



Research Synthetic Biology—Review

Biotechnological Strategies of Riboflavin Biosynthesis in Microbes

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ABSTRACT

Riboflavin is an essential micronutrient for humans and must be obtained exogenously from foods or supplements. Numerous studies have suggested a major role of riboflavin in the prevention and treatment of various diseases. There are mainly three strategies for riboflavin synthesis, including total chemical synthesis, chemical semi-synthesis, and microbial fermentation, the latter being currently the most promising strategy. In recent years, flavinogenic microbes have attracted increasing attention. Fungi, including *Eremothecium ashbyii* and *Ashbya gossypii*, and bacteria, including *Bacillus subtilis*, *Escherichia coli*, and lactic acid bacteria, are ideal cell factories for riboflavin overproduction. Thus they are good candidates for enhancing the level of riboflavin in fermented foods or designing novel riboflavin bio-enriched foods with improved nutritional value and/or beneficial properties for human health. This review briefly describes the role of riboflavin in human health and the historical process of its industrial production, and then highlights riboflavin biosynthesis in bacteria and fungi, and finally summarizes the strategies for riboflavin overproduction based on both the optimization of fermentation conditions and the development of riboflavin-overproducing strains via chemical mutagenesis and metabolic engineering. Overall, this review provides an updated understanding of riboflavin biosynthesis and can promote the research and development of fermented food products rich in riboflavin.

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1. Introduction

Riboflavin (RF), also known as Vitamin B₂ (Fig. 1), is a water-soluble B-group vitamin with high thermostability that melts and decomposes at 280 °C [1]. Despite its thermostability, riboflavin can be easily destroyed by heating in alkaline solutions, whereas it is very stable in hot neutral or acidic solutions for a short period of time [2]. Moreover, riboflavin is a photosensitive molecule that can be triggered with exposure to light, showing photodegradation after exposure to short-wave radiation (< 400 nm) [3]. Riboflavin is one of the essential micronutrients for the human body with a wide range of physiological functions, thus it has been ranked by

the World Health Organization (WHO) as one of the six main indicators for assessing human growth, development, and nutritional status. The average amount of riboflavin required for a healthy person is 0.3–1.8 mg·d⁻¹ for an adult [4], and symptoms of riboflavin deficiency occur when its daily intake is less than 0.2–0.3 mg [5]. The demand for riboflavin increases under certain conditions, such as heavy physical labor, mental strain, pregnancy, and the adolescent growth period [6,7]. Currently, riboflavin deficiency raises concerns in both developing and developed countries.

Riboflavin is an indispensable nutrient for normal cellular function. After uptake, it is intracellularly transformed into flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) by flavokinase and FAD synthetase, respectively. These two major biologically active riboflavin derivatives are involved in a wide range of redox reactions that are crucial for human metabolism. These riboflavin derivatives act as electron carriers, as well as essential cofactors of some enzymes that catalyze redox reactions, such as

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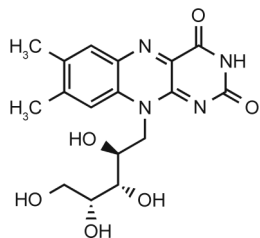


Fig. 1. The chemical structure of riboflavin.

succinic and fumaric dehydrogenase, cytochrome c reductase, and glucose, aldehyde and xanthine oxidases [8–10]. In animal studies, riboflavin is found to affect the absorption and metabolism of iron, and its supplementation can enhance the uptake of both zinc and iron [11]. In view of the crucial role of zinc and iron in cell proliferation, riboflavin has an indirect positive effect on growth. Oppositely, riboflavin deficiency can lead to the reduction of iron absorption, storage and utilization, resulting in human growth retardation [12]. Moreover, it is also known that riboflavin deficiency or defective transport of riboflavin has the ability to cause cataracts, neurological disorders, cardiovascular abnormalities, and even cancers [13]. Therefore, it is necessary to ensure regular dietary intake of riboflavin to prevent deficiency-related disorders.

Like most vitamins, riboflavin cannot be biosynthesized by the human body. It is mainly obtained from the diet and presumably, also from the intestinal microbiota. Riboflavin is naturally present in a wide variety of foods at different levels, including animal sources such as internal organs, milk, and eggs. These are better sources of riboflavin compared to natural plant foods [14]. In addition, some green vegetables and beans also contain certain amounts of riboflavin. Riboflavin is readily available for absorption in the human diet, but it is not stored in significant amounts in the human body due to its water solubility, and can be excreted from the urine and stool if consumed in excessive amounts [15,16].

2. Current strategies of riboflavin production

The annual global production and sales volume of riboflavin reached more than 9000 t in 2012, of which about 1800 t are used in pharmaceuticals and beverages, and about 7200 t in animal feed [17]. With the development of the functional food and animal feed industry, the need for riboflavin also increases [18]. For the production of riboflavin, there are mainly three strategies, including total chemical synthesis, chemical semi-synthesis, and microbial fermentation. Furthermore, the microbial fermentation can be divided into two main types, the traditional flavinogenic microbial fermentation, and fermentation by genetically engineered microbes.

2.1. Total chemical synthesis of riboflavin

The total chemical synthesis of riboflavin employs *D*-ribose or glucose as the starting material, and synthesizes riboflavin via 6–9 chemical reaction steps, including oxidation, displacement, transposition, acidification, lactonization, reduction, condensation, coupling, and cyclization [19,20]. It is obvious that this is a tedious and time-consuming process (Fig. 2), and it also comes with a high cost and is hazardous to the environment [19,20]. Furthermore, the final products usually contain impurities with certain toxicity that are difficult to eliminate. Therefore, total chemical synthesis has been gradually replaced by microbial fermentation strategy [21].

2.2. Chemical semi-synthesis of riboflavin

The chemical semi-synthesis of riboflavin is a combined strategy of microbial fermentation and total chemical synthesis. In this strategy, the microbial fermentation of *D*-glucose is applied first, and the resulting *D*-ribose is then used as the main raw material to chemically synthesize riboflavin. The fermentative production of *D*-ribose from *D*-glucose can simplify and reduce the cost of riboflavin production, which is the primary difference between total chemical synthesis and chemical semi-synthesis strategies. However, chemical semi-synthesis is not suitable for large-scale production of riboflavin due to the difficulty in eliminating chemical additives, resulting in high levels of residues in the final products.

2.3. Production of riboflavin by microbial fermentation

Microbial fermentation has been applied in the commercial production of riboflavin since the middle of last century, with three riboflavin-producing microbes initially employed, including one bacterium (*Clostridium acetobutylicum* (*C. acetobutylicum*)) and two fungi (*Ashbya gossypii* (*A. gossypii*) and *Eremothecium ashbyii* (*E. ashbyii*)) [22,23]. However, the initial microbial fermentation processes could not compete with chemical synthesis due to the long cycle and low yield. Thus, it was difficult to commercialize on a large scale [24]. Later, with the research and development of genetic engineering technology, genetically engineered bacteria have been successfully constructed for riboflavin production, such as with *Bacillus subtilis* (*B. subtilis*) and *Corynebacterium ammoniagenes* [24]. These bacteria have the capability to effectively transform *D*-glucose into riboflavin, which can therefore significantly shorten the production cycle and enhance the riboflavin yield. Therefore, their utilization can finally promote the commercialization of riboflavin production by microbial fermentation into reality [25,26].

Overall, the present riboflavin production by microbial fermentation has several advantages including the short production cycle, the use of simple raw materials, the high yield, and a production cost that is much lower compared to total chemical synthesis and chemical semi-synthesis.

