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Review

Recent advances in screening amino acid overproducers

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ABSTRACT

Microbial fermentation has contributed to 80% of global amino acid production. The key to microbial fermentation is to obtain fermentation strains with high performance to produce target amino acids with a high yield. These strains are primarily derived from screening enormous mutant libraries. Therefore, a high-throughput, rapid, accurate, and universal screening strategy for amino acid overproducers has become a guarantee for obtaining optional amino acid overproducers. In recent years, the rapid development of various novel screening strategies has been witnessed. However, proper analysis and discussion of these innovative technologies are lacking. Here we systematically reviewed recent advances in screening strategies: the auxotrophic-based strategy, the biosensor-based strategy, and the latest translation-based screening strategy. The design principle, application scope, working efficiency, screening accuracy, and universality of these strategies were discussed in detail. The potential for screening nonstandard amino acid overproducers was also analyzed. Guidance for the improvement of future screening strategies is provided in this review, which could expedite the reconstruction of amino acid overproducers and help promote the fermentation industry to reduce cost, increase yield, and improve quality.

1. Introduction

Amino acids have a multibillion-dollar market with applications in the food, animal feed, pharmaceutical, and cosmetic industries. The worldwide market for amino acids reached an overall 10.3 million tons, with gross sales of $28 billion in 2021. The global amino acid market is expected to expand at a compound annual growth rate of 6.76% in the next decade [1]. Amino acids can be synthesized by various methods, such as extraction from protein hydrolysate, chemical synthesis, enzymatic synthesis, and microbial fermentation [2,3]. Compared with other amino acid-producing methods, microbial fermentation is considered economical, efficient, and environmentally friendly [4]. Currently, it has become the primary production method for most amino acids, contributing to 80% of the global amino acid yield [5]. The key to fermentation is obtaining high-performance microbial cell factories (MCF) to produce the desired amino acids [6]. Currently, the breeding of amino acid overproducers mainly relies on constructing a mutant library and subsequent screening [7]. Besides host strains, functional elements, such as rate-limiting enzymes, transporters, or transcriptional regulators in the amino acid synthetic pathway, also require engineering by directed evolution [8]. The screening strategy determines whether ideal amino acid overproducers could be obtained.

An excellent screening strategy for amino acid overproducers should satisfy four requirements. The primary one is high throughput. Traditional MCF breeding methods rely on the direct quantitative detection of products using chromatography and other methods. Sample preparation and detection operations are time-consuming and labor-intensive, leading to low screening throughput. Meanwhile, the size of mutant libraries is rising with the continuous upgrading of mutagenesis approaches. A high-throughput screening strategy is needed to rapidly screen amino acid overproducers from an ever-growing and diversifying mutant library. The second requirement is high fidelity. Otherwise, the screening system will lead to a high false-positive rate. The amino acid low-producing strains or non-target amino acid-producing strains will accidentally be screened out. The third requirement is a simple operation. An ideal screening strategy should take a few steps and not rely on special equipment. Last but not least, previous screening strategies focused only on a few bulk amino acids. Recently, the demand for other amino acids, such as tryptophan, branched-chain amino acids, and even various nonstandard amino acids, such as 5-hydroxytryptophan [9], has been increasing. A screening strategy that could respond to multiple proteinogenic amino acids and even nonstandard amino acids is urgently needed. Meanwhile, in addition to traditional amino acid-producing strains, such as *Escherichia coli* and *Corynebacterium glutamicum*, the screening system should also be applied to other microorganisms with industrial potential for future development. Screening strategies in recent years have moved in these directions [3].

Regarding breeding amino acid overproducers, methods for constructing a mutant library have been intensively reviewed [10–13]. However, there are few discussions on the screening strategies for amino

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acid overproducers. In this review, recent advances in screening strategies, including auxotrophic-based and biosensor-based strategies, were discussed. Special attention was given to the translation-based strategy, including amino acid analog-based, rare codon-based, aminoacyl-tRNA synthetase (aaRS)-based, and artificial aaRS/tRNA pair-based screening approaches. The target amino acids, compatibility with microbial species, screening efficiency, and current applications of these strategies were discussed. Finally, we make some forward suggestions for developing a screening strategy for amino acid overproducers. This review would guide the choice of a strain screening approach, inspire the development of novel screening strategies, and finally aid in reconstructing amino acid-overproducing strains.

2. Different strategies for screening amino acid-overproducing strains

2.1. Auxotrophic strain-based strategy

Auxotrophic strains cannot synthesize essential metabolites, such as amino acids, nucleotides, and vitamins, and must be supplemented with such nutrients to survive [14]. Generally, the growth of auxotrophic strains is positively correlated with the concentration of the corresponding metabolites in the environment [15,16]. Additionally, if a fluorescent protein is integrated into the chromosome of an auxotrophic strain, the growth signal can be converted to a more intuitive fluorescent signal [17]. There are two major auxotrophic strain-based screening strategies (Table 1) [18]. The first is a two-step screening. The target amino acid overproducers could be identified by establishing an auxotrophic strain for a specific amino acid and feeding this indicator strain with the fermentation broth or cell lysate of the strains from the mutant library. Such a library could be constructed by standard physical or chemical mutagenesis methods. For example, the knockout of the *hisL* gene in the L-His-synthesis pathway could establish an L-His-auxotrophic strain. By feeding this indicator strain with cell lysates of strains in the mutant library, the growth of the indicator strain could indicate the amount of L-His, and the corresponding amino acid overproducer could be screened [19].

