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Review

Genetically encoded biosensors enabled high-throughput screening of microbial cell factories

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ABSTRACT

Genetically encoded biosensors provide powerful tools for coupling desired phenotypes to detectable outputs and have been extensively developed to detect a wide range of natural and unnatural products. When integrated with diverse high-throughput screening (HTS) approaches, these biosensors enable efficient product-driven screening across various throughputs, thereby expediting the engineering and optimization of microbial cell factories to produce various target compounds. For effective HTS of microbial cell factories, biosensors need to possess certain crucial characteristics. The performance features of biosensors significantly influence their application potential in HTS and can be precisely engineered through synthetic biology strategies. Furthermore, to ensure biosensor-driven HTS, additional engineering and optimizations of the biosensors are often required to increase the success rate and reduce false positives in the screening process. This review discusses the essential features of genetically encoded biosensors designed for HTS and then summarizes the latest advances in biosensor engineering for HTS purposes via synthetic biology strategies. Following this, the challenges and optimization of biosensors to adapt to different HTS processes are also discussed and exemplified. Finally, the key concerns and research prospects of developing biosensors for HTS applications are highlighted. Overall, this review provides comprehensive guidance on the engineering of genetically encoded biosensors and their applications in HTS for developing microbial cell factories to produce diverse target compounds.

1. Introduction

Utilizing microbial cell factories to transform bio-based materials into chemicals offers a promising pathway towards a sustainable economy [1]. Numerous advances have demonstrated the potential of discovering new molecules and optimizing metabolite production from microorganisms through various rational and random engineering strategies. Additionally, genome-scale genotype-phenotype analyses is becoming a research focus for identifying beneficial gene targets for metabolic engineering and exploring efficient biosynthesis mechanisms of target products [2]. Depending on the strategies for generating genetic diversification, mutant libraries can reach sizes of 10^9 variants [3], among which only a very limited population represents significantly improved performance. As the capacity for developing strains to produce various target products and constructing vast and intricate strain libraries increases, efficient screening method to identify desired variants often becomes a bottleneck in the Design-Build-Test-Learn (DBTL) cycle of metabolic engineering and synthetic biology [3,4].

High-throughput screening (HTS) is a basic enabling technology to pinpoint the most efficient whole-cell biocatalysts in vast genetically diverse strain libraries. Main HTS approaches include agar plate-based screening, multi-well microtiter plate (MTP)-based screening, fluorescence-activated cell sorting (FACS), and fluorescence-activated droplet sorting (FADS), with throughputs ranging from 10^2 to 10^7 events/h [5]. Many advances have demonstrated the effectiveness and efficiency of HTS approaches in processing vast mutant libraries in engineering microbial cell factories to produce target compounds, although different analysis techniques are employed for precise evaluation of production after pre-screening. Traditionally, for target molecules lacking chromogenic or fluorescent characteristics, researchers have to resort to chromatography or mass spectrometry to evaluate variant performance, which is time-consuming and usually involves complex sample preparation [4]. To address this challenge, genetically encoded biosensors have been devised to quantify diverse products without the need for direct titer evaluation. Biosensor-based HTS can convert the production level of target products in microbial cell factories into noticeable traits like

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growth, color, and fluorescence, which will significantly simplify and accelerate performance evaluation process of a large number of variants.

Genetically encoded biosensors are typically characterized by their significant programmability, flexibility, and versatility [6]. When integrated with various HTS approaches, these biosensors enable efficient screening at different throughput levels, thereby accelerating the DBTL cycle for the rational construction of microbial cell factories. However, due to differences in construction principles and genetic circuits, various biosensors may represent different detection threshold, induction concentrations, operational conditions, and analyte types, leading to distinct application scenarios. Some specific biosensors are particular suitable for the use in the product-driven HTS of microbial cell factories. The performance characteristics of biosensors, such as sensitivity, ligand specificity, operational range, and dynamic range [7], are crucial for assessing their suitability in practical applications. These features can be carefully tailored to meet different screening requirements through synthetic biology strategies. Furthermore, biosensors integrated with different HTS approaches often require specific characteristics. For instance, biosensors that assist FACS and FADS processes often need to report fluorescence signals for detection [8]. Moreover, biosensors typically require further genetic modifications to enhance the precision and success rate of screening, while also reducing false positives.

In this review, we will begin by discussing the crucial characteristics of genetically encoded biosensors tailored for HTS applications. We will then summarize the advancements made over the past five years in the engineering and modification of these biosensors through synthetic biology strategies specifically aimed at HTS in the context of microbial cell factories. Subsequently, we will detailly discuss the optimization of genetically encoded biosensors to accommodate various HTS processes, which is the primary focus of this review. Finally, we will highlight the challenges and outline the research prospects associated with the development of biosensors for HTS applications in engineering microbial cell factories.

2. Types of biosensors and their suitability for HTS

Genetically encoded biosensors are versatile tools for monitoring the production of target products in microbial cell factories, converting otherwise undetectable production details into measurable outputs. In the context of HTS for microbial cell factories, the types of target compounds and their titer ranges are diverse, and the physical conditions of fermentation, as well as the physicochemical environment of the fermentation broth, are complex. Consequently, biosensors used in HTS for microbial cell factories should possess certain characteristics. The sensitivity and operational range should be moderate, typically in the micromolar to millimolar range, to enable precise concentration-dependent changes in response to the target product yields while avoiding oversensitivity [9]. A broad dynamic range is required to effectively distinguish varying production levels of target compounds. High ligand specificity is essential to ensure accurate detection of target compounds in the complex fermentation cultures [10]. Robustness is also valuable for conferring stability to biosensors in challenging physicochemical environments. Besides, programmability and flexibility are highly desirable attributes, allowing for parameter adjustments through synthetic biology to meet the requirements of different titer ranges and ensuring adaptability across various microbial chassis.

Genetically encoded biosensors typically consist of sensing and reporting elements [11]. The sensing element undergoes specific changes upon detecting the presence of the target compound and transduces this signal to the reporting element, which then produces a measurable output. Depending on the type of sensing element, genetically encoded biosensors can be categorized into several types: single-component protein biosensors [12], two-component system (TCS)-based biosensors, riboswitch-based biosensors, and transcription factor (TF)-based biosensors (Fig. 1). Due to the differences in sensing and reporting elements, each type of biosensor possesses unique characteristics and exhibit different extent of suitability in HTS applications.

