

Institute of Cell Biology
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UDC: 576.343:582.282.232

Ph.D. dissertation

**NEW FINDINGS ON THE ROLE OF *GND1*, *RIB6*, *RFE1* AND SOME OTHER
GENES ON RIBOFLAVIN OVERSYNTHESIS OF THE YEAST *CANDIDA*
*FAMATA***

09 – Biology

091 – Biology

Applying for the Doctor of Philosophy degree

The dissertation contains the results of my research. The use of ideas, results, and
texts of other authors are linked to the appropriate source

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Lviv – 2025

ANNOTATION

LIU W. New findings on the role of GND1, RIB6, RFE1 and some other genes on riboflavin oversynthesis of the yeast Candida famata. – Qualifying scientific work on the rights of the manuscript.

Thesis for PhD degree in specialty 091 - Biology. – Institute of Cell Biology of the National Academy of Sciences of Ukraine, Lviv, 2025.

The dissertation highlights metabolic engineering approaches to investigating genes and factors in the regulation of riboflavin biosynthesis by the flavinogenic yeast *Candida famata* and further applying this knowledge to construct stable overproducers of riboflavin.

Riboflavin (vitamin B₂) is an essential water-soluble vitamin for the nutrition of living organisms, serving as a precursor of coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which plays fundamental roles in energy metabolism, cellular antioxidant potential, and metabolic interactions with other micronutrients, including vitamin B₆, vitamin B₁₂, and folate (Abbas and Sibirny, 2011; McNulty et al., 2023).

Riboflavin deficiency, known as ariboflavinosis, typically presents with cheilosis, angular stomatitis, glossitis, redness and swelling of the mouth and throat, and severe anemia with erythroid hypoplasia, as well as other symptoms, such as corneal vascularization, seborrheic dermatitis, and neurological alterations may also occur (Hoppel and Tandler, 1990; Thakur et al., 2017; Suwannasom et al., 2020).

Although this vitamin is essential for properly functioning metabolic pathways, humans and animals cannot synthesize riboflavin on their own and must supplement it

from exogenous sources (Lim et al., 2001; Abbas and Sibirny, 2011; Schwechheimer et al., 2016).

Thus, approximately 70% of industrially produced riboflavin is used as a feed additive, while 30% as a yellow colorant in the food industry and as a drug in medicine (Abbas and Sibirny, 2011; Kato and Park, 2012; You et al., 2021). A market analysis reported by *Straits Research* indicates that the global riboflavin market reached approximately US\$ 13.46 billion in 2023 (Riboflavin market size, share 2023-2031, n.d.).

Riboflavin production by microbial fermentation has the advantages of low production cost, short production time, and using renewable resources such as sugars or plant oil compared to chemical synthesis (Richter et al., 1997; Hohmann et al., 1998; Stahmann et al., 2000; Liu et al., 2023). Therefore, chemical synthesis of riboflavin was completely replaced by microbial fermentation in the past decade (Schwechheimer et al., 2016).

Currently, industrial riboflavin production is performed using engineered strains of the filamentous fungus *Ashbya gossypii*, and the Gram-positive bacterium *Bacillus subtilis* (Abbas and Sibirny, 2011; Kato and Park, 2012; Liu et al., 2020; You et al., 2021; Wang et al., 2021). Previously, ADM company (USA) terminated commercial production of riboflavin by strain *Candida famata* dep8 in 2007 due to the low genetic stability of the industrial strain (Abbas and Sibirny, 2011).

Despite this limitation, *C. famata* offers several advantages over strains of *B. subtilis* and *A. gossypii*. For instance, unlike *B. subtilis*, *C. famata* is not sensitive to phage infections (Abbas and Sibirny, 2011). It also has a shorter fermentation time compared to *A. gossypii* (Abbas and Sibirny, 2011; Dmytruk and Sibirny, 2012).

Furthermore, many yeasts can tolerate a wide range of environmental conditions, including fluctuations in pH, temperature, and pressure (Cavka and Jönsson, 2014; Ding and Ye, 2023).

In our group's previous work, effective genetic transformation of *C. famata* was developed (Voronovsky et al., 2002). Subsequently, the non-reverting strain *C. famata* AF-4 was isolated from the wild-type strain VKM Y-9 through classical selection methods. Strain AF-4 showed genetically stable and exhibited comparable levels of riboflavin production compared to industrial strain dep8 (Dmytruk et al., 2011). Further, the riboflavin overproducer *C. famata* BRP (Best Riboflavin Producer) was constructed by overexpression of the native structural genes *RIB1* and *RIB7*, as well as the transcription activator gene *SEF1* based on strain AF-4. It can overproduce riboflavin up to 16.4 g/L in a 7 L laboratory bioreactor during fed-batch fermentation (Dmytruk et al., 2014).

The pathway of riboflavin biosynthesis starts from two immediate precursors, guanosine triphosphate (GTP) derived from the purine biosynthesis pathway and ribulose-5-phosphate (Ru5P) derived from the oxidative pentose phosphate pathway (PPP) (Bacher et al., 2000; Abbas and Sibirny, 2011).

Previously, some beneficial changes were demonstrated due to increased synthesis of purine precursor, GTP. The advanced riboflavin overproducer, BRPI (Best Riboflavin Producer Improved), via overexpression of the modified heterologous genes *PRS3* and *ADE4* based on the strain BRP, led to an increase of GTP pool and 2-fold enhanced riboflavin production (Dmytruk et al., 2020).

Therefore, the purpose of this work was to study the effects of key genes involved in the pentose phosphate pathway, as well as new regulation factors in the riboflavin biosynthesis of *C. famata*.

Ru5P is the end-product of the oxidative branch of the PPP. In the PPP, glucose-6-phosphate dehydrogenase (G6PDH, encoded by gene *ZWF1*) and 6-phosphogluconate dehydrogenase (6PGDH, encoded by gene *GND1*) are two consecutive NADP⁺ dependent dehydrogenases, their reactions convert glucose-6-phosphate into Ru5P, and at the same time generates NADPH, which serves as the major source of reducing power for biosynthetic reactions (Nogae and Johnston, 1990; He et al., 2007).