3. Microbial biosynthesis of riboflavin

In flavinogenic organisms such as fungi, yeasts, and eubacteria, one molecule of guanosine triphosphate (GTP) and two molecules of ribulose-5-phosphate are required for the biosynthesis of one riboflavin molecule [27]. As shown in Fig. 3, the riboflavin biosynthetic pathway starts from GTP (**1**) as the substrate, which is catalyzed by GTP cyclohydrolase II (**enzyme 1**). This step involves the ring-opening of the imidazole ring, and the hydrolytic release of inorganic pyrophosphate from the side-chain of the ribose moiety of GTP, which yields 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone 5'-phosphate (DARPP, **2**). DARPP (**2**) is then transformed into 5-amino-6-ribitylamino-2,4(1*H*, 3*H*)-pyrimidinedione 5'-phosphate (ARPP, **5**) via two successive reactions including reduction and deamination. The sequence of these two reactions varies with different microbes. In fungi, the ribosyl side-chain of DARPP (**2**) is first reduced to the ribityl side-chain, and the resulting intermediate 2,5-diamino-5-ribitylamino-4(3*H*)-pyrimidinone 5'-phosphate (DARPP, **4**) is then deaminated to ARPP (**5**). In eubacteria, however, DARPP (**2**) is first deaminated to 5-amino-6-ribosylamino-2,4(1*H*,3*H*)-pyrimidinedione 5'-phosphate (ARPP, **3**), and the ribofuranose moiety of the intermediate ARPP (**3**) is then reductively ring-opened, giving ARPP (**5**). The order of the reduction and deamination reactions occurs oppositely in bacteria

and fungi. In the subsequent reactions, ArPP (**5**) is further dephosphorylated into 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (ArP, **6**) by the catalytic action of a non-specific phosphatase (**enzyme 4**), which is still elusive. Recently, it was found that haloacid dehalogenase (HAD) superfamily members catalyze the dephosphorylation of ArPP (**5**) in some species, including *B. subtilis*, *Escherichia coli* (*E. coli*), and *Bacteroides thetaiotaomicron* [28,29], filling the role of the **enzyme 4**. On the other hand, another initial substrate ribulose-5-phosphate (**7**) can be transformed into 3,4-dihydroxy-2-butanone-4-phosphate (DHBP, **8**) through a skeletal rearrangement catalyzed by the DHBP synthase (**enzyme 5**). Further lumazine synthase (or riboflavin synthase β -chain, **enzyme 6**) catalyzes the condensation of ArP (**6**) with DHBP (**8**) to give 6,7-dimethyl-8-ribitylmazine (DMRL, **9**), which can be ultimately transformed into riboflavin (**10**) and ArPP (**5**) via a highly unusual dismutation that is catalyzed by the riboflavin synthase (or riboflavin synthase α -chain, **enzyme 7**) [30,31], and the product ArPP (**5**) may be recycled for the biosynthesis of another riboflavin molecule.

Fungi and bacteria are commonly used for riboflavin synthesis via the microbial fermentation [32]. The current studies are mainly focused on the utilization of two yeast-like fungi, including *E. ashbyii* and *A. gossypii*, and three bacteria, including *B. subtilis*, *E. coli*, and lactic acid bacteria [33–39]. For the fungi such as *E. ashbyii* and *A. gossypii*, the riboflavin yield may be improved by the regulation of riboflavin biosynthesis, which can be achieved by directed evolution of the fungi via chemical mutagenesis [40] and genetic engineering [41,42]. However, the resulting fungi usually have a long fermentation process and high viscosity, and should be cultured in a growth medium that contains numerous components [43,44]. All these negative factors will result in an increase in the difficulty of the separation and purification of riboflavin in the late stage, as well as increasing the production cost. In addition, the growth medium should be continuously supplemented with exogenous matter (unsaturated fatty acid), in order to increase the biosynthesis ability of the fungi. By comparison, the use of bacteria has several advantages, such as short fermentation process, simple culture medium, and the availability of genetic engineering technologies applicable to bacteria that have been well established [44].

3.1. Fungi

3.1.1. *E. ashbyii*

E. ashbyii is one of the main strains currently used in industrial riboflavin production by fermentation, and its fermentation has therefore been studied in detail. Kalingan and Krishnan [45] demonstrated the effect of different carbon and nitrogen sources on riboflavin production by *E. ashbyii* NRRL 1363, as well as the effect of their initial concentrations. On the basis of their results, an optimal industrial riboflavin production process has been designed. Kolonne et al. [46] reported the effect of pH on the exocellular riboflavin production by *E. ashbyii*, and the results showed that high riboflavin production yield could be obtained at pH 4.5 and 5.5, whereas almost no riboflavin was produced at pH 3.5 and 8.5. Pujari and Chandra [40] demonstrated a high-yield *E. ashbyii* DT1 mutant produced by ultraviolet (UV) radiation-induced mutation. However, further development for production of this strain has been blocked by its low yield and unstable genetic characteristics. According to the metabolic regulation mechanism in microorganisms, the feedback inhibition of some key enzymes involved in riboflavin biosynthetic pathway can be reduced by utilization of mutants with resistances to analogs of riboflavin and its metabolites. This might increase the accumulation of the final products [47]. However, *E. ashbyii* is not able to grow in inorganic salt culture medium, and it is also difficult to find a suitable

synthetic culture medium for its growth [47]. Therefore, finding a suitable synthetic culture provides a good chance for screening *E. ashbyii* mutants with high and stable riboflavin production capacity, the further utilization of which can enhance the production yield of riboflavin.

3.1.2. *Candida famata*

The yeast *C. flarerii* is a natural riboflavin overproducer, and it was previously used for industrial riboflavin production. Although this yeast has not been used at industrial scale in recent years, there are still a number of research advances in its genetic engineering, suggesting that it may develop into a new platform for riboflavin overproduction.

Yatsyshyn et al. [48] constructed a plasmid pTFMN1-FAD1 that contained the *FAD1* gene (encoding FAD synthetase) and an additional copy of the *FMN1* gene (encoding riboflavin kinase) from *Debaryomyces hansenii* (*D. hansenii*) under the control of the *TEF1* promoter, and transformed it into *Candida famata* (*C. famata*) strain T-OP 13–76, a recombinant strain with an increased expression of the *FMN1* gene. One of the resulting strains, designated as *C. famata* T-FD-FM 27, accumulated 451 mg·L⁻¹ of FAD in a 40-h batch fermenter cultivation under the optimized conditions. This was the first report on obtaining yeast strains overproducing FAD.

In another study, Dmytruk et al. [49] constructed a riboflavin overproducing strain by co-overexpression of the genes *SEF1*, *RIB1*, and *RIB7* in a non-reverting riboflavin producing *C. famata* AF-4 strain. Under a fed-batch fermentation in a 7 L laboratory bioreactor, the constructed strain accumulated up to 16.4 g·L⁻¹ of riboflavin in optimized medium, representing one of the most known active riboflavin producers. Furthermore, when the two modified genes *PRS3* (encoding Phosphoribosyl pyrophosphate (PRPP) synthetase) and *ADE4* (encoding PRPP amidotransferase) from *D. hansenii* were introduced and co-expressed in this constructed strain, a two-fold increase in riboflavin production is observed [50].

3.1.3. *A. gossypii*

The filamentous hemiascomycete *A. gossypii* was originally isolated from diseased cotton plants (*Gossypium* sp.), and it was further found to be a natural riboflavin overproducer [51]. Under nutritional stress, the mycelium developed by this fungus differentiates into sporangia, and then riboflavin is produced in parallel with the generation of spores by sporangia, whose function is probably to protect the hyaline and UV-sensitive spores [52]. After decades of strain improvements, high specific productivity was achieved by establishing the biotechnical riboflavin production using *A. gossypii* in 1990, and the previous multi-step chemical production of riboflavin was then replaced [53]. *A. gossypii* has already been exploited in industrial riboflavin production for more than 20 years for several reasons. It has natural riboflavin-producing ability, along with high scientific prominence, not only because of its biotechnological potential, but also because of its extensive use as a model organism in the biology studies of fungal development and evolution [54,55]. The fruitful results of the industrially applied and basic research on *A. gossypii* obtained during these years have promoted the development of molecular and *in silico* tools for its high-level genetic engineering, which have allowed the rational exploitation of its full biotechnological potential [51].

