Journal Pre-proofs

Review

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PII: S2095-8099(21)00200-9
DOI: https://doi.org/10.1016/j.eng.2021.03.018
Reference: ENG 700

To appear in: *Engineering*

Received Date: 23 May 2020
Revised Date: 25 January 2021
Accepted Date: 3 March 2021

Please cite this article as: J-R. Zhang, Y-Y. Ge, P-H. Liu, D-T. Wu, H-Y. Liu, H-B. Li, H. Corke, R-Y. Gan, Biotechnological Strategies of Riboflavin Biosynthesis in Microbes, *Engineering* (2021), doi: https://doi.org/10.1016/j.eng.2021.03.018

Biotechnological Strategies of Riboflavin Biosynthesis in Microbes

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Column of article (Research)

Type of article (Review)

Subject: Riboflavin Biosynthesis in Microbes

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.

Keywords: Vitamin B_2; *Bacillus subtilis*; lactic acid bacteria; fungi; microbial fermentation.

1. Introduction

Riboflavin (RF), also known as Vitamin B_2 (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/day 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.

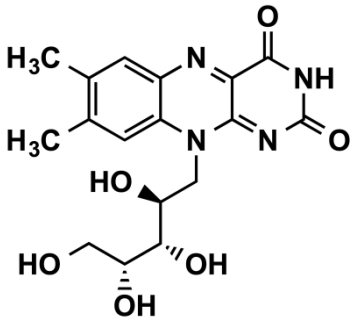

Fig. 1. The chemical structure of riboflavin.

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 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

9,000 T in 2012, of which about 1,800 T are used in pharmaceuticals and beverages, and about 7,200 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].

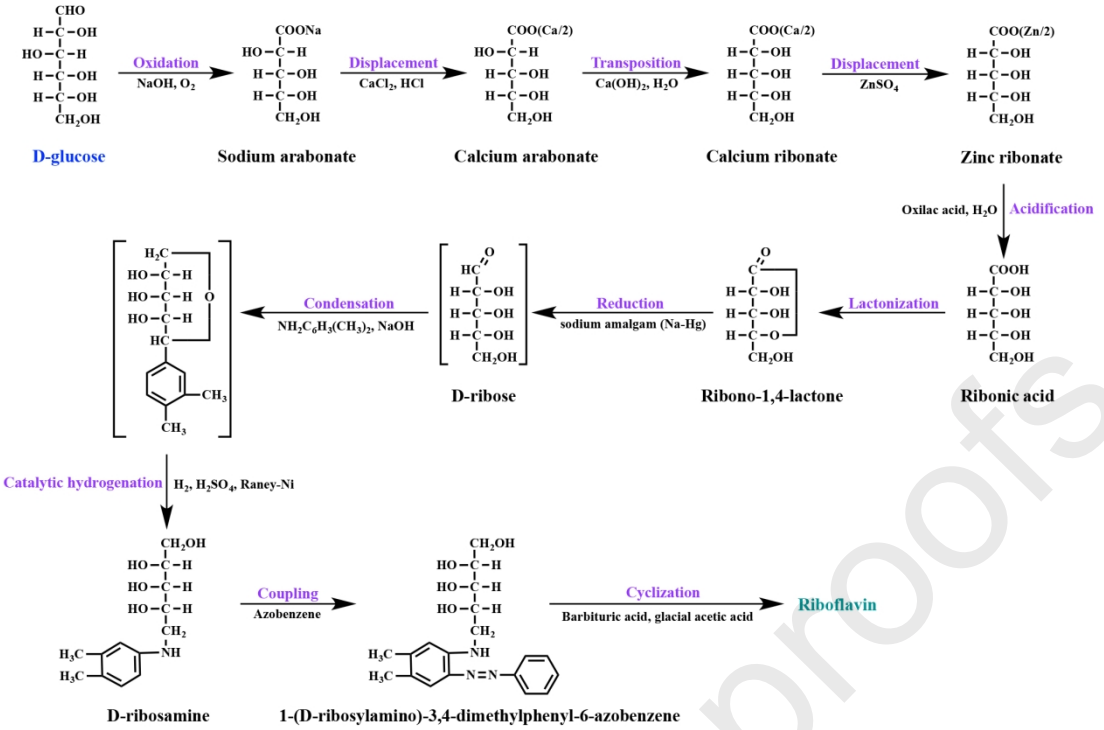

Fig. 2. The total chemical synthesis of riboflavin.

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*) and two

fungi (*Ashbya gossypii* and *Eremothecium 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* 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*, *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-ribityllymazine (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.

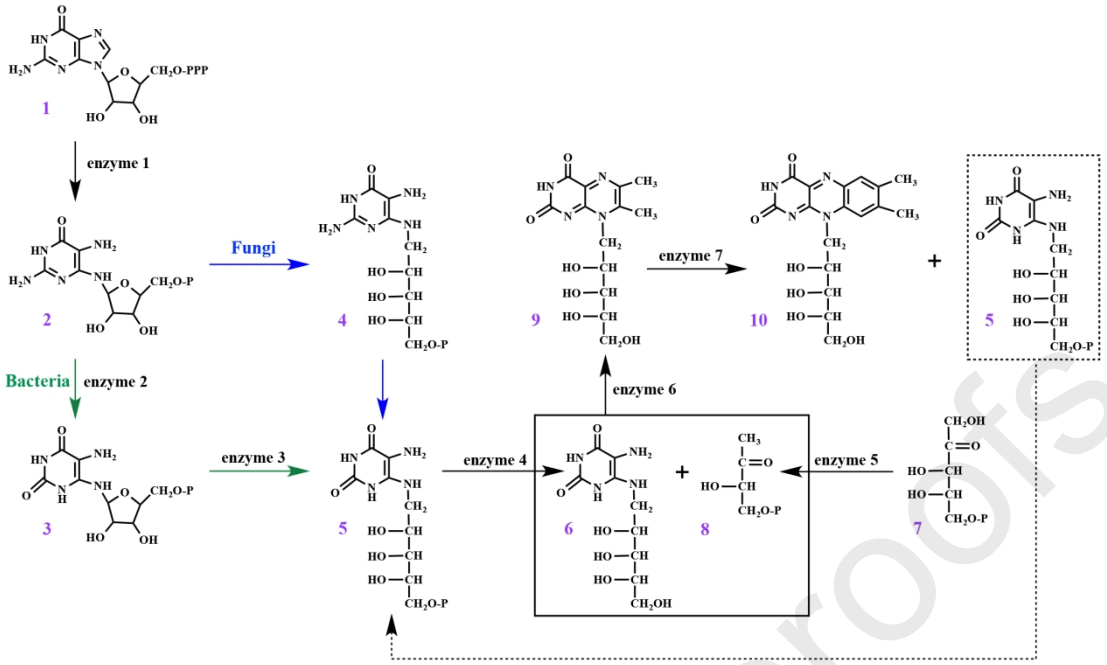

Fig. 3. The biosynthetic pathway of riboflavin adapted from Bacher et al. [31]. Compound 1 is guanosine triphosphate (GTP), compound 2 is 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone 5'-phosphate (DARPP), compound 3 is 5-amino-6-ribosylamino-2,4(1*H*,3*H*)-pyrimidinedione 5'-phosphate (ARPP), compound 4 is 2,5-diamino-5-ribitylamino-4(3*H*)-pyrimidinone 5'-phosphate (DArPP), compound 5 is 5-amino-6-ribitylamino-2,4(1*H*, 3*H*)-pyrimidinedione 5'-phosphate (ArPP), compound 6 is 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (ArP), compound 7 is ribulose-5-phosphate, compound 8 is 3,4-dihydroxy-2-butanone-4-phosphate (DHBP), compound 9 is 6,7-dimethyl-8-ribityllymazine (DMRL), and compound 10 is riboflavin. Enzyme 1 corresponds to GTP cyclohydrolase II, enzymes 2 and 3 correspond to the same enzyme, which is the bifunctional riboflavin-specific pyrimidine deaminase/reductase, enzyme 4 corresponds to a non-specific phosphatase, enzyme 5 corresponds to DHBP synthase, enzyme 6 corresponds to lumazine synthase, and enzyme 7 corresponds to riboflavin synthase.

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 *Eremothecium ashbyii* and *Ashbya gossypii*, and three

bacteria, including *Bacillus subtilis*, *Escherichia coli,* and lactic acid bacteria [33-39]. For the fungi such as *Eremothecium ashbyii* and *Ashbya 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. Eremothecium ashbyii (E. ashbyii)

Eremothecium 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 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 (C. famata)

The yeast *C. famata* (*C. flareri*) 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* under the control of the *TEF1* promoter, and transformed it into *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 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 fermenation in a 7 L laboratory bioreactor, the constructed strain accumulated up to 16.4 g/L of riboflavin in optimized medium, representing one of the most known active riboflavin producers. Furthermore, when the two modified genes *PRS3* (encoding PRPP synthetase) and *ADE4* (encoding PRPP amidotransferase) from *Debaryomyces hansenii* were introduced and co-expressed in this constructed strain, a two-fold increase in riboflavin production is observed [50].

3.1.3. Ashbya gossypii (A. gossypii)

The filamentous hemiascomycete *Ashbya 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 ^{13}C 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 ^{13}C 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 ^{13}C-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 guanosine-5-triphosphate (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 (*ADE12*) and controls the first step of the 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 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 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* ATTC 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 *Bacillus subtilis* and *Escherichia 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 bi-functional 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 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 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].

