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Stochastic Models To Study the Impact of Mixing on a Fed-Batch Culture of *Saccharomyces cerevisiae*

F. Delvigne,* A. Lejeune, J. Destain, and P. Thonart

Centre Wallon de Biologie Industrielle, Unité de Bio-industries, Faculté Universitaire des Sciences Agronomiques de Gembloux, Passage des déportés, 2, B-5030 Belgium

The mechanisms of interaction between microorganisms and their environment in a stirred bioreactor can be modeled by a stochastic approach. The procedure comprises two submodels: a classical stochastic model for the microbial cell circulation and a Markov chain model for the concentration gradient calculus. The advantage lies in the fact that the core of each submodel, i.e., the transition matrix (which contains the probabilities to shift from a perfectly mixed compartment to another in the bioreactor representation), is identical for the two cases. That means that both the particle circulation and fluid mixing process can be analyzed by use of the same modeling basis. This assumption has been validated by performing inert tracer (NaCl) and stained yeast cells dispersion experiments that have shown good agreement with simulation results. The stochastic model has been used to define a characteristic concentration profile experienced by the microorganisms during a fermentation test performed in a scale-down reactor. The concentration profiles obtained in this way can explain the scale-down effect in the case of a *Saccharomyces cerevisiae* fed-batch process. The simulation results are analyzed in order to give some explanations about the effect of the substrate fluctuation dynamics on *S. cerevisiae*.

Introduction

In stirred bioreactors, microorganisms are submitted to concentration fluctuations (substrate, oxygen, pH, etc.) with an intensity and a frequency depending on the operating scale. In a scale-up procedure, it is thus important to characterize the concentration gradient, as well as the way in which microorganisms are exposed to these gradients. We propose two modeling approaches based on the compartment principle. This kind of model is generally expressed by a set of ordinary differential equations (*1*). The originality here lies in the stochastic expression of these models, this approach providing new potentialities. Indeed, by using this model, fluid mixing and particle circulation can be simulated with the same model structure. In this study, the descriptive analysis of a fed-batch culture of *Saccharomyces cerevisiae* (microbial growth and glucose consumption) performed in a small-scale stirred bioreactor (20 L) and in a scale-down reactor comprising a mixed part and a nonmixed part will be followed by two structured hydrodynamic modelization procedures in order to give more insight about the mixing impact on *S. cerevisiae* productivity. In particular, the study will be focused on the microorganism−fluid mixing interactions. The scale-down reactor (SDR) used in this study makes it possible to obtain at a small scale the environment heterogeneity encountered in large-scale bioreactors. This kind of system has been previously used in several applications (*2*−*7*).

The originality here lies in the fact that the microorganism−environment interactions will be entirely modeled by a stochastic approach. The concentration gradient developed when running in fed-batch mode will be represented by a Markov chain, which is a special kind of stochastic model. The Markov chain has been previously developed in the case of a stirred bioreactor and has shown a lot of potentialities compared with classical deterministic compartment models (*8*). The microorganism circulation paths inside the bioreactor are modeled by a stochastic model involving the same transition matrix as for the Markov chain. The superimposition of the gradient field and the microorganism circulation paths leads to the concentration profiles experienced by the microbial cells. These concentration profiles are discussed in function of the biomass yield and the operating conditions of the fermentation tests performed in scale-down reactor.

* To whom correspondence should be addressed. Tel: ++32 81 62 23 11. Fax: ++32 81 61 42 22. Email: bioindus@fsagx.ac.be.

Materials and Methods

Cultures in Stirred Bioreactor and in Scale-Down Reactor (SDR). *Saccharomyces cerevisiae* (MUCL 43341; misapplied name: *boulardii*) strain is stored at −80 °C. Cultures are performed in 20 L stirred bioreactors ($D = 0.22$ m) (Biolaffite-France) equipped with a RDT6 rushton turbine ($d = 0.1$ m). The regulation of the culture parameters (pH, temperature, etc.) is ensured by a direct control system (ABB). Dissolved oxygen level is maintained above 30% of saturation by modulating the stirrer speed. For the scale-down tests, the previously described stirred vessel is connected to a glass bulb of 1 L (diameter 0.085 m; length 0.25 m). During fermentation runs, the broth is continuously recirculated between the stirred vessel (mixed part) and the glass bulb (nonmixed part) by a peristaltic pump (Watson Marlow 323S/D). In the case of the scale-down reactor (SDR) experiments, glucose addition is performed at the level of the nonmixed part. In the case of the classical bioreactor experiments, glucose is fed by the top of the stirred vessel. Glucose addition is controlled by an exponential feeding algorithm according to the equation $F = F_0 \exp(\mu t)$, with F being the feed flow rate (m^3/s), F_0 the initial feed flow rate (m^3/s), μ the microorganism growth rate (h^{-1}), and t the culture

time (h). The two parameters $\mu = 0.005$ min^{-1} and $F_0 = 0.086$ mL/min are calculated from growth data of *S. cerevisiae* in a batch bioreactor.

Tracer Tests: Inert Tracer and Biological Tracer. Two kinds of experiment have been performed using the SDR: tracer experiments in classical mode and in "open system" mode, that is, with the SDR reactor running in continuous mode. Liquid is injected at the top of the mixed section and is extracted at the outlet of the nonmixed section at a given flow rate. Tracer tests are performed by injecting a short pulse (injection time <3 s) at the top part of the mixed section.

Two kinds of tracers have been used: an inert tracer (saturated NaCl solution) and a biological tracer (suspension of microbial cells). Biological tracer experiments are performed by injecting a pulse of a solution containing stained cells in the bioreactor. The cells are stained with a fluorescent dye (Vybrant CFDA SE cell tracer kit V-12883), which facilitates the detection by epifluorescent microscopy. The staining protocol consists of performing a preculture in a 500 mL Erlenmeyer flask in order to obtain the required amount of biomass for further staining. An aliquot of the preculture is centrifuged (5 min at 4000 rpm). The precipitate is washed with 10 mL of sterile PBS buffer (NaCl 8 g/L; KCl 0.2 g/L; K_2HPO_4 1.44 g/L; KH_2PO_4 0.24 g/L; adjusted to pH 7.5 with K_2HPO_4 and KH_2PO_4). Three successive centrifugation/washing sequences are performed. After this, microbial cells are stained by addition of 1 mM of CFDA SE (carboxyfluorescein diacetate succinimidyl ester), followed by an incubation during 3 h at 30 °C. After incubation, the solution is centrifuged and the precipitate is washed with PBS buffer. When performing a biological tracer test, 5 mL of the stained cells suspension (2×10^8 cells/mL) are poured at the level of the nonmixed part of the SDR, whereas samplings are taken at the level of the mixed part. Cells are directly counted by fluorescent microscopy. For each sample, three aliquots of 10 μL each are placed on a microscopic plate for further counting. For each aliquot, three counts are performed for three widths of the microscopic plate. Mean and standard deviation are calculated for each sample.