In addition to the two-step screening, the coculture-based strategy could also be used to detect amino acid overproducers (Fig. 1A). For example, knocking out the L-Trp-synthesis pathway gene *trpB* of the indicator strain makes it rely on the L-Trp produced by the production strain. At the same time, knocking out the L-His-synthesis pathway gene *hisD* of the production strain makes it dependent on the L-His produced by the indicator strain for survival. The dominant number of indicator strains determined by the initial inoculation could be maintained by this cross-feeding strategy to achieve a stable screening effect. After coculturing the mutant library of the production strain with the indicator strain, the amino acid overproducers could be screened in two ways. The first method is colony morphology observation. By spreading a dominant number of indicator strains mixed with an individual production strain on the agar plate, the coculture with the L-Trp overproducer would develop a large colony and could be identified. The second method is based on microplate or microdroplet screening. Wells with L-Trp overproducers would produce higher cell density than others. Fluorescent proteins could be introduced into the indicator strain to increase the screening throughput further. The coculture is encapsulated in the microdroplets, and the droplets containing amino acid overproducers would exhibit strong fluorescence and could be identified [20]. In theory, screening methods based on auxotrophic mutant strains could also be used to screen optimal enzyme variants in amino acid synthesis pathways (Fig. 1B). The mutant library of the enzyme could be transformed into a host strain deficient in this enzyme. The mutant with enhanced catalytic performance could be screened out by coculturing with the indicator strain unable to produce the catalytic product of the target enzyme. The response range of the screening signal could also be adjusted by regulating the ratio between the two strains.

Auxotrophic strain-based strategies have been used to screen overproducers of amino acids, such as L-His, L-Trp, and L-Met (Table 1). The primary limiting factor is that this strategy is inconvenient for accurately identifying individual overproducers. A supernatant or cell lysate feeding-based strategy must extract amino acids produced by individual strains. The coculture-based method should rely on expensive sorting equipment to sort individual overproducers in the microdroplets. The second limiting factor is establishing auxotrophic strains separately to screen the overproducers of 20 proteinogenic amino acids. For nonstandard amino acids, this strategy is usually inapplicable. Additionally, this strategy may be more suitable for screening mutants with higher abilities to export amino acids.

2.2. Biosensor-based strategies

A biosensor is a biological device that can sense specific chemical substances in the environment and convert their concentrations into detectable signals. Biosensors generally consist of a recognition module and a signal conversion module. The recognition module can include various biologically active parts, such as transcription factors, enzymes, antibodies, antigens, and nucleic acids. The signal conversion module is generally comprised of antitoxic proteins, growth-related proteins, chromogenic proteins, and fluorescent proteins. The biosensor has been widely used to detect various small molecules, such as amino acids, alcohols, sugars, vitamins, and antibiotics [21], providing an ideal tool for screening amino acid overproducers. According to the recognition module, amino acid biosensors could be roughly divided into the following categories: enzymatic reaction-coupled biosensors, fusion protein-based biosensors, transcription factor-based biosensors, and riboswitch-based biosensors (Fig. 2A–E) [22].

2.2.1. Enzymatic reaction-coupled biosensors

Amino acids lack high-throughput chemical detection methods. The concentration of amino acids can be converted into detectable color and fluorescence by coupling with enzymes [46,56]. Enzymatic reaction-coupled biosensors have been used to screen amino acid overproducers. For example, molecular oxygen may be used by tyrosinase to catalyze the orthohydroxylation of L-Tyr to 3,4-dihydroxyphenylalanine (L-DOPA), which can be polymerized nonenzymatically to form melanin with a dark color. A method for screening L-Tyr overproducers was established by introducing tyrosinase capable of producing melanin from tyrosine [46]. In addition to a single enzyme-catalyzed reaction, a two-enzyme coupled transformation assay can also be utilized. L-Met can be converted to pyruvate by coupling adenosylmethionine synthase and pyruvate orthophosphate dikinase. The produced pyruvate can then be easily measured using ultraviolet, colorimetry, or fluorescence detection [35]. This offers a possible approach to screen L-Met overproducers.

2.2.2. Fusion protein-based biosensors

Proteins typically undergo conformational changes upon ligand binding. This structural change could be used to build biosensors based on fluorescence resonance energy transfer (FRET) and circularly permuted proteins. FRET-based biosensors consist of a donor fluorescent protein and an acceptor fluorescent protein, which link to the N- and C-termini of the ligand-binding protein, respectively. The wavelength emitted by the donor fluorescent protein could excite the acceptor fluorescent protein. When the ligand-binding protein is in the apo state without binding to the metabolite, the long distance between the donor fluorescent protein and the acceptor fluorescent protein prevents light from the donor from being transmitted to the acceptor. When the metabolite binds to the ligand-binding domain, a change in the conformation of the binding domain was induced, bringing closer the donor fluorescent protein and the acceptor fluorescent protein, enabling the latter to capture the light emitted by the donor to fluoresce. For example, the cyan fluorescent protein that works as the donor and the yellow

Table 1
Screening strategies for 20 proteinogenic amino acid overproducers.