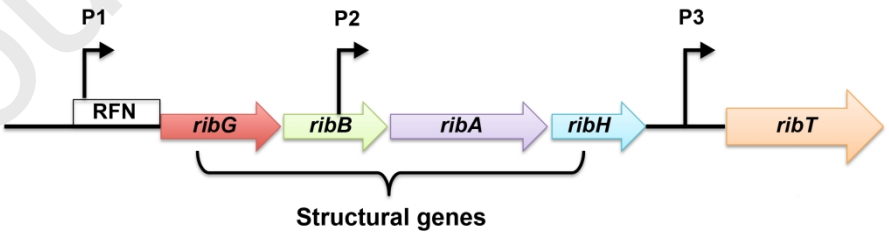

Fig. 4. Structure of the *rib* operon in bacteria. The structural genes encode the metabolism-related proteins, and the transport gene *ribT* encodes the transport-related protein. The fine arrows show the transcription direction of the promoter (P)1, P2, and P3.

Upstream of the *rib* operon in *B. subtilis*, three regulatory regions exist including

392 *ribC*, *ribR*, and *ribO* [67,68]. Mutation in each of these three regions leads to
393 riboflavin overproduction, therefore, they are considered as presumptive repressors of
394 riboflavin biosynthesis. Among them, *ribC* and *ribR* respectively encode a
395 bifunctional flavokinase/FAD-synthetase and monofunctional flavokinase. These two
396 enzymes are involved in the sequential conversion of riboflavin into FMN and FAD.
397 *RibO* is a noncoding region located between the *ribP1* promoter and the gene *ribG*
398 [69], whose transcriptional product can be folded into a conserved RNA secondary
399 structure with a base stem and four hairpins, designated as FMN riboswitch. The
400 interaction of flavins with the FMN riboswitch plays a key role in the regulation of *rib*
401 operon. In the relieving state, the concentration of FMN is too low, thus it does not
402 bind to the FMN riboswitch, and an antiterminator is formed to ensure the ready
403 read-through of the following sequence, whereas, under repression condition, FMN
404 binding with the FMN riboswitch leads to the formation of a transcription terminator,
405 leading to the termination of transcription [70].

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

415 ***3.2.1. Clostridium acetobutylicum (C. acetobutylicum)***

416 *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 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 mM 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 (0.53 mM). 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. Escherichia coli (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 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 riboflavin in shake-flask fermentation, with a riboflavin production yield of 44.8 mg/g 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 (Ptrc) and transferred it into wild-type *E. coli* MG1655, and the resulting strain RF01S could accumulate 229.1 mg/L riboflavin. Subsequently, the deletion of genes *pgi* (encoding glucose-6-phosphategluconolactonase), *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. 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 of riboflavin in 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 in shake-flask fermentation under the optimal conditions, which was nearly twelve-fold higher than that of RF01S, with a riboflavin production yield of 136 mg/g 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 pentose phosphate (PP) pathway. 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 of riboflavin in MSY medium containing 10 g glucose per liter, and in fed-batch fermentation, a riboflavin titer of 10.4 g/L was achieved, with a production yield of 56.8 mg/g 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

517 indeed a potential efficient species for riboflavin production.

518 ***3.2.3. Bacillus subtilis (B. subtilis)***

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

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

535 Ribulose-5-phosphate (Ru5P), the initial precursor of riboflavin, is produced in
536 the oxidative branch of pentose phosphate pathway from the reactions catalyzed by
537 two consecutive $NADP^+$-dependent enzymes, glucose-6-phosphate dehydrogenase
538 (G6PD) and 6-phosphogluconate dehydrogenase (6GPD) [97-99]. However, the
539 activities of these two enzymes are inhibited by Ru5P, as well as several other
540 intracellular metabolites such as NADPH and fructose-1,6-biphosphate (FBP)
541 [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* ATCC13032 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 pentose phosphate pathway 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 P_{43} 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 mg/L), which was 1.4-fold higher than that of BS77 (210 mg/L). 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'-UTR of *pur* operon with a guanine-sensing riboswitch; 2) removal of the product-feedback inhibition of PRPP aminotransferase (encoded by *pur*F 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 mg/L in the engineered stain 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 pentose phosphate (PP) pathway 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 gluconeogenesis 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 phoaphoenolpyruvate carboxykinase) and *fbp* (encodes fructose-1,6-biphosphatase class 3). 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 g/L in shake-flash 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 PP pathway 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 g/L 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 gene *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 g/L) 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 g/L 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* (encodes G6PD) 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 g/L 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*

642 *davawensis* is an energy-independent functional riboflavin transporter in *B. subtilis*,
643 which could mainly catalyze riboflavin export. By introducing the gene *ribM* from *S.*
644 *davawensis* into a high-performance *B. subtilis* riboflavin production strain, riboflavin
645 synthesis by the recombinant RibM overproducing strain was increased significantly
646 depending on the amount of the inducer IPTG. This was the first successful example
647 of optimizing the riboflavin production in *B. subtilis* on the basis of riboflavin export,
648 suggesting that the enhancement of riboflavin excretion is also a useful strategy to
649 increase the riboflavin yield in *B. subtilis*.

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

653 Oxygen dissolved in the culture medium is one of the most important parameters
654 in the fermentation process, and is closely related to cell growth and product
655 formation [110-112]. Its supply is thus a critical factor for riboflavin production in *B.*
656 *subtilis*, which can be easily enhanced by the change of agitation speed [113]. Man et
657 al. [114] investigated the effects of agitation speed on riboflavin production by
658 recombinant engineered *B. subtilis* RF1 in fed-batch fermentation. The results of
659 kinetic analysis showed that low agitation speed (600 rpm) in the initial phase of
660 fermentation process facilitated cell growth and riboflavin production, whereas a
661 higher agitation speed of 900 rpm was favorable in the later phase. Based on these
662 results, a two-stage control strategy aiming at keeping high cell growth and riboflavin
663 production in the whole fermentation process was developed, in which the agitation
664 speed was set at 600 rpm for the first 26 h, and then 900 rpm until the end. However,
665 a negative impact on cell growth and riboflavin production within a short period was
666 observed due to the sudden switch of agitation speed occurring in the two-stage

667 control strategy, so a strategy of gradually increasing in agitation speed from 600 to
668 900 rpm was subsequently established, with which the maximum riboflavin titer
669 reached 9.4 g/L in a 48-h fermentation, with a production yield of 0.051 g/g glucose.
670 Compared to the best result obtained from a fed-batch fermentation using a
671 single-agitation speed (600 rpm), an increase of 20.5% and 21.4% in riboflavin titer
672 and production yield was obtained, respectively.

673 With the aim of searching for a riboflavin production accelerant, Wan et al. [115]
674 investigated the effects of individual addition of seven additives on riboflavin
675 production by *B. subtilis* in shake-flask fermentation, including calcium gluconate,
676 citric acid, sodium citrate, calcium chloride, alanine, malic acid and
677 fructose-1,6-diphosphate (FDP). The bacterial density (OD_{600}) and riboflavin
678 production yield were used as indexes to evaluate the effects. The results indicated
679 that the most significant improvements of riboflavin production were observed with
680 the addition of calcium gluconate, sodium citrate, and alanine. Therefore, these three
681 additives were selected for further study of their combined effects on riboflavin
682 production by orthogonal analysis. The results showed that the optimal concentration
683 ratio of calcium gluconate, sodium citrate and alanine was 7.5-5-1.5 g/L, under which
684 the highest riboflavin titer of 6.46 g/L was achieved, with an approximate increase of
685 40% compared to that obtained without addition of additives.

686 In a recent work, Oraei et al. [116] evaluated the effects of 13 different minerals
687 ($CaCl_2$, CuCl, $FeCl_3$, $FeSO_4$, $AlCl_3$, Na_3MoO_4, $Co(NO_3)_2$, NaCl, KH_2PO_4, K_2HPO_4,
688 $MgSO_4$, $ZnSO_4$, and $MnSO_4$) on riboflavin production by wild-type *B. subtilis* ATCC
689 6051 in shake-flask fermentation, in order to develop an appropriate fermentation
690 medium for improving riboflavin production. Plackett-Burman (PB) design was first
691 carried out to screen minerals with significant influence on riboflavin production, with

692 the results revealing that the concentration of three minerals showed greatest
693 influence, i.e. $MgSO_4$, K_2HPO_4 and $FeSO_4$. Subsequently, an optimization test was
694 performed using response surface methodology (RSM) to determine the optimal
695 concentrations (g/L) of the five selected medium components, including two carbon
696 sources used in all treatments, fructose and yeast extract, and three minerals, $MgSO_4$,
697 K_2HPO_4 and $FeSO_4$. The highest riboflavin titer in a 72-h shake-flask fermentation
698 was obtained (11.73 g/L) when the concentration of these five components was 38.10,
699 4.37, 0.85, 2.27 and 0.02 g/L, respectively.

700 ***3.2.4. Lactic acid bacteria***

701 Lactic acid bacteria (LAB) comprise a heterogeneous group of Gram-positive,
702 non-sporulating, non-respiring, usually non-motile rods or cocci [117,118]. They
703 produce lactic acid as the principal or sole end product of carbohydrate fermentation,
704 and are closely related to human life. The growth of many spoilage and pathogenic
705 bacteria in fermented foods is inhibited by both the acidic environment created by
706 LAB and the production of antimicrobial compounds by LAB such as bacteriocins
707 [119,120]. Since ancient times, these bacteria have been empirically used as
708 fermenting agents for improving the bio-preservation of food [121,122]. Nowadays,
709 LAB is rationally used worldwide as lactic starter cultures in food industries, being
710 chiefly responsible for the production of a large variety of fermented dairy foods such
711 as yogurt and cheese, fermented sausages, and pickles (fermented vegetables) [123].

