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Dynamic metabolic engineering: New strategies for developing responsive cell factories

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Abstract

Metabolic engineering strategies have enabled improvements in yield and titer for a variety of valuable small molecules produced naturally in microorganisms, as well as those produced via heterologous pathways. Typically, the approaches have been focused on up- and downregulation of genes to redistribute steady-state pathway fluxes, but more recently a number of groups have developed strategies for dynamic regulation, which allows rebalancing of fluxes according to changing conditions in the cell or the fermentation medium. This review highlights some of the recently published work related to dynamic metabolic engineering strategies and explores how advances in high-throughput screening and synthetic biology can support development of new dynamic systems. Dynamic gene expression profiles allow trade-offs between growth and production to be better managed and can help avoid build-up of undesired intermediates. The implementation is more complex relative to static control, but advances in screening techniques and DNA synthesis will continue to drive innovation in this field.

Keywords

Dynamic gene expression; metabolic control; metabolic engineering

1. Introduction

Metabolic engineering focuses on the manipulation of cellular metabolism in order to maximize production of valuable products such as biofuels, biochemicals, and proteins. Much of the work in this field has focused on gaining an in-depth understanding of flux distributions in core metabolic pathways and how these distributions can be altered to direct metabolite fluxes toward a particular product of interest. The desired flux distributions are typically in conflict with natural regulatory patterns in the cell, meaning the outcome cannot be achieved by process changes alone, but requires genetic manipulation of the host organism. Aided by both computational and experimental tools, metabolic engineers have been very successful in altering steady-state flux distributions in the cell through the use of gene knockouts, promoter replacements, and introduction of heterologous genes. High-

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Conflict of Interest

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throughput screening of combinatorial promoter and gene libraries has allowed the production space to be more fully explored and aided in optimization of steady state flux distributions. [1, 2].

As interest has grown in production of a wider variety of products, especially ones involving more complex pathways, interest has also grown in dynamic approaches to cellular engineering [3]. Metabolic engineering exists in interplay with the complex regulatory networks of the cell and the native physiology. Native pathway fluxes may differ depending on nutrient availability and growth rate, resulting in changes in the ideal metabolic engineering strategy throughout the course of the fermentation. Additionally, exploiting the capacity of the cell to sense and respond to changing conditions could provide an advantage at large scale, where significant heterogeneity exists within fermenters with respect to nutrient availability, dissolved oxygen, and pH [4].

There is substantial added complexity associated with implementation of dynamic metabolic control. The strategy requires an understanding of time course behavior of the system, identification of appropriate sensor systems, and appropriate tuning for “high” and “low” states, representing maximum and minimum required levels, respectively, of target enzyme expression. Often the high and low baselines are unclear, and much like the static case, require combinatorial tuning to identify. However, with the availability of better and more inexpensive methods for high-throughput screening, the ability to develop systems for dynamic metabolic engineering will continue to increase.

2. Static manipulation of metabolic pathways

As previously noted, significant success has been achieved with static manipulation of metabolic pathways. Rational deletions and up- and downregulation of native genes have been used to enhance production of compounds naturally occurring in the cell, such as lysine in *Corynebacterium glutamicum* [5]. These strategies have also been used to truncate and repurpose natural pathways in concert with expression of novel enzymes, allowing production of completely new products. One such example is metabolic engineering for biodiesel production in *Escherichia coli*, where after the natural production of fatty acids was enhanced via selected knockouts, a heterologous pathway was added to utilize fatty acids for production of fatty acid ethyl esters [6]. To facilitate screening across a range of physiological conditions, a variety of promoter libraries have been developed for different organisms, allowing rapid selection of relevant expression levels [7–9].