Amino acid	Auxotrophic strain-based strategy	Biosensor-based strategy	Analogs based strategy	Other translation-based strategies[b]
L-Ala			D-α-aminobutyric acid [23]	
L-Val		Lrp-regulated promoter P_{brnF} fused with *eyfp* [7,24]	α-aminobutyric acid, 2-thiazole alanine, α-amino-β-hydroxy valeric acid [25]	
L-Leu		Lrp-regulated promoter P_{brnF} fused with *eyfp* [26], A FRET system consists of a fused YFP-LivK-CFP protein [27]	4-azaleucine, nor-leucine, threon-L-β-hydroxyleucine [28]	Rare codon-rich kan^R [3]
L-Ile		Lrp-regulated promoter P_{brnF} fused with *eyfp* [26]	isoleucine hydroxamate [29]	An isoleucine-tRNA synthetase mutant of *E. coli*, $IleRS^{G94R}$ [30]
L-Pro			4,5-didehydroproline, L-azetidine-2-carboxylic acid [31]	Rare codon-rich kan^R and amp^R [32]
L-Met	Natural L-Met-auxotrophic strain *Lactococcus lactis* [33]	A FRET system consists of a fused YFP-MetN-CFP protein [34], an enzymatic reaction-coupled biosensor that uses two enzymes to convert L-Met to pyruvate with detectable UV absorbance [35]	ethionine, methionine sulfoxide, proleucine, and methyl mesylate [36]	
L-Cys		L-Cys-responsive promoter P_{cys} controlling the *gfp* [33], CcdR regulated promoter P_{ccdA} fused with *gfp* [37]		
L-Trp	*E. coli* PJW3087 Δ*tdcC* was used to indicate the L-Trp in the supernatants of overproducers [19]; *E. coli* K12 Δ*trpB* as the sensor strain and L-His as the secondary cross-feeding molecule) Δ*tdcC* [20]	An artificial L-Trp aptamer combined with *gfp* [38]	p-fluorotryptophan, 5-methyl tryptophan, fluorotryptophan, 5-fluoro-DL-tryptophan [39]	
L-Phe		TyrR-regulated promoter P_{tyr} or P_{mtr} fused with *yfp* [40]	Fluorophenylalanine, chlorophenyl alanine [41], m-fluoro-phenylalanine [42]	
L-Ser		LysR-type TF, NCgl0581 fused with *eyfp* [43]		Rare codon-rich kan^R [3]
L-Thr			α-amino-hydroxyvaleric acid, threonine hydroxamic acid, α-aminolauryl lactam [44]	
L-Tyr		TyrR-regulated promoters, P_{aroF}, P_{aroL}, or P_{aroP}, fused with *mCherry* [45], an enzymatic reaction-coupled biosensor that used tyrosinase to produce melanin with the dark color from L-Tyr [46]		
L-Asn		AsnC[a], Lrp[a]		
L-Gln		A FRET system consists of a fused mTFP1-glnH-YFP protein [47]	methionine sulfoxide, sulfaguanidine [48]	
L-Arg		LysG-regulated promoter P_{lysE} fused with fluorescent protein [49]		Rare codon-rich kan^R [3]
L-Lys		LysG-regulated promoter P_{lysE} fused with fluorescent protein [49], A FRET system consists of a fused YFP-LAO-ECFP protein [50,51], an inhibitory riboswitch lysC that coupled with the tetracycline/H^+ antiporter) [38]	S-2-aminoethyl-L-cysteine [52]	
L-His	*E. coli* JW2000 Δ*hisL* [19]	LysG-regulated promoter P_{lysE} fused with fluorescent protein [49,53]	D-histidine, 6-mercaptopurine, 1,2,4-triazolealanine [54]	
L-Asp			aspartate hydroxamate, sulfaguanidine	
L-Glu		A FRET system consists of a fused YFP-YbeJ-CFP protein [55]		
L-Gly	Currently mainly chemically synthesized, but overproducers can theoretically be screened out by using translation-based screening strategies			

[a] They are natural transcription factors for these amino acids, which can potentially be employed for screening overproducers.
[b] This emerging strategy has the potential to be extended to all proteinogenic amino acids and even nonstandard amino acids.

fluorescent protein that works as the acceptor were fused with the L-Lys binding periplasmic protein from *Salmonella typhimurium* serotype LT2. This protein device was successfully employed in *E. coli* and *Saccharomyces cerevisiae* to detect the production of L-Lys [51]. In another example, the L-Leu binding periplasmic protein from *E. coli* K12 was fused with cyan fluorescent protein as the donor and yellow fluorescent protein as the acceptor, creating a biosensor that allows the dynamic measurement of L-Leu concentrations in bacterial and yeast cells [27]. FRET-based biosensors have been used for various amino acid detections, such as branched-chain amino acids [57], L-Glu [58], L-Met [59], and L-Gln [60].

A circularly permuted protein-based biosensor was a rearranged fluorescent protein. It was created by first segmenting the original protein at the chromophore region and fusing the original N- and C-termini by a linker, then connecting the newly formed N- and C-termini, respectively, to two subunits of a ligand-binding domain. Upon binding to the target molecule, an interaction occurs between the two ligand-binding subunits to induce a conformational change in the permuted fluorescent protein. This change could alter the electrostatic potential of the chromophore to produce fluorescence. Such a strategy has been successfully applied to the screening of small molecules. A formaldehyde-responsive transcriptional regulator HxlR monomer from *Bacillus subtilis* was fused