712 Although LAB are usually auxotrophic for several vitamins, it is now commonly
713 known that certain LAB species have the ability to produce some B-group vitamins,
714 such as folates (or vitamin B_9), riboflavin (or vitamin B_2) and cobalamin (vitamin B_{12})
715 [124,125]. The production of vitamins by LAB varies considerably between different
716 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* 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 *Lactobacillus 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 HPLC, five of these strains with relatively high riboflavin producing capacity (extracellular concentrations of riboflavin in the range of 190–260 ng/mL) were selected and then inoculated into soymilk to evaluate both their growth and riboflavin production in this food matrix. After 12-h of fermentation at 37°C, only the strain *Lactobacillus plantarum* CRL 725 showed a significant increase in the concentration of riboflavin from the initial 309 to 700 ng/mL) [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 8 were able to grow in a riboflavin-free medium, and produced riboflavin within a total concentration range of 173–532 ng/mL.

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. subtilis* [136,137], *Lactococcus lactis* [138], *Lactobacillus plantarum* [127], *Leuconostoc mesenteroides* and *Propionibacterium 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 μg/g, which is higher than that of the yogurt produced without addition of adjunct culture (12.9 μg/g), as well as the yogurt produced with *P. freudenreichii* NIZO B374 (10.5 μg/g). 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 ng/mL). 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 mg/L of riboflavin. The best riboflavin-overproducing mutant, named *L. fermentum* PBCC11.5 (which produced the highest level of riboflavin, 1.203 mg/L), 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 μg/g 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 mg/L of riboflavin [130]. Subsequently, roseoflavin-induced spontaneous mutation was carried out using this strain, and the N0.10 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 mg/L 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 *Lactococcus 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 mg/L) 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-hydroxymethyldihydropteridine 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 *Lactococcus 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 cycohydrolase II (encoded by *ribA*) for riboflavin biosynthesis or by GTP cycohydrolase 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 *Lactobacillus 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 mg/L 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 mg/L 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 Table 1). 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 *Bacillus subtilis* and *Ashbya 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 Table 2). 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.

886 **Table 1** Overview of riboflavin over-production in various microorganisms

Strain	Description	Inoculation amount	Carbon source	Nitrogen source / other medium components	Initial pH	Fermentation Temp./ Time	Shaking speed	Riboflavin content	Cause of increased or decreased riboflavin production	Ref.
***Eremothecium ashbyii* (*E. ashbyii*)**										
E. ashbyii NRRL-1363	A highly flavinogenic strain	5% (v/v) of a 48-h culture of mycelium	molasses (50 g/L)	Yeast extract (2.0 g/L) and peptone (8.0 g/L)/ KH_2PO_4 (2.0 g/L), $MgSO_4$ (0.1 g/L), NaCl (1.0 g/L) and Tween 80 (1.8 mL/L)	6.5	30 °C/ 7 d	200 rpm	2.85 (g/L)	-	[45]
			molasses (50 g/L)	Deoiled peanut seed cake (50 g/L)/ KH_2PO_4 (2.0 g/L), $MgSO_4$ (0.1 g/L), NaCl (1.0 g/L) and Tween 80 (1.8 mL/L)			200 rpm	2.85 → 2.45 (g/L)	Change of the nitrogen source: from yeast extract and peptone to deoiled peanut seed cake	
			molasses (50 g/L)	Deoiled peanut seed cake (50 g/L)/ KH_2PO_4 (2.0 g/L), $MgSO_4$ (0.1 g/L), NaCl (1.0 g/L) and Tween 80 (1.8 mL/L)			160 rpm	2.45 → 3.5 (g/L)	Decrease of the agitation speed: from 200 to 160 rpm	
E. ashbyii ATCC 12995	N/M	N/M	glucose (30 g/L)	Ammonium citrate tribasic (3.0 g/L)/ KH_2PO_4 (1.0 g/L), $MgSO_4$ (0.5 g/L), NaCl (0.5 g/L), $CaCl_2$ (0.2 g/L), thiamin hydrochloride (4.0 mg/L) and biotin (40 μg/L)	5.0 controlled at 4.5 during fermentation	28 °C/ 144 h (fed-batch fermentation)	500 rpm	0.08 (g/L) 0.08 → 0.27 (g/L)	- Fermentation carried out at the optimal and constant pH of 4.5	[46]
E. ashbyii UV-18-57	A highly flavinogenic mutant strain derived from *E. ashbyii* DT1 by UV-irradiation	1% (v/v) of the mycelia from GPY broth	glucose (30 g/L)	Yeast extract (2.0 g/L) and peptone (8.0 g/L)/KH_2PO_4 (2.0 g/L), NaCl (1.0 g/L), $MgSO_4 \cdot 7H_2O$ (0.1 g/L) and Tween-80 (1.8 %, v/v)	6.0	30 °C/ 7-8 d	200 rpm	0.132 → 1.315 (g/L)	Possibly because of the high level of sporulation of the mutant *E. ashbyii* UV-18-57, according to previous reports: 1. sporulation has been correlated with riboflavin overproduction；2. nonsporulating *E. ashbyii* produced less riboflavin	[40]
E. ashbyii U_{95-0}	A commercial riboflavin producing strain	Single colony of the strains	glucose (10 g/L)	Asparagine (1.0 g/L)/ KH_2PO_4 (1.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), *DL*-Methionine (20.0 mg/L), L-Histidine hydrochloride (20 mg/L) and inositol (0.1 g/L)	5.8-6.2	28 °C/ 5-7 d	200-250 rpm	3.55 (g/L)	N/I	[47]
E. ashbyii U_{95-1}	A 8-AG (Azaguanine)-insensitive mutant strains derived from *E. ashbyii* U_{95-0} by UV-irradiation							3.55 → 4.1 (g/L)	Partly reduced metabolites inhibition because of their insensitivity to the metabolic analogue 8-AG	

E. ashbyii U_{95-2}	A 8-AG (Azaguanine)-insensitive mutant strains derived from *E. ashbyii* U_{95-0} by UV-irradiation							3.55 → 3.9 (g/L)		
E. ashbyii	N/M	10% (v/v) of a 36-h liquid seed culture	molasses (104.20 g/L)	KH_2PO_4 (2.0 g/L), $MgSO_4$ (1.0 g/L) and NaCl (1.0 g/L)	6.5-7.0	28 °C/ 120 h	180 rpm	0.68 (g/L)	-	[143]
				Corn steep liquor (10 g/L, initially added)/ KH_2PO_4 (2.0 g/L), $MgSO_4$ (1.0 g/L) and NaCl (1.0 g/L)				0.68 → 0.91 (g/L)	Addition of corn steep liquor (optimal concentration of 10 g/L) to the initial fermentation medium as a stimulator	
				Yeast extract (25 g/L, initially added)/ KH_2PO_4 (2.0 g/L), $MgSO_4$ (1.0 g/L) and NaCl (1.0 g/L)				0.68 → 1.32 (g/L)	Addition of yeast extract (optimal concentration of 25 g/L) to the initial fermentation medium as a stimulator	
				Yeast extract (40 g/L in total, 20, 10 and 10 g/L added at 0 h, 24 h and 48 h, respectively)/ KH_2PO_4 (2.0 g/L), $MgSO_4$ (1.0 g/L) and NaCl (1.0 g/L)				0.68 → 1.59 (g/L)	Addition of yeast extract as a stimulator and optimization of the yeast extract feeding strategy	
				Bean oil (3.0-5.0 g/L, added at 0h)/ KH_2PO_4 (2.0 g/L), $MgSO_4$ (1.0 g/L) and NaCl (1.0 g/L)				0.68 → ≈ 0.85 (g/L)	Addition of bean oil (optimal concentration of 3-5 g/L) to the initial fermentation medium as a stimulator	
E. ashbyii	N/M	10% (v/v) of a 36-h liquid seed culture	glucose (30 g/L)	Peptone (10 g/L)/ KH_2PO_4 (2.0 g/L), $MgSO_4$ (1.0 g/L) and NaCl (1.0 g/L) (30 mL/ 250 mL flask)	6.5-7.0	28 °C/ 120 h	180 rpm	0.34 (g/L)	-	[144]
			glucose (50 g/L)	Yeast extract (20 g/L) and corn steep liquor (20 g/L)/ KH_2PO_4 (2.0 g/L), $MgSO_4$ (1.0 g/L) and NaCl (1.0 g/L) (30 mL/ 250 mL flask)				0.34 → 1.15 (g/L)	Optimization of the fermentation medium and conditions	
***Ashbya gossypii* (*A. gossypii*)**										
A. gossypii B2	A mutant strain derived from the wild type strain *A. gossypii* ATCC 10895	3.5% (v/v) of the pre-culture	rapeseed oil (156.5 g/L)	Yeast extract (28.6 g/L)/glycine (9.5 g/L), sodium glutamate-monohydrate (7.4 g/L), L-methionine (1.1 g/L), m-inositol (0.2 g/L), urea (9.0 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), KH_2PO_4 (1.9 g/L) and sodium formate (2.0 g/L, initially added)	7.0	30 °C/ 144 h (under 80% humidity)	200 rpm	≈ 5.2 (g/L)	-	[57]
				Yeast extract (28.6 g/L)/glycine (9.5				5.2 → 7.54	Feeding formate at an early phase (12 h)	

