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## Control strategies to manage trade-offs during microbial production

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### Abstract

When engineering microbes to overproduce a target molecule, engineers face multiple layers of trade-offs to allocate limited cellular resources between the target pathway and native cellular systems. These trade-offs arise from limited free ribosomes during translation, competition for metabolic precursors, as well as the negative relationship between production and growth rate. To achieve high production performance, microbes need to spontaneously make decisions in the dynamic and heterogeneous fermentation environment. In this review, we discuss recent advances in microbial control strategies that are used to manage these trade-offs and to improve microbial production. This review focuses on design principles and compares different implementations, with the hope to provide guidelines to future microbial engineering.

### Introduction

Microbial fermentation has provided an environment-friendly and versatile platform for manufacturing various bio-based products. Typical fermentation products include alcohols, organic acids, amino acids, vitamins, commodity chemicals, antibiotics, antibodies, and industrial enzymes [1–4]. Recent advances in metabolic engineering and synthetic biology have added an increasing number of products to this list, including fragrances, pharmaceuticals, nutraceuticals, and advanced materials [5–10]. The ability to biologically produce diverse products could have profound societal impacts on multiple industries, only if cost-effective bioproduction can be achieved. This challenge demands the development of effective strategies to improve the production performance (i.e. titers, yields, productivities, and robustness) of engineered microbes.

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When engineering microbes to overproduce a specific molecule at high productivity and yield, one needs to consider multiple layers of trade-offs rather than simply overproduce pathway molecules to the highest level. The first layer stems from ribosomal cost of translating target proteins (Figure 1a). Sequestered ribosomes by mRNAs of target proteins reduce a cell's ribosomal budget to make native proteins for biomass generation and energy synthesis [11–14]. Furthermore, the allocation of limited translational power between multiple modules within a target pathway affects the overall catalytic efficiency of the pathway [15,16]. The second layer is metabolic trade-offs that involve both carbon cost and energy cost (Figure 1b). Conversion of precursor metabolites (e.g. acetyl-CoA) to target products can lead to insufficient material and/or energy supply for the synthesis of cellular structures [17]. Protein synthesis and enzymatic reactions from engineered pathways also consume energy molecules (i.e. ATP and NAD(P) H), which can be otherwise used to support cell growth. Besides, molecules in a target pathway can be toxic to cells, particularly when they are accumulated to high concentrations. Thus a balanced allocation of metabolites and energy molecules is required for optimizing microbial production [18]. The third layer of trade-offs comes between growth rate and product yield. High producers usually have a slower growth rate than low producers. The difference in single-cell growth rate caused by mutations or molecular noise allows low producers to accumulate, lowering overall yields (Figure 1c).

Over the past few years, many genetically encoded control strategies have been developed to improve microbial production by managing these trade-offs [19–23]. These control strategies help engineered cells to adjust their metabolism to combat dynamic and heterogeneous environments in large fermenters as well as stochastic cellular processes. In this review, we discuss recent research advances in control strategies with the focus on design principles. Here, we divide these control strategies into three categories based on their mode of operation: feedback control, two-stage metabolic switch, and population quality control.

## Feedback control

Feedback control can alleviate the impact of ribosomal cost on the expression of specific genes of interest (GOIs). When the expression of GOI is uncontrolled, its protein level decreases as free ribosomes are depleted [14,24]. A common strategy in feedback control is to co-express a regulator with the GOI or to use an antithetic topology, where two components that sequester each other can be designed [25•,26,27]. Alternatively, orthogonal ribosomes can be engineered to feed back to the expression of orthogonal 16S rRNAs and to translate the GOI (Figure 2a) [28••], leading to robust expression of pathway enzymes and steady metabolic flux. Furthermore, *in silico* feedback control can be built using optogenetic regulation to achieve robust gene expression [29]. Additionally, stress-responsive promoters induced by burdensome genes can be used to control the expression of a single guide RNA (sgRNA), which in turn represses the expression of the burdensome gene via dCas9 (Figure 2b) [30•]. This CRISPR-based controller does not require genetic modification to the promoters of target genes, thus can be easily applied in many systems.

