

## **MINI REVIEW : RELATIONSHIP BETWEEN HYDRODYNAMIC CONDITIONS AND SUBSTRATE INFLUX TOWARD CELLS.**

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**Abstract.** This paper proposes a review of studies investigating the coupling of hydrodynamics and bioreaction and highlights the complex relationship between energy dissipation, substrates uptake rate and cell physiology. Many lab-scale studies were conducted to understand the effect of substrate heterogeneities on the cell metabolism caused by insufficient mixing. Beyond large scale heterogeneities, small scale turbulent motion which controls the substrate distribution at the microscale also seriously modifies the behavior of the population. Since these studies tend to demonstrate that mixing at small scales plays an important role, segregation (especially in the feed zone) should be therefore taken into consideration in models used for the scale up of bioprocesses. As an illustration of this, the Engulfment mixing model coupled with a model for bioreaction is used to study the effects of imperfect mixing. Results are in good agreement with the experimental observations from the literature.

**Key words:** Bioreactors; Substrate transfer; Biological reaction; Micromixing; local hydrodynamic.

### **1. INTRODUCTION**

The modelling of biological reaction at different scales is a real challenge for the research community. Despite many efforts, numerous questions among which the integration of micromixing in turbulent flow with biokinetics and the dynamics of the metabolism triggered by concentration fluctuations are still opened [1]. The study of the interaction between mixing and biological reactions is complicated by the fact that cells adapt their behaviour to the environmental condition they encounter. In particular for fed-batch cultivations the residence time of cells is sufficiently important for physiological adaptation to take place. As noted by Bailey and Ollis, [2] “effects imposed at a certain length scale can influence the observed kinetics of cell population in different ways. It is important to recognize this connection so that kinetic measurements and models can be developed under conditions which will resemble in some senses those encountered in the large scale reactor.”

Recent investigations in this domain include on the one hand the understanding of the mechanisms provoking reduced yields and on the other hand, the possibilities to obtain a better modelling of such large scale processes and heterogeneities. In this paper we will focus on the latter aspects.

#### **1.1. Experimental evidence of mixing issue in bioreactors**

Large scale bioreactors have been shown to exhibit heterogeneous concentration fields : cells circulating through such reactors are submitted to an extracellular fluctuating environment [3,

4]. As a result, large scale cultivations exhibit lower carbon conversion yields on biomass than expected from lab-scale experiments : Bylund et al. [3] observed a reduced yield of 20% for cultures of *Escherichia coli*; same results were reported by Enfors et al. [5] for the production a Baker's yeast *Saccharomyces cerevisiae*. Generally, substrate is fed at the top of the reactor and air is injected at the bottom. Because of imperfect macromixing both oxygen and substrate gradients are formed (low oxygen and high substrate concentrations at the top and low substrate and high oxygen concentration at the bottom). The effects of such heterogeneities on microbial metabolism were studied in scale down reactors, composed of a two continuous stirred reactors (in the early work of Oosterhuis et al. [6, 7]) or a continuous stirred tank and a plug flow reactor with a view of periodically exposing cells to sudden variation of the substrate concentration in their environment [3-5, 8-10]. Insufficient macromixing represented as periodic exposure to excessive substrate concentration induces some modifications on the cell metabolism, leading to the formation of by-products and decreasing the overall reactor performance.

Dunlop and co workers pointed out the influence of intermediate mixing scales (i.e. mesomixing) on continuous cultures of *S. cerevisiae* [11, 12]. The conversion yield of substrate into biomass is found to be dependent on the number and position of injection point in a 3L aerated bioreactor. Furthermore, Hewitt et al. [13], using flow cytometry, showed that imperfect macromixing (periodic exposure to high glucose concentration at low frequency, 1/60 Hz) strongly influences the cell physiology in terms of membrane permeability and cell viability.

## 1.2. The coupling between hydrodynamic and bioreaction

Bioreactors are characterized by a complex three phase flow (liquid, gas, biomass). The modelling of a bioreactor requires a model for the flow field, a model for the reactions taking place in the biomass and a model for the mass transfer between phases (from gas to liquid and from liquid to biomass). Despite the fact that biological reactions are conditioned by the mass transfer between the liquid and the biomass, hydrodynamics and bioreactions are generally coupled through conservation equations for the dissolved species. Only a few examples exist where the population of microorganisms is treated with a Lagrangian approach [9, 14, 15]. The conservation equation for the scalar  $\alpha$ , written over a control volume, accounts for convective, diffusive, turbulent transport and reaction:

$$\frac{\partial \overline{C_\alpha}}{\partial t} + \overline{u_j} \frac{\partial \overline{C_\alpha}}{\partial x_j} = \frac{\partial}{\partial x_j} \left[ D_\alpha \frac{\partial \overline{C_\alpha}}{\partial x_j} + \overline{u'_j C'_\alpha} \right] + R_\alpha \quad (1)$$