It was shown that overexpression of the gene *GND1* led to approximately 2 times increased riboflavin production by constructed strains. For the first time describe the positive effect of the 6-phosphogluconate dehydrogenase on riboflavin biosynthesis by flavinogenic yeast. Moreover, it was discovered that overexpression of the gene *ZWF1* led to opposite effect and decreased biomass and riboflavin accumulation in constructed strains, however, the reasons have not yet been proven.

In the riboflavin biosynthesis pathway, Ru5P converts to the 3,4-dihydroxy-2-butanone 4-phosphate (DHBP) by DHBP synthase (encoded by gene *RIB6*), then DHBP forms the pteridine precursor of riboflavin (Fischer et al., 2005; Abbas and Sibirny, 2011). Thus, as a continuous of the above work, we decided to construct recombinant *C. famata* strains by the simultaneous overexpression of the three genes: *GND1*, *RIB6*, and *RFE1*, which encode, 6PGDH, DHBP synthase, and riboflavin excretase, respectively. The expression of various combinations of two genes, as well as the co-expression of all three genes, resulted in increased riboflavin production in *C. famata* VKM Y-9 in different media. It was shown that the recombinant strain

engineered for the co-overexpression of all three genes exhibited up to a 3.3-fold increase in riboflavin production in cheese whey, compared to the parental strain.

Another part of the work was the construction of *C. famata* mutants with a disruption gene *VMA1*, which codes for α -subunit of vacuolar ATPase (Förster et al., 1999, 2001). Obtaining mutant L2 *vma1Δ* showed a 9.4-fold increase in riboflavin production compared to the parental strain L2. Disruption of gene *VMA1* suggested that the introduction of appropriate mutations in *C. famata* may lead to a positive effect on riboflavin biosynthesis. Similar effect has been previously demonstrated for the flavinogenic yeast *Pichia guilliermondii* (Boretsky et al., 2011).

It should be noted that the transcription factor Sef1 (encoded by gene *SEF1*) is one of the positive regulators of riboflavin biosynthesis in flavinogenic yeasts, in particular in *C. famata* (Dmytruk et al., 2006). The dissertation also investigated the effects of promoters of gene *SEF1* from different yeasts on riboflavin production. We found that recombinant strains with overexpression gene *SEF1* under control of *C. famata*, *Candida albicans*, and *Candida tropicalis* promoters increased 18.8-, 19.4-, and 13.5-fold on riboflavin production compared to strain L2, respectively.

In this dissertation, we highlighted data about recombinant strain with overexpression of genes *REF1*, *RIB6*, and *GND1*, namely V9/RFE1-RIB6-GND1. The cultivation of strain V9/RFE1-RIB6-GND1 in cheese whey, in which lactose is the only source of carbon, contributed to more riboflavin production.

The potential for the application of *C. famata* at the commercial level of riboflavin production has been confirmed since the implementation of the industrial strain dep8. The presented experimental data are important for understanding certain mechanisms of control over riboflavin biosynthesis. The contribution of our group to

this progress is important and may soon construct robust riboflavin overproducers, thereby increasing the profitability of the production process. In particular, the application of cheese whey as a substrate for the growth of *C. famata* provides greater opportunities to make the process cheaper and more environmentally friendly.

Keywords: *Candida famata*, riboflavin, vitamin B₂, ribulose-5-phosphate, yeast, cheese whey.

АНОТАЦІЯ

ЛЮ В. Нові дані щодо ролі *GND1*, *RIB6*, *RFE1* та деяких інших генів у надсинтезі рибофлавіну дріжджами *Candida famata*. – Кваліфікаційна наукова праця на правах рукопису.

Дисертація на здобуття наукового ступеня доктора філософії за спеціальністю 091 – Біологія. – Інститут біології клітини НАН України, Львів, 2025.

У дисертаційній роботі досліджено вплив низки генів, що беруть участь у синтезі вітаміну B₂, його попередника рибулозо-5-фосфату та екскреції цього вітаміну в середовище, на продукцію рибофлавіну флавіногенними дріжджами *Candida famata* та подальшого застосування цих знань для створення стабільних надпродуцентів рибофлавіну.

Рибофлавін (вітамін B₂) є незамінним водорозчинним вітаміном що є попередником коферментів флавінмононуклеотиду (FMN) і флавінаденіндинуклеотиду (FAD), які відіграють ключову роль в енергетичному метаболізмі клітини (Abbas and Sibirny, 2011; McNulty et al., 2023).

Дефіцит рибофлавіну, відомий як арібофлавіноз, зазвичай проявляється хейлозом, ангулярним стоматитом, глоситом, почервонінням і набряком ротової порожнини та горла, та анемією з еритроїдною гіпоплазією, а також іншими симптомами, такими як васкуляризація рогівки, себореїний дерматит та неврологічні зміни (Hoppel and Tandler, 1990; Thakur et al., 2017; Suwannasom et al., 2020).

Незважаючи на те, що цей вітамін необхідний для правильного функціонування метаболічних шляхів, люди та тварини не здатні синтезувати

рибофлавін і повинні отримувати його з екзогенних джерел (Lim et al., 2001; Abbas and Sibirny, 2011; Schwechheimer et al., 2016). Так, приблизно 70% промислово виробленого рибофлавіну використовується як додаток до кормів у сільському господарстві, а 30% – як жовтий барвник у харчовій промисловості та як лікарський засіб у медицині (Abbas and Sibirny, 2011; Kato and Park, 2012; You et al., 2021). Аналіз ринку, опублікований Straits Research, показує, що світовий ринок рибофлавіну досяг приблизно 13,46 мільярда доларів США у 2023 році (Riboflavin market size, share 2023-2031, n.d.).