Mathematical Models. The structured or compartment models can be divided in two categories: stochastic or deterministic. Stochastic models have a discrete nature and are generally used when considering small populations where heterogeneities and fluctuations are important. Up to now, they have been especially used to simulate particulate mixing processes (*9*). Here, these stochastic models are employed to simulate microbial cell circulation in bioreactors and, in a second time, are adapted to simulate fluid mixing. Deterministic compartment models are used to represent fluid mixing and are mathematically expressed by continuous ordinary differential equations. In this study, a deterministic model will be used to simulate the mixing of a salt tracer pulse and as a reference for comparison with the performances of stochastic models for fluid mixing. The goal here is to find a stochastic model that can be used both in the case of particle flow and fluid mixing, to simulate concentration profiles experienced by microorganisms travelling in bioreactors. It implies that the relation between microbial cells circulation and fluid mixing in bioreactor is to be carefully examined.

Model 1. Stochastic and Deterministic Expression from a Simplified Structure. A simplified model structure is used at first to validate the equivalency assumption for the fluid mixing and microorganisms circulation processes in bioreactors and to validate the assumption that the biomass yield loss in SDR can be attributed to the passage of microorganisms at the level of the nonmixed part of the reactor.

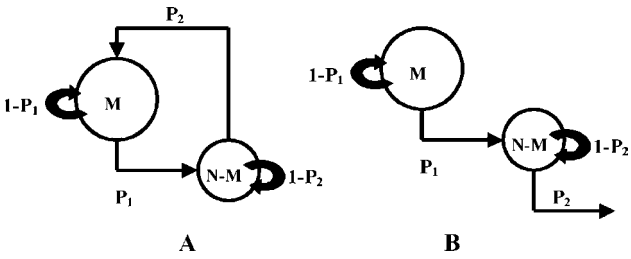

Figure 1. Simplified structures for the SDR model (M = mixed section; N-M = nonmixed section) with the corresponding transition probabilities: (A) classical SDR (closed system); (B) open system. P = probability to shift from a compartment to another; 1-P = probability to remain in the actual compartment.

Figure 1 presents the simplified model structures. The nonmixed part of the SDR has been represented by a single compartment. This approach can be validated by the high dispersing effect of the reactor, which strongly perturbs the plug-flow inside this part of the reactor. The tracer experiments relative to the "closed" SDR have been first considered. The probabilities between the two compartments have been calculated from the real flow rate by the equation $P = Q/V$ (where Q represents the flow rate in m^3/s and V the compartment volume in m^3). This simplified model will be used in its stochastic form to express particles dispersion in the SDR. This is numerically done by comparing random numbers and the respective transition probabilities that allow displacement from one compartment to another. A deterministic expression with the same model structure has also been used to characterize fluid mixing in the SDR. The deterministic compartment model has been widely used in the area of fluid mixing in stirred bioreactors (*1*, *10*−*13*). Its principle is based on the tank-in-series concept, each compartment being assumed to be perfectly mixed. The mathematical expression of the model involves a set of ordinary differential equations representing the mass balance for each compartment considered. The deterministic compartment model will be used in this study as a reference for the stochastic expression of the simplified model structure. The same model structure (i.e., the same well-mixed compartments arrangement) presented in Figures 1 and 2 can be used, but the mathematical expression is fundamentally different. Indeed, in the case of a deterministic formulation, the probabilities are replaced by flow rates between compartments. The mathematical expression of the model comprises a set of ordinary differential equations (ODEs) for the mass balance for each compartment. For example, in the case of the model presented at Figure 1A, the mathematical expression is

$$\frac{dC_M}{dt} = Q_{IN} \cdot C_{NM} - Q_{OUT} \cdot C_M \quad (1)$$

$$\frac{dC_{NM}}{dt} = Q_{IN} \cdot C_M - Q_{OUT} \cdot C_{NM} \quad (2)$$

where C_M is the tracer concentration in the mixed compartment, C_{NM} is the tracer concentration in the nonmixed compartment, Q_{in} is the flow rate (m^3/s) entering in the compartment, and Q_{out} is the flow rate (m^3/s) from the compartment. The set of ODEs is numerically resolved by a Runge−Kutta algorithm.

Model 2. Complex Structure Allowing a Better Resolution on the Hydrodynamic Mechanisms in SDR. The second model structure used in this study is more elaborated and allows a higher resolution at the level of the concentration gradient and

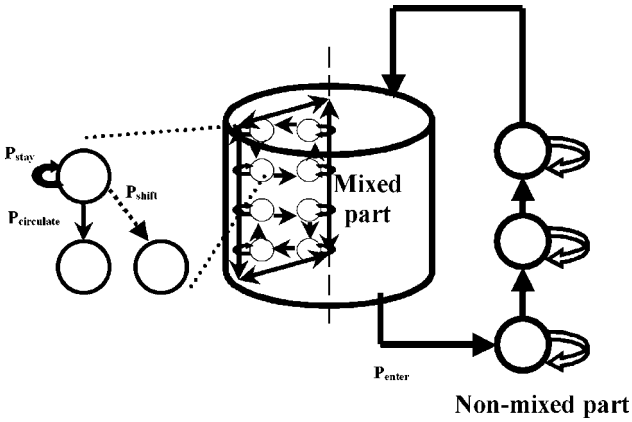

Figure 2. Complex three-dimensional model structure. P_{stay} = probability to stay in the actual compartment; $P_{circulate}$ = probability to be convoyed by the impeller circulation flow; P_{shift} = probability to shift from the circulation loop; P_{enter} = probability to enter in the nonmixed part.

the microorganism circulation path. The expression of this model is stochastic, but the algorithm differs according to whether this is fluid mixing or particle circulation that must be simulated. These procedures will be further described in detail. The structure of the model is presented at Figure 2. The stirred vessel constituting the mixed part of the SDR has been modeled by using eight vertical planes, each of them comprising eight compartments. The interconnections between compartments are in accordance with the radial flow developed by the rushton turbine used for the experiments.

Two possibilities can be envisaged. First, the direct translation of the equivalent deterministic compartment model in a probabilistic context. In this case, the transition probabilities are calculated from the ratio of flow rates allocated to a given compartment. This approach has been tested in a previous study that has shown the great potentialities of Markov chain models in the case of fluid mixing (*8*). Second, and it is this option that has been chosen, we can completely adapt the model structure by considering four kinds of probabilities: the probability to remain in a given circulation flow loop, the probability to remain in the same compartment, the probability to shift to another plane, and the probability to switch from the main flow loop (this method has been proposed in ref 14). The circulation probability can be estimated from dimensionless correlation by the circulation flow rate (m^3/s):

$$Q_c = N_{qc} \cdot N \cdot d^3 \tag{3}$$

where N_{qc} is the dimensionless circulation number having a constant value of 1.51 in the turbulent flow regime for a rushton impeller with six blades, N is the impeller speed (s^{-1}), and d is the impeller diameter (m), and

$$P_{circulation} = \frac{Q_c}{V_{compartment}} \cdot \Delta t \tag{4}$$

where $V_{compartment}$ is the respective volume occupied by a compartment in the model (m^3), and Δt is the time step chosen to run the simulation (in our case $\Delta t = 1$ s).

Two components are involved in the fluid mixing process in stirred vessel: a circulation component and a turbulence component. The circulation component has been described by a probability expressed in eq 4. For each compartment of the model, a particle or a fluid element has a probability to follow the global circulation pattern induced by the impeller but can also switch from this flow pattern under the influence of the turbulence forces. This is expressed by the following equation:

$$P_{switch} = P_{tangential} = P_{stay} = \frac{1 - P_{circulation}}{3} \tag{5}$$

where P_{stay} is the probability to stay in the actual compartment, $P_{tangential}$ is the probability to shift to another plane, and P_{switch} is the probability to shift from the main circulation flow loop. The assumption has been made that all of these probabilities have an equal value. This assumption can be accepted since it makes it possible to match the experimental tracer curves (as will be shown in Figure 6B).

