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Current and future modalities of dynamic control in metabolic engineering

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Metabolic engineering aims to maximize production of valuable compounds using cells as biological catalysts. When incorporating engineered pathways into host organisms, an inherent conflict is presented between maintenance of cellular health and generation of products. This challenge has been addressed through two main modalities of dynamic control: decoupling growth from production via two-phase fermentations and autoregulation of pathways to optimize product formation. However, dynamic control can offer even greater potential for metabolic engineering through open-loop and closed-loop control modalities of the production phase. Here we review recent applications of dynamic control strategies in metabolic engineering. We then explore the potential of integrating biosensors and computer-assisted feedback control as a promising future modality of dynamic control.

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Introduction

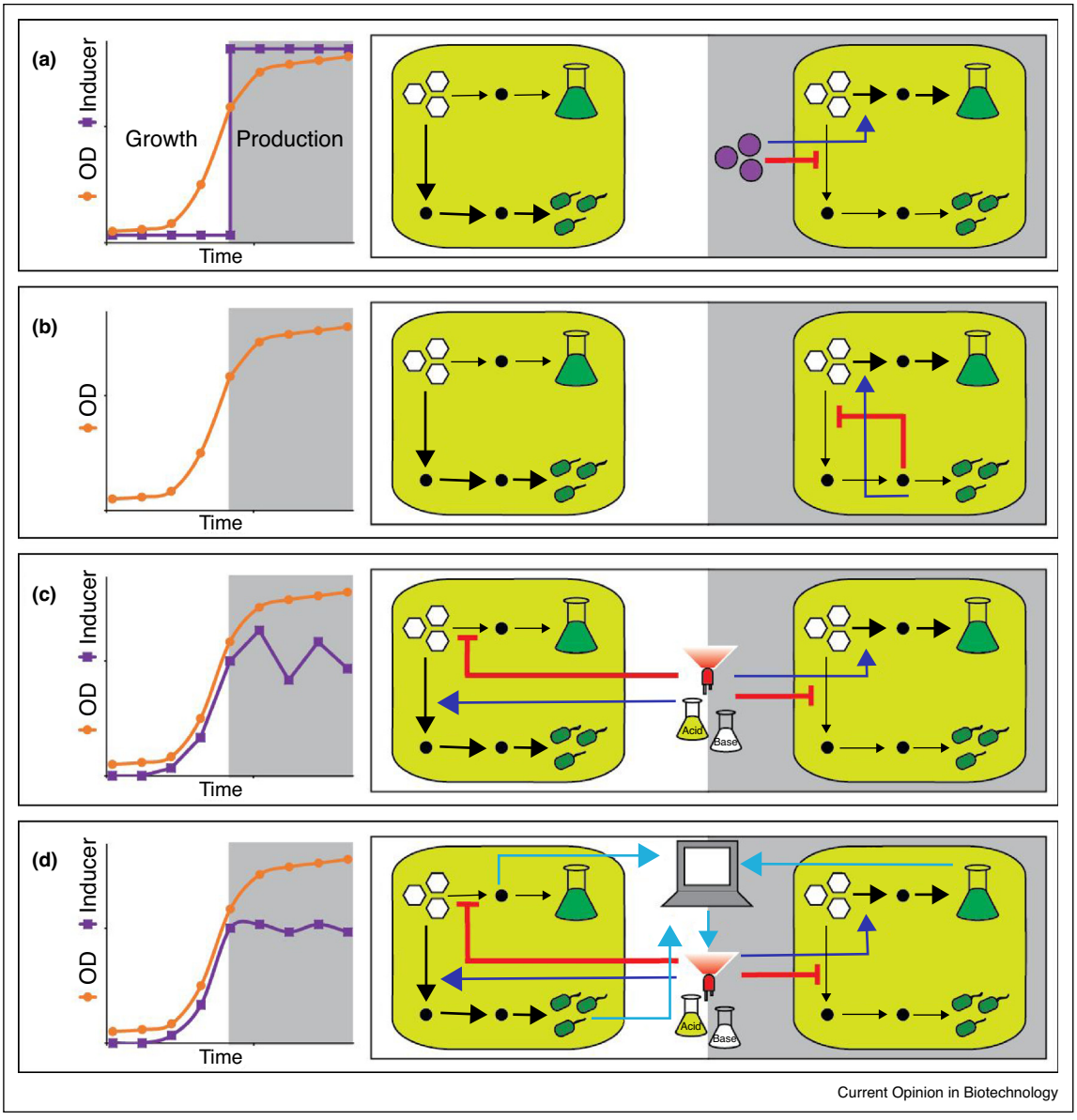
Metabolic engineering uses cells, most commonly microorganisms, as biocatalysts for the production of biofuels, therapeutics, and commodity chemicals. Organisms have successfully been engineered to produce valuable products that are otherwise difficult to obtain, such as plant secondary metabolites [1]. However, maximizing flux towards non-native pathways places tremendous burden on a strain by draining resources from endogenous metabolism. Such engineering can lead to growth defects and loss of production, which is exacerbated if the final product or pathway intermediates are toxic [2].

