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External control of microbial populations for bioproduction: A modeling and optimization viewpoint

François Bertaux^{1,2}, Jakob Ruess^{2,1}, Grégory Batt^{2,1}

Abstract

When engineering microbes for bioproduction, one is necessarily confronted to the existing tradeoff between efficient bioproduction, and maintenance of the cell physiology and growth. Moreover, because cellular processes at the single-cell level are coupled with population dynamics via selection mechanisms, this question should be investigated at the population level. Identifying the temporal induction profile that maximizes production on the long term is highly challenging. External control allows to dynamically adapt the strength of the induction from the outside based on intracellular readouts. It allows benchmarking various regulation functions and, coupled with modeling approaches, identifying and applying optimal strategies. In this review, we describe recent advances using quantitative approaches, modeling and control theory that pave the way to compute external stimulations maximizing long term production.

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Highlights

- External control allows adapting the production demand to the cell physiology
- Rational approaches are needed to find the optimal balance between production and growth
- Coarse-grained models of cell physiology and optimal control can help assessing strategies
- Coupling stochastic cellular models with population models can predict emergent dynamics

Introduction

Bioproduction is a field of major economic importance [1,2]. Bio-manufactured products include a variety of chemicals, such as alcohols, organic acids, fragrances, and antibiotics, and a large range of industrial or pharmaceutical proteins, such as enzymes and antibodies [3–7]. Despite decades of research in bioengineering and in synthetic biology, obtaining robustly high yields and titers remains highly challenging for the industry [1,8,9].

By essence, bioproduction requires diverting away cellular resources normally used by the host cell for growth and self-replication towards the production of the molecules of interest. Host resources consumed by the bioproduction process typically include energy, metabolites and other precursor molecules, enzymes, and cellular machineries. Therefore, there exists a competition between efficient bioproduction and maintenance of cell physiology and cell growth, and channeling as much resources as possible towards production is seldom an optimal strategy. Regulation of bioproduction and growth is critical and identifying optimal trade-offs is needed for maximizing production [10].

A lot of efforts have been dedicated to engineering regulations that trigger a switch from a growth phase to a production phase in the context of two-stage fermentation processes. Such sequential controls can be implemented using metabolites from the environment [11] or using quorum sensing molecules [12]. In the

context of continuous production processes, efforts were made to develop regulations that *couple* cellular growth and bioproduction [13]. Such regulations are implemented to prevent escapers from invading the population of producers, thus favoring the genetic stability of the production system.

Despite all these efforts, developing effective regulations for a novel system remains a considerable challenge. Firstly, the identification of appropriate biological building blocks is not always trivial (*e.g.*, sensors with high specificity, actuators with large dynamical ranges). Secondly, the control device should be genetically-stable and have a modest resource cost for the cell. And thirdly and most importantly, the regulation function is implemented by means of biological building blocks and is therefore hard-wired in the genome. It cannot be easily tuned to be used in different operating conditions.

Cybergenetics can be defined as the application of control theory to engineer control devices steering the dynamics of cellular systems. In this context, the regulation devices mentioned above are seen as feedback controllers inside cells. Alternatively, one can employ external feedback control approaches. In real time, cellular processes are observed, typically by means of fluorescent reporter proteins, and external actions that modify these processes are applied so as to stir the process of interest to its target behavior. The strength of the regulation is dynamically adapted from the outside based on intracellular readouts. The regulation effects can be mediated by means of a change in the chemical environment of the cell (*e.g.*, tuning the concentration of an inducer) or more conveniently by means of light stimulations using optogenetics devices [14]. Models of the cell response to dynamic stimulations are generally used to investigate the effects of a range of possible control actions in a model predictive control framework. In this setup, changing the regulation function is as easy as changing a few lines of code in a program. Yet, assessing the effectiveness of a possible regulation function still necessitates to run costly and time-consuming experiments. Rational approaches are needed to solve this problem in an efficient and robust manner.