In our model, particles are allowed to switch diagonally from the flow loop in the same plane or to switch perpendicularly from the actual plane to the adjacent planes. All of the calculated transition probabilities are stored in a transition matrix **T** that will be used to run simulations. The stochastic simulation procedure is the same as the one described for the simplified model and consists to generate a random number that governs the displacement of a particle according to the respective probability of the transition matrix **T**.

The second step, after simulating particle circulation, is to describe the concentration gradient inside the reactor. This can be achieved by considering a large amount of particles, but this method is fastidious because of the important computational time required. It has also been previously shown that it is possible to use a Markov chain stochastic model to obtain the value of the concentration gradient. This approach has been previously used to model fluid mixing in stirred bioreactors (*8*). In this case, the transition matrix previously presented for the non-Markovian model is multiplied by a state vector representing the concentration in each compartment. This multiplication leads to the state vector of the system at the following time increment. The evolution of the concentration gradient in the modeled bioreactor can be expressed in this way by performing a cascade of state vector−transition matrix multiplications. The advantage of this method lies in the fact that the same transition matrix as that used for particle circulation can be used.

The Markov chain stochastic model consists of an initial state vector S_0, which is multiplied with a transition matrix **T** to give a new state, S_1. This can be written as

$$\text{for the first transition: } S_1 = \mathbf{T} \cdot S_0 \tag{6}$$

The next step involves the multiplication of the new state vector S_1 with the same transition matrix **T** until a steady-state is reached:

$$\text{for the second transition: } (S_2 = \mathbf{T} \cdot S_1) \text{ or } (S_2 = \mathbf{T}^2 \cdot S_0) \tag{7}$$

$$\text{for the } i\text{th transition: } (S_i = \mathbf{T} \cdot S_{i-1}) \text{ or } (S_i = \mathbf{T}^i \cdot S_0) \tag{8}$$

In our case, the state vector contains the tracer concentration values for all compartments.

Results and Discussion

SDR Experiments. Fed-batch cultures of *S. cerevisiae* have been performed in different bioreactor configurations. For each culture, the biomass yield has been calculated (Table 1), and the growth curves are plotted in Figure 3.

The analysis of the results presented in Table 1 reveals a clear impact of the exposure of microorganisms to glucose concentration fluctuations. Indeed, the yield coefficient Y_{xs} has a value of 0.48 in the case of the classical bioreactor (stirred bioreactor

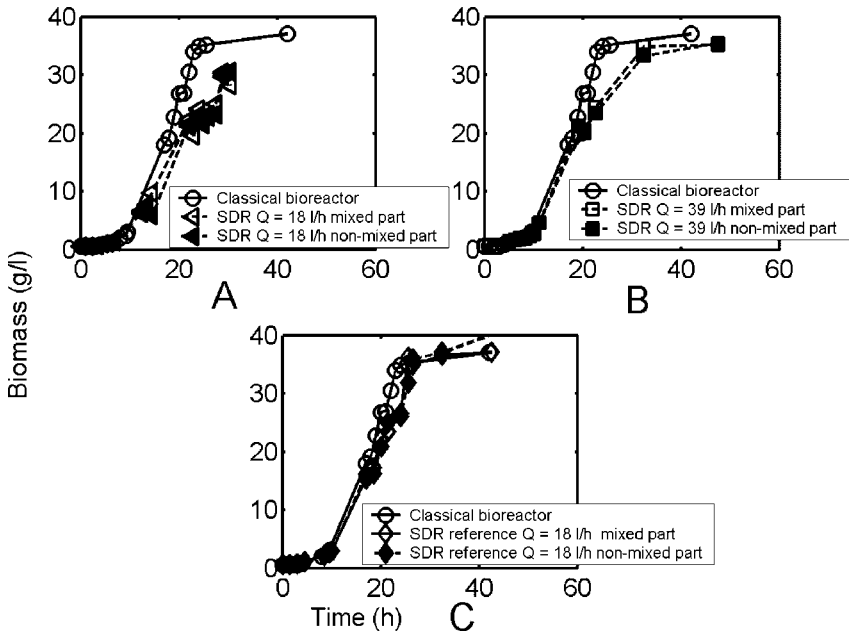

Figure 3. Comparison of microbial kinetic in a classical stirred bioreactor and in a scale-down reactor (SDR). The SDR tests have been performed by varying the recirculation flow rate (A, $Q = 18$ L/h; B, $Q = 39$ L/h) or the glucose feed location (C, SDR with $Q = 18$ L/h but with a glucose feed point located in the mixed part of the reactor instead of the nonmixed as for experiments A and B). For all of the experiments, the growth curve is compared to an "ideal" growth curve obtained in a classical stirred bioreactor.

Table 1. Biomass Yield Coefficients Obtained for Fed-Batch Cultures of *S. cerevisiae* and for Different Bioreactor Configurations

reactor	biomass yield, Y_{xs}
classicial bioreactor	0.48
reference SDR (glucose fed at the level of the mixed part)	0.47
SDR recirculation flow rate $Q = 18$ L/h	0.36
SDR recirculation flow rate $Q = 39$ L/h	0.45

without the nonmixed part). This value drops to 0.36 in the case of the SDR with recirculation flow rate of 18 L/h and to 0.45 when the flow rate is increased to 39 L/h. This drop can be attributed to the passage of microorganisms through the nonmixed part where the pH, the temperature, and the dissolved oxygen concentration can be heterogeneous and where yeast cells are exposed to flocculation.

This effect has been tested by performing a SDR test in which glucose was injected at the level of the mixed part. The results can be viewed in Figure 3C, and it can be concluded that this effect is very slight (Y_{xs} equal to 0.47 comparatively to 0.48 in the case of the classical bioreactor).

Differences between the growth curves obtained in classical and in scale-down reactors appear after approximately 20 h of cultures (Figure 3). This lapse of time includes the batch phase (4 h) and the first 15 first h of the fed-batch phase for which the glucose pulses are very spaced according to the exponential regulation of the feed flow rate. After 15 h, the pulse frequency becomes significative by comparison with the mixing time of the SDR reactor.

From the results showed at Figure 3, we can make the assumption that the loss of productivity can be attributed to the exposure of microorganisms to a fluctuating environment and more precisely to fluctuating glucose concentrations. The scale-down effect can be attributed to the liquid recirculation flow rate between the mixed and the nonmixed section of the SDR. It also depends on the nonmixed section configuration and notably on the diameter of the nonmixed section. Other authors have recorded productivity losses in function of the recirculation flow rate and the nonmixed section configuration (*5*, *6*). This assumption will be tested in the following sections by the use of a structured modeling strategy.

First Modeling Approach (Model 1): Circulation of a Set of Microorganisms inside the Bioreactors. This first modeling approach is coarse and is based on the basic assumption that the loss of productivity in SDR can be attributed to the passage of microorganisms through the nonmixed part. The explanations relative to this model can be found in Materials and Methods (model 1).

When simulating the mixing of a small amount of particles, the results are greatly influenced by the probability effect. To study the impact of this effect, several simulations have been performed by varying the number of particles (Figure 4).