A significant advance in the exploitation of *A. gossypii* for other biotechnological applications beyond riboflavin production has been seen in recent years. Despite this, the optimization of riboflavin production process using *A. gossypii* and the development of high riboflavin-producing *A. gossypii* strains remain the key targets of many researchers, with the current studies being mostly performed at the molecular level [38,56]. With the help of ¹³C isotope experiments, Schwechheimer et al. [57] resolved carbon fluxes in

the riboflavin-producing fungus *A. gossypii* B₂. In this study, the production process was carried out under complex industrial cultivation conditions, and vegetable oil was used as raw material. According to the ¹³C labeling data, formate and serine were identified as one-carbon donors and had positive effects on riboflavin biosynthesis, whereas glycine was exclusively used as a two-carbon donor of the pyrimidine ring of riboflavin. Moreover, a severe bottleneck of the one-carbon metabolism inside the cells was observed during the initial riboflavin production phase, which could be overcome by accumulation of intrinsic formate in the later phases of production. Moreover, time-resolved supplementation of small amounts of formate and serine could enhance their intracellular availability, leading to a 45% increase in riboflavin titer [57]. In subsequent work, Schwechheimer et al. [58] have successfully realized for the first time the quantitative calculation of carbon fluxes in the growth phase of *A. gossypii* B₂ on rapeseed oil and complex medium, as well as that in the riboflavin biosynthetic phase in an industrial riboflavin production set-up. The resulting data showed that yeast extract, the ubiquitous industrial medium component, was the main carbon source for strain growth, and it also had a significant impact on riboflavin biosynthesis. However, rapeseed oil was identified as the main carbon source for riboflavin production, which has, therefore, the greatest impact on riboflavin production. These results highlighted the importance of carbon source for riboflavin production, which should be carefully selected. The study brought some novel insights into the metabolism of *A. gossypii* B₂ under complex industrial cultivation conditions, which were valuable for further strain improvement and process optimization. In order to characterize the riboflavin biosynthetic pathway, Jeong et al. [59] performed ¹³C-metabolic flux analysis (MFA) in an *A. gossypii* wild strain (ATCC 10895) and an *A. gossypii* mutant strain (W122032, a riboflavin overproducer obtained from *A. gossypii* ATCC 10895 by disparity mutagenesis), and differences in their central carbon pathway were analyzed. It was shown that metabolic flux into pentose-5-phosphate through the pentose phosphate pathway (PPP) in the mutant strain was 9% higher than that in the wild strain, and the metabolic flux into riboflavin from purine synthetic pathway (PSP) in the mutant strain was 1.6%, which is sixteen-fold higher than that in the wild strain (0.1%). These results demonstrated that riboflavin production in the mutant strain of *A. gossypii* was increased by the increase of GTP flux via the PPP and PSP, which also indicated that expression reinforcement of the genes related to the PPP and PSP is one option to improve riboflavin production. Silva et al. [60] investigated the effect of blockage of the *de novo* pyrimidine biosynthetic pathway in *A. gossypii* Agura3, a uridine/uracil auxotrophic strain which was recently generated [61]. It was found that the production of riboflavin by this strain on standard solid complex medium was improved, and extra supplementation of uridine/uracil repressed the riboflavin production. Furthermore, it was also found that the growth of this strain and its parent strain (*A. gossypii* ATCC 10895) was hampered by high concentration of uracil, while excess uridine accelerated its growth. This was the first time that the influence of genetic changes in the pyrimidine biosynthetic pathway on riboflavin production by *A. gossypii* was characterized based on experimental results.

As a natural overproducer of riboflavin, *A. gossypii* must ensure a strong flux through the guanine nucleotide pathway in order to increase the bioavailability of GTP, which is a limiting precursor of riboflavin, and an exhaustive characterization of the key enzymes involved in GTP biosynthetic pathway is necessary for the further improvement of riboflavin production in this fungus. Within the purine biosynthetic pathway, inosine-5'-monophosphate (IMP) is the common precursor of adenine and guanine nucleotides, and the enzyme inosine-5'-monophosphate

dehydrogenase (IMPDH) catalyzes its oxidative transformation into xanthosine-5'-monophosphate (XMP), a reaction step which is rate-limiting [41]. The resulting XMP is finally converted into GTP in successive steps. Therefore, IMPDH is an important metabolic bottleneck in the purine pathway. Moreover, from a technical point of view, this enzyme is susceptible to further genetic manipulation by means of metabolic engineering. Buey et al. [41] performed an exhaustive functional and structural characterization of the enzyme IMPDH from the fungus *A. gossypii*. The data showed that the metabolic flux through the guanine biosynthetic pathway in *A. gossypii* ATCC 10895 was increased by the overexpression of the IMPDH gene, and a 40% increase in riboflavin production was finally achieved. This study significantly contributed to the development of a metabolic engineering toolbox aimed at increasing riboflavin production in *A. gossypii*. By analyzing the relative contribution of each of the *rib* genes to riboflavin production in *A. gossypii*, Ledesma-Amaro et al. [42] found that the production of riboflavin in *A. gossypii* was hindered as a result of the low transcription levels of the *rib* genes and the bioavailability of GTP substrate. Moreover, a significant increase in the production yield of riboflavin was achieved by combined overexpression of the *rib* genes (up to five). It was also found that the inactivation and underexpression of the *ADE12* gene, which encodes adenylosuccinate synthase and controls the first step of the adenosine-5'-monophosphate (AMP) branch, showed positive effects on riboflavin production. Furthermore, an *Ashbya* strain (A330) combining the overexpression of all the *rib* genes and the underexpression of the *ADE12* gene was generated using metabolic engineering, and a total yield of 523 mg·L⁻¹ of riboflavin was reached with this strain, which was 5.4 folds higher than that of the strain *A. gossypii* ATCC 10895. This study provided a controllable and scalable strategy to increase industrial riboflavin production.

With the help of disparity mutagenesis, a new genetic mutation technique, Park et al. [62] obtained a mutant strain (W122032) of *A. gossypii* with enhanced riboflavin productivity. In an optimized medium, this strain produced 13.7 g·L⁻¹ of riboflavin in a 3-L fermenter, and this riboflavin yield represents an increase of nine-fold with respect to yield of the wild strain (*A. gossypii* ATCC 10895). Proteomic and DNA microarray analysis demonstrated the upregulation of genes involved in purine and riboflavin biosynthetic pathways, and the downregulation of pathways related to carbon source assimilation, energy generation and glycolysis. These results indicate that the improvement of riboflavin production in the generated mutant is related to a shift in carbon flux from β -oxidation to the biosynthetic pathway of riboflavin.

3.2. Bacteria

Riboflavin biosynthesis has been studied in detail in both Gram-positive and Gram-negative bacteria, particularly in *B. subtilis* and *E. coli*. In riboflavin-producing bacteria, the riboflavin production is controlled by the so-called riboflavin biosynthesis operon (*rib* operon), which consists of five genes (*ribGBAHT*) encoding the riboflavin biosynthetic enzymes (Fig. 4) [63], and the gene order of the *rib* operon differs from the enzymatic reactions in riboflavin biosynthetic pathway (Fig. 3). *RibA* is the third *rib* gene and encodes a bifunctional enzyme with the activities of GTP cyclohydrolase II and 3,4-dihydroxy phosphobutanone synthase, which acts as a rate-limiting enzyme [64]. The GTP cyclohydrolase II activity of the enzyme catalyzes the first enzymatic reaction. *RibG* is the first *rib* gene that encodes another bifunctional enzyme. The riboflavin-specific deaminase/reductase catalyzes sequentially the second and third enzymatic reactions. *RibH* is the last gene of the operon, and it codes for lumazine synthase (or riboflavin synthase β subunit), which catalyzes the penultimate enzymatic reaction. As the second gene of the operon, *RibB* codes for riboflavin synthase α

