Chapter 7

Conclusions

7.1 Synthesis of results and specific conclusions

We present in this section a synthesis of the main results and conclusions concerning each specific objective of this thesis.

A) Conceptual approach

1. To identify the important variables for studying the lag phase. These variables should be appropriate to undertake the study of other transient processes.
Historically, the lag phase has been defined in terms of cell density. Therefore, it has been the most widely used variable in the study of the bacterial lag. We have seen that, minimally, there are two groups of different variables whose study provides interesting information about the lag phase: those related with the biomass, and those related with the growth rate. The biomass distribution dynamics reflect the processes that take place at an individual and population level (Chapter 3): • A backwards shift in the biomass distribution shows that the bacteria are subjected to conditions that have an energetic cost larger than the energy obtained from nutrient uptake.

- If this backwards shift takes place during the lag phase, it means that the bacteria are carrying out a specific metabolic adaptation to the new conditions. If a culture that is growing exponentially shows a backwards shift, it reflects the end of this phase and the beginning of the stationary phase.
- Stability in biomass distribution is a necessary condition for a culture which is in exponential growth and in balanced conditions in terms of biomass distribution. This distribution depends on the bacteria characteristics and on the culture medium conditions. Cultures in late stationary phase may also show a stable biomass distribution but with a mean mass lower than the exponential one.
- Any inoculum with an initial biomass distribution that differs from the characteristics of the exponential phase shows a dynamic evolution of this variable during the lag phase as it adapts to the exponential growth conditions.
- Therefore, any change in a culture's biomass distribution is proof of the culture's adaptation to a change in the environmental growth conditions.
- This kind of information can be partially obtained from the evolution of the total biomass (Chapter 4) or the mean mass (Chapter 5).
- A faster or slower evolution of these variables reflects a faster or lower adaptation of the culture to the new conditions (Chapter 5).

The evolution of the growth rate is also a good indicator of the growth phases. It allows the splitting of the lag phase into two stages: initial phase and transition phase (Chapter 4):

- During the initial phase the growth rate may be negative or equal to zero. Once in the transition stage, the growth rate and its derivative are positive. The growth rate increases until reaching the characteristic exponential value.
- During the lag phase, the growth rate has its maximum value and is more or less constant.
- The beginning of the stationary phase may be seen as a decrease in the growth rate.
- The evolution of the total biomass growth rate and the evolution of the cell density growth rate may be different, providing complementary information about the culture's evolution.

2. To distinguish and analyze two of the five causes of the lag phase mentioned in Section 1.1.3.

Two causes of the lag phase have been assumed and studied separately: (i) the initial state of the inoculum (low mean mass), and (ii) a metabolic adaptation to a new nutrient source (Chapter 3).

- During the lag phase, the biomass distribution evolves to the exponential characteristics. In the first case (i), there is a forwards shift while the culture mean mass increases. In the second case (ii), the metabolic adaptation produces an initial backwards shift in the biomass distribution, followed by a forwards shift to reach the exponential characteristics.
- The two causes are often inseparable in real systems, but IbM simulations have allowed them to be studied independently.
- The main results have been experimentally verified by means of flow cytometry (Chapter 5).

3. To analyze the relationship of the lag phase duration with different variables such as temperature or the inoculum state.

The relationship between the lag phase and different variables has been tackled (Chapter 3):

- With temperature: the experimental results of the literature show that when the temperature increases the growth rate also increases and the lag duration decreases. The microscopic model assumed in this study provides this relationship at a macroscopic level.
- With the initial mean mass: the lower the ratio of the initial mean mass to the mean mass to initiate the reproduction cycle, the higher the *distance to be covered* and, therefore, the longer the lag period. The initial mean mass does not change the culture growth rate.
- With the maintenance energy: an increase in the maintenance energy constant produces a decrease in the growth rate, resulting in an increase in the lag duration.
- With the inoculum size: in the simulation results no variability in the lag is associated with the inoculum size. Nevertheless, when the inoculum size increases there is a decrease in the first division time and the detection time.

These results are in accord with the experimental results found in the literature.

4. To study the relationship between the individual lag and the population lag.

INDISIM simulations have been shown to be very useful in the study of individual lags, since the simulator controls every bacterium of the inoculum. It has been seen that the population lag is lower than the mean of the individual lag, taking as individual lags the time to first division of each bacterium of the inoculum (Chapter 3). This result is in agreement with the specialized literature.