In this review, we will investigate how models, quantitative approaches and control theory can be used to compute external stimulations that maximize bioproduction. In a first part, we will review how resource allocation models and optimal control can help to find optimal trade-offs at the cell population level. In a second part, we will mention several strategies to mitigate the effects of burden in bioproduction. In a third part, we will discuss the impact of cellular heterogeneity and revisit our previous viewpoint using stochastic models coupling single-cell processes with population dynamics to capture dynamic selection effects at the population level.

Using resource allocation models to capture population-level trade-offs

To start our discussion, we assume that one has full control over the bioproduction process, that is, can freely divert away natural resources used by the host for growth towards the production of molecules of interest. We therefore neglect the highly challenging problem of implementing the *production knob* to focus on the regulation question. Even then, a fundamental question remains. How *strongly* and *when* (along the bioproduction process) this production knob should be activated? To address this optimization problem, theoretical models and tools are needed. It is first and foremost critical to have a reliable quantitative model capturing the trade-off between the production process and the impact on the host. The most important host physiological variables to capture are the growth rate, biomass yield and product yield, but other variables such as viability and genetic stability could also strongly impact the efficiency of the bioproduction process.

It is only since recently that such modeling efforts have been undertaken. They all build upon the more established field of microbial resource allocation [15–21], which aims at capturing the endogenous cellular

trade-offs faced by microbes in different environments and at uncovering the regulatory principles used by cells to solve these trade-offs nearly optimally [22,23]. In particular, whole-cell coarse-grained models [15,18,21] are appealing starting points. Indeed, their holistic nature facilitates the explicit modeling of exogenous bioproduction circuits and how these circuits interact with the host processes, modifying endogenous trade-offs and creating new ones. For externally controllable circuits, the resulting models allow representing the *production knob* as a time-varying input variable. Different scenarios of induction profiles (in time and strength) can then be tested, leading to different dynamics of host physiology, product formation and nutrient consumption. Using tools from control theory, it is possible to compute the optimal (in terms of production) time-varying induction profile for a given production process. The specific instantiation of the problem depends on the operation mode, the starting cell density and nutrient concentrations, and the harvest time, to name but a few factors.

This was done recently by Yegarov and colleagues [24]. These authors build upon a model previously published by Giordano and colleagues [20]. In essence, this model is a simple self-replicator model of bacterial growth where the endogenous resource allocation trade-off between metabolism and biosynthesis is represented by a single control variable (the fraction of biosynthesis devoted to producing metabolic machinery, the remainder being devoted to more biosynthesis machinery) [20]. Yegarov and colleagues extended the previous model to investigate a metabolite production application. The host metabolism, normally solely dedicated to the conversion of nutrients into precursors for biosynthesis, is now also converting precursors into the metabolite of interest. The *production knob* is here the same knob as in the starting model: the fraction of biosynthesis devoted to metabolism. However, while endogenous regulation dynamically controls this knob so as to maximize growth [20], here the goal is to maximize metabolite production. Their main qualitative result is simple: the optimal strategy starts with biomass maximization and switches to full production (here, full investment in metabolic machinery at the expense of biosynthesis machinery) towards the end of the time horizon (Figure 1A, optimal timing).

This temporal separation of a biomass accumulation phase followed by a product accumulation phase matches common practice in bioproduction applications [10]. Therefore, synthetic biologists and metabolic engineers have not waited for such theoretical results to invest in the development of efficient, robust host engineering strategies to implement such strong rewiring from biomass maximization to maximal product formation. A remarkable example is the demonstration in yeast of a light-controlled metabolic valve for isobutanol production [25]. It is worth noting that in this work, the choice of induction time was not model-guided and it is possible that other choices would have led to higher titers. In contrast, the model-based approach described earlier does provide an optimal time of induction. It would therefore be of high interest for the field to experimentally validate such model-based optimization approaches. We can already anticipate that their validity domain (in terms of time horizon and production scale for example) will be affected by physiological phenomena not accounted for in the model.

