

**UNIVERSITATEA NAȚIONALĂ de ȘTIINȚĂ și
TEHNOLOGIE „POLITEHNICA” BUCUREȘTI**
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Metodologie de elaborare a modelelor cinetice complexe, structurate pe mai multe niveluri de detaliere (hibride), folosite in calcule de inginerie (bio)chimica privind optimizarea unor reactoare industriale biochimice sau biologice

„Methodology for developing complex kinetic models, structured on multiple levels of detail (hybrid), used in (bio)chemical engineering calculations regarding the optimization of industrial biochemical or biological reactors”

**TEZĂ DE DOCTORAT
PHD THESIS**

Domeniul fundamental de doctorat: Științe Inginerești
Domeniul de doctorat: Inginerie Chimică

Conducător de doctorat
PhD supervisor:
Acad. Prof. Dr. Ing. Gheorghe MARIA

Doctorand
PhD student:
Ing. Laura RENEĂ (căsătorită CERNENCU)

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Chap. I. Thesis topics opportunity and main objectives of the doctoral thesis

I.1.1.- The opportunity of the topics

Advantages and disadvantages of using biocatalysts such as enzymes or cellular cultures (free or immobilized on porous solid supports)

In recent decades, there has been a growing trend to replace complex fine chemical synthesis processes, which are highly energy-intensive and generate large amounts of toxic waste, with biosynthesis processes (using isolated and purified enzymes or cell cultures as biocatalysts). This shift is motivated by the multiple advantages offered by enzymatic processes (**Fig. 1-1**): i) very high selectivity; ii) high conversion efficiency; iii) no generation of toxic byproducts; iv) mild reaction conditions (close to ambient), easily achievable without high costs (low temperatures of 20–60°C, normal pressure, and controllable pH levels); v) the use of low concentrations of biocatalysts [Moulijn et al., 2001; Gavrilescu and Chisti, 2005].

Currently, considerable efforts are being invested in several directions for developing biosynthesis processes, namely:

A) Protein engineering, at the molecular design level, to obtain enzymes with desired properties;

B) Genetic engineering to obtain genetically modified microorganisms (GMO);

C) Physicochemical treatments coupled with the fabrication of bioactive nanostructures to optimize the support material used for immobilizing enzymes or biomass in the bioreactor.

All these efforts (A–C) aim to develop biocatalysts that are as active, selective, and stable as possible.

These efforts attempt to overcome current challenges in the biosynthesis industry, particularly the high costs of producing enzymes or cell cultures that are sufficiently stable and resistant to impurities, slight variations in operating conditions, and chemical stress (due to bioreactor additives) as well as mechanical stress (caused by liquid agitation in the bioreactor). Addressing these challenges requires advanced control of the industrial bioprocess.

As a result, in recent years, a significant number of enzymatic or biological industrial processes have been reported for the production of chemical products/derivatives in fine organic synthesis, pharmaceuticals, food, and detergent industries, using various bioreactors with cell cultures or enzymatic systems [Liese et al., 2006; Wang, 2009; Straathof and Adlercreutz, 2005; Blanch and Clark, 1997; Buchholz and Kasche, 1997; Deckwer et al., 2006; Buchholz and Hempel, 2006; Jiménez-González and Woodley, 2010].

Among these new processes, notable examples include the production of monosaccharide derivatives (synthetic sweeteners), organic acids, alcohols, amino acids, etc., using mono- or multi-enzymatic reactors or bioreactors with cell cultures for the production of yeast, food additives, recombinant proteins (enzymes, vaccines), and biopolymers [Straathof and Adlercreutz, 2005; Liese et al., 2006; Maria et al., 2024B; 2024C; Maria and Renea, 2021; Gijiu and Renea, 2025].

The development of a sustainable biological process must consider several aspects related to the characteristics of the biocatalyst, process integration, and cost minimization, while also meeting economic, environmental/safety, and social objectives [Jiménez-González and Woodley, 2010; Khamseh and Miccio, 2012; Tsangaris and Baltzis, 1996].

When scaling up a new biological process, the first step is to experimentally determine the characteristics of the biological process (kinetic model, optimal reaction conditions for the enzyme/biomass, enzyme/biomass deactivation kinetics). Next, several **biochemical engineering challenges** must be addressed, including:

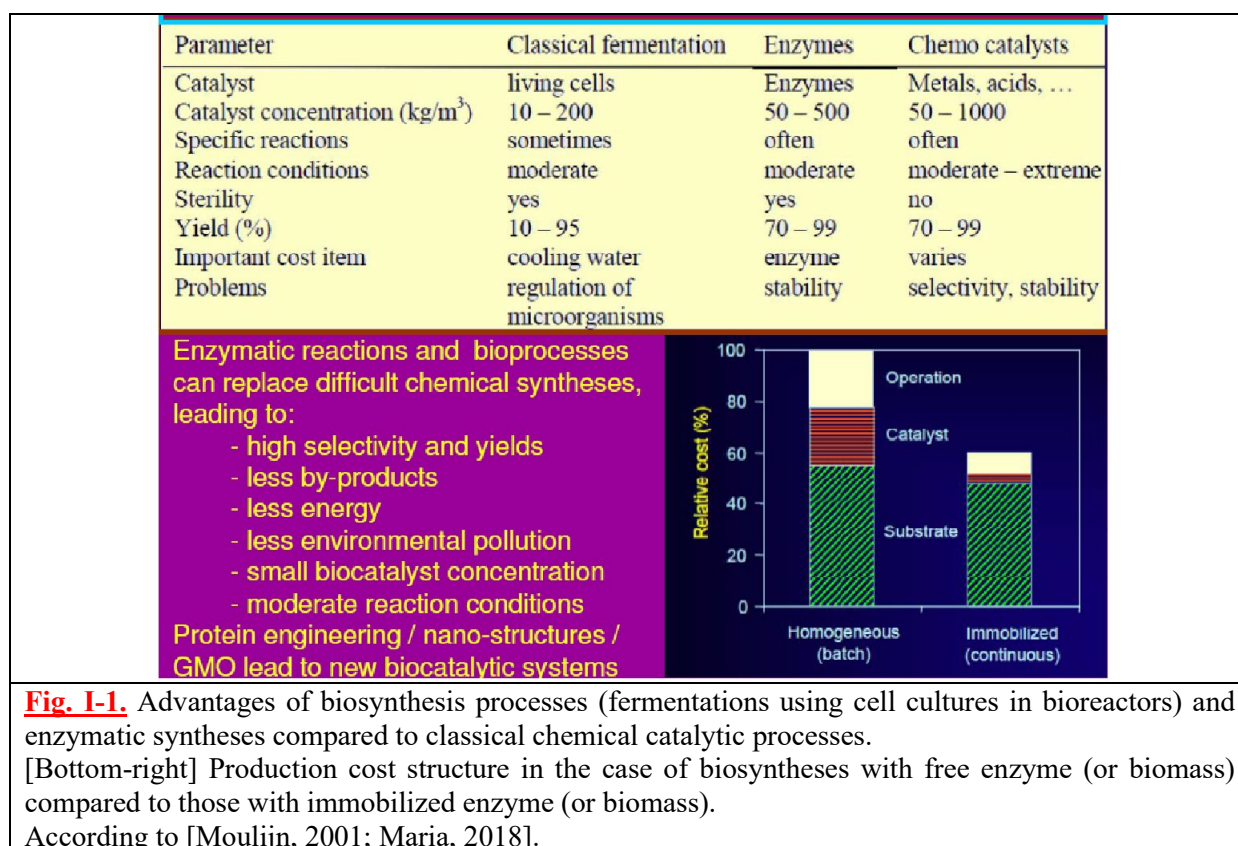
- i) Selecting the most suitable type of enzymatic or biological reactor (with cell cultures) for the studied bioprocess (either perfectly mixed or plug flow if the biocatalyst is immobilized in a fixed bed) [Maria, 2020D; Maria and Renea, 2022B].
- ii) Selecting the optimal operating mode for the chosen reactor [batch (**BR**), semi-continuous (**FBR**), batch with intermittent addition of enzyme/biomass/substrate (**BRP**), or continuous ("chemostat")] [Maria, 2020D; Moser, 1988; Deckwer, 1991];
- iii) Choosing the method of biocatalyst utilization (enzyme/biomass in free form or immobilized on a suitable solid/gel support). Immobilization of the biocatalyst is desirable as it increases its stability and activity duration. Despite the high immobilization costs, the economic effects are highly favorable, as the cost of the non-immobilized biocatalyst contributes the most to production costs, as shown in (**Fig. I-1**) [Nedovic and Willaert, 2005].

Industrial biosynthesis

Industrial biosynthesis has become one of the most significant branches of the pharmaceutical and food industries, playing a crucial role in medicine and having a major social impact. Globally, the enzymatic and biological production of chemicals ranges from 10^2 to 10^6 tons per year, proving to be highly profitable, with an average cost of approximately $10^2 - 10^3$ \$/kg of product [Moulijn et al., 2001]. Additionally, it is noteworthy that production costs depend not only on the complexity of biosynthesis, the cost of raw materials, biocatalysts, and operational expenses but also on the production capacity of the facility, being inversely proportional to it.

Considering that biotechnology, as a whole, represents a multidisciplinary field encompassing natural sciences, engineering, technology, and industrial production, advancements in genetic and genetic engineering research, cellular and molecular biology, biochemistry, biophysics, and microbiology have fostered the development of this technological sector. This field has led to some of the most revolutionary concepts and industrial applications, such as the production of vaccines and medicines (the so-called "recombinant DNA technology"), food products and nutritional supplements, biosynthesis of chemical intermediates, enzymatic/biological energy production processes, and pollutant and waste treatment.

Currently, the application of biotechnologies that involve the use of naturally occurring microorganisms—selected through conventional techniques or reprogrammed through genetic engineering—or the use of cellular components from plants and animals is experiencing exponential growth. This expansion has a profound impact on improving quality of life through economic growth driven by industrial biosynthesis of "fine chemicals" [Buchholz and Hempel, 2006; Hempel, 2006].



The choice of the most suitable reactor type for the studied (multi-)enzymatic or biological process, with either free (suspended) biocatalyst (enzymes or living biomass) or immobilized on solid (or gel) porous granules, refers to the reactor's structural type, namely [Moser, 1988; Lübbert and Jørgensen, 2001; Engasser, 1988; Maria and Renea, 2022B]:

- perfectly mixed **CSTR** (continuous or semi-continuous),
- plug flow reactor, if the immobilized biocatalyst is arranged in a fixed bed column, or
- fluidized bed / mechanically stirred system in a three-phase configuration (with the catalyst immobilized on a solid support).

Secondly, the optimal operating mode of the selected bioreactor must be chosen, namely:

- batch (**BR**),
- semi-continuous / fed-batch (**FBR**), or
- continuous, in the case of immobilized biomass, either in a mechanically stirred **CSTR** or in fixed-bed columns of granular biocatalyst [Maria, 2020D].

This analysis can be conducted much more efficiently and systematically for each case study through an in-silico, offline approach (as in this thesis), using mathematical models of the biological process and the bioreactor. By comparing optimal operating policies determined for each type of bioreactor while considering the optimization objectives formulated in mathematical terms, a well-founded decision can be made [Wang, 2009; Maria, 2012; Khamseh and Miccio, 2012; Jiménez-González and Woodley, 2010].

