

## Chapter 4

# Theoretical analysis of the stages in bacterial lag phase

### 4.1 Introduction

The lag phase of a bacterial culture is usually defined in terms of cell density, since during this phase the number of individuals remains approximately constant or increases slowly, and it is quantified by the lag parameter  $\lambda$ . In the previous chapter, the differences between the cell concentration growth curve and the biomass growth curve were introduced. The study of the metabolic adaptation has shown an initial decrease in total biomass, besides the lag phase in population. Therefore, it seems that the total biomass and the cell concentration evolutions may be isolated and studied separately.

The aim of this chapter is to provide a more in-depth analysis of the different stages that shape the bacterial lag phase. The evolution of cell density and total biomass during these stages is studied through their growth rates, setting an initial phase and a transition phase. We also propose a generic non-specific mathematical analysis of the lag phase in cell density.

The defined stages and the new mathematical model are tested with INDISIM simulations: the lag phase in cell density and the lag phase in total biomass growth are conceptually differentiated, and discriminated in the INDISIM simulation output results. Consequently, we have a unique opportunity to study them separately. At the end of the chapter the definition of the lag phase is tackled, as well as the balanced growth conditions of the exponential phase.

This is a practical example of the usefulness of simulations as the *third way*, to test new theories when the experimental approach is not yet possible (Hilker et al., 2006). In this chapter INDISIM simulations are used as an experimental system to check the theoretical approach.

Before introducing the theoretical analysis of the stages in the lag phase, a brief review of some important concepts and variables of this study is presented, as well as an outline of the INDISIM simulations.

## 4.2 Concepts, variables and simulation setup

### 4.2.1 The bacterial lag phase duration

When an inoculum is added into a culture medium under the appropriate conditions, the number of bacteria increases exponentially. When the medium conditions allow balanced growth, the population achieves a steady state in which all its intensive variables remain stable and well defined (Fishov et al., 1995). Under balanced growth conditions, all cellular constituents, as well as the total biomass, have the same growth rate.

In the first sections of this chapter (4.2 to 4.4), the exponential phase will be considered under balanced growth conditions. So each bacterium is individually changing when growing and reproducing, but the mean values for the variables describing the whole population remain constant. Specifically, the total biomass (B) growth rate (Eq. 4.1)

$$\mu_B = \frac{1}{B} \frac{dB}{dt} \quad (4.1)$$

and the cell density (N) growth rate (Eq. 4.2)

$$\mu_N = \frac{1}{N} \frac{dN}{dt} \quad (4.2)$$

are constant and equal to a maximum value ( $\mu_B = \mu_N = \mu_{EXP}$ ) (see Section 4.3), which is specific for the environmental conditions and the bacterial strain.

As was seen in Chapter 3, whenever the variables of the inoculum differ from those characteristic of the exponential phase, a lag phase precedes exponential growth. Thus, during this initial phase of adaptation, the individual and population variables evolve until they reach the exponential values. Lag phase is usually characterized by its duration, and is measured with a single lag parameter. Its value depends on the chosen definition. That most commonly used is the geometric definition, which graphically sets the lag parameter as the intersection between the initial cell concentration and the prolongation

of the exponential growth phase line in a logarithmic representation of the growth curve. Although many deficiencies have been found in this definition over the last few years (Baranyi and Roberts, 1994), it is still in use for its simplicity. There are publications that use different expressions to calculate the lag phase parameter (Zwietering et al., 1992), thereby yielding different lag times for the same set of data. The difficulty in establishing a general definition of the lag phase hinders the correct understanding and comparison of different existing models.

### 4.2.2 Relevant variables: bacterial mean mass, biomass distribution and maximum growth rate

An important variable in characterizing bacterial cultures is the bacterial mean mass. Helmstetter et al. (1968) presented an empirical expression that relates the mean mass of a culture,  $\bar{m}$ , with the mean of duplications per hour that an individual carries out,  $R$  (Eq. 4.3):

$$\bar{m} = \frac{2^R m_0}{\ln 2} \quad (4.3)$$

INDISIM simulations reproduce this behaviour (López, 1992). Thus, during the exponential phase of a culture the bacteria grow at a fixed growth rate and, therefore, there is a constant mean mass that is a characteristic of the culture (bacterial strain and environmental conditions).

Another population-level variable is central to our study: biomass distribution among the bacteria. Biomass distribution is characterized by the mean mass value and by the shape of the distribution. Both may vary through time in bacterial cultures. The biomass probability distribution is obtained by normalizing the biomass distribution among bacteria.

Wagensberg et al. (1988) proposed a mathematical expression for the biomass probability distribution of a bacterial culture,  $p(m_i)$ , in the exponential phase (Eq.4.4):

$$p(m_i) = Z^{-1}(m_i - m_0)^\gamma \exp(-\beta m_i) \quad (4.4)$$

where  $m_0$  is considered to be the absolute minimum value of mass required for a bacterium to exist, and in a discretization defined by the interval  $\Delta m$ , a bacterium belongs to a class of biomass  $m_i$  which can be written as  $m_0 + i\Delta m$ . This expression is deduced from the Maximum Entropy (MAXENT) principle. The values of the constants  $Z$ ,  $\gamma$  and  $\beta$  are determined according to three constraints regarding the normalization of the

distribution, the definition of mean mass, and the maximization of the system's entropy (Wagensberg et al., 1988a). This theoretical distribution fits the experimental data very well, as do INDISIM simulations (López, 1992). The *three ways* converge on the same point: experiments, theory and IBM simulations show that during exponential growth the biomass distribution in bacterial cultures has a characteristic shape. In conclusion, biomass distribution is a good variable for characterizing bacterial cultures at any one time.

A backwards shift and narrowing of the biomass distribution is commonly observed when a bacterial culture enters the stationary phase (Montesinos, 1982). This behaviour was well reproduced by INDISIM simulations in Chapter 3. Several biological processes operating at a cellular level are often suggested to explain this observation. Nyström (2004) highlights two of them: firstly, the growth rate decreases because of starvation conditions; in consequence, as we stated above, the mean mass decreases. Secondly, in starvation conditions bacteria may resort to self-digestion or cryptic growth to obtain extra energy and survive, which also produces a decrease in the bacterial mass. Consequently, a decrease in the mean mass is found in bacterial cultures during the stationary phase, even though the total biomass and number of bacteria are conserved.

To summarize:

1. Cultures in the exponential growth phase present a characteristic biomass distribution, which is determined by the individual minimum biomass, the culture's mean mass or growth rate, and the variability of the individual masses at division;
2. changes in the characteristic distribution may only take place if drastic and substantial genetic or metabolic modifications occur; and
3. whenever an inoculum is added to a new medium, if its biomass distribution is different from the exponential one, it will go through a lag phase until the distribution reaches the characteristic shape (Chapter 3).

Inocula from exponential phases that differ in growth rate due to a different substrate concentration or incubation temperature generate a lag phase. According to Eq. 4.3, in this case the characteristic mean mass of the inoculum in preculturing conditions is different from the characteristic mean mass of the exponential growth in the new conditions. We have shown by the INDISIM simulations of the previous chapter, as well as the reported results in this chapter, that the necessary adaptation of the cell mean mass is a deep and substantial basis of this kind of lag phase, which is only monitored through bacterial concentration. In the same way, if an inoculum is taken from a starved pre-culture and

put into a rich medium with similar conditions, its mean mass will have to increase until it reaches the characteristic value. This adaptation will also entail a lag phase. We must point out that only a lag phase in cell density growth is found in these cases, since no effects on total biomass growth are observed. This adaptation is tackled in detail in Section 4.3.2.

When an inoculum coming from a different medium adapts to a new environment, growth takes place. This adaptation to the new nutrient status will produce a lag phase both in biomass synthesis and in cell density growth, as described in Section 4.3.4.

### 4.2.3 INDISIM simulations

In this chapter, we tackle the above-mentioned issues from an IbM approach. We propose a model for whole culture behaviour, and we test its outcomes by means of INDISIM simulations. The results and conclusions of this work will be used in further studies to design specific experiments that may be used to validate the model.

The INDISIM version that is used in the simulations of this chapter is detailed in Section 2.3. The intracellular enzyme model is also taken into account in some simulations, when the lag phase in total biomass growth is tackled. The shuffling of the cells and the mixing of the medium at each time step are taken into account to reproduce a shaken batch culture. Thus, no diffusion processes are modelled.

We simulate the growth of an inoculum taken from a previous simulation. Past and present environments will henceforth be denoted as  $ENV^-$  and  $ENV^+$ , according to the nomenclature used by Dens et al. (2005b). Usually, the simulations end when the nutrient is exhausted. Thus, we take the inoculum from the stationary phase of the  $ENV^-$  environment, which has experienced a fall in the mean mass. The initial population has  $N_0 = 1000$  cells. Periodic boundary conditions are used.

