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Review Article

Ergothioneine biosynthesis: The present state and future prospect

Li Liang *, Xu Shan-Shan, Jiang Yan-Jun **

School of Chemical Engineering and Technology, Hebei University of Technology, Tianjin, 300401, China

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ABSTRACT

Ergothioneine (ERG), a rare natural thio-histidine derivative with potent antioxidant properties and diverse biological functions, is widely utilized in food processing, cosmetics, pharmaceuticals, and nutritional supplements. Current bioproduction methods for ERG primarily depend on fermenting edible mushrooms. However, with the advancement in synthetic biology, an increasing number of genetically engineered microbial hosts have been developed for ERG production, including *Escherichia coli*, *Saccharomyces cerevisiae*, and *Corynebacterium glutamicum*. Given the involvement of multiple precursor substances in ERG synthesis, it is crucial to employ diverse strategies to regulate the metabolic flux of ERG synthesis. This review comprehensively evaluates the physiological effects and safety considerations associated with ERG, along with the recent advancements in catalytic metabolic pathway for ERG production using synthetic biology tools. Finally, the review discusses the challenges in achieving efficient ERG production and the strategies to address these challenges using synthetic biology tools. This review provides a literature analysis and strategies guidance for the further application of novel synthetic biology tools and strategies to improve ERG yield.

1. Introduction

Ergothioneine (ERG) was first discovered and isolated by Charles Tanre in 1909 from the fungus *Claviceps purpurea* in rye grains [1]. ERG is predominantly synthesized by specific bacteria and fungi in nature, making it a rare naturally occurring chiral amino acid. In recent years, ERG, as an essential physiological compound, has been widely used in food, cosmetics, medicine and other industries because of its potent antioxidant properties and various biological roles [2–4]. In food, ERG can be used by two ways: exogenous addition and endogenous addition. Compared to synthetic chemical inhibitors, ERG is non-toxic and exhibits superior stability. Therefore, ERG can be used as an effective antioxidant, color-retention agent, and dietary supplement in various high-value foods [2,5]. In cosmetics, ERG is a hot ingredient in cosmetics industry because of its safety, unique antioxidant ability, targeted anti-inflammatory ability, source-based anti-aging ability, noncompetitive whitening ability, and excellent stability [6–9]. In medicine, due to ERG's antioxidant and anti-inflammatory activities, ERG has the huge therapeutic or preventive potential for many oxidative stress-mediated diseases, such as diabetes, kidney disease, cardiovascular diseases, liver diseases, and neurodegenerative diseases [10–14] (Fig. 1).

Currently, commercially available ERG is primarily produced through chemical synthesis. Compared to chemical synthesis methods, microbial cell factory synthesis offers advantages such as high yield and low cost, making it suitable for industrial-scale production. Through metabolic engineering and process optimization, production efficiency can be significantly enhanced, and this approach has gradually become the mainstream method for ERG production. Numerous companies worldwide are engaged in the production and sale of ERG, including major biotech firms and those specializing in health products. For instance, companies like Blue California in United States, Lonza Group in Switzerland, and GeneIII in China are actively developing and promoting ERG products. Through ongoing technological innovation and process optimization, these enterprises have enhanced the production efficiency and quality of ERG. According to market research statistics, global ERG market sales reached 463 million yuan in 2022 and are projected to reach 893 million yuan by 2029, reflecting a compound annual growth rate of 14.46 %. The global ERG market is anticipated to sustain significant growth in the coming years, particularly in the functional foods, dietary supplements, and cosmetics sectors. For instance, Estee Lauder's creams containing ergothioneine are priced at $310.00 per 48 g, while Life Extension's capsules containing ERG cost

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* Corresponding author.
** Corresponding author.
E-mail addresses: liangli@hebut.edu.cn (L. Liang), yanjunjiang@hebut.edu.cn (J. Yan-Jun).

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$20 per 5 mg. In the future, as new manufacturers enter the market, a significant decline in the price of ERG is anticipated. Nevertheless, ERG remains an outstanding product that is relatively high-priced.

Previous reviews have mainly focused on the physiological functions [15–18] and applications [5–7,10,19,20] of ERG as well as the detection and purification methods [3,4]. However, no review has systematically summarized the biosynthesis of ERG driven by synthetic biology. This paper not only reviewed the biological functions and safety of ERG, but also reported the latest progress of ERG production by edible fungi, *in vitro* biocatalysis and transformation. In addition, the challenges of de novo ERG synthesis with engineered strains such as *Escherichia coli*, *Saccharomyces cerevisiae*, and *Corynebacterium glutamicum* were analyzed and valuable synthetic biology strategies were provided.

2. Physiological functions and safety of ERG

ERG is a naturally occurring antioxidant that offers substantial cellular protection. In aqueous solution, ERG exists in tautomeric forms, namely thiol and thione (Fig. 2). Under physiological conditions, ERG primarily exists in its thione form because the thiocarbonyl group is more stable than the sulfhydryl group [21]. Additionally, ERG, with a standard redox potential ($E^0 = -0.06$ V), exhibits lower reactivity, greater resistance to autooxidation, and higher chemical and thermal stability compared to most thiols in organisms, such as glutathione ($E^0 = -0.24$ V), lipoic acid, vitamin C, and vitamin E ($E^0 = -0.2$ to -0.32 V) [16,22]. The antioxidant properties of ERG have been confirmed by numerous studies, and its mechanism can be categorized into four primary aspects (Fig. 3): (1) Reducing the concentration of hydroxyl radicals (-OH) and removing hypochlorous acid (ClO^-) and nitrite, thereby inhibiting the production of reactive oxygen species (ROS) and singlet oxygen [23]; (2) Activating or inhibiting endogenous antioxidant enzymes, thereby enhancing cellular antioxidant capacity [15]; (3) Chelating with various bivalent metal cations (Cu^{2+}, Zn^{2+}, Co^{2+}) to form non-redox active complexes, thereby inhibiting ROS formation and

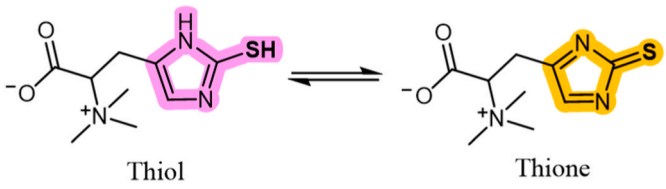

Fig. 2. Two tautomeric forms of ERG.

protecting DNA and proteins from metal-induced oxidative damage [24]; (4) Influencing the oxidation of heme proteins to prevent lipid peroxidation, such as by inhibiting myoglobin oxidation [25].

Human cells cannot synthesize ERG and instead rely on a specific transporter known as OCTN1 to facilitate the transportation of dietary ingested ERG to various parts of the body [19,26,27]. The expression levels of ERG transporters are closely linked to the physiological states of different body regions. Research indicates that liver injury significantly upregulates the expression of ERG transporters [28], whereas OCTN1 deficiency is associated with increased susceptibility to kidney injury and intestinal inflammation in ischemic-reperfusion models [29,30]. Recent reports suggest that a specific ERG transporter, EgtUV, present in *Helicobacter pylori* within the gastrointestinal tract, absorbs ERG, thereby regulating microbial redox homeostasis [31]. Since the discovery of ERG, researchers have been exploring its properties and potential biological roles; however, the complete physiological functions of ERG require further investigation. While the antioxidant properties of ERG are well-established, evidence supporting its biological functions within the body is still limited [18].