Виробництво рибофлавіну шляхом мікробної ферментації має такі переваги, як низька вартість виробництва, короткий час виробництва та використання відновлюваних ресурсів, таких як цукор або рослинна олія, порівняно з хімічним синтезом (Richter et al., 1997; Hohmann et al., 1998; Stahmann et al., 2000; Liu et al., 2023). Тому в останні десятиліття хімічний синтез рибофлавіну був повністю заміщений мікробною ферментацією (Schwechheimer et al., 2016).

На сьогодні, промислове виробництво рибофлавіну здійснюється з використанням рекомбінантних штамів цвілевих грибів *Ashbya gossypii* та бактерій *Bacillus subtilis* (Abbas and Sibirny, 2011; Kato and Park, 2012; Liu et al., 2020; You et al., 2021; Wang et al., 2021). Флавіногенні дріжджі *C. famata* також використовувались для промислового виробництва цього вітаміну. Проте, через низьку генетичну стабільність промислового штаму дріжджів, виробництво рибофлавіну було припинено у 2007 році (Abbas and Sibirny, 2011).

Незважаючи на цей недолік, дріжджі *C. famata* виявляють кілька переваг перед штамми *B. subtilis* і *A. gossypii*. Наприклад, на відміну від *B. subtilis*, *C. famata* не чутливі до фагових інфекцій (Abbas and Sibirny, 2011). За рахунок

синтезу рибофлавіну в експоненційній фазі росту, дріжджі характеризуються коротшим час бродіння порівняно з *A. gossypii* (Abbas and Sibirny, 2011; Dmytruk and Sibirny, 2012). Крім того, дріжджі здатні переносити широкий діапазон умов навколишнього середовища, включаючи коливання рН, температури та тиску (Cavka and Jönsson, 2014; Ding and Ye, 2023).

У попередній роботі, нашою групою було розроблено ефективну систему трансформації *C. famata* (Voronovsky et al., 2002). Згодом, за допомогою методів класичної селекції було селекціоновано штам AF-4, стабільний за ознакою надсинтезу рибофлавіну. Штам AF-4 продукував вітамін B₂ на рівні промислового продуцента, штаму *der8* (Dmytruk et al., 2011). Крім того, на основі штаму AF-4, було сконструйовано надпродуцент рибофлавіну BRP (Best Riboflavin Producer) шляхом надекспресії нативних структурних генів *RIB1* і *RIB7*, а також гена-активатора транскрипції *SEF1*. Продукція рибофлавіну цим штамом становила 16,4 г/л у лабораторному біореакторі об'ємом 7 л під час періодичної ферментації з підживленням (Dmytruk et al., 2014).

Шлях біосинтезу рибофлавіну починається з двох безпосередніх попередників, гуанозинтрифосфату (GTP), що походить від шляху біосинтезу пуринів, і рибулозо-5-фосфату (Ru5P), що походить від окисної ланки пентозофосфатного шляху (PPP) (Bacher et al., 2000; Abbas and Sibirny, 2011). Посилення експресії модифікованих гетерологічних генів *PRS3* і *ADE4* шляху біосинтезу пуринів на основі штаму BRP приводило до збільшення пулу GTP. Сконструйованих штам BRPI (Best Riboflavin Producer Improved) характеризувався двократним зростанням продукції рибофлавіну (Dmytruk et al., 2020).

Тому метою цієї роботи було дослідження впливу генів, залучених у пентозофосфатний шлях, а також нових факторів в регуляцію біосинтезу рибофлавіну *C. famata*.

Ru5P є кінцевим продуктом окисної ланки PPP. У PPP глюкозо-6-фосфатдегідрогеназа (G6PDH, кодується геном *ZWF1*) і 6-фосфоглюконатдегідрогеназа (6PGDH, кодується геном *GND1*) є двома послідовними NADP^+ залежними дегідрогеназами, їхні реакції перетворюють глюкозо-6-фосфат на Ru5P, і в той же час генерує NADPH, який служить основним джерелом відновної енергії в реакціях біосинтезу (Nogae and Johnston, 1990; He et al., 2007).

Ми виявили, що надекспресія гена *GND1* забезпечує підвищення синтезу рибофлавіну трансформантами приблизно в два рази. Вперше описано позитивний вплив 6-фосфоглюконатдегідрогенази на біосинтез рибофлавіну флавіногенними дріжджами. Надекспресія гена *ZWF1* викликає протилежний ефект і зумовлює зниження швидкості росту та рівня продукції рибофлавіну. Причини цього феномену залишаються нез'ясованими.

На шляху біосинтезу рибофлавіну Ru5P перетворюється на 3,4-дигідрокси-2-бутанон 4-фосфат (DHBP) за допомогою DHBP-синтази (кодується геном *RIB6*), який перетворюється на птеридиновий попередник рибофлавіну (Fischer et al., 2005; Abbas and Sibirny, 2011).

Таким чином, як продовження вищезазначеної роботи, було сконструйовано рекомбінантні штами *C. famata* шляхом одночасної надекспресії трьох генів: *GND1*, *RIB6* та *RFE1*, які кодують 6PGDH, DHBP-синтазу та екскретазу рибофлавіну, відповідно. Експресія різних комбінацій двох генів, а

також коекспресія всіх трьох генів призводила до збільшення продукції рибофлавіну в *C. famata* VKM Y-9 у різних середовищах. Було показано, що рекомбінантний штам, з коекспресією трьох генів, характеризувався 3,3-кратним зростанням продукції рибофлавіну в сироватці порівняно з вихідним штамом.

Іншою частиною роботи було конструювання мутанта *C. famata* з пошкодженим геном *IMA1*, який кодує α -субодиницю вакуолярної АТФ-ази (Förster et al., 1999, 2001). Мутант *ima1 Δ* виявляв 9,4-кратне збільшення продукції рибофлавіну порівняно з вихідним штамом L2. Подібний ефект було продемонстровано для флавіногенних дріжджів *Pichia guilliermondi* (Boretsky et al., 2011).