Feedback control can also be built to mediate the metabolic costs raised by engineered metabolic pathways. In this case, a metabolite-responsive transcription factor (MRTF) can be used to detect metabolic overflow of an engineered pathway and then downregulate the expression of upstream enzymes, forming a metabolic feedback loop (Figure 2c). Such metabolic feedback controls have been developed for the malonyl-CoA biosynthetic pathway [31,32]. While malonyl-CoA is an essential metabolic precursor for many important pathways, excess malonyl-CoA produced from engineered pathways can inhibit cell growth. By using a malonyl-CoA-responsive transcription factor FapR, feedback controllers allowed excess malonyl-CoA to repress its own synthesis (via acetyl-CoA carboxylase), maintaining its cellular concentration at desirable levels. The feedback-controlled metabolic systems alleviated cellular toxicity otherwise caused by malonyl-CoA accumulation, leading to improved production of downstream metabolites, such as fatty acids [31,32]. Moreover, metabolic feedback loops can accelerate metabolite biosynthesis [33••] and recovery from metabolite depletion [34]. As demonstrated in a feedback-regulated fatty acid pathway, a layered negative metabolic loop can shorten the fatty acid rise-time (the time needed to reach half of the steady-state concentration) by as much as 12-fold [33••]. This negative feedback control topology has effectively accelerated metabolic response that would otherwise cause uncontrollable overproduction if a positive autoregulatory loop is used. In these metabolic feedback controls, the dose-response curve of MRTF can be rationally designed to coordinate the enzyme level and the metabolic flux [35]. If MRTFs are unavailable, stress-response promoters can be used to control the level of toxic intermediates. Stress-response promoters that are downregulated by the toxic intermediate can be used to drive the expression of enzymes leading to the synthesis of the intermediate to prevent its accumulation (Figure 2d) [36].

Several issues need to be considered to construct an effective feedback controller. First, the engineered feedback controller should be compatible with the natural regulatory network. Second, the feedback controller itself should not pose a significant burden on cell growth. Third, the controller should have suitable feedback strength. While too weak feedback can lead to a long delay, too strong feedback can cause overshoot and create noise [33••,37]. Fourth, the regulator should be easily scalable when multiple genes need control. Lastly, most existing feedback controls were developed and validated during exponential growth phase. Effective controls for use beyond exponential growth phase have not been extensively demonstrated and may require additional engineering efforts.

## Metabolic switch in two-stage fermentation

During microbial production, metabolic flux can be spontaneously shifted from growth-related essential pathways to production pathways by a time-dependent trigger signal. Here we refer this strategy as metabolic switch that is often used in two-stage fermentation (Figure 2e). The trigger signal can be a quorum-sensing molecule that indicates the growth phase via cell density. Quorum-sensing metabolic switches have been developed in both *Escherichia coli* and *Saccharomyces cerevisiae*, showing improved production for multiple compounds [38–41]. Attention needs to be paid to avoid the accumulation of quorum-sensing molecules, which may cause problems in downstream purification processes. Beside quorum-sensing, the target product can also serve as the trigger signal. This has been

demonstrated in muconic acid (MA) production with a metabolic switch embedding an MA sensor [42••]. As MA accumulated over time, cells gradually shift to the production phase by diverting flux from pyruvate and oxaloacetate to MA production. Other trigger signals include nutrient concentration [43,44], temperature change [45], and light [46]. For example, the native ergosterol pathway in *S. cerevisiae* was repressed by glucose limitation during the production phase, so that the common precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) can be channeled to terpenoid production in engineered yeast [43,44]. While most metabolic switches control cell growth and pathway activity through transcriptional regulation by affecting promoter activities of target genes, other regulatory mechanisms, such as using RNAi [41,42••], mRNA attenuation [47], and protease-based regulation [48••] have also been developed. Among them, post-translational controls using protease-based regulation have the advantage of remaining functional in stationary phase, during which transcriptional and translation controls may not be effective. On the other hand, protease-based controls are associated with high ATP consumption during proteolysis and may compete with natural proteins for limited proteolysis machinery.

The kinetics of the trigger signal and the behavior of the controllers are essential to design a good metabolic switch. First, it is important to match the response range of the sensor with the concentration of the trigger molecule so that flux can be switched at the right time or growth stage [39]. Second, the promoter controlling the metabolic switch should have strong 'on' and 'off' modes (i.e. ultrasensitivity) [39]. This can be done by incorporating cooperative binding of the regulator at the promoter or using positive autoregulation [42••].