The safety of ERG has been acknowledged by numerous international organizations and institutions. In 2014, the State Administration for Market Regulation in China officially added ERG to the list of approved cosmetic ingredients. In 2017, the European Commission approved ERG as a novel food ingredient [32]. Subsequently, the range of applications

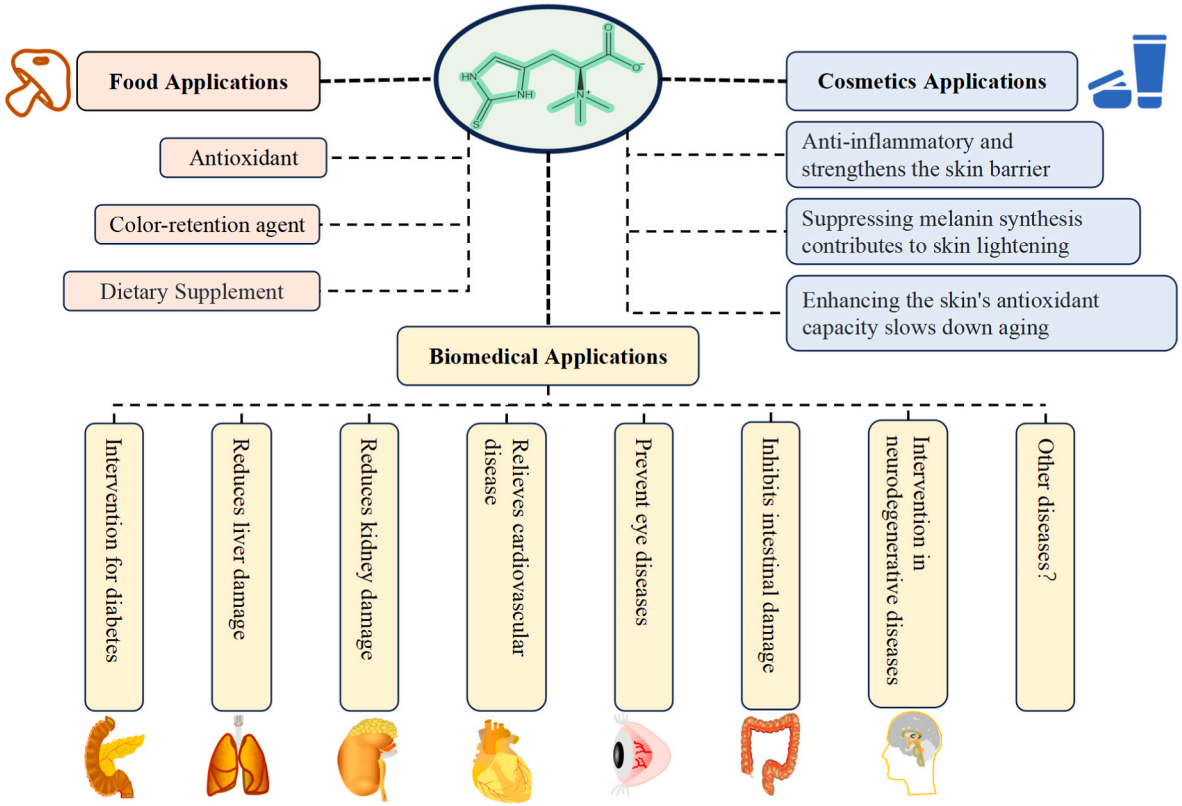

Fig. 1. Application prospect of ergothioneine in different industries.

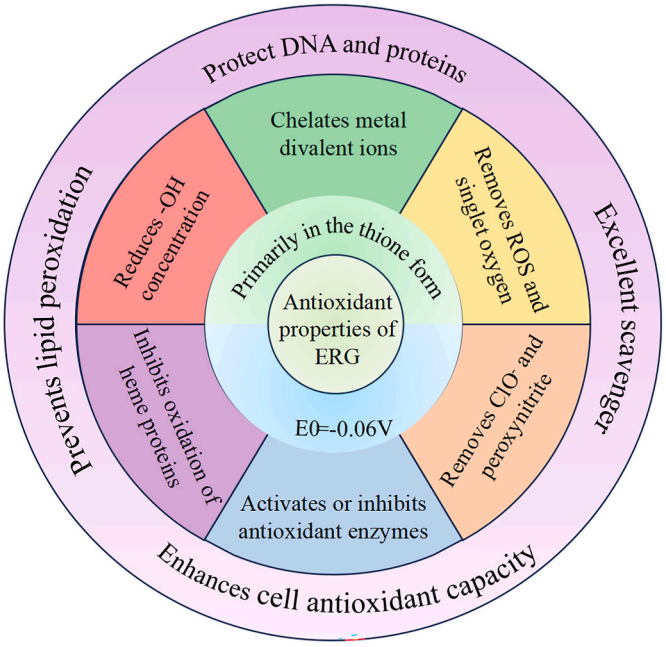

Fig. 3. Antioxidant properties of ERG.

for ERG as a novel food resource broadened to include non-alcoholic beverages, cereal bars, milk, fresh dairy products, and chocolate. ERG has been deemed safe for consumption by infants and adults, including pregnant and lactating women, when consumed within the recommended intake levels [33]. The Food and Drug Administration (FDA) of U.S. has also granted ERG GRAS (Generally Recognized as Safe) status, enabling its incorporation into a diverse range of consumer products, including nutraceuticals, foods, and cosmetics [34]. The safety certification of ERG has undoubtedly facilitated its adoption across various market segments.

3. Progress in the bioproduction of ERG

3.1. ERG production by natural producers

3.1.1. Preparation of ERG from natural producers

Natural edible mushrooms possess ERG synthesis pathways and are commonly used in ERG production including species such as *Flammulina velutipes* [35], *Lentinus edodes* [36,37], *Ganoderma neo-japonicum* [38], *Agaricus bisporus* [39] and *Pleurotus eryngii* [40]. Various strategies have been employed to enhance cell growth and ERG yield in macrofungal fermentation, including optimizing carbon and nitrogen sources, adjusting temperature, and timing the harvest appropriately. Tepwong et al. [36] utilized *L. edodes* mycelium for immersion fermentation in a synthetic medium, achieving a maximum ERG production of 0.913 mg/L on the 15th day. Furthermore, the 70 % ethanol extract of the synthetic medium demonstrated higher 2, 2-diphenyl-1-trinitrohydrazine (DPPH) radical scavenging activity and reducing ability compared to the agricultural waste-supplemented medium. Subsequently, the fermentation process for ERG production using *L. edodes* was further investigated [37]. By incorporating 25 g/L of fructose and 1 g/L of aspartic acid as carbon sources, the mycelium ERG yield was 3.15 times greater than that observed in the control group [37]. Additionally, by introducing 2 mmol/L L-methionine, the ERG yield of the mycelium increased to 3.45 mg/g of dry weight (DW) after a 15-day fermentation period. In addition, the production of ERG from *P. eryngii* mycelium on a 10-Liter scale and the optimal temperature for mycelium growth were further explored [40]. The ERG content in the mycelium reached 5.76−5.84 mg/g DW between the 18th and 20th days of fermentation after the addition of a combination of amino acids under optimal culture conditions, corresponding to approximately 64.2 mg/L in the fermentation broth. Furthermore, the immersion fermentation of *Pleurotus citrinopileatus* was investigated [41]. When a combination of 8 mmol/L L-cysteine, 4 mmol/L L-histidine, and 0.5 mmol/L L-methionine was added, the maximum ERG content reached 14.57 mg/g DW, representing a 39.53 % increase compared to *P. eryngii*. High-yield ERG production strains of *Pleurotus ostreatus* were identified, and the effects of amino acids, exogenous nutrients, and precursors on the synthesis of ERG were investigated. By using safe and edible medium components, an ERG production exceeding 500 mg/L in shake flask fermentation broth was achieved [42]. In addition to certain edible mushrooms, some yeast strains, such as *Rhodotorula mucilaginosa*, have been reported to have the highest ERG yield among non-recombinant strains [43]. ERG was successfully synthesized in *R. mucilaginosa* DL-X01 using untreated and crude molasses as substrates, achieving a yield of 216.25 mg/L in a 5 L fermenter in the latest report [44].

