Layered and multi-input autonomous dynamic control strategies for metabolic engineering

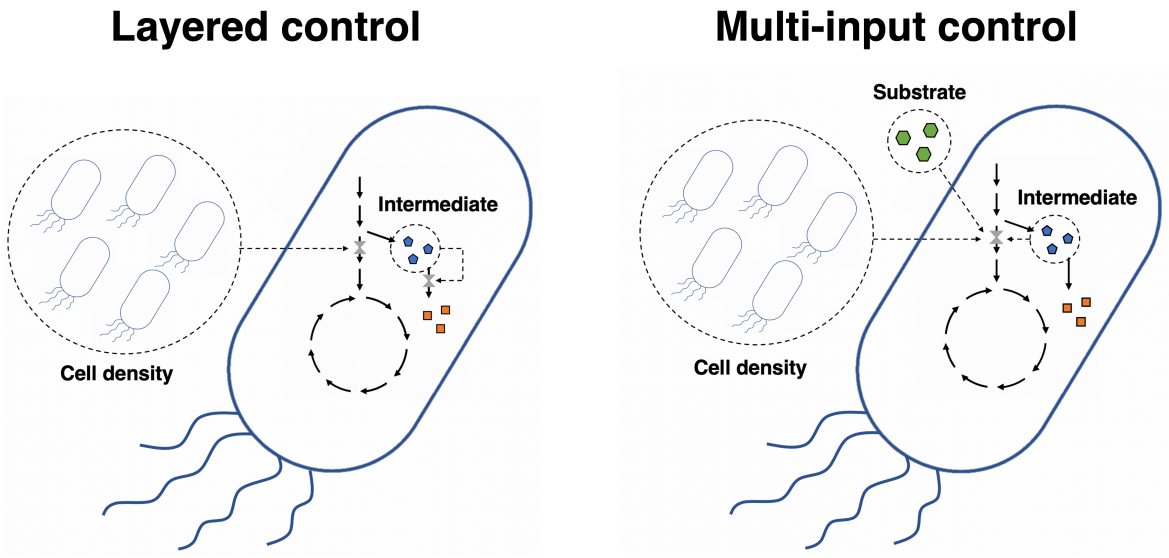
Christina V. Dinh[1] and Kristala L.J. Prather[1]

[1]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, USA

Correspondence should be directed to kljp@mit.edu

Abstract

Metabolic engineering seeks to reprogram cells to efficiently produce value-added chemicals. Traditionally, this is achieved by overexpressing the production pathway and/or knocking out competing endogenous pathways. However, limitations in some pathways are more effectively addressed through dynamic metabolic flux control to favor different cellular objectives over the course of the fermentation. Dynamic control circuits can autonomously actuate changes in metabolic fluxes in response to changing fermentation conditions, cell density, or metabolite concentrations. In this review, we discuss recent studies focused on multiplexed autonomous strategies which (1) combine regulatory circuits to control metabolic fluxes at multiple nodes or (2) respond to more than one input signal. These strategies have the potential to address challenging pathway scenarios, actuate more complex response profiles, and improve the specificity in the criteria that actuate the dynamic response.



Highlights

- Autonomous dynamic control circuits have been developed to balance trade-offs in microbial synthesis systems in a cost-effective manner.

- Significant improvements in production have resulted from implementing layered autonomous control strategies that regulate metabolic fluxes at multiple nodes.

- Synthetic biology tools have enabled the construction of multi-input control circuits which result in more favorable regulation dynamics in some production contexts.

Introduction

Metabolic engineering seeks to take advantage of cellular machinery to produce value-added compounds [1], which can vary widely from biofuels [2,3] to pharmaceuticals [4,5]. Typically, improvements in the production pathway are realized by increasing pathway enzyme levels and/or down-regulating competing endogenous pathways [6,7]. However, some pathways are subject to challenges that are better addressed through dynamically regulating metabolic fluxes [8,9]. For example, down-regulating essential endogenous pathways may result in poor growth, which can be restored by delaying pathway down-regulation until there is sufficient biomass accumulation [10]. This idea of beginning a fermentation with a growth phase before transitioning to a production phase has been shown to improve production in a number of different pathway contexts [11–14].