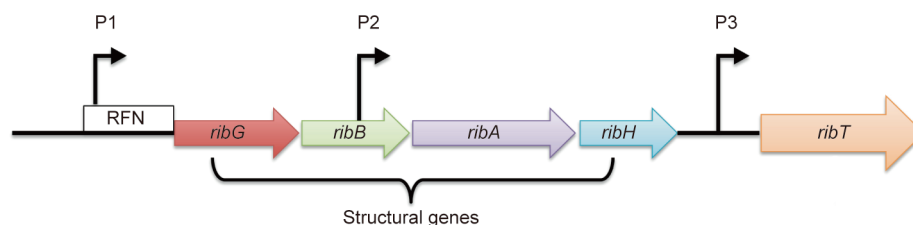


Fig. 4. Structure of the *rib* operon in bacteria. The structural genes encode the metabolism-related proteins, and the transport gene *ribT* encodes the transport-related protein. The fine arrows show the transcription direction of the promoter (P)1, P2, and P3. RFN: riboflavin-element (which is an FMN-dependent riboswitch).

subunit which, however, catalyzes the last enzymatic reaction. Besides, the operon also contains a gene *ribT* whose function has not yet been clarified. This gene was annotated to code for a putative *N*-acetyltransferase, and it was demonstrated that its inactivation could not lead to riboflavin auxotrophy, but a significant decrease in the riboflavin yield [65]. A recent structural study revealed that the gene *ribT* in *B. subtilis* is a member of general control nonderepressible-5 (GCN5)-related *N*-acetyltransferase (GNAT) superfamily, and the enzymes of this superfamily catalyze the transfer of acetyl group from acetyl coenzyme A (acetyl-CoA) to a variety of substrates [66].

Upstream of the *rib* operon in *B. subtilis*, three regulatory regions exist including *ribC*, *ribR*, and *ribO* [67,68]. Mutation in each of these three regions leads to riboflavin overproduction, therefore, they are considered as presumptive repressors of riboflavin biosynthesis. Among them, *ribC* and *ribR* respectively encode a bifunctional flavokinase/FAD-synthetase and monofunctional flavokinase. These two enzymes are involved in the sequential conversion of riboflavin into FMN and FAD. *RibO* is a noncoding region located between the *ribP1* promoter and the gene *ribG* [69], whose transcriptional product can be folded into a conserved RNA secondary structure with a base stem and four hairpins, designated as FMN riboswitch. The interaction of flavins with the FMN riboswitch plays a key role in the regulation of *rib* operon. In the relieving state, the concentration of FMN is too low, thus it does not bind to the FMN riboswitch, and an antiterminator is formed to ensure the ready read-through of the following sequence, whereas, under repression condition, FMN binding with the FMN riboswitch leads to the formation of a transcription terminator, leading to the termination of transcription [70].

In addition, as shown in Fig. 4, the *rib* operon also contains three promoters, including a major promoter P1 (*ribP1*) and two additional internal promoters P2 and P3 (*ribP2* and *ribP3*) [71]. The *ribP1* promoter is located upstream of the first structure gene *ribG*, whereas the *ribP2* promoter lies in the distal region of the *ribB* gene. In addition, the *ribP3* promoter situates between the genes *ribH* and *ribT*, which separates these two genes. Transcription of the four *rib* genes (*ribGBAH*) is mainly controlled by the *ribP1* and regulatory region located at the 5' terminus of the *rib* operon, and the last two genes, *ribA* and *ribH*, are transcribed from the *ribP2* promoter and the RFN regulatory region [72].

3.2.1. *C. acetobutylicum*

C. acetobutylicum is a strictly anaerobic, Gram-positive bacterium, which has been extensively used for the production of biobutanol by fermentation for more than a century [73]. Typically, this bacterium synthesizes the solvents butanol, acetone, and ethanol at an average mass ratio of 6:3:1 [74]. In addition to its main role as a butanol overproducer, *C. acetobutylicum* was also recognized as a natural riboflavin producer since the 1940s due to observation of some riboflavin in its butanol–acetone–ethanol (ABE) fermentation [25,75]. From an economic point of view, if the production yield of riboflavin, a second product or by-product with

high value, could reach 0.5–1 g·L⁻¹ on an industrial scale, the economic value of ABE fermentation process would be improved significantly [76]. However, after decades of research, the production yield of riboflavin in the ABE fermentation process by *C. acetobutylicum* remains insufficient. In recent work, Zhao et al. [77] showed that riboflavin production in the ABE fermentation by *C. acetobutylicum* ATCC 824 was strongly stimulated by extra supplementation of sodium acetate (NaAc) to the bacterial growth medium containing xylose as carbon source. It was found that after supplementation with 60 mmol·L⁻¹ NaAc, the riboflavin biosynthesis rate (*ribA* flux rate) was almost ten-fold more than that of the control, and the final concentration of riboflavin which was initially undetectable increased to 0.2 g·L⁻¹ (0.53 mmol·L⁻¹). This work provided a novel strategy for the co-production of riboflavin with biobutanol by use of *C. acetobutylicum*, which will enhance the commercial value of ABE fermentation process.

3.2.2. *E. coli*

As a common workhorse in fundamental biological research, the genome of the Gram-negative bacterium *E. coli* is well-characterized, and a wide variety of mature molecular tools suitable for its genetic manipulation are established [78,79]. In addition, this bacterium has been long used as a common host for efficient production of various substances, such as amino acids, biofuels, and bulk chemicals [80,81] due to its numerous advantages. For example, *E. coli* grows fast with a doubling time of 20 min under optimal culture conditions, and it can readily grow in culture medium composed of inexpensive components [82]. Based on these attractive features, *E. coli* was considered as a potential efficient host for riboflavin production, although wild-type *E. coli* cannot accumulate riboflavin under natural conditions [78], and a large number of metabolic engineering strategies were carried out in a series of studies to develop *E. coli* as a riboflavin-producing strain.

Currently, almost all laboratory *E. coli* strains are derived from non-pathogenic K-12 or B strains [83]. Although many cellular metabolic and physiological differences were found between the strains B and K-12, comparative genomic analysis showed that they are very similar and closely related [84,85]. Compared to K-12 strains, B strains have a faster growth rate in minimal media and higher recombinant protein expression levels, but a lower acetate production [86]. *E. coli* BL21 (DE3) is a specifically engineered B strain which is the most widely used in scientific studies and industrial applications [85]. Clarification of the differences in the regulative mechanism and metabolism between K-12 and B strains will lead to their improved utilization in metabolic engineering.

Wang et al. [86] showed for the first time that *E. coli* BL21 (DE3) could accumulate riboflavin under normal culture conditions. Subsequently, a single site mutation on the 115 residues of *ribF* gene was identified in BL21 (DE3) by comparing the sequence of the enzymes involved in riboflavin biosynthesis between BL21 (DE3) and K-12 MG1655, and this His115Leu mutation caused an

inadequate activity of the bifunctional riboflavin kinase/FMN adenylyltransferase encoded by *ribF* gene, which might contribute to the riboflavin accumulation in this strain. However, further quantitative polymerase chain reaction (PCR) analysis showed upregulation of all riboflavin biosynthetic genes, suggesting that the excessive riboflavin accumulation could also be caused by a certain regulative mechanism which upregulates all riboflavin biosynthetic genes, and this reason was more plausible. This work suggested that *E. coli* BL21 (DE3) may also be used for riboflavin production.