5. To analyze the temporal behaviour of the system during the lag phase.

The temporal evolution of the population during the lag phase has been tackled (Chapter 4):

- The lag phase has been split into an initial stage and a transition stage.
- A simple mathematical model has been proposed to shape the transition phase in cell density. This model is applicable when no synchronism is found in the first stages, that is, when the cell density growth rate can be defined from the beginning of the growth cycle.
- This mathematical model has been validated by means of INDISIM simulations, and has proved to be very useful to distinguish the two above-mentioned stages in the lag phase: initial stage (time to first division) and transition stage (between the first division and the complete exponential growth).

6. From the obtained results, to carry out a critical revision of the lag phase concept.

The concept of lag phase has been reviewed, and its weak spots have been commented upon (Chapter 4). From the definitions reported in Chapter 1, it is not clear whether the lag time corresponds to the period before the first division (t_1), the period before the growth is fully exponential (t_2) or an intermediate period given by the lag parameter λ. Then:

- In the first case (t_1), the transition phase between the lag and the exponential should be defined and assumed.
- We think that it is better to identify the lag with the global period before the exponential growth (t_2), that is, the time that the growth rate takes to reach the maximum value.
- The lag parameter λ gives a time value between $t_1$and t_2 (when the above-mentioned mathematical model can be applied, $\lambda = \frac{t_1+t_2}{2}$). Although it is useful to analyze experimental results or to compare different models, lag parameter can not be used as a universal definition of the lag phase; it must be taken as an indicator.
- The λ value for a certain culture changes if we are working with an experimental technique that distinguishes viable from non-viable or if the experimental technique does not, giving two different values for the same culture.
- When an important synchronism exists during the first stages of the growth cycle (i.e., small inoculum), the lag parameter is lower than the first division time ($\lambda < t_1$), which is not consistent with any lag phase definition or theoretical intepretation.

B) Methodological approach

1. To improve INDISIM in order to study the bacterial lag phase:
(a) to improve the model with the required adaptations,
(b) to implement the new improvements in the simulator, and
(c) to incorporate the specific calculus and the appropriate outputs in the simulator.

INDISIM has been improved in order to undertake the study of the bacterial lag phase:

- A simple enzyme model has been added to the bacteria model in order to simulate the metabolic adaptation to a nutrient change.
- This model has been implemented with the simulator and validated (Chapter 3).
- The lag parameter (geometric definition) and the maximum growth rate calculation, as well as the estimation of the instantaneous growth rate, have been implemented with the simulator.
- The *distances* assessment has also been added to the simulator, in order to obtain temporal evolution throughout growth as a regular output.

2. To verify the suitability of the IbM approach and, specifically, the suitability of INDISIM in the study of transient processes in bacterial growth such as the lag phase.

INDISIM's usefulness and suitability to undertake the study of a transient phase such as the lag phase have been demonstrated (Chapter 3). The strong points are:

- It allows the isolation of the causes of the lag phase, as well as their independent study to assess their contribution to the lag dynamics. This is not possible in real systems.
- Low inocula are a classic problem in the experimental approach because they require specific techniques, as well as in continuous models because of the diversity and the effects of synchronism. In these cases, INDISIM is especially useful because it allows the study of these inocula dynamics in detail.

3. To develop mathematical tools for assessing the dynamics of the transient processes.

The *mean mass distance*, *mass distribution distance* and *product distance* have been developed in order to assess the evolution of the culture biomass distribution during the growth cycle:

- These *distance* functions have proved to be useful in both the simulations (Chapters 3 and 4) and the experiments (Chapter 5).
- The *product distance* allows the identification of the different growth phases (Chapters 3 and 5), as well as the identification of the balanced growth conditions with regard to the biomass distribution.
- The *product distance* is also a measurement of the adaptation rate of the culture to the new growth conditions (Chapters 3 and 5).
- Simulations and flow cytometry results show a linear decrease in the *product distance* during the lag phase (Chapters 3 and 5).