3.1.2. Modification of natural producers to improve the ERG production

Currently, besides natural edible fungi, various microorganisms in nature have been found to be able to produce ERG directly. Advances in genetic engineering have made it possible to modify other natural producers, thereby providing a viable strategy to enhance ERG production.

Initially, Alamgir et al. [45] identified a high-yielding ERG-producing strain, *Methylbacterium* 22A, from moss based on metabolomic analysis, which produced 6.3 mg/g DW of ERG on the 38th day. Subsequently, the ERG yield increased to 7.0 mg/g DW by increasing the gene copy number of *egtB/D* and knocking out the L-histidine ammonia gene *hutH* [43]. Previous studies have shown that by integrating multiple copies of the *egt1* and *egt2* genes into its genome, the filamentous fungus *Aspergillus oryzae* achieved an ERG yield of 231 mg/kg in culture medium, which is 20 times higher than the wild type [46]. Furthermore, researchers identified the presence of ERG synthase in *Cordyceps militaris* and reintroduced this pathway into its genome, successfully increasing the ERG yield to 2.5 g/kg DW [47]. Another research team [48] demonstrated that overexpression of ERG synthesizing gene clusters and key enzymes promoting L-histidine synthesis in *Mycobacterium neoaureus* resulted in a 100 mg/L ERG. Additionally, by knocking out the putative ERG lyase and overexpressing homocysteine hydrolase, the ERG yield reached 1.56 g/L after 216 h of culture. Subsequently, the proportion of extracellular ERG increased from 18.7 % to 44.9 % by introducing the putative transporter gene *mfsT1*. Subsequently, three copies of the *egtA/B/C/D/E*, *hisG*, and *mfsT*1 genes were integrated into the *M. neoaureus* genome using genome integration tools to achieve an ERG yield of 85.9 mg/L. Consequently, plasmid-free genetically engineered strains exhibiting high stability and yield were developed [49]. Given the rapid advancement of synthetic biology, additional strategies can be employed, such as identifying more microorganisms capable of naturally synthesizing ERG and creating effective genetic engineering platforms to systematically regulate the ERG synthesis pathway at the genomic level, thereby further enhancing the efficiency of ERG synthesis. The levels of ERG synthesized by natural producers through fermentation are summarized in Table 1.

3.2. Production of ERG through in vitro biocatalysis and transformation

Biocatalysis and transformation involve the use of free enzymes or whole cells as catalysts to convert substrates into desired products (Fig. 4). Unlike traditional chemical synthesis, biocatalysis can catalyze both single-step and multi-step reactions, as well as multi-component processes [53]. Biocatalysis typically operates under mild conditions, with pH levels close to the natural environment. The primary advantage of biocatalysts is their superior specificity, including chemical specificity and regioselectivity [54].

However, there are few reports on the synthesis of ERG through biocatalysis and conversion *in vitro*. This scarcity may be attributed to issues such as protein instability, low activity, and the high costs of

Table 1
Summary of natural producers producing ERG.

Producers	Characteristics	ERG concentration	Fermentation period	Reference
Naturally produced				
Lentinula edodes	Soak for fermentation in synthetic medium	0.913 mg/L	15 d	[36]
Lentinula edodes	Fructose and aspartic acid are combined to supplement l-methionine	3.45 mg/g DW	15 d	[37]
Ganoderma neo-japonicum	Adding l-methionine, l-cysteine, l-histidine	15.42 mg/L	10 d	[38]
Agaricus bisporus	Selection of cultivation conditions and post-harvest storage	1.3 mg/g DW	–	[39]
Pleurotus eryngii	Optimization of fermentation process and adding l-methionine, l-cysteine, l-histidine	5.76–5.84 mg/g DW (64.2 mg/L)	18–20 d	[40]
Pleurotus citrinopileatus	Optimization of medium and adding l-methionine, l-cysteine, l-histidine	14.57 mg/g DW	16 d	[41]
Pleurotus ostreatus	Optimization of medium and fermentation process	>500.00 mg/L	18 d	[42]
Rhodotorula mucilaginosa Z41C	Optimize culture conditions	24 mg/L	7 d	[43]
Rhodotorula mucilaginosa DL-X01	Molasses and fishmeal hydrolysate were used as substrates	216.25 mg/L	7 d	[44]
Rhodotorula toruloides	–	79.0 mg/L	7 d	[50]
Methylbacterium strain 22A	–	6.3 mg/g DW	38 d	[45]
Ustilago siamensis CBS9960	Culture in YM medium and raise precursor l-histidine	75 mg/L	5 d	[51]
Panus conchatus	Add molasses and soy peptone and amino acid precursors	148.79 mg/L	6 d	[52]
Genetic modification to produce				
Methylbacterium strain 22A	Increasing the gene copy number of *egtB/D* and knocking out *hutH*	7.0 mg/g DW	7 d	[43]
Rhodotorula toruloides	A CRISPR-assisted Cre recombination (CACR) method and a high-throughput screening method were established	267.4 mg/L	168 h	[50]
Mycobacterium neoaureus	introducing the transporter gene *mfsT1* and integrating the *egtA/B/C/D/E*, *hisG*, and *mfsT1* genes into the genome	85.9 mg/L	168 h	[49]
Cordyceps militaris	ATMT method was used to construct recombinant strains and optimize fermentation culture conditions	2.5 g/kg DW	10 d	[47]
Aspergillus oryzae	Integrating multiple copies of the *egt1* and *egt2* genes	231 mg/kg	–	[46]
Mycobacterium neoaureus	Overexpression of ERG synthesis genes and genes promoting l-histidine synthesis; knockout of *Mn_3042* gene; overexpression of the homocysteine hydrolase gene	1.56 g/L	216 h	[48]

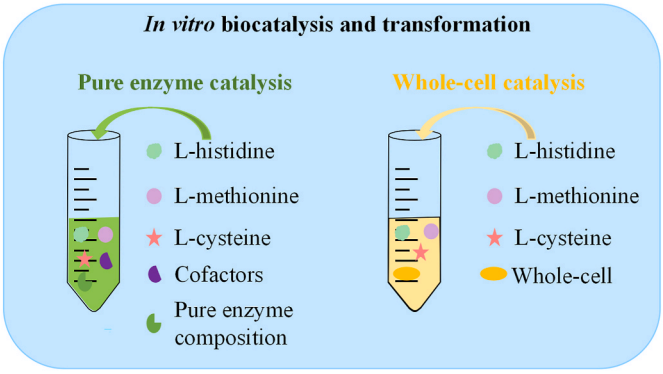

Fig. 4. Two primary modalities of *in vitro* biocatalysis and transformation. In a pure enzyme catalytic system, it is necessary to add specific substrates (such as L-cysteine, L-histidine, and L-methionine), cofactors (such as ATP and pyridoxal-5′-phosphate (PLP)), and precise proportions of enzyme compositions. In a whole-cell catalytic system, only the addition of a specific substrate and the expression of the enzyme-catalyzed system within whole cells are required.

purification when using naturally pure enzymes, which limit the application of pure enzyme catalysis in biotransformation *in vitro* [55]. Additionally, enzymes involved in the target product biosynthesis pathway exhibit a wide range of functional and enzymatic properties. This diversity presents challenges such as protein incompatibility, unsuitable reaction conditions, and intermediates or cofactors that inhibit enzyme activity *in vitro*, severely affecting catalytic efficiency [56]. The stability and compatibility of enzymes can be enhanced by optimizing reaction conditions, employing immobilization, and utilizing directed evolution [57,58]. Sequential tandem or multi-enzyme-catalyzed flow systems are employed to improve protein stability and minimize the inhibition of enzyme activity by intermediates and cofactors [59].