It was previously reported that modification of some key genes in the purine biosynthetic pathway in *B. subtilis* could lead to enhanced supply of the precursors of riboflavin, which would subsequently facilitate riboflavin biosynthesis [87,88]. In recent years, similar studies with *E. coli* were also performed using metabolic engineering. Xu et al. [79] performed a six-step modification of *ribB* gene and five key genes involved in purine pathway (including *ndk*, *gmk*, *purA*, *purF*, and *prs*) in a riboflavin-producing *E. coli* RF01S engineered from wild-type *E. coli* MG1655, and the final strain RF18S, which combined overexpression of all the six genes, produced 387.6 mg·L⁻¹ riboflavin in shake-flask fermentation, with a riboflavin production yield of 44.8 mg·g⁻¹ glucose. Compared to the strain RF01S, RF18S produced a 72.2% higher riboflavin titer and a 55.6% higher yield. This study demonstrated that simultaneously modifying the DHBP synthase and GTP biosynthetic pathway by rational metabolic engineering is an efficient strategy to significantly improve the production of riboflavin in *E. coli*.

By using the method of metabolic engineering, Lin et al. [78] constructed a plasmid p20C-EC10 containing a riboflavin operon under the control of the inducible *trc* promoter (*P_{trc}*) and transferred it into wild-type *E. coli* MG1655, and the resulting strain RF01S could accumulate 229.1 mg·L⁻¹ riboflavin. Subsequently, the deletion of genes *pgi* (encoding glucose-6-phosphate isomerase), *edd* (encoding phosphogluconate dehydratase) and *eda* (encoding multifunctional 2-keto-3-deoxygluconate-6-phosphate aldolase/2-keto-4-hydroxyglutarate aldolase/oxaloacetate decarboxylase) and insertion of *acs* (encoding acetyl-CoA synthetase) promoter in strain RF01S were performed in a stepwise way, and the generated strain RF05S showed a riboflavin titer of 585 mg·L⁻¹. Finally, the expression of *ribF* gene (encoding a bifunctional riboflavin kinase/FMN adenylyltransferase) in strain RF05S was modulated in order to reduce the transformation of riboflavin into FMN, and the final engineered strain RF05S-M40 could produce 1036 mg·L⁻¹ of riboflavin in Luria–Bertani (LB) medium at 37 °C. The optimization of the fermentation conditions was then performed, and riboflavin production by strain RF05S-M40 reached 2703 mg·L⁻¹ in shake-flask fermentation under the optimal conditions, which was nearly twelve-fold higher than that of RF01S, with a yield of 136 mg riboflavin per gram of glucose. In the case of shake-flask fermentation, the riboflavin production yield of *E. coli* strain RF05S was the highest among all reported riboflavin-producing strains. In subsequent work from the same laboratory [89], another novel riboflavin-producing *E. coli* strain engineered from the wild-type MG1655 was successfully constructed, in which the genes *pfka* (encoding 6-phosphogluconolactonase I), *edd* and *eda* were deleted in order to increase the carbon flux through PPP. This engineered strain LS02T harbored a riboflavin operon expression plasmid pLS01, which was also newly constructed in this work and which has higher stability than the plasmid p20C-EC10 [78]. In shake-flask fermentation, this strain produced 667 mg·L⁻¹ of riboflavin in maximum sustainable yield (MSY) medium containing 10 g glucose per liter, and in fed-batch fermentation, a riboflavin titer of 10.4 g·L⁻¹ was achieved, with a production yield of 56.8 mg·g⁻¹ glucose. This work reported for the first time an engineered *E. coli* strain that could accumulate more than 10 g of riboflavin per liter culture medium in a fed-

batch process. The results from the above two studies demonstrated that *E. coli* is indeed a potential efficient species for riboflavin production.

3.2.3. *B. subtilis*

B. subtilis, a ubiquitous, naturally occurring bacterium, is one of the best-characterized species of all the Gram-positive bacteria [90]. This bacterium has been granted qualified presumption of safety (QPS) status by European Food Safety Authority (EFSA) for certain applications in animal feeds and in the production of foods and supplements consumed by humans, such as vitamin K₂, and is therefore regarded as a safe and stable producer [91–93]. In addition, based on the detailed characterization of the enzymes involved in riboflavin biosynthetic pathway at the biochemical, physiological and genetic levels, *B. subtilis* was also successfully engineered as a cell factory for the production of riboflavin [94]. Therefore, *B. subtilis* has been the species of choice for commercial bacterial production of riboflavin [65], and currently, it is also the most important one.

During the past two decades, numerous studies on the improvement of riboflavin production in *B. subtilis* were performed with the help of metabolic engineering. Most of these studies focused on precursor supply and regulation of expression of the key bottleneck enzymes, which were considered to be the two major limiting factors in riboflavin production [95,96].

Ribulose-5-phosphate (Ru5P), the initial precursor of riboflavin, is produced in the oxidative branch of PPP from the reactions catalyzed by two consecutive oxidized form of nicotinamide adenine dinucleotide phosphate (NADP⁺)-dependent enzymes, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6GPD) [97–99]. However, the activities of these two enzymes are inhibited by Ru5P, as well as several other intracellular metabolites such as reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and fructose-1,6-biphosphate (FBP) [100,101]. This allosteric inhibition by intracellular metabolites can cause an insufficient precursor supply for riboflavin production. With the purpose of increasing the availability of Ru5P by genetic modifications, the genes *zwf* (encodes G6PD) and *gnd* (encodes 6GPD) from *Corynebacterium glutamicum* ATCC 13032 were cloned by Wang et al. [101], followed by the successful elimination of their feedback inhibition by site-directed mutagenesis. Further individual expression or co-expression of the two mutant genes in *B. subtilis* RH33 was performed to quantify the effects of their expressions on riboflavin production. The resulting data showed that compared to the parent strain RH33, metabolites from riboflavin biosynthetic pathway were strongly increased in engineered strains, such as Ru5P (increased 46%), DMRL, aminoimidazole, and intracellular riboflavin. In shake-flask fermentation, individual expression of the gene *zwf* (engineered strain denoted as *B. subtilis* SVZ) and *gnd* (engineered strain denoted as *B. subtilis* SVG) resulted in an approximate increase of 18% and 22% in riboflavin production, respectively. Moreover, co-expression of the two genes (engineered strain denoted as *B. subtilis* VGZ) led to a 31% increase in riboflavin production. Furthermore, an average improvement of 39% in riboflavin production was obtained during further fed-batch fermentation using *B. subtilis* VGZ. In this work, precursor supply was demonstrated to be the main limiting factor of riboflavin production in *B. subtilis*, and the redirection of carbon flux toward PPP by metabolic engineering was an effective strategy for the production of riboflavin.

In order to increase the level of carbon flux through the riboflavin biosynthetic pathway, Shi et al. [102] performed genetic manipulation of the *rib* operon in the riboflavin-producing *B. subtilis* strain BS77, including overexpression of *ribA* gene, substitution of the native promoter *ribP1* for the strong promoter P₄₃ derived from *B. subtilis* 168, and deletion of the RFN regulatory region *ribO*.

The strain with overexpression of *ribA* gene (strain BS89) was found to have the highest riboflavin production yield ($506 \text{ mg} \cdot \text{L}^{-1}$), which was 1.4-fold higher than that of BS77 ($210 \text{ mg} \cdot \text{L}^{-1}$). Subsequently, a sequential optimization strategy was performed to deregulate purine biosynthetic pathway in the *rib* operon of strain BS89, including ① elimination of transcription repression by deletion of the *pur* operon repressor PurR (encoded by *purR* gene) and the 5'-untranslated region (UTR) of *pur* operon with a guanine-sensing riboswitch; ② removal of the product-feedback inhibition of PRPP aminotransferase (encoded by *purF* gene) by means of site-directed mutagenesis. With these genetic manipulations, the metabolic flux through the purine biosynthetic pathway was successfully achieved, which consequently resulted in the highest riboflavin production yield of $827 \text{ mg} \cdot \text{L}^{-1}$ in the engineered strain with *purF*-VQW mutation (BS110) in shake-flask fermentation. This study indicated that rational deregulation of purine biosynthetic pathway in *B. subtilis* for the elimination of both transcription repression and product-feedback inhibition is a viable strategy for the improvement of metabolite yield.