Слід зазначити, що фактор транскрипції Sef1 (кодується геном *SEF1*) є одним із позитивних регуляторів біосинтезу рибофлавіну у флавіногенних дріжджів, зокрема у *C. famata* (Dmytruk et al., 2006). У дисертації також досліджено вплив промоторів гена *SEF1* різних дріжджів на продукцію рибофлавіну. Ми виявили, що рекомбінантні штами, які експресують ген *SEF1* під контролем промоторів з дріжджів *C. famata*, *Candida albicans* і *Candida tropicalis* збільшували продукцію рибофлавіну в 18,8, 19,4 і 13,5 разів порівняно з вихідним штамом L2, відповідно.

У дисертаційній роботі описано конструювання рекомбінантного штаму V9/RFE1-RIB6-GND1 з коекспресією генів *REF1*, *RIB6*, та *GND1*. Штам V9/RFE1-RIB6-GND1 характеризувався збільшеною продукцією рибофлавіну при культивуванні в середовищі на основі молочної сироватки.

Дріжджі *C. famata* тривалий час використовувалися у промисловому виробництві вітаміну B₂, що підкреслює їх потенціал у промисловому

застосуванні. Триває робота в напрямку ідентифікації нових факторів, що залучені в регуляцію синтезу рибофлавіну. Експериментальні дані, отримані при виконанні цієї дисертаційної роботи, є важливими для розуміння окремих механізмів контролю біосинтезу рибофлавіну. Отримані результати є передумовою створення високопродуктивних стабільних продуцентів рибофлавіну. Важливим напрямком мікробного промислового виробництва рибофлавіну є пошук та використання дешевих субстратів. Зокрема, застосування молочної сироватки, що належить до відходів молочної промисловості, як субстрату для культивування дріжджових продуцентів вітаміну B₂, здешевлює процес виробництва та водночас вирішує питання утилізації відходів сирного виробництва.

Ключові слова: *Candida famata*, рибофлавін, вітамін B₂, рибулозо-5-фосфат, дріжджі, сироватка.

List of applicant's publications:

Eleven scientific works have been published on the dissertation topic, including four manuscripts in international publications, as well as seven abstracts of reports in the materials of conferences, scientific congresses, and congresses.

Articles in periodical scientific publications indexed in databases Web of Science Core Collection and/or Scopus:

1. **Liu, W.**, Tsyurulnyk, A., Dmytruk, K., Fedorovych, D., Kang, Y., and Sibirny, A. (2025). Co-overexpression of genes *RFE1*, *GND1*, and *RIB6* enhances riboflavin production in yeast *Candida famata*. *Cytology and Genetics*, 59(1), 63–70. <https://doi.org/10.3103/S0095452725010074>. Q4, Scopus and WoS. (*The author, together with co-authors, conducted research, analyzed and summarized the obtained data, took part in the writing, and design of the publication*).
2. Ruchala, J., Andreieva, Y., Tsyurulnyk, A., Sobchuk, S., Najdecka, A., **Liu, W.**, Kang, Y., Dmytruk, O., Dmytruk, K., Fedorovych, D., and Sibirny, A (2022). Cheese whey supports high riboflavin synthesis by the engineered strains of the flavinogenic yeast *Candida famata*. *Microbial Cell Factories*, 21(1), 161-169. <https://doi.org/10.1186/s12934-022-01888-0>. Q1, Scopus and WoS. (*The author, together with co-authors, conducted research, analyzed and summarized the obtained data, took part in the writing, and design of the publication*).
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5. **Liu, W.** Exploration of Role of Overexpressed Genes *ZWF1* and *GND1* in Riboflavin Synthesis by *Candida famata* (*Candida flareri*) // 1st International Conference of Young Scientists of the Institute of Cell Biology and the University of Rzeszów "Current Issues in Cell Biology and Biotechnology", June 02, Lviv, Ukraine. – 2021. – P. 25.

6. **Liu, W.** Role of the pentose phosphate pathway in riboflavin oversynthesis of the flavinogenic yeast *Candida famata* (*Candida flareri*) // Conference of Young Scientists of Institute of Cell Biology, June 08, Lviv, Ukraine. – 2021. – P. 21.

7. Andreieva, Y., **Liu, W.**, Dmytruk, K., Sibirny, A. Evaluation of the effect of overexpressed genes *ZWF1* and *GND1* on riboflavin synthesis by flavinogenic yeast *Candida famata* (*Candida flarerii*) // 8th International Conference "Human – Nutrition – Environment", October 13-14, Rzeszow, Poland. – 2021. – P. 66.

8. **Liu, W.**, Tsyrlunyk, A., Dmytruk, K., Fedorovych, D., Sibirny, A. Development of platform for constructing of riboflavin overproducers based on the

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LIST OF CONDITIONAL DESIGNATIONS

FAD – flavin adenine dinucleotide

FMN – flavin mononucleotide

GTP – guanosine triphosphate

Ru5P – ribulose-5-phosphate

PPP – pentose phosphate pathway

6PG – 6-phosphogluconate

6PGDH – 6-phosphogluconate dehydrogenase

G6P – glucose-6-phosphate

G6PDH – glucose-6-phosphate dehydrogenase

NADP⁺ (H) – nicotinamide adenine dinucleotide phosphate (reduced)

DHBP – 3,4-dihydroxy-2-butanone-4-phosphate

DARRP – 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone-5'-phosphate

ARRP – 5-amino-6-ribosylamino-2,4(1*H*,3*H*)-pyrimidinedione-5'-phosphate

DArPP – 2,5-diamino-6-ribitylamino-4(3*H*)-pyrimidinone-5'-phosphate

ArPP – 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione-5'-phosphate

ArP – 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione

DMRL – 6,7-dimethyl-8-ribityllumazine

DRL – 6,7-dimethyl-8-ribityllymazine

ATP – adenosine triphosphate

ATPase – adenosine triphosphatase

BRP – best riboflavin producer

BRPI – best riboflavin producer improved

Sfu1 – suppressor of ferric uptake

Vma1 – vacuolar membrane ATPase

Rfe1 – riboflavin excretase

BCRP – breast cancer resistance protein

YNB – yeast nitrogen base

YPD – yeast extract peptone dextrose

ORF – open reading frame

PCR – polymerase chain reaction

AMP – adenosine-5'-monophosphate

IMP – inosine-5'-monophosphate

XMP – xanthosine-5'-monophosphate

PRPP – 5-phosphoribosyl-1-pyrophosphate

RFN – riboswitch FMN-specific element

CRISPR – clustered regularly interspaced short palindromic repeats

INTRODUCTION

Relevance of the topic

Riboflavin, (vitamin B₂ or lactoflavin) is essential for growth and health of humans and animals, that cannot synthesize this vitamin by themselves to cover their needs (Capone and Sentongo, 2019; Suter, 2020). The best-known biochemically active coenzymes formed from riboflavin are flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are involved in numerous enzymatic reactions of oxidative metabolism (Bacher et al., 2000; Abbas and Sibirny, 2011; Schwechheimer et al., 2016).