Single-component protein biosensors, equipped with reporting elements such as Förster resonance energy transfer (FRET) or circularly permuted fluorescent proteins (cpFPs), can directly convert the binding of target compounds into measurable outputs [13–15]. These biosensors enable real-time response of target compounds, typically within milliseconds to minutes, making them well-suited for intracellular imaging of ions and compounds [12]. However, their limited dynamic range restricts their applications in HTS, where a broad signal variation is often necessary. TCS-based biosensors are applied in the fields of heavy metal monitoring, gastrointestinal disease diagnostics, and biorefinery processes [16–18]. They can be valuable for detecting extracellular compounds due to differences between intracellular and extracellular concentrations of target metabolites. However, their limited detection range may restrict their broader use in HTS of microbial cell factories [19]. Riboswitch-based biosensors are widely used in food safety detection and pollutant monitoring [20,21]. This type of biosensors usually exhibits high sensitivity at nanomolar levels [22,23], and this may lead to an unsuitable operational range for HTS of microbial cell factories, whose production levels are typically above the micromolar range [9]. Currently, TF-based biosensors are highly valued in HTS applications. They amplify molecular recognition events through gene expression changes [24], providing the broad dynamic range needed to distinguish varying production levels of target compounds. The modular configuration of TF-based biosensors confers high programmability, allowing customized engineering through synthetic biology approaches to meet various specific screening requirements [6]. Additionally, the conserved molecular recognition mechanism among the TF, the operating DNA sequence, and the effector molecule provides a robust modular foundation for cross-species transfer [25]. Although chassis-specific tuning is often requisite, this intrinsic orthogonality facilitates their widespread adoption in various HTS scenarios. To provide a clear guide for selecting the appropriate sensing module, the key characteristics, advantages, and limitations of these four biosensor categories are systematically compared in Table 1.

3. Rational engineering of biosensors for better performances

To meet HTS demands, optimizing biosensor characteristics is essential for reliable and efficient detection. Key performance parameters of biosensors include ligand specificity, sensitivity, operational range, and dynamic range [26] (Fig. 2a). This section will discuss the strategies to enhance the performance features of single-component protein biosensors, riboswitch-based biosensors, and transcription factor (TF)-based biosensors for HTS usage. Cao et al. have comprehensively summarized and introduced the design and fine-tuning methods for TCS-based biosensors [19], which will not be reiterated here.

Generally, the engineering of biosensors relies on two mainstream strategies: rational design and directed evolution. Rational design is a knowledge-driven approach that relies on structural information and computational modeling. It typically involves site-directed mutagenesis of key residues to predictively tune sensing parameters such as affinity and specificity [27]. This strategy is efficient but requires high-resolution structural data and a deep understanding of structure-function relationships. In contrast, directed evolution mimics natural selection and is particularly powerful when structural information is lacking or when complex phenotypes are desired. This approach involves creating genetic diversity through random mutagenesis followed by HTS to isolate variants with improved properties [28,29]. While labor-intensive, directed evolution allows for the exploration of a broader sequence space beyond intuitive design.

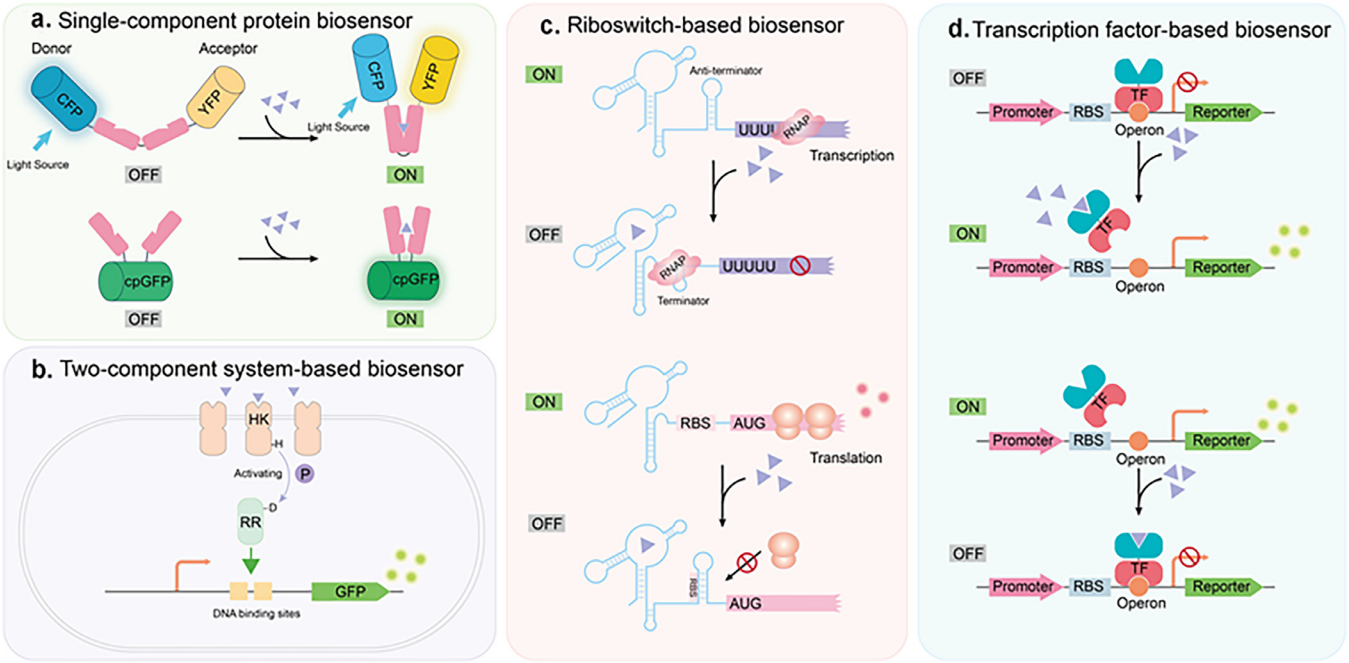

Fig. 1. Illustration of construction principles and working mechanisms of three types of genetically encoded biosensors. (a) Single-component protein biosensor schematic. (b) TCS-based biosensor schematic. (c) Riboswitch-based biosensor schematic. (d) TF-based biosensor schematic. CFP, cyan fluorescent protein. YFP, yellow fluorescent protein. cpGFP, circularly permuted green fluorescent protein. HK, histidine kinase. RR, response regulator. H, histidine residue. P, phosphoryl. D, aspartate residue. GFP, green fluorescent protein. RNAP, RNA polymerase. RBS, ribosome-binding site.

Table 1
Comparison of characteristics, advantages, and limitations of different genetically encoded biosensors.