## 4.3 Theoretical analysis of the stages in bacterial lag phase

### 4.3.1 Growth rates calculated through total biomass and cell density

The evolution of a culture may be characterized through the total biomass (B) growth rate  $\mu_B$  (Eq. 4.1) and by the cell density (N) growth rate  $\mu_N$  (Eq. 4.2), among others.

We can relate both parameters with the mean mass of the culture  $\bar{m}$  (Eq. 4.5),

$$\bar{m} = \frac{B}{N} \quad (4.5)$$

If we derive this equality and divide by the mean mass, we obtain Eq. 4.6:

$$\frac{1}{B} \frac{dB}{dt} = \frac{1}{N} \frac{dN}{dt} + \frac{1}{\bar{m}} \frac{d\bar{m}}{dt} \quad (4.6)$$

which can be rewritten as Eq. 4.7:

$$\mu_N = \mu_B - \frac{1}{\bar{m}} \frac{d\bar{m}}{dt} \quad (4.7)$$

When a previously adapted inoculum does not have any growth restrictions, after a variable amount of time, the population and biomass reach the exponential growth phase. During the exponential phase the growth rates  $\mu_B$  and  $\mu_N$  are constant and equal to a maximum value (Eq. 4.8).

$$\mu_B = \mu_N = \mu_{EXP} \quad (4.8)$$

Sometimes, a lag in the growth is observed before the exponential phase is achieved. We define *lag phase in cell density* as the interval of growth when the number of individuals  $N$  remains approximately constant or grows slowly. Sometimes, a lag in the increase in total biomass  $B$  is also found, which indicates that some internal adaptation of the bacteria is taking place. This period will be denoted *lag phase in total biomass*. These lags may be divided into different phases, depending on the values and evolution of the growth rates  $\mu_B$  and  $\mu_N$ .

In Sections 4.3.2 and 4.3.4 we will study separately the different phases in the lags in cell density and in total biomass, respectively.

### 4.3.2 Lag phase assessed through cell density

The lag phase according to cell density criteria is characterized by a standstill or a slight increase in bacteria concentration. Actually, reference to lag phase usually refers to the lag phase in cell density. It is also equivalent to the concept of 'collective lag' that is used to distinguish it from the 'individual lag'.

This lag appears when the inoculum state differs from the characteristic state of the exponential phase, so that the initial cell density growth rate is lower than the exponential one (Eq. 4.8). The cell density growth rate is related to the evolution of the mean mass (Eq. 4.7), and to the shape of the biomass distribution as described in Section 4.2.2.

During the lag phase in cell density,  $\mu_N$  will increase until it reaches the characteristic exponential value.

Cultures subject to environmental starvation or biological stress will present a lag in cell density linked to a lag in total biomass (Section 4.3.4), increasing the complexity of the global lag phase.

During the lag phase in cell density we can distinguish two subsequent stages: an initial phase  $P_{I(N)}$  and a transition phase  $P_{T(N)}$  (Table 4.1). The existence and duration of each phase is determined mainly by the changes in the environmental conditions between past  $ENV^-$  and present  $ENV^+$  culture media, and the initial mean mass and biomass distribution of the inoculum. The existence of any lag phase implies, minimally, the existence of  $P_{T(N)}$ . In summary:

1. Initial phase: during  $P_{I(N)}$  the cell density growth rate  $\mu_N$  is equal to zero or even negative, if some cells of the inoculum are damaged or too small. The cellular biomass must increase until the first duplication takes place ( $t_{FD}$ ).
2. Transition phase: once the first duplication has occurred, the cell density growth rate increases until it reaches the exponential value  $\mu_N = \mu_B = \mu_{max} = \mu_{EXP}$ .

When the cell density growth rate  $\mu_N$  has reached the maximum growth rate in the provided conditions ( $\mu_N = \mu_{EXP}$ ), the culture enters the exponential phase ( $P_{EXP}$ ).

Note that the concavity of the cell density curve at the initial phase,  $\frac{d\mu_N}{dt}$ , has not been given in Table 4.1. We left this value out because the initial phase can be very complex, depending on the inoculum history, so this derivative can potentially show the three behaviours (positive, negative, zero). Moreover, the discrete essence of  $N$  makes it difficult to extract continuous properties when the absolute value of the growth rate is too low or a synchronism in divisions is shown.

Table 4.1: *Phases in cell density growth (N) and in biomass growth (B).*

<i>Phase in population growth (N)</i>		<i>Growth rate <math>\mu_N</math></i>
Lag phase - Initial phase	$P_{I(N)}$	$\mu_N \leq 0$
Lag phase - Transition phase	$P_{T(N)}$	$0 < \mu_N < \mu_{EXP}, \frac{d\mu_N}{dt} > 0$
Exponential phase	$P_{EXP}$	$\mu_N = \mu_{EXP}, \frac{d\mu_N}{dt} = 0$
<i>Phase in biomass growth (B)</i>		<i>Growth rate <math>\mu_B</math></i>
Lag phase - Initial phase	$P_{I(B)}$	$\mu_B \leq 0$
Lag phase - transition phase	$P_{T(B)}$	$0 < \mu_B < \mu_{EXP}, \frac{d\mu_B}{dt} > 0$
Exponential phase	$P_{EXP}$	$\mu_B = \mu_{EXP}, \frac{d\mu_B}{dt} = 0$

### 4.3.3 A mathematical model for the transition phase in cell density

#### Definition and characterization of the model

We want to establish a general model for describing the evolution of  $\mu$  during the transition phase. We want to explain the shift from its value when the first cellular division takes place to its final value  $\mu_{EXP}$ , at the beginning of the exponential growth phase.

We focus on the cell density growth rate. Obviously, we are assuming continuous behaviour of this variable. When there is growth synchronism in the cell density (low inocula or highly homogeneous inocula), the  $\mu_N$  cannot be defined until the synchronism is lost. Following the Principle of Parsimony (McCullagh and Nelder, 1989), we start with the simplest assumptions. We assume the hypothesis that  $\mu_N$  linearly increases until it reaches the value  $\mu_{EXP}$ . We take the particular case in which its value at the initial phase is equal to zero. We consider three phases that have a direct correspondence with the phases defined before (initial, transition and exponential). That is:

1. Initial phase ( $P_{I(N)}$ ),  $t \leq t_1$  (Eq.4.9):

$$\mu_N = 0 \quad (4.9)$$

2. Transition phase ( $P_{T(N)}$ ),  $t_1 \leq t \leq t_2$  (Eq.4.10):

$$\mu_N = a \cdot (t - t_1) \quad (4.10)$$

3. Exponential phase ( $P_{EXP}$ ),  $t \geq t_2$  (Eq.4.11):

$$\mu_N = \mu_{EXP} \quad (4.11)$$

where  $t_1$  is the time at which the first division takes place and when, therefore, the culture enters the so-called transition phase, and  $t_2$  is the beginning of the exponential phase.

If we integrate these equations for cell density growth ( $\mu_N = \frac{1}{N} \frac{dN}{dt}$ ):

1. Initial phase ( $P_{I(N)}$ ),  $t \leq t_1$  (Eq.4.12):

$$N = N_0 \quad (4.12)$$



2. Transition phase ( $P_{T(N)}$ ),  $t_1 \leq t \leq t_2$  (Eq.4.13):

$$N = N_0 \cdot e^{\frac{\alpha}{2} \cdot (t-t_1)^2} \quad (4.13)$$

3. Exponential phase ( $P_{EXP}$ ),  $t \geq t_2$  (Eq.4.14):

$$N = N_0 \cdot e^{\frac{\alpha}{2}(t_2-t_1)^2} \cdot e^{\mu_{EXP} \cdot (t-t_2)} \quad (4.14)$$

Therefore, the logarithmic representation of the transition phase can be modelled as a quadratic curve (Eq.4.13). A graphical representation of the model is plotted in Figure 4.1.