To avoid deficiency symptoms like skin diseases, damage to the nervous system, and glaucoma, a nutritional requirement of 1.4–1.8 mg riboflavin per day for humans and 1.1–1.3 mg/kg per day for animals is recommended (Hoppel and Tandler, 1990; Capone and Sentongo, 2019; Suwannasom et al., 2020). Thus, riboflavin has great commercial value due to it is used on a large scale as an animal feed additive, in the food industry as a colorant, and in pharmaceutical applications. According to a report by Straits Research, the global riboflavin market size has reached approximately US\$ 13.46 billion in 2023 and will reach US\$ 23.34 billion in 2031 with a compound annual growth rate (CAGR) of 6.30% during the forecast period (Riboflavin market size, share 2023-2031, n.d.).

Microbial fermentation has the advantages of low production cost, short production time, and using renewable resources compared to chemical synthesis (Richter et al., 1997; Hohmann et al., 1998; Stahmann et al., 2000; Liu et al., 2023). Therefore, it has completely replaced chemical synthesis to commercial produce riboflavin in the past decade (Schwechheimer et al., 2016).

Initial studies to develop a fermentation process to produce riboflavin were established in 1940s with anaerobic bacterium *Clostridium acetobutylicum*, *Eremothecium ashbyi*, and *Ashbya gossypii* (Schopfer and Guilloud, 1945; Wickerham et al., 1946; Tanner and Van Lanen, 1947; Peppler and Perlman, 1979)

In 1965, several commercial fermentation processes were established for riboflavin, but a few years later, the production plants were shut down, because they were not competitive with the chemical synthesis (Smiley and Leonard, 1955; Peppler and Perlman, 1979; Lago and Kaplan, 1981).

A production plant with *A. gossypii* was started by BASF (Germany) in 1990, most experience has been gained with mutants of *A. gossypii*, riboflavin yielding up to 15 g/L, and after 6 years of being run in parallel, the chemical process was finally shut down in 1996 (Schmidt et al., 1996; Stahmann et al., 2000).

A second natural riboflavin overproducer is *Candida famata*. The industrial strain *C. famata* dep8 (ATCC 20849) is described as producing riboflavin more than 20 g/L. (Heefner et al., 1992, 1993). It was improved by Coors Brewing Company (USA) and was used for industrial production of riboflavin by Archer Daniels Midland (USA) (Heefner et al., 1992, 1993; Stahmann et al., 2000). However, genetic instability terminated its industrial fermentation (Abbas and Sibirny, 2011).

In 2000, Hoffmann-La Roche (Switzerland) proposed to replace its chemical production by fermentation process with the Gram-positive bacterium *Bacillus subtilis*, which is not a natural riboflavin overproducer, but is efficient in applications involving genetic engineering (Stahmann et al., 2000; Lim et al., 2001; Sun et al., 2020). Until now, companies such as Hubei Guangji Pharmaceutical Co. Ltd. (China) and DSM®

(Netherlands) have used engineered strains of *B. subtilis* to produce riboflavin (Abbas and Sibirny, 2011; Liu et al., 2023).

Although, ADM company stopped commercial production of riboflavin by *C. yeast famata* in 2007. Unlike *B. subtilis*, *C. famata* is not sensitive to phage infections. It has a shorter fermentation time compared to *A. gossypii* (Abbas and Sibirny, 2011; Dmytruk and Sibirny, 2012). Furthermore, yeast can tolerate a wide range of environmental conditions (Cavka and Jönsson, 2014; Ding and Ye, 2023).

Our research group have been committed to developing and improving the *C. famata*, effective genetic transformation methods (Voronovsky et al., 2002) and insertional mutagenesis (Dmytruk et al., 2006) for *C. famata* was developed. In addition, structural and regulatory genes of the riboflavin biosynthesis pathway in *C. famata* have been cloned (Voronovsky et al., 2004; Dmytruk et al., 2004, 2006). Subsequently, the non-reverting strain AF-4, which showed genetically stable and exhibited comparable levels of riboflavin production compared to industrial strain dep8, was isolated from the wild-type strain VKM Y-9 through classical selection methods (Dmytruk et al., 2011). Further, overexpression of the native structural genes *RIB1* and *RIB7*, as well as the transcription activator gene *SEF1* in strain AF-4 to constructed riboflavin overproducer *C. famata* BRP (Best Riboflavin Producer), which can produce riboflavin up to 16.4 g/L in a 7 L laboratory bioreactor (Dmytruk et al., 2014).

The activation of the biosynthesis of GTP via overexpression of the genes *PRS3* (coding for phosphoribosyl pyrophosphate synthetase) and *ADE4* (coding for phosphoribosyl amidotransferase) to avoid the feedback inhibition of both enzymes by purine nucleotides. Subsequently, the study by Dmytruk et al. showed that the advanced strain designed as BRPI (Best Riboflavin Producer Improved) via overexpression of the modified heterologous genes *PRS3* and *ADE4* from

Debaryomyces hansenii in the strain BRP, led to an increase of GTP pool and 2-fold enhanced riboflavin production (Dmytruk et al., 2020).

