



Biosensor-enabled pathway optimization in metabolic engineering

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Microbes can convert inexpensive renewable substrates to valuable metabolites by their natural metabolic pathways. To maximize the productivity, the pathways yet require further optimization, which remains challenging for our limited knowledge of complex biology. Genetically encoded biosensors are able to detect metabolite concentrations or environmental changes and transfer these inputs to measurable or actionable outputs, thus providing enabling regulation and monitoring tools for complicated pathway optimization. Here, we review recent advances in biosensor-mediated dynamic regulation and strain screening for the highest microbial production of diverse desirable products.

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Introduction

Metabolic engineering harnesses microbes to produce value-added chemicals and maximize the productivity to fulfill the requirement of industrial production. However, microbial cellular machinery is naturally designed to seek for optimal cell fitness rather than maximal metabolite yield [1,2]. While advancements have been made with the emerging of revolutionary strategies and technologies, metabolic engineering of microorganisms remains laborious and difficult due to the complexity of metabolic systems [3].

Biosensors, including transcriptional factor-based, nucleic acid-based and protein level biosensors, throw light on simplifying the engineering process [4–6]. These protein, DNA or RNA molecules can sense various metabolites or environmental signal changes and generate measurable or

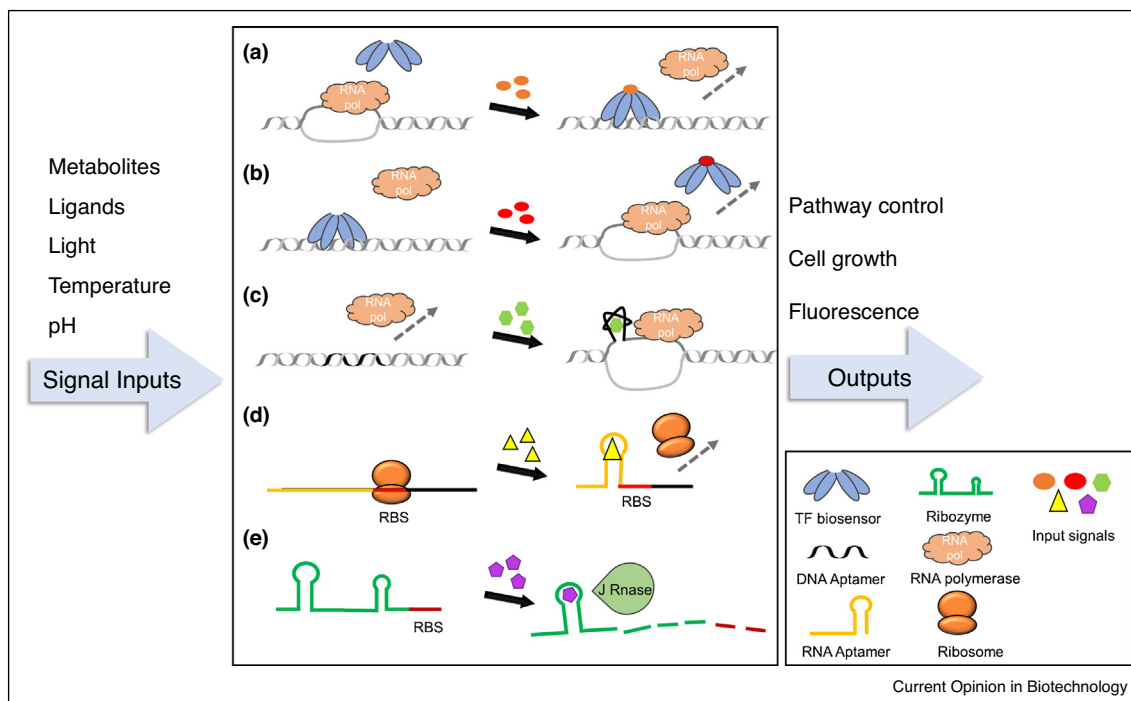
actionable responses. In this review, we focus on the most commonly used transcriptional factor-based biosensors (TF biosensors) and nucleic acid-based biosensors. Specifically, TF biosensors experience conformational changes once the ligand-binding domains are stimulated by inducers or environmental signals, relieving or triggering the interaction with their corresponding promoters, and thus activating or repressing the expression of downstream genes (Figure 1a, b) [4]. Similarly, nucleic acid-based biosensors such as riboswitches, ribozymes and aptamers also change their structures as responses to certain ligands, further regulating their interacting mRNAs or downstream genes at transcriptional or translational level (Figure 1c, d and e) [6]. Although biosensors usually cannot be directly used for their limited sensitivity and dynamic range, biosensor engineering can optimize the properties and fulfill the requirements for application [7,8]. These naturally developed regulation mechanisms can subsequently be utilized to manipulate gene expression by manually controlling the signal inputs [9,10], or endow microbes the intelligence to implement self-control by installing biosensors recognizing the signals generated by their own cell metabolisms [11,12]. Such dynamic regulation strategy has been proven to be effective in balancing carbon fluxes to address the conflicts and stresses brought by the intention shifting from growth to production [13,14]. Meanwhile, when controlling the expression of easily detectable reporter genes, biosensors can easily be repurposed as monitors to rapidly sense and estimate chemical production for high-throughput library screening. In addition, genetic circuits using biosensors to connect productivity with growth fitness allow efficient evolution to yield overproduction strains [15].