One important limitation of the self-replicator model described above is that it assumes that efficient, maximal resource diversion can be maintained for arbitrary long durations at no cost in terms of cell viability and production efficiency. In reality, with current genetic implementations of resource diversion for bioproduction, full induction often leads to detrimental effects ranging from inefficient production to cell death and genetic stability issues. Given the complex nature of the physiological determinants underlying these burden effects, properly accounting for them in mathematical models is a very challenging task, which is still insufficiently addressed. A recent attempt was made in this direction by Nikolados and colleagues [26] using an extension of the seminal whole-cell coarse-grained model of bacterial growth by Weiße and colleagues [18]. The authors predict that ribosome shortage could be responsible for a brutal decrease of effective production when the *production knob* is pushed too hard. Clear experimental demonstrations for such non-monotonic effects are however still largely missing.

Strategies to mitigate the effects of burden in bioproduction

Several strategies can be proposed to mitigate the effects of burden in bioproduction. In what follows, we describe three of them. An appealing first approach to prevent such detrimental effects is to implement in cells a negative feedback regulation of the production knob in response to burden (Figure 1A, host-aware regulation). A remarkable example of such an approach has been developed and demonstrated in *E. coli* by Ceroni and colleagues [27]. The designed regulation leverages endogenous stress response predictive of production-caused burden as an input and *dCas9* transcriptional repression as an effector. A clear advantage of such internal regulation is to prevent the production knob to go over the sustainability limits of the host. The authors demonstrate that the regulated system outperforms the non-regulated one in terms of protein yields in batch. However, to which extent and under which conditions the resulting regulation can enable optimal production remains to be investigated. Still, we can envision that combining such built-in host-preserving synthetic regulations while keeping an external control over the production knob could enable model-based optimal control strategies with a broader application range.

An alternative way to get the benefits of the temporal separation between biomass maximization and production while mitigating issues with the burden associated to full resource diversion is to switch off the resource diversion at regular time intervals during the production phase (Figure 1A, start-and-stop). These regular ‘breaks’ for host cells enable them to regenerate resources and preserve their integrity. This strategy was implemented experimentally by Izard and colleagues [28] with a chemically controllable transcriptional machinery in *E. coli*. It was also used in a fed-batch demonstration of the yeast metabolic valve for isobutanol production mentioned earlier [25]. However, again the choice of the frequency and duration of these ‘breaks’ were not model-guided and their optimality is not proven.