Statistics indicate that whole-cell catalytic synthesis is more commonly used than pure enzyme-catalyzed reactions [60]. Whole-cell biocatalysis facilitates enzyme cascades in multiple reactions and integrates the supply of numerous cofactors necessary for complex biological transformation [61], offering benefits such as straightforward metabolite generation, high conversion rates, and low energy consumption [55]. Moreover, the presence and protective properties of the cell envelope contribute to enzyme stabilization, allowing their application under harsh reaction conditions [62,63]. Additionally, the close proximity of reactants and catalysts, along with the inherent presence of otherwise costly external cofactors, significantly enhances the efficiency of biocatalysts [63]. However, in whole-cell catalysis, cell membranes restrict the permeability of substrates and products, resulting in slower reactions, numerous side reactions, and difficulties in product extraction and purification. Research has shown that the permeability of cell membranes and cell walls can be chemically enhanced to facilitate substrate transfer [61].

3.3. Modification of model strains for the ERG production

3.3.1. Bacterium

E. coli has become the most extensively studied bacterium in industrial biology due to its advanced genetic modification capabilities. It is used for the industrial production of numerous bulk and fine chemicals, including L-homoserine, glutaric acid, and ectoin, among others [64,65]. The ERG synthesis pathway has been genetically engineered into *E. coli*. For example, by heterologously expressing EgtB/C/D/E synthetase and the EgtA isoenzyme GshA in *E. coli*, along with the identification of intermediates and optimization of precursor supply, ERG production significantly increased from 0.2 mg/L to 24 mg/L after 72 h of fermentation, achieving a 120-fold increase [66]. Subsequently,

co-expression of EgtA in this strain, by overexpressing feedback inhibition-insensitive CysE and SerA, resulted in high-yielding L-cysteine strains. Additionally, the disruption of the transcriptional repressor gene *metJ* enhanced the metabolic flux of L-methionine and S-adenosyl-L-methionine (S-SAM), contributing to an ERG yield of 1.3 g/L [67]. Chen et al. [68] developed a novel ERG production system in *E. coli* through the co-expression of two ERG biosynthetic genes (*tregt1* and *tregt2*) from *Trichoderma reesei*, resulting in a yield of 4.34 g/L ERG after 143 h of incubation in a 2 L tank fermenter. In recent studies, *egtB/D/E* from *Methylobacterium aquaticum* has been used to reconstruct the ERG biosynthesis pathway in *E. coli*. Through plasmid copy number optimization and a series of metabolic engineering strategies, the precursor amino acid metabolic pathway was reconstructed, successfully enhancing the supply of precursors for ERG biosynthesis [69]. Overexpression of truncated Egt1 from *N. crassa*, along with the *egtD* and *egtE* genes from *M. smegmatis* in *E. coli* resulted in a final ERG yield of 5.4 g/L [70]. In their study, a combined method based on random mutation and rational design improved the activity and catalytic efficiency of key enzymes for ERG synthesis, significantly enhancing ERG yield and achieving high-efficiency production in a short time.

In addition to the model strain *E. coli*, *C. glutamicum* exhibits enhanced stress resistance, improved safety, reduced pathogenicity, and efficient production of precursor amino acids, making it an exceptional host for ERG biosynthesis. The genes *egt1* and *egt2* from *Schizosaccharomyces pombe* were first introduced into *C. glutamicum* by Kim et al. [71]. By optimizing sulfur assimilation and pentose phosphate pathways and increasing the accumulation of L-histidine and L-cysteine precursors, a yield of 264 mg/L ERG was achieved after 36 h of fermentation. The genetically modified *C. glutamicum* has effectively simplified fermentation conditions. However, it still produces lower ERG yields and requires longer production cycles. Considering its industrial application, further improvements are necessary for the production of ERG from *C. glutamicum*.

3.3.2. Yeast

Yeast, a single-celled fungus, was initially found to naturally synthesize ERG in certain strains [43]. Among these, *S. cerevisiae* has been extensively studied due to its well-characterized genetic tools and established genetic background. Egt1 from *N. crassa* and Egt2 from *C. purpurea* were co-expressed in *S. cerevisiae* by deleting the *TOR1* or *YIH1* genes to regulate nitrogen metabolism and optimizing the media composition, resulting in 598 mg/L ERG after fed-batch fermentation in a 1 L bioreactor [72], whose work marks the first documented production of ERG using *S. cerevisiae*. Subsequently, the researchers screened the genes involved in nitrogen metabolism regulation system in *S. cerevisiae* and identified target genes to improve ERG yield. After optimizing the medium and conducting batch fermentation for 160 h, the ERG yield reached 2.39 g/L [73]. Furthermore, *Gf*Egt1 and *Gf*Egt2, the ERG synthases derived from *Grifola frondosa*, were effectively co-expressed in *S. cerevisiae*, leading to an ERG concentration of 20.61 mg/L after optimizing the fermentation conditions [74].

Unlike *S. cerevisiae*, *Yarrowia lipolytica*, a Crabtree-negative yeast, does not exhibit extensive spillover metabolism under sugar overload conditions, making it more amenable to large-scale fermentation. After 220 h of fed-batch fermentation, the combination of Egt1 derived from *N. crassa* and Egt2 from *C. purpurea* resulted in an ERG production of 1.63 g/L in *Y.lipolytica* [75]. However, in comparison to the production of fat-soluble astaxanthin from *Y. lipolytica* [76], the production of water-soluble ERG appears to be suboptimal. Therefore, researchers established an iterative genome editing approach involving the CRISPR-SpCas system and the Cre-loxp system in *Rhodotorula toruloides*, using the ERG synthesis gene clusters composed of RtEGT1 and RtEGT2, to achieve 267.4 mg/L ERG in *R. toruloides* via shake flask culture [50]. Besides genetic modification, the application of ultraviolet and lithium chloride for random mutagenesis in *S. pombe* led to the generation of a highly efficient ERG synthesis mutant, OMK-79, achieving an ERG yield of 12.5 g/L after 148 h of optimized culture [77], which represents the highest level of ERG production in yeast chassis strains to date. The concentrations of ERG produced by fermentation in current model strains are summarized in Table 2.