During the past several years, advancements in discovering or engineering biosensors for novel signal recognition, higher sensitivity, or expanded dynamic range further contributed to the development of metabolic engineering [16,17]. Here, we review recent studies regarding the application of TF and nucleic acid biosensors in dynamic regulation and overproducer screening and discuss the future perspectives on the biosensor-boosted metabolic engineering.

Biosensor enabled dynamic regulation Metabolite-responsive biosensor

Biosensors response to wide variety of metabolites existing among various types of living microbes to balance

Figure 1



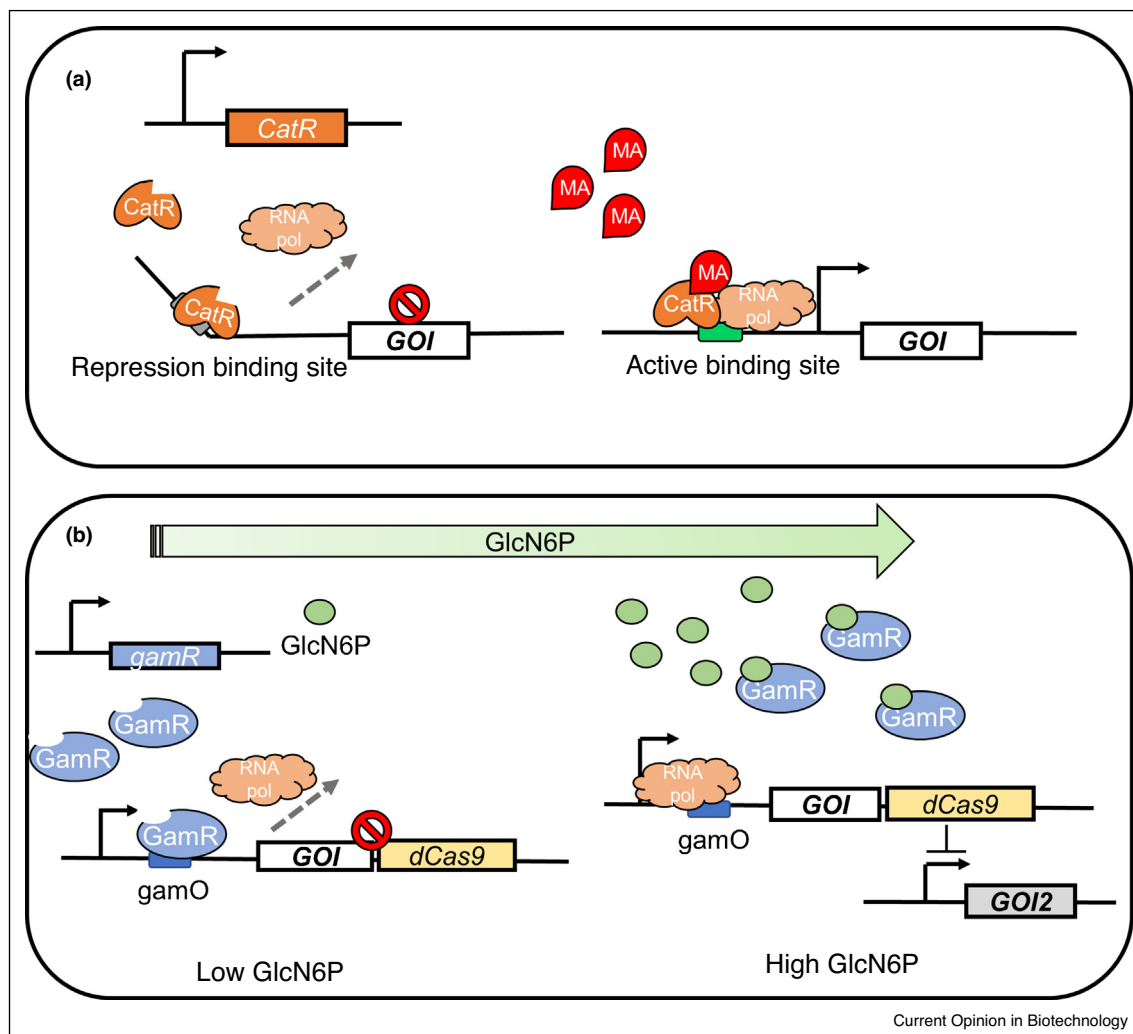
Application of TF and nucleic acid biosensors in pathway optimization and the regulation mechanisms. **(a)** TF biosensor-enabled transcription switch-off. The signal input activates the TF biosensor to interact with its corresponding promoter sequence, blocking the accession of RNA polymerase. **(b)** TF biosensor-enabled transcription switch-on. The signal causes some conformational changes of the TF biosensor, releasing it from the promoter and thus allowing the transcription by RNA polymerase. **(c)** DNA aptamer-promoted transcription switch-on. Signal input triggers the conformational changes of the DNA aptamer, promoting the unwinding of promoter sequence for RNA polymerase binding. **(d)** RNA aptamer-enabled translation switch-off. The conformational changes of the RNA aptamer inhibit the targeting of ribosome to RBS site. **(e)** Ribozyme-mediated translation switch-off. The ribozyme experiences self-cleavage with the signal input, leading to the digestion of the downstream mRNA. Input signals include diverse metabolites and environmental changes, enabling the generation of versatile regulation and screening strategies for metabolic engineering.