## Population quality control

Another important aspect of microbial production is the relationship between growth and production, which is often negatively related. As a result, low producers, caused either by genetic mutations or molecular noise, grow faster than high producers and tend to dominate the culture over time, lowering overall titers and yields [49•,50–54]. This problem cannot be simply solved by the two types of controls discussed above but can be addressed by population quality control, which aims to alter the growth-production relationship (Figure 2f). Once high producers grow faster than low producers in a population, the overall titers and yields will be improved. Sensor-selector and growth-coupled production are two typical strategies to enable population quality control.

### Sensor-selector

Sensor-selector leverages genetically encoded biosensors to sense intracellular metabolites and regulate genes that confer fitness advantages to cells enriched with the detected metabolites (Figure 2g). The selection can be achieved by controlling the expression of an antibiotic resistance gene, a toxin-antitoxin pair, or an auxotrophic marker [49•,50,55]. Sensor-selector has been traditionally used to select for the best genetic variant from large mutagenic libraries [56–58]. It has also been used to eliminate undesirable genetic mutants formed during long-term production, as shown for improved mevalonic acid production [49•]. Recent advances have exploited it to continuously select for high-producing variants from genetically identical populations. One of the first examples was demonstrated in fatty-

acid producing *E. coli*, where enhanced fatty acid yield from the overall cell culture was achieved via enriching for non-genetic, high-producing cells using a fatty acid sensor-regulator [50]. The effect of selection on non-genetic variation suggests that some traits in the high producers could be epigenetically inherited to their offspring. With sensor-selector, low producers have a slow growth rate and are diminished during the selection, regardless of whether they are genetically different from high producers or not.

Sensor-selector has several limitations, some of which may be overcome by other methods. First, the method can be limited by the availability of a product-specific biosensor [59,60]. Instead of developing a new sensor, which can be challenging by itself, an alternative approach is to use a metabolite-dependent enzyme for auxotrophic selection. For example, a coenzyme B<sub>12</sub>-dependent methionine synthase, which links the end product B<sub>12</sub> to cell growth, has been used to increase production [61]. Second, the cross-membrane transport of the product cannot be faster than the response time of sensing-selection. For example, if product transport is mediated by a membrane protein, strong expression of the transporter may allow low-producing cells to obtain product metabolites from high-producing cells. In this case, reducing the expression level of the transporter can be an effective strategy to eliminate crosstalk between single cells. Third, the sensor-selector strategy works effectively when sensing the end product. If the sensor-selector can only sense an intermediate metabolite of the target product, this strategy may select cells that accumulate the intermediate rather than those consuming the intermediate. This issue could be potentially solved by exploiting a co-culture system, with a second microbial strain converting the accumulated intermediate to a final product, as demonstrated in the production of phenol [62] and tryptamine [63]. When using sensor-selectors in a co-culture system, additional attention needs to be paid to both the diffusion of the intermediate and competition between multiple populations [64].

### Growth-coupled production

The design principle of growth-coupled production is to turn the product, an intermediate, or a by-product from the target pathway into a growth-essential molecule (Figure 2h). A common strategy to build growth-coupled production is to eliminate or over-accumulate an essential metabolite followed by rescuing this metabolite through the target pathway [65–68]. In the case of engineering *E. coli* for 1,4-butanediol production, model guided gene knockouts were performed to deliberately accumulate intracellular NADH to toxic levels, which reduced cell growth. The engineered 1,4-butanediol production pathway was then used to consume NADH and balance redox potential, thus providing a mechanism to positively relate production to cell growth [69]. In another example, an *E. coli* strain was engineered with pyruvate generated solely by biosynthesis of anthranilate [70]. Introducing different anthranilate-derived biosynthetic pathways in this strain showed enhanced production of MA and tryptophan, with nearly no accumulation of anthranilate.