Biosensor type	Sensing mechanism	Response time	Key advantages	Main limitations
Single-component protein biosensors	Ligand-induced conformational change (FRET/cpFP)	Fast (ms to min)	• Real-time *in situ* monitoring • High spatial resolution • No transcription/translation lag	• Limited dynamic range • Difficult to engineer for new ligands • Often restricted to intracellular analytes
Riboswitch-based biosensors	RNA aptamer binding & conformational switching	Medium (Co-transcriptional)	• Compact genetic size • Low metabolic burden	• Narrow dynamic range • Prone to early saturation • Complex rational design
TF-based biosensors	Ligand-dependent transcriptional activation/repression	Slow (min to hours)	• Broad dynamic range • Modular and high programmability • Abundant natural candidates	• Metabolic burden from protein expression • Response delay • Cell-to-cell heterogeneity
TCS-based biosensors	Phosphorylation cascade (Histidine kinase + regulator)	Slow (min to hours)	• Excellent for sensing extracellular environments • High sensitivity and signal amplification	• Complex genetic architecture • Potential crosstalk with chassis signaling • Slower than allosteric biosensors

3.1. Single-component protein biosensors

Single-component protein biosensors consist solely protein modules, including both ligand-binding domains and signal-transduction domains [30]. This intrinsic feature makes them highly amenable to optimization through protein engineering, enabling application-oriented improvements across multiple performance dimensions. Parameters of specificity, sensitivity, and operational range are fundamentally associated with ligand-binding affinity of protein (Fig. 2b), typically characterized by the dissociation constant (K_d) or the half-maximal effective concentration (EC_{50}). Ligand specificity is reflected in the relative differences of K_d/EC_{50} values across structurally similar molecules; sensitivity is largely determined by the absolute affinity for the cognate ligand; and operational range is governed by the shape and span of the dose–response curve. Accordingly, engineering strategies that remodel the ligand-binding pocket—via directed evolution or structure-guided site-saturation mutagenesis of key residues—enable rational modulation of K_d/EC_{50} values, thereby fine-tuning biosensor selectivity, affinity, and concentration-dependent responsiveness [31–35]. In contrast, optimization of the dynamic range often requires engineering of linker sequences and insertion sites [36,37]. For FRET-based biosensors, the length and flexibility of the donor–acceptor linker strongly influence the conformational coupling efficiency [38]. Similarly, in cpFP-based biosensors, the choice of the insertion site within the sensing domain and the properties of the connecting peptide determine how efficiently ligand-induced conformational changes are transmitted to the fluorescent protein [39]. By constructing libraries of linkers with varied lengths and sequences, followed by HTS, it is possible to identify variants that maximize conformational responsiveness while maintaining proper protein folding and stability [40].

Overall, the engineering of single-component protein biosensors generally follows an iterative cycle of module selection, mutagenesis-driven

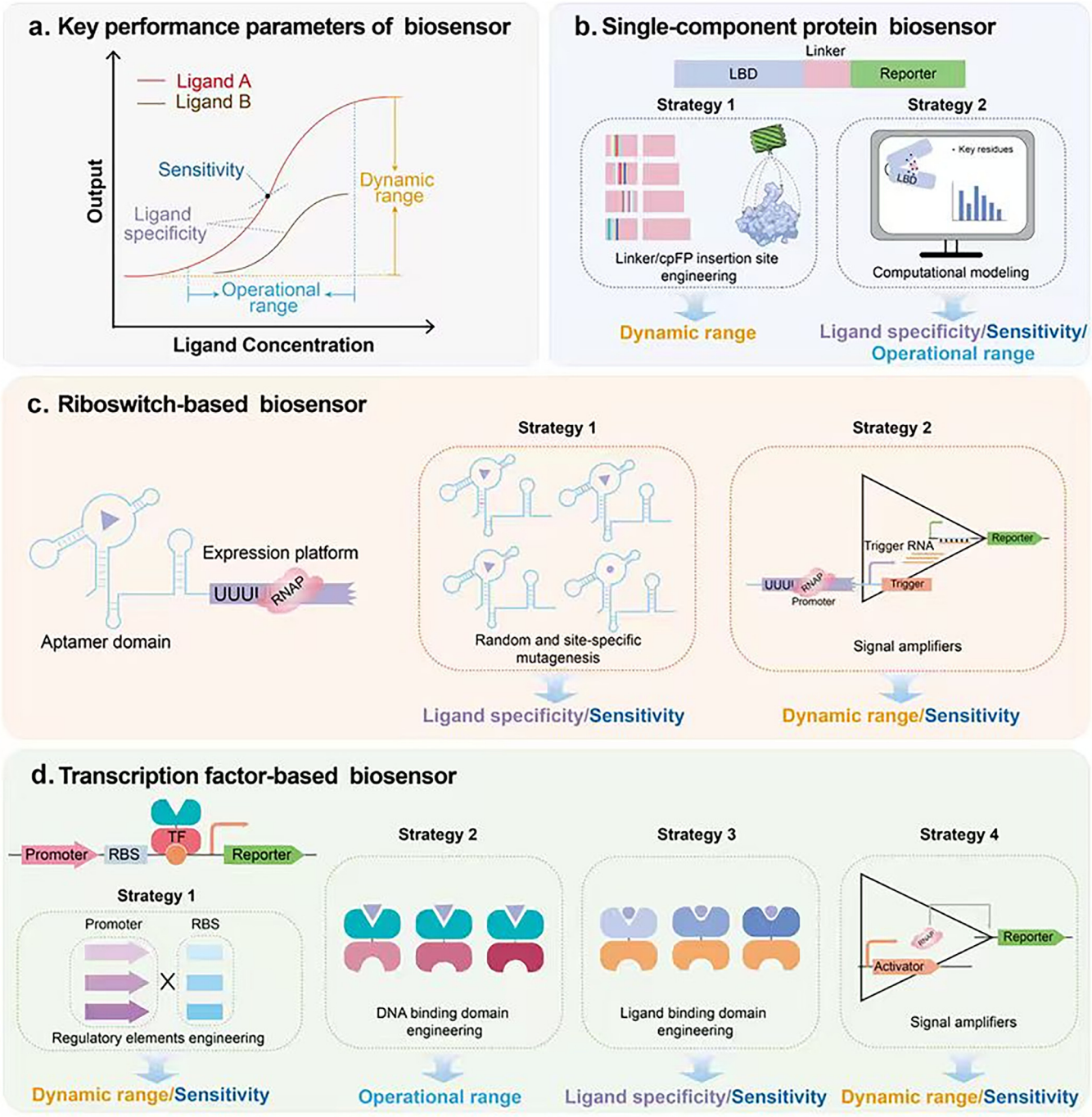

Fig. 2. Strategies for biosensor engineering to improve their performance characteristics. (a) Key performance parameters. Schematic definitions of sensitivity, dynamic range, operational range, and ligand specificity. (b) Single-component protein biosensors. Strategy 1: Engineering linker properties (length, sequence, or composition) optimizes the dynamic range. Strategy 2: Computational modeling of the LBD tunes ligand specificity and sensitivity. (c) Riboswitch-based biosensors. Strategy 1: Aptamer mutagenesis alters ligand specificity (*e.g.*, shifting recognition from ligand A [triangle] to ligand B [circle]) or sensitivity. Strategy 2: Signal amplifiers enhance dynamic range and sensitivity. (d) Transcription factor-based biosensors. Strategy 1: Tuning regulatory elements adjusts dynamic range and sensitivity. Strategy 2: Engineering the DNA-binding domain shifts the operational range. Strategy 3: Engineering the ligand-binding domain tunes ligand specificity and sensitivity. Strategy 4: Signal amplification circuits boost both dynamic range and sensitivity. Engineering strategies for TCS-based biosensors are not depicted here to avoid redundancy, as they have been comprehensively summarized recently by Cao et al. [19].

diversification, HTS, characterization and refinement. HTS is critical for identifying functional variants from vast libraries, yet conventional approaches are limited by low throughput, high labor requirements, and insufficient resolution of subtle performance differences. To overcome these bottlenecks, droplet-based microfluidics and other advanced high-throughput technologies have emerged as powerful solutions [41–43]. By rapidly generating and sorting millions of variants with single-cell resolution, these platforms accelerate enrichment of high-performance biosensors and markedly expand the design space for rational engineering.

