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Transcription factor-based biosensor: a molecular-guided approach for advanced biofuel synthesis

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Abstract

As a sustainable and renewable alternative to petroleum fuels, advanced biofuels shoulder the responsibility of energy saving, emission reduction and environmental protection. Traditional engineering of cell factories for production of advanced biofuels lacks efficient high-throughput screening tools and regulating systems, impeding the improvement of cellular productivity and yield. Transcription factor-based biosensors have been widely applied to monitor and regulate microbial cell factory products due to the advantages of fast detection and in-situ screening. This review updates the design and application of transcription factor-based biosensors tailored for advanced biofuels and related intermediates. The construction and genetic parts selection principle of biosensors are discussed. Strategies to enhance the performance of biosensor, including regulating promoter strength and RBS strength, optimizing plasmid copy number, implementing genetic amplifier, and modulating the structure of transcription factor, have also been summarized. We further review the application of biosensors in high-throughput screening of new metabolic engineering targets, evolution engineering, confirmation of protein function, and dynamic regulation of metabolic flux for higher production of advanced biofuels. At last, we discuss the current limitations and future trends of transcription factor-based biosensors.

Key words: Transcription-factor-based biosensor, advanced biofuels, synthetic biology, high-throughput screening, dynamic control, adaptive evolution

1. Introduction

Climate change arising from increasingly excessive consumption of fossil fuels has necessitated a rethinking of transportation infrastructure (Keasling et al., 2021; Pfleger and Takors, 2023). Inclusive focus was given to greener transport fuel to cut down the net carbon emissions. Biofuels produced by microorganisms promise to replace carbon-polluting fuels (Ashokkumar et al., 2022; Lin and Lu, 2021). Compared to the well-established biofuel ethanol, advanced biofuels like butanol, isobutanol, fatty acid derivatives and isoprenoid-derivatives are more suitable alternatives to replace fossil fuels due to similar energy density, transportation, and storage characteristics (Das et al., 2020; Liu, Z. et al., 2022; Rana et al., 2022). Thus, the biosynthesis of advanced biofuels is of great benefit in tackling the economic and environmental threats caused by reliance on fossil fuels.

The feasibility of microbial-assisted bioconversion relies on the inherent metabolic facet of the host strain. An ideal microorganism is supposed to have high productivity, high yield, and high titer. However, some advanced fuels are naturally produced in low concentration or low efficiency by the exogenous pathway (Ruffing et al., 2022). These shortcomings can be tackled by tailoring or redesigning metabolic pathways in the host strains (Madhavan et al., 2023; Zhang, Jianzhi et al., 2021). The synthetic biology “design-build-test-learn” principle enables rapid design and construction of vast microbial cell libraries, while the test phase remains a bottleneck for lacking effective high-throughput approaches to associate genotype with phenotype (Patwari et al., 2023). Genetically encoded biosensors based on transcription factors (TFs) stand out for realizing cheaper and faster in-situ high-throughput screening of desired properties by transforming the intracellular concentration of a target metabolite to a detectable signal.

The components of a TF-based biosensor encompass a TF that senses environment stimuli (end product, precursor, or other metabolites), a TF binding promoter which is regulated by TF-inducer complex, and a reporter gene under the control of cognate promoter (Yu et al., 2023). Upon the existence of biofuels or their

precursors, TFs undergo conformation change and bind to the transcription factor binding site (TFBS) of the cognate promoter, controlling the expression level of downstream reporter genes (Liu, C. et al., 2022). With the development of synthetic biology, plenty of TFs have been discovered and developed as biosensors for the biosynthesis of biofuels (Morgan et al., 2016). However, wild-type biosensors rarely meet the practical requirements because of the low sensitivity, specificity, and dynamic range. These properties can be enhanced by modifying the TF and promoter of biosensors (Gao et al., 2022; Gong et al., 2022). Coupling with different output reporters, the optimized biosensor satisfies various applications, including precise product detection and dynamic metabolic flow regulation, thereby further increasing the production of advanced biofuels and other chemicals (Ding et al., 2021).

Herein, we summarized TF-based biosensors that detect advanced biofuels or their intermediate metabolites biosynthesis. The principles of construction and validation of the genetic circuits were highlighted, especially the selection and optimization of genetic parts. We also reviewed the applications of biosensors in enhancing the production of advanced biofuels by mining potential engineering targets, high-throughput screening evolved enzymes and hyperproducers, confirming functional proteins and fine-tuning metabolic flux. Lastly, this review discusses the current limitation of exploring and optimizing genetic encoded biosensor and outlines the potential solution in the future.

2. Construction and development of TF-based biosensor monitoring advanced biofuels and their derivatives

2.1 The mechanism of transcription-factor-based biosensors

Transcription factors (TFs) are important proteins in the sensory systems that microorganisms have evolved in response to intracellular metabolite changes or environmental perturbation (Fiume et al., 2016; Zhang and Shi, 2021). By taking advantage of TFs' regulatory characteristics, TF-based biosensors that can

quantitatively identify the specific metabolite (inducer) concentrations have been widely studied in different microorganisms (De Paepe et al., 2017; Pham et al., 2022).

TF-based biosensors are executed based on the conformational change of TFs after binding to the inducer. As shown in Figure 1a, TFs can be categorized into activator and repressor according to their regulation mode when induced by the interaction of inducer and TFs (Deng et al., 2022; Nasr et al., 2023). TFs typically contain a C-terminal ligand-binding domain (LBD) and an N-terminal DNA-binding domain (DBD) (Matilla et al., 2022; Zhang, Jian et al., 2021). In the presence of an inducer, the activation function of activator is triggered. The activator's LBD combines with the inducer and causes the conformational change of TF, making its DBD bind to upstream of promoters and recruit RNA polymerase (RNAP) to initiate the downstream gene transcription (Cosma, 2002). Conversely, the presence of inducer represses the suppressive function of repressor. The inducer-repressor complex impacts the binding of repressor's DBD and promoter, releasing the steric hindrance caused by the repressor on the promoter of RNAP, thus activating the downstream gene expression. (He et al., 2023; Zhou et al., 2022). Overall, upon detecting different inducers, both TFs undergo protein conformation alterations, altering the binding affinity between the TFs and promoter, thus achieving the up- or down-regulation of target gene expression.

2.2 Design of TF-based biosensors

Generally, the architecture of TF-based biosensors can be divided into two modules: the sensing module and the reporting module (Zhang, Jian et al., 2021) (Figure 1b). The sensing module serves to detect the input signals, such as various metabolites and environmental signals, through the interactions between the inducers and TFs. Then, the TFs-inducer complex affects the cognate promoter, enabling the reporting module to translate the input signals into output signals, such as fluorescence intensity, cell fitness, or the yield of desired product (Alvarez-Gonzalez and Dixon, 2019; Chiang and Hasty, 2023; Zhou et al., 2021). Hence, the functions of

TF-based biosensors are determined by genetic parts, including TFs, cognate promoters, and reporters. Selecting and mining of these genetic parts are critical for transcription-factor-based biosensor design. Some TF-based biosensors sensitive to advanced biofuels are developed based on reported native or heterologous regulons, while others rely on novel technologies like omics analysis to expand the biosensor toolbox. Several approaches have been used for the mining and developing of genetic parts for advanced biofuel biosensors.