				g/L), sodium glutamate-monohydrate (7.4 g/L), L-methionine (1.1 g/L), m-inositol (0.2 g/L), urea (9.0 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), KH_2PO_4 (1.9 g/L) and sodium formate (2.0 g/L, added after 12 h of cultivation)				(g/L)	of cultivation	
A. gossypii W122032	A mutant strain derived from the wild type strain *A. gossypii* ATCC 10895 by disparity mutagenesis	Cells harvested from the preculture (inoculated by 10% (v/v) of the seed culture)	glucose (50 mM)	Asparagine (1.5 g/L), KH_2PO_4 (0.75 g/L), myo-inositol (0.1 g/L), and mineral ions [$CoCl_2 \cdot 6H_2O$ (4.4 mg/L), $MnCl_2 \cdot 4H_2O$ (18 mg/L), $ZnSO_4 \cdot 7H_2O$ (44 mg/L), $MgSO_4 \cdot 7H_2O$ (10.1 mg/L), $FeCl_3 \cdot 6H_2O$ (27 mg/L) $CaCl_2 \cdot 6H_2O$ (21.9 mg/L) and $CuSO_4 \cdot 5H_2O$ (2.7 mg/L)]	6.8	28 °C/ 10 d	120 rpm	0.01 → 0.10 (g/L)	A higher supply of guanosine triphosphate (GTP) in the mutant strain leading to an improved riboflavin production	[59]
A. gossypii Agura3 (*Agura3*Δ::*lox*P)	A uridine/uracil auxotrophic mutant strain derived from the strain *Agura3GEN3* (*Agura3*Δ::*lox*P-*GEN3*-*lox*P), which was genetically engineered from *A. gossypii* ATCC 10895	10^5 spores	glucose (20 g/L)	Yeast extract (10 g/L), tryptone (10 g/L)/myo-inositol (1.0 g/L)	N/M	30 °C/ 3 d	Solid-state cultivation on *Ashbya* Full Medium (AFM)	1.0 → 7.5 mg/g mycelium	The *de novo* pyrimidine biosynthetic pathway blocked at the orotidine-5'-phosphate decarboxylase (AgUra3P) level, leading to an increased riboflavin production in the mutant strain	[60]
A. gossypii P_{GPD}-*IMPDH* strain	A mutant strain overexpressing AgIMPDH, engineered from the wild type strain *A. gossypii* ATCC 10895	N/M	glucose (10 g/L)	Yeast extract (2.0 g/L) and peptone (20 g/L)/ myo-inositol (0.6 g/L)	6.8	28 °C/ 5 d	150 rpm	0.09 → 0.12 (g/L)	Overexpression of the enzyme guanosine 5'-monophosphate dehydrogenase (*IMPDH*) from *A. gossypii* (AgIMPDH) increased the metabolic flux through the guanine pathway	[41]
A. gossypii A260	A mutant strain overexpressing the *RIB1* gene, engineered from *A. gossypii* ATCC 10895	N/M	glucose (10 g/L)	Yeast extract (2.0 g/L) and peptone (20 g/L)/ myo-inositol (0.6 g/L)	6.8	28 °C/ 7 d	120 rpm	0.11 → 0.23 (g/L)	Overexpression of the *RIB1* gene alone	[42]
A. gossypii A263	A mutant strain overexpressing the *RIB2* gene, engineered from *A. gossypii* ATCC 10895							0.11 → 0.14 (g/L)	Overexpression of the *RIB2* gene alone	
A. gossypii A262	A mutant strain overexpressing the *RIB3* gene, engineered from *A. gossypii* ATCC 10895							0.11 → 0.16 (g/L)	Overexpression of the *RIB3* gene alone	

A. gossypii A273	A mutant strain co-overexpressing the genes *RIB3* and *RIB1*, engineered from *A. gossypii* ATCC 10895							0.11 → 0.26 (g/L)	Combination of the overexpression of both *RIB3* and *RIB1* genes	
A. gossypii A286	A mutant strain co-overexpressing the genes *RIB3*, *RIB1* and *RIB5*, engineered from *A. gossypii* ATCC 10895							0.11 → 0.30 (g/L)	Combination of the overexpression of the genes *RIB3*, *RIB1* and *RIB5*	
A. gossypii A289	A mutant strain co-overexpressing the genes *RIB3*, *RIB1*, *RIB5* and *RIB2*, engineered from *A. gossypii* ATCC 10895							0.11 → 0.31 (g/L)	Combination of the overexpression of the genes *RIB3, RIB1*, *RIB5* and *RIB2*	
A. gossypii A329	A mutant strain co-overexpressing the genes *RIB3*, *RIB1*, *RIB5*, *RIB2* and *RIB7*, engineered from *A. gossypii* ATCC 10895							0.11 → 0.33 (g/L)	Combination of the overexpression of the genes *RIB3, RIB1*, *RIB5*, *RIB2* and *RIB7*	
A. gossypii A307	A *ADE12*-gene-deleted (*ade12Δ*) mutant strain co-overexpressing the genes *RIB3*, *RIB1*, *RIB5*, *RIB2* and *RIB7*, engineered from *A. gossypii* ATCC 10895							0.10 → 0.50 (g/L)	Deletion of the *ADE12* gene and combination of the overexpression of the genes *RIB3, RIB1*, *RIB5*, *RIB2* and *RIB7*	
A. gossypii A330	A mutant strain underexpressing the gene *ADE12* (*pRIB7-ade12*), and co-overexpressing the genes *RIB3*, *RIB1*, *RIB5*, *RIB2* and *RIB7*, engineered from *A. gossypii* ATCC 10895							0.10 → 0.52 (g/L)	Underexpression of the *ADE12* gene and simultaneous co-overexpression of the genes *RIB3, RIB1*, *RIB5*, *RIB2* and *RIB7*	
A. gossypii W122032	A high riboflavin-producing mutant strain derived from the wild type *A. gossypii* ATCC 10895 by disparity mutagenesis	10% (v/v)	rapeseed oil (90.5 g/L)	Corn steep liquor (40.3 g/L), yeast extract (36.1 g/L), and soybean mill (15 g/L)/ KH_2PO_4 (1.5 g/L), glycine (2.0 g/L), alanine (0.2 g/L), glutamic acid (0.47 g/L) and mineral ions [CO^{2+} (2.0 μg/L), Mn^{2+} (5.0 μg/L), Zn^{2+} (10 μg/L) and Mg^{2+} (1.0 μg/L)]	6.8	28 °C/ 9 d (fermentation in a 3-L fermentor)	600 rpm	1.5 → 13.7 (g/L)	Significant overexpression of the genes involved in the riboflavin biosynthetic pathway during both riboflavin production and stationary phases, such as the genes *RIB1* and *RIB3*	[62]

***Clostridium acetobutylicum* (*C. acetobutylicum*)**									
C. acetobutylicum ATCC 824	N/M	5% (v/v) of the seed culture	xylose (230 mM, filter sterilized solution added after autoclave)	Yeast extract (5.0 g/L)/ KH_2PO_4 (0.75 g/L), K_2HPO_4 (0.75 g/L), $MgSO_4{\cdot}7H_2O$ (0.7 g/L), $MnSO_4{\cdot}5H_2O$ (0.017 g/L), $FeSO_4{\cdot}7H_2O$ (0.01 g/L), $(NH_4)_2SO_4$ (2.0 g/L), L-asparagine (2.0 g/L), *p*-aminobenzoic acid (0.004 g/L), $CH_3COONa\ {\cdot}3H_2O$ (30 mM), resazurin (4.0 mL/L), NaCl (1.0 g/L) and NaAc (60 mM)	6.5	37 °C/ 100 h (fermentation in a 3.6-L bioreactor)	100 rpm	0.02 → 0.2 (g/L)	Supplementation of NaAc (60 mM) in the culture medium	[77]
C. acetobutylicum ATCC 824 (pJpGN)	A recombinant strain derived from *C. acetobutylicum* ATCC 824, which contains the plasmid pJpGN (constructed by inserting P_{ptb}-*ribGBAH*-original into the shuttle vector pJIR750)	An appropriate amount from the seed cultures OD_{600} = 0.1	*Clostridium* growth medium (CGM) with thiamphenicol (25 mg/L)		N/M	37 °C/ 3 d	Static culture	Undetectable amount → 0.027 (g/L)	Overexpression of riboflavin biosynthetic genes *ribGBAH* from *C. acetobutylicum*	[76]
			Clostridium growth medium (CGM) with thiamphenicol (25 mg/L) and MES (50 mM)		5.5			0.027 → 0.07 (g/L)	Addition of 50 mM MES by setting the medium pH to 5.5
***Escherichia coli* (*E. coli*)**									
E. coli MG1655	N/M	N/M	glucose (10 g/L)	$(NH_4)_2HPO_4$ (19.92 mmol/L), $NH_4H_2PO_4$ (7.56 mmo/L), KCl (2.0 mmo/L), $MgSO_4{\cdot}7H_2O$ (1.5 mmo/L), betaine·KCl (1.0 mmo/L), $FeCl_3{\cdot}6H_2O$ (8.88 mmo/L), $CoCl_2{\cdot}6H_2O$ (1.26 mmo/L), $CuCl_2{\cdot}2H_2O$ (0.88 mmo/L), $ZnCl_2$ (2.2 mmo/L), $Na_2MoO_4{\cdot}_7H_2O$ (1.24 mmo/L), H_3BO_3 (1.21 mmo/L) and $MnCl_2{\cdot}4H_2O_2$ (2.5 mmo/L)	N/M	(N/M)/ 65 h	N/M	≈ 0.017 (g/L)	-	[86]
E. coli BL21(DE3)	N/M							≈ 0.085 (g/L)	Compared to *E. coli* MG1655, the increase in riboflavin production most probably because of an up-regulation of FAD synthesis pathway and the reduced RibF enzyme activity (caused by a site mutation on the 115 residue of RibF)
E. coli RF18S	A recombinant riboflavin-producing strain engineered from the wild type *E. coli* K-12 MG1655	An appropriate amount of the secondary seed cultures OD_{600} = 0.01	glucose (10 g/L)	Yeast extract (5.0 g/L) and tryptone (10 g/L)/ NaCl (10 g/L)	N/M	37 °C/ 30 h	250 rpm	0.39 (g/L)	Combination of the overexpression of *ribB* gene and the genes involved in *de novo* purine biosynthetic pathway (*ndk*, *gmk*, *purA*, *purF* and *prs*)	[79]
E. coli LS02T	A recombinant riboflavin-producing	1% (v/v)	glucose (10 g/L)	yeast extract (5.0 g/L)/ Na_2HPO_4 (3.8	N/M	37 °C/ 23 h	N/M	0.67 (g/L)	-	[89]