Transitioning from growth to production phase requires a shift in metabolic fluxes, which typically results from a change in enzyme levels. Enzyme levels can be regulated using engineered gene circuits that control transcription, translation, or enzyme degradation rates. This review is focused on transcriptional control circuits that employ repressor or activator proteins that bind or release from a promoter sequence in the presence of a small molecule. Until recently, regulation of enzyme levels was most frequently controlled through circuits responding to exogenous chemical inducers such as isopropyl-β-D-1-thiogalactopyranoside (IPTG), anhydrotetracycline (aTc), or L-arabinose [15,16]. While feasible in academic settings, these strategies are not practical in many industrial processes due to the high cost of inducer molecules. With the goal of developing more industrially feasible methods of dynamic control, recent studies in this field have explored autonomous control systems. Instead of responding to an exogenous signal, autonomous control circuits respond to a stimulus that results from cell metabolism such as substrate depletion [3], pathway precursor or product generation [3,17–20], or increased cell-density [13,14,21–23]. Application of these circuits to regulating metabolic fluxes has resulted in significant improvements in a number of products including glucaric acid [13,18], lycopene [24], and amorphadiene [25].

With the rapid development of synthetic biology tools, it is possible to design, construct, and characterize more complex control circuits. This has led to a recent focus on (1) “layered”

autonomous strategies capable of controlling more than one metabolic flux node [18,22,26] and (2) "multi-input" autonomous strategies which sense multiple stimuli to influence one metabolic flux node [27–29]. For single-input autonomous control systems that regulate flux at one metabolic node, we suggest the excellent reviews by Xu, Shen, Lalwani, and Tan [30–33]. This review will be focused on more recent studies on layered and multi-input strategies.

Layered control methods

Some pathways are subject to limitations at two or more metabolic nodes that can be addressed through implementing dynamic control. Figure 1A illustrates an example regulation scheme that uses one regulation module to delay production of an intermediate and a second to increase availability of an endogenous precursor. In this context, controlling both metabolic nodes may improve production over a system that only addresses one. To implement this regulation scheme, recent studies have developed autonomous bifunctional control circuits [18,22,26]. These systems achieve dual regulation, either by employing one control circuit that regulates expression of multiple genes, or by employing two control circuits each of which is responsible for regulating one metabolic node. This section presents three illustrative examples of recent work on layered dynamic control, highlighting the differences in tunability that result from the module choices. Studies by Dahl [25], Zhang [17], and Xu [3] are excellent studies that controlled multiple metabolic fluxes or employed two sensor variants, but will not be discussed here (Table 1).

When one control circuit is used to regulate metabolic fluxes at both metabolic nodes, the switching dynamics of these nodes are fully coupled. That is, both regulation modules switch at the same time. Figure 1C shows that whether Circuit A, B, or C is used to control both reactions, there are significant areas of the switching time search space that cannot be explored. While this can be limiting for cases for which production is highly sensitive to the switching time of both regulated reactions, Yang et al. [26], Soma and Hanai [14], and Williams et al. [23] showed that this regulation scheme is well-suited to overcoming challenges in certain pathways (Table 1). For example, Yang et al. aimed to improve muconic acid production by employing a regulation system that would allow the cells to adapt to the fermentation environment before gradually turning ON