Prioritization of both growth and production can be invaluable in achieving industrially viable yields and titers. This can be accomplished through dynamic control: genetically modifying an organism to shift its metabolism using an inducing agent [3,4]. Dynamic control has most commonly been implemented in a modality that splits fermentations into two phases: a growth phase to cultivate microbial cultures to high cell densities, followed by a production phase in which heterologous pathways are expressed (Figure 1a). The shift from growth to production has been controlled using a variety of strategies, most commonly chemical inducers [5,6•]. Other systems use genetically encoded autoregulation programs, in which the intracellular concentrations of key metabolic intermediates dynamically control the expression of enzymes to shift flux at key metabolic branch points during the fermentation without human input [7,8••].

Although two-phase fermentations and autoregulation have greatly benefited metabolic engineering, both have limitations. Two-phase fermentations give the user control over when growth is shifted to production, but act as step functions with limited ability for further optimization once the inducer is added (Figure 1a). While autoregulation addresses this issue, it shifts control of the pathway away from the human operator to the microbe (Figure 1b), thereby preventing process corrections should the need arise. One offers control but limited dynamism; the other, dynamic regulation but limited control. The full potential of dynamic control can only be realized through combining the two: continuous, rapid, tunable, and user-controlled regulation.

Two recent reviews have excellent discussions on the mechanisms of two-phase regulation and autoregulation [9,10]. In this review, we discuss dynamic control in the context of the current and future modalities in which it can be applied to metabolic engineering (Figure 1). We first cover strategies for inducible two-phase control of metabolic valves. Next, we analyze examples of control within the production phase via pathway autoregulation. Lastly, we discuss the potential of new dynamic control modalities involving open-loop (Figure 1c) and closed-loop (Figure 1d) controls for metabolic engineering.

Figure 1



Different modalities for dynamic control in metabolic engineering. **(a)** Two-phase fermentation, in which an external inducing agent is used as a step function to switch from growth to production. **(b)** Autoregulation of metabolism, using intracellular regulatory strategies to separate growth from production without human input. **(c)** Open-loop control of production, allowing user-mediated dynamic regulation of metabolism. **(d)** Closed-loop control of production, using system output and feedback algorithms to assist user input. Light blue arrows represent system outputs (e.g. growth, product and intermediate concentrations) that are used to determine feedback response.

Two-phase fermentation dynamic control modality: step function to switch from growth to production phase

The predominant application of dynamic control in metabolic engineering has been as a binary switch: cultures are grown to certain cell densities, then induced (most commonly with chemicals) to activate production pathways that would otherwise slow growth. Recent metabolic engineering studies utilizing common inducing agents are listed in Table 1.

Chemicals and nutrients comprise the most common inducers used to determine the growth and production phases of fermentation. An excellent review article describes several chemical induction systems for regulation of metabolism in *Escherichia coli* [29]. In particular, anhydrotetracycline (aTc) and isopropyl β-D-1-thiogalactopyranoside (IPTG) are common inducers that have been utilized to improve production of compounds such as isopropanol, anthocyanin, malate, 1,4-butanediol [11,14–16], and many others. The carbon source, which

Table 1

Induction strategies used to separate growth and production phases for metabolic engineering in *E. coli* and *S. cerevisiae*