Computational tools in this area are also well-developed. As the focus is on altering steady-state flux distributions, flux balance analysis (FBA) combined with a genome-scale stoichiometric model can be used to predict changes in flux distribution as a result of gene knockouts. Metabolic optimization algorithms, such as OptKnock [10], seek to maximize flux toward product while maintaining the maximum possible biomass formation rate, which is a function of the fluxes of a variety of key metabolites. This strategy has been successful for predicting knockout strategies to increase yields of products such as succinic acid in *E. coli* [11] and ethanol in yeast [12]. Extending this, additional algorithms have been developed, allowing predictions of required fine-tuning of fluxes through up- and

downregulation of target genes [13] and incorporating experimental data from metabolic flux analysis to better estimate the true flux variability in the wild-type strain [14]. Genetic manipulations predicted by these algorithms to improve product yields have been shown to be consistent with experimentally successful strain designs for production of fatty acids and malonyl CoA-derived products in *E. coli* [15, 16].

3. Metabolic models to support dynamic control

Although there are many examples of the successful implementation of static changes to metabolism in order to increase product yields, it is clear that reducing expression of key metabolic enzymes often results in decreased cellular growth rate. While the capacity exists in such knockout strains to produce high levels of product, the productivity is limited by lack of biomass formation. Computational models that integrate an ability to switch flux distributions in the cell between biomass formation and product formation have been used to explore the potential benefits of dynamic control.

In case studies on glycerol and ethanol production, Gadkar *et. al.* demonstrated the theoretical improvements in productivity that could be achieved via dynamic control of enzyme levels in contrast to static knockout or upregulation [17]. By allowing a phase of biomass production before diverting flux through glycerol kinase, their model predicted that production of glycerol could be improved by over 30% in a fixed 6 hour batch time. Similarly, it was shown that dynamically manipulating *ackA* expression in the case of ethanol production could be expected to improve productivity. Subsequent studies have examined how a similar model framework based on dynamic flux balance analysis (dFBA) could be used to predict optimal switching strategies for improved production of succinic acid and serine [18, 19].

In addition to managing trade-offs between growth and production, dynamic control of enzymes in heterologous pathways might offer a way to balance fluxes and minimize protein expression burden. A number of studies have examined how temporal control of enzyme expression within a production pathway could be used to achieve maximum formation of product with the minimal cost of enzyme production [20–22]. For a simple, two-step pathway converting substrate to product, Klipp *et. al.* showed that the fastest conversion of substrate into product was expected to occur when all available protein was first allotted to the initial pathway enzyme, with later switching to more balanced expression of both enzymes [21]. Not surprisingly, similar dynamic controls also appear to have developed in natural systems. Zaslaver *et. al.* examined amino acid biosynthesis pathways in *E. coli* and found that promoters for enzymes closer to the beginning of the amino acid synthesis pathways showed both a shorter response time and higher maximal promoter activity in response to amino acid starvation, in agreement with a mathematical model for maximizing product formation while minimizing enzyme production [20]. Oscillatory patterns of enzyme expression are another potential route to minimize protein expression burden or to match expression with systems showing a natural oscillatory cycle, such as the cyanobacterial Kai proteins [23]. A kinetic model incorporating oscillatory expression of sets of glycolytic proteins showed that this strategy could be used to increase phosphoenolpyruvate pools by 1.86-fold [24]. Aside from protein expression burden, a

number of pathway-specific constraints also make temporal control of enzyme expression favorable, including instability of downstream enzymes, toxic pathway intermediates, and product inhibition of upstream enzymes.

4. Experimental demonstration of dynamic control in metabolic engineering

Farmer and Liao [25] demonstrated an early example of engineering dynamic controls into central metabolism for improvement of pathway productivity. In lycopene production, phosphoenolpyruvate synthase (*pps*), controls the balance between the precursors glyceraldehyde-3-phosphate and pyruvate, but overexpression of this enzyme causes growth inhibition during glycolytic growth. Recognizing that acetyl-phosphate (AcP) buildup was a signal of excess metabolic capacity, a strain was constructed capable of sensing acetyl-phosphate levels via a transcriptional regulator from the native Ntr regulon in *E. coli*. By controlling expression of *pps* and isopentenyl diphosphate isomerase (*idi*) from the AcP responsive promoter, those enzymes were expressed only when excess glycolytic flux toward acetate occurred. In the strain utilizing this system, yields of lycopene were improved 18-fold over a strain with constitutive expression of all pathway genes. In addition to showing improved lycopene yields, the strain utilizing the AcP responsive promoter instead of the Tac promoter for *pps* expression also showed a growth profile more comparable to the host control, which could help contribute to the final improvement in observed lycopene titers.