In summary, ERG is synthesized through various production methodologies, including natural biosynthesis, *in vitro* biocatalysis or transformation, as well as simulated microbial cell factories, to accommodate diverse production requirements and conditions. It is acknowledged that each of these approaches possesses distinct advantages and disadvantages. First, the main advantages of natural biosynthesis include: natural producers can obtain directly from nature, the production process is simple, does not require complex equipment and technology, and the naturally generated ERG is conducive to market acceptance. However, low yields and over-reliance on natural producers can cause environmental stress and resource depletion, which hinders large-scale production. In addition, natural producers have long production cycles and are significantly influenced by environmental conditions, leading to instability and lack of control in production [51,80]. Although the use of genetic engineering techniques to modify natural producers can improve the rate of ERG production, due to the unclear genetic background of these natural producers and the lack of gene editing tools, only a few strains have been effectively modified to improve ERG production [43,45–50]. Secondly, the production of ERG by biocatalysis and transformation *in vitro* has the advantages of high efficiency, strong specificity, mild reaction conditions, low energy consumption, few by-products and environmental protection standards. However, the production and purification of enzymes is costly, and the stability and activity of enzymes may be reduced under different conditions. Additionally, the cell membrane of the whole-cell catalyst limits substrate and product permeability, leading to slow reaction rates and numerous side reactions. Product extraction and purification are challenging, necessitating continuous optimization of enzyme or whole-cell performance and reaction conditions to enhance economic viability and feasibility [55,61,63]. Finally, the microbial cell factory of ERG produced by the model strain has the characteristics of high yield and good sustainability. The genetic modification of microorganisms effectively improves the production efficiency of ERG, allowing large-scale production under controlled fermentation conditions, which is ideal for industrial applications and can meet the demands of large-scale markets. However, microbial cell factories require significant development investment, the discovery of complex metabolic engineering, and process optimization [81,82]. In addition, the use of GM technology may encounter regulatory challenges and market acceptance issues, thereby a comprehensive assessment of safety and ethics is required. With future technological advances, *in vitro* biocatalysis and transformation, as well as microbial cell factory synthesis of ergothionein, may become more competitive and environmentally friendly mainstream methods.

4. Enhancing ergothioneine production: synthetic biology tools and strategies

Although many microorganisms can synthesize ERG, the natural production by wild-type strains falls significantly short of industrial requirements. Therefore, it is crucial to employ engineering strategies and synthetic biology techniques to optimize biosynthetic pathways of ERG in microorganisms for enhancing the yield of target products, although this approach may impact microbial cell growth [83].

4.1. Key enzyme modification and mutant library construction

Enzyme engineering is a crucial strategy for maximizing pathway flux toward desired natural products. Modifying key enzymes involved in ERG biosynthesis is essential to increase ERG production. When the structural information and catalytic mechanism of enzyme proteins are not understood, a random mutant library with sequence diversity can be effectively generated through technologies such as site-directed

Table 2
Summary of model strains producing ERG.

Producers	Characteristics	ERG concentration	Fermentation period	Reference
Bacterium				
E. coli BW25113	Expression of EgtBCDE synthetase and EgtA isoenzyme GshA	24 mg/L	72 h	[66]
E. coli BW25113	Overexpression of *egtDE* and *egtB* genes; Expression of *cysE**, *SerA** and *ydeD* genes; Knock out the *metJ* gene	657 mg/L	192 h	[78]
E. coli BW25113	Overexpression of *egtABCDE* genes; Expression of gshA, *cysE**, *serA** and *ydeD* genes; Knockout the *metJ* gene	1.3 g/L	216 h	[67]
E. coli BW25113	Overexpression of *tregt1* and *tregt2* genes	4.34 g/L	143 h	[68]
E. coli BL21 (DE3)	Overexpression of *serA** and *thrA* genes; Knock out *metJ* and *sdaA* genes	130 mg/L	72 h	[69]
E. coli BL21 (DE3)	Expression of *egtE* gene; Expression of EgtD and ^{T}NcEgt1 with semi-rational design and random mutations	5.4 g/L	96 h	[70]
C. glutamicum	Overexpression of *egtDE* and *egtB* genes	100 mg/L	336 h	[79]
C. glutamicum	Expression of *egt1* and *egt2* genes; Overexpression of *cysE*, *cysK*, and *cysR* genes; Strengthens sulfur assimilation and pentose phosphate pathways; Knockout of the *sdaA* gene	264 mg/L	36 h	[71]
Yeast				
S. cerevisiae	Overexpression of *Gf*Egt1 *and Gf*Egt2	20.61 mg/L	168 h	[74]
S. cerevisiae	Co-expression of double-copy *egt1* and *egt2* genes	598 mg/L	84 h	[72]
S. cerevisiae	Co-expression of double-copy *egt1* and *egt2* genes; Overexpression of MET14; Knockout of the *SPE2* gene	2.39 g/L	160 h	[73]
Y. lipolytica	Co-expression of double-copy *egt1* and *egt2* genes	1.63 g/L	220 h	[75]
R. toruloides	iterative genome editing	267.4 mg/L	7 d	[50]
S. pombe	After multiple rounds of ultraviolet and lithium chloride mutation treatment	12.5 g/L	148 h	[77]

saturation mutagenesis, error-prone PCR and DNA recombination. The target mutants with specific traits can then be expressed and screened [84]. When the structural information of the enzyme protein is known, computer-aided analysis of the conserved sites and crystal structure could be used to select several amino acid sites as the engineered targets in a non-random way. Combined with the rational selection of effective codons, a "small but fine" mutant library is constructed to introduce potentially beneficial mutation sites. Subsequently, the mutant library is screened for beneficial mutants using analytical assays or high-throughput screening (HTS) methods. Finally, with the assistance of computers, a series of computational methods such as molecular docking, molecular dynamics simulation, and quantum mechanical methods are used to predict and evaluate the changes in beneficial mutants in terms of structure, free energy, and substrate binding energy [85]. Experimental verification is then carried out to determine whether the mutants can express normally, fold correctly and perform the expected functions. Based on the experimental results, the next round of calculations is formulated, and the cycle continues until the desired enzyme is obtained (Fig. 5).

Among the different biosynthetic pathways found in extant organisms, ERG synthesis pathways mainly include anaerobic biosynthesis pathways and aerobic biosynthesis pathways (including prokaryotic and eukaryotic pathways), as shown in Fig. 6. Recent reports have revealed that the strictly anaerobic green sulfur bacterium *Chlorobium limicola* can also synthesize ERG autonomously. The genome of this microorganism can encode two proteins, EanA and EanB (ergothioneine anaerobic biosynthetic enzyme A and B), with EanA having histidine-specific methyltransferase activity that converts histidine to hercynine (HER) and EanB having sulfur transfer function and can catalyze the conversion of HER to ERG [86]. The structures, kinetics, and mechanisms of action of EgtB, EgtC, EgtD, and EgtE from *M. smegmatis* have been thoroughly analyzed [87–94]. EgtD and EgtB are key enzymes in the ERG synthesis pathway. EgtD catalyzes the initial step of ERG synthesis by converting L-histidine and S-SAM into HER, while EgtB catalyzes the formation of γ-glutamyl-hercynylcysteine sulfoxide (γGC-HER) from γ-glutamyl-hercynylcysteine (γ-GC). Additionally, homologous genes of these two key enzymes are found in various prokaryotes, including firmicutes and proteobacteria [95–98]. Furthermore, the mechanisms action of NcEgt1 and NcEgt2 from *N. crassa* have been elucidated [99,100]. NcEgt1, a bifunctional enzyme, is hypothesized to originate from a fusion of two genes, *egtB* and *egtD* [99]. Kinetic studies have shown that the preferred substrates for Egt1 are HER and L-cysteine [101], bypassing the involvement of γ-GC and eliminating competition with glutathione in eukaryotes (Fig. 6).