Inducer	Organism	Control node	Control strategy	Product	Improvement	Reference
aTc	*E. coli*	*gabD*, *ybgC*, *tesB*	CRISPRi	1,4-BDO	~2-fold (titer)	[11]
aTc	*E. coli*	Pfk	SspB	Glucaric acid	42% (titer)	[12]
aTc	*E. coli*	Pfk	SspB	Myo-inositol	2-fold (titer, yield)	[13]
Doxycycline	*S. cerevisiae*	*HXK1*	P_{7xtetO}	Gluconate, Isobutanol	50-fold (yield), 3-fold (yield)	[6•]
IPTG	*E. coli*	*metJ*	CRISPRi	P3G	~21-fold (titer)	[14]
IPTG	*E. coli*	*gltA*	CRISPRi	Isopropanol	3.7-fold (titer), 3.1-fold (yield)	[15]
IPTG/aTc	*E. coli*	*PC, CS, ACN, ICL, MS*	CRISPRi	Malate	2.3-fold (titer)	[16]
Arabinose	*E. coli*	*vioABCE*	*araBAD*	Deoxyviolacein	5-fold (titer)	[17]
Galactose/ methionine	*S. cerevisiae*	*ERG9*	P_{GAL1}, P_{MET3}	Amorphadiene	3–6-fold (titer)	[18]
Galactose/glucose	*S. cerevisiae*	*ERG9*, *tHMG1*, *CrtE*, *CrtYB*, *CrtI*	P_{HXT1}, $P_{GAL1/10}$	Carotenoids	1156 mg/L	[19]
Methionine/copper	*S. cerevisiae*	*ERG9*	P_{CTR3}, P_{MET3}	Artemisinic Acid	10-fold (titer)	[20]
Methionine	*S. cerevisiae*	*ERG9*	P_{MET3}	Sesquiterpenes	Various	[21–23]
β-Estradiol	*S. cerevisiae*	*CrtE*, *CrtB*, *CrtI*, *CrtY*, *CrtZ*	P65-Gal-ER, modified P_{GAL10}	Zeaxanthin	50-fold (titer)	[24]
Temperature	*E. coli*	*ldhA*, *icd*	pR, pL	D-Lactate, itaconic acid	122.8 g/L, 48% (productivity)	[25,26]
Oxygen	*E. coli*	Various production genes	*nar*	D-Lactate, 2,3-BDO, 1,3-PDO	Various	[27•]
pH	*S. cerevisiae*	*ldhL*	P_{YPG1}, P_{CCW14}	Lactic acid	2.9–7.9 g/L	[28]

heavily regulates metabolism and gene expression profiles, presents another popular induction strategy. For instance, the *araBAD* promoter system in *E. coli*, which allows for tight and tunable transcriptional induction via L-arabinose, has been used to initiate the production phase of fermentations. Because *E. coli* can metabolize L-arabinose as a carbon source, Rodrigues *et al.* engineered a strain to prevent arabinose catabolism and stabilize induction, achieving five-fold improvement in deoxyviolacein titers [17].

In contrast to *E. coli*, inducible systems in the baker's yeast *Saccharomyces cerevisiae* primarily use specific carbon sources, nutrients and ions to control transcription. The galactose-activated and glucose-repressed *GAL1* and *GAL10* promoters have been widely used to induce heterologous pathways, following growth phases in glucose, by switching to galactose-containing media [18–20]. Nutrients can also be used to control gene expression. For instance, methionine, which represses genes under control of the P_{MET3} promoter, has been ubiquitously used for controlled inhibition of *ERG9* to direct farnesyl pyrophosphate (FPP) flux away from sterol biosynthesis [18,21–23]. Another commonly used inducer in yeast is copper (II) ion, which activates or represses transcriptional expression via the native yeast promoters P_{CUP1} and P_{CTR3}, respectively [20,30•].

Exogenous chemicals have also been developed for dynamic control in yeast. For example, using the Tet-Off system, which enables transcription only in the absence of tetracycline antibiotics, Tan *et al.* overproduced gluconic acid and isobutanol by redirecting glucose flux away from central carbon metabolism via repression of *HXK1* [6•]. Similarly, Liang *et al.* fused the native *GAL4* DNA-binding domain, an estrogen receptor, and the p65 activation domain of human NF-κB to create a tightly regulated system inducible by β-estradiol. Using this inducible system, they achieved 50-fold improvements in production of zeaxanthin over use of constitutive promoters [24].