Figure 4A shows that when operating with only 10 particles, variation from one simulation to another is important (represented by the standard deviation), but the standard deviation rapidly drops when the number of particles involved in the simulation reaches 50 (Figure 4B). For the cell tracking experiments, the microscopic direct counting technique involves a maximum of about 100 stained yeast cells per microscopic plate. Thus, simulations with 50 or 100 particles can be used to match the experimental results (Figure 4B and 4C show a good correspondence between simulated and experimental values). However, this number of cells does not include the entire microbial population in the bioreactor. In reality, the microbial population is important and its repartition in the bioreactor can be represented by a deterministic model or by a stochastic model involving an important number of particles (Figure 4E). This fact highlights the relation existing between stochastic and deterministic or discrete and continuous formulation of the models.

The parameters considered for the previous tracer experiments can be used to perform simulations when the system is running in continuous ("open system") mode (Figure 5). The model structure corresponding to this operating mode can be found in Figure 1B.

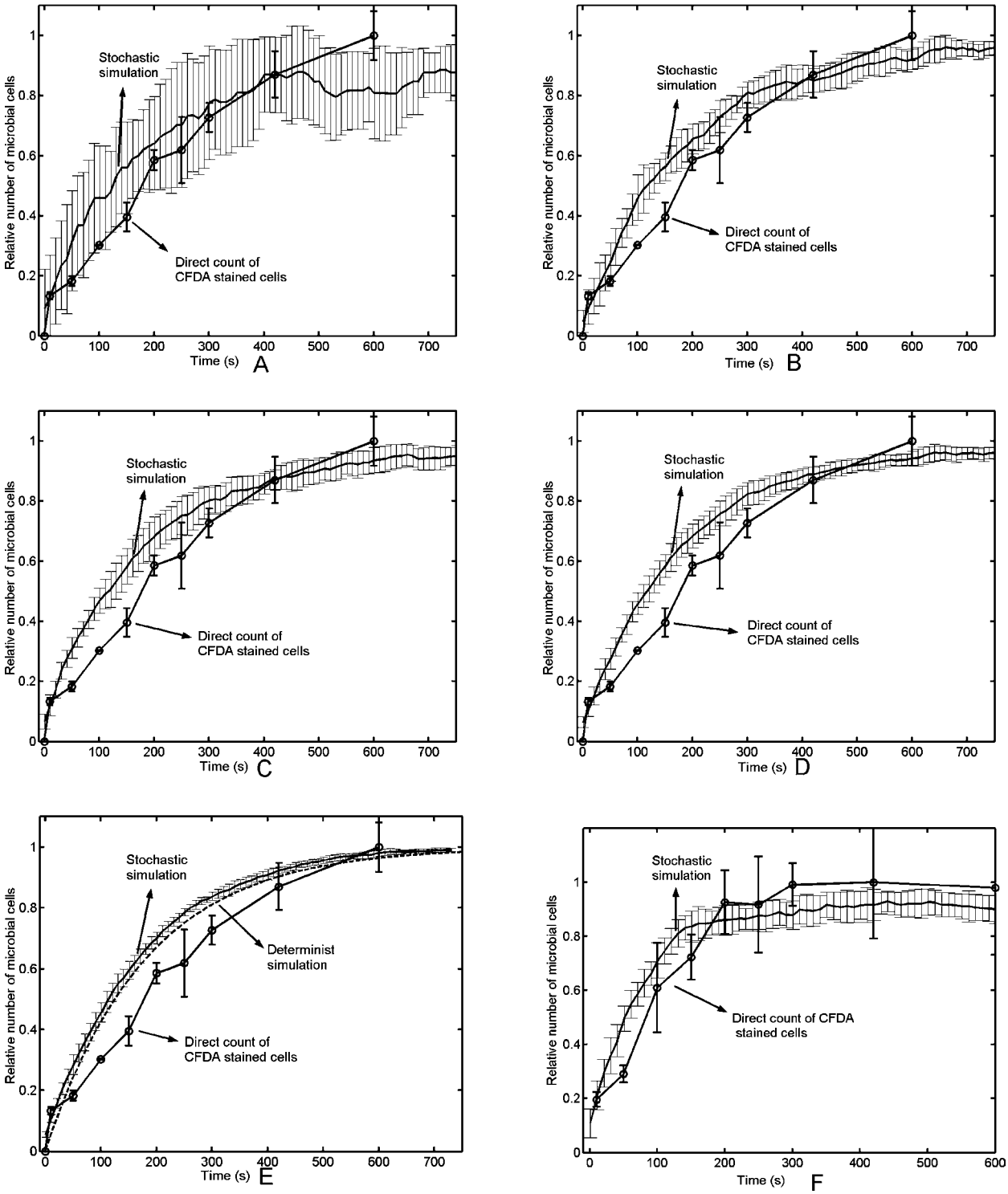

Figure 4. Stochastic simulations performed with 10 (A), 50 (B), 100 (C), 200 (D) and 500 (E) particles with a recirculation flow rate of 18 L/h, or with 50 particles with a recirculation flow rate of 39 L/h (F). The particles are starting from the nonmixed part of the SDR (compartment N-M in Figure 1) and the relative number evolution is recorded at the level of the mixed part (compartment M in Figure 1). For each conditions, 10 simulations have been performed and the mean as well as the standard deviation are represented on the graphs. Comparison with experimental yeast cells tracer test is shown. In the case of simulation E, comparison with the equivalent deterministic compartment model has been done.

The results shown in Figure 5 highlight other important phenomena such as the time required for a microbial population to leave the mixed or the nonmixed section of the SDR. From a methodological point of view, the inert tracer is used to validate the fluid mixing−deterministic models and the cell suspension is used to implement the particle circulation−stochastic models, but from the obtained results (Figure 5), it can be seen that the two kinds of experiments can be explained both in a deterministic or stochastic context. This is quite important, since a single model can be used to simulate fluid mixing and particle circulation. Some precautions must be taken, however, concerning for example the importance of microbial population investigated. Indeed, for a reduced population, the probabilistic aspect has an important role (see Figure 4) and

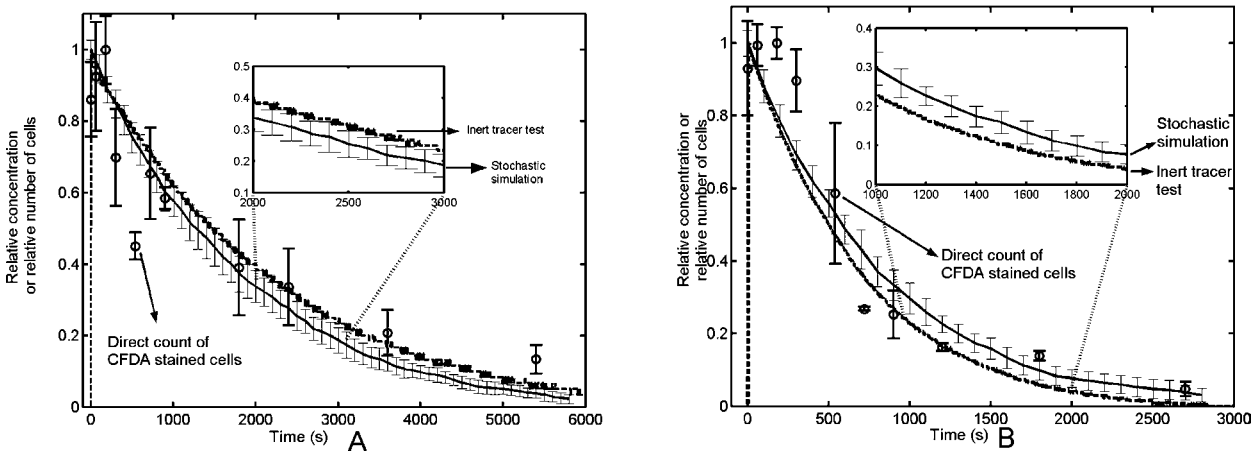

Figure 5. Stochastic simulations performed in the "open system" with a recirculating and exit flow rate of 18 L/h (A) and 39 L/h (B). Simulations represent the tracer concentration evolution at the level of the mixed part of the SDR (compartment M in Figure 1). For each condition, 10 simulations are performed and the mean as well as the standard deviation are represented on the graphs. Comparison with experimental yeast cells tracer test, as well as with inert tracer tests (NaCl) is given.