The optimization of a biological reactor follows a similar approach to that of an enzymatic reactor, with optimal operating policies depending on process characteristics (kinetics, raw material purity, number of control variables, number of time intervals chosen for control variable variation, and optimization objectives), biomass characteristics (activity range in terms of temperature and pH, stability, and source), and the chosen bioreactor design [Maria, 2012; Maria, 2020D].

Additionally, for each specific case, one of two possible alternatives must be chosen: the use of free biomass or biomass immobilized on a suitable porous support.

I.1.2 The importance of optimal operation of biological reactors using deterministic MCSMD kinetic models with continuous variables, hybrid approaches, and multi-level structured representations of the bioprocess.

In the case of the biological reactor (with biocatalyst, either free cell cultures or immobilized on a porous solid support), the trend in industry is to use complex systems with genetically modified organisms (**GMOs**) and efficient immobilization systems (which prevent biomass inactivation due to mechanical and chemical stress in the culture medium) in order to successfully replace energy-intensive and polluting chemical syntheses, by using milder reaction conditions and generating very little waste [Gavrilescu and Chisti, 2005; Hempel, 2006; Buchholz and Hempel, 2006]. Thus, modern biological processes, together with enzymatic ones, prove to be efficient in the biosynthesis of numerous chemical compounds, competing in terms of efficiency with organic chemical synthesis, resulting in high selectivity and specificity, producing fewer by-products, reducing energy consumption, and generating less environmental pollution (**Fig. I-1**). This characteristic of industrial biosynthesis is exploited for various economic purposes (industry, medicine, environment, agriculture, fuel production) [Buchholz and Hempel, 2006; Hempel, 2006; Moulijn et al., 2001].

However, industrial bioprocesses still have limited spread due to the high costs of isolating and stabilizing biomass/enzyme on a suitable support, as well as its high sensitivity to operating conditions, the relatively low reproducibility of the biological process due to biomass changes from one cell cycle to another, and the difficult control of the bioreactor. Many of these inconveniences can be overcome through i) efficient biomass immobilization on suitable supports, ii) the use of bioprocesses with **GMOs** exhibiting superior catalytic activity, and/or iii) **the optimization of working conditions and operation modes of the selected biological/enzymatic reactor**, using advanced mathematical models of the biological/enzymatic process and the reactor.

This thesis focuses on the last aspect of the engineering problem, using original computational techniques (**deterministic, modular, hybrid kinetic models**, structured on multiple levels of detail, denoted **MCSMD**) inspired by chemical and biochemical engineering practices (IChB) [Maria, 2017, 2017B, 2018]. Such a **structured, modular, deterministic kinetic modeling approach** of the central carbon metabolism (**CCM**) allows the use of advanced tools, namely numerical algorithms and classical modeling concepts from **IChB**, as summarized by [Maria, 2017A; Maria, 2017B; Maria, 2018; Maria, 2023B].

Thus, **this thesis makes a remarkable contribution** to solving, using **IChB** computational tools, the engineering problems that arise when scaling up new biological/biochemical processes, knowing that the engineering part (bioreactor/plant design and **optimal operation**, all performed **in-silico**, **off-line**, through biochemical engineering calculations based on a mathematical model) is perhaps the most difficult stage in the development of a new bioprocess and in operating an industrial bioreactor.

As demonstrated in the specialized literature, in-silico numerical analysis (based on mathematical/kinetic models) of biochemical (enzymatic) or biological (cell cultures) processes has proven to be not only an essential tool but also extremely beneficial for engineering assessments aimed at determining the optimal operating policies for biological reactors [Maria, 2020A; Maria and Renea, 2021; DiBiasio, 1989; Maria et al., 2018C; Scoban and Maria, 2016] or for complex multi-enzyme ones [Maria, 2012; Maria and Crisan, 2017; Maria, 2020B; Maria and Peptanaru, 2021; Maria and Renea, 2022C; Straathof and Adlercreutz, 2005].

The classical (routine, "default") approach in biochemical engineering practice for solving design, optimization, and control problems based on mathematical models of industrial

biological reactors uses unstructured models such as Monod-type models (for bioreactors with cell cultures) or Michaelis-Menten-type models (for biochemical, enzymatic reactors), which ignore detailed representations of cellular processes. The engineering rules applied are similar to those used for chemical processes (**IChB**) and are inspired by nonlinear systems control theory. However, by only considering the key macroscopic variables of the process (concentrations of biocatalyst, substrate, and product), these unstructured (apparent, global) models do not adequately reflect the metabolic changes at the biomass level in the bioreactor, making them unsuitable for predicting the cellular response to environmental perturbations through self-regulated cellular metabolism. These unstructured models can be satisfactory for approximate modeling of the biological process but not for modeling cellular metabolic processes, as they cannot correlate the operation of the bioreactor with the continuous adaptation of biomass metabolism to the variable conditions within the bioreactor. Additionally, the predictive accuracy of these global kinetic models is quite low. In the case of multi-enzyme processes, these unstructured models do not account for the dynamic interactions between the modules of enzymatic reactions or the connection of all species with the dynamics of the enzymatic reactor.

The current trend. This thesis follows the current trend to solve such engineering problems with greater accuracy, namely by using deterministic, modular, hybrid kinetic models (with continuous variables and based on cellular metabolic reaction mechanisms) (**MCSMD**), structured on multiple levels of process detail according to the application, which take into account the cellular metabolic reactions in the CCM involved in the studied bioprocess (at the nanoscale) in connection with the dynamics of the macroscopic state variables of the bioreactor.

In the case of biological reactors, these hybrid MCSMD models, structured on at least two levels of biological process representation, can numerically simulate, with greater accuracy, both the key stages in the central carbon metabolism (**CCM**) at the cellular scale responsible for the synthesis of cellular metabolites of interest in industrial biosynthesis, as well as the dynamics of the macroscopic state variables of the bioreactor.

As demonstrated by the results in this thesis, the **MCSMD** models of the **ODE** type (ordinary differential equation systems) for **CCM**, even though they are complex and often over-parameterized, present a significant number of advantages. They can reproduce in detail, through numerical simulations, the molecular interactions at the cellular level, and the continuous slow or fast response of the cell to continuous exo/endo-genic perturbations [Styczynski and Stephanopoulos, 2005; Maria, 2017A]. Moreover, the use of **ODE** kinetic models offers the advantage of being easily approached from the perspective of numerical computation, being flexible, easily expandable, and suitable to be characterized using **IChB** tools and nonlinear systems theory [Heinrich and Schuster, 1996]. Thus, the properties of the cellular regulation system are taken into account, namely: dynamics, direct and feedback regulation loops, and optimality. Such an **ODE** kinetic modeling approach for **CCM** allows the use of powerful tools, namely numerical algorithms and the classic modeling concepts of **IChB**, as summarized in Chapter I.4.

In the case of multi-enzyme reactors, the same type of multi-level hybrid **MCSMD** models couples the kinetic models of interconnected enzymatic reaction modules, taking into account all key reaction intermediates and inhibitory processes, all connected with the dynamics of the macroscopic state variables of the analyzed reactor.

On the other hand, the use of **MCSMD** kinetic models with multiple control variables and conflicting objectives increases the difficulty of the optimization problem. In this case, using the Pareto front technique and comparing a larger number of optimal operating alternatives leads to superior results compared to the classical approaches for optimizing biological or multi-enzyme reactors.

The thesis brilliantly demonstrates the value of *hybrid* **MCSMD** kinetic models in biochemical engineering calculations by solving two case studies regarding the optimization of reactor operating regimes in the pharmaceutical/food industries: a) the biosynthesis of an essential amino acid (tryptophan-TRP) using genetically modified *E. coli* cultures [Maria and Renea, 2021], and b) the production of mannitol from fructose using a mixture of enzymes (MDH and FDH) [Maria and Renea, 2022A,B; Renea et al., 2022; Gijiu, Renea, et al., 2025].

Numerical simulation of **CCM** dynamics using **MCSMD** kinetic models

In the case of biological reactor processes that use viable cell cultures, for solving complex optimization problems of their operation using *hybrid, deterministic, structured* **MCSMD** kinetic models (with continuous variables and based on cellular metabolic reaction mechanisms), it is absolutely essential to include, as the central mode, a reduced kinetic model of the central carbon metabolism (**CCM**) of the respective cell. In *hybrid MCSMD* models, structured on *multiple levels of detail*, according to the application, the cellular metabolic reactions and the dynamics of the key cellular species of **CCM** involved in the studied bioprocess (at the nanoscale) must be considered, in connection with the dynamics of the macroscopic state variables of the bioreactor due to the species imported/excreted by the cells.

According to the definition from the literature [Creative Proteomics, 2024], **CCM** is as follows: "The central carbon metabolism, also known as the central pathway of metabolic reactions of carbon, or the central metabolic pathway, refers to a series of interconnected biochemical reactions that occur in living cells. It involves the conversion of carbon-containing molecules, such as sugars and other organic compounds, into energy and precursor molecules necessary for cell growth, proliferation, and survival."

The most important enzymatic reaction modules of **CCM** (**Fig. I-3, I-4**), considered in this thesis (grouped or detailed), are the following: i) Glycolysis (**Fig. I-5**), which includes the PTS system (phosphotransferase system, GLC $\rightarrow$ F6P sequence) responsible for glucose (GLC) import into the cell; ii) the PPP module ("pentose phosphate pathway"); iii) the citric acid cycle - TCA (Krebs); iv) the ATP regeneration system; v) the NADPH regeneration system; vi) lipid metabolism, etc. It is important to emphasize that each glycolysis intermediate (the central module of **CCM**) is a precursor for cellular biosynthesis. For example, G6P is a precursor for nucleotide synthesis (gene fragments); PEP is a precursor for amino acid synthesis; PYR is a precursor for the TCA cycle, etc.

On the other hand, it is well known that the cells of microorganisms widely used in industry contain $O(10^3-4)$ cellular compounds, $O(10^3-4)$ transcription factors, activators, inhibitors, and at least one order of magnitude more biochemical (enzymatic) reactions, all ensuring a rapid adaptation of cellular metabolism to an environment in constant change, using complex "genetic regulatory circuits" (**GRC**), which include chains of individual "genetic expression regulation reaction modules" (**GERM**), etc. [Maria, 2017B, 2018, 2023B].

This is the reason why, despite numerous attempts in the literature, the development of very detailed structured kinetic models for cellular **CCM**, on a deterministic basis, to adequately and in detail simulate cellular metabolism self-regulation, cell growth, and replication for such astronomical complexity of cellular metabolism is practically impossible due to the lack of structured and comprehensive experimental data and computational limitations resulting from the high complexity of the cellular system [Maria, 2017B, 2018, 2023B].

Despite the previously mentioned difficulties, there have been numerous successful attempts to simulate the dynamics of **CCM** in a convenient reduced form [Chassagnole et al., 2002; Jahan et al., 2016; De and Tomar, 2012; Cerbon et al., 2024; Costa et al., 2010; Costa et al., 2009]. A review of several such attempts is provided by [Styczynski and Stephanopoulos,

2005; Maria, 2017B; Maria, 2018; Maria, 2014A; Visser et al., 2004]. These models can include from a few dozen to several hundred enzymatic reactions (see chapter III). For example, the **CCM** model in *E. coli* by [Edwards and Palsson, 2000] contains 436 cellular species participating in 720 cellular metabolic reactions.