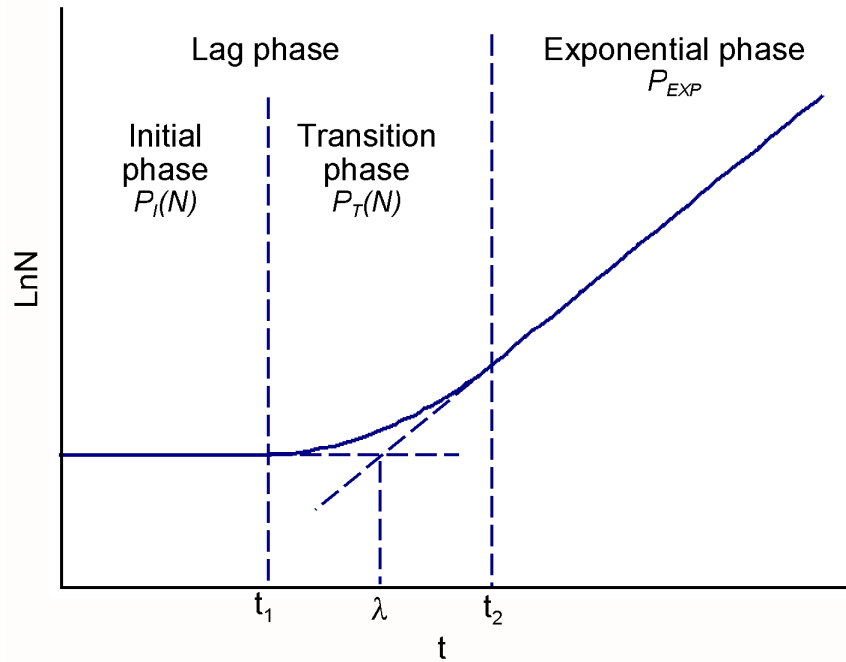


Figure 4.1: Graphical representation of the mathematical model for the cell number curve. The three phases described in Table 4.1 are represented: initial phase (Eq. 4.12), transition phase (Eq. 4.13) and exponential phase (Eq. 4.14).  $t_1$  and  $t_2$  are the mathematical parameters of the model, and  $\lambda$  is the lag parameter (see Section 4.3.3).

This model has four parameters:  $t_1$ ,  $t_2$ ,  $a$ , and  $N_0$ . We can relate them to four independently measurable experimental quantities. The inoculum size  $N_0$  is a controlled initial condition of the experimental cultures. The first division time  $t_1$  can be measured with microscopic techniques (Wu et al., 2000), as well as the maximum growth rate  $\mu_{EXP}$ , which can also be measured as the slope of the cell density growth curve at the exponential phase.

Using the definitions of the transitional and exponential phases, we can determine  $a$  as a function of  $t_1$ ,  $t_2$  and  $\mu_{EXP}$  (Eq. 4.15):

$$a = \frac{\mu_{EXP}}{t_2 - t_1} \quad (4.15)$$

Finally, with Eq. 4.14 and Eq. 4.15 the coordinates of any point of the exponential phase curve can be used to set  $t_2$  (Eq. 4.16). These coordinates will be  $t_e$  and  $N_e$ , with  $t_e$  being the time of measurement ( $t_e > \lambda$ ) and  $N_e$  the measured cell density at this point.

$$t_2 = 2t_e - t_1 - \frac{2}{\mu_{EXP}} \cdot \ln \frac{N_e}{N_0} \quad (4.16)$$

#### Relationship between the model parameters and the geometrical definition of the lag phase

It is also important to relate the parameters of this model to the usual geometrical definition of the lag phase  $\lambda$ . As we showed in Section 4.2.1,  $\lambda$  is the intersection of the straight lines given by Eq. 4.12 and Eq. 4.14. If  $\lambda$  is isolated from this intersection, we obtain Eq. 4.17.

$$\lambda = \frac{1}{2} \cdot (t_2 + t_1) \quad (4.17)$$

Note that since we have modelled the transition phase as a quadratic curve, it is natural that the lag parameter is the middle point of this symmetric transition. If the value obtained from Eq.4.17 is the same as the measured geometrical value, the assumption of a linearly increasing  $\mu_N$  is a good approximation of the behaviour of the system at a population level.

#### 4.3.4 Lag phase according to total biomass criteria

When  $ENV^+ \neq ENV^-$ , bacteria may find the environment stressful because their metabolism is not adapted to the new conditions. Then, while this adaptation takes place, there is a lag phase in total biomass growth, or even a decrease in the total biomass

of the culture (see Section 4.2.2). This behaviour is correctly reproduced by INDISIM simulations, as was seen in Chapter 3.

Again, different stages can be distinguished. The metabolic or physiologic adaptation, if necessary, is carried out during the first phases called  $P_{I(B)}$ , the initial phase, and  $P_{T(B)}$ , the transition phase (Table 4.1). Their characteristics depend on several factors.

1. Initial phase: during  $P_{I(B)}$ , the total biomass may initially decrease. The reason for this behaviour is the consumption of cellular reservoirs to generate new macromolecules, which will allow the bacteria to metabolize the nutrients found in the new environment. Nevertheless, there is also the possibility that some external nutrients may be used as this adaptation takes place; in some cases, total biomass could remain constant, without showing a decrease. The initial phase ends when the total biomass begins increasing, either after an initial decrease or a lag. Again, the concavity of the biomass growth curve at the initial phase,  $\frac{d\mu_B}{dt}$ , has not been given due to the complexity of this phase.
2. Transition phase: during  $P_{T(B)}$ , the total biomass growth rate  $\mu_B$  increases until it reaches the maximum value  $\mu_B = \mu_{EXP}$ .

It should be noted that the lag in total biomass is always accompanied by a lag in cell density.

### 4.3.5 The lag phase as a result of the biomass and cell density interaction

The above-defined phases allow different and distinguishable culture behaviours. As stated in Section 4.2.1, the growth rates at the exponential phase keep the equality  $\mu_N = \mu_B = \mu_{max} = \mu_{EXP}$ . During the lag phase, if present, the growth rates evolve to reach the characteristic exponential value. At an individual level, during the lag phase bacteria in the inoculum have to increase their biomass in order to begin duplicating. Thus, the total biomass evolution determines the cell density evolution: if the increase in biomass is too slow, the bacteria cannot reach the appropriate mass to initiate duplication.

If the biomass increases at its maximum rate from the very beginning, there is no lag phase in terms of biomass, and the duration of the lag phase in cell density will be fixed by the initial mass of bacteria. A culture lag will be observed if the inoculum mass distribution is different from the exponential distribution; this means that, minimally, the culture goes through the transition phase in cell density ( $P_{T(N)}$ ). This case is studied in Section 4.4.1.

In other situations, the total biomass may show an initial lag. While  $\mu_B$  is lower than  $\mu_{EXP}$ , bacteria are not adding biomass fast enough to maintain their maximum cell density growth rate, and the culture can not enter the exponential phase ( $P_{EXP}$ ). When both total biomass and cell density growth rates ( $\mu_N$  and  $\mu_B$ ) have reached the maximum growth rate in the provided conditions ( $\mu_N = \mu_B = \mu_{max} = \mu_{EXP}$ ), the culture finally enters the exponential phase. This is the case presented in Section 4.4.3. In such situations, the four above-defined phases ( $P_{I(N)}$ ,  $P_{T(N)}$ ,  $P_{I(B)}$  and  $P_{T(B)}$ ) may be found.

It must be pointed out that the potential lags in biomass and in cell density may be either consecutive or overlapped. This implies that, under certain specific conditions, biomass and cell density may evolve simultaneously. In this case,  $P_{I(N)} = P_{I(B)}$  and  $P_{T(N)} = P_{T(B)}$ , and it would not be necessary to separate the two evolutions: the lag in terms of biomass and the lag in terms of cell density would be the same.

## 4.4 Simulation results on the stages in bacterial lag phase

In this section we will present several results of the simulations performed with IN-DISIM. These simulations have been used as a virtual experimental system in two different ways. Firstly, two possible culture lag phases are analyzed separately: lag phase in cell density, and lag phase in both cell density and total biomass. The evolution of  $\mu_N$  and  $\mu_B$  are observed in each case to illustrate the phases defined in Sections 4.3.2 and 4.3.4. Secondly, the model presented in Section 4.3.3 is adjusted to the simulations of lag in cell density to test its consistency.

### 4.4.1 Transition between lag and exponential phases in cell density

The simplest adaptation that an inoculum has to overcome corresponds to the case where the mediums  $ENV^-$  and  $ENV^+$  are the same, but where the nutrient of  $ENV^-$  has been exhausted. Consequently, the mean mass of the cells in culture  $ENV^-$  decreases. However, the characteristic exponential growth rates and biomass distributions are the same in both cultures. Since no metabolic adaptation is required by the cells to grow in the new medium, the total biomass increases at its maximum rate ( $\mu_B = \mu_{EXP}$ ) from the very beginning. Thus, there is no lag in total biomass evolution (Fig. 4.2a), and only a lag in cell density is observed (Table 4.2).

Table 4.2: *Description of the observed phases in simulated growth for the study of the lag phase in cell density.*

Number of bacteria ( $N$ )			Total biomass ( $B$ )		
$P_{I(N)}$	$\mu_N = 0$				
$P_{T(N)}$	$\mu_N < \mu_{EXP}$	$\frac{d\mu_N}{dt} > 0$	$P_{EXP}$	$\mu_B = \mu_{EXP}$	$\frac{d\mu_B}{dt} = 0$
$P_{EXP}$	$\mu_N = \mu_{EXP}$	$\frac{d\mu_N}{dt} = 0$			

While the mass of the largest bacteria is too small to allow any bacterial division, the cell density growth rate is equal to zero ( $\mu_N = 0$ ). After the first cellular division, simulations show that  $\mu_N$  increases almost linearly until  $\mu_N = \mu_{EXP}$  (Fig. 4.3a), according to the theoretical model presented.