It was performed that overexpression of gene *RFE1* (homolog of gene *BCRP*), which encodes putative riboflavin efflux protein Rfe1 responsible for the excretion of riboflavin from the yeast cell in strain BRP to generated strain BRP/RFE1, had a 1.5–1.8 fold increase in riboflavin production (Tsyurulnyk et al., 2020). Furthermore, overexpression of the riboflavin biosynthesis structural gene *RIB6* (encoding DHBP synthase) increased riboflavin production in strains AF-4 and BRP (Petrovska et al., 2022).

In parallel, the advanced riboflavin overproducer, BRPI, via overexpression of the modified heterologous genes *PRS3* and *ADE4* based on strain BRP, led to 2-fold enhanced riboflavin production (Dmytruk et al., 2020).

Finding cheap and readily available substrates is one of the goals of industrial fermentation. Previously, riboflavin producers were cultivated in the medium of glucose as the carbon sources (Ishchuk et al., 2008). Riboflavin biosynthesis in cheese whey was activated in *C. famata* strains, which overexpressed gene *SEF1* under the control of lactose-induced promoter of gene *LAC4* (encoding β -galactosidase) (Tsyurulnyk et al., 2021). It was found that *C. famata* strains also grow and overproduce riboflavin in cheese whey (Ruchala et al., 2022). Recently, it was found that lignocellulosic hydrolysate supports robust growth and riboflavin biosynthesis in *C. famata*. The engineered strain BRPI/XYL1, which overexpressed gene *XYL1* coding for xylose reductase, exhibited increased riboflavin production and reached 1.5 g/L during a bioreactor using bagasse hydrolysate as the carbon source (Dzanaeva et al., 2024).

Through our colleagues' efforts for more than 20 years, we have obtained many positive results and made great improvements to *C. famata*, but our goal is to construct robustly engineered strains that can have strong competitiveness.

We assume that deepening knowledge about the factors that regulate the processes of riboflavin biosynthesis in *C. famata* can help to carry out targeted modifications of genomes. The use of these approaches towards an increase in riboflavin production is promising for the creation of competitive industrial strains. In the future, the results of this dissertation could optimize riboflavin production and meet the needs of the market.

Connection of work with scientific programs, plans, topics

The presented work was carried out as one of the parts of fundamental research in the Department of Molecular Genetics and Biotechnology of the Institute of Cell Biology of the National Academy of Sciences of Ukraine on the topics: "Genetic control of riboflavin biosynthesis and transport in flavinogenic yeast" (state registration number 0115U001362, 2015-2019); "Genetic and biochemical aspects of the regulation of some catabolic and anabolic processes in microorganisms: alcoholic fermentation, methanol catabolism, biosynthesis of flavins, glycerol, hydrogen, and glutathione" (Decision of the Expert Council on Evaluation of Fundamental Research Topics at the National Academy of Sciences of Ukraine dated 17.06.2015 No. 3, 2016-2019); "Study of the mechanism of action of new genes in the regulation of riboflavin synthesis in the flavinogenic yeast *Candida famata*" (Resolution of the Bureau of the VBFMB of the National Academy of Sciences of Ukraine dated October 2, 2018 No. 5, state registration number 0119U001677, 2019-2020); "Identification and elucidation of the role of new structural and regulatory genes in the oversynthesis of riboflavin in flavinogenic yeasts" (Resolution of the Bureau of the National Academy of Sciences

of the National Academy of Sciences of Ukraine dated November 26, 2020 No. 4, state registration number 0121U10926, 2021-2025).

Part of the experimental work was carried out within the scope of research under the grant of the Polish National Science Center, Opus UMO-2018/29/B/NZ1/01-497 - "Regulatory mechanism involved in riboflavin overproduction in the flavinogenic yeast *Candida famata*". The author of the dissertation is one of the executors of the aforementioned studies.

The aim and objectives of the research

Aim

The work aims to use metabolic engineering approaches to investigate genes and factors in the regulation of riboflavin biosynthesis by the flavinogenic yeast *C. famata* and further apply this knowledge to construct robust overproducers of riboflavin.

In order to achieve the goal, the following tasks were expected to be fulfilled in the dissertation work:

1) To study the role of genes encoding key enzymes of the oxidative branch of the pentose phosphate pathway in riboflavin biosynthesis by *C. famata* strains L2, AF-4, and BRP.

2) To construct strains via co-overexpression of the genes *RFE1*, *GND1*, and *RIB6* in *C. famata* VKM Y-9 and analyze the level of riboflavin production in recombinant strains.

3) To investigate the ability of promoters of gene *SEF1* from various flavinogenic (*C. famata*, *Candida albicans*, *Candida tropicalis*) and non-flavinogenic

(*Scheffersomyces stipitis*, *Saccharomyces cerevisiae*) yeasts to regulate expression levels of gene *SEF1* in *C. famata* L2 *sef1Δ*.

4) To obtain *C. famata* L2 with deletion of gene *VMAl* and find out whether this gene affects the regulation of riboflavin biosynthesis in recombinant strains.

The object of research: regulation genes and factors of riboflavin biosynthesis in yeast *C. famata*.

The subject of research: the mechanism of regulatory genes and factors of riboflavin biosynthesis in yeast *C. famata*.

Research methods

Conventional genetic, biochemical, and microbiological research methods were performed in this work (Sambrook et al., 1989). The construction of recombinant plasmids was carried out using methods of molecular biology, such as hydrolysis of DNA by restriction endonucleases, elution of DNA fragments from agarose gel, dephosphorylation of linearized plasmids, ligation of the plasmid with an insert. To introduce recombinant plasmids into recipient cells (bacterial or yeast cells), transformation was performed by electroporation. Plasmid isolation from *E. coli* was performed with the Wizards Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA). The genomic DNA of *C. famata* was isolated using the Wizards Genomic DNA Purification Kit (Promega, Madison, WI, USA). Transformants (bacteria or yeast) were isolated on a selective medium and PCR (polymerase chain reaction) to analyze the obtained transformants in a GeneAmps PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). The cell biomass was determined turbidimetrically with a Helios Gamma spectrophotometer (OD, 600 nm; cuvette, 10 mm) with gravimetric calibration. The riboflavin concentration was

determined by measuring fluorescence (TurnerQuantechFM109510-33 fluorometer, excitation maximum = 445 nm, emission maximum = 535 nm) after cultivation in batch culture in flasks for 5 days. The biochemical characterization of recombinant strains was carried out by determining the activity of enzymes in cell-free extracts. For modeling genome modification cassettes, processing results, etc. computer analysis methods, bioinformatics Internet resources and software, and electronic databases of known genes were used.