A reduced form of **CCM**, with only 72 metabolites and 95 reactions, is schematically represented in (**Fig. I-3**). This model was used by [Maria et al., 2011; Maria, 2014A; Chassagnole et al., 2002; Usuda et al., 2010; Kadir et al., 2010; Ceric and Kurtanjek, 2006; Tusek and Kurtanjek, 2009] for various purposes. Similarly, a review of how a cellular **GRC** is constructed, including a certain number (usually under 25) of individual modules (**GERM**) for regulating genetic expression, organized in chains with multiple cellular functions, such as genetic switches, operon expression (gene clusters), etc., is made by [Maria, 2017A, 2017B, 2018; Xiong, 2006; Rocha et al., 2010].

A simplified representation of the **CCM** model by [Edwards and Palsson, 2000] includes a stoichiometry considering 72 metabolites and 95 reactions, which is schematically represented in (**Fig. I-3**). Deterministic kinetic models using continuous variables have been developed for glycolysis by [Maria, 2014A] (**Fig. I-4, I-5**, chapter III) and other researchers (see review [Maria, 2020A]). Notable are also the kinetic models for simulating CCM by [Schmid et al., 2004; Chassagnole et al., 2002; Costa et al., 2009, 2010; Machado et al., 2015]. Such models can adequately reproduce the cell's response to continuous perturbations, with the structure and size of the **CCM** kinetic model adapted based on the quantity/quality of available bio-omic information. Even though such extended structured models are predominantly used for research purposes today, being difficult to identify (due to incomplete cellular kinetic data), it is only a matter of time before they are adapted for industrial/engineering purposes as reduced structured hybrid MCSMD models [Maria, 2023B]. The two case studies presented and discussed in this thesis brilliantly illustrate this aspect and follow this trend in modern biochemical engineering and bioengineering.

In this context, the thesis makes a *remarkable contribution* by developing a methodology for constructing **hybrid, structured, deterministic MCSMD kinetic models**, combining the characteristics of the cellular metabolic process at the nanoscale of the essential **CCM** modules with the macroscopic variables of the industrial bioreactor state. In this way, more precise and detailed predictions are obtained for both the dynamics of the biological process at the cellular level and for the state variables of the analyzed bioreactor. The immediate application of these models is to determine the more accurate optimal operating regime of an industrial bioreactor. The advantages of such an approach are mentioned in Chapter I.3.

I.2 The main objectives of the thesis

To summarize, **the main objective of the thesis** is to develop hybrid, structured kinetic models of the **MCSMD** type, to be used in engineering calculations for the design and optimal operation of biological reactors (containing cell cultures) or multi-enzymatic reactors (containing free or immobilized enzymes). More specifically, the following will be pursued:

- **A).** The development of a **methodology for constructing a hybrid structured mathematical (kinetic) model (MCSMD)**, deterministic, suitable for the characteristics of the bioprocess carried out in the analyzed bioreactor, which combines the features of the cellular metabolic process at the nanoscale level of essential species and reactions from the central carbon metabolism (**CCM**) of the living cell, with the macroscale features at the level of the state variables of the industrial bioreactor. In this way, more precise and detailed predictions are obtained for both levels of representation of the studied bioprocess.
- **B).** A more precise determination, based on **MCSMD** models, of an **optimal operating policy** for an industrial bioreactor, as well as predicting how the biomass

metabolism changes and self-adapts over the course of dozens of cellular cycles, i.e., dozens of hours of operation of an industrial bioreactor. [Maria, 2021; Maria and Renea, 2021; Maria and Renea, 2022C; Maria and Renea, 2024B, 2024C].

○ **C).** The **optimization of the biological or enzymatic reactor** will take into account both economic criteria, such as productivity (maximizing yield), minimizing raw material consumption, as well as criteria related to its mode of operation (discontinuous or semi-continuous), and the influence of variations in operating parameters (control variables – such as flow rate and substrate or biocatalyst feeding concentration, etc.) on the reactor's performance.

All of these aspects will be addressed and solved using original dynamic models of the **MCSMD** type, suitable for the addressed bioprocess. The optimization problems, "translated" into a mathematical form, will be solved using numerical procedures for parametric estimation and high-performance optimization, which will be described in detail for each individual case study.

In this way, the thesis stands out for its **high degree of originality and novelty**, with **wide applicability** in biochemical engineering calculations required in the biosynthesis industry. The **MCSMD** model adapted for simulating glycolysis and essential stages of cellular **CCM** in *E. coli* cells can be easily extended and adapted to other industrially relevant bacteria.

To better illustrate these new concepts of modeling cellular processes for immediate engineering purposes [Maria and Renea, 2022C], the thesis also aims to develop and test **a simulation and optimization platform for bioreactors using MCSMD**. This platform includes, in a modular form, kinetic models of essential stages of cellular CCM processes. This computational framework, supplemented with cellular reactions related to the synthesis of an industrially relevant metabolite (e.g., TRP here), is designed to be easily integrated into the dynamic mathematical models of the main types of industrial biological reactors tested (discontinuous - **BR**; semi-continuous, "fed-batch" - **FBR**; three-phase, with mechanical stirring - **MACR**). Repeated simulations with **MCSMD** models and the application of specific optimization procedures from biochemical engineering will allow the selection of the best operational alternative for the biological or biochemical reactor (as applicable). The optimization procedure for the operation regime of the industrial reactor will consider one or more economic objectives (maximize yield of useful product, minimize raw material consumption), with evaluations made for sufficiently long operating periods that highlight biomass adaptability and the dynamics of key cellular species and fluxes.

The thesis exemplifies these new proposed concepts for developing **MCSMD** kinetic models through the solution of several complex case studies, namely:

➤ **(Glycolysis)**. Adaptation into an **MCSMD** form of a kinetic model of glycolysis in bacteria of industrial interest (*E. coli* here). This model (**Fig. I-3, I-4, I-5**) described in detail in Chapter III is an essential step in developing any cellular **CCM** model. An appropriate kinetic model of glycolysis provides the ability to predict operating conditions that ensure a balanced stationary CCM (cellular homeostasis), as well as those that lead to an oscillatory glycolytic regime (**Fig. I-5**) [Maria, 2014A; Maria et al. 2018; Maria, 2020A, 2021; Maria and Renea, 2021] with immediate implications for the efficiency of cellular metabolic synthesis, translated at the macromolecular scale into the efficiency of industrial bioreactors. The importance of the glycolytic cellular dynamic model adapted in this thesis is extraordinary because glycolysis is the central element of **CCM**, being responsible for: **(1-a)** glucose import (the main substrate) into the cell, **(1-b)** proper functioning of the citric acid cycle (TCA, Krebs), which provides "cellular energy" in the form of ATP, reducing agents (NADH, NADPH), etc., and **(1-c)** the concentration level of glycolytic intermediates which, in their entirety, are precursors for the reaction pathways leading to the synthesis of most cellular metabolites, many of which are excreted through the cell membrane and have

industrial importance, such as various amino acids (tryptophan- TRP here), succinic acid (SUCC), citric acid (CIT), monoclonal vaccines (MABS), etc. [Maria et al., 2019A; Maria et al., 2011; Maria, 2018, 2020A, 2023B; Maria and Renea, 2021, 2024B].

➤ **(Case Study 1)** Adaptation from the literature [Maria, 2014A] of a detailed structured kinetic model of glycolysis (Chapter III, **Fig. I-4, I-5**), which will be coupled with the cellular module of tryptophan (TRP) synthesis reactions (specifically, the expression of the TRP operon, **Fig. I-4**). This structured cellular kinetic model was coupled into an **MCSMD** hybrid model with the one describing the dynamics of state variables in a semi-continuous bioreactor ("fed-batch", **FBR**) [Maria and Renea, 2021; Maria, 2021]. The resulting hybrid model was used **for the first time** to optimize the operation of the FBR bioreactor with the goal of maximizing TRP production (**Fig. I-4**, Chapter IV).

➤ **(Case Study 2).** For cases involving multi-enzyme reactions, determining an optimal operating policy for the discontinuous reactor (**BR**) often translates into a difficult multi-objective optimization problem. The example is provided in Chapter V for the bioreductive conversion of D-fructose to mannitol using the enzyme MDH (mannitol dehydrogenase) and the cofactor NADH as a proton donor. NAD(+) regeneration is achieved *in situ* through the enzymatic degradation of ammonium formate in the presence of formate dehydrogenase (FDH). For such a coupled multi-enzyme system, *in-silico* engineering evaluations will consider multiple competing (opposite) optimization objectives and various technological constraints. This thesis presents an **original methodology** for generating the **optimal operating policy of the enzymatic reactor** using, in an original way, the Pareto front technique or the classic nonlinear programming (**NLP**) method, considering several pairs of opposing economic objectives and multiple control variables. The optimal operation of the **BR** reactor thus obtained is compared with the optimal operation of a semi-continuous reactor ("fed-batch", **FBR**) with variable feeding or a series of equal-volume **BRs** (**SeqBR**). As demonstrated, the "breakpoint" (slope change) of the optimal Pareto front has proven to be a favorable option [Maria and Renea, 2022B; Gijiu and Renea, 2025].

I.3 The significance and advantages of the proposed MCSMD hybrid models in the thesis

A reasonable compromise, adopted in this work as well, is the use of *hybrid MCSMD kinetic models* for enzymatic or biological processes that combine the characteristics of the unstructured process with a structured representation of it (detailed, based on the reaction mechanism) to generate more accurate predictions of the dynamics of the (bio)reactor under study [Maria, 2023B; Maria and Renea, 2022C]. In practice, hybrid models use a two-level dynamic simulation hierarchy: a) macroscopic state variables of the bioreactor, connected to b) nanoscale state variables that describe the dynamics of the cellular metabolic processes of interest [see case study no.1, Chapter IV]. This is also the case for interconnected multi-enzyme reactions, which describe the dynamics of the reactor on two hierarchical levels: a) the evolution of intermediates in the enzymatic reactions, observable or unobservable, but b) connected to the macroscopic state variables of the reactor [see case study no. 2, Chapter V].