A deeper understanding of the central carbon metabolism is critically required for developing rational metabolic engineering strategies aiming at improving the production of metabolites of interest. Gluconeogenesis (GNG), a biological process leading to the generation of glucose from non-sugar carbon substrates, is one of the most important pathways in central carbon metabolism. Its modification has been successfully applied to reroute the carbon flux toward the PPP in *Corynebacterium glutamicum* [103,104]. In addition, previous study has demonstrated that further improvement of riboflavin production by deregulating the key enzymes involved in gluconeogenic pathway could be feasible [96].

Wang et al. [94] therefore performed a systematic study to investigate the effects of deregulation of GNG on the improvement of riboflavin production, through overexpression of the three critical gluconeogenic genes in parental strain *B. subtilis* RH33, including the genes *gapB* (encodes NADPH-dependent glyceraldehyde 3-phosphate dehydrogenase), *pckA* (encodes phosphoenolpyruvate carboxykinase) and *fbp* (encodes fructose-1,6-bisphosphatase). The resulting data showed that compared to the strain RH33, co-overexpression of the genes *gapB* and *fbp* led to a significant increase in riboflavin titers up to $4.89 \text{ g} \cdot \text{L}^{-1}$ in shake-flask fermentation, and increases of 21.9% and 27.8% in riboflavin production were obtained respectively from co-overexpression of the genes *gapB* and *fbp* in shake-flask and fed-batch fermentation. This study implied that deregulation of gluconeogenesis is an effective strategy to further improve riboflavin production, as well as other metabolites directly from PPP and NADPH-dependent compounds using glucose as carbon source.

Zhang et al. [105] studied the effects of modifications of the genes *ribA* and *ribH* on riboflavin production in *B. subtilis*. The results showed that compared to the parent strain LXZ-1, individual overexpression of the gene *ribA* in engineered strain LXZ-2 led to an increase of 99% in riboflavin production, with a riboflavin titer of $0.47 \text{ g} \cdot \text{L}^{-1}$ during a 60-h shake-flask fermentation. However, a decrease of 30% in biomass was observed, and cell autolysis occurred because of the accumulation of 5-amino-6-(1-D-ribitylamino) uracil based on mass spectrometry analysis. In the case of co-overexpression of the genes *ribA* and *ribH* in engineered strain LXZ-3, riboflavin production increased by 280% and 91% compared to that of the strains LXZ-1 and LXZ-2, respectively, and neither loss in biomass nor cell autolysis was observed. Subsequently, a low copy-number plasmid containing a complete *rib* operon (pMX45) was transformed into the strain LXZ-3, and the resulting strain LXZ-3/pMX45 was then used to study the effects of different carbon sources on riboflavin production, including glucose, sucrose, xylose, and mixture of xylose and sucrose in different ratios. It was found that in a 60-h shake-flask fermentation, the

highest riboflavin titer ($1.6 \text{ g} \cdot \text{L}^{-1}$) was obtained when a mixture of sucrose and xylose in a ratio of 1.5/6.5 (total addition of sugar, 8%, w/v) was used as carbon source. During large-scale fermentation carried out in a 5-L fermenter, the highest riboflavin titer of $3.6 \text{ g} \cdot \text{L}^{-1}$ was obtained from the use of the same mixed carbon source after 70-h incubation. This study demonstrated that co-metabolism with sucrose and xylose can increase precursor supply, thus leading to an improvement in riboflavin production.

Norseothricin (NTC) is a streptothricin antibiotic that can inhibit protein biosynthesis. Thus, it is widely used for resistance screening of bacteria, fungi, yeast, and plant cells. Cheng et al. [106] constructed a novel NTC-resistant plasmid with the insertion of the gene *sat* (encodes streptothricin acetyltransferase) in expression plasmid pMA5, namely pMA5-*sat*. Subsequently, the gene *zwf* was cloned and inserted in the plasmid pMA5-*sat*. The generated plasmid pMA5-*sat*-*zwf* was then transferred into a previously engineered riboflavin-producing *B. subtilis* strain RF1, in order to study overexpression of the gene *zwf* on riboflavin production. The results revealed that the activity of G6DP in recombinant *B. subtilis* strain RF1-pMA5-*sat*-*zwf* was fifty-fold higher than that in the strain RF1, indicating successful overexpression of this enzyme. In addition, a final riboflavin titer of $12.01 \text{ g} \cdot \text{L}^{-1}$ was achieved with this recombinant strain in a large-scale fermentation performed in a 5-L fermenter, which increased by 30.3% compared to that of the strain RF1. These results indicated the potential applications of this newly constructed NTC-resistant plasmid pMA5-*sat* in genetic modifications of riboflavin-producing *B. subtilis* strains.

Besides, Hemberger et al. [107] reported that RibM from *Streptomyces davawensis* (*S. davawensis*) is an energy-independent functional riboflavin transporter in *B. subtilis*, which could mainly catalyze riboflavin export. By introducing the gene *ribM* from *S. davawensis* into a high-performance *B. subtilis* riboflavin production strain, riboflavin synthesis by the recombinant RibM overproducing strain was increased significantly depending on the amount of the inducer isopropyl β -D-thiogalactoside (IPTG). This was the first successful example of optimizing the riboflavin production in *B. subtilis* on the basis of riboflavin export, suggesting that the enhancement of riboflavin excretion is also a useful strategy to increase the riboflavin yield in *B. subtilis*.

In addition to the method of metabolic engineering, optimization of fermentation conditions was also reported as a viable method to improve riboflavin production in *B. subtilis* [108,109].

Oxygen dissolved in the culture medium is one of the most important parameters in the fermentation process, and is closely related to cell growth and product formation [110–112]. Its supply is thus a critical factor for riboflavin production in *B. subtilis*, which can be easily enhanced by the change of agitation speed [113]. Man et al. [114] investigated the effects of agitation speed on riboflavin production by recombinant engineered *B. subtilis* RF1 in fed-batch fermentation. The results of kinetic analysis showed that low agitation speed ($600 \text{ r} \cdot \text{min}^{-1}$) in the initial phase of fermentation process facilitated cell growth and riboflavin production, whereas a higher agitation speed of $900 \text{ r} \cdot \text{min}^{-1}$ was favorable in the later phase. Based on these results, a two-stage control strategy aiming at keeping high cell growth and riboflavin production in the whole fermentation process was developed, in which the agitation speed was set at $600 \text{ r} \cdot \text{min}^{-1}$ for the first 26 h, and then $900 \text{ r} \cdot \text{min}^{-1}$ until the end. However, a negative impact on cell growth and riboflavin production within a short period was observed due to the sudden switch of agitation speed occurring in the two-stage control strategy, so a strategy of gradually increasing in agitation speed from 600 to $900 \text{ r} \cdot \text{min}^{-1}$ was subsequently established, with which the maximum riboflavin titer reached $9.4 \text{ g} \cdot \text{L}^{-1}$ in a 48-h fermentation, with a production yield of 0.051 g per gram of glucose. Compared to the best result obtained from a fed-batch fermentation using a

single-agitation speed ($600 \text{ r} \cdot \text{min}^{-1}$), an increase of 20.5% and 21.4% in riboflavin titer and production yield was obtained, respectively.

With the aim of searching for a riboflavin production accelerant, Wan et al. [115] investigated the effects of individual addition of seven additives on riboflavin production by *B. subtilis* in shake-flask fermentation, including calcium gluconate, citric acid, sodium citrate, calcium chloride, alanine, malic acid and fructose-1,6-diphosphate (FDP). The bacterial density optical density (OD_{600}) and riboflavin production yield were used as indexes to evaluate the effects. The results indicated that the most significant improvements of riboflavin production were observed with the addition of calcium gluconate, sodium citrate, and alanine. Therefore, these three additives were selected for further study of their combined effects on riboflavin production by orthogonal analysis. The results showed that the optimal concentration ratio of calcium gluconate, sodium citrate and alanine was 7.5, 5.0, and $1.5 \text{ g} \cdot \text{L}^{-1}$, under which the highest riboflavin titer of $6.46 \text{ g} \cdot \text{L}^{-1}$ was achieved, with an approximate increase of 40% compared to that obtained without addition of additives.