### Cautions in the population quality control

When designing population quality control, stringent selection pressure can only be turned on after high producers have accumulated enough signal molecules to differentiate themselves from low producers. Furthermore, the controller should pose minimal additional

burden on growth rate to prevent the sacrifice of productivity. The production threshold for high producers and the fraction of high producers in the population are important parameters determining the effect of the controller. In sensor-selector, these parameters can be tuned by engineering biosensors or applying external control to the selector [49,50,71••]. In growth-coupled production, an iterative knockout approach may reach the same goal [69,72]. Measuring a distribution of production in single cells is beneficial in determining optimal parameters for the controller.

## Conclusions

Considerable progress on various control strategies has been made to improve microbial production in recent years. Different control strategies were developed to solve different problems in bioproduction. Feedback control is a versatile tool to provide robust production of burdensome proteins and metabolites, avoid the accumulation of toxic intermediates, and shorten the rise-time of metabolites. Metabolic switch can replace the traditional inducible system used in two-stage batch fermentation to avoid resource competition between cell growth and engineered metabolic pathways. Population quality control can effectively prevent low-producing cells from dominating cell cultures, thus improving overall yields and titers, especially during large-scale production. Understanding the strengths and weaknesses of each control strategy can help obtain the most effective control for optimal performance enhancements. Different types of control strategies can also be potentially combined in one engineered strain to fulfill distinct functions for bioproduction. As demonstrated in a recent study on naringenin production, a metabolic feedback control was used to prevent overflow from malonyl-CoA to lipid biosynthesis, while a sensor-selector was used to couple naringenin production with cell growth [71••]. Combination of these two control strategies has increased both naringenin titer and strain stability. With the development of more sophisticated control technologies, the era of intelligent manufacturing in biology is coming.