The mathematical model has been adjusted to the cell density curve. The values of the parameters are indicated in Figure 4.2a. The fitting of the mathematical model to the INDISIM simulation results has a high correlation factor of  $r = 0.9959$ . It is important to point out two aspects of this: i) this global behaviour emerges from the individual traits, and ii) the mathematical model is not imposed on the laws that govern the bacteria at the individual level. The high correlation between the theoretical predictions and the simulated outcomes can be explained by the essential nature of the physical assumptions taken into account when building both models: the theoretical model proposes the simplest evolution from a low value of  $\mu_N$  to a higher one, a linear increase. The IbM reproduces the expected behaviour by assuming a simple model for the individual increase in biomass and cellular cycle.

The mean cellular biomass increases through the lag phase until it reaches its expected value at the exponential growth phase. Meanwhile, the biomass distribution evolves to reach its characteristic shape, as described in Section 4.2.2 (Fig. 4.4a). This evolution shows the adaptation of the system from a transient state (the initial state) to a balanced growth state (the exponential growth phase) characterized by the stability of the intensive properties (e.g. mean biomass) and their distribution among the population (e.g. biomass distribution).

When the culture reaches the exponential growth phase, its macroscopic state is the least correlated with the initial state of the system. This means that the population-level characteristics of the culture during the exponential phase (i.e., shape of the biomass distribution) do not depend on the initial characteristics (i.e., shape of the biomass distribution of the inoculum), but on the environmental conditions of and physical and biological constraints on the system. This means that different inocula in similar mediums will reach similar biomass distributions in the exponential phase.

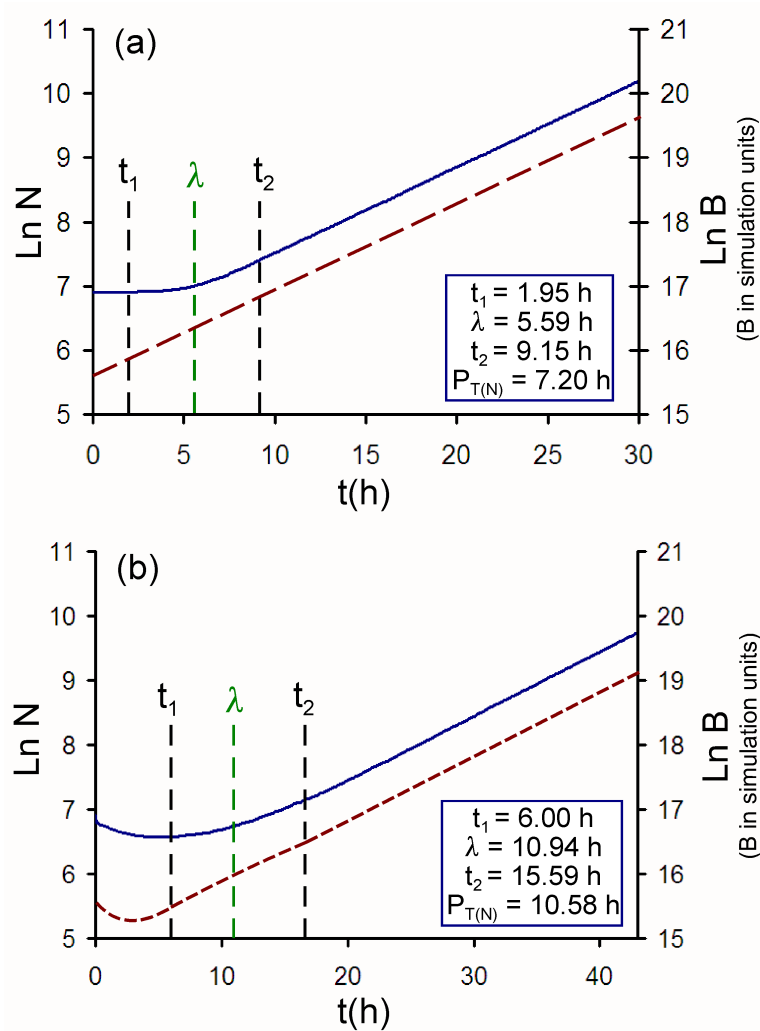


Figure 4.2: *INDISIM* simulation results for a batch culture. The solid lines depict the cell density evolution and the dashed lines show the total biomass evolution for (a) a culture with no initial enzymatic adaptation (Section 4.4.1) and (b) a culture with an initial enzymatic adaptation (Section 4.4.3). The text boxes give the parameters of the mathematical model that describe the cell density evolution (Section 4.3.3) and the duration of the transition phase in cell density ( $P_{T(N)}$ ).

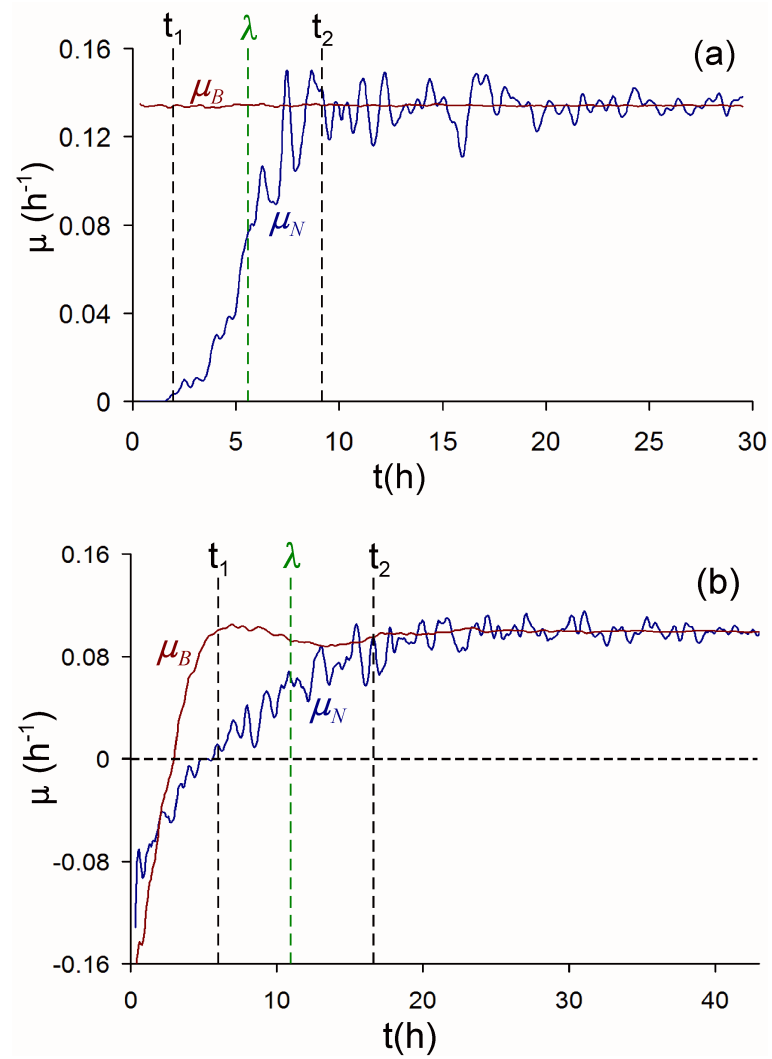


Figure 4.3: *INDISIM* simulation results for a batch culture. The lines depict the evolution of the cell density growth rate ( $\mu_N$ ) and the evolution of the biomass growth rate ( $\mu_B$ ) for (a) a culture with no initial enzymatic adaptation (Section 4.4.1) and (b) a culture with an initial enzymatic adaptation (Section 4.4.3). The dashed lines depict the parameters of the mathematical model that shape the cell density evolution (Section 4.3.3).