Through the case studies addressed, this work highlights the *advantages* of the *structured hybrid MCSMD* kinetic models used for simulating the dynamics of bioreactors and, implicitly, the dynamics of cellular metabolic processes of the biomass in the bioreactor, as follows:

i.)- The high level of detail and precision in the predictions of the *hybrid MCSMD* models are remarkable, both at the macroscopic level (bioreactor state variables) and at the nanoscale level (dynamics of cellular species related to **CCM**), as demonstrated by several examples below.

ii.)- For the study of glycolysis dynamics in *E. coli* (Chapter III), the **MCSMD** model by [Maria, 2014A, 2020A; Maria et al., 2018B, 2018C; Maria and Renea, 2021] provides a greater level of detail in predicting the dynamics of species in the bioreactor, i.e., it can predict, through simulation, the dynamics of [9 (cellular species) + 1 species (in bioreactor liquid)], compared to the dynamics of only 1 species (in bioreactor liquid) predicted by a classical macroscopic **FBR** model. At the same time, the structured model covers a broader operating range, namely $[GLC]_{ext} = [0-1]$ mM, and $[GLC]_{feed} = 100-1000$ mM.

iii.)- For case study no. 1 (Chapter IV), concerning the maximization of TRP production in an **FBR** using a *E. coli* “wild” or **GMO** culture [Maria, 2021; Maria and Renea, 2021], the **MCSMD** model by [Maria, 2021; Maria and Renea, 2021; Maria and Gheorghe, 2024] provides a higher degree of detail in predicting the bioreactor behavior, characterizing the dynamics of [14 (cellular species) + 4 (species in bioreactor liquid)] compared to only [3 (species in bioreactor liquid)] in a classic macroscopic (unstructured) **FBR** model. At the same time, it covers a broader operating range and a wider range of control variables and different strains of *E. coli* (“wild” or **GMO**), such as: bioreactor dilution (feeding/evacuation rate relative to the bioreactor liquid volume) = $[10^{-5} - 0.01]$ 1/min; $[GLC]_{feed} = [200-3500]$ mM; $[GLC]_{ext} = [0-10]$ mM; $[X] = 10-100$ gDW/L; $[GLC]_{initial} = [1-200]$ mM; $FL = [0.015-0.040]$ L/h [Maria and Renea, 2021; Maria, 2021]. For comparison, the **FBR** bioreactor model by [Chassagnole et al., 2002] simulates the behavior of this system with a similar **MCSMD** model (but with a different glycolysis model), however, with much narrower operating ranges (approximately one order of magnitude smaller, see [Maria et al., 2018C]).

iv.)- For the same case study no. 1 (Chapter IV), regarding the maximization of TRP production in an **FBR** using a *E. coli* “wild” or **GMO** culture [Maria, 2021; Maria and Renea, 2021], this thesis proves the superiority and enhanced capabilities of the hybrid structured **MCSMD** kinetic models in offering more accurate predictions when used for biochemical engineering calculations (off-line bioreactor operation optimization) compared to unstructured Monod-type models. For example, **(a)** the **MCSMD** model can predict an optimal operating policy for an **FBR** bioreactor to maximize TRP production with a constant feeding that simultaneously satisfies multiple economic objectives (using the Pareto front technique) [Maria et al., 2018A; Maria et al., 2018C; Maria and Gheorghe, 2024]; **(b)** the **MCSMD** model predicts an optimal variable “stepwise” feeding policy (in flow rate and substrate concentration) of an **FBR** bioreactor for maximizing TRP production, net superior to a constant feeding operation [Maria and Renea, 2021; Maria, 2021]. **(c)** The same conclusion holds for a bi-enzymatic **FBR** reactor (case study no. 2). In a similar case study, the **MCSMD** model can predict an optimal operating policy for an **FBR** bioreactor to maximize mannitol (M) production from fructose, with multiple operating alternatives, using the Pareto front technique [Gijiu, Maria, and Renea, 2025].

v.)- The scientific literature also contains other case studies that support the use of **MCSMD** models, despite their complexity and the substantial experimental and computational effort required to develop such extended dynamic models. Notable examples include: **(a)** the **MCSMD** model [72 (cellular species), involved in 95 cellular reactions + 1 (bulk species, i.e., biomass)] used to in-silico design **GMO** strains of *E. coli* for optimizing succinic acid (SUCC) production simultaneously with biomass production in a discontinuous reactor (BR) [Maria et al., 2011]; **(b)** the **MCSMD** model [26 (cellular species) + 3 (bulk species)] used for optimizing an **FBR** for mercury ion retention by *E. coli*. The same model was also used to predict the plasmid cloning level with mercury of the *E. coli* **GMO** cell to maximize the mercury ion removal yield from wastewater, without endangering cellular metabolism [Maria, 2023B; Maria and Luta, 2013]; **(c)** an **MCSMD** model used for optimizing an **FBR** for monoclonal antibody (mAb) production [Maria, 2020D; Maria and Renea, 2024B].

vi.)- *Structured* cellular models of the **MCSMD** type are also useful for understanding the cellular bioprocess in direct relation to the way the bioreactor operates. For example, in case study no. 1 (Chapter IV), *in-silico* (through mathematical model simulation) the conditions for the emergence of glycolytic oscillations can be determined in direct relation to the substrate concentration in the culture medium [GLC]_{env} and with cellular characteristics, [Maria et al., 2018B; Maria, 2020A], as well as their influence on the oscillations in the TRP operon expression [Maria, 2020A; Maria et al., 2018A], or those leading to balanced cell growth (in a quasi-steady state - QSS, i.e., at homeostasis) [Maria et al., 2018C].

In conclusion, **by using a modern and original approach** of the difficult biochemical engineering problems, this thesis stands out for its **high degree of originality and novelty**, with a wide applicability in the biosynthesis industry.

Last but not least, it should be mentioned that the numerical techniques for estimating kinetic model parameters, simulating the dynamics of bioprocesses, as well as those used for optimizing the bioreactor, are of high complexity, employing nonlinear optimization in the presence of multiple constraints, as well as the Pareto front technique.

Chap. II.- Bioreactors and industrial enzymatic reactors

This chapter is dedicated to a brief description of the main types of biochemical (enzymatic) or biological (with live cell cultures) reactors used in the biosynthesis industry. Their characteristics, advantages, and disadvantages are reviewed, along with examples of their use. Additionally, the most commonly used optimization objectives found in the specialized literature for engineering calculations dedicated to optimizing the operation of enzymatic or biological reactors are discussed. The author's effort to systematize the specialized literature is highly commendable.

Chap. III – GLYCOLYSIS - An essential step of the CCM. Description of a kinetic model from the literature applied to E. coli cells.

Glycolysis is the essential step of the central carbon metabolism (**CCM**) in a living cell (**Fig. I-3**). It ensures the import of substrate (**GLC**) necessary for all cellular metabolic syntheses. For this purpose, all glycolysis intermediates are starting points (precursors) for the cellular synthesis of the majority of its metabolites.

When developing a hybrid structured kinetic model of the **MCSMD** type, it must necessarily include the essential reaction modules of the **CCM**.

This chapter reviews the main types of kinetic models of glycolysis found in the specialized literature, with a focus on the reduced model of [Maria, 2014A] (**Fig. I-5**) for the E. coli cell – an experimentally identified model, which is also adopted in this thesis. Special attention will be given to the environmental conditions in the bioreactor and those related to the E. coli bacterial phenotype that induce glycolytic oscillations.

Using a model for tryptophan (TRP) synthesis (described in detail in Chapter IV; **Fig. I-4**), coupled with the glycolysis model, this chapter exemplifies how glycolytic oscillations also affect the oscillations and yield of TRP synthesis [Maria et al., 2018A, 2018B, 2018C].

Chap. IV - Case study 1. Modeling the dynamics of essential steps of CCM and the metabolic process of tryptophan (TRP) synthesis in E. coli cells. Contributions to the optimization of the industrial FBR bioreactor for TRP synthesis using a multi-level hybrid MCSMD-type model.

Modular deterministic kinetic models (with continuous variables, and based on cellular metabolic reaction mechanisms) (**MCSMD**), *hybrid* models *structured* on multiple levels of detail (chapter I.1.2), connect the dynamics of cellular metabolic processes (enzymatic

reactions at the nanoscale) with the dynamics of macroscopic state variables of the bioreactor. These mathematical (kinetic) models, developed in this chapter as well, are increasingly used in biochemical or biological engineering assessments to obtain more precise predictions of the dynamics of the bioreactor and biomass under variable operating conditions. Depending on the complexity of the cellular model, such a mathematical tool can be used to evaluate cellular metabolic fluxes (i.e., reaction rates in quasi-stationary conditions) in relation to the operating conditions of the bioreactor, thus suggesting genetic modification strategies for microorganisms to improve the efficiency of the industrial bioreactor. Even though such an extended model requires more experimental and computational efforts to be constructed, its use is advantageous. The case example addressed in this chapter refers to a hybrid MCSMD model that describes the dynamics at the nanoscale of key species related to the central carbon metabolism (CCM) in *E. coli* cells, connected with the dynamics of the macroscopic state variables of a semi-continuous industrial bioreactor ("fed-batch" – FBR) used for tryptophan (TRP) production. This results in much more precise and detailed predictions of the cellular bioprocess and bioreactor dynamics (in terms of the number of species considered in the model) compared to using a classical, unstructured kinetic model ("default") (Monod).

The immediate application of such a hybrid structured MCSMD model is its use in more accurately determining the optimal operating policies of BR or FBR bioreactors. This chapter brilliantly exemplifies the value of hybrid, structured MCSMD models in solving these optimization problems for industrial bioreactor performance. The case study analyzed addresses the issue of maximizing tryptophan (TRP) production in an FBR-type reactor [Maria and Renea, 2021].

The natural *E. coli* strain ("wild") used was genetically modified to maximize the cellular synthesis of excreted TRP. To this end, the PTS system in CCM (reaction V1 in [Figures I-3, I-5](#)) for glucose (GLC) import into the cell was replaced with a more efficient system based on the galactose permease/glucokinase (GalP/Glk) controlled import system, by modulating the gene expression of GalP/Glk. The study presents multiple novel elements: (i) the proposed hybrid MCSMD modular mathematical (kinetic) model is original; (ii) it was validated on an experimental basis, using the experimental data from a research team at TU Hamburg, with which the Ph.D. supervisor collaborates (see "Acknowledgements" below); (iii) the efficiency of the hybrid MCSMD (kinetic) model was tested in determining the optimal in-silico operating policy of the FBR.

After emphasizing the importance and advantage of the *in-silico* approach for the analyzed case study, the characteristics of the FBR bioreactor analyzed, operated at TU Hamburg (Germany), as well as the sets of experimental kinetic data obtained from the foreign collaboration partner, are described, for which the following "Acknowledgements" were addressed.

The authors of the papers [Maria and Renea,2021; Maria, 2021] are deeply grateful to Prof. An-Ping Zeng and to their research group (dr. Minliang Chen, dr. Lin Chen, dr. Chengwei Ma) from the Hamburg University of Technology (Institute of Bioprocess and Biosystems Engineering, Germany) for providing on a free basis, and without any claim of any kind (including no claim of authorship) experimental data from their published works on the TRP synthesis in a fed-batch bioreactor using a genetically modified *E.coli* (T5 strain) cell culture. Particularly, we want to thank to Prof. An-Ping Zeng for the constructive comments and discussions on the manuscript above mentioned papers.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. This work did not receive any kind of funding under any circumstances, from any source. The authors confirm that his paper has **no conflict of interest** of any kind, and of any nature. While the advice and information in this work are believed to be true and accurate at the date of publication, the entire responsibility for the work content lies with the authors. The authors' institutions have no responsibility for the content of this work.

Next, the *hybrid, structured MCSMD* kinetic model of the cellular bioprocess and the **FBR** bioreactor is described. Emphasis is placed on describing the main modules of the *hybrid MCSMD structured* model, namely: i) Biomass growth [X]; ii) The dynamic model of the FBR; iii) Module [A] – Glycolysis; iv) Module [B] – ATP regeneration system; v) Module [C] – TRP synthesis. The process of how the 49 rate constants were estimated for the entire *hybrid MCSMD* kinetic model [A+B+C+X+ **FBR**] is then described, according to [Maria, 2021].

The optimization problem of the FBR bioreactor is addressed gradually, detailing the steps in applying the calculation algorithm, namely: i) Formulation of the optimization problem; ii) Selection of control variables; iii) Choice of the objective function; iv) Constraints of the optimization problem; v) Selection of the most suitable value for the parameter “Ndiv” (the number of equal divisions of the batch duration used for variable feeding of the FBR in each division); vi) Numerical algorithms used for solving the optimization problem; vii) Particularities of the optimization solution in the case of the **FB** bioreactor; viii) **FBR** optimization results and their discussion.