2.2.1 TF-based biosensor constructed by natural TF

TFs are natural regulatory proteins existing in microorganisms. Many native TFs and their cognate promoters have been discovered and leveraged for biosensor construction. Recent advances in biosensor development and engineering have accelerated the production of advanced liquid biofuels, including short-chain alcohols (Wu, T. et al., 2022), isoprenoid derivatives (Li, M. et al., 2020), and fatty acid derivatives (Li, C. et al., 2022).

Transcription factor BmoR and the cognate promoter P_{bmo} are a TF-promoter pair for butanol biosensor construction. P_{bmo} is a σ^{54} -dependent promoter of alkane monooxygenase in *Pseudomonas butanovora*, mediated by the alcohol-regulated transcription factor BmoR (Kurth et al., 2008). The transcription of P_{bmo} requires the σ^{54} -RNAP holoenzyme binding to the promoter. In the presence of n-alcohol, the BmoR-alcohol complex remodels the σ^{54} -RNAP holoenzyme into an open complex and initiates the transcription of P_{bmo} . Based on this mechanism, a BmoR-based biosensor was developed in *Escherichia coli*, which exhibits a detection range for C3-C6 alcohols (Dietrich et al., 2012). This biosensor was applied to sense n-butanol and isobutanol overproducers (Dietrich et al., 2012; Yu et al., 2019a; Yu et al., 2019b).

Transcription factor TbuT and its promoter P_{tubA1} were applied for a whole-cell biosensor that monitors the intracellular isoprene, the simplest isoprenoid. TbuT regulates the *tub* operon encoding a toluene-3-monooxygenase in *Ralstonia pickettii* PKO1 (Byrne and Olsen, 1996). In the presence of toluene or benzene, the TbuT binds to its ligand and activates the P_{tubA1} promoter of *tub* operon, promoting the

degradation of toluene and benzene. Since the TbuT recognizes monosubstituted benzene derivatives isoprene as a less-preferred ligand, a T7 RNA polymerase-mediated transcriptional cascade was utilized to increase the chromosomal isoprene biosensor sensitivity (Kim et al., 2018). Using this enhanced biosensor, a high isoprene producer *E. coli* was identified.

FapR and fadR are TFs sensing malonyl-CoA and acyl-CoA, respectively, which are both the building blocks for fatty acid derivatives synthesis (Fujita et al., 2007). FapR is a transcriptional repressor of *fap* regulon in the fatty acid pathway of *Bacillus subtilis*. In the absence of malonyl-CoA, FapR negatively regulates *fap* expression by binding to the *cis*-regulatory element *fapO* (Machinandiarena et al., 2020). Similarly, FadR is a transcriptional repressor controlling the genes involved in fatty acid metabolism in bacteria while it is induced by acyl-CoA (Teo et al., 2013). FapR and FadR can also act as transcriptional activators by fusing with a transcription activation domain (Qiu et al., 2020). Both fapR- and fadR-based biosensors were widely used for screening overproducing strains and dynamic regulation to enhance the production of fatty acid and its derivatives (Johnson et al., 2017; Teo et al., 2013).

The endogenous War1p/PDR12 regulation pair was applied to construct a fatty acid biosensor in *Saccharomyces cerevisiae*. War1p constitutively binds to the weak acid response element (WARE) region of the promoter of ATP-binding cassette transporter gene *PDR12* and is phosphorylated under weak acid stress to activate PDR12 expression (Baumann et al., 2018). This biosensor showed a linear response to hexanoic, heptanoic and dodecanoic acid (Baumann et al., 2021).

The alkane-responsive transcription factor AlkR in *Acinetobacter baylyi* ADP19 and AlkS in *Pseudomonas oleovorans* sense the environmental alkane. They bind to the promoter P_{alkM} and P_{alkB} , respectively, activating the alkane-degrading gene expression in the presence of alkane (Ratajczak et al., 1998; van Beilen et al., 2001). Thus, the alkane biosensors AlkR- P_{alkM} and AlkS- P_{alkB} have been developed. However, the AlkR- P_{alkM} biosensor has a wide alkane-detection spectrum but cannot function in *E. coli* (Zhang, D. et al., 2012). In contrast, the AlkS- P_{alkB} biosensor only detects the medium-chain alkanes in *E. coli* (Reed et al., 2012), in which the long-

chain alkanes are usually the main product. To develop a long-chain alkane sensor in *E. coli*, the two elements were reassembled into a chimera cARE. The constitutive promoter P_{alkS} controls the expression of AlkR, and AlkR activates promoter P_{alkM} in the presence of alkane. This genetically assembled biosensor could detect endogenous long-chain alkanes in an alkane-producing *E. coli* strain (Wu et al., 2015).

2.2.2 Mining and selecting novel genetic parts by transcriptome analysis

Most of the biosensors are based on the adaptation of well-studied regulons, which consist of a clear TF and its cognate promoter. However, the characterized TFs in nature are limited. High-throughput genomic, transcriptomic, and proteomic analyses are widely used to expand the TF-based biosensor toolbox by screening uncharacterized native TFs and promoters for specific input signals (Hanko et al., 2020). These approaches have been exemplified in identifying TFs in response to 1-butanol (Shi et al., 2017) and FPP (Dahl et al., 2013). Briefly, the omics analysis was used to identify promoters and their binding TFs that respond to the inducers, and then the TF-promoter pairs can serve as the genetic components for biosensor construction. For example, the toxicity of accumulated intermediate metabolite in the cell triggers a native stress response, typically a change in promoter activity, to deal with the assault. Based on this mechanism, Dahl et al. (Dahl, 2013) compared the genome-wide gene expression profile of an FPP accumulated *E. coli* strain to a non-accumulated strain at different growth phases. It was found that 462 genes were differentially expressed. The promoters highlighted in the microarray experiment were cloned upstream of the fluorescent gene for further test. The strength of two promoters, P_{restA} and P_{gadE} , showed dynamically change in response to different concentrations of FPP. However, the regulation mechanism of the two promoters and their corresponding TFs haven't been discovered.

A similar strategy was applied to recognize alcohol-responsive promoters in *S. cerevisiae* (Shi et al., 2017). The lag-phase yeast was exposed to 1-butanol, 1-propanol, and ethanol with different concentrations. Sample were taken for transcriptome analysis by RNA-seq. It was found that 50 out of 435 genes that were

solely up-regulated in response to 1-butanol were randomly selected for further analysis. The 1 kb upstream from the translation start codon of these genes was defined as the promoter and fused with mCherry to evaluate promoter response to 1-butanol. Four promoters positively respond to 1-butanol, while only two specifically have dose-response to 1-butanol. Unlucky, the two promoters behaved like “black boxes” without any known metabolite-responsive mechanism. Altogether, this method is suitable for discovering stress-responsive genetic parts of TF-based biosensors. However, it is only applicable in specific or direct transcriptional regulation and the tedious work of identifying and confirming the screened candidate. The selected responsive promoter is more like a black box. The lack of information about its underlying regulation mechanism hinders further improvement of the sensor system.

Compared with TFs and promoters, the selection of reporters is more straightforward. The reporters were usually selected as fluorescence, resistant genes, or target pathway genes (Cui et al., 2021; Li, J.-W. et al., 2020). Fluorescent genes are generally combined with fluorescence-activated cell sorting techniques to enable high-throughput screening of hyperproducing strains. GFP and mCherry are commonly used fluorescent proteins (Wang, M. et al., 2023). On the other hand, applying resistant genes or metabolic pathway genes as reporter is mainly for dynamic control of genetic circuits. Therefore, the genetic parts of biosensors selected to construct biosensors are essential to the performance and usage purpose of biosensors.