The scalar transport can be described using compartment models or Computational Fluid Dynamics (CFD) codes. Compartment models are very popular in the domain of bioreactor simulation because multiphase aspects and metabolic models can be easily implemented in the overall model for the bioreactor [16-18]. However, when a compartment model is used for the hydrodynamics, all transport terms in equation (1) have to be modelled. Recent progress in computer power now allows for the computation of the 3D turbulent, multiphase flow by CFD calculations [1, 4, 5, 19].

Whatever the approach (Eulerian or Lagrangian) two assumptions are made:

- the control volume is homogeneous; a possible occurrence of segregation under the resolved scale is not considered.
- the reaction rate is computed from kinetic laws that are strictly valid for steady state conditions with a high degree of mixing (small scale reactor). More details are presented in the next section.

Yet, it is well established that the applicability of the biokinetic models to temporally changing conditions experienced by microorganisms in large scale bioreactors is not guaranteed [18]. Frequently, the parameters of the biokinetic model (including metabolic model) obtained at the lab scale have to be adapted in order to fit the results in large scale bioreactors [17]. It has already been pointed out that the interaction between mixing and biological reaction (physiological response to imperfect mixing) has to be taken into account in order to improve the predictive capabilities of the models [1]. To begin with, recent progresses in the description of the substrate assimilation capacity of the microorganisms are presented. This brief presentation is important since the substrate assimilation is not characterized by a constant rate but depends on the cell physiology which is conditioned by the encountered fluctuating concentrations. This is a significant increase in complexity by comparison to turbulent reacting flows.

## **2. THE IMPACT OF MIXING ON BIOREACTION**

### **2.1 Consequences of imperfect mixing on the metabolism**

Considering that cells growing in bioreactors are exposed to two kinds of fluctuating concentrations, rapidly changing concentration due to small mixing scales and low frequency fluctuation inhering in the circulation time in the bioreactor, the aim of this paragraph is to highlight the impact of periodic fluctuations on bacterial metabolism. Microbiologists all agree with the fact that starvation and suboptimal levels of substrate induce non negligible consequences on bacterial metabolism [20]. Among them, one can note the fact that bacterial cells adapt their substrate uptake capacities to the concentration fields they undergo [21]. In other words when the substrate concentration changes abruptly in time, or equivalently when cells are transported through a gradient of substrate, relation (2) is not applicable locally because it is a law of equilibrium.

Neubauer et al. [8, 10] showed that heterogeneous conditions caused by insufficient mixing influences cell physiology. They noticed that cells cultivated under limiting conditions (starvation period during 27 minutes) but regularly exposed to substrate excess (for 2 minutes) respond by an increase of their substrate uptake capacity. As a result the instantaneous uptake rate measured after the limitation relief is up to six times higher than the maximum uptake rate measured in a batch culture. This faculty to exhibit instantaneous uptake rate that are not correlated to the growth rate was also reported by Leegwater et al.[22]. Natarajan and Srienc [23, 24] confirmed by recent techniques that growth rate and substrate uptake rate were decoupled in the range of growth rates studied. Ferenci explains that when exposed to limiting concentration cells develop additional systems for the transport of glucose characterized by a high affinity for the substrate (very low  $K_S$  value) [20, 21, 25]. From these experimental results, it is possible to conclude that cells can develop an extra assimilation capacity, suggesting that they assimilate a certain amount of excess substrate when suddenly exposed to high substrate concentration. The actual instantaneous uptake rate of the cell population is therefore a consequence of the culture history [10]. Thus, the maximum uptake rate can not be readily set to a constant independently from the scale of the reactor if imperfect mixing conditions occur. Then, the instantaneous uptake rate in heterogeneous concentration field can not be calculated from the average growth rate.