More recently, several successful examples of dynamic control have appeared, focusing both on knockdown of native enzymes and balancing of heterologous pathways. An overview of the typical implementations of these types of dynamic control is shown in Figure 1. The studies focusing on control of native enzyme levels have generally been concerned with pathway redirection, splitting carbon flux between pathways critical to cellular growth and heterologous pathways for production of valuable small molecules. This focus on essential genes makes sense, as these pathways offer the most direct ability to benefit from a controlled tradeoff between biomass formation and product. Areas recently investigated in *E. coli* include both direct transcriptional control of the metabolic enzyme of interest, and controlled degradation of the enzyme. In the area of direct transcriptional control, Solomon *et. al.* modulated glucokinase (*Glk*) levels via a genetic inverter in order to redirect glucose into gluconate production, improving titers by 30% [26]. Another recent study focused on control of citrate synthase (*gltA*) to redirect acetyl CoA into isopropanol production [27]. As with *glk*, deletion of *gltA* results in no growth on glucose minimal medium [28], making it a poor target for knockout. Using a genetic toggle switch from Gardner *et. al.* [29], a strain was developed capable of shutting off citrate synthase expression in response to IPTG. Leaky expression of *gltA* still allowed growth and isopropanol production even in the “off” state, but dynamically shutting off expression at 9 hours still resulted in a 10% increase in yields and titers of isopropanol relative to downregulation from the start of the fermentation and more than a two-fold improvement over expression of *gltA* from the native promoter.

Similar results have been achieved via controlled degradation of essential enzymes, relying on addition of a modified SsrA degradation tag to the coding sequence of the gene and expression of an additional adaptor protein, SspB, to increase the rate of proteolysis [30].

For example, induced degradation of FabB was used to stop elongation of fatty acids and improve production of octanoate [31]. Controlled degradation of phosphofructokinase (Pfk) was also applied to increase yields and titers of *myo*-inositol produced from glucose-6-phosphate [32]. A reduction in Pfk levels could be used to switch cells growing in glucose minimal medium between a “growth mode” with low pools of glucose-6-phosphate and fructose-6-phosphate and a “production mode”, with increased pools of those sugar phosphates. Dynamic control of Pfk activity resulted in more than a two-fold improvement in titers of *myo*-inositol when compared to the static case. While the *ssrA*/*SspB* system is designed to function in *E. coli*, it was recently shown that the Lon protease from *Mesoplasma florum* can function as a host-independent system, with expression of the protease resulting in degradation of proteins containing the cognate *ssrA* tag in *Lactococcus lactis* as well as in *E. coli* [33]. Protein degradation strategies still require control at the transcriptional level to induce expression of the protease or adaptor protein required for degradation of the target to occur. However, compared to transcriptional switching, they offer the advantage of very rapid depletion of the protein of interest even under conditions of slow growth, when removal via dilution is slow, and the possibility to add degradation tags to genes in their native context, without requiring adjustment of transcription from an inducible promoter to native levels.

The development of such dynamic systems for pathway redirection has not been limited to applications in *E. coli*. In yeast, the native promoters such as the repressible *MET3* promoter have been used to conditionally repress expression of essential native genes [34–36]. Recent work has also focused on developing dynamic control in yeast where knockdown is decoupled from outside inducer addition and instead tied to natural changes in culture conditions. For example, Scalcinati *et. al.* have used the *HXT1* promoter to couple the expression level of squalene synthase (*ERG9*) to glucose concentration in the culture medium, with lower expression during glucose limitation [37]. *ERG9* is essential for production of ergosterol during growth [38], but diverts farnesyl diphosphate (FPP) flux away from production of the desired sesquiterpene, α -santalene. This system was tested under fed-batch conditions, with the expectation that *ERG9* expression levels would decrease at the start of glucose-limited feed, effectively redirecting FPP consumption. The strain utilizing the *HXT1* promoter in place of the native *ERG9* promoter showed more than a three-fold increase in α -santalene production rate in fed-batch and a decreased ergosterol production rate.