3. Evaluation and optimization of TF based-biosensor towards advanced biofuels

3.1 Evaluation of TF-based biosensor

A constructed TF-based biosensor requires the evaluation of several key performance factors: specificity, sensitivity, operating range, and dynamic range, which are important for incorporating this biosensor into the production of advanced

biofuels (Ding et al., 2021; Liu et al., 2023). The detailed information of these performance factors can be presented by the input-output response curve fitted by the Hill equation (Mannan et al., 2017). As shown in Figure 1c, the specificity refers to the capability of biosensors to specifically identify the target signal in a complex environment. A high-performance biosensor should have high specificity to distinguish the target chemical from other background signals (Sun et al., 2023). Sensitivity is an inseparable parameter from specificity. It reflects the response speed of biosensor when the input signals were changed from low to high, which is presented by the slope of the response curve. A biosensor with stable and wide sensitivity is desired. The operation range is the detectable range of the concentration of the input signals, which reflects the working flexibility of the biosensor (Wang, Y. et al., 2023). A biosensor with a wide operating range can respond to the input signals especially with the low abundance. Likewise, the dynamic range is defined as the ratio of the maximum output signal (ON state) to the minimum output signal (OFF state) when the input signal is present or absent (Hartline and Zhang, 2022). The high dynamic range is beneficial to distinguish the input signal from the background noise, thus reducing the probability of inaccurate output signals and providing higher accuracy for biosensor detection. Furthermore, the response time, stability and biosafety of transcription-factor-based biosensors are also important parameters in applying biosensors (Ding et al., 2021). Overall, the knowledge concerning these performance factors not only provides information for biosensor evaluation, but also serves as a guideline for further rational engineering of biosensor.

3.2 Optimization of sensing performance of TF-based biosensor

The performance of biosensors is related to their application objectives. However, most wild-type biosensors suffer disadvantages such as narrow dynamic range, low signal output, poor sensitivity, and restricted detection range. Furthermore, since natural TFs are limited, a majority of metabolites in the biofuel synthesis pathway are still lack of corresponding TF, which also holds back the popularity of

biosensors in the production of advanced biofuels. Therefore, genetic modification of TF-based biosensors is needed to satisfy practical applications. Strategies to enhance the dynamic range and sensitivity, broaden the detection range and alter the specificity of biosensors are concluded as a guideline for superior biosensor construction.

3.2.1 Strategies to increase dynamic range and sensitivity of biosensor

Due to the characteristic of turning chemical input into easily detectable output, a TF-based biosensor with outstanding properties is desired in screening excellent strains for target metabolite biosynthesis. However, the applications of many wild-type or recombinant TF-based biosensors are hijacked by the narrow dynamic range and low sensitivity. As a result, many efforts have been made to improve the dynamic range and sensitivity of biosensors including promoter strength optimization, plasmid copy number modulation, RBS strength optimization and genetic amplifier utilization.

Changing promoter strength, which regulates the expression of reporter, is one of the most widely used approaches to optimize the dynamic range and sensitivity of TF-based biosensors (Figure 2a). Changing the number and position of TFBS in the promoter that drives the reporter affects the interaction between the promoter with TF and RNAP, thus achieving expanded dynamic range and improved sensitivity of biosensors. For example, Liu et al. (Liu et al., 2014) mutated the TFBS of FapR and the -35 and -10 regions of the promoter, leading to a variety of promoter strengths and expanding the dynamic range of biosensors. Dabirian et al. systematically studied the effect of TFBS *fapO* at different sites of different promoters (Dabirian et al., 2019a). The *fapO* was placed in the upstream and downstream of either the TATA box or the transcription start site of the promoter individually or in combination. The engineered promoters resulted in biosensors with various dynamic ranges and sensitivities. Importantly, the position of TFBS in the promoter should be selected carefully because inserting TFBS into the core promoter region also takes the risk of weakening the promoter strength. To overcome this limitation, TFBS were placed in the upstream region of the promoter P_{TEF} and can be recognized by a chimeric repressor containing

FapR and native repressor Mig (Dabirian et al., 2019b). The resulting synthetic P_{TEF} promoter maintained its strength and showed a 5-fold repression strength for the FapR-Mig repressor than that of the FapR repressor. Furthermore, engineering promoter strength also enables the dynamic range of biosensors with controllable induced and leakage-expressed states. Low basal expression level in the absence of inducers is achieved by constructing hybrid promoters to tightly regulate reporter gene expression. For example, a hybrid promoter containing two TFBSs that respond to both fatty acid and IPTG was constructed to eliminate the leaky expression (Zhang, F. et al., 2012). The TFBS of FadR and LacI were individually placed upstream of the -35 region, between the -35 and -10 region, and downstream of the transcription start site, respectively. All the hybrid promoters were tightly repressed in the absence of IPTG and induced by the endogenous fatty acid/acyl-CoA levels.

The plasmid copy number can affect the dynamic range and sensitivity of biosensors by regulating the intracellular concentration of TF and reporter (Figure 2b). The genetic circuits of TF-based biosensors at a high expression level may also cause leaky expression, while at a low expression level, they may be unable to sense the target metabolites. To avoid the leaky expression and keep the biosensor operate properly, the plasmid copy number of isobutanol-sensing genetic circuit BmoR- P_{bmo} was optimized, thereby increasing the sensitivity of biosensors (Yu et al., 2019b). Apart from optimizing plasmid copy number, modulating RBS strength, aiming at tuning the translation level of TF, is also effective for broadening the dynamic range. For example, to obtain an optimal expression level of the FapR, a malonyl-CoA responsive transcriptional repressor, the RBS of which was mutated to create an RBS mutant library (Kalkreuter et al., 2019). Most variants provided significantly higher output than the original one, and some even increased their dynamic range by 60 times.

In addition to the above strategies, employing genetic amplifiers to enhance the biosensor response is more predictable and rapid. Genetic amplifiers based on transcriptional cascade are commonly used to increase dynamic range and sensitivity (Figure 2c). For example, a T7 RNA polymerase (T7 RNAP) based signal amplifier

was adopted to improve the signal-to-noise ratio of the isoprene biosensor (Kim et al., 2018). T7 RNAP is an RNAP specific to the T7 promoter with high transcription efficiency. The isoprene-responsive biosensor TbuT/P_{tbuA1} orthogonally interacted with the T7 RNAP/P_{T7} pair. In this genetic construct, the isoprene-TbuT complex activates the promoter P_{tbuA1} in front of T7 RNA. Then, the expressed T7 RNAP trans-activates the T7 promoter, which initiates the expression of a fluorescent signal. The fold-change in fluorescent with T7 RNAP-based signal amplification cascade was 3.8-fold higher than in the original version.

Overall, synthetic approaches, including optimizing promoter strength, modulating plasmid copy number, optimizing RBS strength and implementing genetic amplifier, can effectively improve the dynamic range and sensitivity of TF-based biosensors. However, most of the biosensors with enhanced performance are obtained *via* trial-and-error experiments. In the future, it is hoped that the strength of the variant biosensors can be accurately predicted by algorithms.

3.2.2 Protein engineering to alter detection range and substrate specificity

Detection range and specificity are the other two essential evaluation criteria for the performance of biosensors. TF-based biosensors with low detection range and specificity have limited practical application in advanced biofuel production. The detection range and specificity are affected by the binding affinity between target metabolites and TF, which is determined by the ligand binding domain (LBD) of TF (Snoek et al., 2020). Tailoring the TF's protein structure for perceiving the desired product is an effective strategy to improve the detection range and specificity of biosensor. Protein engineering through rational design or directed evolution are common methods to optimize the detection range and change the substrate specificity of biosensors (Li, J. et al., 2022; Wu, T. et al., 2022).