However in most papers the evaluation of  $R$  in equation (1) is obtained from a Monod type law described in all microbiology textbook, which relates bacterial growth rate  $\mu$  to the substrate concentration  $S$ . This relationship is obtained from steady state experiments and perfect mixing is assumed. Therefore the local value  $S(x,t)$  around each cell, the temporal average  $\overline{S(t)}$  and the volume average  $\langle S \rangle$  are all equal. The substrate uptake rate  $q_S$  is defined by equation (2) where  $K_S$  is defined as the affinity constant for the substrate (a few

$\text{mg.l}^{-1}$ ),  $Y_{XS}$  the conversion yield of substrate into biomass (around  $0.5 \text{ g}_X \cdot \text{g}_S^{-1}$ ) and  $\mu_{max}$  the maximal growth rate.

$$q_S = Y_{XS}^{-1} \cdot \mu = Y_{XS}^{-1} \cdot \mu_{max} \frac{\langle S \rangle}{K_s + \langle S \rangle} = q_{S \max} \frac{\langle S \rangle}{K_s + \langle S \rangle} \quad (2)$$

By definition this relation is valid for cells that are adapted to their environment, i.e. that have been facing a constant concentration  $S(t)$  for a long time. At steady state, the uptake rate is found to be proportional to the growth rate, which is itself related to the averaged volume concentration in the bioreactor. Because it is related to growth, the substrate assimilation rate as defined in (2) is indeed an averaged value over the time scale for growth. Yet, the literature reported above clearly indicates that the maximum uptake rate is not constant but is a function of the culture history. Therefore, equation (3) should be preferred.

$$q_{S \max}(t) = f \left( \frac{1}{\tau} \int_{t-\tau}^t q_S(u) du \right) \quad (3)$$

In order to account for such a memory effect, the most natural approach is to treat the biomass as a dispersed phase. Recently, Lapin et al. [14, 15] have integrated a dynamic metabolic model for the glucose uptake (adapted from Chassagnole et al. [26]) in an Euler-Lagrange simulation of a bioreactor in order to compute the local substrate uptake rate as a function of the cell composition. This modelling approach allows for the decoupling of the substrate uptake rate from the local concentration. It must be noted however that the local concentration here is still an averaged value over the control volume which size is determined by the spatial resolution of the hydrodynamic model.

## 2.2. Turbulent mixing and substrate influx

The mixing phenomena in turbulent flows can be decomposed into three steps (macro-, meso- and micromixing) occurring simultaneously, whose characteristic time and length scales are summarized in table 1 [27, 28].

For usual reactor configurations, the Kolmogorov scale varies from 50 to 300  $\mu\text{m}$ , thus being far larger than bacterial cell (2  $\mu\text{m}$ ) or yeast (10  $\mu\text{m}$ ) [11]. Microbial cells are placed in molecular diffusion controlled environment and the phenomenon responsible for providing substrate to the cell remains the molecular diffusivity. However the concentration in the environment of cells is controlled by mesomixing.

Al Homoud and Hondzo studied the effect of very low turbulent dissipation rate  $\varepsilon$  on the rate of assimilation of oxygen and glucose [29, 30]. They described an experimental device which consisted in a batch reactor with an oscillating grid, whose frequency varied from 0 to 6 Hz resulting in an averaged  $\varepsilon$  ranging from 0 to  $4.6 \cdot 10^{-5} \text{ m}^2 \cdot \text{s}^{-3}$ . The measured assimilation rates are always below the maximum uptake rate which means that the uptake is limited by the transport towards the cell. In such conditions, i.e. no segregation at the macroscopic scale, very low  $\varepsilon$ , limiting substrate concentration, they found that the Sherwood number was correlated to  $\varepsilon$ . However, such low values are not encountered in industrial bioreactors and therefore, diffusion is not the limiting step for assimilation in stirred bioreactors.

Merchuk and Asenjo [31] proposed a reinterpretation of the Monod's law as the result of the competition between fast reaction at the cell interface and limiting transport. They showed that the  $K_s$  value is indeed an apparent constant that depends on the rate of mass

transport. As a consequence, the parameter  $K_S$  also should not be set independently from mixing conditions.