Researchers utilized error-prone PCR to create a mutation library for the key enzyme sulfoxide synthase TNcEgt1 (truncated NcEgt1) and screened for five positive strains. Following saturation mutagenesis of the mutant residues in the positive strains, the improved mutant strain M1 was identified. Building on M1, the key enzyme EgtD was further modified through rational design and random mutation, yielding the optimal mutant MD4, which produced 5.4 g/L ERG after 96 h incubation in a 5L fermenter [70]. Therefore, modifications of enzyme activities, such as those in L-histidine methyltransferase and sulfoxide synthase,

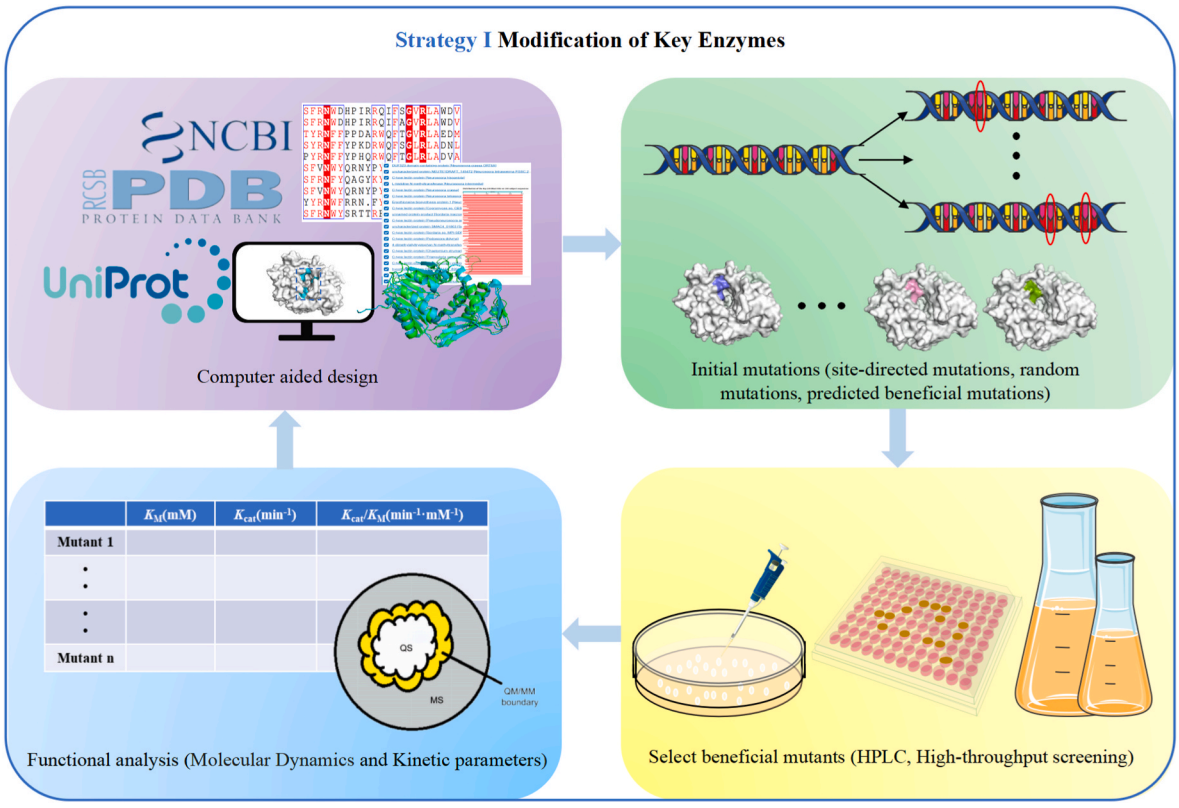

Fig. 5. Exploration, modification, screening, and analysis of key enzymes in the synthetic pathway. Using various databases to screen and align key enzymes in the ERG biosynthetic pathway; Utilizing computer simulations to predict possible beneficial mutation sites, and employing kits to introduce random mutations; Relying on HPLC detection methods or HTS to identify beneficial mutants; Performing functional analysis of molecular dynamics and kinetic parameters on the identified beneficial mutants.

have promoted the conversion of precursor amino acids into ERG. However, modifications of key enzymes in ERG synthesis pathway are less frequently reported. The low activity and poor substrate selectivity of natural enzymes hinder the biosynthesis rate of ERG. Advancements in bioinformatics and protein engineering have led to an increasing array of methodologies for enzyme design evolution [102–104], enabling the redesign of native enzymes to enhance their catalytic activity, substrate selectivity, and stability. Future research should focus on developing more rational enzyme engineering strategies to enhance key enzyme activity for ERG production.

4.2. Increase the synthesis of ERG precursors and balance cell growth

In recent years, metabolic engineering techniques have been employed to optimize and enhance substrate production pathways, resulting in a series of high value-added product strains [102–104]. By synergistically regulating precursors and eliminating competitive bypasses, the synthesis capacity of the target product is enhanced due to the abundant supply of precursors and the reduction of bypass pathway metabolic flux. The expression levels of L-histidine, L-methionine and L-cysteine, important precursors in the ERG biosynthetic pathway, determine the accumulation of ERG. In recent years, CRISPR-derived genome editing technologies [105–107], including CRISPR-Cas, CRISPRi, CRISPR-related transposase systems, and CRISPR-mediated base editing systems, have provided new technical supports for specific studies. These studies focus on improving the metabolic flux of precursors and coordinating metabolic balance in ERG biosynthesis.

Studies have demonstrated that the transfer of EgtA/B/C/D/E genes from *M. smegmatis* into *E. coli*, which has high yields of L-cysteine and L-methionine, significantly enhances ERG production [67]. One of the primary challenges in microbial metabolic engineering is the inhibition of substrates by enzymes, which impacts the production efficiency of high value-added products. Zhang et al. [69] addressed this issue by eliminating the transcriptional suppressor gene *metJ* and overexpressing genes related to the L-histidine and L-cysteine synthesis pathways, thereby alleviating substrate inhibition in the precursor synthesis pathway of ERG, resulting in an increase in ERG output from 35 mg/L to 130 mg/L. These studies demonstrate the necessity of precise regulation of the precursor metabolic network, as it not only ensures sufficient availability for synthesizing target products, but also simplifies medium composition, reduces costs, and enhances ERG yield [69,73].

4.3. Enhancing the expression of membrane transporters

From the perspective of boosting productivity, it is crucial to regulate substrate uptake, augment product efflux, and enhance transporter activity. However, due to the challenge of identifying transporters capable of carrying target compounds, limited progress has been made in functional analysis, and only a few transporters have been utilized in practice [108].