In a recent work, Oraei et al. [116] evaluated the effects of 13 different minerals (CaCl_2 , CuCl , FeCl_3 , FeSO_4 , AlCl_3 , Na_3MoO_4 , $\text{Co}(\text{NO}_3)_2$, NaCl , KH_2PO_4 , K_2HPO_4 , MgSO_4 , ZnSO_4 , and MnSO_4) on riboflavin production by wild-type *B. subtilis* ATCC 6051 in shake-flask fermentation, in order to develop an appropriate fermentation medium for improving riboflavin production. Plackett–Burman (PB) design was first carried out to screen minerals with significant influence on riboflavin production, with the results revealing that the concentration of three minerals showed greatest influence, that is, MgSO_4 , K_2HPO_4 , and FeSO_4 . Subsequently, an optimization test was performed using response surface methodology (RSM) to determine the optimal concentrations of the five selected medium components, including two carbon sources used in all treatments, fructose and yeast extract, and three minerals, MgSO_4 , K_2HPO_4 , and FeSO_4 . The highest riboflavin titer in a 72-h shake-flask fermentation was obtained ($11.73 \text{ g} \cdot \text{L}^{-1}$) when the concentration of these five components was 38.10, 4.37, 0.85, 2.27, and $0.02 \text{ g} \cdot \text{L}^{-1}$, respectively.

3.2.4. Lactic acid bacteria

Lactic acid bacteria (LAB) comprise a heterogeneous group of Gram-positive, non-sporulating, non-respiring, usually non-motile rods or cocci [117,118]. They produce lactic acid as the principal or sole end product of carbohydrate fermentation, and are closely related to human life. The growth of many spoilage and pathogenic bacteria in fermented foods is inhibited by both the acidic environment created by LAB and the production of antimicrobial compounds by LAB such as bacteriocins [119,120]. Since ancient times, these bacteria have been empirically used as fermenting agents for improving the bio-preservation of food [121,122]. Nowadays, LAB is rationally used worldwide as lactic starter cultures in food industries, being chiefly responsible for the production of a large variety of fermented dairy foods such as yogurt and cheese, fermented sausages, and pickles (fermented vegetables) [123].

Although LAB are usually auxotrophic for several vitamins, it is now commonly known that certain LAB species have the ability to produce some B-group vitamins, such as folates (or vitamin B_9), riboflavin (or vitamin B_2), and cobalamin (vitamin B_{12}) [124,125]. The production of vitamins by LAB varies considerably between different strains, being species- or strain-specific, which is generally related to the interruption (partial or complete) of genetic information for the biosynthesis of vitamins. For example, Thakur et al. [126] performed a PCR-based screening for riboflavin biosynthesis genes in 60 putative riboflavin-producing *Lactobacilli* strains, of which only 14 strains were found to grow in a commercial

riboflavin-free medium. It was further observed that the presence of *rib* operon was strain-specific across different species of *Lactobacilli*. Furthermore, an interrupted or incomplete *rib* operon in LAB is usually associated with the loss of riboflavin production ability [127]. As previously reported by Kleerebezem et al. [128] and Burgess et al. [129], the *Lactobacillus plantarum* (*L. plantarum*) strain WCFS1 contains an incomplete *rib* operon, in which the entire *ribG* gene and part of the *ribB* gene are absent, and as expected, this strain could not grow in the absence of riboflavin. However, it was also reported that several wild-type strains of *L. plantarum* harbor the complete *rib* operon and are able to produce riboflavin, such as *L. plantarum* strain RYG-GYY-9049 isolated from traditional Chinese pickle juices [130], *L. plantarum* strain NCDO 1752 isolated from pickled cabbage [129], and *L. plantarum* strains UNIFG1 and UNIFG2 isolated from natural sourdoughs [127]. Overall, the integrity of *rib* operon in LAB is essential for riboflavin production.

Riboflavin is one of the most studied vitamins produced by LAB because of its importance in human health and the frequency of its deficiency [131]. Although riboflavin is normally present in a wide variety of foods, a high incidence of its deficiency is found worldwide, mainly because of an inadequate diet. Therefore, riboflavin-bio-enriched foods have attracted great attention due to several factors. They represent a more natural, consumer-friendly and less expensive alternative to chemically produced riboflavin, and fulfill the increased consumer demand for natural foods [39]. The adaptability of LAB to industrial food fermentation and their riboflavin production ability make them ideal candidates for riboflavin production in foods [72]. This improved function of LAB opens up new opportunities for developing novel functional foods with enhanced riboflavin content. Therefore, screening of wild-type riboflavin-producing LAB from various food sources has gained the interest of numerous researchers. It was previously reported that only 42 strains could grow in a commercial riboflavin-free medium among 179 strains of LAB isolated from a wide variety of food products [132]. On the basis of the concentrations of riboflavin determined by high performance liquid chromatography (HPLC), five of these strains with relatively high riboflavin producing capacity (extracellular concentrations of riboflavin in the range of $190\text{--}260 \text{ ng} \cdot \text{mL}^{-1}$) were selected and then inoculated into soy-milk to evaluate both their growth and riboflavin production in this food matrix. After 12-h of fermentation at 37°C , only the strain *L. plantarum* CRL 725 showed a significant increase in the concentration of riboflavin from the initial 309 to $700 \text{ ng} \cdot \text{mL}^{-1}$ [132]. In another study [131], screening of wild-type riboflavin-producing LAB from raw goat milk and cheeses was performed, and a total of 179 strains of LAB were isolated. Among these strains, only eight were able to grow in a riboflavin-free medium, and produced riboflavin within a total concentration range of $173\text{--}532 \text{ ng} \cdot \text{mL}^{-1}$.

However, the riboflavin titer and productivity obtained from fermentations using the wild-type strains of LAB screened from foods or other sources were relatively low, since they are not naturally optimized for maximal production rates of some specific bioactive compounds such as riboflavin [133]. Thus, improving riboflavin-producing capacity of these strains in order to achieve the overproduction of riboflavin is still a challenge. For this purpose, two parallel strategies have been commonly used, including screening of roseoflavin-resistant strains and development of recombinant strains based on metabolic engineering [39,124].

Roseoflavin, a natural toxic analog of FMN and riboflavin, can directly bind to the FMN riboswitch [134], resulting in inhibition of transcription of the *rib* operon [135]. Exposing riboflavin-producing strains of LAB to this antibacterial compound can cause spontaneous mutations in LAB thus generating riboflavin overproducers. This strategy has been successfully used to select natural riboflavin-overproducing mutants of food-grade strains of *B. sub-*

tilis [136,137], *Lactococcus lactis* (*L. lactis*) [138], *L. plantarum* [127], *Leuconostoc mesenteroides* and *Propionibacterium freudenreichii* (*P. freudenreichii*) [129], and has proven to be reliable.

The strain *P. freudenreichii* NIZO B2336 is a spontaneous roseoflavin-resistant mutant screened from wild-type *P. freudenreichii* NIZO B374, which produces a higher level of riboflavin than the parent strain [139]. These two strains were tested as adjunct cultures for yogurt fermentation, and were added 24 h prior to the addition of the yogurt starter culture *Campina* MUH306. The results showed that the final amount of riboflavin in the yogurt produced with *P. freudenreichii* NIZO B2336 as an adjunct culture reached $19.7 \mu\text{g}\cdot\text{g}^{-1}$, which is higher than that of the yogurt produced without addition of adjunct culture ($12.9 \mu\text{g}\cdot\text{g}^{-1}$), as well as the yogurt produced with *P. freudenreichii* NIZO B374 ($10.5 \mu\text{g}\cdot\text{g}^{-1}$). This study demonstrated that *P. freudenreichii* NIZO B2336 could be used for developing novel fermented milk products with high levels of riboflavin, which may convey additional health benefits.