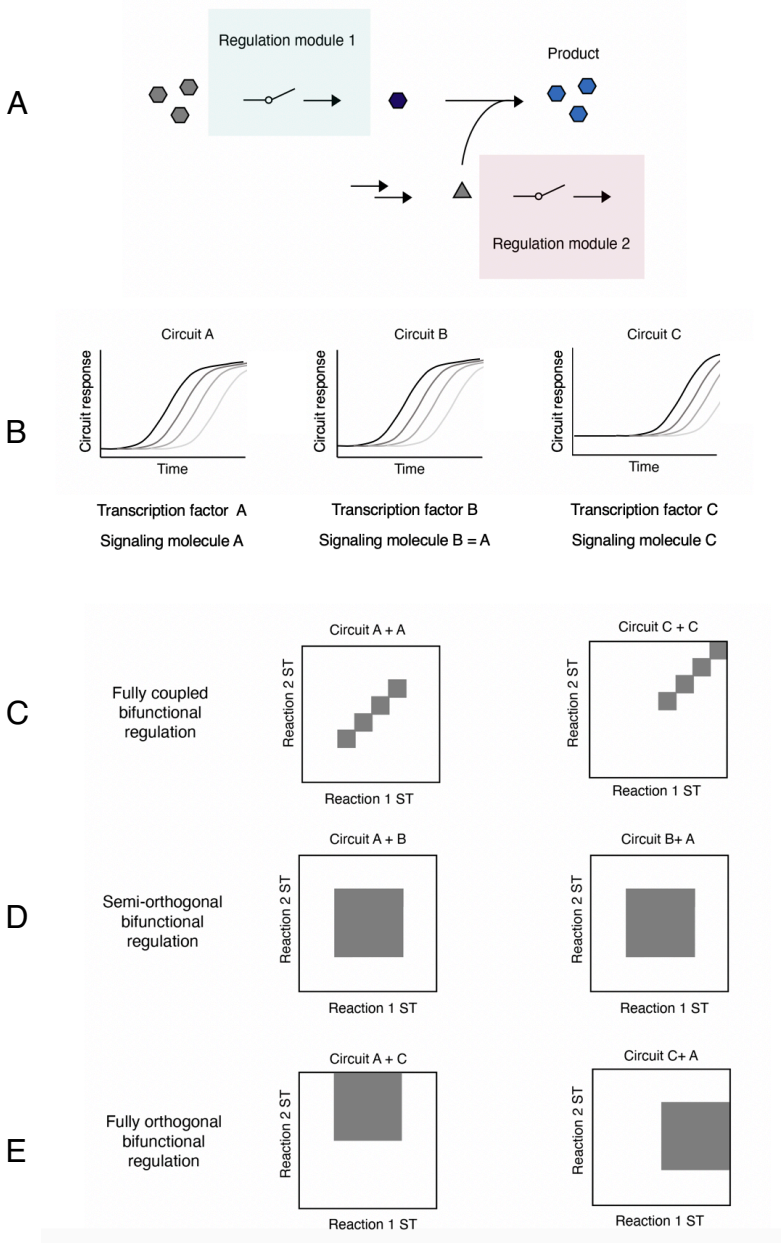
the first two steps of the muconic acid pathway and turning OFF carbon flux towards the TCA cycle. To actuate the switch from the adaptation phase to the production phase, a sensor was derived from the natural muconic acid response machinery in *Pseudomonas putida.* This sensor contains a regulatory protein, CatR, which binds to the P_{MA} muconic acid-responsive promoter. CatR underdoes a conformational change in the presence of muconic acid to allow transcription from the P_{MA} promoter [34]. This circuit was used to control expression of the first two pathway genes [35], along with anti-sense RNA to down-regulate *ppc* expression to achieve a muconic acid titer of 1.8 g/L, a substantial improvement compared to static and both single-layer controls.

In some pathway contexts, production is highly dependent on the switching time of both regulation modules. These situations benefit from regulation schemes that employ orthogonal circuits to control each module, increasing the accessible area of the switching time search space from a line to a rectangle (Figure 1E). While implementation of this control scheme adds complexity by requiring additional regulatory elements, Doong et al. demonstrated the importance of accessing an expanded search space in achieving efficient production of glucaric acid from glucose [18]. The first regulation module of this system increased the availability of glucose-6-phosphate for the production pathway by down-regulating *pfk* expression using a quorum-sensing (QS) circuit [13]. The second module used a biosensor to express the pathway gene, *MIOX*, only in the presence of its substrate to address enzyme instability (E Shiue, PhD thesis, Massachusetts Institute of Technology, 2014). The switching dynamics of both modules were tuned by varying the expression level of key circuit components – the AHL synthase for the QS circuit and the regulator protein for the biosensor. A combinatorial screen through the switching dynamics of both modules showed that the switching dynamics of both circuits were key parameters that drastically influence glucaric acid titers. Implementation of only the *pfk* control layer results a greater than four-fold titer increase and addition of the *MIOX* control layer led to an additional two-fold increase.