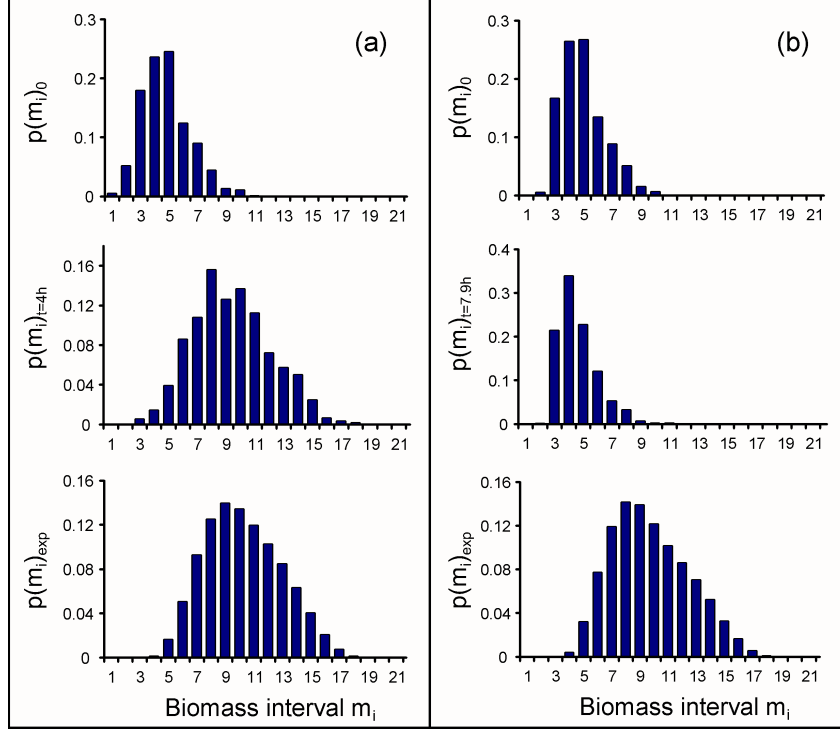


Figure 4.4: *INDISIM* simulation results for a batch culture that show the evolution of the biomass distribution for (a) a culture with no initial enzymatic adaptation (Section 4.4.1) and (b) a culture with an initial enzymatic adaptation (Section 4.4.3). They show the biomass distribution of the inoculum ( $p(m_i)_0$ ), the biomass distribution of the culture at a certain  $t$  ( $t_1 < t < t_2$ ) and the characteristic balanced biomass distribution during the exponential phase ( $p(m_i)_{exp}$ ). The enzymatic adaptation generates a backward shift of this distribution during the lag phase.

#### 4.4.2 On the duration of the transition phase in cell density

The transition phase in cell density,  $P_{T(N)}$ , is limited by  $t_1$  and  $t_2$ . The length of this phase may vary from one culture to another, although they may have similar lag parameters. As an example of this variation, we present the results of two simulations. We simulate the growth of two different inocula in the same culture medium and environmental conditions. Therefore, their characteristic distributions of biomass during the exponential growth phase will be almost the same. The two inocula used in these simulations have the same initial mean mass, but different biomass distributions within the population. The first is a homogeneous inoculum, in which all the bacteria have similar initial masses (Fig. 4.5a). The second simulation corresponds to a heterogeneous inocu-



lum, in which the initial population shows a greater variability among bacterial masses, although the mean biomass of the inoculum is the same as in the first case (Fig. 4.5b).

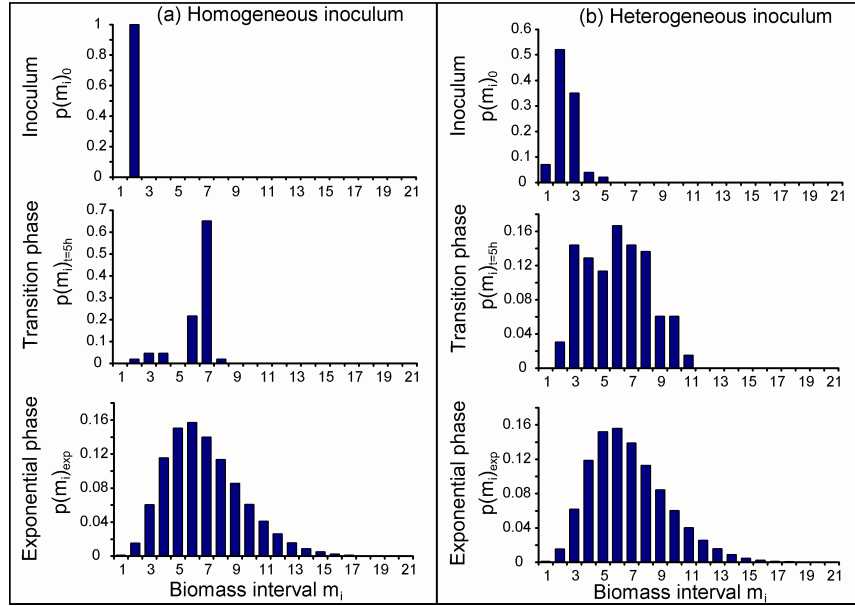


Figure 4.5: *INDISIM* simulation results for a batch culture that show the evolution of the biomass distribution of (a) a homogeneous inoculum and (b) a heterogeneous inoculum. They show the biomass distribution of the inoculum ( $p(m_i)_0$ ), the biomass distribution of the culture at  $t = 5h$  ( $t_1 < t < t_2$ ) and the characteristic balanced biomass distribution during exponential phase ( $p(m_i)_{exp}$ ).

In both cases, the lag phase is caused by the low value of the mean mass of the inoculum, which is insufficient to confer the capacity cell for division to the *average* bacterium. The model does not assume any metabolic adaptation to a substrate change in these particular cases. Therefore, we find a lag in cell density growth, but the total biomass increases from the beginning. The above presented theoretical model has been fitted to both cases, showing a good correlation ( $r = 0.988$  for the homogeneous inoculum and  $r = 0.999$  for the heterogeneous one).

In Figure 4.6, the lag parameter of the homogeneous inoculum is slightly greater than that of the heterogeneous inoculum. However, bacteria start dividing earlier in the heterogeneous culture. The main difference between these simulations is the length of the transition phase; in this particular case, the heterogeneous inoculum presents a transition phase that is rather longer than that of the homogeneous culture. In consequence, the lag

parameter of the homogeneous culture is greater than that of the heterogeneous one, but the homogeneous culture reaches the exponential phase first. This example shows that the lag parameter is a useful tool for describing the whole system macroscopically (i.e., at a population level), but it is not adequate for explaining its behaviour at a mesoscopic (i.e., from cellular to population) or microscopic (cellular or individual) level.

This different behaviour of the two cultures can be explained by means of individual lag variability. The INDISIM simulations show that the heterogeneity of the second inoculum gives bigger cells that will begin dividing earlier. The first divisions of bigger cells occur soon ( $t_{1,heterogeneous} < t_{1,homogeneous}$ ), and cell density starts to increase; the culture enters the transition phase earlier. However, there are also small bacteria that have longer lags, as shown in Figure 4.5b. This prolongs the transition. The homogeneity of the first inoculum means smaller variability between individual lag times. Once the first bacteria reach their mass to initiate the reproduction cycle, the smaller ones will not be far behind (Fig. 4.5a). Thus, the transition phase is shorter.

The classical definition of the lag phase as given by the classical lag parameter  $\lambda$  (Fig. 4.1) partially loses sense when we look at the system more closely. Consequently, a more accurate definition of the lag phase at a mesoscopic and cellular level of description is required. In such circumstances, a basic question arises: does the lag phase in cell density end when cellular reproduction begins, or when the culture fully enters the exponential growth phase? If we define the lag as the arrest of bacterial divisions, then the longest lag phase will be found in the homogeneous culture. On the other hand, if we define the lag as the time that it takes the culture to start its exponential growth phase, then the longest lag phase will be found in the heterogeneous culture.

### 4.4.3 Transition between lag and exponential phases both in cell density and total biomass

If the composition of medium  $ENV^+$  differs from that of medium  $ENV^-$ , the inoculated bacteria must carry out a metabolic adaptation in order to metabolize the new nutrient. This adaptation can be individually modelled as an enzymatic synthesis (see Section 2.3.3) that temporally drives the nutrient catabolism, and that may cause an initial decrease in the total biomass. In extreme situations, cellular death may be the terminal outcome for those cells that initially contained very small amounts of energy and skeleton carbon sources.