These recent studies have focused on dynamic knockdown of essential genes, which represent the clearest benefit of this strategy, because the corresponding gene knockouts are lethal. As the ability to design dynamic systems increases, exploration will likely also expand to genes that are not essential, but could still benefit from time-dependent control of expression. For example, global regulatory proteins could be placed under defined dynamic control, allowing native regulatory pathways to be rewired to generate a response to a metabolite of interest in lieu of their natural control.

There is also significant interest in controlling the interplay of multiple genes and managing multiple pathway fluxes. In a “genetic switchboard” developed in *E. coli*, Callura *et. al.* [39] demonstrated control of multiple native metabolic enzymes. Addition of different inducer

combinations allowed switching of flux between glycolysis, the pentose phosphate pathway, and the Entner-Doudoroff pathway, resulting in changes in relative metabolite pools. While not directly connected to a heterologous pathway, the altered intracellular metabolite pools demonstrate the potential of more complex approaches for dynamically rerouting carbon fluxes.

The concept of multi-gene control can be expanded to heterologous pathway balancing. Two recent examples from the Keasling lab focused on dynamic control of multi-gene expression modules. In the first case, the transcriptional regulator FadR was used to control expression of genetic modules involved in the synthesis of fatty acid ethyl esters (FAEE) [40]. Because the native *E. coli* promoters interacting with FadR have limited dynamic range, more responsive synthetic promoters were designed by placing FadR and LacI binding sequences in the phage lambda and T7 promoters, resulting in up to a 60-fold change in fluorescence when tested for RFP expression in the presence and absence of oleic acid. These promoters were placed upstream of the modules involved in ethanol production, so that biosynthesis was only induced in the presence of fatty acids, avoiding wasting carbon for excess ethanol production and resulting in improved titers of FAEE. Importantly, a series of constitutive promoters was also tested for driving expression of the modules, to see whether a static balancing of expression could have achieved the same result; in this case, it was found the dynamic system was still superior with 2-fold higher FAEE titers than any of the thirty constitutive promoter combinations tested.

A modular approach was also employed to avoid buildup of the toxic intermediate FPP in the production of amorphadiene [41]. With no known FPP responsive transcription factors, whole-genome transcriptional analysis was used to identify candidate FPP responsive promoters. Promoters were identified that showed both up and downregulation in response to FPP, allowing a system to be developed with approximately a three-fold decrease in expression of the upstream FPP production module and four-fold increase in expression of the downstream consumption module upon FPP buildup. A similar strategy was recently used by Xu *et. al.* to balance malonyl-CoA pools for fatty acid production [42]. Malonyl-CoA responsive promoters were designed based on FapR, a malonyl-CoA responsive transcription factor from *Bacillus subtilis*, allowing both upregulation and downregulation of gene expression in response to increasing malonyl-CoA levels [43]. These promoters were used to decrease expression of the upstream malonyl-CoA production operon (*accADBC*) and increase expression of the downstream consumption operon (*fabADGI tesA'*) upon buildup of malonyl-CoA, resulting in oscillatory levels of intracellular malonyl-CoA and a 2.1 fold improvement in fatty acid titers over the unregulated pathway. With an appropriate choice of metabolic modules, for instance splitting modules at a metabolic branch point or at points where intermediate buildup has already been observed, a dynamic approach can prove very valuable. The optimal balance for a set of static promoters will represent some balance over the average of all cellular conditions in a fermentation, which may not be the best balance at any given time point.

