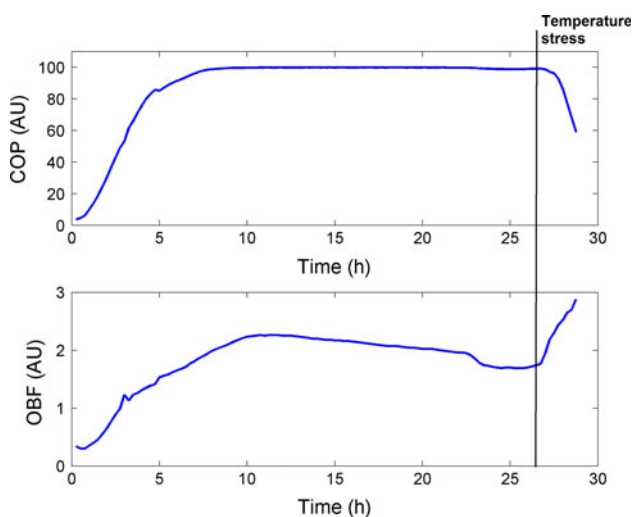

Fig. 4 Effect of a rise of temperature on the COP and OBF parameters during a fed-batch culture of *E. coli* in well-mixed conditions. Temperature has been raised to 45 °C after 26 h of culture

performed in order to correlate the OBF parameter (Fig. 5). A first sample has been collected before performing the heat stress as a standard (Fig. 5a). Several other samples have been collected after 10 min of exposure to the three different heat stress intensities (Fig. 5b–d). It can be logically observed seen that the amount of cells located in the $R3$ (damaged) region increases when temperature is raised. *E. coli* is not able to grow above 46 °C and the different temperatures considered in the heat stress procedure can be considered as lethal. However, some differences can also be noticed between these different lethal temperatures. First the repartition of cells between the different regions is not the same for the three temperature values. More particularly the cells located on the $R2$ (intermediate) region are always observed for all the temperatures tested, indicating that some cells among the population are more resistant to heat stress. On the other hand, the sharp increase observed at the level of the OBF parameter is correlated to the increase of the amount of cells located in the $R3$ region. As stated before, this region corresponds to damaged cells; and it confirms our previous hypothesis that the OBF parameter is strongly affected by lethal injuries and to a lower extent by sub-lethal injuries. Second, a modulation at the level of the FL1 channel of the flow cytometer can also be observed. Indeed, our *E. coli* cells carry a GFP reporter system that is constitutively expressed. In this case, The GFP signal can, thus, be used as an indirect reporter of cell rupture during heat stress. Decrease of the GFP signal has been observed for the heat stress carried out at 60 °C and to a larger extent to the one carried out at 70 °C but not for the experiment at 50 °C. This result is in accordance with the COP profile recorded since it decreases only when 60 °C is reached in the bioreactor.