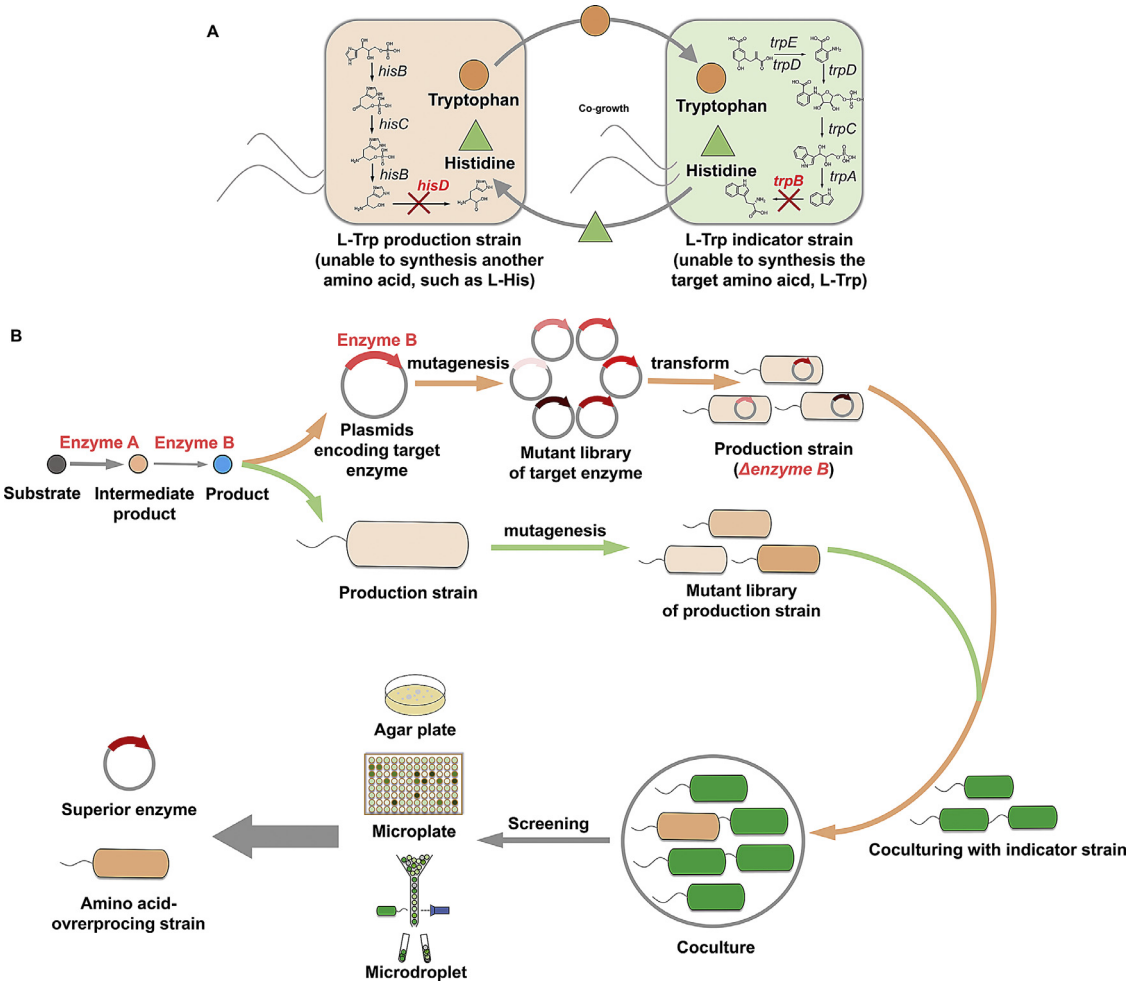

Fig. 1. *Auxotrophic strain-based strategy*. Screening strategy based on the coculture of the production strain and the indicator strain. In the coculture process, it is necessary to maintain dominance of the indicator strain by using strategies such as cross-feeding to obtain a stable screening effect. (A) To screen the L-Trp overproducers while knocking out the L-Trp-synthesis pathway gene *trpB* of the indicator strain, knocking out the L-His-synthesis pathway gene *hisD* of the production strain makes it rely on the L-His produced by the indicator strain for survival. (B) Proposed screening strategy for identifying optimal enzyme variants in amino acid synthesis pathways.

to the two ends of the circularly permutated yellow fluorescent protein, and a formaldehyde biosensor was successfully established [61]. These circularly permuted protein-based biosensors can achieve high spatial-temporal resolution and could be developed as a screening strategy for amino acid overproducers. However, the design of such fusion proteins is complex. It is necessary to find a particular ligand-binding protein and to exclude the interference of external factors, such as medium acidity and small ion concentrations. Fusion protein-based biosensors link the recognition module and the signal conversion module into a single protein device, which allows the binding of small molecules to co-occur with fluorescence generation, thus enabling nanosecond responses to amino acids.

2.2.3. *Transcription factor-based biosensors*

Transcription factor-based biosensors have been applied to detect various fermentation products, such as carbohydrates [62,63], alcohol [64,65], esters [66], organic acids [67,68], and amino acids [69]. Such biosensors generally use transcription factors (TF) as the recognition module. TFs could act as activators or inhibitors after binding to metabolites, thus initiating or inhibiting the transcription of downstream genes [70]. These TFs have similar structures, a C-terminal ligand-responding domain, and a conserved N-terminal DNA-binding domain. When the C-terminal binds ligands, the spatial structure and protein conformation of the DNA-binding domain change dramatically to alter the binding of TF to the promoter. This change will block or promote the binding of RNA polymerase with the promoter to regulate the expression of downstream genes. The TFs that have been used in the screening of amino acid overproducers are mainly from the L-Leu responsive regulatory protein (Lrp) family, the L-Lys regulator (LysR) family, and the L-Tyr regulator (TyrR) family.

The Lrp family is the most widely used recognition module, responding to various amino acids, such as L-Leu, L-Ile, L-Val, and L-Met. Closed-loop/open-loop conformational change or octamer/hexamer transition of the Lrp may be induced by the amino acids, consequently adjusting the Lrp regulation on the expression of target genes [71]. For example, the enhanced yellow fluorescent protein was ligated downstream of the Lrp-regulated promoter P_{brnF}. Branched-chain amino acids bind to Lrp and activate the P_{brnF}-controlled fluorescent protein [26]. This Lrp biosensor has been used to screen L-Val overproducers [24].

LysR-type transcriptional regulators may be the most prominent family of TFs in prokaryotes. When bound to ligands such as amino acids, they often form multimers to regulate the transcription of target genes. LysR was found to bind to three basic amino acids. Therefore, it was used to screen L-Lys overproducers. Enhanced yellow fluorescent protein was cloned downstream of the promoter P_{lysE}, which was regulated by a LysR-type TF from *C. glutamicum*. Production of the basic amino acids (L-Lys, L-Arg, and L-His) would activate the P_{lysE}-controlled fluorescent protein to enable screening [49]. By mutating the amino acid-binding