cellular metabolism [14]. Over the past few decades, increasing number of metabolites have been identified as signal molecules for application of biosensor in dynamic circuits as multiple technologies being explored for biosensor mining and optimizing [7,8]. Metabolites-responsive biosensors transfer input molecular signals to the expression levels of downstream operons. During this process, production is enhanced as the result that carbon flux is rewired to balance cell fitness and production dynamically, also to prevent the accumulation of toxic intermediates [14,18]. GlcN6P responsive sensor *glmS* ribozyme switch was developed as a tool to dynamically regulate the production of *N*-acetylglucosamine (GlcNAc) (Figure 1e). With the control of *glmS* ribozyme, *pfkA* and *glmM* or *pgi* were inhibited according to GlcN6P accumulation to balance the GlcN6P level and enhanced GlcNAc titer up to 18.45 g/L [11]. HucR is a natural biosensor responsive to uric acid. A library of HucR variants was constructed by saturation mutagenesis, and effector specificity changed variants that response to ferulic acid and vanillin were identified. By using the

modified HucR system, enhanced vanillin production was achieved through balancing the growth phase and production phase.

In recent years, bifunctional dynamic regulation circuits have been designed by applying antisense RNA and CRISPRi. With the strategy of bifunctional dynamic regulation, muconic acid (MA)-responsive biosensor CatR was used to activate genes within the MA synthesis pathway and simultaneously guide a RNAi system to inhibit the central metabolism (Figure 2a). The MA titer reached 1.8 g/L under such dynamic regulation [19]. Another bifunctional circuit was designed using TF biosensor GamR (Figure 2b) to control the expression of GlcN6P *N*-acetyltransferase transferring GlcN6P to GlcNAc while GlcN6P accumulating. GamR also regulated a CRISPRi system inhibiting genes related to cell growth and byproduct generation. Such dual-control circuit constructed in *Bacillus subtilis* improved GlcNAc production to 131.6 g/L [20]. Central metabolism can also be the target of dynamic regulation. A pyruvate-

Figure 2



Application of metabolite-responsive biosensors for dynamic pathway regulation. **(a)** The mechanism of MA-responsive biosensor CatR. The CatR tetramer binds to a specific repression site, triggering the bending of DNA chain and blocking its interaction with RNA polymerase when the signal molecule MA is absent. Once MA combines with CatR and causes its conformational changes, CatR binds to another active site to release the bending of DNA and retrieve transcription. MA, muconic acid. **(b)** GlcN6P-responsive biosensor enabled bifunctional dynamic regulation. When the intracellular concentration of GlcN6P is low, TF biosensor GamR binds to *gamO* sites to block the binding of RNA polymerase, inhibiting the transcription of *GOI* (gene of interest) and CRISPR/*dCas9*. When GlcN6P concentration reaches high level, GamR combines with GlcN6P, and the allosteric effect of GlcN6P decreases the affinity of GamR to DNA and activates the expression of *GOI* and CRISPR/*dCas9*. The *dCas9* coupled with sgRNA represses *GOI2*, thus realizing simultaneous activation and repression.

responsive biosensor PdhR was developed to regulate glucaric acid synthesis, *ino1* gene expression was activated to enhance yield, and antisense RNAs targeting *pgi* and *zwf* were employed to inhibit glycolysis and pentose pathway to construct bifunctional circuit based on PdhR [21^{••}].