	strain engineered from the wild type *E. coli* K-12 MG1655, by deleting the genes *pfkA*, *edd* and *eda*, and introducing a riboflavin operon expression plasmid (pLS01)	of the seed culture		g/L), KH_2PO_4 (1.5 g/L), $(NH_4)_2SO_4$ (1.0 g/L), $MgSO_4$ (0.2 g/L) and trace element solution (2%, v/v)					
		10% (v/v) of the seed culture	glucose (10 g/L)	yeast extract (5.0 g/L)/ Na_2HPO_4 (3.8 g/L), KH_2PO_4 (1.5 g/L), $(NH_4)_2SO_4$ (1.0 g/L), $MgSO_4$ (0.2 g/L) and trace element solution (2%, v/v)	Controlled at 7.0 during fermentation	37 °C/ 71 h (fed-batch fermentation)	N/M	10.4 (g/L)		
E. coli RF05S	A recombinant strain engineered from the wild type *E. coli* K-12 MG1655, with an enhancement of the flux from 6-phophogluconate to Ru-5-P by deleting the genes *pgi*, *edd* and *eda*, and overexpressing the *acs* gene (encoding acetyl-CoA synthetase)	1% (v/v) of the seed culture	glucose (10 g/L)	Yeast extract (5.0 g/L) and tryptone (10 g/L)/ NaCl (10 g/L)	N/M	37 °C/ 48 h	220 rpm	0.59 (g/L)	-	[78]
E. coli RF05S-M40	A mutant strain of *E. coli* RF05S, obtained by fine-tuning the native ribosome-binding site (RBS) of *ribF* on the chromosome of RF05S	1% (v/v) of the seed culture	glucose (10 g/L)	Yeast extract (5.0 g/L) and tryptone (10 g/L)/ NaCl (10 g/L)	N/M	37 °C/ 48 h	220 rpm	0.59 → 1.04 (g/L)	Increased riboflavin production by the decrease in the expression level of flavokinase (encoded by the gene *ribF*), caused by modulating the RBS of *ribF*	
				Yeast extract (5 g/L) / NaCl (10 g/L)		31 °C/ 55 h		1.04 → 1.51 (g/L)	Change of the fermentation temperature from 37 °C to 31 °C	
				Non-optimized MS medium: yeast extract (5.0 g/L)/ Na_2HPO_4 (3.8 g/L), KH_2PO_4 (1.5 g/L), $(NH_4)_2SO_4$ (1.0 g/L), $MgSO_4$ (0.2 g/L) and trace element solution (2%, v/v)		31 °C/ 55 h		1.2 (g/L)	-	
				Optimized MS medium		31 °C/ 60 h		1.2 → 2.7 (g/L)	Optimization of the fermentation medium	
***Bacillus subtilis* (*B. subtilis*)**										
B. subtilis BS77	A recombinant strain derived from the wild type *B. subtilis* 168, which contains mutations in regulatory gene *ribC* and RFN regulatory element *ribO*	2% (v/v) of the pre-culture	glucose (100 g/L)	Yeast power (20 g/L)/ KH_2PO_4 (0.5 g/L), K_2HPO_4 (0.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L)	N/M	41 °C/ 72 h	240 rpm	0.21 (g/L)	-	[102]
B. subtilis BS89	A recombinant strain derived from *B. subtilis* BS77, by inserting the P_{43} promoter (derived from *B. subtilis* 168)							0.21 → 0.51 (g/L)	Overexpression of the gene *ribA* leading to an increase in the metabolic flux of rate-limiting step	

	to upstream of the gene *ribA* in *B. subtilis* BS77			
B. subtilis BS93	A recombinant strain derived from *B. subtilis* BS89, by replacing the native promoter *ribP1* and *ribO* in *B. subtilis* BS89 with the P_{43} promoter		0.51 → 0.34 (g/L)	Decrease in the relative transcription levels of the genes *ribG* and *ribA*, probably caused by the disruption of the gene *ribO*
B. subtilis BS102	A recombinant strain derived from *B. subtilis* BS89, by deleting the gene *purR* (encoding *pur* operon repressor PurR) in *B. subtilis* BS89		0.51 → 0.53 (g/L)	-
B. subtilis BS103 (-10*)	A recombinant strain derived from *B. subtilis* BS102, by replacing the original -10 sequence (TAAGAT) of *Ppur* in *B. subtilis* BS102 with frequently used -10 sequence (TATAAT)		0.53 → 0.51 (g/L)	-
B. subtilis BS104 (Δ*att*)	A recombinant strain derived from *B. subtilis* BS102, by deleting a 75-bp region (defined as *att*, corresponding to the attenuator region in 5'-UTP) in *B. subtilis* BS102		0.53 → 0.63 (g/L)	*att* deletion leading to a decrease in the relative transcription levels of the genes *guaC* and *purA*, which was in favor of riboflavin accumulation
B. subtilis BS107 (-10*, Δ*att*)	A recombinant strain derived from *B. subtilis* BS103, by deleting a 75-bp region (defined as *att*, corresponding to the attenuator region in 5'-UTP) in *B. subtilis* BS103		0.51 → 0.58 (g/L)	
B. subtilis BS106	A recombinant strain derived from *B. subtilis* BS104, by inserting the P_{43} promoter to upstream of the *purF* in *B. subtilis* BS104		0.63 → 0.69 (g/L)	Overexpression of the gene *purF*
B. subtilis BS110	A recombinant strain with *purF-VQW* mutation module, generated by introducing the three residue mutations (D293V, K316Q and S400W) into *B.*	41 °C/ 96 h	0.69 → 0.83 (g/L)	It was suggested that the insertion of *purF-VQW* mutation led to an increase in the concentrations of GMP, GDP and GTP, which was in favor of the

	subtilis BS106							production of riboflavin	
B. subtilis BS111	A recombinant strain derived from *B. subtilis* BS106, by replacing the wild-type gene *purF* in *B. subtilis* BS106 with mutated gene *purF* from *E. coli* (containing mutations K326Q and P410W)					41 °C/ 72 h	0.69 → 0.64 (g/L)	-	
B. subtilis RF1	A recombinant *B. subtilis* strain with deregulated riboflavin biosynthetic pathway, in which the native chromosomal riboflavin operon is replaced by a copy of the constitutively expressed recombinant *B. subtilis* riboflavin operon containing an additional chloramphenicol resistance marker	Overall of an 15-h seed culture	glucose·H_2O (initial concentration 20 g/L, maintained constantly at 10-15 g/L during fermentation)	Yeast extract (20 g/L)/ KH_2PO_4 (5.0 g/L), $(NH_4)_2SO_4$ (6.0 g/L), $MgSO_4{\cdot}7H_2O$ (1.5 g/L), $ZnSO_4{\cdot}7H_2O$ (0.03 g/L), $MnCl_2{\cdot}4H_2O$ (0.05 g/L), $FeSO_4{\cdot}7H_2O$ (0.02 g/L) and antifoaming agent (0.3 g/L)	Controlled at 6.9 during fermentation	40 °C/48 h (fed-batch fermentation)	500 rpm 600 rpm 700 rpm 800 rpm 900 rpm 600 rpm for 26 h, then 900 rpm for 22 h 600 rpm for 26 h, then 700 rpm for 5 h, 800 rpm for 5 h, and finally 900 rpm for 12 h	5.3 (g/L) 7.2 (g/L) 7.2 (g/L) 7.3 (g/L) 7.8 (g/L) 8.7 (g/L) 9.4 (g/L)	Optimization of the agitation speed	[114]
B. subtilis RH33	A recombinant strain resistant to 8-azaguanine (8-AG), decoyinine and roseoflavin, which has multiple copies of deregulated rib operons in the chromosome	2% (v/v) of the pre-culture	glucose (100 g/L)	Yeast power (20 g/L)/ $MgSO_4{\cdot}7H_2O$ (0.5 g/L), KH_2PO_4 (0.5 g/L) and K_2HPO_4 (0.5 g/L)	N/M	41 °C/ 72 h	250 rpm	4.0 (g/L)	-	[94]
B. subtilis TPA	A recombinant strain derived from *B. subtilis* RH33, by individually							4.0 → 3.8 (g/L)	-	