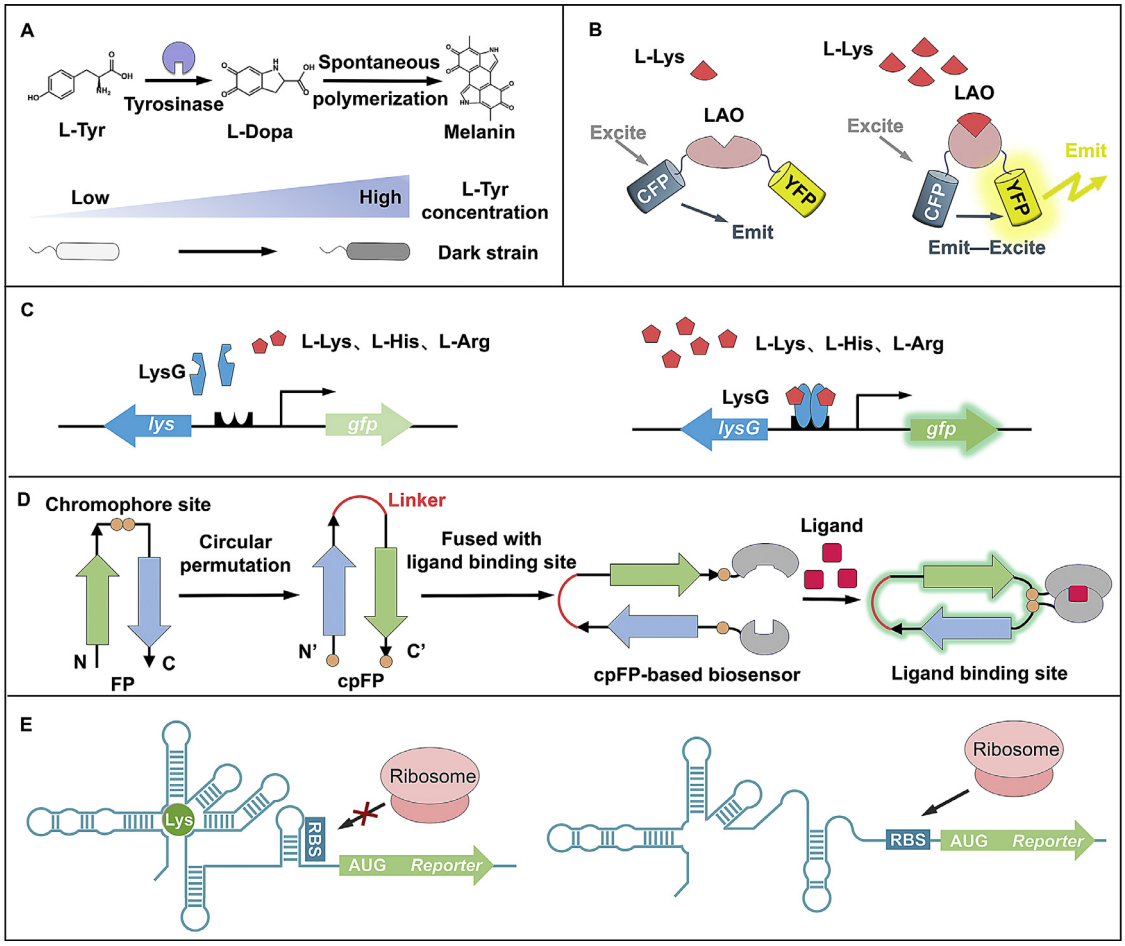

Fig. 2. *Biosensor-based strategy for amino acid overproducers.*(A) L-Tyr biosensor based on a tyrosinase-coupled color reaction. (B) L-Lys biosensor based on substrate-binding domain-fused proteins. (C) Basic amino acid biosensor based on transcription factor LysG. (D) Circularly permuted protein-based biosensor. (E) L-Lys biosensor based on riboswitch. LAO, lysine-/arginine-/ornithine-binding protein; FP, fluorescent protein; cpFP, circularly permuted fluorescent protein; RBS, ribosome binding site.

region and the non-conserved regions of LysR-type TFs, biosensors can be transformed to respond solely to L-His [53].

TyrR-type transcriptional regulators could respond to aromatic amino acids, and TyrR from *E. coli* has been used to screen L-Tyr overproducers [45]. This biosensor has also been used to screen L-Phe overproducers [40]. A TyrR-type TF coupled with P_{tyr} or P_{mtr} promoter could sense elevated concentrations of L-Phe and activate the expression of the downstream yellow fluorescent protein.

Additionally, TFs from other families can also be used to construct amino acid biosensors. Recently, a putative feast/famine regulatory protein-type transcription factor, CcdR, from the *Pantoea ananatis* AJ13355, responsive to L-Cys, was used to establish an L-Cys-specific biosensor to screen L-Cys overproducers. Through multilevel optimization, such as increasing CcdR dimerization and regulating the expression of CcdR, the sensitivity and response strength of the biosensor were significantly improved [37]. The TF-based biosensor has been used to establish a screening system for overproducing strains of at least 10 amino acids (Table 1).

2.2.4. *Riboswitch-based biosensors*

Riboswitch consists of two parts: an aptamer and an expression platform. The aptamers directly bind to small molecules, and the expression platform could undergo structural changes in response to changes in aptamers, thereby regulating the expression of downstream genes [72]. One of the most typical mechanisms is that, after binding with ligands, the disruption and formation of the stem-loop structure of the ribosome binding site region within the expression platform will regulate the translation of the entire mRNA. Riboswitch-based biosensors have been applied to screen amino acid overproducers. For example, the inhibitory riboswitch lysC, which turns off the expression of downstream genes upon binding to L-Lys combined with the tetracycline/H^+ antiporter, was used to screen L-Lys overproducers [38]. In addition to the natural aptamer, artificial aptamers could also be used to construct a riboswitch that responds to non-native molecules. For example, by introducing an artificial L-Trp aptamer [73], a riboswitch capable of recognizing the L-Trp overproducer was established [38].

Compared with auxotroph-based screening strategies, the operability and sensitivity of biosensor-based screening strategies have been greatly improved. However, biosensor-based screening strategies face several challenges. First, natural biosensors are often multifunctional, especially the most widely used TFs that recognize multiple amino acids. Therefore, biosensors must be engineered to respond specifically to target amino acids. Second, some amino acids do not have a suitable biosensor, and there are no biosensors for nonstandard amino acids. Third, the response of natural elements to amino acid concentrations is generally within the physiological range. However, the concentration of amino acids in industrial production far exceeds the physiological upper limit. Therefore, the operational range must be expanded to screen amino acid overproducers. Fortunately, the affinity of TF to the substrate could be changed by modifying the substrate-binding domain of TF, thereby obtaining various sensors with different substrate specificities, broad responding ranges, or high sensitivity [74]. In addition to the biosensors

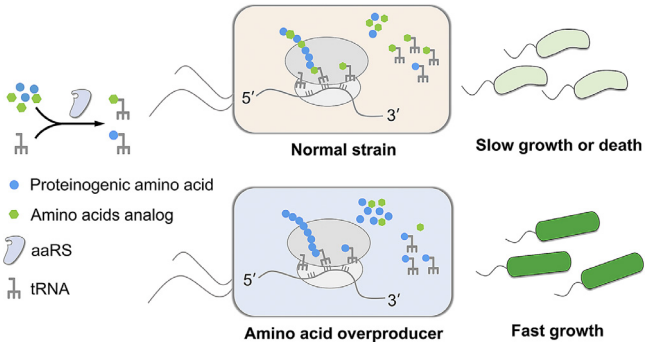

Fig. 3. Mechanism of using amino acid analogs to identify overproducers.