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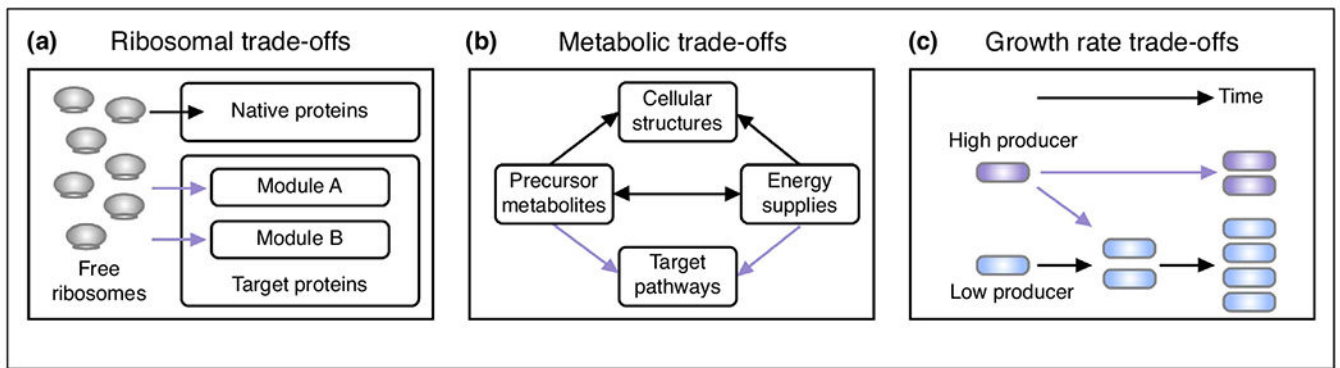
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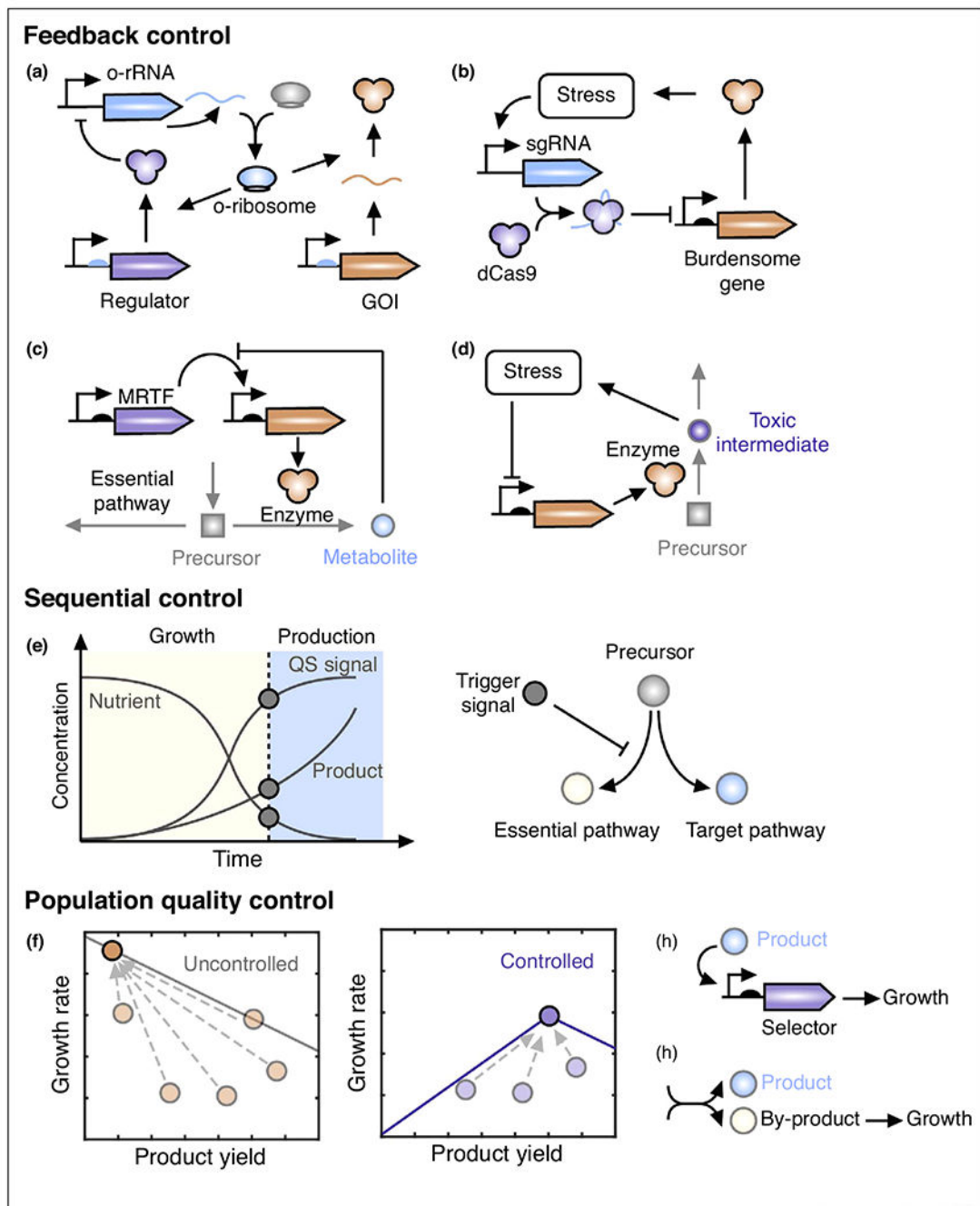
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**Figure 1.**

The presence of trade-offs during microbial production. **(a)** Ribosomal trade-offs.

Overexpressing one protein decreases the number of free ribosomes available to synthesize native proteins and other target proteins within an engineered pathway. **(b)** Metabolic trade-offs. Precursor metabolites are used for the synthesis of cellular structures, energy supplies, as well as target pathways. Consumption of precursor metabolites by a target pathway can affect both cell growth and maintenance. **(c)** Growth rate trade-offs. Low producers usually grow faster than high producers. Without control, low producers gradually dominate the culture over time.

**Figure 2.**

Design principles of control strategies that improve microbial production. **(a)** Feedback control of orthogonal rRNA provides robust gene expression from orthogonal ribosomes. **(b)** Stress-mediated feedback control to limit the burdensome protein synthesis. **(c)** Metabolic feedback control via MRTF to prevent metabolic overflow. **(d)** Stress-mediated feedback control to reduce the accumulation of the toxic intermediate. **(e)** Dynamics of trigger signals used to inhibit competing but essential pathways. **(f)** Setting an evolutionary stable point for high producers through population quality control. **(g,h)** Implement population quality

control. Linking production and cell growth via sensor-selector (g) and by-product (h). GOI, gene of interests; o-rRNA, orthogonal rRNA; o-ribosome, orthogonal ribosome; MRTF, metabolite-responsive transcription factor; QS, quorum sensing.

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