Timed expression of recombinant proteins is also an area of interest in engineering mammalian cells. In cases where transgene expression has a negative effect on the host cell such as growth inhibition, proper timing of expression is important to maximize

productivity. Small molecule inducers can be added exogenously, but to reduce costs and simplify processing, intracellular signals and expression patterns of natural promoters can be exploited. Sensors for intracellular redox state have been explored in CHO cells [44], as well as cell-cell communication systems for protein induction at specified cell density [45]. Le *et al.* recently demonstrated that by using a native CHO promoter with a natural upswing expression pattern that resulted in higher stationary phase expression, transgene expression could be enhanced 4- to 16-fold in stationary phase relative to exponential phase [46]. This promoter was then utilized to drive expression of the mGLUT5 fructose transporter, allowing differential consumption of glucose and fructose during the course of the culture.

5. Strategies for development of new dynamic systems

A number of examples of dynamic control have been successfully implemented. However, to date, many of these required time-consuming screening for appropriate biosensors and balancing of gene expression through promoter engineering. The rapid development of new tools in synthetic and systems biology will help expand the field of dynamic metabolic engineering and streamline the processes needed for implementation.

A key factor in developing a system for dynamic pathway regulation is often finding an appropriate sensor system. Applications based on quorum sensing signals offer the ability to control response based on cell density, an important parameter for many metabolic engineering applications. Quorum sensing promoters have been used to drive protein expression and effect changes in cell physiology for a variety of applications, from delaying recombinant protein synthesis [47, 48] to sensing pathogens [49]. Through protein engineering, the affinity of the regulator protein for its cognate autoinducer can be attenuated, allowing the system to be tuned for control of induction time [50, 51]. Stationary phase promoters [52] and autoinduction medium [53] have also been successfully used as indirect methods of sensing cell density for applications like delayed recombinant protein expression. When sensing of a specific small molecule product or intermediate is desired, it may be possible to utilize one of many previously characterized transcription factors. Several recent reviews have addressed the current state-of-the-art in biosensors and their potential applications for both high-throughput screening and metabolic engineering [54–56]. Protein engineering techniques can be used to alter the specificity of known transcription factors or increase their affinity for molecules of interest [57].

In cases where no responsive promoter/regulator system has been identified for the metabolite or product of interest, decreases in the cost of RNAseq have expanded the opportunities for screening promoter response to larger libraries of small molecules. Additionally, a library is available with GFP expression driven by nearly 2,000 *E. coli* promoters, which can be used in fluorescence-based screening for small molecule responsive promoters [58]. In these screens, it may not be possible to identify the mechanism of promoter regulation, making it more difficult to apply in new systems, and generally limiting the applicability to a single organism. Additionally, even once identified, responsive promoters may not have the desired basal transcription level or dynamic range to be used in the desired metabolic engineering application. However, in this area, utilizing tools from synthetic biology will be very valuable. Rather than directly controlling the

protein of interest from a responsive promoter, the promoter can be integrated into a larger genetic control system, allowing the output response to be modulated. A number of strategies have already been demonstrated for amplifying [59] and inverting [60] a signal from a biosensor or maintaining output even after the initial small molecular inducer disappears [29]. Multiple biosensors can also be integrated into cellular logic gates, allowing the response to be finely tuned against different combinations of signals [61]. The robustness of these logic gates is also being explored in the context of industrial strains and fermentation conditions, which are relevant for eventual application in pathways for large scale production of chemicals [62].

Dynamic control of metabolic enzyme levels and activities can be exerted not only at the transcriptional level, but also at the post-transcriptional and post-translational level. Figure 2 illustrates how implementation of dynamic control might be envisioned at each level. Some of the initial applications of RNA-based control for metabolic engineering purposes have included the use of anti-sense and small RNA constructs [63, 64]. Small RNAs can be designed for a wide variety of targets, providing a useful system for screening the effect of changing expression of multiple proteins, although these still require a responsive transcriptional element to drive expression of the regulatory RNA at the appropriate time. In addition to using anti-sense strategies, there are a variety of other routes for implementing RNA-based control, which could offer the opportunity to utilize RNA in a sensing capacity, through the use of aptamers that bind to small molecules. RNA-based control of gene expression via riboswitches has been demonstrated, both when the regulatory element is included on the mRNA of the gene of interest [65] and in the case of *trans* acting RNAs [66]. In both cases, binding of a small molecule effector to the RNA resulted in changes in the folded structure, which can be exploited to block or unblock the ribosome binding site, resulting in a change in translation of the protein of interest. Other mechanisms of riboswitch action exist, including ligand-dependent self cleavage and transcriptional attenuation due to ligand-dependent formation of a hairpin acting as a transcriptional terminator. Riboswitches have been discovered that bind naturally to purines and their derivatives, amino acids, protein coenzymes, and metal ions [67], but by replacing the sensing domain with RNA aptamers, synthetic riboswitches and ribozymes have been developed which respond to theophylline [68, 69]. A tetracycline aptamer has also been used to control gene expression in yeast by insertion in the 5' UTR without use of a natural riboswitch scaffold [70]. To expand the library of possible ligands, SELEX techniques can be used to screen for novel aptamers [71], although the integration of the binding domain with the existing mRNA structure requires careful development. Overall, RNA-based strategies offer significant flexibility to easily target multiple genes for control of expression, as well as to develop RNA expression cascades, which could be used for more complex genetic controls [72].