	integrating the plasmid pUC18-TPA into the chromosome of *B. subtilis* RH33								
B. subtilis NPB	A recombinant strain derived from *B. subtilis* RH33, by individually integrating the plasmid pUC18-NPB into the chromosome of *B. subtilis* RH33							4.0 → 4.6 (g/L)	Overexpression of the gene *gapB*	
B. subtilis SPF	A recombinant strain derived from *B. subtilis* RH33, by individually integrating the plasmid pUC18-SPF into the chromosome of *B. subtilis* RH33							4.0 → 4.7 (g/L)	Overexpression of the gene *fbp*	
B. subtilis PFB	A recombinant strain derived from *B. subtilis* RH33, by individually integrating the plasmid pUC18-PFB into the chromosome of *B. subtilis* RH33	2% (v/v) of the pre-culture	glucose (100 g/L)	Yeast power (20 g/L)/ $MgSO_4·7H_2O$ (0.5 g/L), KH_2PO_4 (0.5 g/L) and K_2HPO_4 (0.5 g/L)	N/M	41 °C/ 72 h	250 rpm	4.0 → 4.9 (g/L)	Co-overexpression of the genes *fbp* and *gapB*	
		N/M	glucose (20 g/L)	Yeast extract (5 g/L)/ $NaNO_3$ (10 g/L), NH_4NO_3 (5.0 g/L), KH_2PO_4 (4.0 g/L), K_2HPO_4 (7.5 g/L), $MgSO_4·7H_2O$ (3.0 g/L), $FeCl_2$ (0.02 g/L), $MnCl_2·4H_2O$ (0.02 g/L) and $ZnCl_2·7H_2O$ (0.02 g/L)		41 °C/2 d (fed-batch fermentation)	N/M	13.4 (g/L)	-	
B. subtilis PAB	A recombinant strain co-overexpressing the genes *pckA* and *gapB*, which was derived from *B. subtilis* TPA by introducing the plasmid pUC18-NPB into the genome of *B. subtilis* TPA	2% (v/v) of the pre-culture	glucose (100 g/L)	Yeast power (20 g/L)/ $MgSO_4·7H_2O$ (0.5 g/L), KH_2PO_4 (0.5 g/L) and K_2HPO_4 (0.5 g/L)	N/M	41 °C/ 72 h	250 rpm	3.8 → 4.5 (g/L)	Co-overexpression of the genes *pckA* and *gapB*	
B. subtilis PFBA	A recombinant strain co-overexpressing the genes *pckA*, *gapB* and *fbp*, which was derived from *B. subtilis* TPA by introducing the plasmid pUC18-PFB into the genome of *B. subtilis* TPA							3.8 → 4.8 (g/L)	Co-overexpression of the genes *pckA*, *fbp* and *gapB*	
B. subtilis SVZ	A recombinant strain derived from *B.*	2% (v/v)	glucose (100 g/L)	Yeast power (20 g/L)/ $MgSO_4·7H_2O$ (0.5	N/M	41 °C/ 72 h	250 rpm	+ 18%	Overexpression of the genes *zwf243*	[101]

	subtilis RH33, by individually integrating the plasmid pUC18-SVZ into the chromosome of *B. subtilis* RH33	of the pre-culture		g/L), KH_2PO_4 (0.5 g/L) and K_2HPO_4 (0.5 g/L)				(compared to the parent strain)		
B. subtilis SVG	A recombinant strain derived from *B. subtilis* RH33, by individually integrating the plasmid pUC18-SVG into the chromosome of *B. subtilis* RH33							+ 22% (compared to the parent strain)	Overexpression of the genes *gnd361*	
B. subtilis VGZ	A recombinant strain resistant to kanamycin and spectinomycin, which co-overexpresses the genes *zwf243* and *gnd361* in *B. subtilis* RH33	2% (v/v) of the pre-culture	glucose (100 g/L)	Yeast power (20 g/L)/ $MgSO_4·7H_2O$ (0.5 g/L), KH_2PO_4 (0.5 g/L) and K_2HPO_4 (0.5 g/L)	N/M	41 °C/ 72 h	250 rpm	+ 31% (compared to the parent strain)	Co-overexpression of the genes *zwf243* and *gnd361*	
		6% (v/v) of the seed culture	glucose (initial concentration 20 g/L, maintained at 5.0 g/L after the first 10 h)	Yeast extract (5 g/L)/ $NaNO_3$ (10 g/L), NH_4NO_3 (5.0 g/L), KH_2PO_4 (4.0 g/L), K_2HPO_4 (7.5 g/L), $MgSO_4·7H_2O$ (3.0 g/L), $FeCl_2$ (0.02 g/L), $MnCl_2·4H_2O$ (0.02 g/L) and $ZnCl_2·7H_2O$ (0.02 g/L)	Controlled at 6.8 during fermentation	41 °C/ 50 h (fed-batch fermentation)	900 rpm	15.7 (g/L)		
B. subtilis	N/M	6% (v/v) of the seed culture	glucose (80 g/L)	Yeast extract (2.0 g/L), corn steep liquor (16 g/L) and soybean cake powder (10 g/L)/ NH_4Cl (3.0 g/L), $MgSO_4$ (0.5 g/L), K_2HPO4 (1.4 g/L), KH_2PO4 (0.6 g/L), calcium gluconate (7.5 g/L, added at 0 h), sodium citrate (5.0 g/L, added at 0 h) and alanine (1.5 g/L, added at 18 h)	7.0-7.1	40 °C/ 48 h	220 rpm	4.6 → 6.46 (g/L)	Addition of three exogenous additives (calcium gluconate, sodium citrate and alanine)	[115]
B. subtilis LXZ-1	A recombinant, riboflavin-producing *B. subtilis* strain	3% (v/v) of the seed culture	sucrose (80 g/L)	Yeast power (30 g/L)/ MgSO4·7H2O (0.5 g/L) and urea (2.0 g/L)	7.2	37 °C/ 60 h	220 rpm	0.24 (g/L)	-	[105]
B. subtilis LXZ-2	A recombinant strain derived from *B. subtilis* LXZ-1, which overexpresses the gene *ribA*							0.24 → 0.47 (g/L)	Overexpression of the gene *ribA*	
B. subtilis LXZ-3	A recombinant strain derived from *B. subtilis* LXZ-1, which co-overexpresses the genes *ribA* and *ribH*							0.24 → 0.9 (g/L)	Overexpression of the genes *ribA* and *ribH*	

B. subtilis LXZ-3/pMX45	A recombinant, riboflavin-producing strain derived from *B. subtilis* LXZ-3, by transforming a riboflavin operon expression plasmid pMX45 (with a low copy number) into *B. subtilis* LXZ-3	3% (v/v) of the seed culture	sucrose (15 g/L) + xylose (65 g/L) (80 g/L in total)	Yeast power (30 g/L)/ $MgSO_4 \cdot 7H_2O$ (0.5 g/L) and urea (2.0 g/L)	7.2	37 °C/ 60 h	220 rpm	0.9 → 1.6 (g/L)	Change of the carbon source from sucrose to a mixture of sucrose and xylose	
		5% (v/v) of the seed culture	sucrose (15 g/L) + xylose (65 g/L) (80 g/L in total)	Yeast power (30 g/L)/ $MgSO_4 \cdot 7H_2O$ (0.5 g/L) and urea (2.0 g/L)	6.5	37 °C/ 70 h (fed-batch fermentation)	500 rpm	3.6 (g/L)	-	
B. subtilis RF1/pMA5-*sat*	A recombinant, riboflavin-producing strain with resistance to norseothricin (NTC), generated by transforming a recombinant plasmid pMA5-*sat* into *B. subtilis* RF1	N/M	glucose (initial concentration 40 g/L, maintained at 10-15 g/L during fermentation process)	Yeast power (10 g/L)/ trisodium citrate dihydrate (0.11 g/L), $(NH_4)_2HPO_4$ (6.0 g/L), KH_2PO_4 (5.0 g/L), $MgSO_4 \cdot 7H_2O$ (1.5 g/L), $ZnSO_4 \cdot 7H_2O$ (0.03 g/L), $MnCl_2 \cdot 4H_2O$ (0.05 g/L) and $FeSO_4 \cdot 7H_2O$ (0.02 g/L)	Controlled at 6.8	40 °C/ 60 h (fed-batch fermentation)	600 rpm	9.2 → 12 (g/L)	It is suggested that the overexpression of the gene *zwf* (encoding G-6-P dehydrogenase) improved the flux through the pentose phosphate pathway, finally leading to an increase in riboflavin production	[106]
B. subtilis PYZ	A recombinant strain containing an additional copy of the gene *zwf* (encoding G-6-P dehydrogenase), which was generated by introducing the additional *zwf* gene into the *zwf* locus of *B. subtilis* PY	cells harvested from the preculture (inoculated by 1% (v/v) of a 12-h seed culture)	glucose (20 g/L)	$(NH_4)_2SO_4$ (2.0 g/L), $K_2HPO_4 \cdot 2H_2O$ (14 g/L), KH_2PO_4 (6.0 g/L), casein hydrolysate (0.05 g/L), $MgSO_4 \cdot 7H_2O$ (0.02 g/L), $ZnCl_2$ (0.03 g/L), $MnCl_2 \cdot 4H_2O$ (0.04 g/L) and $FeCl_2 \cdot 6H_2O$ (0.02 g/L)	N/M	37 °C/ (N/M)	200 rpm	+ 25% ± 2 (compared to the parent strain)	Overexpression of the gene *zwf* improved the flux of the pentose phosphate pathway, finally leading to an increase in riboflavin production	[98]
B. subtilis RH44	A recombinant, riboflavin-producing strain constructed by increasing both the dosage and expression levels of the riboflavin operon in *B. subtilis* RH13	N/M	glucose (80 g/L)	K_2HPO4 (1.0 g/L), KH_2PO_4 (1.0 g/L) and $MgSO_4 \cdot 7H_2O$ (1.0 g/L)	7.2	41 °C/ 60 h	240 rpm	3.77 (g/L)	-	[108]
			glucose (80 g/L)	$NaNO_3$ (5.0 g/L)/ K_2HPO4 (1.0 g/L), KH_2PO_4 (1.0 g/L) and $MgSO_4 \cdot 7H_2O$ (1.0 g/L)				3.77 → 4.22 (g/L)	Addition of $NaNO_3$ (5.0 g/L) as an inorganic nitrogen source	
			glucose (80 g/L)	$NaNO_3$ (5.0 g/L) and NH_4NO_3 (5.0 g/L)/ K_2HPO4 (1.0 g/L), KH_2PO_4 (1.0 g/L) and $MgSO_4 \cdot 7H_2O$ (1.0 g/L)				3.77 → 4.61 (g/L)	Addition of both $NaNO_3$ (5.0 g/L) and NH_4NO_3 (5.0 g/L) as inorganic nitrogen sources	
			glucose (110.8 g/L)	yeast extract (10 g/L), $NaNO_3$ (14.9 g/L) and NH_4NO_3 (5.0 g/L)/ $MgSO_4 \cdot 7H_2O$	7.2	41 °C/ 60 h	240 rpm	4.61 → 6.65 (g/L)	Optimization of the fermentation medium using statistical designs	
		6% (v/v) of the seed culture	glucose (initial concentration 20 g/L, maintained constantly at 5-10	(3.0 g/L), K_2HPO_4 (5.2 g/L), KH_2PO_4 (1.0 g/L), sodium citrate (0.1 g/L), $ZnSO_4$ (0.03 g/L), $MnCl_2$ (0.05 g/L) and $FeCl_2$ (0.02 g/L)	Controlled at 7.2	41 °C/ 48 h (fed-batch fermentation)	850 rpm	16.4 (g/L)	-	