Correlation of cell viability with protein leakage

Previous studies involving the use of microbial cells expressing GFP have revealed that this molecule can be used as an indirect parameter for cell membrane permeability [7, 9]. In the case of *E. coli*, the outer membrane can be affected by the stresses encountered in bioreactors, leading to the release of intracellular compounds into the extracellular medium [4, 27]. In this context, GFP leakage has been observed for different GFP whole cell biosensors in different bioreactor operating conditions [6]. In order to confirm this hypothesis in the context of this work, supernatant have been analyzed throughout the cultures carried out in the two bioreactor configurations (Fig. 6). Immunoblot analysis reveals that the supernatants coming from the well-mixed bioreactor contain significantly more GFP than those extracted form a scale-down reactor. This effect has to be put directly in relation with the PI-uptake tests showing that cells cultivated in homogeneous
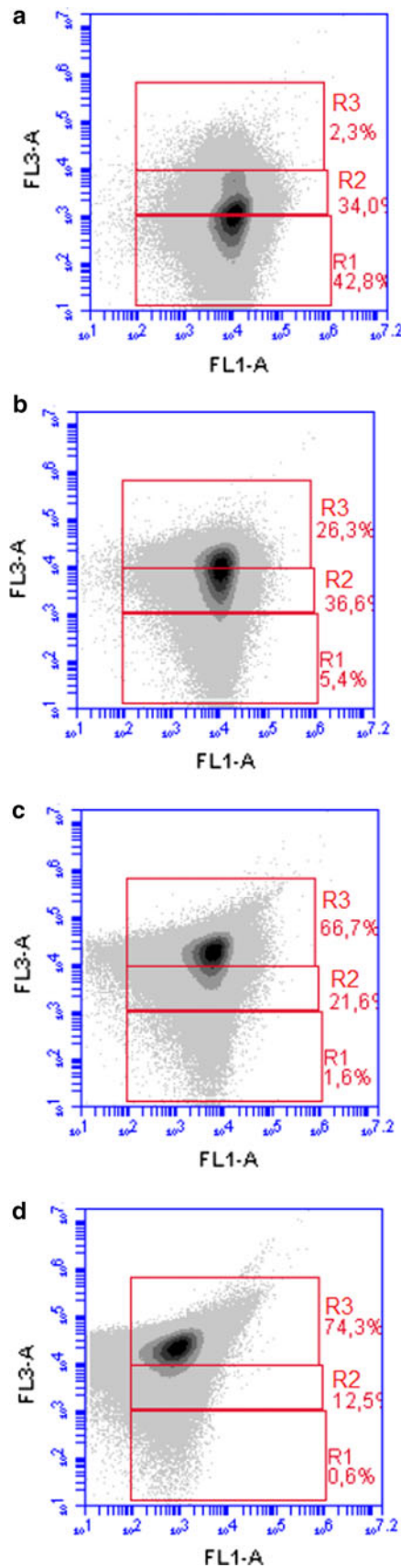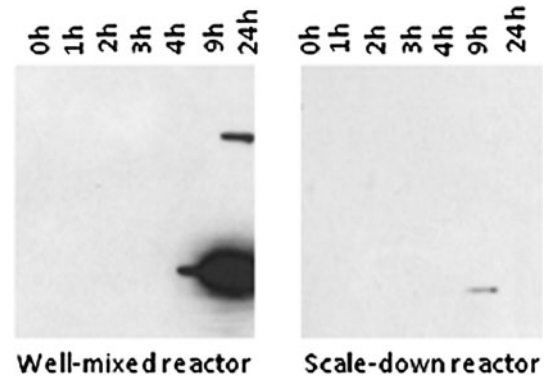
Fig. 6 Immunoblot analyses of the supernatant coming from the well-mixed and the scale-down reactor. Analyses have been carried out with antibody targeted against GFP. The lower band corresponds to GFP with a molecular weight of 27 kDa. The higher band that can be visualized in the case of the well-mixed reactor after 24 h of culture corresponds to GFP aggregates

conditions tend to be more permeable and are subject to protein leakage. This result displays another aspect of the sub-lethal injuries occurring in process-related conditions.

Conclusions

The ORM probe has allowed us to monitor in real-time the evolution of cell viability (OBF) and cell density (COP) in two different bioreactor configurations. The COP can be directly linked with cell density but the description of the OBR parameter necessitate more experiments since microbial viability is a concept that is actually not well defined and parameterized [8]. It has been shown in conjunction with flow cytometry experiments that the OBF can efficiently describe the effects of sub-lethal and lethal injuries on microbial cells. Indeed, the viable and non-viable states are separated by a continuum of "intermediate" states in which the concept of viability is much more complex to define. However, both the flow cytometry analyses and the 3D-ORM probe release a signal which is modulated between these two states. The ORM probe is based on measurement carried out at the single cell level. However, the multi-capture system analysis accompanying the probe has not been configured yet to take into account population segregation and the OBF and COP parameters measured in this work have been averaged over the whole population. The extension of the MCSA software to the analysis of single cells would be a promising perspective since the single cell methodology reported so far does not allow time-resolved cell analysis [16]. In conclusion, the efficiency of the ORM

probe has been assessed in the case of fed-batch cultures of *E. coli*. The main interest of this probe relies on the combination of three advantages which are not encountered simultaneously with other available technologies, i.e., the simultaneous measurement of cell density and viability, the heat resistance of most of the components allowing in situ sterilization and the possibility for the acquisition of single cell measurements. It will also be useful to test the effectiveness of this probe in bioprocesses where cell viability is critical, such as cell cultures [22].

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