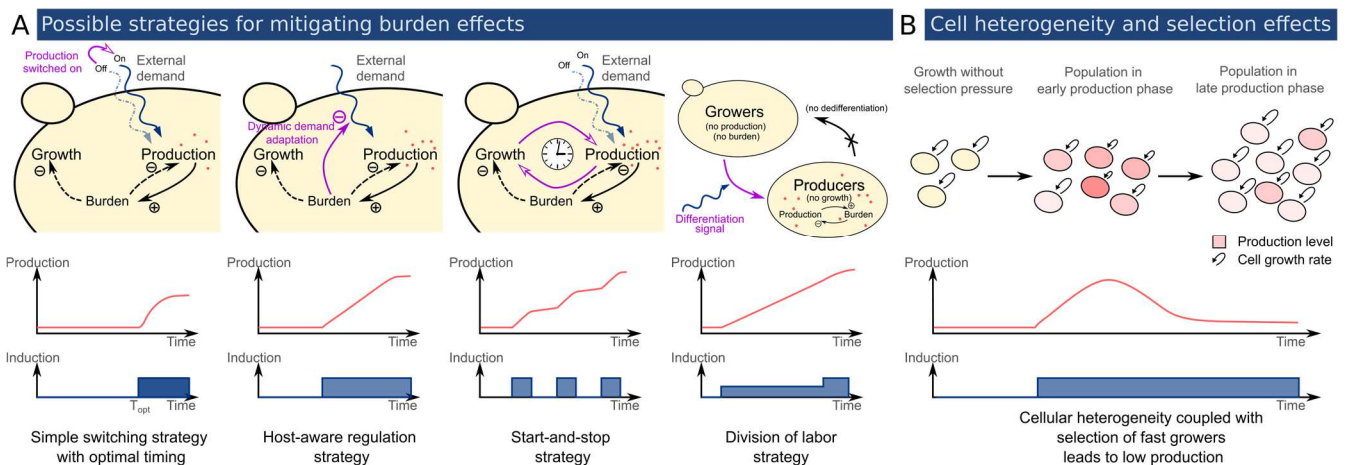


Figure 1. Long-term impact of production burden and mitigation strategies. (A) Strategies for mitigating burden effects. The simplest form of regulation is to turn production on after a phase of growth. Timing needs to be carefully chosen to enable for a long production phase (early switch) made by many producing cells (late switch). If the response to the external demand is tuned down in overly stressed cells by means of internal regulations, production and growth can be concurrent and production can start earlier. Alternatively, growth and production can either alternate in time or happen in different cells via an artificial differentiation strategy. All these strategies tend to limit the growth of cells at reduced rates coming from burden effects. *Strategies that involve highly dynamic changes of the input are more conveniently implemented using light instead of chemicals to act on cellular processes.* **(B)** Cellular heterogeneity and selection effects. Because of non-genetic variability, production, production burden, and ultimately, cell growth rate, differ from one cell to another. Having a less reduced growth rate, lowly producing cells are likely to invade the population. Color intensity and self-loop size encode for production levels and cell growth rate, respectively.

Finally, a third strategy to mitigate issues associated to burdening production is to separate growth and production *between cells* rather than temporally in the *same cells*. For example, provided that an efficient artificial differentiation system enables the externally controlled conversion of ‘grower’ into ‘producer’ cells as described in [29], one can now ask what is the time-varying optimal conversion rate optimizing overall production (Figure 1A, division of labor). The performance of this novel bioproduction paradigm was investigated theoretically by Weill and colleagues [30]. It was found that the differentiation strategy can be advantageous over traditional induction systems for chemostat operation mode. Moreover, the controller identified solutions that are likely to correspond to optimal stationary trade-offs between growth and production. The underlying model did not consider deterioration of the producer cells with time, and we anticipate that more realistic models regarding this important aspect will make the differentiation strategy even more appealing.

Accounting for cell-to-cell heterogeneity and its couplings with population dynamics

In the previous modeling approaches, cellular heterogeneity has been neglected. Indeed, the inherent stochasticity of biochemical reactions inside individual cells leads to the existence of significant differences between cells of the same population. Cells therefore have variable capacities for bioproduction and for withstanding the bioproduction burden. If burden causes a growth defect, the single cell production process becomes inherently coupled to selection processes at the population scale (Figure 1B). Low producers, having a lesser growth reduction, tend to invade the cell population over time, a situation eventually leading to low yields [31]. This effect is even more pronounced when the product of interest causes a particularly large burden for cells, for instance when it is toxic. Even small differences in its amount between cells may create selectable phenotypes in a growing population. For proteins produced from plasmids, variability in cellular burden may emerge readily from the often very large differences in plasmid copy numbers between cells even in cases where the produced protein is just a simple fluorescent reporter. For instance, Gnuegge et al. [32] measured snapshots of the distribution of fluorescent reporter proteins expressed from different types of plasmids and found that, to obtain a faithful model of the system, it was necessary to incorporate a burden and growth penalty that increases with the cells’ plasmid copy number. As a consequence, cells with low plasmid copy numbers are enriched during growth, which implies that the total amount of produced protein in the population is reduced and that the plasmid loss rate of the population is increased.