			g/L after a 6-h cell growth)						
B. subtilis ATCC 6051	N/M	5% (v/v) of the pre-culture	glucose (40 g/L) fructose (40 g/L) maltose (40 g/L) arabinose (40 g/L) fructose (38.1 g/L)	yeast extract (10 g/L) yeast extract (4.37 g/L)/ $MgSO_4$ (0.85 g/L), K_2HPO_4 (2.27 g/L) and $FeSO_4$ (0.02 g/L)	N/M	30 °C/ 72 h	200 rpm	0.002 (g/L) 0.004 (g/L) 0.003 (g/L) 0.001 (g/L) 0.012 (g/L)	Optimization of the fermentation medium using statistical designs	[116]
***Lactococcus lactis* (*L. lactis*)**									
L. lactis NZ9000 (pNZA)	A nisin-induced riboflavin producer strain, which is the wild type strain *L. lactis* NZ9000 containing the plasmid pNZA, overexpressing the gene *ribA*	1% (v/v) of an overnight pre-culture	lactose (5.0 g/L)	K_2HPO_4 (2.5 g/L), KH_2PO_4 (2.5 g/L), $(NH_4)_3$-citrate (0.6 g/L), Na-acetate (1.0 g/L), cysteine-HCl (0.25 g/L), casein hydrolysate (5.0 g/L, salt- and vitamin-free), vitamin solution (10 mL/L, containing no riboflavin and folic acid), metal solution (10 mL/L) and chloramphenicol (5.0 mg/L)	N/M	30 °C/ 3 h (after nisin induction)	Incubated statically	$0 \rightarrow < 1.0 \times 10^{-5}$ (g/L)	Overexpression of the intact *ribA* gene	[138]
L. lactis NZ9000 (pNZB)	A nisin-induced riboflavin producer strain, which is the wild type strain *L. lactis* NZ9000 containing the plasmid pNZA, overexpressing the gene *ribB*							0	-	
L. lactis NZ9000 (pNZBA)	A nisin-induced riboflavin producer strain, which is the wild type strain *L. lactis* NZ9000 containing the plasmid pNZBA, co-overexpressing the genes *ribB* and *ribA*							$0 \rightarrow < 1.0 \times 10^{-5}$ (g/L)	Co-overexpression of the genes *ribB* and *ribA*	
L. lactis NZ9000 (pNZBAH)	A nisin-induced riboflavin producer strain, which is the wild type strain *L. lactis* NZ9000 containing the plasmid pNZBAH, co-overexpressing the genes *ribB ribA* and *ribH*							$0 \rightarrow < 1.0 \times 10^{-5}$ (g/L)	Co-overexpression of the genes *ribB*, *ribA* and *ribH*	
L. lactis NZ9000 (pNZGBA)	A nisin-induced riboflavin producer strain, which is the wild type strain *L. lactis* NZ9000 containing the plasmid pNZGBA, co-overexpressing the genes *ribG ribB* and *ribA*							$0 \rightarrow 1.8 \times 10^{-4}$ (g/L)	Co-overexpression of the genes *ribG*, *ribB* and *ribA*	
L. lactis NZ9000	A nisin-induced riboflavin producer							$0 \rightarrow 0.02$ (g/L)	Co-overexpression of the genes *ribG*,	

(pNZGBAH)	strain, which is the wild type strain *L. lactis* NZ9000 containing the plasmid pNZGBAH, co-overexpressing the genes *ribG ribB*, *ribA* and *ribH*								*ribB*, rib*A* and *ribH*	
***Lactobacillus fermentum* (*L. fermentum*)**										
L. fermentum PBCC11.5	A spontaneous roseoflavin-resistant mutant of the wild type *L. fermentum* PBCC11	N/M	Modified chemical defined medium (CDM) containing no riboflavin, and glucose (10 g/L) used as the sole carbon source		6.2	30 °C/until OD_{600} = 2.5 (stationary phase)	Incubated statically	$0 \rightarrow 1.2 \times 10^{-3}$ (g/L)	An A-to-G substitution at position 240 in the RFN regulatory element	[135]
***Lactobacillus plantarum* (*L. plantarum*)**										
L. plantarum CRL 725 (G)	A spontaneous roseoflavin-resistant mutant of the wild type *L. plantarum* CRL 725	N/M	A commercial riboflavin-free medium (riboflavin assay medium, Difco, Becton, Dickinson, and CO., Sparks, Maryland)		N/M	30 °C/ 16 h	Incubated statically	$0.3 \times 10^{-3} \rightarrow 1.1 \times 10^{-3}$ (g/L)	N/M	[132]
***Leuconostoc mesenteroides* (*Lc. mesenteroides*)**										
Lc. mesenteroides CB207	A spontaneous roseoflavin-resistant mutant of the wild type *Lc. mesenteroides* NCDO2028	N/M	lactose (5.0 g/L)	K_2HPO_4 (2.5 g/L), KH_2PO_4 (2.5 g/L), $(NH_4)_3$-citrate (0.6 g/L), Na-acetate (1.0 g/L), cysteine-HCl (0.25 g/L), casein hydrolysate (5.0 g/L, salt- and vitamin-free), vitamin solution (10 mL/L, containing no riboflavin and folic acid) and metal solution (10 mL/L)	N/M	30 °C/ until the cells entered stationary phase	Incubated statically	$0 \rightarrow 0.5 \times 10^{-3}$ (g/L)	A G-to-C substitution at position 77 within the *rib* operon leader region	[129]

887 Temp.: temperature; N/M, not mentioned; GPY broth, glucose-peptone-yeast extract broth.

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889 **Table 2** Comparison of different riboflavin-producing microorganisms

Strain		Current status	Main method used for advanced riboflavin production	Advantage of the strain	Disadvantage of the strain
Fungi	*E. ashbyii*	1) In large-scale: this stain was previously used for industrial production in USA and Pinsk (Belarus), which has been replaced (by *A. gossypii*) because of its relatively low productivity (yield well below 4.0 g/L) and high genetic instability; 2) In lab-scale: an 8-AG-insensitive mutant strain with 2-4 fold increase in riboflavin production (4.1 g/L) has been isolated	1) Optimization of media and cultivation conditions; 2) Mutagenesis based on UV-irradiation	1) A natural riboflavin overproducer, for which iron inhibition is not a factor; 2) In addition to riboflavin, this strain can produce small amounts of FAD which has pharmaceutical and nutraceutical applications, so it still possesses its own biotechnological potential	1) Low productivity and high genetic instability; 2) Instable in storage, for example during lyophilization or storage in slant at room temperature; 3) No tools for molecular research on this strain have been reported
	C. famata	1) In large-scale: only the strain *C. famata* dep8 (ATCC 20849) was previously used in industrial production, which has not been used at industrial scale in recent years, due to the instability (at that time) of the strain dep8; 2) In lab-scale: *C. famata* with tubercidine- and iron-resistance has been developed and the yield of riboflavin has reached 20-30 g/L	1) Optimization of cultivation conditions; 2) Introduction of several unidentified mutations that lead to resistance against different toxic agents; 3) Increase in the copy number of transcription factor gene *SEF1* and *IMH3* (encoding IMP dehydrogenase) from *D. hansenii*, along with the genes involved in purine nucleotide interconversion and the riboflavin biosynthesis pathway	A natural overproducer of riboflavin, and as a unicellular yeast, it is easier to distribute in a large-scale fermentor than a filamentous fungus growing in mycelial pellets	1) Inhibition due to iron should be overcome to achieve higher levels of riboflavin production using this yeast, and extra effort is required to keep the concentration of iron in the substrate below 15 μM, in order to avoid its negative effect; 2) The industrial strain *C. famata* dep8 is rather unstable, reverting to totally non-flavinogenic variants, and cannot use ethanol as the sole carbon source
	A. gossypii	About 30% of the world's industrial riboflavin output is produced by direct fermentation with this fungus, which has been reported to give riboflavin yields in the range of 15-20 g/L	1) Optimization of fermentation conditions favoring riboflavin over-synthesis; 2) Increase in the riboflavin pathway supply with purine precursors; 3) Activation of the glyoxylic acid cycle which improves catabolism of oil (preferred carbon source for riboflavin biosynthesis) 4) Disparity mutagenesis	1) A strong natural riboflavin overproducer, whose growth is not linked to riboflavin production, so there is no risk of selecting non-producing mutants; 2) Iron inhibition is not a factor for this fungus; 3) A good riboflavin production on plant oil as carbon source	The growth phase of this fungus is separated from its riboflavin production phase.
Bacteria	*B. subtilis*	Widely adopted in the commercial production of riboflavin	1) Impairment of regulation of the riboflavin operon;	1) The fastest-growing organism; 2) Easy to isolate riboflavin producing	1) Wild-type strains of *B. sutilis* are not natural riboflavin producers, the deregulation of purine synthesis and a mutation in flavokinase/FAD synthase