A third category of bifunctional regulation circuits combines features from the fully coupled and fully orthogonal strategies (Figure 1D). An example of this strategy was developed in a study that constructed a bifunctional circuit composed of the *lux* and *esa* QS circuits that respond to the same *N*-acyl homoserine lactone (AHL), but contain different transcription factors [22]. Under this regulation scheme, changing the expression level of the AHL synthase results in a change in the

switching dynamics of both modules, while independent tuning of the *lux* circuit can be achieved by varying the expression level of the *lux* regulator protein that only impacts its cognate promoter. This regulation system was implemented to improve production in the naringenin and salicylic acid pathways. In both applications, the *lux* module controlled expression of CRISPRi components to dynamically down-regulate endogenous pathways that compete for availability of a production pathway precursor [36] and the *esa* module was used to delay expression of heterologous pathway genes to overcome enzyme inhibition [37,38] and product toxicity in the naringenin and salicylic acid pathways, respectively. Implementation of one and two layers of dynamic regulation in the naringenin pathway resulted in an 8-fold and 16-fold titer increase compared to the static system, respectively, and dual-regulation in the salicylic acid pathway resulted in a 2-fold increase over the static case. In both pathway contexts, product titers were highly dependent on the switching time of both modules, confirming the importance of tunability in some contexts.

These early applications of layered dynamic regulation have shown that control of two or more metabolic fluxes can result in significant production improvements by addressing two pathway limitations. These studies show three approaches for bifunctional regulation which primarily differ in the extent to which the two regulation modes are coupled. Fully- or semi-orthogonal control schemes are necessary for achieving the optimal production in some pathways, but since there is a trade-off between simplicity and tunability, the combination of modules should be chosen to suit the application.



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161 **Figure 1.** Illustration of layered control methods. (**A**) Example of a regulation scheme that employs layered dynamic
162 control. The first regulation module (green box) controls flux through the production pathway to delay intermediate
163 and product formation. The second regulation module (red box) regulates consumption of a pathway precursor
164 (triangle) by endogenous pathways. (**B**) Response curves for three different circuits – A, B, and C. In each circuit, the
165 response curve shifts based on the expression level of circuit components such as the transcription factor (or synthase
166 for the signaling molecule in QS circuits). Circuits A and B have different transcription factors, but share a signaling
167 molecule. Circuit C has a unique transcription factor and signaling molecule. (**C**) Possible combinations of switching
168 times (shaded gray) with a fully coupled regulation system in which both modes are under control of the same circuit.
169 (**D**) Possible combinations of switching times with a semi-orthogonal bifunctional regulation system in which the
170 regulation modes respond to the same signaling molecule, but have unique transcription factors. (**E**) Possible
171 combinations of switching times with a fully orthogonal bifunctional regulation system in which the two regulation
172 modes are controlled under circuits with different transcription factors and signaling molecules.

173 **Table 1.** Summary of layered dynamic control strategies

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Target product	Circuit type(s)	Target gene(s) and response	Outcome	Reference
Fatty acid ethyl ester	Fatty acid/acyl-CoA biosensor	*pdc, adhB, atfA, fadD* ON	3-fold yield increase over inducible system from previous study [39]	17
Amorphadiene	Stress-response	*ADS* ON + FPP-production pathway OFF	2-fold titer increase over inducible or constitutive promoters	25
Fatty acids	Malonyl-CoA biosensor	*tesA, fabADGI* ON + *accADBC* OFF	2.1-fold titer increase over no-regulator control	3
Isopropanol	QS circuit	*gltA* OFF + *thlA, atoAD, adc, adhE* ON	3-fold titer increase over no-QS control	14
para-hydroxybenzoic acid	QS circuit	*ARO4, ubiC, TKL1* ON + *CDC19, ARO7, ZWF1* OFF	37-fold titer increase over no-ON or -OFF control	23
Glucaric acid	QS circuit + myo-inositol biosensor	*pfkA* OFF (QS) and *MIOX* ON (biosensor)	4-fold titer increase with OFF + additional 2-fold titer with ON	18
Muconic acid	Muconic acid biosensor	*entC, pchB* ON + *ppc* OFF	3.7-fold titer increase with ON + additional 1.6-fold titer increase with OFF	26
Naringenin	*lux* + *esa* QS circuits	*TAL, 4CL* ON (*esa*) + *fabF, fabB, adhE, sucC, fumC* OFF (*lux*)	8-fold titer increase with ON + additional 2-fold titer increase with OFF	22
Salicylic acid	*lux* + *esa* QS circuits	*entC, pchB* ON (*esa*) + *pheA*, *tyrA* OFF (*lux*)	2-fold titer increase over static expression	22