Post-translational control becomes more complicated, as this relies on changing the structure of the target enzyme. Many natural enzymes that exert significant flux control within a pathway are allosterically regulated by cofactors or pathway products, and a number of attempts have been made to engineer new allosteric sites into existing enzymes. One strategy that has been successfully implemented is that of domain insertion, where a protein

domain that undergoes a conformational change upon exposure to a stimulus, such as a small molecule, is inserted within the sequence of an existing protein [73]. Ideally, the catalytic activity of the original protein is then coupled to the presence of the stimulus. This approach has been successfully implemented in order to couple the activity of β -lactamase to the presence of heme and to couple the activity of *E. coli* dihydrofolate reductase to light exposure [74, 75]. While this approach could potentially offer targeted control of enzyme activity, allowable insertion sites are hard to predict and extensive screening is required to identify protein variants that retain both high activity and significant allosteric response. An alternative strategy for post-translational control is inducible protein degradation, through selective exposure of degradation tags by cleavage [76] or use of tags requiring an additional adaptor protein to facilitate proteolysis [30]. However, as in the case of antisense RNA, transcriptional control is still required to drive expression of the second component required for cleavage or proteolysis.

As an alternative to feedback loops based on intracellular sensors like RNA or proteins, responsive strains can also be combined with computational control systems, allowing feedback loops to be developed based on external process variables such as dissolved oxygen or off gas composition. Inexpensive inducers such as heat, or even pulses of light could then be used to affect the desired changes in cellular state. By fusing the Gal4 DNA-binding and activation domains to the light-responsive PhyB/PIF module, Miliás-Argentis *et. al.* were able to demonstrate a feedback control system for YFP expression in yeast based on pulses of light [77]. Light-based control of gene expression has also been demonstrated in mammalian cells, where a signaling cascade initiated by a conformational change in melanopsin due to photo-isomerization of 11-cis retinal was used to trigger transgene expression in human embryonic kidney cells. Control of transgene expression was successfully demonstrated both in bioreactors and in implants in mice [78]. Future applications will need to address the limited penetration of light in tissues and in high-density bacterial cultures. In the case of mammalian tissues, use of near infrared wavelengths can improve penetration depths from millimeters to a few centimeters [79]; however, application of light-controlled gene expression in fermentation vessels at the meter scale would still present a number of design challenges.

As with static systems, fine-tuning of expression in dynamic systems will still be required. This becomes especially important when considering control of enzymes in central metabolism, where baseline expression levels determine cell physiology. Degenerate oligos generated using tools such as the RBS Library Calculator [80] provide a basis for rationally screening across a range of expression levels. Combining this with high throughput, scarless recombineering techniques such as MAGE [81] can provide a platform for screening libraries of strains.

6. Conclusions

Dynamic strategies for metabolic engineering have shown promise for conditional knockdown of essential genes and for balancing pathway fluxes in response to fermentation conditions. Natural cellular systems exhibit a wide variety of dynamic controls, such as allosteric inhibition or transcriptional repression via feedback from downstream metabolites.

Many of these can be harnessed for metabolic engineering and integrated into novel applications.