To improve the detection range of an isobutanol biosensor, strategies including random and site-directed mutagenesis were applied to the isobutanol-sensitive TF BmoR (Yu et al., 2019a). Error-prone PCR was first utilized to construct a random BmoR mutation library. The single-point mutants were divided into N-terminal

mutant, C-terminal mutant, and central domain mutant and analyzed by modeling the BmoR-isobutanol complex. Functional mutation sites were confirmed by site-directed mutation. Four N-terminal mutants exhibited higher K_m and one C-terminal mutant showed higher output were obtained. Combining these mutation sites on the N-terminal and C-terminal endows BmoR an increased upper detection limit from 40 nM to 100 nM to isobutanol. A similar approach was used to improve the responsiveness towards short-chain alkanes such as pentane or hexane by targeting AlkS for directed evolution (Reed et al., 2012). Through two rounds of mutation, the final AlkS mutant showed higher specificity in response to pentane and hexane than the wild type.

In addition to expanding the detection range, protein engineering through rational design and directed evolution has been applied to alter specificity (Figure 2d). The specificity of TFs with known structures can be tuned by rationally designing their binding domains. Since there have been no natural TFs to sense IPP and DMAPP, two artificial TF-based biosensors were constructed to monitor cellular IPP/DMAPP concentration based on modifying a natural arabinose-responsive transcription factor AraC (Chou and Keasling, 2013). The synthetic IPP-responsive TF consisted of three parts. Part 1 is an IPP binding enzyme (Idi) that replaced the ligand-binding domain (LBD) on the amino-terminal of AraC protein; Part2 is the DNA-binding domain (DBD) of AraC carboxyl-terminal, responsible for binding to the target promoter P_{BAD} . Connecting part 1 with part 2 by the linker (part 3), a synthetic IPP-responsive TF in *E. coli* called IA was constructed. In *S. cerevisiae*, part 2 was the activator domain and DBD of GAL4 protein. The dimerization of Idi initiates the transcription of GAL promoter. In another study, the AraC operon was engineered to respond to DMAPP by substituting its native LBD with *ispS* gene (Liu et al., 2017). The AraC-IspS fusion protein together with P_{BAD} promoter and the downstream *gfp* gene was assembled as a DMAPP-sensing biosensor. On the other hand, the specificity of TFs with no protein crystal structures are available to be engineered by directed evolution (Figure 2d). For example, an isopentanol-responsive biosensor was constructed by directed evolution of AlkS-biosensor to enrich the

biosensor toolboxes (Bahls et al., 2022). The wild type AlkS/P_{alkB} only showed response to n-pentanol. Thus, AlkS was directedly evolved by random mutagenesis for shorter alcohol detection. The C-terminal likely responsible for DNA-binding domain was excluded from mutagenesis. The variant library was enriched in separate batches containing butanol, isobutanol or isopentanol and was selected followed by fluorescence assisted cell sorting (FACS). Two additional rounds of evolutions were applied to further improve the dynamic range of biosensors for the detection of isobutanol. The mutation sites of AlkS variants responding to different alcohols were analyzed and the substrate specificity of variants was tested. The biosensor variants showed higher to isopentanol than to isobutanol. In another study, the AlkS/P_{alkB} based biosensor was further evolved to expand multiple alcohol detection. The biosensor variants showed varied maximum fold-changes in the range of 5-30 mM to n-butanol, isopentanol and isobutanol, respectively, indicating excellent suitability for screening (Wu, T. et al., 2022).

Protein engineering through rational design and direct evolution effectively promotes the detection range and specificity of biosensors by altering the protein structure of TF. With the advancement of protein structure analysis, it is possible to predict and analyze mutation sites, thus guiding us to develop TF-based biosensors with different target metabolites and desired performance.

4. Application of TF-based biosensor in the biosynthesis of advanced biofuels

As important components of the regulatory network of gene expression, TFs play a crucial role in the transcriptional regulation of organisms. As shown in Table 3, transcription-factor-based metabolic engineering regulatory systems have made significant progress in metabolic engineering.

4.1 Biosensor-aided mining for novel metabolic engineering targets

Targeted engineering strategies have been widely applied to enhance microbial production of advanced biofuels. Most of them target pathways directly related to metabolite synthesis, such as strengthening precursor supply pathways and weakening compete pathways (Kumar et al., 2023). However, the production of target compounds is also regulated by many factors indirect to the main synthesis pathway because of the complex cellular network. A large-scale genome-wide library enables unveil new target genes without prior knowledge, but one limitation is the tedious screening process by traditional analysis methods such as gas chromatography. Biosensor, in combination with fluorescence assisted cell sorting (FACS), is a powerful tool to overcome this drawback (Figure 3a).

For example, a genome-wide cDNA library and an acyl-CoA sensor were co-transformed into *S. cerevisiae* and screened by FACS to search candidate genes for enhancing the acyl-CoA pool (Dabirian et al., 2019a). Sixteen candidate genes with high read counts and involved in lipid metabolic processes. Among these candidates, overexpression of 3 undiscovered genes increased the production of fatty alcohol by 80%, as well as changed fatty acid saturation and chain length distribution compared to the control. A similar work was conducted to discover new targets for increasing the production of octanoic acid (Baumann et al., 2021). An octanoic acid biosensor was established, optimized and applied for high-throughput screening of a gene overexpression library via fluorescence-activated cell sorting. Two unexpected genes, *FSH2* and *KCSI*, were identified, neither of which were involved in the fatty acid synthesis pathway. Combinational expression of these two genes increased octanoic acid titers by 55% compared to the parental strain.

To accurately predict possible target genes and fine-tune their expression level to balance metabolic, a combination of genome-scale models (GEMs) that can preliminarily evaluate candidate genes and the CRISPR system to tune gene expression stands out as an attractive solution (Ferreira et al., 2019). The constraint-based flux balance analysis was used to predict candidate gene responsible for

enhancing malonyl-CoA metabolic flux. Then, the malonyl-CoA biosensor was helped to retrieve functional gRNAs targeting candidate genes by multiple rounds of FACS. The most enriched gRNAs enhanced the final product yield by 36% compared to the control. These successful cases demonstrated that TF-based biosensors played a vital role in high-throughput screening for novel genes boosting the product formation by fluorescence-activated cell sorting.

4.2 Confirmation of enzyme function

A synthetic biology platform developed for production of advanced biofuels requires the comprehensive knowledge of each section. Well characterization of the enzymes in the metabolism pathway is the prerequisite for precisely controlling the engineered system. Biosensors play a role in the aid of enzyme function confirmation (Figure 3b). Although the AlkL protein family is widespread in Gram-negative bacteria, little is known about this family. Grant and coworkers (Grant et al., 2014) identified that AlkL facilitates the uptake of C12-C16 alkanes. To further illustrate the role of AlkL in alkane transport, an alkane biosensor based on AlkS TF was utilized to test the rate of uptake of C10, C11, and C14 alkane in an *alkL* expression strain. The biosensor showed an increased output signal upon the presence of C10 and C14 in the *alkL*-induced strain, which indicated the AlkL is involved in alkane uptake.