		2) Amplification of the copy number of the structural gene; 3) Genetic manipulation of central carbon metabolism	mutants; 3) Has been granted QPS (Qualified Presumption of Safety) status by EFSA (European Food Safety Authority) for certain applications in the production of foods and supplements that are consumed by humans	are required to obtain riboflavin production using this bacterium; 2) Also inhibited by iron like the yeast *C. famata*; 3) Riboflavin production by this bacterium is linked to its growth, bearing the risk of selecting non-producing mutants; 4) Very sensitive to high temperature; 5) Riboflavin fermentation using this bacterium suffered the increased osmotic stress due to the accumulation of extracellular riboflavin
C. acetobutylicum	Only in lab-scale as riboflavin producer, which produces 0.2 g/L of riboflavin as a by-product in ABE (acetone, butanol and ethanol) fermentation process	1) Optimization of culture conditions; 2) Genetic improvement, including over-expression of the *rib* operon, and genes involved in the purine pathway of this bacterium, like *purF* gene, which encodes the rate-limiting enzyme PRPP amidotransferase	This bacterium was also recognized as a natural riboflavin producer and was one of the earliest organisms used to produce riboflavin. It provides a novel strategy for the co-production of riboflavin with biobutanol, and if riboflavin could reach 0.5-1 g/L in ABE fermentation on a large-scale, the commercial value of ABE fermentation process will be significantly improved	After decades of work, the yield of riboflavin remains insufficient in this bacterium
E. coli	Only in lab-scale: the engineered strain *E. coli* LS02T could accumulate 10.4 g/L riboflavin in fed-batch fermentation	Many metabolic engineering strategies were carried out in a series of studies to develop this bacterium as a riboflavin-producing strain	1) Has been long used as a common host for efficient production of various substances; 2) This bacterium may be an efficient host to produce riboflavin because of its clear genetic background, fast-growing, low maintenance metabolism and the presence of mature molecular tools suitable for its genetic manipulation; 3) The engineered strain *E. coli* BL21(DE3) was found to be able to accumulate riboflavin under culture conditions	1) Wild-type *E. coli* cannot accumulate riboflavin under natural conditions, which needs to be further developed into riboflavin-producer by metabolic engineering; 2) The expression of the *rib* operon in engineered *E. coli* strain needs to be induced by IPTG, which is unfavorable for industrial processes; 3) Extra glycine may be needed in the cultivation medium, which increases the producing cost to some extent 4) *rib* operon expression plasmid with higher stability is needed
LAB	Only in lab-scale: their riboflavin production was several thousandfold lower than that of industrial riboflavin producers	Selection of roseoflavin-resistant mutants was an efficient approach for the isolation of riboflavin-overproducing strains of several LAB species, including *L. lactis*, *L. plantarum*, *L. fermentum* and *L. mesenteroides*	Ideal candidates for riboflavin production in foods, due to their adaptability to industrial food fermentation and riboflavin production ability, opening new opportunities for developing novel functional foods with enhanced riboflavin content	1) LAB have fastidious nutritional requirements, which need complex nutritional media for normal growth and metabolic activities, and these fastidious nutritional requirements may also limit the ability to optimize and control the metabolic activities of LAB; 2) From a consumer or regulatory point of view, genetic engineering is hard to be accepted, and the genetically engineered strains of LAB so far still cannot be applied in human food fermentation

Acknowledgements

This work was supported by China Central Public-Interest Scientific Institution Basal Research Fund, Chinese Academy of Agricultural Sciences, China (Y2020XK05), and the Shanghai Pujiang Talent Plan, Shanghai, China (18PJ1404600).

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.

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Declaration of Interest Statement

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

Figures

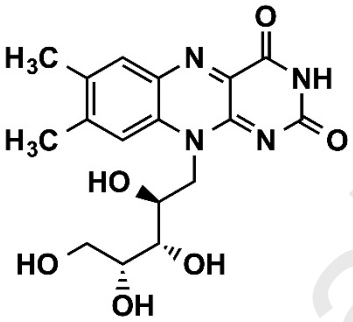

Fig. 1. The chemical structure of riboflavin.

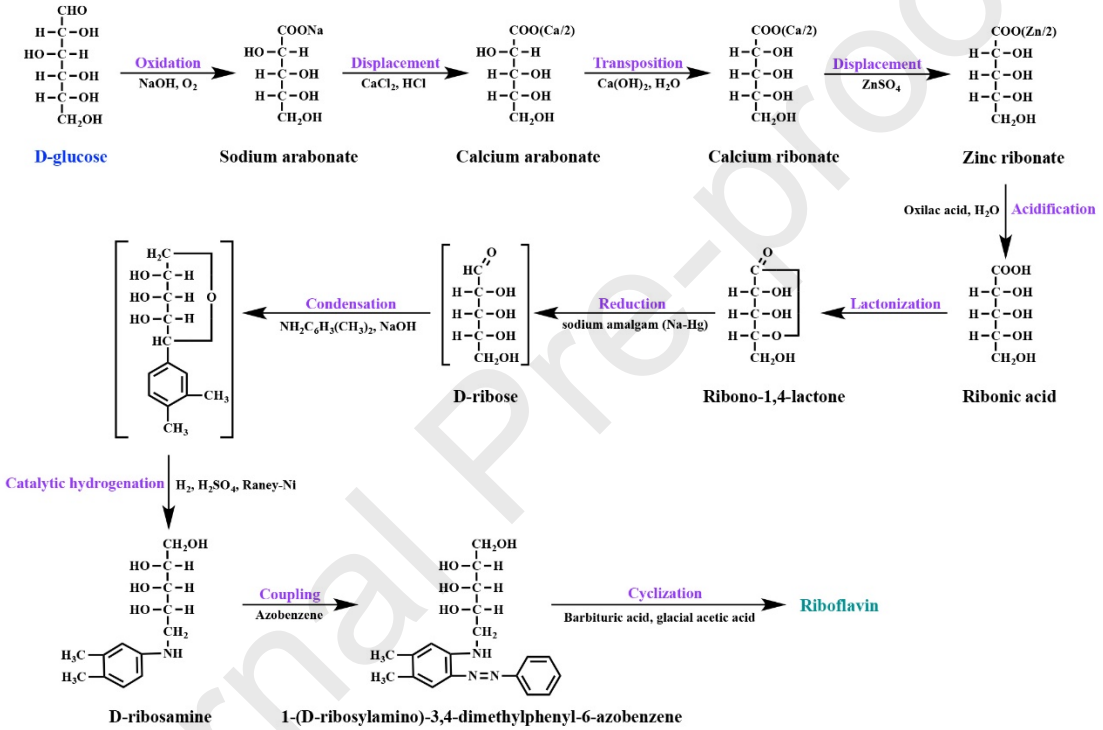

Fig. 2. The total chemical synthetic process of riboflavin.

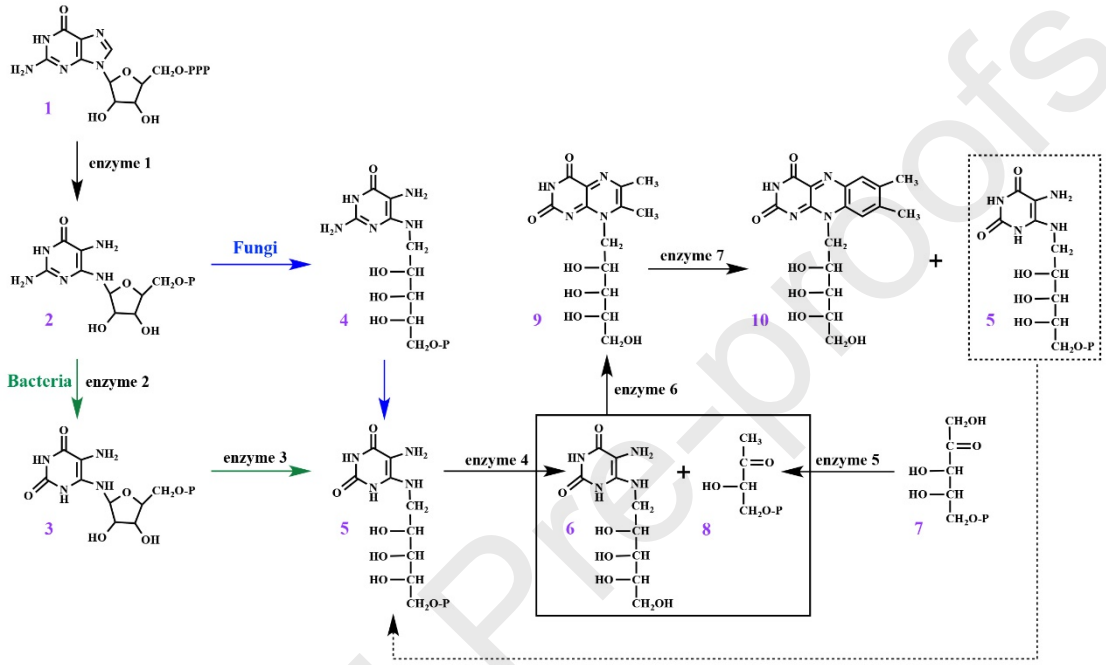

Fig. 3. The biosynthetic pathway of riboflavin adapted from Bacher et al. [31].

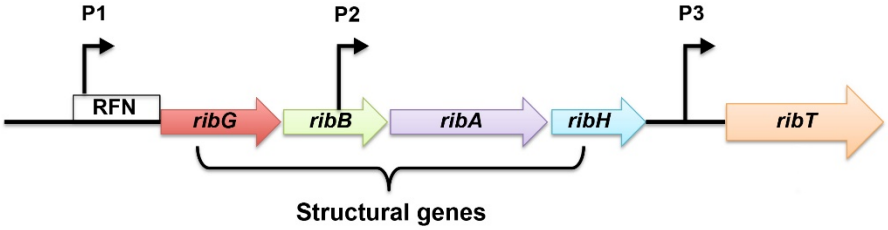

Fig. 4. Structure of the *rib* operon in bacteria.