the stochastic model must be used. On the contrary, for an important population (such as the number of NaCl molecules in an inert tracer pulse), only the general trend will prevail and the deterministic model is recommended. However, in this case, a previous study has shown that the transition matrix can be used in a Markov chain context and gives results similar to those obtained with a determinist model (*8*). In the following section, only the stochastic basis will be used to elaborate the fluid mixing and circulation models, the fluid mixing being simulated with a Markov chain stochastic model and the particle circulation with a stochastic non-Markov model.

At this level, we can thus estimate the transition probabilities between the two parts of the SDR and determinate the repartition of a microbial population in this reactor. But these probabilities does not explain the fluid dynamic effect on microbial growth and it is necessary to refine the model. Indeed, the concentration gradient will be influenced by the recirculation flow rate and by the pulse frequency of the feed pump. A more elaborated model is thus needed to represent precisely the evolution of the concentration field at some points of the reactor.

Another important conclusion is that the yeast cells appear to behave like the inert tracer and consequently must be considered as following the global flow inside the reactor. It is very important in the case of the microbial cell circulation model elaboration. Indeed, we can conclude in our case that the same model structure can be used to model the fluid mixing process and cells circulation, and this property will be exploited in the next section.

Second Modeling Approach (Model 2): Superimposition of Microorganisms Circulation on the Concentration Gradient inside Bioreactors. A more elaborated modeling approach can lead to the visualization of the concentration gradient establishment and to a higher resolution on the path taken by the microorganism during its circulation in the reactor. The idea here is to superimpose the microorganism circulation path on the gradient field to obtain a characteristic concentration profile experienced by the circulating microorganisms. If increasing the number of compartments, we approach the structure of a more elaborated compartment model generally called network-of-zones. This kind of model has been used to represent fluid mixing in bioreactors (*1, 15*), and we will extend its use in a stochastic context. Figure 2 shows the complex network of compartments used here in the case of the SDR (constituted by a mixed part and a nonmixed part).

Figure 6A shows a simulation performed with 3500 particles (50 microbial cells per compartment), which roughly correspond to the number of microorganisms detected during the CFDA stained cells experiments. By running simulations with this model, we observe a probabilistic fluctuation more pronounced than the one that occurs in the case of the simplified stochastic flow models used in the previous section. This can be explained by the increasing number of compartments, which leads to a multiplication of the possible trajectories that can be taken by the flowing particles. It should also be noted that the number of cells at the equilibrium is higher for the compartments located in the nonmixed section of the reactor. This is simply because the volume of these compartments is more important than one of those located in the mixed section.

The Markov chain procedure (described in Materials and Methods) can be used to perform a fluid mixing simulation with the same model structure and with the same transition matrix. Figure 6B shows the results of the simulation. We can see that there is a good agreement between the stochastic random number (Figure 6A) and the Markov simulation results (Figure 6B), this observation validating the fact that the same transition matrix can be used to simulate fluid mixing and particle circulation. As shown before (*8*), the Markov chain model exhibits the same potentialities than the deterministic compartment models and can be used to calculate the concentration field inside the reactor. This fact is reinforced anew by the logical evolution of the simulated tracer curves obtained in this study (Figure 6B). Indeed, concentration variations are very pronounced in the compartments located at the level of the nonmixed, whereas those located in the mixed part exhibit tracer curves that are very close to each other as a result of the high homogenization efficiency in this part of the reactor. From these results, we can say that the model structure is sufficiently elaborate to obtain a good resolution, for both the fluid mixing and the cell circulation simulations. This is important for the circulation simulation, for which the model structure has an impact on the variance of the circulation time distribution.

To obtain a good representation of the concentration field evolution during the bioprocess, the pulse effect of the feed pump too must be taken into account. In our case, the pulse frequency is time varying because of the exponential increase of the feed flow rate during the culture. This pulse frequency can be included in the stochastic model by the

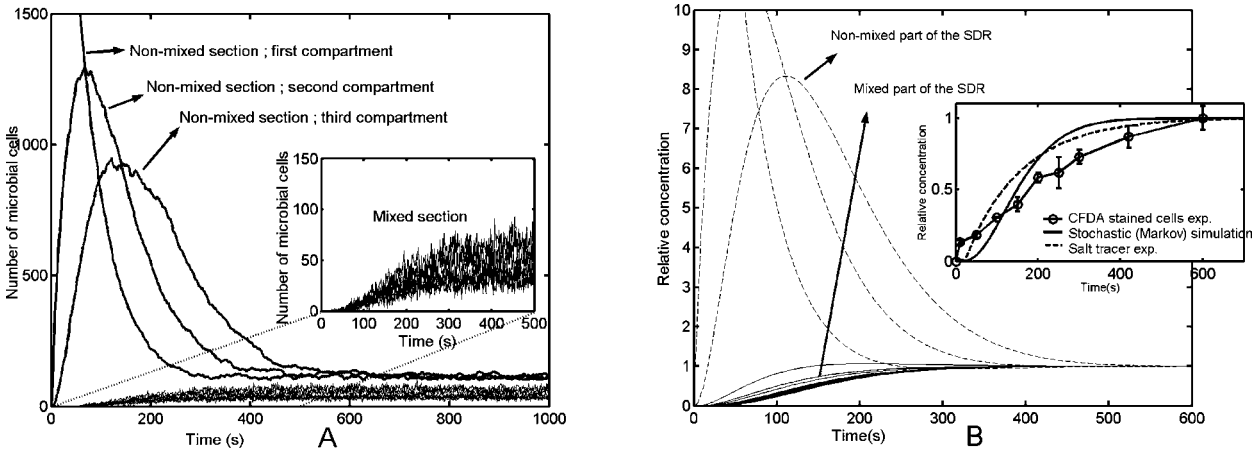

Figure 6. (A) Random number stochastic simulation of microbial cells dispersion in SDR (three-dimensional model with a recirculation flow rate of 18 L/h). Microbial cells (quantity = 3500) were initially located at the level of the first compartment of the nonmixed section. (B) Markov chain simulation of tracer dispersion in SDR reactor (three-dimensional model with a recirculation flow rate of 18 L/h). The pulse injection of tracer was performed at the level of the first compartment of the nonmixed part of the SDR.

use of the following matrix:

$$\mathbf{S}_{\text{pulse}} = \begin{bmatrix} C_{\text{pulse}} & 0 & 0 \ldots & 0 & C_{\text{pulse}} & 0 \ldots \\ 0 & 0 & 0 & & & . \\ . & . & . & & & . \\ . & . & . & & & . \\ . & . & . & & & . \\ . & . & . & & & . \\ 0 & 0 & 0 & . & . & .0 \end{bmatrix} \quad (9)$$

In the first row, the number of zero elements between two pulses (C_{pulse} being the concentration of the tracer pulse) depends of the feed pump activation frequency. The number of elements in a column corresponds to the number of compartments in the model. In the case of this matrix, the pulse is added at the level of the first state of the model (first element in a column). The number of elements in a row corresponds to the number of simulation steps performed.