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Multi-input control methods

While the autonomous methods discussed so far have resulted in meaningful improvements in a number of production pathways, some situations may benefit from regulation dynamics that cannot be achieved by one-input control circuits, motivating exploration of multi-input control. Additionally, multi-input circuits are a promising method of achieving pathway-independent control with non-monotonic response profiles. This section discusses two studies that improve response dynamics [27,28] and one study that achieves a specific and non-monotonic response profile [29] using multi-input circuits (Table 2).

With the goal of developing a control circuit that responds to both cell density and cell physiological state, He et al. constructed an AND gate composed of a QS circuit and a stationary phase sensor, both of which turn from OFF to ON during fermentations [28]. The AND gate is based on a circuit that requires expression of two transcriptional activator genes, *hrpR* and *hrpS* to activate expression from the P_{hrpL} promoter [40]. Regulation of *hrpR* under the QS promoter (P_{QS}) and *hrpS* under the stationary phase promoter (P_{SP}) results in transcription of the P_{hrpL} promoter only when both cell density and physiological state requirements are met (Figure 2A). Control of the polyhydroxybutyrate (PHB) production pathway under the P_{hrpL} promoter resulted in improved growth characteristics over statically-induced and QS-only controls and resulted in a greater-than two-fold improvement in PHB production over QS-only, stationary-phase-only, and static controls.

Lo et al. applied an alternative multi-input circuit architecture that assembles nutrient starvation and substrate-sensing circuits in a layered manner [27]. The first layer of the circuit controls expression of the gene encoding the first pathway enzyme under the P_{csiD} promoter (P_{CS}), which is upregulated under carbon starvation conditions [41,42]. The second layer of the circuit employs a biosensor for the product of the first conversion regulated under the first layer to control expression of the rest of the pathway. While the enzyme-biosensor pair must suit the production pathway, the authors of this study showed that one enzyme-biosensor pair could be used to produce five different compounds by changing the substrate and down-stream production pathway genes. They showed significant growth and titer improvement under two different enzyme-biosensor pairs

compared to nutrient sensor-only and static controls. Both studies demonstrate the benefit of multi-input control dynamics in some pathway contexts.

While studies have shown improved production when a key metabolic node is regulated in a reversible manner [3,43], pathway-independent control circuits discussed so far are only capable of producing monotonic responses. To develop a pathway-independent method for addressing these situations, Moser et al. characterized pathway-independent circuits that respond to changing glucose, oxygen, and acetate levels. These circuits were applied to reducing acetate production while limiting growth effects by assembling multi-input circuits that express silencing components for the key acetate-production pathway genes, *poxB* and *pta*. The authors aimed to designed the circuits to activate transcription of the silencing components during periods of *poxB* and *pta* transcription. Based on time-resolved transcript data for *poxB* and *pta*, the authors used computational models of the characterized circuits to generate predictions of multi-input circuits that would match the transcription profiles of the target genes. Characterization of the predicted circuits (Figure 2C) resulted in non-monotonic activation profiles consistent with the target profiles and significantly reduced acetate generation.