Scientific novelty

Currently, industrial riboflavin overproducers were developed and created based on Gram-positive bacterium *B. subtilis* and fungus *A. gossypii* via using modern metabolic engineering approaches, such as overexpression of structural and regulatory genes of the riboflavin biosynthesis pathway, and activation of synthesis of the precursors of riboflavin biosynthesis. Previously, our colleagues successfully constructed advanced riboflavin overproducer BRPI by overexpressing the key genes of *de novo* purine biosynthesis which led to the activation of GTP supply. However, the role of another precursor, Ru5P, the intermediate of the PPP, in riboflavin overproduction in *C. famata* remains unknown. Therefore, we focused on the effects of the key genes in the oxidative branch of the PPP in riboflavin biosynthesis by the yeast *C. famata*. We first found that overexpressing gene *GND1* increased riboflavin biosynthesis in yeast *C. famata*, while overexpressing gene *ZWF1* significantly reduced riboflavin production and cell growth.

Based on the above results, we decided to increase Ru5P flux towards riboflavin biosynthesis by co-overexpressing genes *GND1*, *RIB6* (encodes DHBP synthase, is responsible for converting Ru5P), and *RFE1* (encodes protein Rfe1 responsible for the

excretion of riboflavin), led to 3.3-fold increase in riboflavin production by yeast *C. famata* VKM Y-9.

In addition, we investigate the mechanisms of regulation factors outside of the riboflavin biosynthesis pathway. It was found that expression of the gene *SEF1* (encodes transcription factor Sef1, which is a positive regulator) only under the promoters of flavinogenic yeast leads to increased production of riboflavin. It was shown for the first time that gene *VMA1* (encodes α -subunit of the vacuolar ATPase) acts as a negative regulator of riboflavin biosynthesis for yeast *C. famata*.

Practical significance of scientific results

The revealed genes and factors of riboflavin biosynthesis can be used for the construction of new robust overproducers on the advanced overproducer BRPI by combining and modifying their expression. The resulting strains should be robust and competitive riboflavin overproducers. This will make it possible to introduce appropriate strains into industrial production.

Personal contribution of the applicant

The graduate student developed a research plan for the fulfillment of specified tasks with the help of a scientific supervisor. Together with the scientific supervisor, the dissertation student analyzed the results of experimental research. The applicant was engaged in the research work of methods and techniques to select the best of them in order to achieve the set goal of the experiment. However, the scientific supervisor was involved in this process if necessary. The preparation of scientific publications was carried out by a graduate student with the advisory support of a scientific supervisor, and the selection of a journal was carried out by a scientific supervisor. The results

presented in the dissertation were obtained by conducting scientific research by a graduate student in cooperation with the co-authors of the publications.

The author of the dissertation expresses sincere gratitude to the scientific director, co-authors of the publications and all employees of the Institute of Cell Biology who participated in obtaining the research results presented in the dissertation.

Approbation of the results of the dissertation

The main provisions of the dissertation work are published in the form of scientific articles in specialized journals and presented in the form of abstracts of oral or poster presentations.

Among the conferences at which the obtained results were presented are XX International Scientific Conference for Students and PhD Students "Youth and Progress of Biology" (Lviv, Ukraine, 2024), VII Congress for All-Ukrainian Public Organization Ukrainian Society of Cell Biology with International Representation (Lviv, Ukraine, 2024), VIII International Conference "Human – Nutrition – Environment" (Rzeszow, Poland, 2021), The first International Conference of Young Scientists of the Institute of Cell Biology and the University of Rzeszów "Current Issues in Cell Biology and Biotechnology" (Lviv, Ukraine, 2021), Conference of Young Scientists of Institute of Cell Biology (Lviv, Ukraine, 2022).

In total, eleven scientific works were published on the topic of the dissertation, including four articles in international editions indexed in the Scopus and Web of Science Core Collection databases, and seven abstracts of reports in the materials of conferences, scientific symposia, and congresses.

The structure and scope of the dissertation

The dissertation contains the following sections: "Introduction", "Literature Review", "Materials and Research Methods", "Results", "Discussion", "Conclusion" and "References", "Appendix". The dissertation is presented on 159 pages of printed text, of which the main part occupies 93 pages. The work contains 34 figures, 4 tables and 1 formula. The list of used literature includes 202 literature sources. One appendix is attached at the end of the work.

1. LITERATURE REVIEW

1.1. Introduction to riboflavin

Riboflavin, as known as vitamin B₂, was first identified in 1879 as a yellow pigment found in milk, and it was initially named lactoflavin (Karrer et al., 1935). In 1930s, a research group from Heidelberg University, including Paul György, Richard Kuhn, and Theodore Wagner-Jauregg, successfully isolated and purified riboflavin for the first time (Kuhn, 1935). They used a bioassay based on the growth response of rats fed a purified diet to measure the activity of the vitamin. Its chemical structure is 7,8-dimethyl-10-ribityl-isoalloxazine, C₁₇H₂₀N₄O₆ (Figure 1.1), which is composed of a heterocyclic isoalloxazine ring system connected to a ribityl side chain. The isoalloxazine ring is responsible for the molecule's yellow color and its ability to absorb light (Karrer et al., 1935; Kuhn, 1935).