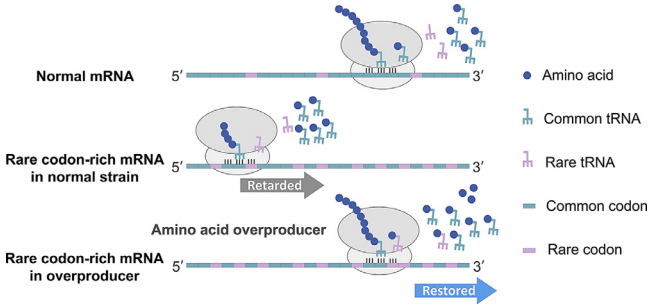

Fig. 4. The rare codon-based strategy for screening amino acid overproducers.

described above, G proteins could also respond to amino acids and potentially be used to construct screening systems [75].

2.3. Translation-based strategies

2.3.1. Strategy based on amino acid analogs

The strategy for screening amino acid overproducers based on amino acid analogs is a traditional method (Fig. 3). These analogs have equivalent size, structure, and charge properties to the proteinogenic amino acids. An analog competes with its corresponding amino acid for the finite tRNAs in protein biosynthesis [76]. Once incorporated into the protein, the analog interrupts translation or inactivates the protein. This could lead to growth retardation or even cell death [77]. However, for amino acid overproducers, excess amino acids could outcompete analogs to generate correctly charged tRNA and thus produce enough functional proteins to survive [78]. For instance, 4-Azaleucine, with an additional dimethylamino group compared with L-Leu, has been successfully applied to select L-Leu overproducers [28]. Other analogs, such as 2-thiazole alanine [25] and ethionine [36], were also used to screen L-Val and L-Met overproducers, respectively (Table 1).

Compared with previous methods, the analog-based strategy requires only the addition of amino acid analogs into the culture medium without strain modification or introducing other elements. However, this strategy suffers from two significant flaws. First, amino acid analogs may interfere with cellular activities other than protein synthesis, disrupting the nucleus regions [78], affecting the structure of cellular membranes, and inhibiting purine and pyrimidine biosynthesis [79]. This could accidentally eliminate potential amino acid overproducers. Second, strains can escape the selection pressure of analogs through several pathways. For instance, analogs could be blocked by amino acid transporters or pumped out of the cell by efflux pumps [77]. Analogs could also be degraded into nontoxic forms or incorporated directly into bacterial proteomes [80]. This results in a high false-positive rate in which the screened strains are not true amino acid overproducers. It should also be noted that although corresponding analogs could be found for each amino acid, limited by the cost, stability, and toxicity of the analogs, not every amino acid has an analog suitable for screening overproducers. Therefore, a safe, accurate, simple, low-cost, and universal screening strategy remains to be developed.

2.3.2. Strategy based on rare codon

The translation process of proteins can be divided into two steps. The first step is aminoacylation. That is, aaRS specifically charges amino acids onto the corresponding tRNA. This step could be considered a double-substrate reaction catalyzed by aaRS, with amino acids and tRNA binding to aaRS, then the aaRS charges amino acids to the 2′- or 3′-hydroxyl group of the last base at the 3′ end of the tRNA. The second step is to decode the mRNA. The tRNA recognizes and pairs with the corresponding codons through its anticodon loop and transfers the loaded amino acid to the extending peptide chain. The majority of amino acids have at least two codons. The use of codons with different frequencies affects the translation rate of proteins. The traditional screening strategy based on amino acid analogs is to create competition by introducing analogs to the substrate-binding step, and amino acid overproducers would gain growth advantage over other strains. Such a competition principle could also be introduced to other translation steps, including the binding of the tRNAs to aaRS, the aminoacylation reaction of the aaRS, and the mRNA decoding process. When artificial competition is created, the natural translation process weakens. Under this pressure, only the amino acid-overproducing strains could gain a competitive advantage in translating the reporter proteins. Following this principle, Zheng et al. [3] proposed a novel strategy for screening amino acid overproducers. By introducing competition to the aminoacylation-decoding process, a rare codon-based screening system was established, which took advantage of the “codon preference” law that is ubiquitous in nature (Fig. 4) [3,81].

During protein translation, the frequency of codon usage differed. Meanwhile, different codons corresponded to varying contents of tRNA. The common codons widely used by organisms are usually read by the most abundant types of tRNAs in cells. Nucleic acid sequences composed of such codons could be translated more efficiently. In contrast, the rare codons that infrequently appear in protein-coding sequences often correspond to the tRNAs with low abundance in the cell. When the total amount of amino acids is limited, such low-abundance tRNAs are challenging to charge amino acids in competition with abundant tRNAs. They are often in an “uncharged” state, leading to decreased or stagnated translation of the rare codons. Only when the intracellular amino acid or the environment amino acid concentration is significantly increased, the rare tRNAs can charge the excessive amino acids and allow normal translation of rare codons [3].