In an application of our IBM model, individual bacteria must achieve metabolic adaptation through enzymatic synthesis. This leads to a collective initial lag in both total biomass and cell density clearly observed in INDISIM simulation outcomes (Fig. 4.2b):

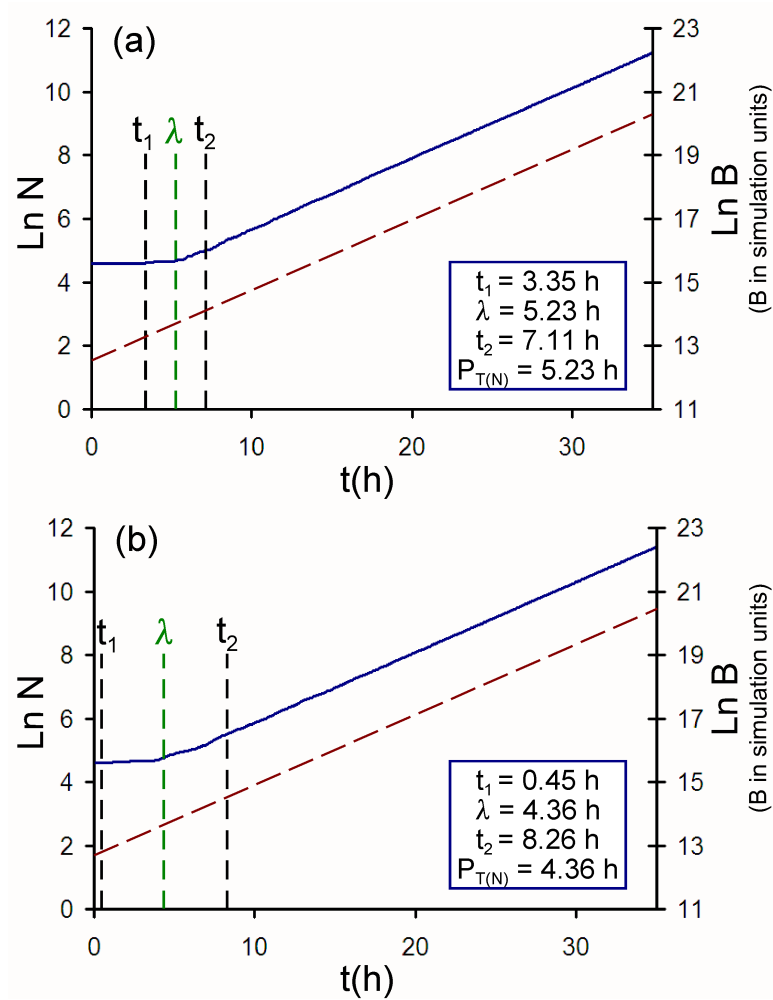


Figure 4.6: *INDISIM* simulation results for a batch culture. The solid lines depict the cell density evolution and the dashed lines show the total biomass evolution of (a) a homogeneous inoculum and (b) a heterogeneous inoculum. The text boxes give the parameters of the mathematical model that describe the cell density evolution (Section 4.3.3). Different durations of the transition phase in cell density ( $P_{T(N)}$ ) can be observed.

the lag phase in total biomass is shorter than the lag phase in cell density. The simulations show that bacterial colonies cannot grow immediately after being inoculated into the new medium. The rates  $\mu_N$  and  $\mu_B$  increase until reaching their maximum value at the exponential growth phase, even if there is an initial decrease either in total biomass

or in cell density ( $\mu \leq 0$ ), or in both of them. The stages in cell density and total biomass lag phases observed are described in Table 4.3.

Table 4.3: *Description of the observed phases in simulated growth for the study of a lag phase both in biomass and cell density.*

Number of bacteria ( $N$ )			Total biomass ( $B$ )		
$P_{I(N)}$	$\mu_N \leq 0$		$P_{I(B)}$	$\mu_B \leq 0$	
$P_{T(N)}$	$\mu_N < \mu_{EXP}$	$\frac{d\mu_N}{dt} > 0$	$P_{T(B)}$	$\mu_B < \mu_{EXP}$	$\frac{d\mu_B}{dt} > 0$
$P_{EXP}$	$\mu_N = \mu_{EXP}$	$\frac{d\mu_N}{dt} = 0$	$P_{EXP}$	$\mu_B = \mu_{EXP}$	$\frac{d\mu_B}{dt} = 0$

Another simulation upshot states that an appropriate value for the mean biomass, and thus a suitable biomass growth rate, is required prior to an optimized cell density growth rate. In the present case, cell density growth rate begins increasing when the biomass growth rate has already reached its exponential value (Fig. 4.3b).

The mathematical model was fitted to the transition phase in cell density growth with a high correlation factor,  $r = 0.9934$ . Again, the high correspondence between the two model outcomes lies in the simplicity of their assumptions. The fitting parameters are shown in Figure 4.2b.

The initial decrease in the total biomass is clearly observed as a backwards shift in the biomass distribution. Then, once  $\mu_B$  begins increasing, the biomass distribution evolves to reach the characteristic exponential mean and shape (Section 4.2.2). This three-step evolution is shown in Figure 4.4b.

## 4.5 On the lag and exponential phase definition

The results presented in this report provide a new description of bacterial axenic cultures during the lag phase and interpretation at a cellular, population and mesoscopic level.

The IbM simulations allow a thorough study of the transition from lag to exponential phase, providing a fresh approach to the complexity and uncertainties of this phenomenon. This description questions the classical identification of the culture lag phase with the cell density lag phase. The lag phase of a culture is usually determined by the lag parameter: the intersection between inoculum level and exponential phase extrapolation in the logarithmic representation of the cell density growth curve. The geometrical evaluation of the lag parameter gives a time value which corresponds exactly to half of the transition phase in cell density, as defined in our mathematical model. But the remaining half of the transition phase is still part of the lag phase, since the culture has not yet entered the

exponential growth phase. Furthermore, the macroscopic (population-level) definition of the lag phase in cell density is usually indistinctly associated with two different states of the culture, namely the moment when the culture starts growing and the moment when the culture enters its exponential growth phase. Therefore, the lag phase does not yet have precise limits.

### 4.5.1 The lag parameter

In the preceding sections we have split the lag phase into an initial phase and a transition phase. The end of the lag phase and initiation of the exponential phase have been defined with the parameter  $t_2$  (Eq. 4.16), which would be an appropriate parameter to define the lag duration. Nevertheless, it is only available when the mathematical model is valid—that is, when the transition phase can be modelled as a quadratic curve in the logarithmic scale. It implies that the inoculum has to be big enough to define the  $\mu$  from the beginning. Moreover, if we want to evaluate  $t_2$  in an experimental dataset, we need to determine the first division time ( $t_{FD} = t_1$ ), which is not possible in most of the available techniques.

One can consider the lag phase to be only the initial phase—that is, the absence of duplications. Then, the transition phase would be included in the exponential phase, and the lag parameter would be the first division time,  $t_{FD}$ . This is probably the more precise and meaningful parameter, but it has an evident difficulty when it comes to being measured with experimental techniques.

The typical lag parameter,  $\lambda$  (geometrical definition), is the easiest one to define and evaluate in any growth curve, either experimental or simulated. Actually, it is equivalent to a hypothetical delay in reaching a limit concentration. But, although it is still the most used and useful, it is the one with the weakest biological meaning. In this chapter it has been seen that the lag parameter is only an indicator of the lag phase, but it can not be used as the unique definition for the lag of a culture. In the following paragraphs, two weak spots in the use of  $\lambda$  to identify the lag of a culture are presented.

#### **Low inocula: problems of the geometrical definition**

Low inocula growth curves have a high synchronism in the first stages of the growth. This synchronism produces a geometrical effect that deforms the geometrical evaluation of the lag parameter. That is, the lag parameter ( $\lambda$ ) gives a time that is shorter than the first division time ( $t_{FD}$ ): this result is not consistent with the lag phase definition, since it is not possible for the lag to end before the first division has been made. As the

inoculum size increases, this effect disappears.

This effect can be observed in the output results of the simulations presented in Section 3.5, where the growth of different inocula were studied to evaluate the effect of the inoculum size. Figure 4.7 shows the relationship between the ratio  $t_{FD}/\lambda$  and the inoculum size. If the lag parameter is lower than the first division time, this ratio is greater than 1.

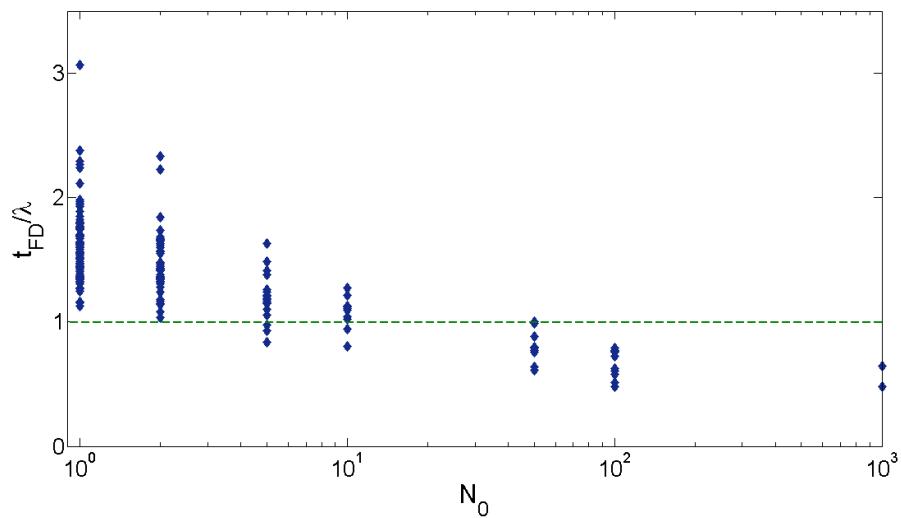


Figure 4.7: *INDISIM* simulation results showing the relationship between first division time ( $t_{FD}$ ) and the lag parameter ( $\lambda$ ) versus inoculum size. The dashed line indicates the level  $t_{FD}/\lambda = 1$ .