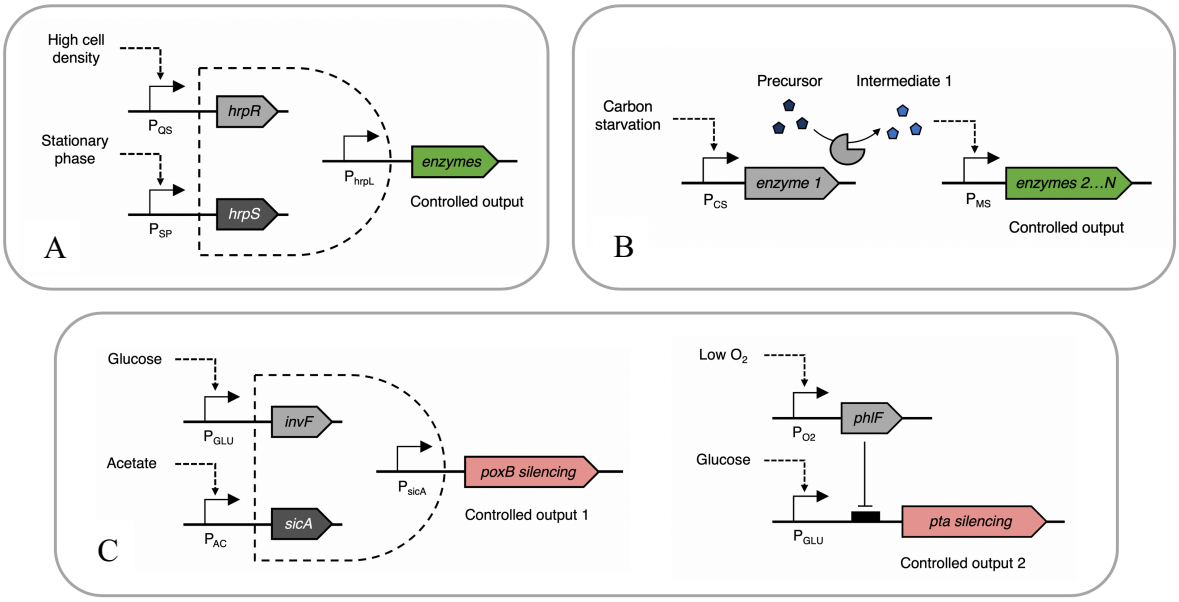

Figure 2. Illustrations of two multi-input control circuits which actuate a response in the presence of two input signals. (**A**) AND gate in which the target gene is expressed when both cell density and stationary phase thresholds are satisfied. The QS promoter (P_{QS}) and the stationary phase promoter (P_{SP}) control expression of the transcription factor genes encoding HrpR and HrpS, both of which are required to activate expression from a third promoter (P_{hrpL}). (**B**) Two-layer regulatory circuit which expresses a target gene when both carbon starvation and precursor requirements are met. The carbon starvation promoter (P_{CS}) controls expression of a gene that encodes an enzyme that produces the metabolite (Intermediate 1) that triggers activation from the metabolite-responsive promoter (P_{MS}). P_{MS} controls expression of the rest of the pathway genes. (**C**) Two multi-input circuits constructed to decrease acetate production. The genes for *poxB* silencing are expressed during high glucose and high acetate conditions by regulation under the P_{GLU} and P_{AC} promoters, respectively. The genes for *pta* silencing are expressed during high glucose oxygen conditions by regulation under P_{GLU} and P_{O2} promoters, respectively.

Table 2. Summary of multi-input control strategies

Target product	Circuit types	Target genes	Outcome	Reference
Vanillic acid	Glucose sensor and hydroxycinnamic acid biosensor	*fcs*, *ech*, *vdh*	5-fold productivity increase over constitutive control	27
Ethyl oleate	Glucose sensor and oleic acid biosensor	*fadD*, *pdc*, *adhB*, *aftA*	2.4-fold productivity increase over single-input inducible control	27
Polyhydroxybutyrate	QS and stationary phase	*phbCAB*	1-2-fold titer increase over QS or stationary phase only	28
Acetate reduction	Glucose, acetate, and oxygen sensors	*poxB*	2-fold reduction in acetate accumulation (mM)	29
Acetate reduction	Glucose, acetate, and oxygen sensors	*pta*	4-fold reduction in acetate accumulation (mM)	29

Conclusions and future outlook

In recent years, layered and multi-input autonomous control schemes have been designed, constructed and implemented to control metabolic fluxes. These autonomous tools have shown promise in early studies. As production pathways and control schemes become increasingly complex, co-culture production becomes a more attractive option for distributing burden between population members or segregating incompatible pathway or circuit components [44–49]. Autonomous control circuits to coordinate cellular behavior between sub-population members [50] and regulate co-culture composition [50] are expected to play a significant role in improving control of co-culture systems.

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Authors employed QS circuits to control the growth rate of a co-culture sub-population and constructed a model to predict the influence of certain parameters on co-culture composition.