As described above, Juarez del Valle et al. [132] screened a strain *L. plantarum* CRL 725 with which a two-fold increase in the initial concentration of riboflavin in soymilk was obtained. Further screening of roseoflavin-resistant mutants of these strains was performed. It was found that one of the mutants, denominated CRL 2130, increased the initial concentration of riboflavin in soymilk six-fold (from 309 to $1860 \text{ ng}\cdot\text{mL}^{-1}$). It was, therefore, demonstrated that roseoflavin-resistant strains could be used to develop new riboflavin-enriched soy products with improved nutritional value.

In addition, Russo et al. [135] isolated a wild-type riboflavin-producing strain of *Lactobacillus fermentum* (denominated *L. fermentum* PBCC11) from sourdough, and screening of roseoflavin-resistant mutants of this strain was subsequently performed. A total of 15 mutants were obtained, seven of which produced more than $1 \text{ mg}\cdot\text{L}^{-1}$ of riboflavin. The best riboflavin-overproducing mutant, named *L. fermentum* PBCC11.5 (which produced the highest level of riboflavin, $1.203 \text{ mg}\cdot\text{L}^{-1}$), was selected and further co-inoculated with commercial yeast *Saccharomyces cerevisiae* to fortify bread. It was found that the final amount of riboflavin reached $6.66 \mu\text{g}\cdot\text{g}^{-1}$ in the bread fermented with co-inoculum of yeast and *L. fermentum* PBCC11.5, corresponding to an approximately two-fold increase compared to that of conventional bread.

Our recent work successfully screened out a native riboflavin-overproducing *L. plantarum* strain RYG-YYG-9049 from traditional Chinese pickle juices, which produced $0.734 \text{ mg}\cdot\text{L}^{-1}$ of riboflavin [130]. Subsequently, roseoflavin-induced spontaneous mutation was carried out using this strain, and the No. 10 mutant (named as *L. plantarum* RYG-YYG-9049-M10) showed the highest riboflavin-producing capacity. This mutant was used to ferment raw soymilk in order to fortify the riboflavin content reaching $2.920 \text{ mg}\cdot\text{L}^{-1}$ riboflavin under optimized conditions.

According to previous reports, roseoflavin-induced spontaneous mutation mainly included gene deletion and/or single-base modification in the upstream regulatory region of the *rib* operon [72,129,140]. Our recent study found a novel mutation type where roseoflavin induced an insertion of a 1059-bp DNA fragment in the upstream regulatory region of the *rib* operon, which was probably the main reason for the increase in riboflavin production [130]. It is suggested that roseoflavin-mediated mutations were able to affect the stability of the terminator structure, leading to a reduced formation of this terminator and thereby allowing a continuous transcription of the *rib* operon [129], although the exact mechanisms need further investigation.

Therefore, it is feasible to increase the riboflavin levels in fermented food products, such as yogurt, cheeses, and bread, by proper selection of LAB species/strains and optimization of fermentation conditions. Novel functional fermented foods with increased

levels of riboflavin would increase their nutritional value and provide health benefits for their consumers.

Regarding metabolic engineering, it is generally used to construct recombinant strains with increased riboflavin-producing capacity. Burgess et al. [138] performed the genetic analysis of *rib* operon in *L. lactis* ssp. *cremoris* strain NZ9000 in order to clarify the effects of the overexpression of *rib* genes on riboflavin production. The four structural *rib* genes, including *ribG*, *ribB*, *ribA*, and *ribH*, were cloned into expression vector pNZ8048 in different combinations and then introduced into *L. lactis* NZ9000. The results showed that a high level of riboflavin ($24 \text{ mg}\cdot\text{L}^{-1}$) was only obtained by simultaneous overexpression of all the four *rib* genes (engineered *L. lactis* NZ9000 harboring the plasmid pNZBAH). Thereby, the strain *L. lactis* NZ9000, which was a riboflavin consumer, was successfully converted into a riboflavin producer.

By exposure to roseoflavin, Sybesma et al. [141] isolated a riboflavin-producing mutant from *L. lactis* strain NC9000, and sequence analysis showed that this mutant *L. lactis* CB010 only contained a base change in the regulatory region upstream of the *rib* genes. Subsequently, the gene *folKE*, which encodes a bifunctional enzyme amino-4-hydroxy-6-hydroxymethylidihydropteridine pyrophosphokinase and GTP cyclohydrolase I, was cloned into plasmid pZ8161, and the resulting plasmid pNZ7017 was then transferred into *L. lactis* CB010. It was found that by overexpression of the gene *folKE* in the engineered riboflavin-producing mutant (*L. lactis* CB010 harboring the plasmid pNZ7017), the production of folate was also enhanced. Therefore, a simultaneous overproduction of both folate and riboflavin in *L. lactis* was achieved with the help of metabolic engineering.

As the common precursor of both riboflavin and folate, GTP can be used either by GTP cyclohydrolase II (encoded by *ribA*) for riboflavin biosynthesis or by GTP cyclohydrolase I (encoded by *folE*) for folate biosynthesis. Therefore, there exists competition for GTP molecules between these two enzymes, and inactivation of the gene *folE* may promote riboflavin production because of the improved GTP supply. By inactivation of the *folE* gene, the riboflavin-producing capacity of *L. fermentum* MTCC 8711, a wild-type riboflavin-producing strain isolated from yogurt, was increased. In a chemically defined medium (CDM), the strain *Lb. fermentum* MTCC 8711 produced $2.29 \text{ mg}\cdot\text{L}^{-1}$ of riboflavin in a 24-h fermentation, which remained stable up to 72 h, and the *folE* disrupted mutant *Lb. fermentum* GKJFE produced $3.49 \text{ mg}\cdot\text{L}^{-1}$ of riboflavin in a 72-h fermentation, representing an approximate 50% increase compared to that of the parental strain [142].

Metabolic engineering proves to be a reliable way to exploit biotechnologically important strains with no or with low riboflavin-producing capacity. However, the genetically engineered strains so far still cannot be applied in the fermentation of food products destined for human consumption, because of consumer or regulatory concerns related to genetic engineering.

4. Conclusions

This review illustrates demonstrated the current advances in the microbial overproduction of riboflavin, which have mainly been achieved by the optimization of fermentation conditions and the use of riboflavin overproducing strains derived from chemical mutagenesis and metabolic engineering (summarized in Appendix A Table S1) [10–42,45–47,57,59,60,62,76–79,86,89,94,98,101,102,105,106,108,114–116,129,132,135,138,143,144]. Compared to chemical and semi-chemical synthesis, riboflavin production by microbial fermentation is more economical and environmentally friendly. In recent years, a number of biotechnological fermentation processes, which are mainly based on the use of *B. subtilis* and *A. gossypii* strains, have been developed, and

are currently applied in industrial-scale to replace the more costly chemical synthetic process. However, it is still far from conclusive which microorganism will prove to be the most advantageous and displace others, since each microorganism has its pros and cons (as described in Appendix A Table S2). Compared to traditional riboflavin-consuming starter cultures, bacteria even with low riboflavin-producing capacity can be better candidates for use as starter cultures for food fermentation. The production of fermented food products with increased levels of riboflavin will increase both the nutritional and commercial value of the foods, and eliminate the requirement for fortification. In addition, it is also expected that consumption of such novel fermented foods will reduce the incidence of riboflavin deficiency. Finally, the concept of *in situ* production of riboflavin with carefully selected strains could open the way to develop novel food products destined at different or specific groups, such as the elderly, children, pregnant women, sportsmen, vegetarians, and adolescents.

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Compliance with ethics guidelines

Jia-Rong Zhang, Ying-Ying Ge, Pin-He Liu, Hong-Yan Liu, Ding-Tao Wu, Hua-Bin Li, Harold Corke, and Ren-You Gan declare that they have no conflict of interest or financial conflicts to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.eng.2021.03.018>.

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