While dynamic systems have shown potential for improvements in yield and titer, gains relative to static control are in many cases limited by the increased difficulty of fully exploring the production space in these systems. Rapid screening using previously developed constitutive promoter libraries facilitates optimization of steady state expression levels. Current dynamic systems often rely on discovery of specific small-molecule responsive promoters, which may not result in ideal expression ranges for system performance, especially when considering modulation of native enzyme expression.

However, methods for rapidly altering expression levels and balancing pathways, which have been already successfully applied for static control, will also facilitate development of dynamic control. Dynamic systems offer a much larger number of “control knobs”, and recent modelling efforts have shown that careful choice of system architecture and expression levels is required for optimal outcomes can be achieved [82]. Moving forward, both experimental and computational tools will be needed to fully exploit the potential of these systems. New technologies for screening biosensors and evolving their specificity will certainly push forward this area as well. Next generation gene synthesis technologies can reduce the cost of screening multiple system architectures. Combinatorial assembly techniques for combining short synthesized pieces of DNA into large expression cassettes have been used in applications like refactoring of complex pathways [83], and provide a platform for screening any type of multipart cellular system.

As lab scale applications are improved, a future challenge will be implementation of dynamic control strategies in industrial strains and fermentation systems [62, 84]. The concept could be quite valuable, as it would allow the cell to adapt in a pre-defined manner to changing conditions within the fermentation. In the laboratory, balancing of growth and production or balancing of pathway intermediate levels typically occurs under well-mixed conditions, where substrate concentrations vary slowly and continuously in one direction. However, at large scale, microorganisms are expected to move quickly through substrate and oxygen gradients. Scale-down studies have shown that for *E. coli*, short cycles of residence between an anoxic zone (1 minute) and a well-mixed zone (9 minutes), resulted in decreases in biomass yield and increases in formic acid production similar to those observed in large-scale fermenters [85]. In design of industrially robust systems, fast response time, reversible response, and genetic stability of components will play a role in future success. Drawing from both natural and engineered systems, we can develop “smarter” cells, in which native metabolism is consistently balanced with heterologous pathways, even under changing conditions.

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Abbreviations

AcP	acetyl phosphate
dFBA	dynamic flux balance analysis
FAEE	fatty acid ethyl ester
FBA	flux balance analysis
FPP	farnesyl diphosphate
IPTG	Isopropyl β -D-1-thiogalactopyranoside

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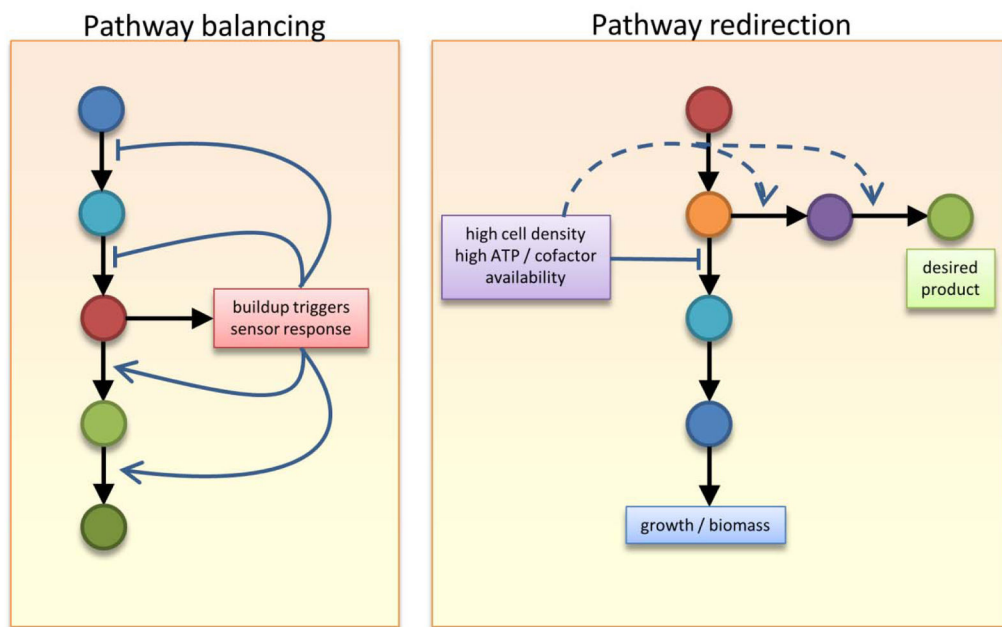


Figure 1.