Another alkane importer, FadL, was verified in a similar way. FadL was hypothesized to be the alkane importer in *E. coli* since it positions in the outer membrane and shares some similar structure and transfer mechanism with AlkL (Call et al., 2016). To confirm this presumption, the alkane biosensor was expressed in both wild type strain and a $\Delta fadL$ strain to sense C8, C10, and C12 alkane. Compared to the wild type strain, the $\Delta fadL$ strain showed decreased fluorescent signals for octane and decan, demonstrating FadL is mainly responsible for medium-chain alkane import.

4.3 Biosensor aided in evolution engineering

The complex genome-wide metabolism of strain restricted rationally engineering approaches to improve microbial metabolite production, which necessitates directed evolution for generating high-yielding strains (Wu, Y. et al., 2022). However, most desired phenotypes of variants are unfavorable to high-throughput screening and selection. Furthermore, directed evolution strategies still suffer from some weakness, such as the accumulation of deleterious mutations or fermentation heterogeneity. TF-based biosensor-mediated evolution engineering effectively improves screening and selection efficiency, as well as compensate for deficiencies in directed evolution (Hwang et al., 2023).

TF-based biosensors have been served as high-throughput screening platform for directed evolution. Implementing fluorescent gene or selective gene as output, biosensors combined with FACS or metabolic addition strategy are contributed to screen and select high yield strain from mutant library. For example, Zhang et al., constructed a α -IPM sensing biosensor, combined with FACS to screen high-producing strains with libraries of mutagenesis enzymes of isobutanol/ isopentanol synthesis pathway (Zhang et al., 2022). The variants exhibiting the top 1% fluorescence output were sorted and iteratively recultivated and reisolated. After the third round of sorting, the strain retransformed with the best mutant achieved 3.2 and 4.8 higher titer of isobutanol and isopentanol, respectively. Similarly, biosensor-aided FACS also enabled high-throughput screening of alkane-producing strains from the enzyme mutant library, leading to a 13-fold increase in the production of long-chain alkanes (Chen et al., 2023). On the contrary, biosensors with selectable markers as output render the product concentration positively correlated with the cell viability. The growth phenotype repeatedly exerts selection pressure on the cell population to enrich overproducers. For example, a tetracycline resistance gene *tetA* was leveraged as a reporter of a butanol-inducible biosensor (Dietrich et al., 2012). This biosensor coupled butanol production with final cell density, identifying a pathway variant yielding a 35% increase in 1-butanol.

Apart from selective function, TF-based biosensors also aid evolution engineering in optimizing mutation process to increase metabolite production. For example, the mutation library generated by strains with unregulated and high mutation rate accumulation of deleterious mutations is amenable to cause cell death. Thus, Chou et al., developed an adaptive control system called feedback-regulated evolution of phenotype (FREP) to achieve variable mutation rates (Chou and Keasling, 2013). This system worked by controlling a mutator gene *mutD5* under the control of a TF-based biosensor responding to isopentenyl pyrophosphate (IPP). Based on this strategy, the mutation rate increased to cause the population diversity when the concentration of IPP is low, while decreased to minimal cell death as concentration IPP is high, thus stabilizing the hyperproducer in the population. The application of FREP in *E.coli* successfully increased IPP production by three times. Beyond the detrimental high mutation rate, heterogeneity induced by naturally inherent nongenetic variation may also cause suboptimal biosynthesis. To unify population heterogeneity, Xiao et al., developed a population quality-control system (PopQC), which uses a fatty acid sensing biosensor to regulate the expression level of survival gene *tetA* (Xiao et al., 2016). Under the selection pressure of tetracycline, the high-performance, nongenetic variants have a growth advantage over the low-performance variants. Thus, the high performers were gradually enriched while the low performers were eliminated. The application of PopQC system demonstrated a 3-fold higher production of FFA than the control.

Overall, TF-based biosensor mediated evolution engineering facilitates the selection of high-yield strains as well as optimizes the mutation process. These examples demonstrated that TF-based biosensors are powerful tools to speed the efficiency of directed evolution and improve metabolite productivity.

4.4 Dynamic control of metabolic pathway

Traditional static methods for improving product titer, such as overexpression of metabolic pathway genes, wakening metabolic flux of competing pathways, and

cofactor balancing, may cause metabolic imbalance, which diminishes the yield of the desired product. On the other hand, dynamic regulation by inducible promoters is susceptible to environmental perturbations and higher operation costs. Thus, cells with the ability to automatically distribute the metabolic flux are urgently needed. Dynamic regulation of TF-based biosensors via the pathway-dependent circuits or cell population responsive strategies opens up new venues for optimizing production efficiency (Mitchler et al., 2021) (Figure 3d).

Pathway-dependent control employs an intracellular biosensor to sense a critical intermediate of the final product, and the cells dynamically adjust the pathway expression according to the metabolic concentrations. Xu et al. (Xu et al., 2014) constructed two malonyl-CoA biosensors with opposing transcriptional activities in *E. coli*. The transcriptional activity of the T7-based malonyl-CoA biosensor was positively related to the level of malonyl-CoA. In contrast, the transcriptional activity of the pGAP-based malonyl-CoA sensor decreased with increased malonyl-CoA. The two gene circuits were integrated as a metabolic switch to balance the supply pathway and the consumption pathway of malonyl-CoA. This metabolic oscillator regulated by intracellular malonyl-CoA level managed the trade-off between cell growth and product formation. Finally, it increased FA titer by 2.1-fold compared to the strain carrying the uncontrolled FA synthesis pathway. In addition, an engineered HpdR/P_{hpdH} biosensor induced by 3-HB was combined with a CRISPRi system for fatty acid pathway repression (Wang, J. et al., 2021). As the intracellular concentration of 3-HB increased, the CRISPRi system was activated and redirected the acyl-CoA flux from fatty acid synthesis to 1,3-butanediol synthesis. This biosensor-CRISPRi circuit resulted in 22.6 g/L production of 1,3-butanediol, achieving 80% of the theoretical maximal yield. Besides balancing the competing and producing pathways, biosensors were also applied as feedback controls to improve the tolerance to biofuel (Siu et al., 2017). Pinene, a cyclic terpene, serves as an attractive precursor for biojet fuel. However, it is toxic to cells. The static expression of efflux pump for pinene export inhibited the cell growth. The expression of the *acrAB* pump was controlled by an optimized pinene inducible biosensor to balance the trade-off between pinene

tolerance and cell growth (Siu et al., 2017).

Since the characterized metabolite-responsive biosensors are limited, a pathway-independent regulatory system is ideal for improving the titer of biofuel. Quorum sensing (QS) genetic circuits respond to cell population, enabling the cell to spontaneously fine-tune gene expression when a certain cell density is reached (Teng et al., 2022). A QS system based on the autoinducer gene *luxR* and transcriptional activator gene *luxI* was applied for the production of biofuel compound bisabolene in *E. coli* (Kim et al., 2017). The pSensor plasmid carries the *luxI* and the *luxR*, and the pRespond plasmid contains the genes of bisabolene synthesis pathway under the control of the P_{lux} promoter. When the autoinducer reaches the threshold level, the LuxR binds to the autoinducer and activates the P_{lux} promoter. Through optimizing the combination of two plasmids, the best-performing group yielded 1.1 g/L bisabolene. Meanwhile, a cytokinin-mediated system from *Arabidopsis thaliana* was integrated with yeast Ypd1-Skn7 pathway for construction of a QS circuit, which was later applied to mediate the protein degradation (Yang et al., 2021). The population-regulated protein degradation system was used to dynamically regulate α -farnesene synthesis, increasing the production by 80%.