4. To develop methods for parameter estimation in IbMs.

We have made progress in IbM parameter estimation methods. Specifically, we have estimated INDISIM input parameters in order to fit the output results of the simulations to an experimental dataset. Three methods have been analyzed (Chapter 6):

- The classic grid search, which may be too time-consuming.
- The Nelder-Mead Threshold Accepting, which reduces the time spent when working with one parameter, but is still too time-consuming when estimating two or more parameters.
- The NEWUOA method, which gives the best results with regard to time spent, and which maintains satisfactory precision in the parameter estimation results.

This study was carried out together with BioTeC research group (Katholieke Universiteit Leuven), working in parallel with BacSim parameter estimation. The results obtained with INDISIM and BacSim were equivalent, which is proof of the consistency of the analyzed parameter estimation methods and an indicator of INDISIM and BacSim validity as IbM simulators.

C) Experimental approach

1. To select appropriate experimental methods for measuring the evolution of the biomass distribution of a culture throughout the growth cycle.

We have worked with two experimental methods that allow size distribution measurements of a bacterial population: flow cytometer and multisizer. These methods have been used not only for assessing the size distribution of the cultures at a particular moment, but also to follow their evolution throughout the growth cycle (Chapter 5).

- We carried out the experimental measurements in *Escherichia coli* cultures growing in an M9 medium at 20°C and 35°C.
- The multisizer used allows rapid measurements of cell concentration, but it has a low threshold at $0.7\,\mu m$ and a significant fraction of the cells is lost.
- The flow cytometer allows rapid, numerous and precise measurements of the forward scatter distribution among a population with small samples. Therefore, the size distribution dynamic evolution throughout the growth cycle may be easily assessed.

2. To adapt the experimental protocols in order to obtain the necessary measurements.

The experimental protocols have been adapted in the following way (Chapter 5):

- In order to ensure an initial lag, we have worked with inocula taken from the stationary phase of the pre-inoculation cultures (between 12 and 18 hours in 35°C experiments, and up to 7 days in 20°C experiments).
- Due to the small size of the bacteria used, it is essential to filter the culture medium ($0.2\,\mu m$) to avoid incorrect measurements.
- It is essential to take measurements at short time intervals ($10^2 - 10^3\,s$) during the first hours of the growth, in order to guarantee enough measurements of the culture during the lag phase.

3. To analyze the experimental results and to develop the necessary mathematical methods for doing this.

The experimental results have been analyzed in the following way (Chapter 5):

- The *distance* functions have been adapted as mathematical tools to analyze the experimental results. The *product distance* has proved to be a useful tool for this purpose.
- The obtained distributions have been graphically represented in order to observe their stability or movement in the different growth phases.
- The temporal evolution of the mean diameter and the temporal evolution of the *product distance* have been used to distinguish the different phases of the growth (lag, exponential and stationary).

4. To interpret the obtained results and to compare them with the previous simulation outputs in order to validate the suitability and soundness of INDISIM simulations.

Several simulation results reported in Chapter 3 for the lag caused by a low initial mean mass have been experimentally verified:

- The temporal evolution of the size distribution during the different phases of the growth has been shown: forwards shift during lag phase, stability during exponential phase and backwards shift at the beginning of stationary phase.
- It has been seen that the initial decrease in *product distance* is linear.
- It has been checked that the lag phase at 35°C is shorter than the lag at 20°C.

Since these results are neither trivial nor accidental, we can say that INDISIM has been validated as a suitable method for the study of the bacterial lag phase.

7.2 General conclusions

The bacterial lag phase has been generally tackled from two generic approaches: at a cellular and intracellular level, which we call the microscopic scale, and at a population level, which we call the macroscopic scale. Studies at the microscopic level undertake the

processes that take place inside the bacterium during its adaptation to the new conditions such as the changes in genetic expression and in metabolism. Studies at the macroscopic scale deal with the description of a population growth cycle by means of mathematical continuous modelling and experimental measurements of the variables related to cell density evolution.

IbMs introduce a mechanistic approach by modelling the cell as an individual unit. IbM simulations deal with 1 to 10^6 cells. They allow the specific study of the phenomena that emerge from the interaction among cells. These phenomena, which are inherent to living systems, belong to the mesoscopic level. Mesoscopic approaches are essential if we are to understand the effects of cellular adaptations at an individual level on the evolution of a population. Thus, they are a bridge between individuals and population, or, to put it another way, between models at a microscopic scale and models at a macroscopic scale.