Other induction strategies have focused on manipulating culture conditions to regulate metabolism. In *E. coli*, growth and production have been decoupled by changing the fermentation temperature. For example, Zhou *et al.* used the temperature-sensitive λ promoters p_R and p_L to downregulate production of lactate (a growth inhibitor) during an initial growth phase at 33 °C, then turned on production at 42 °C, leading to titers of 122.8 g/L D-lactate [25]. Using the same promoters at lower

temperatures, Harder *et al.* attained fast growth at 37 °C, then downregulated the TCA cycle at 28 °C by repressing isocitrate dehydrogenase (*icd*) with the repressor *C1857* to produce 47 g/L of itaconic acid [26]. *E. coli* metabolism has also been regulated through dissolved oxygen (DO), using the oxygen-sensitive *nar* promoter. Hwang *et al.* split fermentations into aerobic and microaerobic stages by controlling rotation speed. In doing so, they achieved titers of lactate, 2,3-butanediol, and 1,3-propanediol that were comparable to those obtained in optimized strains using chemical induction [27•]. In *S. cerevisiae*, Rajkumar *et al.* developed a system to regulate metabolism by inducing gene expression at low pH, using modified P_{YGP1} and P_{CCW14} promoters with binding sites from the stress-responsive transcription factors Msn2/Msn4p, Rlm1p, and Swi4p. These synthetic promoters were then applied in low-pH fermentations to improve production of lactic acid [28]. Since temperature, oxygenation, and pH are much more reversible than chemical inducers, they provide flexibility for implementation of more complex dynamic control strategies.

Development of dynamic control tools in organisms other than *E. coli* and *S. cerevisiae* represents an intriguing area for future exploration. For example, aTc, IPTG, and arabinose-inducible expression systems from *E. coli* have successfully been imported to *Corynebacterium glutamicum*, a commonly used organism for amino acid [31] and organic acid production [32,33]. In addition, promoter systems that are regulated by erythritol/oleic acid and glycerol/glucose have recently been developed in the oleaginous yeasts *Yarrowia lipolytica* and *Pichia pastoris*, respectively [34–36]. These studies offer promise for expanding dynamic control in other industrially relevant organisms.

Autoregulation modality: cell-mediated control of the production phase

Dynamic control can also be implemented without the use of external inducers by genetically engineering autoregulation programs within metabolic pathways, thereby mimicking endogenous pathway regulation. Several studies have taken advantage of native regulatory systems to autonomously balance metabolic flux between growth and production [37,38]. One of the first examples of autoregulation in metabolic engineering was demonstrated by Farmer and Liao, who developed a system to autonomously control *pps* and *idi* in *E. coli* using the acetyl-phosphate responsive promoter AcP [7]. In doing so, excess carbon flux that would normally be used for acetate production was instead shunted towards lycopene production without inhibiting cell growth. This strategy showed that by controlling key metabolic nodes, dynamic autoregulation can often outperform constitutive overexpression.