### 3. INFLUENCE OF ENERGY DISSIPATION RATE, LOCAL HYDRODYNAMIC CONDITION ON SUBSTRATE INFLUX: BIBLIOGRAPHICAL ELEMENTS

#### 3.1. Influx controlled by mixing effects

Table 1 : time and length scales of mixing in homogeneous turbulent flow

	Macromixing	Mesomixing Reduction of integral scale	Micromixing by engulfment	Micromixing by diffusion
Time scales	$t_c = \frac{V}{N_{Qc} \cdot N \cdot d^3}$	$t_s = 2 \left( \frac{L_c^2}{\varepsilon} \right)^{\frac{1}{3}}$	$t_K = \left( \frac{V}{\varepsilon} \right)^{\frac{1}{2}}$	$t_D = 2 \left( \frac{V}{\varepsilon} \right)^{\frac{1}{2}} \cdot \arcsin h \left( 0.05 \frac{V}{D} \right)$
Length scales		$\Lambda = \frac{1}{2} \frac{k^{\frac{3}{2}}}{\varepsilon}$	$\lambda_K = \left( \frac{V^3}{\varepsilon} \right)^{1/4}$	$\lambda_B = \left( \frac{D^2 V}{\varepsilon} \right)^{1/4}$

In fed reactors, substrate is transported from the feeding zone through macro and meso mixing down to the Kolmogorov scale. Besides macromixing, which is correlated to circulation time in the reactor and the averaged dissipated energy, the local energy dissipation rate is a key element for determining the substrate influx to cells. Indeed, local segregation can occur whereas the reactor is perfectly macro mixed.

Dunlop and co-workers [32] have shown that feeding substrate from zones with high energy dissipation rate causes an increase of substrate flux towards cells. Some of their experiments were conducted using a stirred tank reactor with a recirculation loop through a static mixer with a removable grid. Substrate is fed either in the stirred or in the recirculation loop (referred as the *plug flow reactor zone*, or PFR zone) to *S. cerevisiae*. This yeast can exhibit overflow metabolism when exposed to substrate concentration above a critical value (growth repression). When the substrate is fed into the well mixed stirred tank bioreactor no overflow is observed, indicating that most of the cells experiment a concentration that remains below the critical value. The efficient mixing and the dilution of the feed into the whole stirred tank result in a low substrate concentration in the well mixed zone. But, when the substrate is fed through the static mixer the *averaged* concentration in the plug flow reactor zone controlled by the relative mass flow rates of the feed and the recirculation, exceeds the critical value. In the grid-equipped static mixer, the energy dissipation rate is high ( $\varepsilon = 1 \text{ kW} \cdot \text{m}^{-3}$ ) but there is no dilution effect of the feed and thus all cells experience the sub critical substrate concentration which causes overflow and a significant decrease in the reactor performance. When the grid is removed, mixing in the mixer body is poor ( $Re = 940$ ) and although the averaged concentration is the same as in the previous experiment, only few cells face a high concentration and are consequently repressed. This study clearly shows the impact of mixing intensity at the feed point, in a way that it conditions the cell population for further cultivation in the stirred tank. In another lab scale study [11], conducted in well agitated continuous fermentor, overflow metabolism was observed when the feed point is located in a zone of low  $\varepsilon$ . According to the methodology proposed by Bourne [28] for the investigation of mixing effects in chemical reactors, this suggests an effect of mesomixing and/or micromixing

These studies show the importance of considering both the local  $\varepsilon$  and substrate concentration values to calculate the substrate flux to cells.

### 3.2. Micromixing model for the investigation of substrate concentration distribution during first mixing times

We will now examine the source of errors using equation (2) to compute the source term in equation (1) when some segregation in the control volume exists. This was done by using the E model with self engulfment [33]. This model distinguishes two zones in the mixture, reaction only takes place in the substrate rich zone in which cells are incorporated at a rate defined by the parameter  $E=1/t_k$ . The PFR zone from Dunlop's experiments is studied [32]. In figure 1, the averaged concentration in the mixture ( $0.2 \text{ g.L}^{-1}$ ) is far above  $K_S$ , and results are examined in terms of the ratio between the averaged assimilation rate and the assimilation rate based on the averaged concentration (perfectly mixed hypothesis). When mixing is slow, the

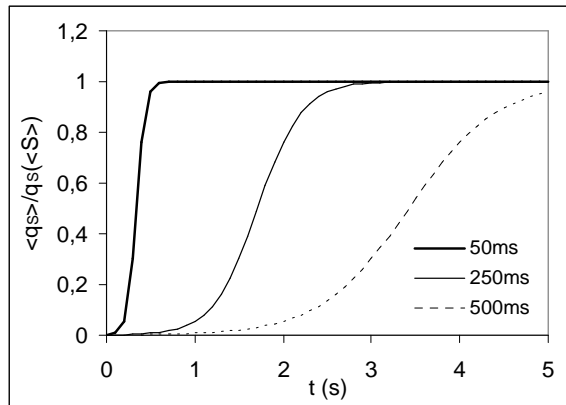


Figure 1 : Effect of segregation on the substrate assimilation rate at high  $\langle S \rangle$  value

