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Engineering metabolism through dynamic control

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Metabolic engineering has proven crucial for the microbial production of valuable chemicals. Due to the rapid development of tools in synthetic biology, there has been recent interest in the dynamic regulation of flux through metabolic pathways to overcome some of the issues arising from traditional strategies lacking dynamic control. There are many diverse implementations of dynamic control, with a range of metabolite sensors and inducers being used. Furthermore, control has been implemented at the transcriptional, translational and post-translational levels. Each of these levels have unique sets of engineering tools, and allow for control at different dynamic time-scales. In order to extend the applications of dynamic control, new tools are required to improve the dynamics of regulatory circuits. Further study and characterization of circuit robustness is also needed to improve their applicability to industry. The successful implementation of dynamic control, using technologies that are amenable to commercialization, will be a fundamental step in advancing metabolic engineering.

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Introduction

Metabolic engineering of microorganisms has proven crucial for improving their ability to produce valuable products, such as pharmaceuticals [1,2], nutraceuticals [3], fuels [4] and commodity chemicals [5–7]. The field of metabolic engineering has been accelerated with the introduction of new computational and experimental methods for the design and construction of plasmids [8–15] and strains [16–20]. Furthermore, synthetic biology has improved our understanding of genetic circuits [21,22] and their applicability to industry [23], while providing new tools, such as rapid and cost effective gene synthesis and sequencing, to expedite the design-build-test cycle.

To produce commercially viable strains, three crucial variables must be optimized: yield, titer and productivity. Heuristic targets proposed by Van Dien are 80% of theoretical maximum yield, 50 g/L and 3 g/(L h), respectively, for products near $1.00/lb [24]. Of these metrics, yield has classically held high priority, and this is particularly exemplified in the development of strain design algorithms (Box 1). These classical metabolic engineering methods have been reviewed at length [25].

One of the first papers to discuss the merits of titer and productivity, early in the strain design workflow, was presented by Zhuang *et al.* [26•]. They demonstrated that a strain designed for optimal yield results in suboptimal productivity, and discussed the inherent trade-off between the two. To overcome this issue, they developed an algorithm that takes into account productivity and titer, in addition to yield (DySSCo). However, this algorithm, along with most others, assumes that enzymes will not be dynamically controlled. We refer to this as *static metabolic engineering*.

In most of these strategies, product pathway flux is maximized. Since these pathways can drain metabolites used in biomass synthesis, this leads to a trade-off between growth and the production of the desired compound (Figure 1). Hence, most strategies to improve yield will also result in strains with low volumetric productivity, due to impaired growth rates. Slow growth rates can also be a result of poor cofactor balance [27], the accumulation of toxic intermediates [28], or an inefficient metabolic network resulting from the elimination of byproducts [29]. In strains with near optimal theoretical yield, optimization of the metabolic network alone is insufficient to significantly improve growth rate, as all substrate is diverted to product; this effect is evident when drawing from an early glycolytic metabolite [30,31•]. Slow growth rates can result in low volumetric productivities and high capital costs for commercial plants, and this must be addressed to ensure commercial viability.

Although growth rates can also be improved using adaptive evolution [29], such a strategy cannot be applied in all cases; the growth rate may not improve to acceptable levels or the initial growth rate can be too low for successful adaptive evolution. Another approach to overcoming the

Glossary
Capital cost: the cost associated with building a commercial fermentation plant.
Deregulation: the modification of native gene regulation through methods such as deletion, attenuation or overexpression.
Dynamic metabolic engineering: the practice of engineering microorganisms to respond to a changing intracellular (e.g. metabolite concentration) or extracellular (e.g. inducer concentration) environment.
Continuous control: control strategies which measure and control variables continuously.
Flux: the flow of metabolites through metabolic pathways (mmol/(gdw h)).
Growth stage: the stage in a batch fermentation in which optimal growth (wild-type growth rate) is targeted.
On–off control: control strategies which are implemented as either fully on or fully off, with no intermediate states (e.g. inducer addition).
Production stage: the stage in a batch fermentation in which maximal flux of the product is targeted.
Productivity: overall rate of production for the entire batch, the concentration of product per unit time (g/(L h)).
Static metabolic engineering: the practice of engineering microorganisms with genetic circuits lacking engineered dynamic control elements.
Titer: concentration of product at the end of a batch ($g_{product}$/L).
Yield: mass of product formed per mass of substrate consumed ($g_{product}/g_{substrate}$).