Overexpression of amino acid transporters facilitates the absorption of precursor amino acids and effectively enhances the synthesis of metabolites. Additionally, overexpression of the ERG output protein effectively mitigates the feedback inhibition of ERG in cells, resulting in increased cell growth and ERG production [73]. In other studies, the introduction of the putative ERG transporter gene *mfsT1* into *M. neoaurum* enhances the transmembrane transport of intracellularly synthesized ERG, thereby reducing the substrate feedback pressure on the cell and ultimately increasing ERG production [49]. Initially, human transporter SLC22A4 was identified as a specific transporter for ERG [26]. The ERG transporter SLC22A4 was employed to enhance ERG transport in yeast. However, due to poor expression of this transporter in

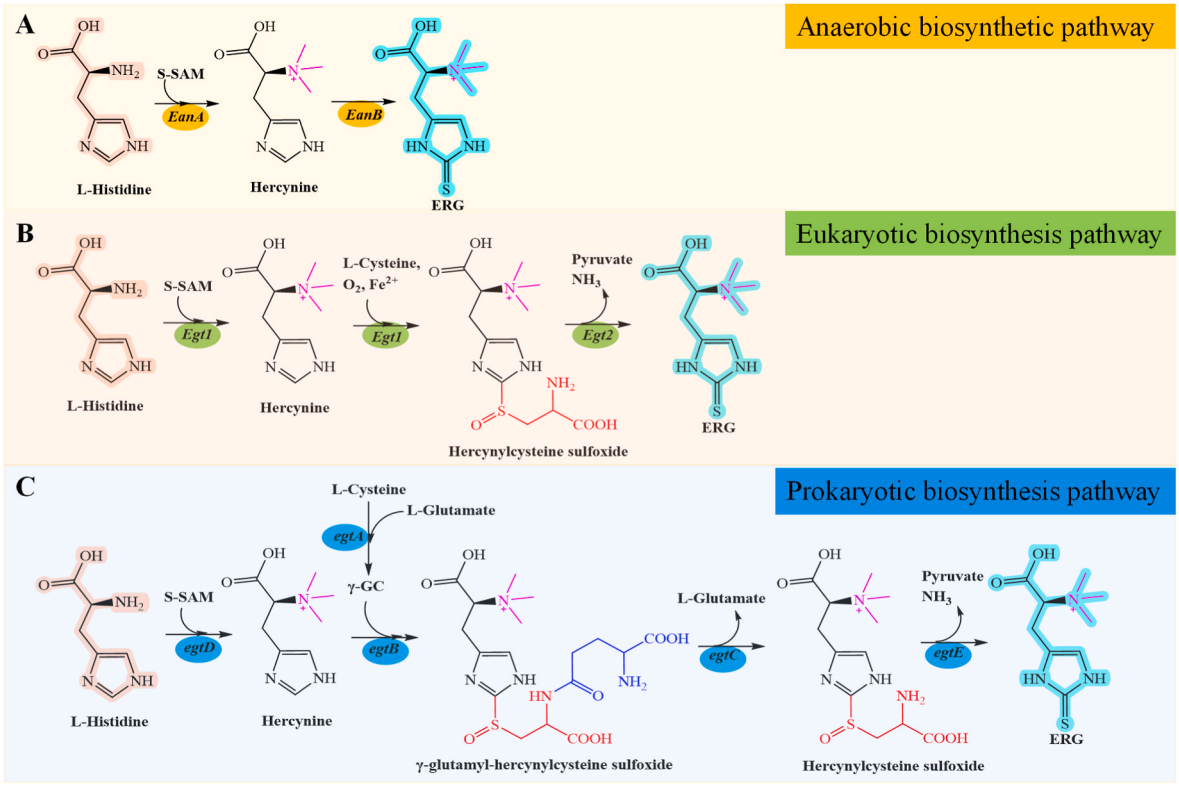

Fig. 6. There are three main ERG biosynthetic pathways. (A) Anaerobic biosynthetic pathway, it occurs mainly in *Chlorobium limicola*, catalyzed by EanA and EanB. (B) Eukaryotic biosynthesis pathway, it occurs mainly in *Neurospora crassa*, catalyzed by Egt1 and Egt2. (C) Prokaryotic biosynthesis pathway, it occurs mainly in *Mycobacterium smegmatis*, catalyzed by five enzymatic steps, involving EgtA, EgtB, EgtC, EgtD, and EgtE.

yeast, an *Arabidopsis thaliana* transporter with high homology to SLC22A4 was selected. Positive effects were observed in yeast strains with *TPO4* deletion and *AQR1* overexpression [72].

Recent studies have identified that *spd_1642–1643* gene from *Streptococcus pneumoniae* encodes the ABC transporter EgtU, which exhibits high affinity and specificity for ERG [109]. In the functional annotation of transporters, information regarding their transport substrates is particularly crucial. The experimental identification and characterization of transporters are currently both expensive and time-consuming. Therefore, developing reliable bioinformatics-based methods to predict the specificity of transporters and their substrates has become an important and urgent task.

4.4. Enhance enzyme catalytic activity by increasing cofactor levels

In addition to enhancing precursors supply through key enzyme modifications and optimization, it is critical to maintain a balanced supply of cofactors essential for enzyme activity. The final step in the ERG synthesis pathway is catalyzed by PLP -dependent β-lyase. The low content of PLP in the cell limits ERG production. Additional PLP supplementation is required during cell catalysis, making it impractical for large-scale production [110]. Liu et al. [111] successfully engineered the *E. coli* strain L18 to produce cadaverine directly from glucose without the need for additional pyridoxal phosphate (PP). In their study, endogenous and heterologous PLP synthesis modules in *E. coli* were enhanced using a dual-pathway strategy. A molecular switch based on growth stage was introduced to dynamically balance precursor competition between PLP synthesis and cell growth. Additionally, a negative feedback loop was constructed using a PLP sensor to precisely regulate PLP synthesis.Therefore, to enhance the catalytic activity of EgtE enzyme and promote the cleavage of hercynylcysteine sulfoxide (Cys-HER) into the final product ERG, more effective cofactor regulatory mechanisms need to be explored and identified to increase PLP production in the future.

4.5. Other strategies

When microbial cells undergo engineered treatment, they are adversely affected by metabolic burdens, compound toxicity, and stressful environments, often resulting in reduced effectiveness and productivity. Therefore, it is urgent to enhance the precise regulation of microbial cell factories. On the one hand, cell metabolism needs to be dynamically regulated through a complex regulatory network to maintain vigorous growth, resist environmental fluctuations, and eliminate excessive production of metabolate intermediates and precursors, ensuring that intermediate substrates and cofactors are efficiently converted into desired products. On the other hand, HTS is essential to promote high yields, concentrations, and productivity of compounds [111–113]. In recent years, intracellular biosensors (Fig. 7) have become powerful tools for monitoring and regulating microbial cell factories. When combined with HTS, they can accelerate the optimization of genetic elements, metabolic pathways, and chassis cells [114]. When biosensors are integrated with the synthetic pathways of target compounds, feedback regulation of metabolic pathways and coupling or decoupling of compound synthesis and cell growth would be achieved [111,113,115]. Thus, the use of intracellular biosensors can enhance the precise regulation of microbial cell factories, addressing key bottlenecks in the biosynthesis of target compounds [82,116].