As more biosensors responsive to various molecules being identified, dynamic regulation circuit design tends to be more complex and multi-layered. Naringenin responsive sensor FdeR is an activator targeting on FdeO site when

binding with naringenin. Leucine-auxotrophic strain with *LEU2* under the control of P_{FdeO} was constructed to achieve a naringenin addicted growth manner. Additionally, fatty acid responsive promoter controlled CRISPRi negatively regulates the competing lipogenic pathway in this study [22^{••}]. *p*-Coumaric acid can inactivate regulator PadR. By coupling FdeR and PadR system, a multiple-layered circuit was constructed to enhance naringenin production [23]. In another layered dynamic regulation circuit, both quorum sensing (QS) system and metabolite-responsive biosensor were applied to increase the

production of glucaric acid. With the QS system dynamically downregulates glycolysis pathway and the TF biosensor IpsA responsive to myo-inositol (MI) inducing the pathway gene *Miox* as MI accumulating, the yield of glucaric acid reached almost 2 g/L [24].

Environmental signal-responsive biosensor

Quorum sensing

Quorum sensing (QS) functions to coordinate gene expression according to population density through TF biosensors responsive to extracellular signaling molecules in cell communication [25]. A typical QS circuit is composed of a synthase responsible for the synthesis of signal molecules as cells grow, and a signal-responsive transcription regulator controlling specific genes [26]. As a pathway-independent dynamic control, QS systems have been applied for the induction of gene expression in metabolic engineering. The reported QS systems mostly utilized in metabolic engineering include EsaI/EsaR system from *Pantoea stewartii* [27] and LuxI/LuxR system from *Vibrio fischeri* [28]. In the EsaI/EsaR system, P_{esaS} promoter can be activated by the binding of transcriptional regulator EsaR. The accumulation of 3-oxohexanoylhomoserine lactone (AHL), which is synthesized by EsaI, can disrupt the EsaR binding and deactivate the transcription of P_{esaS} (Figure 3a). As an example, EsaI/EsaR system was applied in *Escherichia coli* to redirect glycolytic flux from competing pathway into heterologous pathways by switching off *Pfk-1* expression at desired times and cell densities. The final titer of myo-inositol increased 5.5-fold and titer of glucaric acid increased from unmeasurable to over 0.8 g/L [12]. Similar strategy has also been demonstrated for further increasing glucaric acid titer, resulting in a 5-fold increase in glucaric acid titer [24]. To release the toxicity of the heterologous pathway, EsaI/EsaR system was also applied to activate the 4-Hydroxyphenylacetic acid (4HPAA) synthesis pathway with the accumulation of AHL in *E. coli*, and the final titer exhibited a 46.4% improvement compared to the statically controlled pathway [29]. EsaI/EsaR system was optimized continually and applied to the production of 5-aminolevulinic acid (ALA) and poly- β -hydroxybutyrate (PHB). The optimized QS switch can achieve inhibition and activation of specific genes simultaneously, resulting in a 6-fold and 12-fold increase in the titer of PHB and ALA [30]. As another well-studied system, LuxR can bind with AHL, which is synthesized by LuxI, to activate the transcription of P_{LuxI} promoter (Figure 3b). Examples of utilizing the LuxI/LuxR circuit for dynamic regulation include autonomous control of metabolic state to increase the titer of bisabolene (1.1 g/L) [31] and redirection of carbon flux from central metabolic pathway into isopropanol production [32]. For the synthesis of bisabolene, related genes were controlled by P_{LuxI} to activate their expression at specific time with specific concentration of AHL. In another case, LuxI/LuxR circuit was combined with a positive feedback loop to rewire the carbon flux