Chap. V - Case study 2. Modeling the dynamics of the bi-enzymatic mannitol synthesis process from fructose with continuous regeneration of the NADH cofactor. Contributions to the optimization of the industrial batch reactor (BR) or of the semi-continuous reactor (FBR) using a hybrid, multi-level, MCSMD-type model.

Enzymatic reactions can successfully replace complex chemical syntheses, using milder reaction conditions and generating less waste. The *in-silico*, *off-line* numerical analysis developed in this chapter is based on a complex, hybrid, **MCSMD**-type kinetic model (deterministic, with continuous variables, modular, with two levels of detail corresponding to the two interconnected enzymatic processes and connected with the state variables of the reactor in which these reactions occur). This complex kinetic model has proven to be an extremely beneficial and useful *engineering tool* for determining *in-silico*, *off-line*, the optimal operating policies for multi-enzymatic reactors. As demonstrated in the literature, for multi-enzymatic cases, determining an optimal operating policy, even for the simple case of a batch reactor (**BR**), corresponds to a multi-objective, non-convex, multi-modal optimization problem, difficult to solve. This work demonstrates how using a hybrid kinetic model of the **MCSMD** type, along with efficient optimization procedures related to nonlinear programming (**NLP**) or the **Pareto-optimal** front technique (with conflicting objective functions), can lead to the optimization of **BR** or **FBR** reactor operations (with constant or variable feeding).

To exemplify, the complex enzymatic reduction process of D-fructose to mannitol using MDH (mannitol dehydrogenase) and the cofactor nicotinamide adenine dinucleotide (NADH) was chosen. The depleted cofactor NAD⁺ is continuously regenerated *in-situ* through an enzymatic reaction involving the degradation of formate in the presence of FDH (formate dehydrogenase). For such a coupled system, *in-silico* engineering evaluations must consider multiple and opposing optimization objectives, as well as various technological constraints. This paper presents a methodology for generating Pareto-optimal operating policies for **BRs**, considering several opposing economic objectives and involving a moderate number of control variables. Among the multiple elements of novelty are: i) Using the technique of multiple Pareto-optimal fronts can lead to obtaining an optimal operating policy for a **BR** with high performance. ii) Comparing this optimal **BR** with results reported in the literature for the same process, but operated in an **FBR**, or optimally operated in a series of equal-volume **BRs** (“batch-to-batch,” **SeqBR**), revealed the superiority of operating a **BR** using multiple Pareto-

optimal fronts. iii) Choosing the "breaking point" of the slope of the Pareto-optimal front as the solution to the optimization problem for **BR** reactor operation proves to lead to consistent economic benefits. iv) The modular, deterministic, hybrid, structured **MCSMD** kinetic model proves to be an efficient and effective calculation tool in solving such biochemical engineering analyses, even in multi-enzymatic reaction cases.

GENERAL CONCLUSIONS

Achievement of the Main Objectives of the Thesis

Using the concepts, principles, and numerical calculation methods of the **Chemical and Biochemical Engineering (IChB)**, outlined in section I.4 [Maria and Renea, 2022C], this thesis successfully demonstrates, by solving two complex case studies (Chapters IV and V), the multiple advantages offered by the proposed structured, multi-level, hybrid kinetic **MCSMD** models. These models, developed within the thesis, are used to solve biochemical engineering problems—specifically, the optimization of the operating regime of biological reactors (with living biomass) or of biochemical (enzymatic) reactors. In this way, the thesis brilliantly and fully accomplishes its main objectives, as followings:

1). Development of a **methodology for constructing a structured hybrid (MCSMD)** math model.

This modular, deterministic (with continuous variables and based on a reaction mechanism) kinetic/dynamic model integrates at least two levels of detail for the biological process conducted in an analyzed bioreactor.

It combines the metabolic kinetics of essential species and reactions from the central carbon metabolism (**CCM**) at the nano-scale level with the macroscopic kinetic variables of the industrial bioreactor.

This approach leads to more precise predictions of both cellular biological process dynamics and operating parameter (state variable) dynamics of the industrial bioreactor.

The methodology for assembling the **MCSMD** model modules is detailed in Chap. IV (Case Study 1).

2). More precise determination of an **optimal operating policy** for an industrial bioreactor by using **MCSMD** models.

This aspect is proved by the Case Study 1 (Chap. IV), where the developed model predicts how biomass metabolism changes and self-adapts over multiple cell cycles, spanning tens of hours of industrial bioreactor operation [Maria, 2021; Maria and Renea, 2021; Maria and Renea, 2022C; Maria and Renea, 2024B, 2024C].

The superiority of multi-level structured models compared to the classical unstructured models (such as Monod-type models with only three key variables) results by ensuring:

.- A higher accuracy in predicting species dynamics.

.- A significantly higher level of detail (13 nano-cellular species, plus 4 macroscopic liquid-phase species in the bioreactor for Case Study 1, compared to only 3 macroscopic species in an unstructured Monod model).

3). More precise determination of an **optimal operating policy** for an **industrial multi-enzymatic reactor** using structured *hybrid* **MCSMD** kinetic models

This is addressed in Case Study 2 (Chap. V) through a structured, deterministic approach of two interrelated enzymatic processes, by also considering key macroscopic state variables and by optimizing various criteria, as followings:

- Economic: Maximizing product yield, minimizing raw-material consumption (enzymes, cofactors).
- Operational: Evaluating batch vs. semi-continuous reactor operation.
- Controllability: Assessing how feed rate and substrate/biocatalyst concentration variations affect reactor performance.
- Optimization algorithm influence: Examining how different optimization techniques impact solution quality.

Beyond using classical nonlinear programming (NLP) algorithms with a single objective function (e.g., maximizing useful product yield), the study highlights the exceptional value of Pareto-optimal front techniques, which simultaneously consider multiple conflicting optimization objectives.

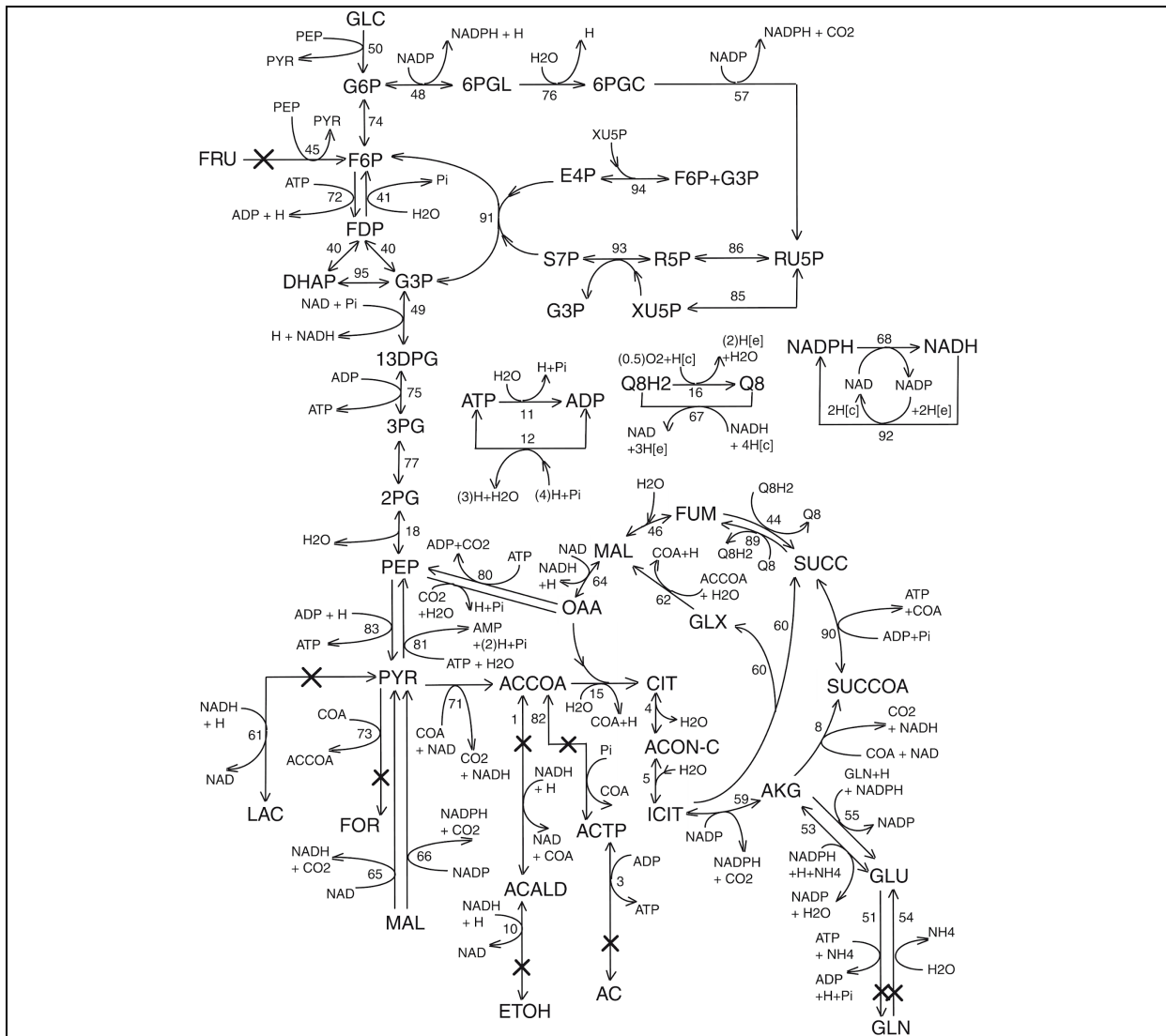


Fig. I-3. Simplified diagram of the main reactions in the central carbon metabolism (CCM) of *E. coli* considered in this thesis and in the kinetic model of [Maria et al., 2011; Maria et al., 2014A]. This model is a simplified version of [Edwards and Palsson, 2000], referring to the “wild” strain, which includes the PTS glucose (GLC) import system and the successive reaction sequence $GLC \rightarrow F6P$. Membrane transport fluxes [Metabolite(envir.)] $\leftrightarrow$ [Metabolite(cyt.)] [Metabolite(envir.)] $\leftrightarrow$

Metabolite(cyt.)][Metabolite(envirn.) $\leftrightarrow$ Metabolite(cyt.)] and exchanges with the environment have been omitted. See [Maria et al., 2011; Maria, 2014A] for details on the numbering of the 95 reactions involving 72 key metabolites in the kinetic model. Adapted from [Maria et al., 2011] with permission from CABEQ JI. The V2 reaction (**Fig. I-4**) serves as the “node” generating glycolytic oscillations, regulated by forward and backward feedback loops, both positive and negative [Maria, 2020C; Maria et al., 2018B; Maria, 2021]. Abbreviations: [e][e][e] = extracellular environment, [c][c][c] = cytosol, **GLC** = glucose, **F6P** = fructose-6-phosphate, **FDP** = fructose-1,6-diphosphate. The V1-V6 reaction rates are grouped (**Fig. I-4**) and discussed in [Maria, 2021, 2014A], where species abbreviations are also explained.