In science experimental research is essential. Thus, experiments at the mesoscopic scale are also indispensable to study the bacterial lag phase. Cytometric measurements allow the assessment of cellular property distributions and their dynamics during culture growth.

The three approaches, *micro-*, *meso-* and *macroscopic*, are essential to completely understand microbial systems. Therefore, IbM and cytometry experimental techniques are indispensable tools in the progress toward global knowledge of microbial system dynamics.

In this thesis we have shown that the study of culture evolution through variables such as the growth rate and the biomass distribution provides a new insight into the processes that take place at the above-mentioned mesoscopic scale. The growth rate evolution shows that the lag phase is not a simple period of time, but rather a dynamic process with different stages: an initial phase, with a growth rate negative or equal to zero, and a transition phase, with an increase of the growth rate up to the maximum value.

We have developed the *distance* functions, which have shown to be a good tool to assess the dynamics of the cellular property distributions during the growth cycle. Specifically, the evolution of the biomass distribution during the lag phase, the exponential phase, and the transition from exponential to stationary phase have been studied by means of the *product distance*. These dynamics have been obtained by INDISIM simulations and verified through cytometry experiments.

Another possibility afforded by the mesoscopic approach is the assessment of balanced growth conditions. These conditions are found in exponentially growing cultures, but we can also find cultures exponentially growing in non-balanced conditions. Balanced growth is characterized by the stability of the cellular property distributions. Thus, the *product distance* becomes a good instrument to distinguish balanced from non-balanced growth

conditions. In this thesis we have applied this methodology to the study of balanced growth in terms of biomass distribution. Again, INDISIM simulations and cytometry measurements have provided the same results.

In this thesis we have basically tackled the study of the lag phase from an IbM approach. Specifically, we have used INDISIM methodology. Therefore, we want to finish these conclusions emphasizing the three achievements of INDISIM methodology in the research reported in this thesis:

1. We have shown that INDISIM is a good tool to improve the understanding of microbial systems.
2. We have shown the suitability of INDISIM simulations as a virtual experimental system to validate continuous mathematical models prior to real experimental verification.
3. We have shown that INDISIM allows us to predict and interpret experimental results, as well as to design new experiments to be carried out.

Above all, the validity of INDISIM as a useful tool to tackle transient processes such as the bacterial lag phase has been amply demonstrated.

7.3 Perspectives and further work

The results gathered in this thesis have answered many questions, but they have also suggested new ones and opened new avenues to explore. Below are a few of the many possibilities for further developments on this field.

In the presented work, the lag phase of axenic cultures has been investigated. In order to progress in the comprehension of the involved processes, the model of bacteria should be improved by distinguishing different *compartments* in each cell: DNA, structural material, proteins, etc. This division should be aimed (i) to improve the cell cycle model, which is essential to make progress in studying the causes of the bacterial lag; and (ii) to examine the stationary phase, which is another important phase of a bacterial culture that implies complex processes of adaptation at a cellular and population level. In fact, since the lag phase of a culture is related with the preinoculation conditions, a complete understanding of the phenomena that take place during the stationary phase is essential for the study of the subsequent adaptation of the bacteria to the new culture medium (lag phase).

A complete understanding of the balanced growth is especially interesting when studying, for instance, the transition between different metabolisms. The division of the cells

into the above-mentioned compartments should allow for greater in-depth study of the balanced growth conditions since it would permit us, for instance, to distinguish the balanced growth in terms of DNA from the balanced growth in terms of proteins.

The experimental assays should follow the same line. Cytometry techniques have proved to be a powerful tool for assessing population dynamics during the culture growth. Since they also allow the measurement of DNA content, this may be a useful way to validate the simulation results after dividing the cell into compartments.

In subsequent studies, more complex systems and situations should be studied from the IbM approach. The interaction of different species during the lag and the stationary phases, as well as the spatial effects in the different phases of the growth, should be taken up in detail. Such approaches would allow the study of bacterial growth in real foods like meat and cheese. Moreover, the quantitative effects of several environmental factors such as temperature, a_w and pH in the lag phase have been widely described at the cellular level, but their effect on the culture dynamics should be addressed from the IbM perspective.