These examples showed that TF-based biosensors served as a preferred strategy to relieve the conflict between cell growth and product synthesis. However, these regulatory systems were the single input circuit, unfavorable for the regulation of complex metabolic networks. Further researches to design layered and multi-input circuits are warranted to address more challenging metabolic flux (Dinh and Prather, 2020).

5. Conclusion and perspectives

Along with the increasing transcription factors excavated, TF-based biosensors would play a more significant role in the production of advanced biofuels. Furthermore, the development of synthetic biology ensures the optimization of

dynamic range, specificity, and sensitivity of TF-based biosensors. However, the design, optimization, and application of genetic biosensor remain challenging. There are still relatively few types of TF-based biosensors employed in the production of advanced biofuels and their intermediate metabolites, as well as the inefficient trial-and-error experiments to lift biosensor performance. Future development of biosensors detecting advanced biofuels requires more precise detection, faster design, more diverse types, and more application forms. Implementing these ideas through advanced technologies can overcome the biosensors' difficulty of transferring from laboratory scale to commercial application.

Because of the one-to-one correspondence between chemical inducer and TF, the identification of novel TFs sensitive to advanced biofuels remains a challenge. Recently, bioinformatics, particularly machine learning, has accelerated the process of biosensor selection and construction. Algorithms can be used to discover unknown TFs, rationally design the existing TFs to broaden or change their specificity, as well as tune the biosensor functionality. For example, machine learning has been used to find the uncharacterized TFs and their regulatory rules in the genome-scale transcriptional regulatory network (Lim et al., 2022) (Gao et al., 2018). In addition, the structural characterization of LysG was revealed by docking process, and then its L-lysine binding site was abolished while its L-histidine binding capability remained (Della Corte et al., 2020). Similarly, an array of engineered XylS with response to acetyl salicylate was generated through site-directed mutagenesis, as predicted by protein homology modeling and molecular docking (Monteiro et al., 2019). Furthermore, the promoter strength and dynamic range of biosensor can also be predicted and tuned by machine learning (Ding et al., 2020; Zhao, M. et al., 2021). Such examples demonstrate the significant value of computational-based design in development of biosensors monitoring advanced biofuels. Future work for custom-made biosensor construction may include the *de novo* design TFs based on *de novo* protein design (Quijano-Rubio et al., 2021).

TF-based biosensors can also function without a specific chemical ligand (Pham et al., 2022). In addition to QS systems, temperature-sensitive biosensors and optical

biosensors provide improved autonomous and user-mediated control of the fermentation process, which are more inexpensive and reversible compared to chemical inducers (Richter et al., 2016; Sun et al., 2018). Two optogenetic gene expression systems based on light-inducible TF EL22 were constructed and applied in yeast. Under the exposure of blue light, EL22 undergoes a conformational shift and binds to the cognate promoter P_{C120} . Fusing a transactivation domain to EL22 enables a blue light-gene expression system OptoEXP. Combining with galactose regulon, the response of OptoEXP was inverted to a light-repressible manner (OptoINVRT) (Zhao, E.M. et al., 2021; Zhao et al., 2020; Zhao et al., 2018). These two systems were applied to optogenetically control the isobutanol pathway, yielding 8.5 g/L isobutanol. Further optimization of the OptoINVRT system enhanced isobutanol titer by more than 15% (Zhao et al., 2020). Thermal controlled biosensors have also been demonstrated to have effective dynamic control on the synthesis of L-threonine and polyhydroxyalkanoates (Fang et al., 2020; Wang, X. et al., 2021). These non-chemically induced biosensors designate reversible dynamic control regulatory strategies for production of advanced biofuels.

Recently, a cell-free system is emerging as a low-cost, easy-to-use platform for detecting difficult-to-test or toxic chemicals, eliminating the issue of porting heterologous components into different host organisms (Jung et al., 2022; Silverman et al., 2020). The cell-free system also speeds up the construction and optimization of TF-based biosensors (Jiang et al., 2018). The open reaction environment permits design-built-test iterations of biosensors without the need to re-engineer the host strain, reducing the cycle time.

In this review, we summarized the transcription-factor-based biosensors that detect advanced biofuels and their intermediates and can be used for the synthesis of advanced biofuels. Transcriptional activators and repressors are different in response mechanisms upon the stimulation of inducers. Genetic parts of sensing module, including transcription factors and corresponding promoters, are selected based on previous studies or mined from omics analysis, while the selection of reporter genes in transduction module relies on the practical application of biosensors. The properties

of TF-based biosensors, including sensitivity, specificity, and dynamic range, could be optimized by various synthetic biology strategies. These biosensors, combined with FACS, are used for high-throughput screening to screen new targets with increased titer from a large-scale genome library or to promote the development of evolutionary engineering for key enzymes or pathways. It can also dynamically regulate the synthesis pathway, such as balancing the supply of precursors between cell growth and product formation or reducing the impact of toxic metabolites in the pathway on cell growth. Future development and optimization of biosensors for production of advanced biofuels will focus on bioinformatics technology to explore new regulon, in combination with non-chemical inducible elements to expand genetic toolbox, or using cell-free systems for compound monitoring and rapid prototyping of biosensors.

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Tables

Table1. Overview of genetically biosensor developed and applied to sense advanced biofuel-related chemicals

Inducer	TF (source)/output	Host	Detection range	Application	Reference
IPP	AraC-Idi (<i>E. coli</i>)/P _{BAD}	<i>E. coli</i>	0-10 mM	Increased lycopene production up to 17-fold with FREP	(Chou and Keasling, 2013)
Butanoyl-homoserine lactone	LuxI,LuxR (<i>V. fischeri</i>)/P _{luxI}	<i>E. coli</i>		Achieved 1.1 g/L bisabolene production by QS system	(Kim et al., 2017)
DMAPP	AraC-IspS (<i>E. coli</i>)/P _{BAD}	<i>E. coli</i>	0-10 mM	Screening two IspS mutants from the mutant library	(Liu et al., 2017)
Isoprene	TbuT (<i>R. pickettii</i>)/P _{tbuA1}	<i>E. coli</i>	0.05-8 mM	Monitor intracellular isoprene concentration	(Kim et al., 2018)
Malonyl-CoA	FapR (<i>B. subtilis</i>)/P _{Gal}	<i>S. cerevisiae</i>		Screen ACC1 mutant with enhanced catalytic activity	(Chen et al., 2018)
Malonyl-CoA	FapRPrm (<i>B. subtilis</i>)/P _{Aox} ,P _{Gap}	<i>K. phaffii</i>	< 1 nmol/mgDCW	Regulated malonyl-CoA flux to increase 6-MSA production by 260%	(Wen et al., 2020a; Wen et al., 2020b)
Malonyl-CoA	FapR (<i>B. subtilis</i>)/P _{TEF}	<i>S. cerevisiae</i>		Select genes to enhance malonyl-CoA from gRNA library	(Ferreira et al., 2019)
Malonyl-CoA	FapR (<i>B. subtilis</i>)/P _{T7} ,P _{Gap}	<i>E. coli</i>	0.1-1.1 nmol/mgDCW	yield 2.1-fold improvement in FA titer by dynamic control	(Xu et al., 2014)
Malonyl-CoA	FapR (<i>B. subtilis</i>)/P _{TEF}	<i>S. cerevisiae</i>	2-7 fold	yield 10-fold improvement in 3-HP titer by dynamic control	(David et al., 2016)
Malonyl-CoA	FapR (<i>B. subtilis</i>)/P _{GPM1}	<i>S. cerevisiae</i>		Screen target gene for improving 3-HP production over 100%	(Li et al., 2015)
Isobutanol	BmoR (<i>P. butanovora</i>)/P _{bmo}	<i>E. coli</i>	100-400 mg/L	Isolate isobutanol overproduction strain	(Yu et al., 2019b)
Alkane	AlkR (<i>A. baylyi</i>)/P _{alkM}	<i>A. baylyi</i>	<40 mM	Selection and optimize different	(Lehtinen et al., 2017)