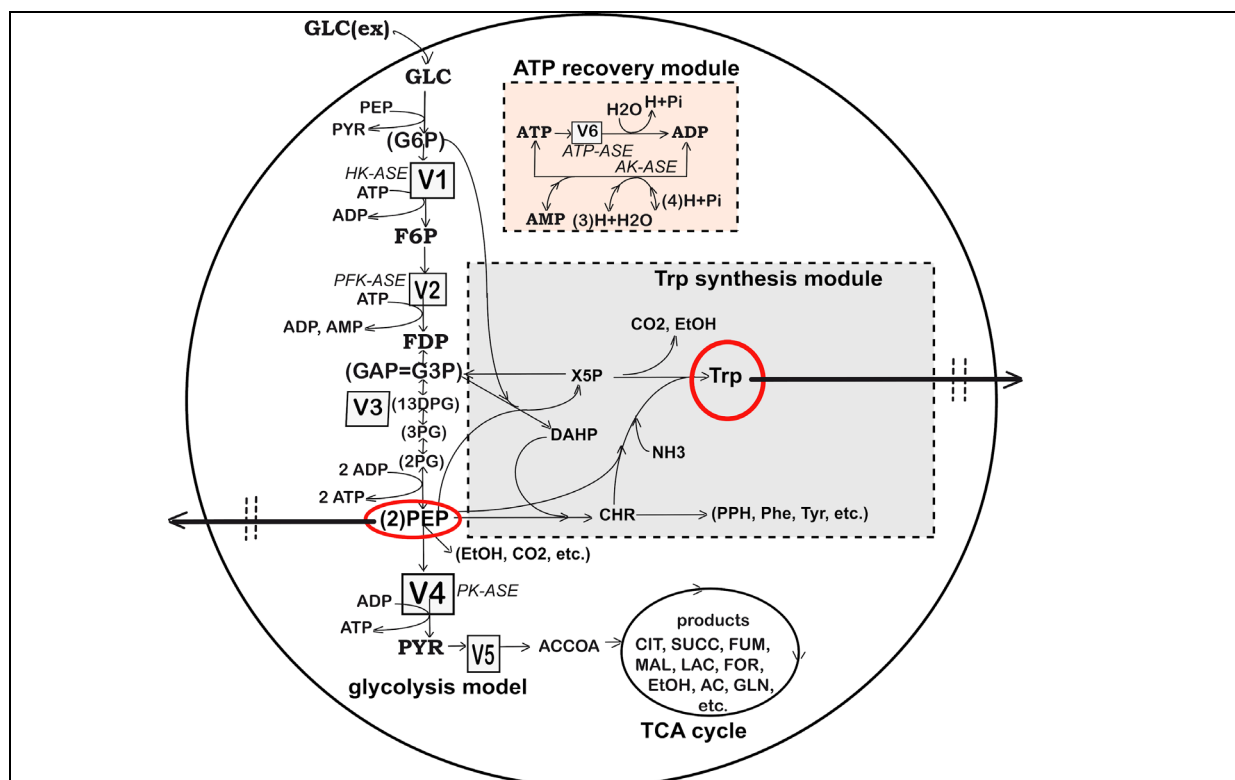


Fig. I-4. Simplified reaction scheme for glycolysis in *E. coli* (adapted from [Maria, 2014A; Maria, 2021]), specifically the vertical sequence of consecutive reactions on the left (GLC $\rightarrow$ PYR), connected to TRP synthesis (gray area) [Maria et al., 2018A; Maria & Renea, 2021]. In the “wild” strain, GLC import is facilitated by the phosphotransferase system (PTS), represented by the global reaction V1 (GLC $\rightarrow$ F6P). This reaction scheme was used by [Maria et al., 2018A, 2018C; Maria, 2021; Maria & Renea, 2021] to develop a structured, hybrid kinetic model (**MCSMD**) for simulating TRP synthesis dynamics. The connection between TRP synthesis and glycolysis occurs through the common PEP node [Maria et al., 2018A; Maria, 2021]. The modular kinetic model structure also includes the synthesis of co-metabolites adenosine ATP, ADP, and AMP as part of the ATP regeneration system (pink rectangle in the figure). Notations: GLC(ex) = glucose in the extracellular environment. Species abbreviations are provided in the abbreviation list of [Maria & Renea, 2021]. Species in parentheses are not explicitly included in the glycolysis model. Italicized letters denote enzymes. Squares indicate enzymatic reaction rate notations V1-V6, as included in the glycolysis model proposed by [Maria, 2014A, 2021; Maria & Renea, 2021]. Expressions for V1-V6 and further details are presented in Chapters III and IV. Adapted from [Maria, 2014A] with permission from CABEQ JI. and supplemented according to the kinetic model of [Maria, 2021]. **GLC(ex)** = glucose in the extracellular space.

4.- Testing for the first-time in the literature the use of multi-level structured *hybrid* kinetic models (**MCSMD**) in optimizing the biochemical/biological reactors leading to highly positive results. These models enabled the *in-silico* generation of a larger number of operation alternatives for the **FBR** reactors, all determined with a higher accuracy compared to classical unstructured models, such as Monod-type models (see Case Study 1, Chap. IV), or Michaelis-Menten-type models (see Case Study 2, Chap. V). The advantages of the proposed **MCSMD**

hybrid models, as demonstrated by the two case studies, are extensively described in the Chap. I.3.

5.- Demonstrating the validity of the principles, concepts, and computational algorithms of Chemical and Biochemical Engineering (**IChB**) (Chap. I.4) used in constructing the structured deterministic *hybrid* kinetic models (**MCSMD**).

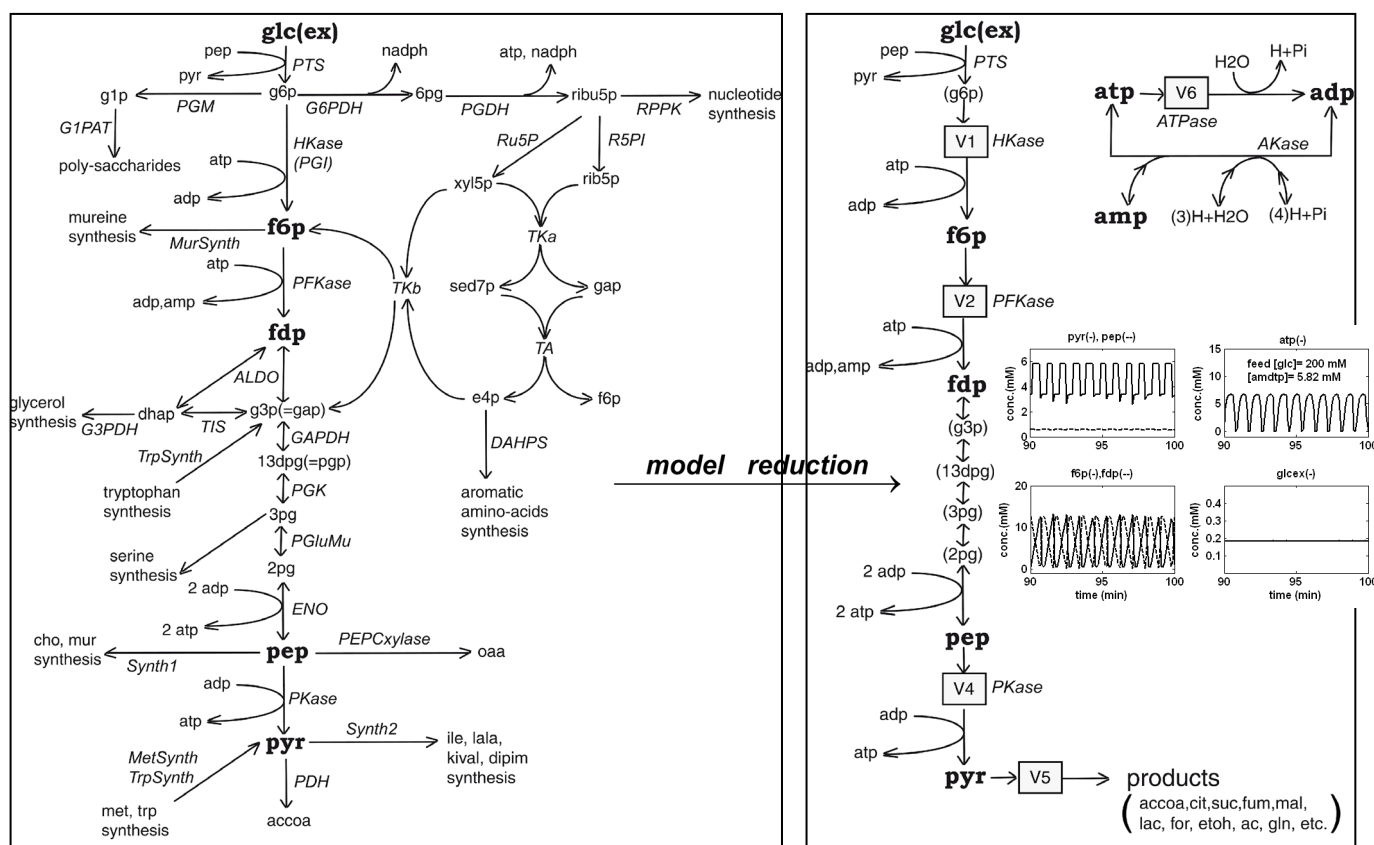


Fig. I-5. Extended and simplified models of glycolysis in *E. coli* used by: (left) the extended **ChassM** model, which includes the pentose-phosphate pathway (PPP) [Chassagnole et al., 2002]; (right) the reduced **mTRM** model by Maria [2014A], which also incorporates the ATP regeneration system via co-metabolite synthesis (ADP, AMP). Squares indicate enzymatic reactions in the **mTRM** model (right). Species in parentheses are not explicitly included in the **mTRM** model. Italicized letters denote enzymes. **Metabolite notations** (not included in the abbreviation list of [Maria, 2014A]): 6pg = 6-phosphogluconate; ac = acetate; cho = chorismate; cit = citrate; dhap = dihydroxyacetonephosphate; dipim = diaminopimelate; e4p = erythrose-4-phosphate; etoh = ethanol; for = formate; fum = fumarate; g1p = glucose-1-phosphate; gln = glutamine; ile = isoleucine; kival = α -ketoisovalerate; lac = lactate; lala = L-alanine; mal = malate; met = methionine; mur = mureine; oaa = oxaloacetate; rib5p = ribose-5-phosphate; ribu5p = ribulose-5-phosphate; sed7p = sedoheptulose-7-phosphate; trp = tryptophan; xyl5p = xylulose-5-phosphate. **Enzyme notations:** AK-ase = adenylate kinase; ALDO = aldolase; DAHPS = DAHP synthases; ENO = enolase; G1PAT = glucose-1-phosphate adenylyltransferase; G3PDH = glycerol-3-phosphate dehydrogenase; G6PDH = glucose-6-phosphate dehydrogenase; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; MetSynth = methionine synthesis; MurSynth = mureine synthesis; PDH = pyruvate dehydrogenase; PEPCxylase = PEP carboxylase; PGDh = 6-phosphogluconate dehydrogenase; PG1 = glucose-6-phosphate isomerase; PGK = phosphoglycerate kinase; PGluMu = phosphoglycerate mutase; PGM = phosphoglucomutase; R5PI = ribose-phosphate isomerase; RPPK = ribose-phosphate pyrophosphokinase; Ru5P = ribulose-phosphate epimerase; Synth1,2 = synthesis 1,2; TA = transaldolase; TKa = transketolase, reaction a; TKb = transketolase, reaction b; TIS = triosephosphate isomerase; TrpSynth = tryptophan synthesis.