### Viable and non-viable cells

Another example of the drawbacks of reducing the lag phase to the determination of the lag parameter is that the observed lag phase of a culture may vary depending on the experimental technique used to evaluate the total population. Major differences are found when comparing counting techniques that discriminate viable from non-viable cells, such as CFU (Colony Forming Unit) counting, with techniques that do not, such as microscopic counts, electric counts and cytometric counts. In addition, it is generally accepted that viable cell counts are lower than the corresponding total cell count in any microbial culture.

We present a simulation showing how different experimental counts affect the calculated value of the lag parameter, when some cells do not survive the adaptation period. Two samples of the same culture (simulation) are counted using two different experimental techniques: one of them (A) distinguishes viable from non-viable cells (e.g. CFU counting); the other one (B) does not (e.g. microscopic counts). As expected, the results show that the cell concentration obtained by method (A) is always lower than that obtained by method (B) (Fig. 4.8). Their outcomes for the lag phase measure (Eq. 4.17) show that the lag phase measured with method (B) is shorter than that measured by method (A). Since both samples belong to the same culture (in this case, an INDISIM simulated culture), the results obtained with different techniques should not be different. Otherwise, the lag parameter is not a general enough tool for characterizing the cultures.

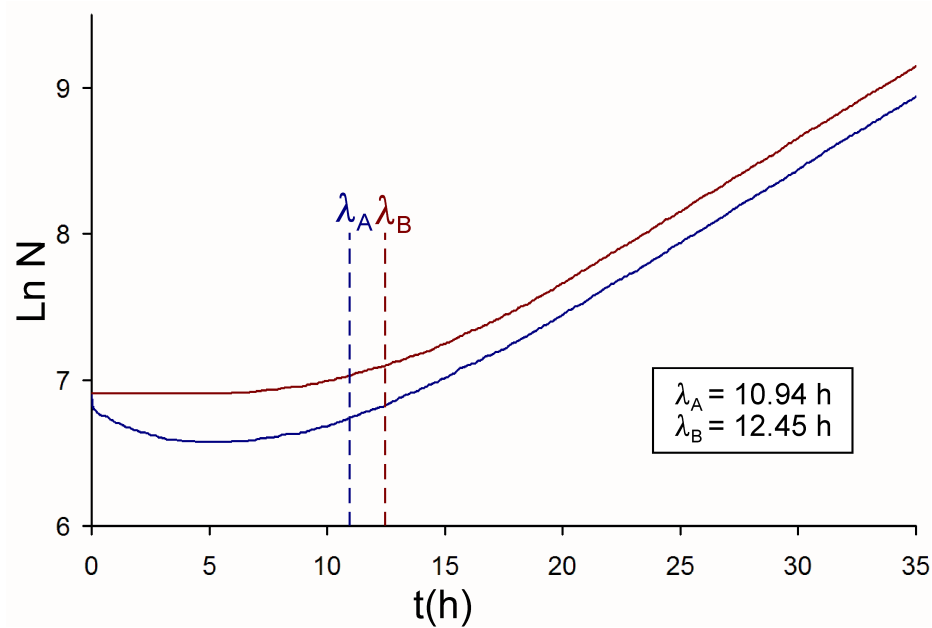


Figure 4.8: *Effect of the experimental technique on the lag parameter determination. The curve A gives the simulated results for an experiment that distinguishes viable from non-viable cells. The curve B shows the simulated results for an experiment that does not. The lag parameters obtained by means of Eq. 4.17 are shown in the figure.*

The effect of the non-growing cells is higher when the culture growth takes place in a really adverse environment—that is, near its growth limits. If we take an inoculum of 10 cells, and we suppose that only one cell is able to adapt to the new medium and grow,

we can obtain differences in lag times such as the one presented in Figure 4.9. In this case, the proportion of non-growing cells is 90%. Then, we observe a clear *apparent* lag or *pseudolag* in the culture, while the only cell that is growing would determine the *real* lag (Pirt, 1975).

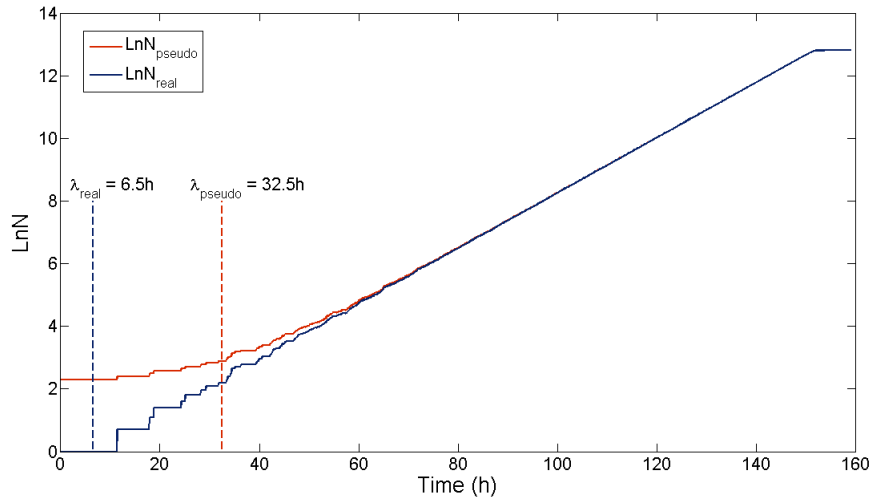


Figure 4.9: *INDISIM* simulation output for an inoculum of 10 cells with a growing fraction of 1/10 and a non-growing fraction of 9/10. If we evaluate the total population lag phase we obtain the pseudolag (32.5 h), and if we evaluate the growing fraction we obtain a real lag (6.5 h). Both lags are calculated by means of the geometrical definition.

### 4.5.2 Exponential phase and balanced growth

The exponential phase is often assumed to be accompanied by balanced growth conditions: all cellular constituents have the same growth rate. Under balanced growth conditions, the distribution of the individual properties in the culture (for instance the biomass distribution or the DNA content distribution) is also constant. In these conditions (exponential and balanced), we talk about a steady state (Fishov et al., 1995).

Sometimes, the culture may be exponentially growing without balanced growth conditions. This is the case of the transition between the lag and exponential phases, or the initial stages of a low inocula growth. *INDISIM* simulations allow precise control of the evolution of the biomass distribution along the growth. The *distances* were defined to visualize and evaluate this evolution (Section 3.2.1). Thus, the *product distance*  $D(t)$



(Eq. 3.3) is the appropriate mathematical tool to distinguish non-balanced from balanced growth in terms of biomass distribution.

While the  $D(t)$  fluctuates, the biomass distribution is not stable: the culture is not growing in a balanced way in terms of the biomass distribution. When  $D(t)$  remains approximately constant around 0, we can be assured that the balanced growth conditions in terms of biomass distribution have been assessed.

Let us take again the series of simulations regarding the inoculum size effects used in Section 3.5. The presence or absence of meaningful fluctuations in  $D(t)$  is subject to an arbitrary decision. Thus, it does not become a universal definition of balanced or non-balanced growth. But it can be used as an indicator of the change.

In this case, we start with the information about the higher part of the growth curve: we take the same interval that has been used for calculating the lag parameter (see Section 2.3.5). In these simulations, the interval that has been used is limited by  $\rho_{inf} = 0.6$  and  $\rho_{sup} = 0.85$ . That is, the lag parameter has been set as the intersection of the inoculum level and the logarithmic regression of the growth curve in the interval  $[\ln N_0 + 0.6 \cdot (\ln N_f - \ln N_0)]$  and  $[\ln N_0 + 0.85 \cdot (\ln N_f - \ln N_0)]$  in each simulation. Moreover, the reference biomass distribution to characterize the exponential growth ( $\bar{p}_{k_m, exp}$ ) for assessing the *mass distribution distance* ( $D_{p_k}(t)$ ) is also fixed by the mean distribution in the interval defined by  $\rho_{inf}$  and  $\rho_{sup}$ . Then, in this interval the *product distance*,  $D(t)$ , is highly stable.

We take the mean value of  $D(t)$  in the interval defined by  $\rho_{inf}$  and  $\rho_{sup}$ . Let us call this mean value  $\bar{D}_{exp}$ . This value is used as a reference: we consider that the fluctuations have disappeared when  $D(t)$  remains under  $(\tau_B \cdot \bar{D}_{exp})$ . Then, the value of the threshold  $\tau_B$  is subject to the arbitrary decision. We have chosen a value  $\tau_B = 10$ , which shows consistent results for all the simulations studied.