deleterious effects of genetic modifications is the use of two-stage dynamic control, whereby fluxes can be restored to wild-type distributions to improve growth rates in a growth stage, followed by a production stage with maximal flux through the product pathway [32,33]. To demonstrate the benefit of dynamic control, we have simulated and compared the productivity for static strategies and two-stage dynamic control using dynamic flux balance analysis [34]. These results show that the use of a dynamic strategy has the potential to further improve the productivity for any hypothetical strain designed using the previously described DySSCo strategy (Figure 2).

In this review, we focus on applications of dynamic metabolic engineering strategies and the different approaches for their implementation. We also highlight some challenges in the context of metabolic and regulatory network dynamics.

Restoring the wild-phenotype in mutant strains

To successfully implement two-stage fermentation and address low growth rates, we require a wild-type flux distribution in the growth stage, and maximal flux through the product pathway in the production stage [32,33]. In order to take advantage of the benefits of each phase, biomass can be quickly generated in the growth phase, before switching to a production phase. To restore a wild-type flux distribution in a mutant strain, an efficient strategy is to eliminate the effect of any genetic modifications. This restoration can be achieved through wild-type level expression of any native genes that have been deregulated, and repression of any heterologous pathways. Following the growth stage, the genetic manipulations must be restored in order to maximize the production rate.

Alternatively, in some cases, growth can be improved by modifying environmental conditions, including dissolved oxygen concentration, inducer concentration, and pH. In these scenarios it is possible to improve productivity by implementing dynamic control at the process level by controlling fermentation conditions; most of the early attempts to implement dynamic control utilized process level methods. More recently, owing largely to progress in synthetic biology, fluxes can be regulated dynamically by controlling the expression of key enzymes using genetic circuits. These methods will be described in the following section.

Dynamic control strategies

Control of fermentation conditions

One common implementation of two-stage fermentation at the process level is to follow an aerobic growth stage by an anaerobic production stage. This strategy has been applied for lactic acid production, where single-stage anaerobic strategies (using minimal media) have productivities ranging from 0.27 to 0.33 g/(L h) [35], and can be improved approximately 10-fold using a two-stage strategy, reaching a productivity of 3.32 g/(L h) [36]. More recently, process level control has been applied for 1,4-butanediol production; cells were grown aerobically to an OD_{600} of approximately 10 before switching to microaerobic conditions and inducing pathway gene expression using isopropyl-1-β-thiogalactopyranoside (IPTG) [7].

An alternative is to use pH for the control of the growth and production phases. For example, α-ketoglutarate was produced in *Yarrowia lipolytica* using two-stage fermentation; a pH of 5.0 and 50% air saturation (aerobic conditions) was used for the growth phase, while a switch to pH 3.8 and 10% air saturation (microaerobic conditions) was used for the production stage [37]. A two-stage strategy is also common in the recombinant protein production industry, where protein production can significantly reduce the growth rate of the production host. Thus, the production stage is induced with IPTG, or other inducers, after reaching optimal cell density [38].

Two-stage fermentation has proven successful for anaerobic products and high-value proteins; however, it has not been thoroughly explored for lower-value products, or for products with pathways which are difficult to link to a process level parameter. In these cases, it is cost prohibitive to use inducers, such as IPTG, and it may not be possible to use oxygen concentration, or pH, as a trigger to switch between states. Furthermore, aerobic growth of strains designed for anaerobic production will not necessarily restore wild-type growth rates, especially when heterologous pathways draw significant flux from biomass