5. Conclusions and future perspectives

ERG has broad application prospects in food, cosmetics, medicine and various other fields due to its potent antioxidant and cell-protective activities. However, due to output and cost constraints, only a few methods can be practically applied to the industrial production of ERG, resulting in high market prices. Production methods for ERG are

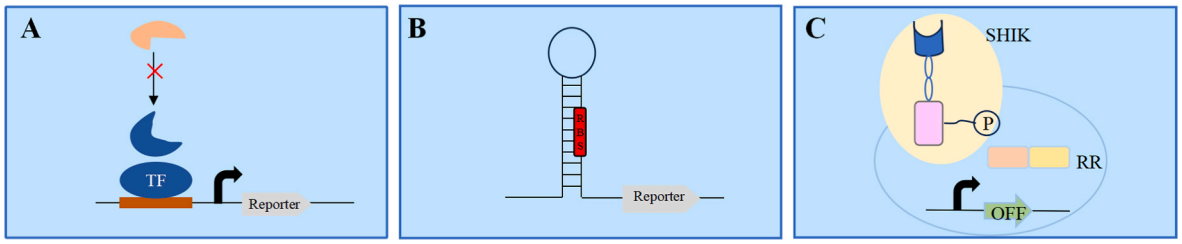

Fig. 7. Applications of intracellular biosensors. (A) Transcription factor-based biosensors: Transcription factors can recognize and bind to specific molecules. When combined, they activate or inhibit the expression of downstream genes. Through changes in reporter gene expression, fluorescent signals are detected and output, allowing for the measurement of the target metabolite concentration. (B) Ribosome switch-based biosensors: Ribosomal switches typically consist of specific RNA domains that bind to target molecules. Upon binding, the RNA structure changes, affecting the translation efficiency of the ribosome on the mRNA. Detection of target molecules is achieved through changes in the yield of downstream products. (C) Protein-based biosensors: Sensor proteins specifically recognize and bind to target molecules. Upon binding, the conformation or function of the protein changes. This change can be converted into a detectable signal through various methods (e.g., fluorescence change, enzyme activity change), enabling the detection of the target molecule concentration.

receiving increasing attention from researchers and product developers. Compared to chemical synthesis, biosynthesis is significantly safer. Therefore, improving ERG production by biosynthesis has become a top priority. The comprehensive application of upstream genetic engineering, enzyme engineering, metabolic engineering and fermentation engineering technology to enhance t microbial production of ERG has gradually become a research focus in recent years.

Future studies can consider the following aspects to improve ERG production (Fig. 8): **Firstly, modification of key enzymes involved in ERG biosynthesis is critical.** The identification of distinct ERG synthases in bacteria and fungi has significantly advanced this research field [47–50]. However, current research outcomes are insufficient to meet market demands. Thus, discovering more highly active ERG synthesis genes is essential. For instance, developing highly efficient enzymes for the synthesis of Cys-HER rate-limiting processes by HER is crucial. Additionally, exploiting enzymes with functions similar to Egt1 can mitigate competition between ERG and glutathione biosynthesis. **Secondly, balancing the precursor synthesis pathway is necessary.** Novel gene-editing and synthetic biology techniques need to be explored.

to optimize the anabolic modules of ERG precursors, orchestrate cell growth, and establish more proficient ERG-producing cell factories. **Thirdly, enhancing extracellular secretion and matrix penetration of ERG is important.** Engineering the ERG secretion and precursor

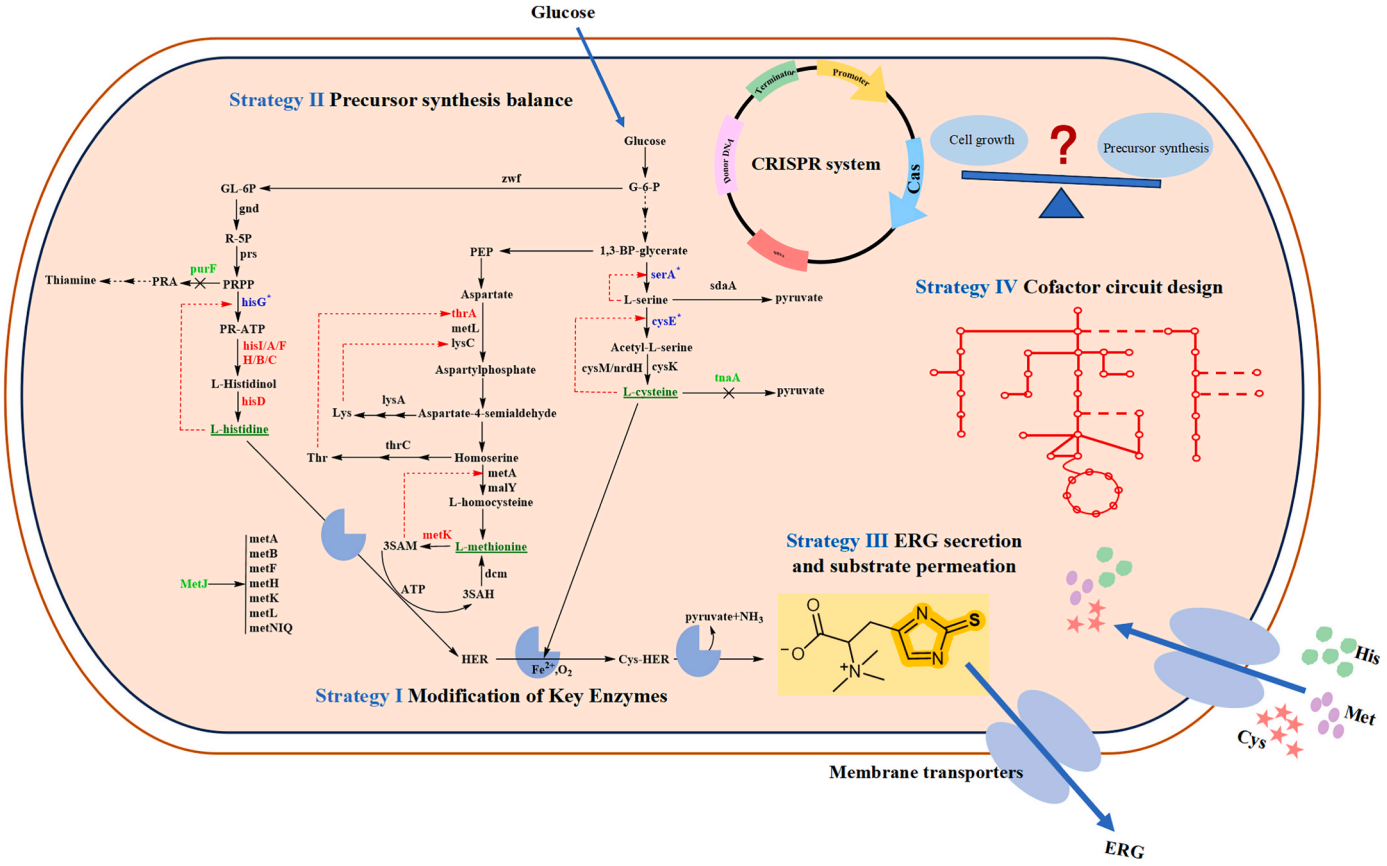

Fig. 8. Strategies for Ergothioneine Synthesis. Modification of key enzymes in the ERG synthesis pathway. Enhance the supply of precursors for ERG synthesis (blue and red genes) and block competing metabolic pathways (green genes) to balance cell growth and promote ERG accumulation using CRISPR-derived genome editing technology. Construct a biosensor to regulate the synthesis of cofactors and coordinate cell growth required for ERG synthesis. Strengthen the precursor intake and ERG secretion pathways to promote ERG accumulation and efflux.

absorption pathways are effective ways to improve ERG production. In addition to mining new specific transporters, improving membrane secretion of ERG through engineering methods provides an effective way to enhance ERG synthesis. **Finally, achieving dynamic cell balance and fine regulation through cofactors is essential.** The dynamic equilibrium of cells and the precise regulation of microbial cell factories are crucial for stable and high-yield ERG production which can be accomplished by modulating the production of ERG intermediates and cofactors through cellular biosensors. Therefore, synthetic biology approaches hold promise for the breakthrough production of ERG with higher purity and lower cost in the future.

CRediT authorship contribution statement

Li Liang: Writing – review & editing, Investigation. **Xu Shan-Shan:** Writing – original draft, Investigation. **Jiang Yan-Jun:** Writing – review & editing.

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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