The Utility of Hybrid Structured MCSMD Models in Biochemical Engineering Calculations

This doctoral thesis presents extensive theoretical and in-silico research (based on kinetic models of biological or biochemical processes sourced from specialized literature and experimentally validated either through multiple studies conducted by referenced researchers or through collaborations of the doctoral advisor (Chapter V)), focusing on both reaction mechanisms and the mathematical expressions of enzymatic reaction rates at the cellular level, particularly within the central carbon metabolism (CCM). The primary objective of this study is to develop a methodology for constructing modular, deterministic, structured, and hybrid mathematical (kinetic) models (MCSMD), similar to those used in chemical and biochemical engineering (IchB), which can accurately characterize the dynamics of essential CCM stages and the reaction modules responsible for synthesizing industrially relevant metabolites. Additionally, this thesis aims to test the efficiency of the new hybrid kinetic MCSMD models in engineering calculations for optimizing bioreactors and enzymatic reactors.

Traditional *unstructured* (“default”) models, which consider only key macroscopic process variables (biomass, substrate, and product concentrations), do not adequately reflect metabolic changes within biomass in a bioreactor, making them unsuitable for accurately predicting cellular responses to environmental perturbations through self-regulated metabolism. While these unstructured models may provide an *approximate* representation of biological processes, they fail to model cellular metabolic processes effectively and cannot correlate bioreactor operation with biomass metabolism adaptation. In multi-enzyme reactors, *unstructured* Michaelis-Menten models cannot properly simulate the interactions between multiple simultaneous enzymatic reactions or their relationship with reactor state variables.

In this context, several conclusions regarding the advantages of using hybrid MCSMD models in biochemical engineering (Chapter I.3) emerge from the *in-silico* study:

6. Based on two case studies, this thesis recommends the use of modular, structured, hybrid kinetic MCSMD models in biochemical engineering calculations. Compared to unstructured models, MCSMD models provide higher precision predictions, account for a greater number of species, and maintain accuracy over extended reaction durations.

This work makes a significant contribution to the modeling of cellular biological processes at a nano-molar scale by integrating a hybrid dynamic model that combines structured cellular kinetic models (which include species dynamics and metabolic reactions within CCM) with the macroscopic dynamic model of industrial bioreactor state variables. Structurally, MCSMD models are deterministic, mechanistic, and continuous-variable-based, as they rely on the studied cellular bioprocess mechanism. They are constructed using principles from biochemical engineering (IchB) and nonlinear control theory to characterize the response of the bioreactor-biomass system to variations in the reactor’s liquid-phase composition (external cellular environment) [Maria and Renea, 2022C].

7. Complex MCSMD models can accurately represent key stages of cellular-scale CCM metabolism. The modular and extensible approach can incorporate, in addition to core CCM modules (glycolysis, phosphotransferase system (PTS) or an equivalent substrate import system, tricarboxylic acid (TCA) cycle, ATP regeneration system, etc.), reaction modules responsible for synthesizing industrially valuable metabolites (e.g., tryptophan synthesis in Case Study 2, Chapter IV). According to the literature [Maria, 2018; Maria, 2023B], structured cellular kinetic models such as MCSMD can deterministically simulate CCM’s self-regulated metabolism, enabling rapid adaptation to continuously changing bioreactor conditions using complex genetic regulatory circuits (GRCs), which include cascades of gene expression regulatory modules (GERMs). These aspects were not explored in this thesis.

8). MCSMD models *provide higher detail (number of species considered) and greater accuracy in predicting* bioprocess dynamics at both macroscopic (bioreactor state variables) and nanoscopic levels (cellular species dynamics within **CCM**). In Case Study 2, concerning TRP production maximization in a bioreactor (**BR**) or fluidized bed reactor (**FBR**) using *E. coli* wild-type [Maria et al., 2018C] or genetically modified (**OMG**) strains [Maria, 2021; Maria and Renea, 2021], the structured hybrid MCSMD model by [Maria, 2021; Maria and Renea, 2021] accounts for the dynamics of 13 cellular species + 4 macroscopic species in the reactor liquid phase, compared to only 3 macroscopic species modeled using a traditional unstructured Monod model. The MCSMD model provides broader operational predictions, accommodating a wider range of control variables and different *E. coli* strains. Control variable ranges in the studied bioreactor are significantly broader [Maria and Renea, 2021; Maria, 2021; Maria et al., 2018C], covering $[GLC]_{ext} = [0-1]$ mM and $[GLC]_{feed} = [100-1000]$ mM], with extensive control variables (GLC, feed rate F_L , dilution rate $D=F_L/V$), and a wide range of phenotypes modulating ATP regeneration via ATPase enzyme characteristics (reaction constants k_6 and K in the cellular process model, **Table IV-2**). In comparison, a *structured* **MCSMD** model used by Chassagnole et al. [2002] for simulating an **FBR** bioreactor exhibited a narrower operational range. In Case Study 2, focused on optimizing TRP production in a continuous FBR, the *hybrid structured* **MCSMD** model predicts various optimal reactor operation strategies:

a) Constant-feed optimal policy [Maria and Renea, 2021; Maria et al., 2018C].

b) Stepwise variable-feed strategy (feed rate and substrate GLC), superior to constant-feed operation [Maria and Renea, 2021].

9). As indicated in the literature, an **MCSMD** *hybrid structured* model is a valuable tool for evaluating cellular metabolic fluxes and determining substrate (GLC) utilization, facilitating the *in-silico* design of **OMG** strains with enhanced excretable metabolite production (e.g., TRP) [Maria, 2021; Maria and Renea, 2021]. In Case Study 2, the relevant species and fluxes pertain to glycolysis, the PTS system for GLC import, ATP regeneration, and TRP operon expression [Maria et al., 2018B; Mihalachi and Maria, 2019]. This enables *in-silico* design of genetically modified cells (**OMG**) with desirable metabolic traits [Maria and Renea, 2021]. Another literature study employed a *hybrid* **MCSMD** model for designing *in-silico* *E. coli* clones with *mercury*-operon plasmids to enhance mercury uptake from wastewater [Maria and Luta, 2013; Maria, 2010].

10). MCSMD kinetic models also *provide insight into cellular bioprocesses* in direct relation to bioreactor function. In Case Study 2, *in-silico* simulations determine conditions leading to glycolytic oscillations in response to culture medium substrate concentration $[GLC]_{env}$ and cellular characteristics [Maria et al., 2018A; Maria et al., 2018B; Maria, 2021]. Understanding these oscillations is crucial since many glycolytic intermediates serve as *precursors* for internal metabolite production (e.g., amino acids, succinic acid, citric acid, etc.) [Maria et al., 2018A; Maria et al., 2011; Maria, 2023B]. The same case study (Chapter III) examines the interference between glycolytic oscillations and TRP operon expression oscillations [Maria, 2020A; Maria et al., 2018A], as well as bioreactor operating conditions that promote balanced cell growth (quasi-steady-state, **QSS**, or homeostasis) [Maria et al., 2018C; Mihalachi and Maria, 2019]. In another study [Maria and Luta, 2013], an extended **MCSMD** hybrid model predicted enzyme *mer* expression levels and mercury ion reduction rates in a bioreactor used for mercury removal from wastewater by *E. coli* biomass, depending on the level of *mer*-plasmid cloning in **OMG** *E. coli* cells. ("**mer**" = "mercury")

Comparison with Similar Literature Results and Perspectives on Using MCSMD Hybrid Models in Biochemical Engineering

The **MCSMD** hybrid kinetic models presented in Case Study 1 (Chapter IV), as well as in Case Studies (1-3) from Maria [2023B], are *structured* and *hybrid* because they extend across two levels of bioprocess representation: 1). A nanoscopic, cellular scale, which uses key intracellular species concentrations as state variables. 2). A bioreactor-level scale, describing the dynamics of measurable metabolite concentrations in the liquid phase. In such a hybrid kinetic model, cellular state variables are directly linked to those of the bioreactor through cellular import/export processes of substrates, nutrients, metabolites across cell membrane.

While the dynamics of macromolecular species can be experimentally determined using standard analytical techniques, measuring the dynamics of intracellular metabolites is significantly more challenging—both in steady-state conditions (QSS, quasi-steady state) and in dynamic conditions. However, recent advances [Niklas et al., 2011] have enabled the simultaneous measurement of a large number (600) of intracellular metabolites and their time evolution. These refined measurements facilitate metabolic flux analysis under QSS or dynamic conditions, using classical calculation methods such as those by Stephanopoulos et al. [1998], applied to an extended or reduced **CCM** scheme (**Fig. 1-3**).

In summary, the case studies presented in this work, along with those in the literature [Maria, 2023B], solved using *structured*, *hybrid* **MCSMD** kinetic models, demonstrate that these models are modern, precise, and valuable computational tools for developing structured numerical simulators for various engineering applications, including:

- (a) Metabolic flux analysis under variable bioreactor operating conditions [Maria, 2021; Maria and Renea, 2021; Maria and Luta, 2013; Maria, 2010].
- (b) Optimization of metabolite synthesis by improving bioreactor operation and/or genetically modifying the cell culture (Case Study 1, Chapter IV) [Maria, 2021; Maria and Renea, 2021].
- (c) *In-silico* reprogramming of cellular metabolism (based on stationary metabolic flux modeling) to design genetically modified organisms (**GMOs**) with desired traits [Maria, 2017, 2017B, 2018; Maria et al., 2019A; Maria, 2021; Maria and Renea, 2021; Maria and Luta, 2013; Maria, 2010; Maria et al., 2011].
- (d) Interpreting metabolic changes in **GMOs** in direct relation to bioreactor operation [Maria and Renea, 2021; Maria, 2023B].
- (e) More precise bioreactor operation optimization (Case Study 1, Chapter IV) [Maria, 2021; Maria and Renea, 2021].
- (f) More accurate extrapolations of bioprocess dynamics over dozens of cell cycles [Maria and Luta, 2013; Maria, 2010; Maria and Renea, 2021, 2024B, 2024C].
- (g) Determining optimal bioreactor operating conditions to minimize substrate consumption while maintaining high productivity for both the target metabolite and biomass. Examples include TRP synthesis in Case Study 1 (Chapter IV) [Maria and Renea, 2021; Maria and Gheorghe, 2024], succinic acid production in Maria [2023B], and monoclonal antibody (mAbs) vaccine production in Maria [2020D; Maria and Renea, 2024B].

ORIGINAL CONTRIBUTIONS

In the specialized literature, there are few systematic studies on the construction of *structured modular hybrid deterministic bi-level kinetic mathematical* models of the **MCSMD** type and their application in solving practical biochemical engineering problems, such as the more precise determination of optimal operating policies for industrial bioreactors.

There is no systematic study on how to construct such a *structured, cellular* **MCSMD** model or how it (which predicts the dynamics of metabolic processes at the nano-molar cellular scale) is coupled with the macroscopic dynamic model of the industrial bioreactor to produce

a *hybrid* model capable of accurately and simultaneously predicting both the dynamics of state variables at the cellular level and those at the macroscopic level of the bioreactor.

In this context, the present study stands out for its *high degree of originality* on multiple levels, addressing and detailing several essential aspects related to the dynamics and performance of the cellular metabolic process in connection with the industrial bioreactor used, employing adapted kinetic models from the specialized literature or original ones (case study 1, Chapter IV).