3.2. Riboswitch-based biosensors

Riboswitches-based biosensors comprise two core functional modules: an aptamer domain (AD) that recognizes the ligand and an expression platform (EP) that converts the ligand-binding event into a gene regulatory signal [44]. Because this cis-acting mechanism operates without protein cofactors, riboswitches as ideal scaffolds for genetically encoded biosensor creation in synthetic biology. Performance optimization focuses on the precise engineering of the sequence, structure, and interaction between the two modules. Specificity and sensitivity are improved by reshaping the AD binding pocket to precisely control molecular recognition of the cognate ligand (Fig. 2c). Directed-evolution or rational-design approaches guided by high-resolution structures help identify key nucleotide mutations that yield new binding profiles [45–47]. A classic example is the synthetic theophylline riboswitch: site-directed mutagenesis and selection at key sites yielded variants that bind caffeine (a structural analogue of theophylline) with high specificity, eliminating cross-reactivity and demonstrating the ability to engineer new recognition specificities [48]. This strategy has been widely applied to develop novel synthetic riboswitches responsive to small molecules, among which the tetracycline aptamer serves as a representative and widely adopted examples [49].

To achieve large gains in dynamic range and sensitivity, a breakthrough strategy employs a modular cascade that decouples sensing from signal amplification (Fig. 2c). In this architecture, an upstream riboswitch serves as a high-specificity sensor that controls production of an intermediate signal molecule, such as a trigger RNA (trRNA). This trRNA subsequently activates a downstream amplifier—typically a toehold switch—that maintains ultra-low background. Given the potent signal amplification of toehold switch, the cascade can boost the signal-to-noise ratio and fold-change by several orders of magnitude [50–52]. Crucially, modular coupling ensures high orthogonality between components, laying a robust foundation for constructing complex, predictable genetic logic circuits.

3.3. TF-based biosensors

TF-based biosensors consist of a sensing element and a signal-transduction element. As allosteric proteins, TFs transduce signals via conformational changes, and minor adjustments to their allosteric pocket directly determine specificity and affinity [25]. Overall performance—sensitivity, dynamic range, and operational range—is also governed by downstream genetic circuit components, including promoter activity, ribosome binding site (RBS) strength, and reporter copy number[53]. These parameters dictate how molecular recognition is converted into quantifiable output and influence both signal gain and basal expression. Optimization must therefore proceed at two interconnected levels: molecular precision of ligand binding and genetic control of signal gain/basal expression (Fig. 2d). Furthermore, high-performance biosensor design often faces intrinsic trade-offs: stringent binding sites demanded for high specificity can shift EC_{50} outside the desired operational range, while expanding the dynamic range often raises basal signal or response threshold. Contemporary strategies therefore aim to mitigate these intrinsic trade-offs through systemic decoupling (*e.g.*, separating recognition from amplification). A systematic summary of performance metrics, challenges, and optimization strategies for TF-based biosensors is provided in Table 2.

3.4. Challenges and artificial intelligence (AI)-driven solutions for developing genetically encoded biosensors in HTS

Genetically encoded biosensors based on various mechanisms have proved powerful for HTS of microbial cell factories, yet their widespread adoption is hindered by both mechanistic and practical limitations. A primary bottleneck is the opacity of signal-to-function coupling: whether signal transduction relies on ligand-induced conformational changes, transcriptional regulation, or RNA structural rearrangements, the underlying pathways are typically nonlinear and lack interpretable, quantitative models. Consequently, biosensor design and optimization remain heavily empirical, relying on random mutagenesis and screening [53,74,75]. A second, intrinsic constraint is the trade-off among dynamic range, specificity, and stability. Enhancing sensitivity may elevate background noise or response latency, while tightening specificity often reduces dynamic range impeding simultaneously optimization across multiple parameters [76,77]. Furthermore, portability remains a critical challenge, particularly when transitioning to emerging industrial chassis such as *Streptomyces* and *Pseudomonas putida*. Distinct from model organisms, these non-model chassis often lack well-characterized orthogonal genetic parts (*e.g.*, promoters and RBS), leading to unpredictable transcriptional activation. Moreover, chassis-specific metabolic backgrounds—such as rapid ligand degradation or high basal metabolite flux—can severely skew the dynamic range of biosensors originally developed in model organisms, necessitating laborious refactoring using native regulatory elements or broad-chassis-range vectors [78]. Finally, current DBTL cycles remain low-throughput and lack standardized, automated workflows, leading to long development times and high labor costs. AI offers transformative strategies to overcome these bottlenecks by shifting the biosensor design paradigm from trial-and-error to data-driven prediction and de novo generation. Specifically, advanced machine learning (ML) technologies—including deep learning, graph neural networks, and generative AI—have been successfully deployed to capture the nonlinear sequence-function relationships of genetic parts.

For single-component protein biosensors, AI approaches fall into two categories: performance optimization and *de novo* design [79,80]. To optimize existing biosensors, ML ensembles are used to predict the effects of mutations on biosensor kinetics and dynamic range. A prominent example is the work by Wait et al., where an ensemble of regression models trained on GCaMP variants enabled an *in silico* screen of >1400 sequences, identifying variants with faster kinetics and larger signal changes ($\Delta F/F_0$) than prior generations [79]. For creating entirely new biosensors, generative diffusion models like RFdiffusion allow for the backbone design of custom binding proteins, while LigandMPNN facilitates the precise generation of amino acid sequences conditioned on specific small-molecule ligands [81,82]. This combination effectively allows for the *de novo* creation of ligand-binding domains for non-natural metabolites, expanding the range of detectable analytes beyond natural templates.

For riboswitch-based biosensors, AI is tackling the complexity of RNA folding thermodynamics. While traditional physics-based algorithms remain vital, deep reinforcement learning frameworks have emerged to tackle the complex constraint satisfaction problems in riboswitch design. These generative approaches can efficiently navigate vast search spaces to create diverse libraries of variable-length candidates that satisfy the specific conformational switching requirements [83,84]. Hybrid strategies are also emerging, for instance, coupling deep batch Bayesian optimization with HTS data has successfully navigated the vast sequence space to engineer hybrid riboswitches that execute precise NAND Boolean logic in yeast [85].

For TF-based biosensors, AI significantly accelerates development by addressing two critical bottlenecks: the discovery of novel sensing modules and the precise prediction and optimization of cis-regulatory elements. First, regarding the mining of new sensing modules, expanding the repertoire of transcription factors is essential for detecting diverse analytes. Deep learning models like DeepTFactor utilize convolutional neural networks to screen vast proteomic datasets for predicted DNA-binding domains. This tool successfully identified 332 candidate TFs in *Escherichia coli* K-12, and similar deep learning strategies are now being extended to mine genomic data from non-model organisms, unlocking native sensing modules for industrial chassis [86]. Second, AI-driven strategies facilitate the precise characterization and engineering of genetic circuits, ranging from TF binding sites (TFBSs) prediction to the fine-tuning of promoters and RBS. For accurate binding site identifi-

Table 2
Performance challenges and engineering strategies for TF-based biosensor optimization.