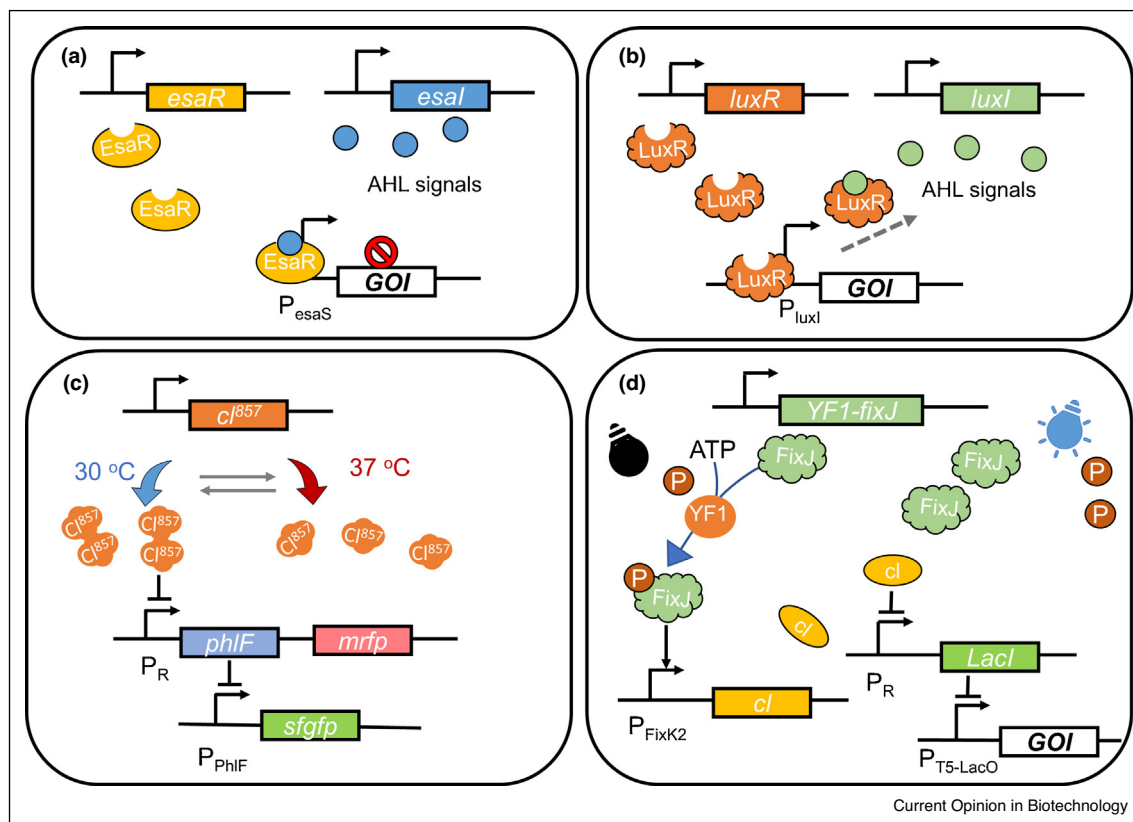
from TCA cycle into isopropanol synthesis pathway. More challenging pathway may require layered dynamic regulation to upregulate and downregulate related genes simultaneously. EsaI/EsaR and LuxI/LuxR systems were also combined to upregulate the genes for naringenin synthesis and downregulate assumption of malonyl-CoA for competing pathway. The final titer of naringenin was $463 \pm 1 \mu\text{M}$ naringenin, exhibiting a 140% increase compared to the original strain [33^{**}]. In addition to the aforementioned systems, there are many other QS circuits used in other model organisms. A bifunctional Phr60-Rap60-Spo0A QS system was applied in *B. subtilis* to fine-tune the synthesis of menaquinone-7 dynamically, which caused a 40-fold improvement in the final titer [34]. Similarly, a bifunctional comQXPA-PsrfA QS system was developed for dynamic control of gene expression in *Corynebacterium glutamicum* [35].

Other environmental changes

Some biosensors are sensitive to environmental stimulus including certain chemical or bio-molecules, pH, lights and temperature, and thus can serve as inducible components to design dynamic regulation circuits. For example, Deng *et al.* [36] reported using human thrombin-responsive DNA/RNA aptamers to design bifunctional dynamic regulation circuits at not only transcriptional but also translational level (Figure 1c, d). They further used the circuits for the biosynthesis of 2'-fucosyllactose (2'-FL) in *B. subtilis*, increasing the titer from 24.7 to 674 mg/L. Bañares *et al.* [37] adopted an engineered transmembrane transcriptional factor CadCA responsive to pH in constructing a dynamic regulation circuit controlling the accumulation of D-xylonic acid to overcome the culture acidization problem in utilizing xylose oxidative pathway, resulting in 170% higher glycerol production.