In this regard, the original contributions of this thesis consist of the following:

1). The first-time development in the literature of a step-by-step methodology for constructing structured, modular, hybrid deterministic kinetic models of the **MCSMD** type. This methodology is brilliantly exemplified in case study 1 (Chap. IV). Thus, the kinetic modules concerning the essential stages of **CCM** are first constructed, interconnected, and linked to the kinetic module of the target metabolite synthesis (TRP). Then, these are connected with the macroscopic state variables of the bioreactor dynamic model through the biomass growth model (X) and through the species imported (GLC) or excreted by the cell (TRP, PYR). Next, the kinetic constants are estimated based on experimental data, considering the entire **MCSMD** model as a whole. [Maria, 2021; Maria and Renea, 2021].

Furthermore, the thesis vividly and convincingly demonstrates how such a structured hybrid kinetic **MCSMD** model can be successfully constructed using the main concepts and rules of **IchB** (Chap. I.4) [Maria and Renea, 2022C].

2). The first-time testing in optimization calculations of biological/biochemical reactors using structured, hybrid, bi-level, complex kinetic models of the **MCSMD** type. These models enabled the *in-silico* generation of a greater number of optimal operating alternatives for **BR** or **FBR** reactors, with all operational policies being determined with increased precision and a higher level of detail (number of species considered in the model) compared to using classical unstructured, simplistic, global "default" models such as Monod (see case study 1, Chap. IV) or simple Michaelis-Menten (see case study 2, Chap. V). The significance and advantages of the *hybrid MCSMD* models proposed in this thesis, as revealed by the solution of the two case studies, are extensively described in Chap. I.3 and I.1.2. [Maria, 2021; Maria and Renea, 2021].

3). The bi-level hybrid kinetic MCSMD models proposed in this thesis contain, for the first time in the specialized literature, multiple features used simultaneously in an original way in biochemical engineering calculations. These include:

a) Structured models incorporating the reaction mechanism and the dynamics of cellular species (considered individually or in groups) that directly and significantly influence cellular metabolic syntheses of industrial interest (e.g., TRP synthesis in the case study 1).

b) Modular models containing inter-connected kinetic modules representing enzymatic reaction modules that govern the essential stages of **CCM**, directly connected with the kinetic synthesis module of a target metabolite (of industrial interest) or controlling genetic regulatory circuits (**GRC**). These kinetic modules at the cellular level are also connected to kinetic modules representing the state variable dynamics of the bioreactor (**BR** or **FBR**). The modular approach in constructing *hybrid MCSMD* models facilitates their extension/adaptation when studying a new bioprocess. This approach is natural since many **GRC** systems or **CCM**-related processes are modularly organized. [Maria, 2018; Maria, 2023B; Kholodenko et al., 2002; Maria, 2017].

c) Deterministic, reaction-mechanism-based models to facilitate numerical simulation interpretation. These models use continuous variables, enabling the application of key concepts, principles, procedures, and algorithms of **IchB** (Chap. I.4) [Maria, Renea, 2022C].

d) Hybrid models. This thesis makes a **remarkable contribution** by developing, for the first time in the specialized literature, a methodology based on **IChB** principles and techniques for constructing deterministic **MCSMD kinetic mathematical models** that combine two levels of process representation. Thus, the nanoscale characteristics of essential **cellular CCM** modules are combined with the macroscopic state variables of the industrial bioreactor. This results in more accurate predictions for both the cellular biological process dynamics and the operational parameter dynamics of the industrial bioreactor analyzed. This is due to species that are connected at both levels of representation. For case study 1, these include the substrate (GLC), biomass (X), and metabolites excreted by the cell (TRP, PYR). The immediate application of these structured hybrid models lies in the more precise determination of the optimal operating regime of an industrial bioreactor. The advantages of using MCSMD models in solving bioengineering problems are described in Chap. I.3.

e) Extendable models. The modular construction of hybrid kinetic **MCSMD** models allows for the attachment of new kinetic modules if a more detailed scheme of the cellular **CCM** is required or if additional cellular metabolite syntheses need to be included.

4). By solving two case studies *in-silico* demonstratively (one of which, from Chapter IV, was verified with experimental data), this work demonstrates the superiority of structured, hybrid MCSMD dynamic models compared to unstructured dynamic models when applied to the simulation of an industrial bioprocess. The structured **MCSMD** models provide a higher level of detail and accuracy in dynamic model predictions, both at the macroscopic level (bioreactor state variables) and the nanoscopic level (dynamics of cellular species related to **CCM**). For example, in Case Study No.1, regarding the maximization of TRP production in an **FBR** bioreactor using a "wild" *E. coli* culture [Maria et al., 2018C] or a **GMO** strain [Maria, 2021; Maria and Renea, 2021], the **MCSMD** model of [Maria, 2021; Maria and Renea, 2021] achieves a greater level of detail in predicting bioreactor behavior. Specifically, it can predict the dynamics of 13 (cellular species), plus 4 (species in the liquid phase of the bioreactor), compared to only 3 species (in the bioreactor liquid) obtained by applying a classic unstructured macroscopic (global) model (Monod). It also covers a broader operational time range and a wider set of control variables, as well as different *E. coli* strains ("wild" or GMO), as follows: **(i)** Bioreactor dilution {feed/evacuation flow rate FL relative to the liquid volume VL in the bioreactor} = $[1e-5 - 0.01]$ 1/min. {identical to cellular dilution, calculated with the relation $\text{Ln}(2)/(\text{cell cycle duration})$ }; **(ii)** Glucose substrate concentration [GLC]_{feed} = $[200-4500]$ mM; [GLC]_{ext} = $[0-10]$ mM; **(iii)** Biomass concentration [X] = 10-100 gDW/L; **(iv)** Initial conditions [GLC]_{initial} = $[1-200]$ mM; FL = $[0.01-0.04]$ L/h [Maria and Renea, 2021; Maria, 2021]. For comparison, the partially structured **MCSMD** model of the **FBR** bioreactor from [Chassagnole et al., 2002] simulates its behavior in narrower operational intervals, approximately one order of magnitude smaller [Maria et al., 2018C]. This high-precision approach developed in this thesis is explained by the numerous original elements in the construction of the structured **MCSMD** model mentioned earlier. Additionally, long-duration simulations of bioreactor operation allow for the visualization of how biomass adapts to the continuously changing environment within the bioreactor [Maria, 2020A; Maria, 2021; Maria and Renea, 2021].

An important observation is that the structured hybrid **MCSMD** mathematical (kinetic) model from Case Study 1 (Chapter IV) was experimentally validated using an extensive set of kinetic data generously provided by TU Hamburg through an international collaboration [Chen, 2020; Maria, 2021; Maria and Renea, 2021]. Similarly, the structured hybrid **MCSMD** mathematical (kinetic) model from Case Study 2 (Chap. V) was validated using large sets of experimental data from the scientific literature [Maria and Renea, 2022B; Slatner et al., 1998].

For the structured hybrid **MCSMD** mathematical (kinetic) model from Case Study 1 (Chapter IV), a modification of the structured kinetic module from the literature was proposed and validated for simulating the oscillatory synthesis of TRP [Maria et al., 2018A, 2018C; Maria and Renea, 2021]. This new structured kinetic module was re-identified using the experimental kinetic data from [Bhartiya et al., 2006].

5) The application of the structured hybrid **MCSMD** mathematical (kinetic) model (with 4 interconnected kinetic modules) in Case Study 1 (Chap. V, optimization of the **FBR** bioreactor for TRP synthesis) presents several novel and original elements compared to the specialized literature:

5i) Demonstration of the modular construction methodology for the structured hybrid **MCSMD** kinetic model. The resulting two-level model is sufficiently complex to adequately represent the dynamics of key species belonging to essential **CCM** cellular modules ([a] glycolysis, [b] ATP recovery system, [c] TRP operon expression, TCA cycle), as well as the liquid phase dynamics of the **FBR**, including biomass growth, GLC depletion, and accumulation of excreted TRP and PYR.

5ii) Although TRP production using **GMO** E. coli strains has been widely studied, the need for multiple precursors in its synthesis and the complex regulation of biosynthetic pathways make achieving high product yields still a major challenge. This engineering problem has been successfully addressed here using an in-silico approach based on a structured hybrid **MCSMD** kinetic model, complemented by a biological improvement with a **GMO** strain [Chen, 2020] of the E. coli culture used.

5iii) The optimal variable operating policy of the **FBR** is defined over time intervals (so-called "time arcs") of equal length and in a small number ($N_{div} = 5$) to facilitate implementation. The control variables have optimal but constant levels within each time arc (varying between time arcs) during **FBR** operation.

5iv) The biomass culture used refers to a genetically modified E. coli T5 strain that replaces the PTS substrate (GLC) import system in the cell with a more efficient system based on the galactose-permease/glucokinase (GalP/Glk) system, by modulating the gene expression of GalP/Glk. The characteristics of this strain were reflected in the estimated reaction rate constants by [Maria, 2021]. This new T5 strain was developed by [Chen, 2016; Chen et al., 2021]. The resulting T5 strain demonstrated a 52.93% increase in TRP production in a sub-optimally operated **FBR** (25.3 mg/gDW biomass/h) compared to the initial "wild" strain [Chen et al., 2021] and a 70% increase for an optimally operated **FBR**, as determined in-silico in this study using a structured hybrid MCSMD model (Case Study 1, Chapter IV) [Maria and Renea, 2021].

6).- Proposal of a systematic methodology (see solved case studies 1 and 2) for selecting the most suitable type of bioreactor for a given biological or enzymatic process and determining, in-silico, off-line, based on a hybrid **MCSMD** kinetic model, structured for the studied process, the optimal operating policy of the selected reactor [Maria, 2021; Maria and Renea, 2021; Maria and Renea, 2022B, 2022C, 2024B].

7).- The original approach in which the two case studies addressed in this thesis, complemented by several similar studies from the specialized literature [Maria and Luta, 2013; Maria, 2023B], eloquently illustrate the usefulness of a moderately sized structured hybrid **MCSMD** kinetic model of the studied process, based on the essential stages of the cellular **CCM**. Such a kinetic model is a modern, precise, and valuable computational tool when developing structured simulators for various engineering and bioinformatics applications, such as:

(7a).- Analysis of cellular metabolic fluxes under variable bioreactor operating conditions [Chen, 2016, 2020; Maria, 2021; Maria and Renea, 2021]. These result from solving the dynamic **MCSMD** model rewritten for steady-state conditions (with the derivatives of the species concentrations in the mass balance equations set to zero). Based on this analysis, it is possible to reprogram cellular metabolism in-silico by designing **GMOs** for industrial applications [Chen, 2020; Maria, 2018, 2021; Maria & Renea, 2021].

(7b).- Optimization of the desired metabolite synthesis by improving the operation of the bioreactor (**BR** or **FBR**) and/or through genetic modification of the cell culture (this thesis, and [Maria, 2021; Maria and Renea, 2021]).

(7c).- More precise optimization of bioreactor operation, as exemplified by case study no. 2 in Chapter IV, and the author's works [Maria and Renea, 2021; Maria and Renea, 2022B; Maria and Renea, 2024B, 2024C]. Moreover, it is possible to determine the optimal operating policies of a bioreactor concerning multiple simultaneous economic objectives, using the Pareto optimal front technique, aiming to minimize raw material consumption (biomass, enzymes) while maintaining high productivity of the desired metabolite (e.g., TRP in case study 2, Chapter IV).

(7d).- *Structured hybrid* **MCSMD** models of the studied bioprocess allow for more precise extrapolations of process dynamics in the studied bioreactor over dozens/hundreds of cellular biomass cycles (approximately 200 for case study no. 1, Chapter IV) [Maria and Renea, 2021; Maria, 2010; Maria and Luta, 2013].