Feature	Challenge	Molecular engineering strategy	Genetic engineering strategy
Ligand specificity	Broad target selectivity	Directed evolution/Structure-guided saturation mutagenesis [54–60]	N/A
Dynamic range	Constrained upper/lower limits	Altering ligand binding affinity [61,62]	RBS/Promoter strength tuning [62–64], signal amplification [65,66]
Sensitivity	High/low limit of detection	Tuning signal transduction efficiency via directed evolution or structure-guided mutagenesis [61,67,68]	Reporter gene optimization, increasing TF/reporter gene copy number [69–71]
Operational range	EC_{5n}–target concentration mismatch	Altering operator or ligand binding affinity [72]	Tuning TF expression via RBS engineering, adjusting regulator–operator binding affinity [69,73]

N/A: Not Applicable.

cation, advanced architectures like MLSNet integrate multi-scale convolutional fusion with DNA shape features to predict TFBS with high precision, outperforming sequence-only models [87]. Building on prediction, AI algorithms have further streamlined the generative design and fine-tuning of regulatory elements to address the portability challenge. For instance, deep generative models have been integrated into toolkits like GPro to design synthetic promoters with tunable strengths across diverse species, while Gaussian process-based active learning has proven effective in optimizing RBS sequences, achieving a 34 % increase in translation rates with minimal screening [88,89]. These data-driven approaches allow for the rapid "refactoring" of biosensors, ensuring robust performance when transferring genetic circuits from model to non-model strains.

Crucially, these AI approaches are often not standalone but integrated into an active learning loop: the model suggests a small batch of high-potential variants, which are then tested via HTS, and the resulting data is fed back to refine the model. This iterative strategy significantly reduces the experimental screening burden and converts biosensor development into a rational, predictive framework.

4. Applications of genetically encoded biosensors in HTS for microbial cell factories

Generally, the accessibility of assembled and modified biosensors does not guarantee successful applications in HTS. Implementing a biosensor in a specific HTS scenario typically requires careful consideration and additional optimization, as their suitability can vary significantly when integrated with different screening methods, depending on the intended use. Furthermore, ensuring effectiveness, efficiency, and accuracy—factors that may vary across applications—is a crucial concern in biosensor-driven screening. Consequently, selecting an appropriate screening platform is pivotal for the success of microbial cell factory engineering. Critical factors such as library size, assay sensitivity, and the localization of the target product (intracellular *vs.* secreted) must be carefully weighed against throughput requirements. To assist researchers in navigating these technical choices, we have summarized the key performance metrics of the four mainstream HTS methods in Table 3. Complementing these quantitative metrics, Fig. 3 provides a schematic overview of these screening modalities and their corresponding biosensor design strategies, illustrating the seamless incorporation of genetically encoded biosensors into diverse HTS systems.

4.1. Biosensor-driven agar plate-based screening

Biosensor-driven agar plate-based screening offers a relatively straightforward procedure, enabling visual detection without the need for specialized equipment. In most cases, biosensors that convert target concentrations into outputs indicating bacterial growth or colorimetric signals can facilitate efficient preliminary screening of large mutant libraries during agar plate incubation [90,104–106]. Mutants with high production tend to survive better under selective conditions or accumulate more pigment for easy visual recognition (Fig. 3a). These promising mutants are then collected for the subsequent round of screening or precise production evaluation. For example, a PYR1-based biosensor system in *Kluyveromyces marxianus* enabled visual screening of monoterpene-overproducing mutants on agar plates. By coupling monoterpene accumulation to a colorimetric growth phenotype, colonies with stronger reporter responses were readily identified without specialized instrumentation, facilitating preliminary selection of improved producers from genome-wide mutant libraries [90]. The reporter gene *lacZ* was coupled to construct the salicylate-responsive TF-based biosensor, enabling rapid and effective identification of optimal gene expression patterns. Colonies were visually screened based on their blue color intensities, leading to the identification of a variant showing a 123 % improvement in salicylate production [91]. Similarly, a chromogenic L-lysine biosensor was constructed in *Corynebacterium glutamicum* by expressing phytoene desaturase under the control of the transcriptional regulator LysG. The red pigment lycopene accumulated in a manner positively related to L-lysine concentration, simplifying the identification of high L-lysine-producing candidates through colorimetric screening on agar plates [92].

When utilizing biosensor-driven agar plate-based screening, significant differences among mutants are crucial for successful screening. This makes precise engineering of biosensors important to generate distinguishable outputs in response to various productions. Additionally, it should be noted that visual recognition of colony size or color intensity is often subjective and can vary depending on different operators and experiment batches in biosensor-assisted agar plate-based screening. This subjectivity can lead to inaccuracies or even false positives. Nevertheless, it remains an efficient and effective method for preliminary screening, capable of roughly filtering a large number of mutants, especially those with a relatively low positive rate resulting from random mutagenesis [8].

4.2. Biosensor-driven MTP-based screening

24-, 48-, and 96-well plates facilitate HTS by reducing the scale of the culture system compared to shaking-flask-based cultivation. Biosensor-driven MTP-based screening can bypass complex post-processing of fermentation broth and are widely used in the preliminary screening of mutants exhibiting enhanced production levels compared to the parental strain. Generally, biosensors can be integrated into MTP-based screening in two main forms.

In one approach, product biosynthesis and signal detection are performed separately, with biosensors introduced as indicators at the endpoint of the fermentation process (Fig. 3b). For instance, an adipic acid-responsive biosensor was incorporated as an indicator into the fermentation supernatant following 24-well-plate cultivation, and fluorescence intensities were measured to pinpoint potential adipic acid overproducers [93]. Similarly, a modified EryD-based biosensor capable of sensing erythritol was added to the fermentation supernatant after cultivation in deep-well plates, enabling rapid screening for erythritol hyperproducers from 1152 *Yarrowia lipolytica* mutants [94]. One significant advantage of this approach is that the biosensor genetic circuits can be designed and expressed in genetically tractable chassis, such as *E. coli* strains.