Alkane	AlkS (<i>P. putida</i>)/P _{alkB}	<i>E. coli</i>		alkane synthesis pathway Verification of alkane importer function	(Lehtinen et al., 2018) (Call et al., 2016; Grant et al., 2014)
Alkane	AlkS (<i>P. putida</i>)/P _{alkB}	<i>E. coli</i>	52-107 mg/L (EC ₅₀)	High-throughput screening of highly long-chain alkane-producing strains	(Chen et al., 2023)
C6 fatty acid C7 fatty acid C8 fatty acid	War1p (<i>S. cerevisiae</i>)/P _{PDR12}	<i>S. cerevisiae</i>	0.01-2 mM 0.01-1.5 mM 0.01-0.75 mM	Genes screened by biosensor increased octanoic titer by 55%	(Baumann et al., 2018) (Baumann et al., 2021)
Acyl-CoA	FadR (<i>E. coli</i>)/P _{lacUV5}	<i>E. coli</i>	0.01-1 mM	Increase FAEE titer to 1.5 g/L by dynamic regulate metabolic pathway	(Zhang, F. et al., 2012)
Acyl-CoA	FadR (<i>E. coli</i>)/P _{TEF}	<i>S. cerevisiae</i>	0.01-0.3 mM	Screen genes for enhancing fatty acyl-CoA pool	(Dabirian et al., 2019a)
Acyl-CoA	FadR (<i>E. coli</i>)/P _{TEF}	<i>Y. lipolytica</i>		Decouple cell growth and production phase	(Park et al., 2021)

Figures

Figure 1

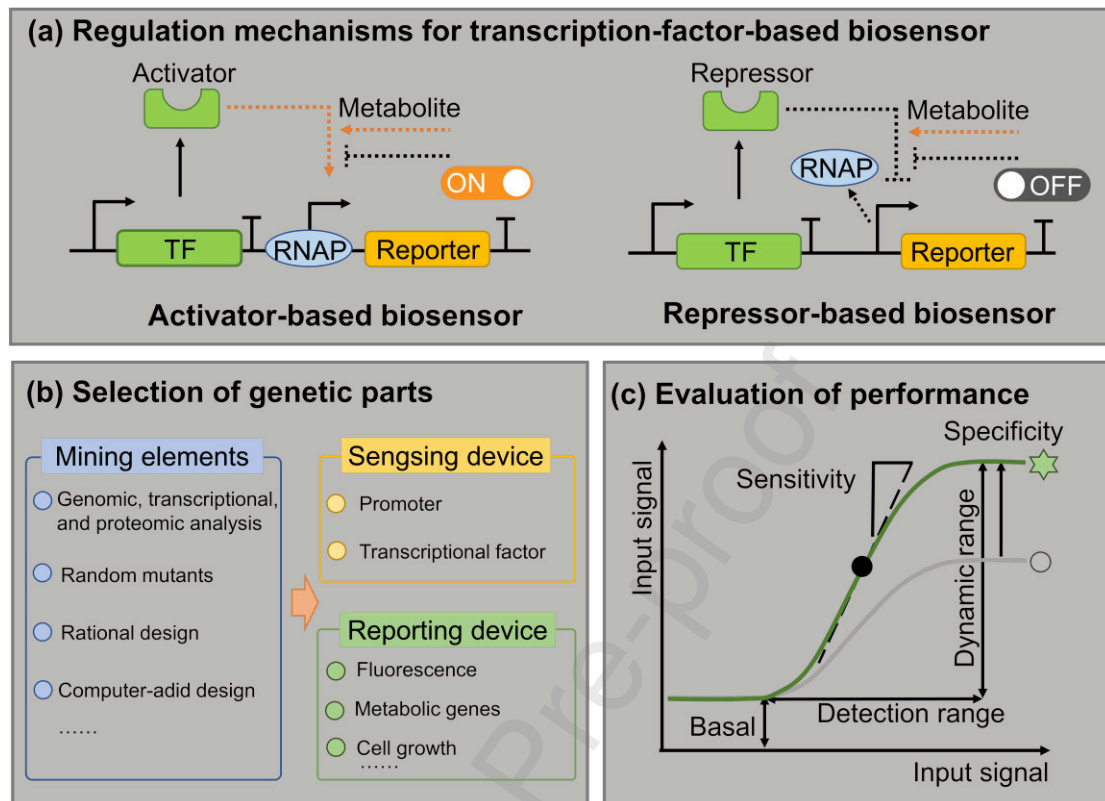
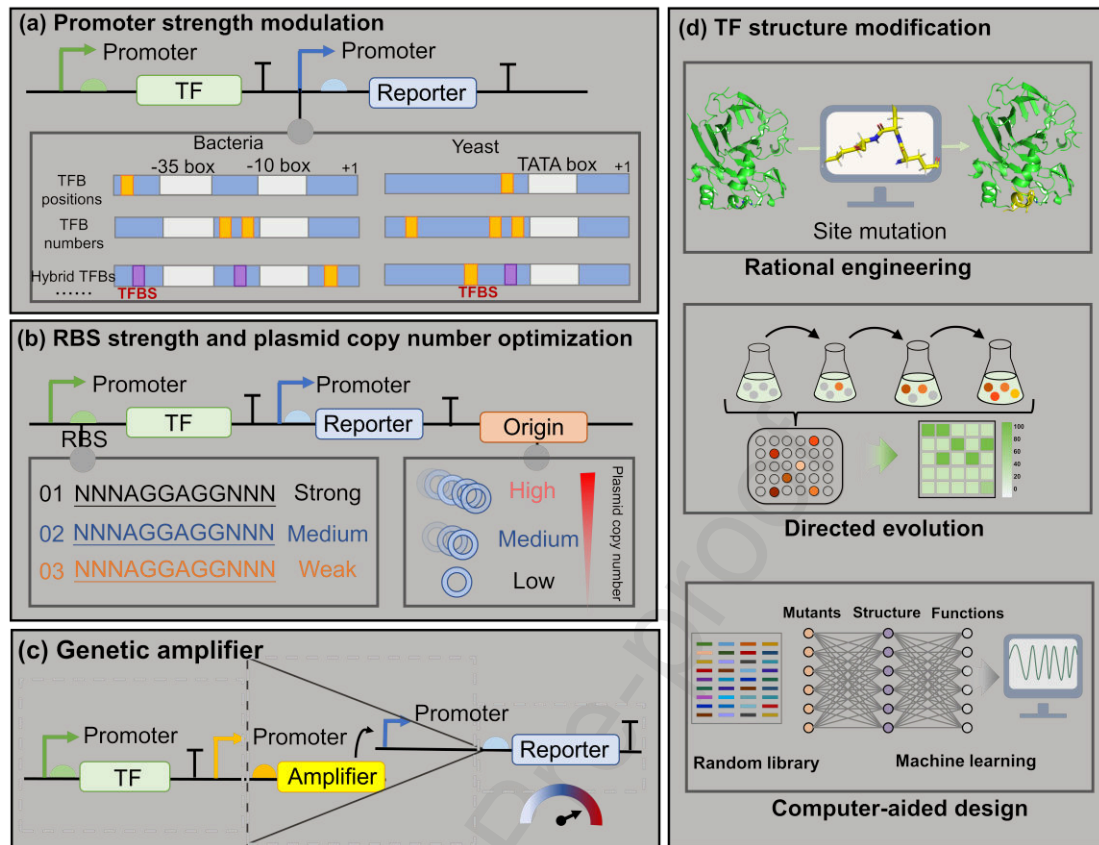