Typical implementations of dynamic control in metabolic engineering. For “pathway balancing”, buildup of an undesired intermediate is used to trigger repression of upstream enzymes and activation of downstream enzymes. The sensing of intermediate buildup may be direct (binding of the target small molecule) or indirect (sensing of cofactor imbalance, growth inhibition). “Pathway redirection” is typically associated with splitting intermediate flux between cellular growth and energy production and a pathway for a desired product. In this case, some information about cellular state, such as biomass concentration, is used to trigger knockdown of enzyme(s) in the cell’s native metabolism and/or upregulation of the pathway toward the desired product.

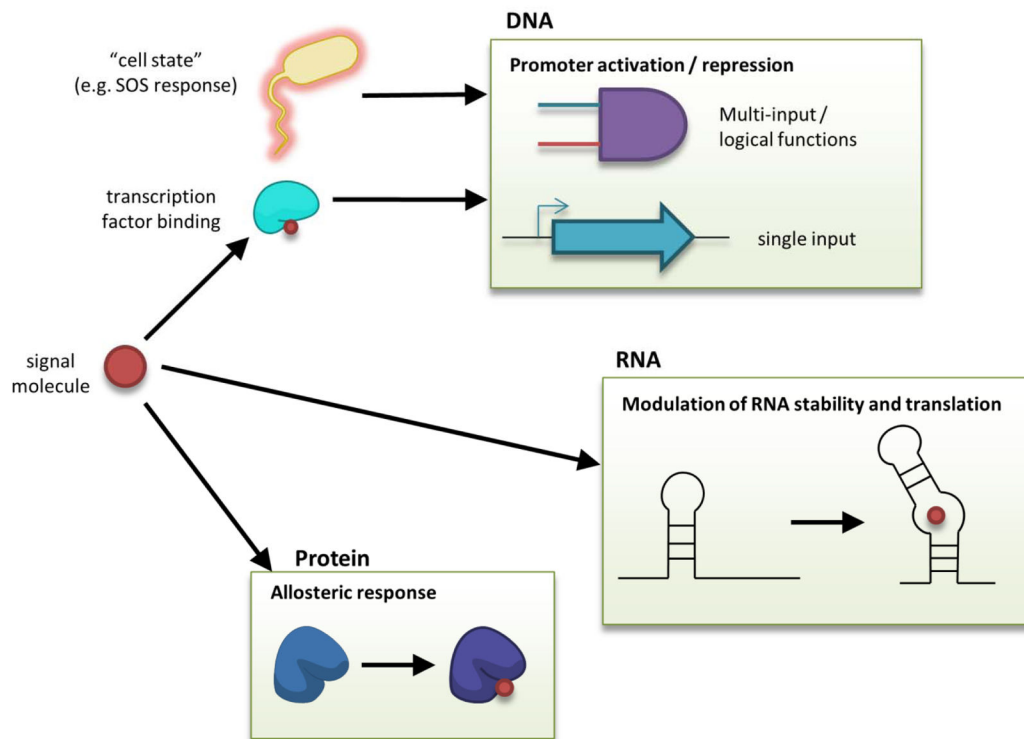


Figure 2. Strategies for dynamically modulating enzyme activity. After sensing of a relevant condition or small molecule, control of enzyme activity can be exerted at the transcriptional, post-transcriptional, and post-translational level. At the transcriptional level, interactions between transcription factors and relevant small molecules can be exploited to activate or repress gene expression. At the post-transcriptional level, use of RNA aptamers can provide a method for controlling translation of the relevant mRNA. Control at the post-translational level is possible in some cases using strategies such as engineered allostery.