Temperature sensitive biosensors and optical biosensors grant researchers the opportunity to use temperature and lights to manually induce dynamic regulation, which are more inexpensive and reversible compared to chemical inducers. The thermal sensitive repressor CI and its modified version CI⁸⁵⁷ have been extensively applied in temperature controlled dynamic regulation circuits. They inhibit transcription at the corresponding P_R-P_L promoter by forming dimer complexes at low temperature, and the inhibition can be eliminated by raising temperature. Fang *et al.* [9] designed a thermal switch system with the CI repressor to overexpress the L-threonine synthesis pathway or switch off the L-alanine synthesis pathway according to temperature. The highest yield of L-threonine reached 124.03% molar yield. Wang *et al.* [38] developed a thermal-controlled bifunctional dynamic regulation system, namely T-switch, by combining CI⁸⁵⁷ and a TetR-family repressor PhlF. This system demonstrated the reversibility of temperature-control by swapping between 30°C and 37°C (Figure 3c) and was successfully applied to regulate

Figure 3



Application of biosensors responsive to environmental changes for dynamic regulation. **(a)** The diagram of *EsaI*/*EsaR* system. The transcription regulator *EsaR* activates the downstream expression from P_{esaS} . The binding of AHL generated by *EsaI* releases *EsaR* to deactivate the transcription from P_{esaS} . **(b)** The diagram of *LuxI*/*LuxR* system. The transcription regulator *LuxR* activates the transcription from P_{luxI} when combining with the AHL produced by *LuxI*. **(c)** Mechanism of the T-Switch system. Dimers of C_{I}^{857} are formed under low temperature (30°C), which inhibit the transcription of *PhlF* from P_R and maintain the transcription of *sfgfp* from P_{PhIF} at normal level. When the temperature rises (37°C), C_{I}^{857} dimers decompose to monomers and thus relieve the repression on *PhlF* and *mrp*. Transcription of *sfgfp* is then blocked since the inhibition of *PhlF* on P_{PhIF} . **(d)** Mechanism of the OptoLAC system. Under dark, *FixJ* phosphorylated by *YF1* (a photosensory histidine kinase) activates the expression of *cl*, which inhibits the expression of *LacI* and allowing the expression of *GOI* from the *LacO* containing promoter. Blue light induces the dephosphorylation of *FixJ*, stopping the *cl* expression and removing the inhibition on *LacI*. Expressed *LacI* thus can repress the expression of *GOI*.

the fraction of 3-hydroxybutyrate and 4-hydroxybutyrate in synthesizing di-block polyhydroxyalkanoates (PHA). Compared to temperature, light-controlled biosensors can generate more tunable regulation circuits. An optogenetic circuit with transcriptional factor EL222 controlling *GAL* system was firstly applied to improve the production of valuable chemicals in *Saccharomyces cerevisiae* [10]. This system was further optimized in recent studies to obtain amplified induction fold (OptoAMP [39]) and rapid response time (OptoINVERT7 [40]). Both systems were applied for the biosynthesis of lactic acid and isobutanol, with the latter improved the production of lactic acid and isobutanol by more than 50% and 15%. Optogenetic switch was also developed for metabolic engineering in *E. coli*. Tandar *et al.* [41] adopted chromatic acclimation sensor/regulator (*CcaSR*), which inhibits gene expression

when exposed to green light and relieves the inhibition with red light, to rewire the carbon fluxes between the Embden-Meyerhof-Parnas and oxidative pentose phosphate pathways with different ratios. Wu *et al.* [42] combined EL222 with dCpf1-mediated CRISPRi system to construct a light controlled CRISPRi platform. With the opto-CRISPRi dynamically repressing central metabolism and competing pathway, muconic acid production was increased by 130%. Very recently, Lalwani *et al.* [43] designed a series of OptoLAC systems using light as the replacement of IPTG to induce the *lac* operon in *E. coli* (Figure 3d). Through this, light generated stronger and more tunable induction than IPTG, and applying the OptoLAC systems resulted in mevalonate and isobutanol productions respectively 24% and 27% higher than IPTG induction.