Figure 2



Figur3

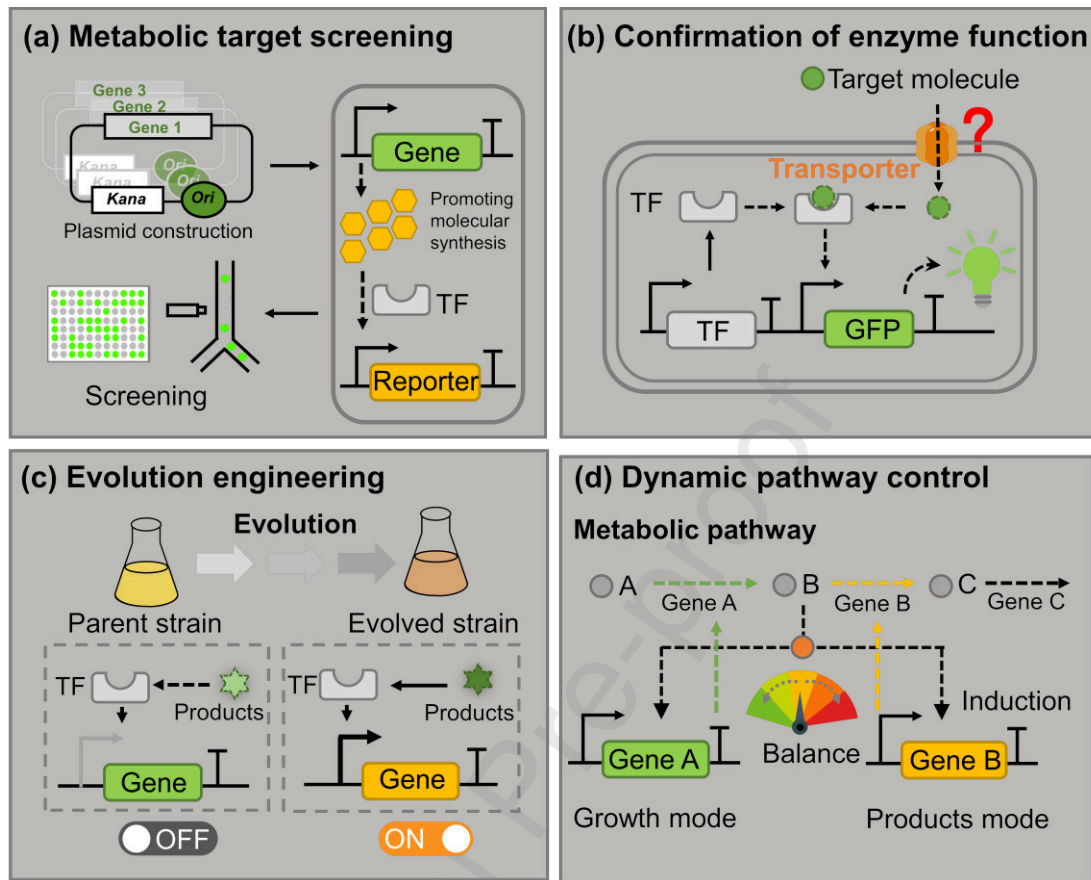


Figure caption

Figure 1. TF-based biosensor construction and performance evaluation. (a) Regulation mechanism of transcriptional activator and repressor. TF-based biosensor contains an activated TF or a repressive TF, the promoter and the reporter gene under the control of promoter. RNA polymerase (RNAP) is considered as a regular configuration in the TF-based biosensor. The activator or repressor undergoes a conformational change upon binding to the inducer metabolite, and the activator/repressor-inducer complex promotes or prevents the binding of RNAP to the promoter of reporter gene, thus initiating or inhibiting the transcription of reporter gene. (b) Selection of genetic parts of TF-based biosensor. Genetic parts of TF-based biosensor are divided into sensing device and reporting device. Sensing device contains TF and its cognate promoter, which are discovered by omics analysis or modulated by protein engineering of existing biosensor; the reporting device is the reporter gene of the biosensor. Depending on the application, various reporting devices are available as biosensor output, including fluorescence cell growth and metabolic flux. (c) Response curve of biosensor. Key properties determine biosensor performance including sensitivity, the slope of response curve; operation range, the detectable concentration range of input signal; dynamic range, the ration of maximum output and minimum signal; specificity, the ability to distinguish target metabolite (green star) from other metabolites (gray circle); basal, the background expression level of biosensor.

Figure 2. Strategies for improving TF-based biosensor performance. Strategies to modify the dynamic range and sensitivity of biosensor (a-c). (a) Modulating promoter strength, including changing the number and position of transcription factor binding sites (TFBS, orange box and purple box) on the promoter, or use hybrid promoter containing two kinds of TFBS to modulate cognate promoter strength, thus regulating the expression level of reporters. (b) Modulating the expression level of TF or the whole biosensor circuit by engineering RBS strength of TF's promoter or by adjusting plasmid copy number, respectively. "NNNAGGAGGNNN" represents general RBS

sequence. (c) Implementing genetic amplifier to amplify the output signal. TF activated by inducer activates the promoter of amplifier gene. In turn, the amplifier gene initiates the expression of reporters. (d) The specificity and detection range of biosensors can be tuned by modifying the structure of TF. A TF variant with enhanced affinity to target metabolite can be obtained by rational engineering, directed evolution or computer-aided design.

Figure 3. Applications of TF-based biosensor in the biosynthesis of advanced biofuels. (a) TF-based biosensor assists in uncovering novel genes to improve product titer. The biosensor and the gene overexpression library were co-transformed to the strain, the resulting mutation library was screened for novel genes boosting the product formation by fluorescence-activated cell sorting (FACS). (b) TF-based biosensor implemented to verify the function of transporter. To demonstrate the transporter (orange rectangle) facilitates uptake of alkane (green circle), the transporter protein was co-expressed alongside an alkane biosensor. If the transporter mediates the import of alkane, then an improved fluorescence intensity would be expected when the extracellular alkane concentration increased. (c) TF-based biosensor driven evolution engineering. The TF-based biosensor activates the promoter controlling the fluorescent gene or resistance gene expression. This fluorescence or cell-dominant phenotype repeatedly put selection pressure on the variant library to enrich high-yield strains. (d) TF-based biosensor dynamically regulates the metabolic pathway. Coupling with pathway gene expression as output, biosensor allows engineered cell factory dynamically regulate metabolic flux between product formation and cell growth pathway by responding to the metabolic intermediate concentration or cell population, thus improving the overall productivity and fitness. Grey circle A, B, C presents pathway intermediates.

Declaration of interests

Authors declare no conflict of interest.



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