The matrix $\mathbf{S}_{\text{pulse}}$ is included in the standard Markov chain procedure (see eq 8) to describe the evolution of the state vector:

$$S_i = \mathbf{T} \cdot S_{i-1} + \mathbf{S}_{\text{pulse}} \quad (10)$$

The strategy adopted here consists of superimposing the gradient field obtained with the Markov chain model on the microorganisms circulation process obtained with the classical stochastic model (i.e., with random number generator), to obtain the concentration profile experienced by a population comprising a given number of microorganisms.

The first step consists of calculating the evolution of the glucose concentration in each compartment of the model. The problem here comes from the reactive behavior of the investigated system. Indeed, a substrate consumption component exerted by yeast cells modifies the glucose gradient field appearance in the bioreactor. Figure 7 shows that glucose concentration at the outlet of the nonmixed section is the same as the one observed in the bulk of the mixed section of the SDR.

As a result of the large sample interval between glucose concentration determination experiments, the oscillatory effect induced by the pulse addition of glucose could be not visible. However, in general, there are no significant glucose concentration differences between the two parts of the SDR. As shown in this study and in others, during a fed-batch culture the average

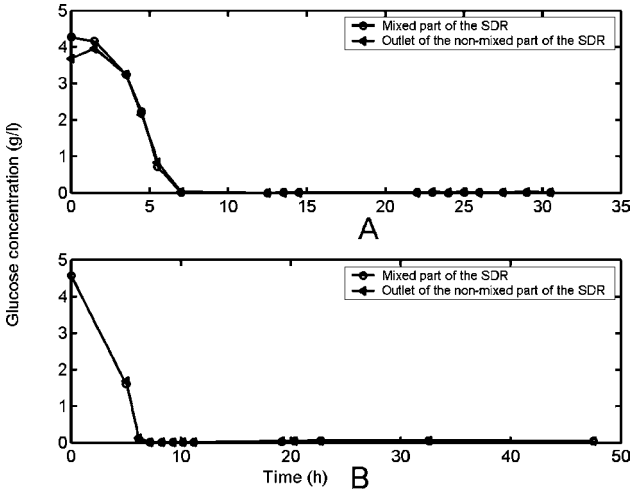

Figure 7. Evolution of glucose concentration during SDR experiments (A, Q_{recirc} = 18 L/h; B, Q_{recirc} = 39 L/h).

glucose concentration in the bioreactor is low, the cell density is high, and the glucose pulses are located in the immediate vicinity of the feed addition point. The concentration profiles in the different compartments of the model will consequently be expressed in terms of relative differences. In others words, the situation in a compartment for a given time interval will be calculated as the difference beteween the relative concentration in this compartment and the one in the compartment exhibiting the smallest relative concentration. This approach makes it possible to calculate a representative normalized concentration field in accordance with the mixing performances of the system. To compare the different SDR tests on the basis of the mixing performance, Markov chain simulations have been performed according to the feed pump pulse profile. The pulse frequency matrix $\mathbf{S}_{\text{pulse}}$ (eq 10) has been extracted from the experimental feed profile recorded by the bioreactor control unit. The feed profile was performed according to an exponential regulation (described in Materials and Methods). Simulations obtained by this way in the case of a SDR operating at a recirculating flow rate of 39 L/h are shown in Figure 8.

On the basis of the intermittent behavior of the feed pump, two limiting cases can be highlighted: the first one being when the mixing time of the SDR is inferior to the time interval between two pulses (T), and the second one arising when the mixing time of the SDR is superior to T. Gradient appearance

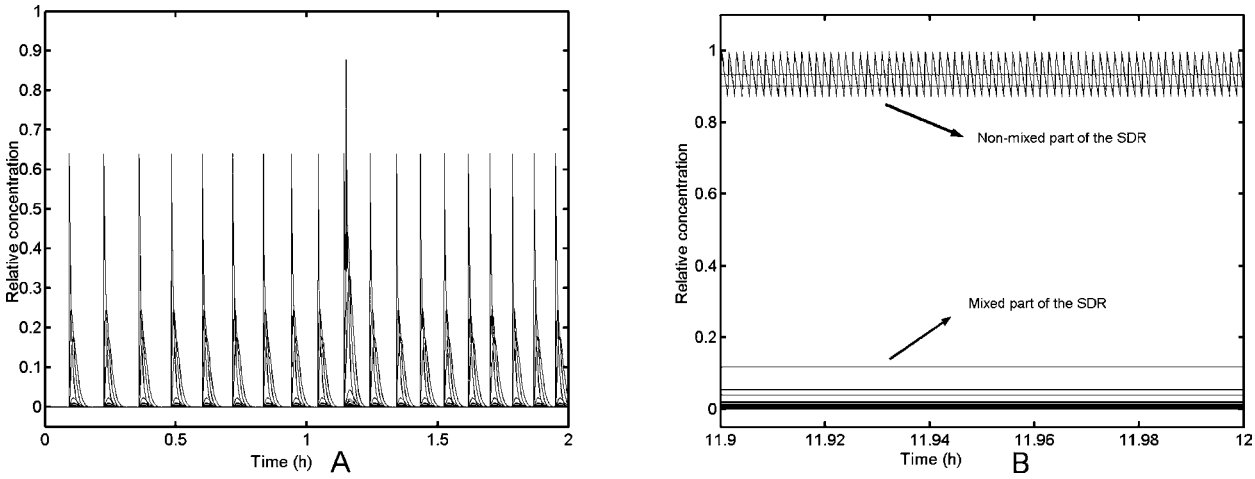

Figure 8. Markov chain simulation of tracer dispersion in SDR (Q_{recirc} = 39 L/h) according to the pulse profile of the feed pump employed during fed-batch test. The system is assumed to be nonreacting (no substrate consumption by the microorganisms). (A) Concentration gradient in the case of an exponential feeding strategy with a time between two pulses always superior to 30 s. (B) Concentration gradient in the case of a constant feeding strategy with a time interval between two pulses equal to 5 s (corresponding in practice to the end of the fed-batch culture). For the sake of simplicity, results are expressed in relative concentration in nonreacting system (no consumption of the tracer), each pulse having a unitary intensity.

and persistence arises when the characteristic mixing time of the bioreactor is larger than *T*. This phenomenom is shown in Figure 10, in which the impact of the increase of the pulse frequency on the concentration gradient is clearly marked. This impact is traduced by an increasing difference of concentration between the mixed and the nonmixed section of the SDR. The gradient is expanded to the mixed section of the SDR when the mixing time related to this section of the reactor is larger than the characteristic time of the feed pump (Figure 8B). These observations highlights the fact that our model is in accordance with the conclusions of a classical regime analysis of the process (*16*). From these observations, we can conclude that when performing a SDR experiment at a lower recirculation flow rate, the concentration gradient appears earlier and tends to be more pronounced. This is a possible explanation for the Y_{xs} drop noted between the two SDR experiments presented in this study (Figure 3).