Biosensor-aided strain screening

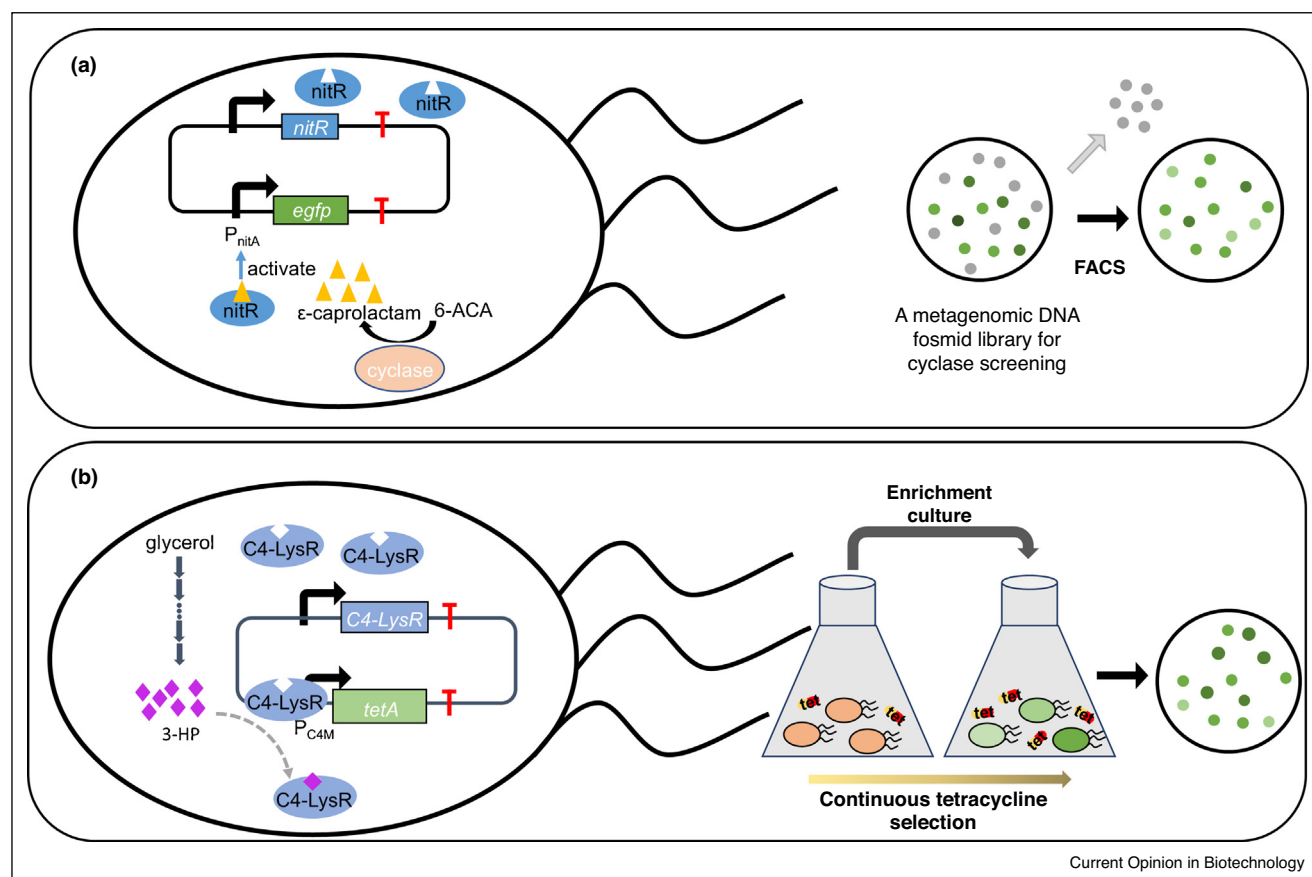
High-throughput screening

In addition to rationally rewiring carbon fluxes, biosensor involved high-throughput screening is a promising approach to dig out the potential productivity of the chassis host [44]. Such strategy shows the superiority of time-saving, high-efficiency, and easy-monitoring compared to the traditional screening methods by transferring productivity to easily readable signals [45]. The well-studied TF biosensors efficiently assisted to screen strains or enzymes for the high production of diverse valuable compounds. Yang *et al.* [46] repurposed type III polyketide synthase as a malonyl-CoA biosensor, which converts malonyl-CoA to a direct colorimetric indicator, flaviolin. The repurposed biosensor enabled the screening of an 1858 synthetic regulatory RNA library to identify knockdown sites that would improve the malonyl-CoA level. The authors then rapidly obtained a strain with the highest production of 6-methylsalicylic acid (440.3 mg/L). Qiu *et al.* [47^{*}] adopted an erythritol-

responsive TF biosensor EryD to control the expression of eGFP (enhanced green fluorescent protein). Such design converts erythritol production to fluorescence level and efficiently screened a mutated strain library of 1152 mutants, receiving an erythritol overproducer reaching over 148 g/L.