Now, for each simulation we can evaluate the lag parameter,  $\lambda$ , and the time at which the culture starts to be in balanced growth conditions (in terms of biomass distribution). This parameter will be the minimum time  $t_B$  where the condition  $D(t > t_B) < \bar{D}_{exp}$  is satisfied. The last part of the growth, when the culture enters the stationary phase, is not taken into account. Figure 4.10 shows a sample of the results obtained from the mentioned simulations: for each inoculum size, one simulation is shown. The values of  $\lambda$  and  $t_B$  are indicated in each plot. The fluctuations before  $t_B$  can be seen in all the curves.

It can be observed that the small inocula have a longer  $t_B$  than the larger inocula. This is an effect of the initial synchronism produced by the small inocula. The biomass distribution can not be stable until the concentration reaches a *statistical* level. It must be

pointed out that the mean mass of the inocula is similar in all cases. Actually, the  $\lambda$  values are also similar, as was seen in Section 3.5 in greater detail. Thus, the differences in the non-balanced *vs.* balanced behaviour are due to the differences in the initial concentration (and, therefore, to the initial biomass distribution).

## 4.6 Discussion

Cell density and total biomass growths have been distinguished and studied separately. According to the analytical outcome of our approach in Section 4.3 and the simulation results in Section 4.4, we observed that the culture lag phase should be defined, at a minimum, by both the lag phases in total biomass and in cell density.

The stages in the bacterial lag phase presented here are a holistic property of the system. The different observed behaviours of the system through the different phases are emergent properties of the individual behaviours, which appear once variability is introduced among the population. Thus, the present work requires a statistical number of cells. Firstly, because at an individual level, the collective effects of variability are lost. Secondly, because randomness in the processes affecting the individual cells (reproducing the genetic and biochemical variability) would provide a central role to the 'history' of the culture. Thus, universality of the outcomes would be lost. If a single cell or a small inoculum is studied, the present analysis cannot be undertaken and the above-mentioned phases cannot be extrapolated. They require specific treatment.

The use of INDISIM as an experimental system to test a new model has given good results. There is not argument that the ultimate proof of a model is the experimental test, but the simulations give a first insight in the soundness of the proposed theory.

Some problems with the geometrical definition of the lag parameter have been identified. The main problem is its biological significance: it gives an intermediate point in the transition between the non-divisions interval and the full exponential growth. The split of the lag phase into two stages, namely initial and transition, offers a more precise interpretation of each phase. Nevertheless, it may be difficult to identify the necessary parameters. On the one hand, the time to the first division,  $t_1 = t_{FD}$ , requires a specific experimental design to measure. For instance, the flow chamber (Elfwing et al., 2004) is an appropriate experimental system to assess this measure. On the other hand, the time at which the growth is fully exponential,  $t_2$ , has a clear mathematical definition for the cases where the presented mathematical model can be used. But it has to be defined for cultures with an initial synchronism such as the growth of low inocula.

The differences between the lag obtained with an experimental instrument that dis-

tinguishes viable from non-viable cells and the lag obtained with an experimental system that does not have been studied. This leads to the definition of the *real* lag and the *pseudolag* of a culture, which may be very different.

A first attempt at a complicated and putatively interesting discussion on the significance of the exponential growth phase and on the meaning of steady state (Fishov et al., 1995) has been made in this chapter. The exponential phase is usually characterized by balanced growth, where the intensive variables remain stable (the extensive variables such as cell density or total biomass are obviously growing). But once it is ascertained that  $\mu$  remains constant during this phase, why do we assume that all the culture's properties behave in the same way? We have shown here that while a culture may be growing exponentially in terms of biomass, it may well be going through a lag phase in cell density. Moreover, if a bacterial population grows exponentially, does this mean that every statistical value concerning any cellular property remains stable? Is it possible to have a culture in the exponential growth phase with a non-stable mean mass? Does a stable biomass distribution reflect a stable DNA distribution (or other individual property distributions) among cells? None of these questions has an immediate response, and they should be tackled from a multidisciplinary point of view. In this chapter the study of the balanced growth conditions during the exponential phase has been approached through the *distances*. The *product distance* has proved to be very useful in distinguishing between non-balanced and balanced growth conditions in terms of biomass distribution.

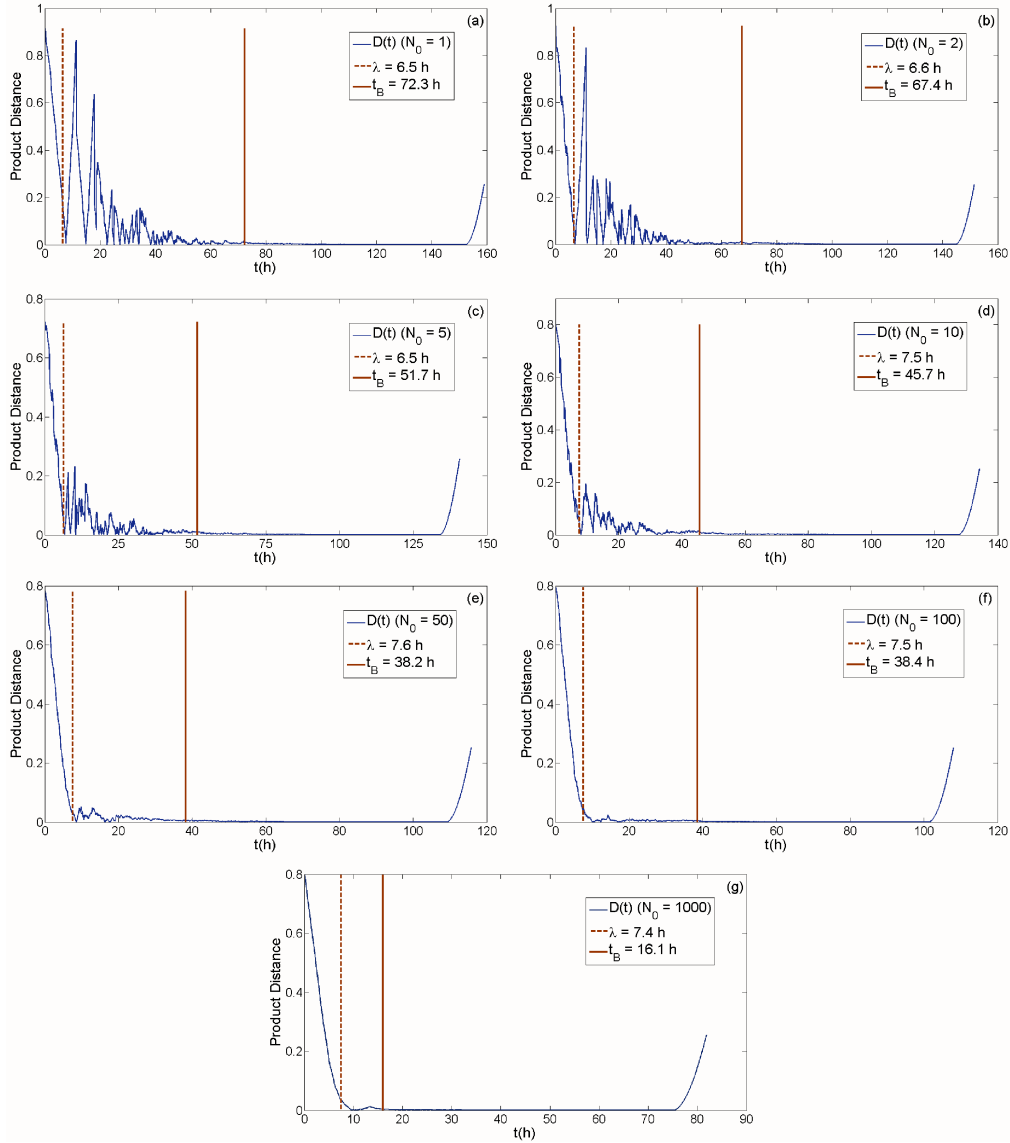


Figure 4.10: Evolution of the product distance ( $D(t)$ ) for INDISIM simulations with different inoculum sizes: (a)  $N_0 = 1$  cell, (b)  $N_0 = 2$  cells, (c)  $N_0 = 5$  cells, (d)  $N_0 = 10$  cells, (e)  $N_0 = 50$  cells, (f)  $N_0 = 100$  cells, (g)  $N_0 = 1000$  cells. Each graph shows the corresponding lag parameter,  $\lambda$ , calculated by means of the geometrical definition, and the time of initiation of balanced growth,  $t_B$ , calculated by means of the method detailed in Section 4.5.2.