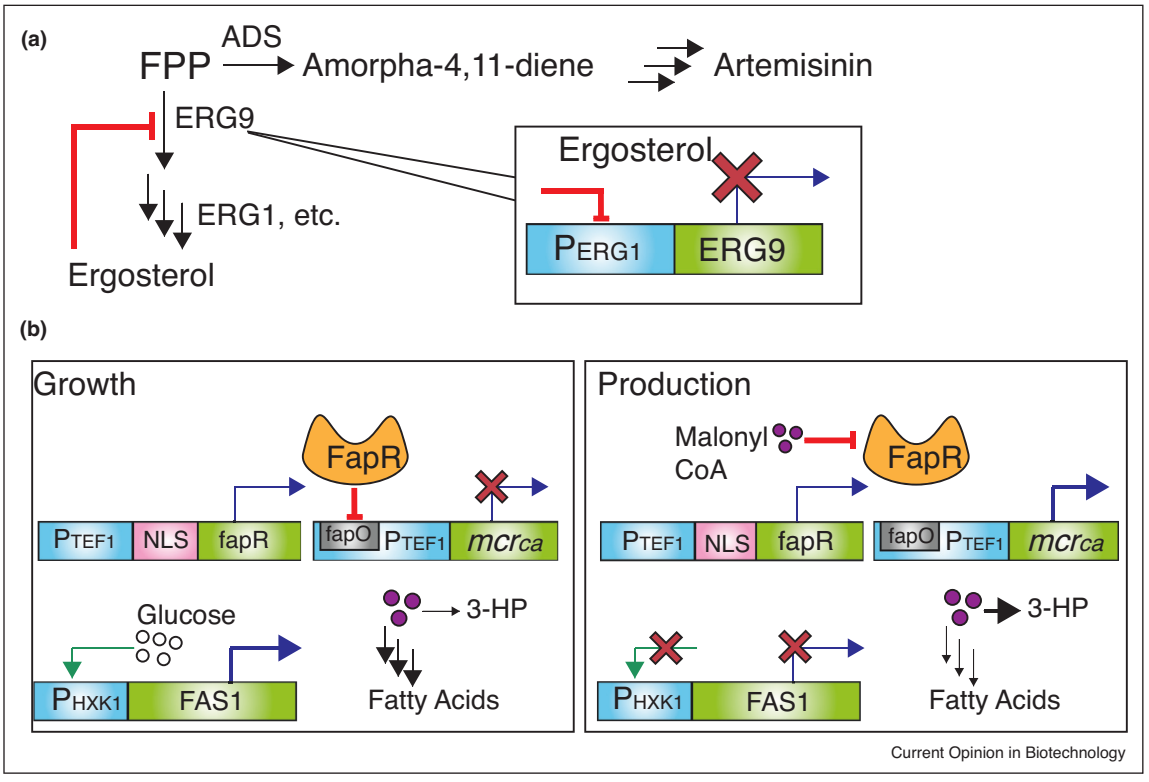
Dynamic control via autoregulation has been implemented using a variety of auto-induction strategies. A common method is using carbon source responsive promoters which automatically tune gene expression between growth (high sugar concentration) and production (low sugar concentration) phases. For instance, in *S. cerevisiae*, the hexose transporter promoters P_{HXT1} and P_{HXT7}, which are activated and repressed by glucose respectively, have been effectively used to temporally regulate gene expression. These promoters have been used to make products such as fatty alcohols and α-santalene autonomously by allowing regular growth in glucose-containing media, then automatically turning on production genes as glucose runs out to convert glucose and ethanol in the media to desired product [30•,39]. Similarly, the P_{ADH2} promoter has been used in autoregulation programs for production of polyketides and triacetic acid lactone [40,41]. This promoter is also repressed by glucose, keeping production pathways turned off during the growth phase; then, after the glucose is consumed, the P_{ADH2} promoter is derepressed and induced by the ethanol produced during the growth phase via glucose fermentation. Other glucose-repressed promoters such as P_{SSA1} and P_{SUC1} offer intriguing alternatives for autoregulation in yeast [42•,43•]. Auto-induction media for *E. coli* follows a similar principle, using regulation of the *lac* operon by glucose and lactose to delay gene expression until glucose is sufficiently consumed [44].

Another strategy to implement metabolic autoregulation is by engineering quorum sensing systems that are regulated by cell density. Recently, Gupta *et al.* imported into *E. coli* the Esa quorum sensing system from *Pantoea stewartii*, which downregulates genes under the P_{esaS} promoter in the absence of 3-oxohexanoylhomoserine lactone (AHL). By varying repression of P_{esaS} through expression of AHL synthase EsaI, gene circuits were designed that allowed knockdown of target genes *pfkA* and *aroK*, which are essential but compete with production. In this way, dynamic autonomous repression of *pfkA* and *aroK* led to significant improvements in production of myo-inositol, glucaric acid, and shikimic acid [45]. An autoregulation system was also developed in *S. cerevisiae* by Williams *et al.* in the form of a genetic AND gate that is activated by quorum sensing and aromatic amino acids [46]. At high cell densities and in the presence of tryptophan, α-pheromone is expressed from the P_{ARO9} promoter and arrests cell growth. At the same time, heterologous production genes are expressed from the quorum sensing-induced P_{FUS1J2} promoter, while an imported RNAi system downregulates competing endogenous genes *ARO7* and *CDC19*. This led to a 37-fold improvement in production of para-hydroxybenzoic acid (PHBA), demonstrating the efficacy of dynamic translational control using an autoregulated system (quorum sensing) that acts in concert with user-mediated induction (with an aromatic amino acid).