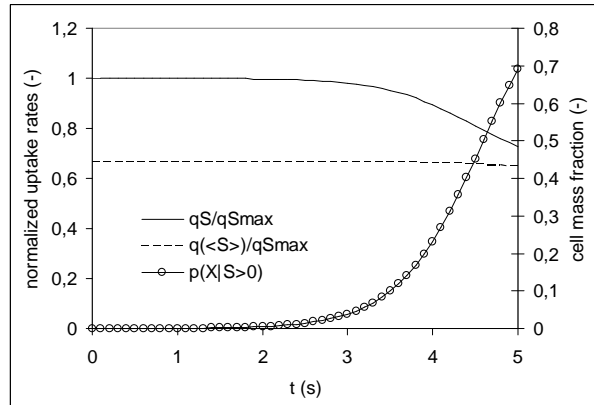


Figure 2 : Effect of segregation at low  $\langle S \rangle$  value.  $t_k=500\text{ms}$

ratio smoothly increases with time. In such case the fraction of cells in the glucose rich zone, where they assimilate the substrate at the maximum rate, is small. When mixing is fast, the fraction of cells facing high concentration reaches 1 after 0.5 s. Then, the entire population assimilates the substrate at the maximum rate for the time remaining in the PFR zone (set to 5 s in the simulations as in [32]). In case of  $q(\langle S \rangle)$  causing overflow, a perfect mixing will be prejudicial to the overall performance of the bioreactor as reported by Dunlop.

In figure 2, the average concentration ( $0.02 \text{ g.L}^{-1}$ ) is only twice the value of  $K_S$ , resulting in  $q_S(\langle S \rangle) = 0.66 q_{Smax}$ . The normalized uptake rate for the fraction of cells in the glucose rich environment (solid line) exceeds the normalized uptake rate based on the average value (dashed line). The fraction of cells in the glucose rich zone (line with circles), increases as mixing proceeds and finally reaches 70% of the whole population at the end of the mixer. From this it is clear that using equation (2) instead of considering segregation would over estimate the fraction of reacting cells and underestimate the actual uptake rate of cells that assimilate the substrate. In case of  $q(\langle S \rangle)$  under the overflow threshold, the model accounting for segregation is the only one to predict that a subpopulation of cells actually facing high concentrations may shift to an overflow metabolism.

## 4. CONCLUSION

Cells cultivated in large scale bioreactors are transported through a heterogeneous environment. Their behaviour is an integrated consequence of all the fluctuations they encounter. The impact of these fluctuations on microbial metabolism and therefore the prediction of microbial compartment remains a crucial issue for microbiologists and engineers. Experiments reviewed clearly establish connections between mixing and biological

reaction but the effects of sub-grid segregation are ignored in the modelling of bioreactors. Coupling biological reaction to a mixing model gave a sound explanation of experimental observations. This review points out the need to consider mesomixing for the prediction of local biological reaction rates in models used for the scale up of bioprocesses.

## NOMENCLATURE

$C_\alpha$	concentration of species $\alpha$ ( $\text{kg}\cdot\text{m}^{-3}$ )
$D_\alpha$	molecular diffusivity of species $\alpha$ ( $\text{m}^2\cdot\text{s}^{-1}$ )
$K_S$	affinity constant for the substrate ( $\text{mg}\cdot\text{l}^{-1}$ )
$N$	agitator speed ( $\text{s}^{-1}$ )
$R_\alpha$	source/sink term due to the biological reaction ( $\text{kg}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$ )
$S$	substrate concentration
$Y_{XS}$	biomass yield on substrate ( $\text{g}_X\cdot\text{g}_S^{-1}$ )
$q_S$	substrate uptake rate ( $\text{g}_S\cdot\text{g}_X^{-1}\cdot\text{h}^{-1}$ )
$q_{S\max}$	maximum substrate uptake rate ( $\text{g}_S\cdot\text{g}_X^{-1}\cdot\text{h}^{-1}$ )
$t_C$	circulation or macromixing time (s)
$\varepsilon$	turbulent kinetic energy dissipation rate ( $\text{m}^2\cdot\text{s}^{-3}$ )
$\varepsilon_V$	energy dissipation per volume unit ( $\text{W}\cdot\text{m}^{-3}$ )
$\mu$	specific growth rate ( $\text{h}^{-1}$ )
$\mu_{\max}$	maximal specific growth rate ( $\text{h}^{-1}$ )

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