A second explanation, which has been related in the literature (*17*), involves the impact of the pulse addition of glucose on the metabolic oscillations inside microbial cells. It is clear that, from the above-mentioned observations, a yeast cell in displacement in the SDR is submitted to a fluctuating extracellular environment, in terms of glucose concentrations. Several reports (*17*) have shown that, in the case of baker's yeast, glucose pulses induce an oscillatory response at the level of the intracellular dynamics. Yeast cells submitted at a given frequency to this stimulus seem to be able to withstand these perturbations. In other words, yeast cells are trained to be exposed further to environmental changes. This training mechanism could also explain the scale-down effect recorded during our SDR experiments.

At the level of the bioreactor, the exposure of microbial cells to a pulsing environment is difficult to represent because of the strong probabilistic nature of the system. However, this can be achieved by using our stochastic model methodology. The gradient field calculated by the Markov chain model is superimposed on the circulation of yeasts cells in the bioreactor in order to obtain the concentration profiles experienced by microorganisms. To achieve this, we will focus our attention on the constant flow rate period of the fed-batch culture, where glucose fluctuations are the most intensive. On the other hand, the size of the microbial population to be considered in the

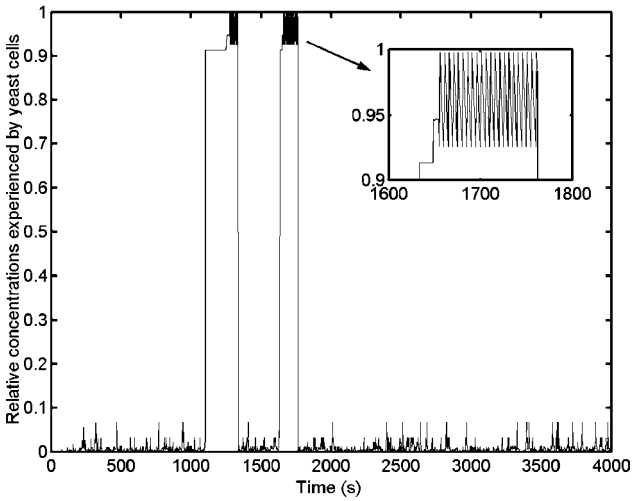

Figure 9. Simulation of the relative concentration profile experienced by a yeast cell in displacement inside the SDR.

stochastic circulation model must be determined in order to obtain a representative simulation. Several approaches can be considered:

- First, simply by considering a single microbial cell. This approach was adopted in a previous work in the case of real large-scale bioreactors (*18*). An example of a simulation performed in the case of a SDR is shown in Figure 9. It can be seen that the microbial cell is submitted to rapid concentration fluctuations, especially when entering in the nonmixed part of the SDR. It can be deduced that the scale-down effect is due to the passage of microorganisms through the nonmixed part of the SDR because of the larger concentration fluctuations encountered in this part of the reactor compared to those encountered at the level of the mixed part.
- Second, by considering a microbial population of n microorganisms. In this case, the concentration profile is calculated as the mean of all the glucose concentration profiles experienced by the microbial population on the time interval considered.

The use of the second strategy would allow us to follow the history of a microbial population inside the bioreactor. The difficulty about the simulation results analysis come from the

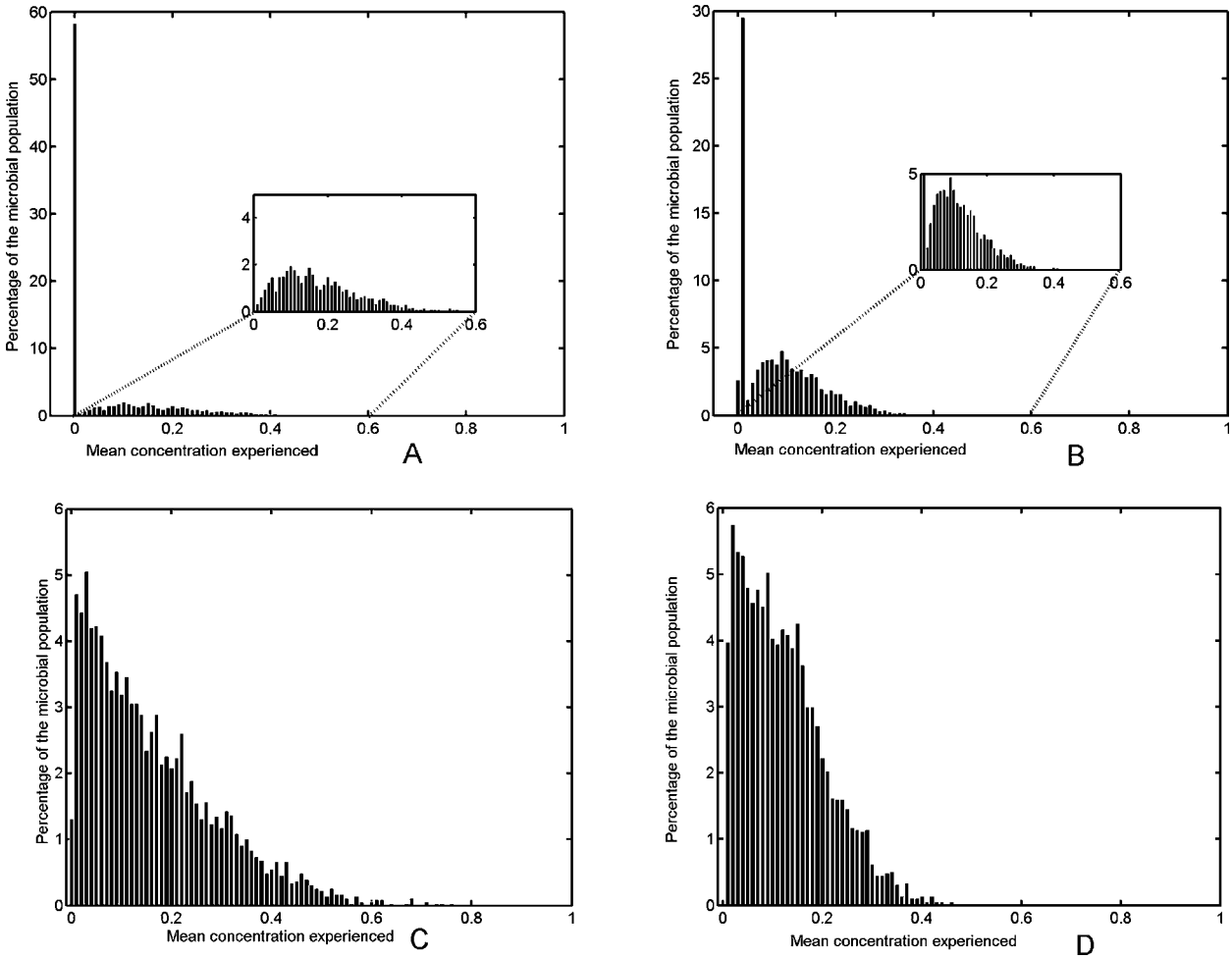

Figure 10. Frequency distributions of the mean relative concentration experienced by microorganisms circulating inside SDR. (A) Q_{recirc} = 18 L/h, 3500 microbial cells starting from the mixed part of the reactor. (B) Q_{recirc} = 39 L/h, 3500 microbial cells starting from the mixed part of the reactor. (C) Q_{recirc} = 18 L/h, 3500 microbial cells starting from the nonmixed part of the reactor. (D) Q_{recirc} = 39 L/h, 3500 microbial cells starting from the nonmixed part of the reactor.

fact that each microbial cell involved in the model (3500 cells in this case) has its own history. To overcome the complexity of this analysis, frequency distributions of the mean relative concentration experienced by microorganisms have to be considered. These distributions have been calculated from the superimposition of the relative gradient field simulation with the circulation simulation of a population of 3500 microbial cells. Four cases have been considered: scale-down tests performed with a recirculation flow rate of 18 or 39 L/h, by considering the microbial cells starting either from the mixed or the nonmixed part of the reactor (Figure 10).