Changes in intracellular metabolite concentrations can also be used to drive engineered autoregulation systems. In one study in *S. cerevisiae*, Yuan and Ching controlled the *ERG9* gene, which drives FPP towards ergosterol, using the P_{ERG1} promoter within the ergosterol pathway (Figure 2a) [47••]. This promoter is downregulated in the presence of ergosterol; by engineering product inhibition into the pathway, sufficient ergosterol is produced to maintain health while excess flux is diverted towards amorpha-4,11-diene. Titers were improved 2–5 fold using this strategy, demonstrating that native regulatory elements can be successfully used to downregulate essential pathways that compete with the product of interest. In another example, David *et al.* imported a malonyl-CoA-regulated transcriptional repressor, FapR, from *Bacillus subtilis* into *S. cerevisiae*, and inserted its binding sites into the constitutive P_{TEF1} promoter to design a malonyl-CoA activated promoter (Figure 2b) [8••]. This system was used to autoregulate the *Chloroflexus aurantiacus* malonyl-CoA reductase (mcr_{ca}), producing 3-hydroxypropionic acid (3-HP) in the presence of excess malonyl-CoA. At the same time, fatty acid synthase *FAS1*, which is essential for growth but competes with product formation, was autoregulated by glucose levels using the P_{HXK1} promoter to allow flux towards fatty acid synthesis during growth but repress it in the production phase. By simultaneously autoregulating 3-HP production and malonyl-CoA concentrations, it was possible to produce 0.8 g/L of 3-HP (Figure 1b). This study effectively combines external carbon source-mediated two-stage fermentation with autoregulation of an intracellular metabolite to increase production of a valuable molecule.

Autoregulation in metabolic engineering has not been limited to *E. coli* and *S. cerevisiae*. Yin *et al.* utilized a low-pH-induced promoter, P*gas*, to regulate *Aspergillus niger* fermentations, which turn acidic over time [48]. Using P_{gas} to autoregulate expression of *CAD*, which encodes for an enzyme that converts cis-aconitate to itaconate, led to a 5.3-fold improvement in itaconate titers. In another example, Le *et al.* performed transcriptomic analyses of CHO cells to identify the dynamic Txnip promoter, which is upregulated as a culture reaches stationary phase. By using this promoter to drive expression of the fructose transporter GLUT5, the CHO cells were able to consume fructose in stationary phase, reducing formation of lactate, which hinders the production of protein therapeutics. Finally, Zhou and Zeng have developed riboswitches activated and repressed by L-lysine for *Corynebacterium glutamicum*. Using these riboswitches to autoregulate *gltA*

Figure 2



Genetic circuits for autoregulation of gene expression. **(a)** The P_{ERG1} promoter is used to autoregulate ergosterol biosynthesis through ergosterol-mediated downregulation of *ERG9*, which competes with isoprenoid production [47••]. **(b)** The bacterial transcriptional repressor FapR, which is inactivated by malonyl CoA, was engineered into yeast to control expression of mcr_{ca}, in combination with glucose-mediated control of *FAS1* using the P_{HXK1} promoter to autoregulate malonyl-CoA flux between essential fatty acid biosynthesis and 3-HP production [8••].

(repressed by L-lysine) and *lysE* (activated by L-lysine), encoding citrate synthase (entry into the TCA cycle) and L-lysine export genes respectively, improved L-lysine yields by 63% and 21% [49,50]. These examples highlight the potential for further development of autoregulation systems in less commonly used host organisms.

Potential of open-loop and closed-loop modalities for dynamic control of the production phase of fermentation

Continuous user-inputted or computer-inputted control using open-loops or closed-loops is potentially a powerful feature of dynamic control for metabolic engineering. This would allow for continued optimization within the production phase, while maintaining the ability to adjust fermentation conditions and respond to perturbations. Current implementations of two-phase fermentations act as step functions that cannot be readily modified once switched to the production phase (Figure 1a), while autoregulation of pathways takes control entirely out of the hands of the user (Figure 1b). One obstacle to achieving user-inputted or computer-inputted control of the production phase of fermentation is that it is difficult to reverse signals from chemical inducers due to their persistence in the media, which limits their use in temporal control to step functions [29]. A potential solution is using systems inducible by temperature, pH, and oxygenation, which offer more reversibility; however, these systems generally offer little dose-dependent control of expression.