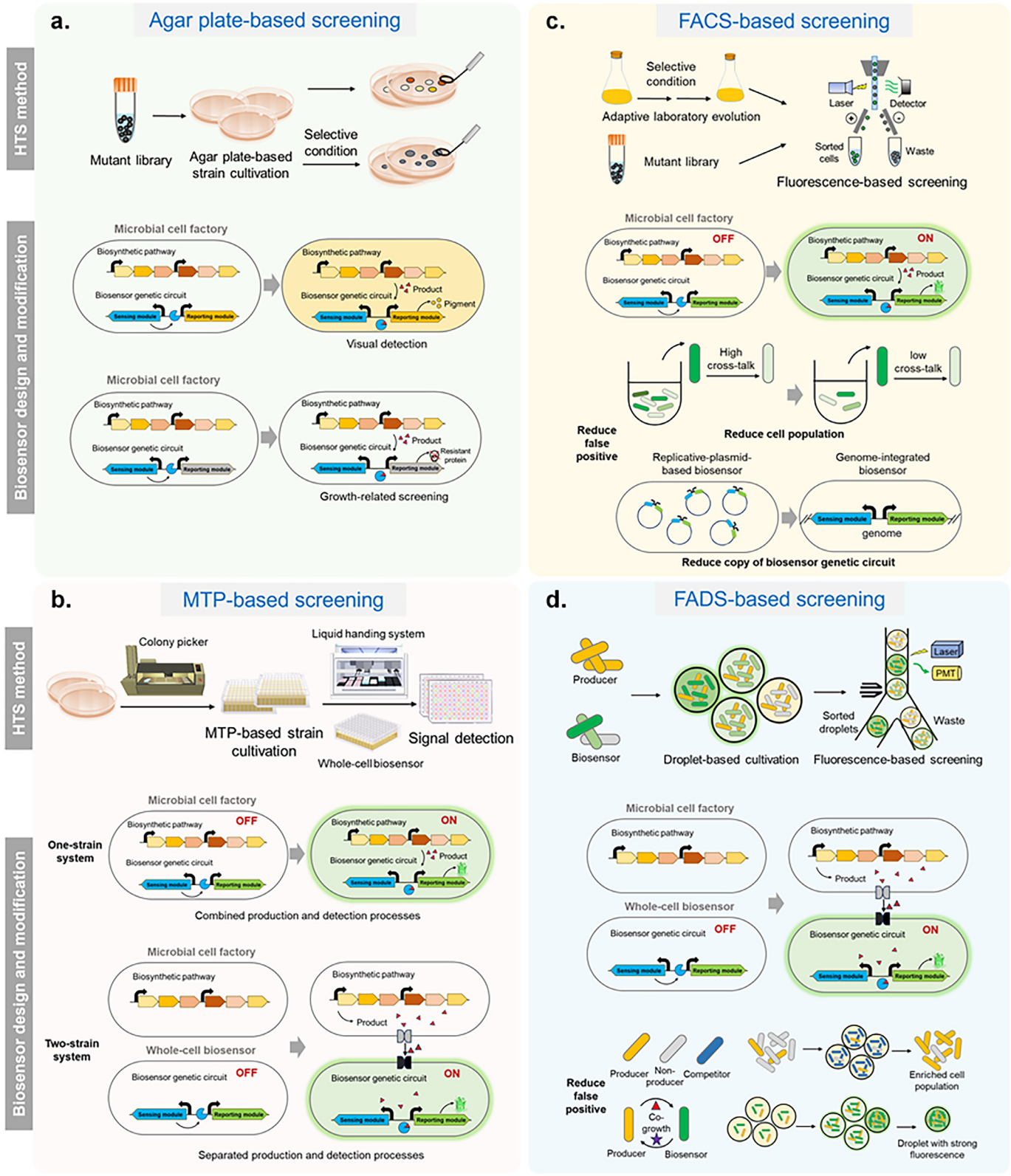

Fig. 3. Different high-throughput methods and biosensor design strategies for screening and engineering better-performing microbial cell factories. (a) Agar plate-based screening utilizes either visual detection or growth-coupled selection to isolate mutants. (b) MTP-based screening employs automated colony picking and liquid handling systems. The schematic contrasts two biosensor configurations: a "one-strain system" where the biosensor circuit operates intracellularly within the producer, and a “two-strain system” where a separate whole-cell biosensor detects secreted products (red triangles) released by the microbial cell factory. (c) FACS-based screening sorts individual cells based on fluorescence. Strategies to mitigate false positives include reducing cell density to minimize signal cross-talk (top) and integrating biosensor circuits into the genome (bottom) to reduce expression noise compared to plasmid-based systems. (d) FADS-based screening encapsulates cells in microdroplets. The bottom panel illustrates a syntrophic co-culture strategy to reduce false positives. In this mutualistic interaction, the red triangle represents the target product secreted by the producer, which triggers the biosensor. In return, the biosensor secretes an essential cross-fed nutrient (purple star) required for the producer’s survival. This growth-coupling ensures that only producers synthesizing the target product (triangle) receive the nutrient (star) necessary for co-growth, thereby eliminating non-producing competitors.

Table 3
Comparison of key performance metrics for different HTS methods.

Screening method	Screening throughput	Required volume	Detection sensitivity	Key advantages	Main limitations	Refs.
Agar plate	Medium (10^4–10^5 clones/day)	N/A (Solid phase)	Medium	Simple, low-cost, no equipment required	Low quantitation accuracy, lower throughput	[90–92]
MTP	Low (10^2–10^3 clones/day)	μL to mL range	Medium	Simple, automated handling, time-resolved monitoring	High reagent cost, lower throughput, high biological variability	[93–95]
FACS	Ultra-High (>10^7 events/hour)	Single cell	Very High	Single-cell resolution, extremely high speed, multi-parameter analysis	Difficult to screen secreted products, reliance on the fluorescence reporting system, cross-talk risk	[96–99]
FADS	High (10^6 –10^7 events/hour)	pL to nL range	High	Single-cell resolution, capability of screening secreted products, low reagent consumption	Requirement of specific equipment, reliance on the fluorescence reporting system, leakage risk	[100–103]

N/A: Not Applicable.

Additionally, whole-cell biosensors can be cultured separately according to their specific requirements, eliminating the need to consider the cultivation and product biosynthesis conditions of different microorganisms. For example, an *E. coli* whole-cell biosensor, which converts D-glucaric acid concentration into a fluorescence signal, was utilized for rapid screening of high D-glucaric acid producers in both *E. coli* and *Saccharomyces cerevisiae* chassis [95].

In the alternative approach, both the biosensor genetic circuit and the biosynthetic pathway are integrated into microbial cell factories to achieve real-time and *in situ* conversion between compound concentrations and readouts (Fig. 3b). For example, the EAC103–3H biosensor specifically responding to 5-aminolevulinic acid (5-ALA) concentration was successfully integrated into an engineered *E. coli* strain for HTS. Microplate-based fluorescence measurements provided a rapid, quantifiable readout for large-scale libraries, yielding a 4.78-fold increase in 5-ALA production [59]. Here, product biosynthesis and signal transduction occur concurrently within the intracellular microenvironment. This co-existence necessitates careful consideration of the metabolic burden on cellular resources and potential signal crosstalk arising from metabolite exchange between modules. It has been reported that greater reproducibility was observed in the “one-pot, two-strain” system, where biosynthesis and biosensor vectors were located in different strains, compared to the “one-pot, one-strain” system, where multiple vectors were harbored together in a single chassis [95].