Figure 10 shows that there are significant differences according to the initial location of the microbial population in the SDR. When the microorganisms are initially located at the level of the mixed part of the reactor, a significant fraction of the microbial population experiences very low substrate concentration: about 60% of the microbial population is submitted to a mean relative concentration centered on 0 for a Q_{recirc} of 18 L/h and about 30% when the Q_{recirc} reaches 39 L/h. This is a logical result since when the recirculation flow rate Q_{recirc} is doubled, the microbial fraction that is submitted to a small concentration variation centered on 0 drops from 60% to 30%. Indeed, in the stochastic model, the probability for a microorganism to enter in the nonmixed part of the SDR, where concentration fluctuations are very high, is multiplied by 2. However, this phenomena is at the opposite of the fermentation test results performed in SDR. Indeed, for these fermentation tests, the biomass yield increases when the recirculation flow rate increases. The frequency distributions in Figure 10A and 10B must not be analyzed by observing the fraction of the microbial population subjected to very low relative concentration centered on 0, but by observing their dispersion. Indeed, we can see that the frequency distribution is more dispersed in the case of Figure 10A than in the case of Figure 10B, suggesting that the increase of the recirculation flow rate diminishes the intensity of the relative concentration experienced by the microorganisms. This fact is in good accordance with the fermentation tests performed in the respective SDR. The same dispersion phenomena can be viewed more easily at Figure 10C and 10D. Indeed, it can be seen that when passing from a recirculation flow rate of 18 L/h to 39 L/h, there is a shift of the distribution to the left, which means that the microbial cells are exposed to less pronounced environment fluctuations. A percentage of about 7% of the microbial population is exposed to a relative mean concentration higher than 0.4 when operating with a recirculation flow rate of 18 L/h, whereas less than 0.8% are exposed to such elevated concentration when the recirculation flow rate is increased to 39 L/h. These results permit to explain the scale-down effect induced by increasing the recirculation flow rate between the two parts of the SDR. There is a strong probabilistic aspect involved in this effect that can be represented by stochastic models. Intuitively, by increasing

the simulation time, the entire microbial population would have the time to visit the entire volume of the SDR and thus to be exposed to the high fluctuating environment of the nonmixed part of the reactor. The resulting simulations will be thus expressed by identical frequency distribution whatever the initial position of the microbial population in the reactor. These considerations will be taken into account in a further study.

Conclusions

Our contribution has consisted of the elaboration a stochastic structured model allowing the description of both the fluid mixing and the microbial cell circulation in stirred bioreactors and its use to provide valuable insight about what happens to the microbial cells during a fed-batch process performed in a SDR. The following conclusions can be made:

- This study highlights the impact of the probabilistic aspect of both fluid mixing and particle circulation in a stirred vessel. The probabilistic nature of such phenomena has a direct impact in the case of a bioprocess in the sense that it will affect not only the environment heterogeneity in a bioreactor but also the way in which microbial cells are exposed to these environment fluctuations. The bioreactor mixing and circulation probabilistic components can be efficiently studied by stochastic models.
- The analysis puts in evidence a strong relation existing between microorganisms circulation and fluid mixing. This property has been tested by carrying out inert tracer and stained cells retention time experiments. The comparison of the two kinds of tracer experiments has shown strong similarities, which implies that yeast cells are submitted to fluid dynamics constraints similar to those of the classical inert tracer. Because of the above-mentioned observations, the transition matrix of the stochastic model exposed in this study can be used both for the microbial cell circulation and the fluid mixing simulations.

The use of the stochastic model has made it possible to describe the concentration fluctuations experienced by the microbial cells during the culture. From these observations, some assumptions can be advanced to explain the scale-down effect:

- The increase of the recirculation flow rate between the mixed and the nonmixed parts of the SDR can enhance the frequency at which yeast cells are exposed to glucose fluctuations. This phenomena can induce the adaptation phenomena reported in ref 17. A similar adaptation phenomena has also been noted in the case of *E. coli* (*19*), which is also a gradient-sensitive microorganism (*6*).
- The superposition of the circulation of 3500 microbial cells on the gradient field simulations has shown that the cells are submitted to higher mean relative concentrations in the case of a lower recirculation flow rate (results are presented in the form of frequency histograms at Figure 10). These simulation results are in accordance with the real scale-down fermentation tests.

Nevertheless, several issues are required before being able to fully explain the yeast cells metabolism−fluid mixing interaction mechanisms. Some other components of the problematic have been described in the literature and the following considerations involve some of them:

- Our work is limited to the study of bioprocess in scale-down reactors, which are easy to modelize from a fluid dynamics point of view. In large-scale bioreactors, circulation is more difficult to represent and experimental data are hard to be found. Nevertheless, some circulation time distributions are available in the literature and can be helpful for the stochastic modelization of microbial cells circulation in large-scale bioreactors. The second difficulty come from the determination of the effective volume of the feed zone. This determination is easy in the case of a SDR because of the physical retention of the feed in the PFR section. In the case of a large-scale bioreactor, the volume of the feed zone depends on the fluid mechanics and of the feeding strategy. Some data are available in the literature but are limited (*5*).
- In the perspective of a microorganism−fluid mixing interaction modelization, the inclusion of a sufficiently realistic microbial kinetic model is the key factor. To go further, it can be said that the importance of this model exceeds the hydrodynamic part of the problem. This fact has been highlighted by several authors, and some observations made in this study show the same way. In ref 20 a deterministic compartment model has been coupled with a Monod kinetic to determine concentration gradient appearance in a stirred vessel. The same methodology was adopted in ref 21, but involving a structured metabolic model. This last approach has led to more reliable results about the impact of the bioreactor heterogeneity on bioprocesses. In the case of *S. cerevisiae*, it is known that several glucose transporters are involved in function of the extracellular glucose concentrations (*17*). This phenomena induces some variations concerning the affinity constant of the microorganism for the substrate that cannot be taken into account by a simple Monod kinetic model.
- The characterization of the glucose gradient concentration and its perception by the microorganism is an efficient structured attempt to study the scale-down effect on microbial growth, but there are many other factors that also play a role in this effect. Among these, shear stress and dissolved oxygen gradient influence strongly the microbial process. It is possible to characterize these respective components by the use of the stochastic models presented in this study (e.g., shear stress can be characterized by calculating the passage frequency of microorganisms at the level of the compartments close to the impeller, and the dissolved oxygen gradient field can be calculated by adding to our model the computational method proposed in ref 22).

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