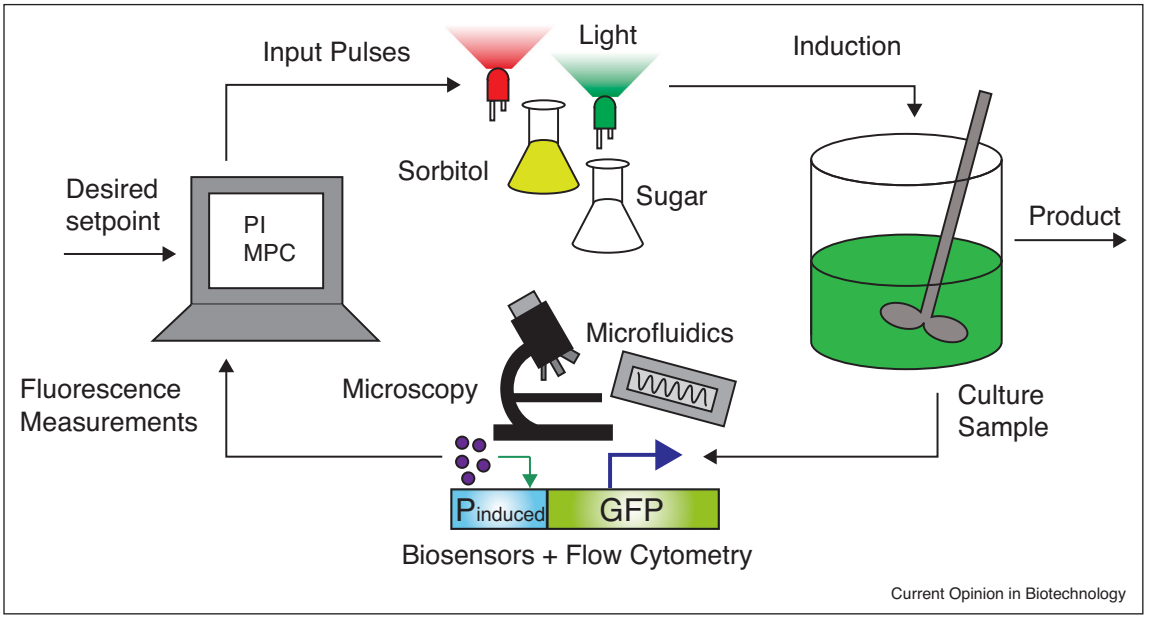
An enticing possible solution is to use optogenetics: light-mediated control of gene expression [51,52]. Using optogenetics to control expression levels of key metabolic enzymes would allow open-loop and closed-loop controls of engineered metabolic pathways using light inputs. Light has several advantages. It can be instantaneously and reversibly applied in highly tunable doses determined by either light intensity or pulse frequencies. Light is also relatively inexpensive, and has minimal toxicity and off-target effects. However, before optogenetics can be applied to metabolic engineering, the limitations in light penetration imposed by high cell densities need to be understood and resolved. Promising new technologies, involving highly light-sensitive proteins and optogenetic circuits, allow for robust and homogeneous gene expression in fermentations of at least 50 OD_{600} in 5-L bioreactors [53••]. Optogenetic systems stimulated by red [54], green [55,56], and blue [52,53••,57] light could be used simultaneously for orthogonal multichromatic control, provided that cross-talk between the optogenetic systems is sufficiently minimized. Optogenetics offers a powerful induction strategy for dynamic control in metabolic engineering.

Dynamic open-loop control during the production phase of fermentation represents a promising new frontier for optimization of chemical production [53••]. However, a potential drawback of open-loop control is that there is no active feedback to determine if the operation is on track to attaining the desired outcome, thus requiring trial and error to find optimal operating conditions or the development of computer models for each specific strain. This problem can be addressed in the form of computer-assisted automated control to regulate gene expression using real-time output measurements to provide feedback to control inputs (Figure 3). Several groups have demonstrated computer-assisted feedback regulation of gene expression. However, this technology has thus far only been used in proof-of-concept-studies that showcase dynamic control of cell growth or fluorescent reporters, and not yet to metabolic engineering. Nevertheless, real-time control of cellular metabolism offers tremendous potential for metabolic engineering applications, such as to prevent the accumulation of toxic intermediates or depletion of essential metabolites.

The choice of algorithm for feedback control depends on the complexity of the system. For simple pathways or maintenance at a static set-point, computationally inexpensive Proportional-Integral (PI) control would likely be sufficient. However, Model Predictive Control (MPC) or other advanced control algorithms may be needed to implement dynamic signal tracking or regulate complex systems [58]. While these algorithms are more computationally expensive, they are necessary to accurately capture the dynamism of metabolic pathways of interest, which contain complex regulation networks and thus exhibit highly nonlinear behaviors.