Inspired by this theory, the amino acid concentration required for translation could be intentionally increased by replacing codons in reporter genes with their rare alternatives. When the concentration of amino acids produced by the strain was low, the translation of the rare codon-rich reporter gene was hindered. On the contrary, when the concentration of amino acids produced by the strain is increased, expression of the reporter gene can be rescued, resulting in easily detectable phenotypes. Accordingly, a selection system of amino acid overproducers was established using rare codon-rich antibiotic-resistance genes. A screening system was established using rare codon-rich chromogenic or fluorescent protein-encoding genes. The screening and selection systems based on the rare codons were successfully applied to screen *E. coli* overproducers of L-Leu, L-Arg, and L-Ser. Taking L-Leu as an example, in the selection system, the 29 L-Leu codons in the kanamycin resistance gene (kan^R) were all replaced by the rare alternative CTA. In a diluted Luria-Bertani medium containing kanamycin, this rare codon-rich kan^R gene was introduced into the random mutant library of *E. coli*. The strain with a growth advantage is the L-Leu overproducer and can be selected. In addition, by replacing the L-Leu codons in the gene encoding green fluorescent protein or purple protein with their rare forms, overproducers can be picked by visual observation of color changes. This system could also

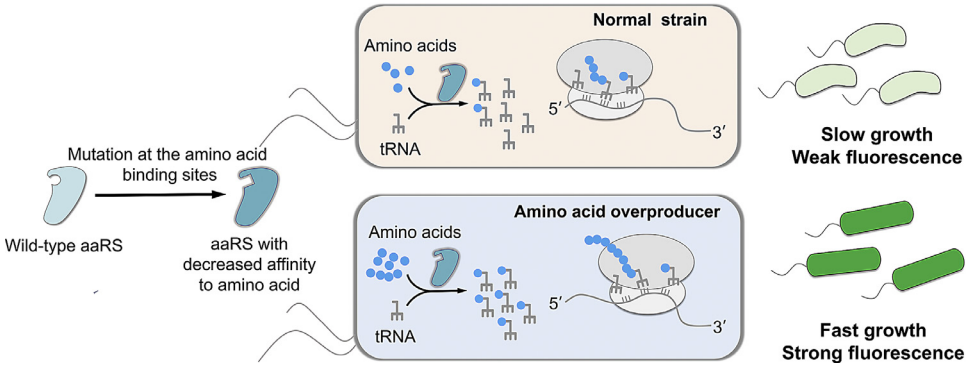

Fig. 5. The aaRS-based strategy for screening amino acid overproducers.

be extended to screening other common amino acid-producing hosts. For instance, the L-Arg codon in *kan*^{R} was replaced with its rare form in *C. glutamicum* and several *C. glutamicum* strains overproducing L-Arg were successfully identified [81].

This rare codon-based strategy was subsequently applied to screen L-Pro overproducers in both *E. coli* and *Serratia marcescens* JNB5 by replacing the codon of L-Pro on the resistance gene with the corresponding rare alternatives [32]. This system could also assist in screening overproducers for nonstandard amino acids, such as the L-Pro-derivative trans-4-hydroxy-L-proline [82].

It is considered that the rare codon-based screening strategy is feasible, simple to operate, and universal [83]. This strategy requires only a single rare codon-rich marker gene. At the same time, the screening strength could be adjusted by changing the frequency of rare codons and the copy numbers of markers. Although this strategy cannot be directly applied to amino acids with only one tRNA, such as L-Lys, L-His, and L-Phe, it provides a promising alternative to amino acid analog-based screening strategies in breeding amino acid overproducers [84].

2.3.3. Strategy based on aaRS

Following the idea of translational competition, selection pressure could also be created by regulating the affinity of aaRS with amino acids. Aminoacyl-tRNA synthetase is responsible for the specific recognition of its corresponding amino acid and tRNA and then generates aminoacyl-tRNA through a two-step catalytic mechanism. Since the amino acid-binding domain of aaRS and the corresponding tRNA are two separate domains, mutations to the amino acid-binding domain of aaRS can reduce the affinity to amino acids without affecting the binding to tRNA. When the total amount of amino acids is limited, aaRS with poor affinity to amino acids is difficult to form an aaRS-amino acid complex. This leads to decreased or even stagnated cell growth. When the intracellular amino acid or the environmental amino acid concentration is increased, this aaRS with poor affinity to amino acids could charge enough tRNAs, and cell growth can be compensated. Under amino acid starvation, using a mutant of *E. coli* isoleucine-tRNA synthetase IleRS^{G94R}, which has an affinity for isoleucine 10^4 times lower than the wild-type IleRS, protein synthesis will be hindered, thus affecting cell growth. Cells producing sufficient L-Ile can resist this interference and show a growth advantage. Therefore, they could be screened out [30] (Fig. 5). Theoretically, for each proteinic amino acid, constructing a mutant aaRS with low affinity for an amino acid could extend this method to 20 natural amino acids. However, the wild-type aaRS on the genome should be knocked out to apply this method, which might bring unknown interference to the overall metabolic network of the cell. At the same time, the application of this strategy is confined to strains that are difficult to perform genetic manipulation.

2.3.4. Strategy for screening nonstandard amino acid overproducers

Translation-based screening strategies have the potential to screen nonstandard amino acid overproducers. The genetic codon expansion technology that emerged in recent years can realize the reassignment of one or more specific codons to nonstandard amino acids and introduce nonstandard amino acids into proteins. This technique requires the establishment of artificial aaRS/tRNA pairs [85]. First, the amino acid-binding domain of exogenous aaRS was engineered to recognize specific nonstandard amino acids [86]. Meanwhile, the anticodon loops of the exogenous tRNAs were replaced with complementary bases of the reassigned codons, usually the amber codon UAG. A low charging efficiency of the aaRS/tRNA pair for nonstandard amino acids [87–89], together with the competition from the release factor RF1 in reading the UAG codon [90], would contribute to the screening effects of this system. Only the strains capable of producing high concentrations of nonstandard amino acids could allow sufficient charging of the tRNA to translate the UAG-containing marker genes. This strategy has been used to indicate the concentration of nonstandard amino acids, such as selenocysteine (Sec) [91,92].