Importantly, robotic automation can significantly enhance biosensor-driven MTP-based screening, improving both the strain cultivation and the signal detection processes. Automated equipment, such as colony pickers, liquid handling systems, and multifunctional microplate readers, can be integrated under software control to execute repetitive experimental procedure efficiently [5,107]. This integration will greatly reduce labor and time costs while substantially increasing the screening throughput of a single experimental batch.

4.3. Biosensor-driven FACS

Currently, FACS stands as the predominant method for biosensor-driven HTS. FACS enables the analysis of extensive variant libraries at the single-cell level and facilitates the sorting of cells with desired fluorescence intensities from a bulk culture. The throughput of FACS can reach up to 10^6 cells per second [96], significantly surpassing other screening methods in speed. The quantitative monitoring of intracellular metabolites with *in vivo* biosensors provides an efficient means of identifying high-yield strains and observing product accumulation in real time (Fig. 3c).

During the screening and rational engineering of microbial cell factories for the production of target natural products, biosensor-driven FACS offers versatile assistance in many aspects. These include the screening of expression regulatory elements [108], directed evolution of key enzymes [97,109,110], pathway engineering [111,112], and identification of potential gene targets for metabolic engineering [57,98,113–117]. In protein directed evolution, biosensor-driven FACS screening are efficient and effective for evaluating a large number of variants generated by various mutagenesis techniques. An HTS platform coupling an (*2S*)-naringenin-responsive biosensor with FACS was developed to enable directed evolution of chalcone synthase (CHS), the rate-limiting enzyme in the flavonoid biosynthetic pathway. By screening a CHS mutant library using a TtgR-based transcriptional biosensor, high-activity CHS variants were identified. The best mutant exhibited a 2.1-fold increase in catalytic efficiency and doubled (*2S*)-naringenin production compared to the wild-type enzyme, thereby enabling the construction of a high-yielding microbial strain [97]. In pathway engineering, a biosensor-assisted iterative FACS screening approach was employed to optimize the expression and activity of key enzymes in the pathway, enhancing the biosynthesis of branched-chain higher alcohols in the mitochondria and cytosol of *S. cerevisiae* [111]. Furthermore, biosensor-based FACS has significantly contributed to mining potential metabolic engineering targets from genome-wide random mutations. Notably, this approach can identify beneficial mutations in genes that may not intuitively appear directly connected to the target biosynthetic pathway [118]. This method is frequently used for the isolation of improved mutants after adaptive laboratory evolution, typically followed by genome resequencing to identify beneficial mutations enriched by the variants for subsequent rational strain engineering [113,114,119]. Utilizing adaptive laboratory evolution and a biosensor-driven HTS strategy, a *Klebsiella oxytoca* strain capable of withstanding high concentrations of xylose and producing a high yield of 2,3-butanediol, 57.5 g/L, was successfully isolated [114]. With the rapid development of genome editing tools, generating libraries related to a collection of genes of interest or at a genome scale is becoming increasingly feasible and convenient [90,115,120]. In this context, biosensor-based methods will play a pivotal role in HTS libraries to explore efficient biosynthetic mechanisms and identify potential gene targets for the rational construction of microbial cell factories.

Although powerful and convenient, there are limitations to applying biosensors to FACS. The biosensor and biosynthesis modules should be integrated into a single chassis to facilitate *in situ* monitoring of metabolites, and different fluorescence systems are typically relied upon to report measurable outputs. These implies that the microbial chassis should be genetically tractable for plasmid transformation or genome modification. Therefore, biosensors employed in FACS screening are usually developed in model microorganisms such as *E. coli* [55,121,122], *C. glutamicum* [61,108] *Pseudomonas putida* [113], and yeasts [111,115,117]. Additionally, some emerging microbial chassis with accessible genetic tools, like *K. oxytoca* [114] and *Vibrio natriegens* [116], which are promising platforms for synthesizing value-added bioproducts, are also attracting efforts to develop compatible biosensors.

When biosensors are employed in FACS, false-positive results can occur during the screening process due to heterogeneity caused by genetic variation, diverse expression of fluorescent proteins, and different growth states of individual cells. Thus, a re-screening step following FACS is essential to distinguish truly improved variants from false positives. Various strategies have been documented to mitigate cell heterogeneity during biosensor-based FACS screening (Fig. 3c). Recent biosensor-guided strain engineering in the industrial chassis *Pseudomonas putida* integrated a stable, product-responsive reporter circuit into the chromosome, rather than relying on variable-copy plasmids. This strategy constrained cell-to-cell variability in biosensor output and reduced false positives during sorting, thereby enhancing the reliability of downstream FACS-based selection for isoprenol overproducers [98]. Moreover, numerous small molecules can easily permeate biological membranes through diffusion or be transported via carrier-mediated mechanisms. This characteristic presents a significant hurdle in the biosensor-based FACS screening, as it may result in cross-talk between producing and non-producing variants, potentially leading to a considerable number of false-positive variants in practical applications. This problem can be partially addressed by reducing the cell population of samples in FACS screening. It has been reported that a diluted starting OD_{600} of strains and reduced cultivation time can successfully alleviate biosensor cross-talk in FACS screening [99]. Despite these limitations, the positive outcomes reported with the utilization of biosensor-driven FACS suggest its potential as a promising and straightforward screening method suitable for most scenarios.

4.4. Biosensor-driven FADS

Recently, droplet-based microfluidic systems, such as FADS, have become increasingly popular for analyzing microorganisms at the single-cell resolution. Droplet microfluidics create independent microenvironments for cultivating individual microorganism cells, serving as reactors for complex morphological differentiation and a variety of biochemical reactions [100]. Thus, FADS is particularly suitable for screening multicellular microorganisms with complex morphological differentiation, such as filamentous fungi [123,124] and actinomycetes [125,126]. Similar to FACS, FADS sorts desired variants based on the fluorescence signal they generate (Fig. 3d). However, FADS shows reduced cross-talk between compartments compared to FACS due to the independence of microdroplets surrounded by the oil phase. Moreover, it has been reported that when enabled by a biosensor, FADS demonstrated an enhanced positive mutant enrichment rate and improved performance of the sorted mutants compared to FACS [101].

In biosensor-driven FADS screening, product biosynthesis and sensing modules can be incorporated into a single chassis. In this scenario, the enriched fluorescence of multiple cells can be detected due to cell division and proliferation over extended culture periods, potentially amplifying subtle signal differences between mutants [127]. In contrast, in the majority of applications, the producer and the biosensor are separate strains co-cultured within droplets (Fig. 3d). This approach usually utilizes biosensors constructed in well-established chassis to avoid excessive genetic modification of the producing strain, making it particularly suitable for screening genetically intractable industrial strains from random mutant libraries [125]. An *E. coli* whole-cell biosensor integrated with FADS was used to screen industrial *Saccharopolyspora erythraea* strains with hyper erythromycin production, and a mutant demonstrating a 50 % increase in production was successfully isolated [125]. Beyond strain optimization, FADS has also been applied to enzyme engineering. For example, Li et al. programmed an L-tryptophan-responsive biosensor to direct the evolution of tryptophan hydroxylase (TPH) via high-throughput droplet sorting [128]. By coupling biosensor fluorescence to in-droplet enzymatic conversion, they rapidly screened iterative saturation mutagenesis libraries and identified a TPH variant exhibiting 4.25-fold higher catalytic activity and 3.2-fold improved thermostability relative to the parental enzyme [128].