So far, three induction strategies have been used for *in silico* feedback control in microbial systems. Several studies in *S. cerevisiae* use fluorescence measurements from a reporter driven by P_{GAL1} to controls feed rates of media containing either glucose or galactose [59••,60,61]. In another study, Uhlendorf *et al.* controlled osmotic pressure in yeast using MPC to add media containing concentrated sorbitol [62]. In this system, osmotic stress induced the high osmolarity glycerol (HOG) signaling cascade, including a fluorescent reporter engineered under the control of the P_{STL1} promoter. Other groups have employed optogenetic dimerizing systems to regulate expression of a fluorescent protein from the P_{GAL1} promoter: the red/infrared responsive PhyB-PIF3 with MPC control [63], or the blue light responsive CRY2-CIB1 using bang-bang (on-off) control [64]. Feedback control of optogenetic systems has also been demonstrated in mammalian cells to clamp fluorescent protein expression at desired set points via PI control [65], as well as to dynamically track mathematical functions in *E. coli* [56]. Recently, Milias-Argeitis *et al.* demonstrated, for the first time, optogenetic feedback control of microbial growth rates by regulating expression of the *metE* auxotrophic marker in *E. coli*, using PhyB-PIF3 [66]. This

Figure 3



Integration of computer-assisted feedback control algorithms with *in vivo* processes for dynamic regulation of fluorescence. Culture samples are measured for fluorescence outputs, which are fed to a chosen algorithm. Based on the difference between the readings and the desired set-point, the algorithm returns input pulses which are used to induce the culture and minimize the output offset. PI, Proportional-Integral; MPC, Model Predictive Control; ZAD, Zero Average Dynamics.

study utilized both PI and MPC control, finding that PI control was sufficient for growth control due to the slow dynamics, whereas MPC control was necessary for accurate dynamic signal tracking of GFP fluorescence.

For rapid and accurate feedback control, it is essential to have easily and frequently measurable system outputs. The studies above implemented dynamic control through tracking fluorescent reporters either via flow cytometry or microscopy imaging using microfluidic devices (Figure 3). For metabolic engineering applications, this strategy can be adapted to use fluorescent reporters controlled by genetically encoded biosensors of specific products, precursors, byproducts, or cofactors, allowing user-operated or computer-operated closed-loop controls of engineered metabolic pathways [67,68]. This represents a challenging but exciting frontier in metabolic engineering.

Future outlooks

Endogenous metabolic networks are naturally regulated at the DNA, RNA, and protein levels to provide tight and robust control, yet most control exerted in metabolic engineering has predominantly relied on transcriptional regulation. In particular, developing expansive sets of post-translational tools to control enzymes would significantly boost dynamic control and optimization of engineered metabolic pathways. In *E. coli*, inducible ClpXP-mediated proteolysis has been used to improve titers of glucaric acid and myo-inositol [12,13]. While post-translational control has thus far not been implemented in yeast for metabolic engineering, the development of degrons highlight the potential for future progress [69–72].

Dynamically regulated gene circuits are another research area with great potential for metabolic engineering. For example, the GAL expression system in yeast controls metabolism of galactose through a transcriptional activator Gal4p, a repressor Gal80p, and sensor Gal3p. Interactions between these proteins have been used to create galactose-regulated gene circuits [73]. These circuits could provide powerful metabolic engineering tools, such as amplified expression of bottleneck enzymes or inverted activation/repression of competing pathways. Sets of promoters with inverted induction/repression responses, such as the aforementioned P_{HXT1}/P_{HXT7} and P_{CUP1}/P_{CTR3} in yeast, could provide additional opportunities for deployment of orthogonally inducible gene circuits.

Conclusion

Dynamic control has long served as a critical component of the metabolic engineering toolbox. Two-phase fermentations and autoregulation have been used ubiquitously in metabolic engineering to achieve impressive improvements in titers and yields of valuable products. However, there remains significant potential to bridge the current gaps that remain between ‘dynamic’ and ‘control’

seen in these two control strategies. This can only be achieved through the development of easily applied and rapidly reversible induction methods. Optogenetic control of gene expression could prove a promising tool for user-mediated dynamic control of the production phase [53••]. In the future, tighter and more robust control may be achieved through computer-aided feedback regulation of fermentations, using genetically encoded biosensors that provide rapid measurements of the state of the system. In our opinion, this seems to be a logical next step forward to achieve full dynamic control in metabolic engineering.

Conflicts of interest

None.

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