Compared with the above strategies, the fidelity of the system for screening nonstandard amino acid overproducers is low. Although artificial tRNA/aaRS pairs are generally used across taxonomic domains and have undergone orthogonality optimization, they still have a probability of mis-incorporating their original amino acids. Specific amino acids can be selected to avoid this problem in selecting the substitution sites of marker genes. First, amino acid residues structurally similar to target nonstandard amino acids could be selected as candidate targets. Next, among these candidate targets, the residues on the marker protein essential for protein function could be selected as insertion sites. For example, an antibiotic-degrading enzyme, β-lactamase (NmcA), was selected as the indicator protein to detect Sec incorporation. Since the aminoacylation of Sec is dependent on seryl-tRNA synthetase, there will be Ser interference in the synthesis of selenocysteine. Proteins with specific residues capable of distinguishing between Sec and Ser could be selected as indicator proteins. NmcA requires a disulfide bond adjacent to the active site for activity. When Cys is replaced by a highly similar Sec instead of Ser, the newly formed Cys-Sec or Sec-Sec bond can maintain protein activity, while a Ser insertion will inactivate this enzyme. Therefore, the codon of Cys can be replaced by the UAG codon to identify Sec production. This may happen with the aid of an artificial EF-Tu-dependent $tRNA^{Sec}_{CUA}$ [91]. When the strain produces high concentrations of Sec and inserts Sec into the UAG site on the *nmcA*, the strain survives in the medium containing ampicillin. Theoretically, a screening system for Sec overproducing strains based on the *nmcA* resistance gene could be established.

Alternatively, a DnaB intein that relies on selenocysteine insertion to maintain function can be used as an indicator protein. This DnaB mini-intein mutant of only 16 amino acids requires Cys at position 1 to splice the protein. When Cys at this site is substituted by Sec with equivalent properties, it can retain its function. By incorporating this engineered intein, any marker gene, such as GFP, could be modified to respond specifically to Sec [92]. That is, when Sec is inserted, the intein has cleavage activity, which can splice and reactivate GFP. Otherwise,

Table 2
Comparison between different screening strategies.

Screening strategies		Resolution (to distinguish individual cell)	Specificity	Responding speed	Potential compatibility with 20 proteinogenic amino acids	Compatibility with nonstandard amino acids	Compatibility with different microbial species	Simplicity of system construction	Toxicity
Auxotrophic strain-based	Cell lysate-feeding	+	++	+	+++	–	++	++	N
	Co-culture	+	++	+	+++	–	+	+	N
Biosensor-based	Coupled enzymatic reaction	++	+	+	+	–	+	+	Y
	Fusion protein	++	+	+++	+	–	++	+	N
	Transcription factor-based biosensor	++	+	++	++	–	+	++	N
	Riboswitch	++	++	++	+	–	+	+	N
Translation-based	Amino acid analogs	++	+	++	++	–	++	++	Y
	Rare codon-rich marker	++	++	++	++	–	++	++	N
	Mutated aaRS	++	++	++	+++	–	+	+	N
	Artificial aaRS/tRNA pair	++	+	++	+++	+	++	+	N

–: It is difficult to achieve.
+: This screening strategy performs well in this aspect and more "+" represent better performance.
Y/N: "Y" for Yes and "N" for No.

when other amino acids are inserted, the intein has no cleavage activity, and the reporter gene cannot be expressed normally. A new solution for the high-fidelity screening of nonstandard amino acid overprducers is provided by this strategy. However, obtaining target tRNA/aaRS orthogonal pairs and selecting high-fidelity markers for various nonstandard amino acids still face challenges.

3. Conclusion

Microbial fermentation has become the primary approach for producing amino acids. The screening strategy for amino acid overproducers is one of the most crucial factors in obtaining MCF with high performance to produce the desired amino acids. Establishing a universal, efficient, and easy-to-operate strain screening strategy has become a current focus. The choice of screening strategies is suggested considering the advantages, disadvantages, and applicability of these strategies. First, auxotrophic-based, fusion protein-based biosensors, and translation-based screening strategies are more applicable for uncommon host species. The auxotrophic-based strategy responds to amino acids *in vitro* without introducing sensing elements into the production strain. The fusion protein-based biosensor has no particular dependence on the transcriptional regulatory system of the host strain. Translation-based strategies apply principles of translation that are ubiquitous in species. Therefore, these three strategies have the potential to be extended to uncommon host species. Second, when the diversity of the mutant library is large, the screening strategy needs to have high throughput and time efficiency, and biosensor-based and translation-based screening strategies are better choices. In particular, the fusion protein-based biosensor enables nanosecond responses to amino acids. Third, to expand the response range, the auxotroph-based screening strategy is more convenient for adjusting the response threshold. A simple approach to adjusting the response range by diluting fermentation broth is offered by this strategy. Fourth, auxotrophic-based and translation-based strategies could be used for different amino acid species to screen overproducers of 20 natural amino acids, with the latter being much easier to operate. When codon expansion technology is introduced, the translation-based strategy could be particularly extended to screening nonstandard amino acid overproducers (Table 2).

Although translation-based screening strategies have been significantly improved in universality, efficiency, and operability, there is still much room for improvement. First, in the aminoacylation step of the translation process, tRNA is also a critical substrate. Reducing the efficiency of aminoacylation by reducing the affinity of tRNAs to the corresponding aaRS might also create a screening pressure, which could offer a new method for developing a screening strategy based on the translational process. Secondly, coupling the screening strategy with high-throughput detection technologies, such as microfluidics and flow cytometry, or even the combination of the automatic workstation, may significantly improve the throughput and speed of strain screening. It should be noted that expanding the screening strategy to screen more complex nonproteinogenic amino acid overproducers and further enhancing the universality of the screening strategy still face challenges. In addition, the response range and accuracy of the screening strategy also need further improvement.

Following the design-build-test-learn cycle, analysis of the overproduction mechanism of the identified overproducers could aid in reconstituting amino acid-overproducing strains. Combining whole-genome sequencing, systems biology techniques, and bioinformatics techniques, amino acid overproducers could be systematically analyzed. Exploring potential targets that are indirectly related to amino acid synthesis and utilization could further the understanding of the mechanism of amino acid overproduction. Integrating these new targets into the currently used producing strains could break through the production ceiling of the existing strains and further help the amino acid fermentation industry to reduce costs, increase production capacity, and accelerate industrial growth.

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.

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