Biosensor-coupled fluorescence activated cell sorting (FACS) system can sort high and low-productive variants automatically and accurately. Yeom *et al.* [48] reported a caprolactam-detecting genetic enzyme screening system (CL-GESS) with an optimized lactam-detecting biosensor NitR_{L117F} and FACS (Figure 4a). By applying this rapid screening toolbox to screen an enormous marine metagenome, they successfully found and characterized a cyclase, CF3HBD, that had the capacity to converted 6-ACA (6-aminocaproic acid) to ϵ -caprolactam. Wang *et al.* [49] built a screening system based on a BenM biosensor system coupled with FACS system in *S. cerevisiae* to obtain high *cis,cis*-muconic acid (CCM) production

Figure 4



Highly efficient strain screening enabled by biosensors. (a) Schematic process of nitR enabled high-throughput enzyme screening method. ϵ -Caprolactam binding with nitR activates the *egfp* expression. Only cyclases efficiently producing ϵ -caprolactam from 6-ACA can generate intense fluorescence to be screened out by the FACS system. 6-ACA, 6-aminocaproic acid; FACS, fluorescence activated sorting system. (b) Schematic process of 3-HP responsive biosensor driven adaptive evolution. 3-HP induces the expression of tetracycline resistance gene, *tetA*. With continuous tetracycline selection, only strains with high 3-HP yield can survive. Tet, tetracycline; 3-HP, 3-hydroxypropionic acid.

variants. As a result, a variant Mut131 possessing 49.7% higher CCM titer was screened which could be used for related pathway optimization. By similar strategy, Bentley *et al.* [50] screened a muconate-productive variant by harnessing another muconate biosensor CatM with FACS system in *Pseudomonas putida*. Aptamer-riboswitch-based biosensors were also used to rapidly screen strains with increased titer of target products. Liu *et al.* [51] constructed a high-throughput screening platform combining a riboswitch-based tryptophan biosensor and fluorescence-activated droplet sorting (FADS). After screening the ARTP (atmospheric and room-temperature plasma)-based whole-genome random mutation library, 192 colonies with higher fluorescence readout were chosen as overproducer candidates. The best strain K3mu1 produced 2.19 g/L tryptophan, which is 155.1% higher than the parent strain.

Adaptive evolution

Adaptive evolution driven by biosensors is another significant screening application for productive phenotypes, in which genes affecting cell growth are controlled by biosensors to promote high producers to become dominant under selection pressure [52]. An example is the population quality control (PopQC) system using biosensors to control the tetracycline resistance gene *tetA*. In tetracycline-added culture, the PopQS system allows the continuous enrichment of high performers to overcome nongenetic cell-to-cell variation. The application of PopQS system employing FadR for fatty acid biosynthesis reached the titer of 21.5 g/L in fed-batch fermentation [53]. Similar continuous cell selection systems were designed with 4-hydroxybenzoate (4HB) sensor PobR [54], and with tryptophan sensor Tnac [55,56]. Guo *et al.* [57] attempted to replace exogenous antibiotic with a toxin/antitoxin system hip A/hip B in cell selection, and demonstrated that it can be used in combination with a phenylalanine biosensor TyrR or a tryptophan biosensor TrpR for production improvements.

In addition to screening target overproducers, adaptive evolution also provides a unique opportunity to uncover the mechanism underlying a phenotype and provide guidance for strain engineering [58,59]. Seok *et al.* adopted a synthetic biosensor C4-LysR responsive to 3-hydroxypropion (3-HP) to design similar adaptive evolution (Figure 4b). Upon further investigation, they found that mutations in global transcriptional factors rewired central metabolic flux towards 3-HP, and combining the mutations reached almost theoretical maximum 3-HP titer [60**].

Conclusion and perspectives

In this paper, we summarize recent studies on applying biosensors in versatile dynamic regulation and strain or enzyme screening for pathway optimization. Various metabolite-responsive biosensors, in cooperation with

other booming technologies, generated not only genetic circuits with diverse regulation functions, but also efficient overproducer screening methods. Meanwhile, environmental-responsive biosensors present the possibility to introduce autonomous regulation according to cell density, or the use of signals like light or temperature rather than chemical inducers that usually affect cell fitness. Overall, biosensors markedly assisted the pathway optimization process, leading to impressive production enhancements or novel biosynthesis of desired chemicals.

However, challenges remain mainly for the limited number of biosensors. The variety of recognizable signal inputs including environmental changes and more importantly cellular metabolites, are crucial for the development of biosensor-powered pathway optimization. Advances in computational methods applicable for biosensor mining and engineering can promisingly overcome these challenges. Tools such as MD (Molecular dynamics) simulations and molecular docking not only can generate guidance for experimental engineering of biosensor, but also allow *in silico* design of ligand-binding domains of biosensors to alter their specificity to target chemicals [61,62]. Genome mining has been serving as an effective approach to discover various novel biosensors [62]. Further improvements might be made by coupling bioinformatic tools with recently released machine learning-based structure prediction program AlphaFold [63]. In the future, we expect that a large number of biosensors with distinct specificities can be obtained more efficiently with the assistance of these advanced computational methods.

Conflict of interest statement

Nothing declared.

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