Whenever stochasticity of the single cell circuit creates selectable phenotypes, predicting emergent population level outcomes mandates coupling a description of circuit dynamics inside cells to a model of population growth and selection. While recognized as one of the major challenges for “future systems and control research in synthetic biology” [33], mathematical modeling approaches that allow one to couple mechanistic single cell models based on stochastic chemical kinetics to population level processes are still in their infancy. The main challenge is that, in the presence of selection at the population scale, an exact description of the full system dynamics requires tracking each chemical reaction in each cell of the population simultaneously. This implies that simulation of the system using adapted versions of approaches such as Gillespie’s stochastic simulation algorithm becomes immensely cumbersome except for very small cell populations, while a representation of system dynamics based on the chemical master equation (CME) scales exponentially in the population size and is impossible to solve for all practical purposes. Duso and Zechner used population moment equations to alleviate the computational burden [34] but the approach requires accurate moment closure methods that may not be available for many practical problems. Thomas investigated the particular problem of coupling cell growth and division to stochastic reaction networks inside cells [35], focusing mostly on emerging stationary distributions in the infinite time limit. Lunz and

colleagues used a Fokker-Planck continuum approximation of the CME coupled to discrete states that can be used to capture a variety of different population level processes [36]. A numerical solver was also developed that can be used to accurately track the full multi-scale dynamics of single cell and population processes for sufficiently large population sizes at a computational cost that is comparable to the cost of calculating with standard chemical master equations of isolated single cell processes. All of the discussed approaches, however, remain limited to fairly simple single cell processes. In the future, it will be important to develop further methodology for tracking coupled single cell and population dynamics for more complex intracellular processes.

Conclusions

Recent developments in optogenetics are opening up avenues for bioproduction applications (see the recent review [14]). Light induction is cheap and particularly convenient to use. It is also easily compatible with the small-volume bioreactor platforms that are rapidly gaining popularity in the field and that allow for probing various light induction profiles in parallel and in a cost effective manner [37–41]. The actual applicability of optogenetics for industrial applications is not demonstrated yet, but bioreactor technologies coming from algae bioproduction should help addressing the issue of limited light penetration in dense cell cultures [42].

Yet, it is still unclear how the tighter control of intracellular processes offered by optogenetics technologies can be leveraged to improve production yields and titers. The space of all possible regulation functions cannot be efficiently explored by empirical approaches. Indeed, even if changing the regulation function simply amounts to changing one or a few lines of code, assessing the effectiveness of this novel regulation strategy still necessitates to run time-consuming experiments.

We therefore believe that model development and rational approaches will likely play a critical role to address this challenge. However, this field is still in infancy. The challenges ahead include better understanding the fundamental nature of the growth/productions tradeoffs, better predicting the emerging dynamics of cell population with selection processes at play, and better characterizing bioproduction processes together with their impact on the host cell for various induction profiles and growth conditions.

Conflict of interest statement

Nothing declared.

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Highlighted citations

Papers of outstanding interest

- Zhao et al.: This work is a striking demonstration that dynamic external control can be beneficial for bioproduction. In comparison to the classical two-phase production strategy, significantly higher yields of isobutanol are obtained in *S. cerevisiae* using a start-and-stop strategy in fed-batch mode. The optimality of the proposed strategy is not investigated however.

Papers of special interest

- Izard et al.: Implementation and characterization in *E. coli* of a circuit enabling external ON-OFF control over global transcription via inducible expression of RNA polymerase, effectively resulting in a growth switch. When applied to glycerol bioproduction, the growth switch showed promising results in terms of yield.
- Yegorov et al.: First application of optimal control theory on a (simple) whole-cell coarse-grained model of microbial physiology to compute optimal induction strategies maximizing heterologous production of batch processes.
- Duso and Zechner: Development of a mathematical framework based on counting processes and derivation of moment equations for population statistics in cases where both intracellular processes and population dynamics are stochastic. The authors used the approach to study the dynamics of biochemical reaction networks in subcellular compartments.
- Thomas: Development of an analytical agent-based modeling framework that tracks growth and division of cells as well as intracellular biochemical processes governed by a master equation. The author used the methodology to study extrinsic and intrinsic noise in population snapshot distributions and the statistics of lineage tree data.
- Lenz et al.: Development of an augmented chemical master equation framework that can be used to jointly track stochastic biochemical processes inside single cells and population level processes such as selection or growth. The authors used a continuum approximation of the reaction network and provided open source software that automatically computes dynamics from a specification of the system.