Most importantly, unlike FACS, which tends to prioritize sorting producers with high intracellular accumulation, FADS is particularly valuable for screening targets related to extracellular products. Therefore, biosensor-based FADS can facilitate screening for both high production and high secretion [102,129], both of which are crucial characteristics for microbial cell factories in industrial biotechnology. For instance, *E. coli* strains with improved secretion of 3-hydroxypropionic acid were successfully screened using a producer-biosensor co-culture FADS system from an overexpression library, and the genes responsible for 3-hydroxypropionic acid secretion were successfully identified [102]. In another example, a growth-based biosensor combined with FADS screening contributed to the successful isolation of three mutated *Lactococcus lactis* strains with 5–10 times increased secretion capacity of amino acids compared to the wild-type strain [129].

When employing biosensor-driven FADS screening, false positive results may arise from various factors, such as cell heterogeneity, co-encapsulation of multiple producing cells during droplet generation, or even the accumulated mutations that could disable the biosensors. These false positives can significantly impact the overall screening and consequently lead to a need for considerable additional effort in the re-screening process. To address this issue, introducing a growth connection between the producers and the biosensors is an alternative approach to enhance the differentiation among bacteria-containing droplets, thereby aiding in distinguishing true positives from false ones (Fig. 3d). A biosensor-mediated selection strategy was developed by co-culturing competing and producing strains. The competition between two strains in the co-cultivation droplets conferred a selective advantage to the producers over the non-producers, successfully enriching the muconate-producing strains from a large population of non-producers[130]. In another case, the metabolic cross-feeding circuit targeting 2-ketoisovalerate or L-tryptophan was introduced to construct the auxotrophic producer and the biosensor, enabling the co-growth of two strains in the syntrophic co-culture. This design amplified the production difference into significant growth phenotypes and facilitated the successful FADS screening of a mutant with 5-fold higher isobutanol production from a random mutagenesis library [131]. The connecting metabolic nodes between two strains to build a growth-dependent relationship can be both the final products and the intermediate metabolites [129,131]. Biosensor-driven FADS screening serves multiple purposes. Beyond isolating promising mutants, it enables researchers to explore biosynthesis and secretion mechanisms by interrogating the relationship between bacterial phenotypes and genotypes [102,125,129,132].

Despite the specific advantages of FACS and FADS, both high-throughput methods face the persistent challenge of false positives arising from cellular heterogeneity and signal noise. To systematically address this, several advanced strategies have recently emerged across different screening platforms. First, output normalization effectively alleviates cell-to-cell variability. For instance, in a droplet-based system, Zhao et al. employed a dual-color strategy to calibrate biosensor signals against cell density, successfully distinguishing true hyperproducers [103]. Second, logic-gate circuits can enhance state specificity. Kelvin et al. engineered hybrid riboswitches executing NAND Boolean logic, which ensures reporter expression only occurs under strict dual-ligand conditions, thereby minimizing leaky signals in fluorescence-based sorting [85]. Third, expanding on the concept of growth-coupling, Krüger et al. linked intracellular product formation to essential cell survival, creating a strong evolutionary pressure that effectively eliminates non-producing “cheaters” before measuring fluorescence [104]. Finally, mitigating genomic heterogeneity is crucial for stable phenotypes. Using FACS, Menasalvas et al. demonstrated that integrating biosensor circuits into the chromosome, rather than relying on variable-copy plasmids, significantly reduced signal noise and false positives during the selection of isoprenol producers [98]. Collectively, these multi-layered strategies—ranging from circuit design to genomic integration—significantly enhance the precision and reliability of biosensor-driven HTS.

5. Conclusions and future perspectives

Biosensor-driven HTS can greatly enhance the efficiency of screening a large number of variants and is a powerful tool in microbial cell factory engineering. However, each screening method has its applicable scenarios. For different target metabolites, the selection of a suitable biosensors-driven HTS strategy is a prerequisite for successful screening, and different choices of methods may lead to different outcomes. The adaptability of biosensor performance characteristics is crucial for successful screening. Each biosensor represents unique detection thresholds and linear ranges for detecting target products, and the requirements for biosensor characteristics often vary when integrated with different HTS processes. To this end, the modular "plug and play" design concept of synthetic biology may be applied in biosensor optimization [6]. Genetic circuits of biosensors can be modularly optimized using synthetic biology strategies. One example is to divide biosensors into induction and reporting modules, in which the expression of key genetic elements can be separately optimized to improve biosensor performance [133]. Besides, in the engineering of microbial cell factories, several crucial factors can influence the suitability of biosensors for different HTS demands, such as the production range [69], the temporal and spatial accumulation of products [134], the culture conditions of producing strains, and the tolerance of biosensor strains to target products. Therefore, there is no universal method to guarantee successful outcomes in all applications. In many cases, customized adjustments and optimizations are still required when combining biosensors with HTS processes to meet the actual screening needs or reduce false positive rates.

Currently, access to biosensors adapted for diverse product-driven HTS purposes is still limited, which is in stark contrast to the numerous natural and unnatural compounds that can be synthesized in microbial cell factories. So, there is an urgent need to develop novel biosensors to assist in screening and optimizing microbial cell factories. For specific compounds, their related metabolite-sensing elements, as well as genetic regulatory circuits, may be successfully characterized in native producing chassis [135], and in microorganisms capable of degrading [136], tolerating [137–139], or utilizing [114,140,141] of target compounds. These regulatory components can be explored using multi-omics guided approaches and subsequently used as the core elements for constructing novel biosensors [142,143]. Additionally, leveraging advanced technologies such as protein design and AI to develop universal biosensors applicable to screening for specific classes of compounds may be a crucial approach to addressing the shortage of biosensors for the growing demand for HTS. Starting from the chemical structures of target products, AI technologies can be employed to design biologically functional proteins with high affinity to small molecules from scratch [82,144], which will significantly accelerate biosensor development to meet diverse application scenarios.

With the fast-developing metabolic engineering and synthetic biology approaches, more natural or unnatural compounds can be biosynthesized by microbial cell factories. The adoption of biosensor-driven HTS technologies can accelerate the building and optimization of microbial cell factories, enabling efficient and cost-effective access to high-value compounds, which is significant for promoting sustainable development without reliance on fossil fuels.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Jin Wang: Writing – review & editing, Writing – original draft, Visualization, Data curation. **Xueyan Liu:** Data curation. **Longqian Zhao:** Data curation. **Yue Zhang:** Writing – review & editing, Writing – original draft, Visualization, Funding acquisition, Data curation, Conceptualization. **Meng Wang:** Writing – review & editing, Supervision, Funding acquisition.

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