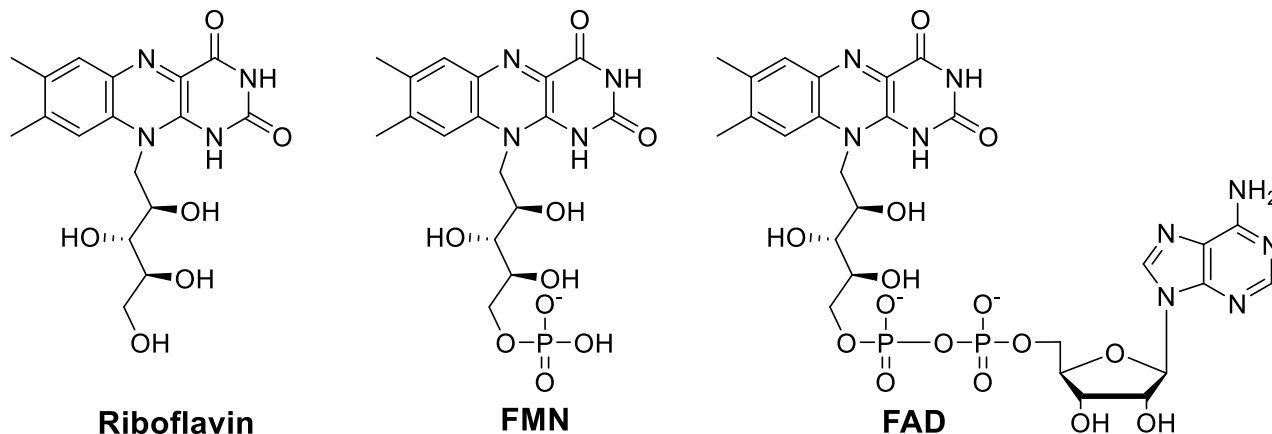


Figure 1.1 Chemical structures of riboflavin, FMN, and FAD.

Riboflavin is a water-soluble vitamin with high thermostability (Al-Shammmary et al., 1990), whereas it can be easily destroyed by heating in alkaline solutions (Brown and Reynolds, 1963). Furthermore, it is a light-sensitive molecule that undergoes photodegradation when exposed to short-wavelength radiation, particularly below 400 nm (Weber, 1950).

Riboflavin is an important compound for the nutrition of living organisms, serving as a precursor of coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Figure 1.1), which are involved in numerous enzymatic reactions of oxidative metabolism (Abbas and Sibirny, 2011).

The Food and Nutrition Board recommends that the daily intake of riboflavin for humans is 1.4 – 1.8 mg (Food and Nutrition Board, 1998). Riboflavin deficiency led to retarded growth, failure to thrive, cardiovascular disease, impairment of iron metabolism, etc. (Foraker et al., 2003; Powers, 2003).

All plants and most microorganisms can synthesize riboflavin on their own, however, animals and rare prokaryotic and eukaryotic microorganisms cannot synthesize riboflavin and need to obtain it from supplements (Abbas and Sibirny, 2011; Schwechheimer et al., 2016).

Over the past decade, the industrial production of riboflavin has been exclusively accomplished by microbial fermentation replacing chemical synthesis, because microbial fermentation has advantages such as low cost, short production cycle, and environmentally friendly relative to chemical synthesis (Averianova et al., 2020; Zhang et al., 2022).

The industrial strains used for riboflavin production mainly are mainly derived from the Gram-positive bacterium *Bacillus subtilis*, the filamentous fungus *Ashbya gossypii*, and yeast *Candida famata* (Stahmann et al., 2000). The Archer Daniels Midland (USA) has stopped yeast industrial process due to the low genetic stability of the engineered *C. famata* dep8 (Abbas and Sibirny, 2011). Subsequently, Dmytruk et al. demonstrated that introduction of additional copy of gene *SEF1* (encoding a putative transcription factor) into *C. famata* dep8 could greatly stabilized and increased

riboflavin production (Dmytruk et al., 2011). This result reignited interest in *C. famata* and demonstrated its considerable potential in riboflavin production.

Nowadays, riboflavin has great commercial value due to its use on a large scale as an animal feed additive, in food industry as a colorant and in pharmaceutical applications (Abbas and Sibirny, 2011; Schwechheimer et al., 2016; Zhang et al., 2022). According to a market analysis report by Straits Research, the global riboflavin market size has reached approximately US\$ 13.46 billion in 2023 with a compound annual growth rate (CAGR) of 6.30% during the forecast period (2023-2031), that is revenue of US\$ 23.34 billion in 2031 (Riboflavin market size, share 2023-2031, n.d.).

1.2. Microbial biosynthesis of riboflavin and its derivatives

The earliest research on riboflavin biosynthesis can be traced back to 1950s (Goodwin and Pendlington, 1954; Bacher et al., 2000). Most of the research on riboflavin synthesis was carried out on microorganisms. At the initial stages, all attention was focused on filamentous fungi, namely *Eremothecium ashbyii* and *A. gossypii*, as well as on yeasts, including several species of the genus *Candida* (Goodwin and Pendlington, 1954; Goodwin and McEvoy, 1959; Wendland and Walther, 2005; Fischer et al., 2005). This was due to the natural occurrence of flavinogenic representatives of these organisms (Bacher et al., 2000; Abbas and Sibirny, 2011).

Only later did certain eubacteria, including *Escherichia coli* and *Bacillus subtilis*, as well as the non-flavinogenic yeast *Saccharomyces cerevisiae* gain popularity in studies of riboflavin biosynthesis. As for studies of riboflavin synthesis in plants, they were started on a large scale only in the 1990s (Bacher et al., 2000; Fischer et al., 2005; Abbas and Sibirny, 2011).

In flavinogenic organisms like fungi, yeasts, and eubacteria, the biosynthesis of one molecule of riboflavin necessitates one molecule of guanosine triphosphate (GTP) and two molecules of ribulose-5-phosphate (Ru5P) (Bacher et al., 2000; Abbas and Sibirny, 2011). However, the regulatory mechanism of riboflavin biosynthesis differs significantly among different species. For example, FMN riboswitches regulate the expression of riboflavin operons in only a few strains (Abbas and Sibirny, 2011; García-Angulo, 2017).

The terminal riboflavin biosynthesis pathway comprises a total of seven enzymatic steps starting from two different branches: the purine biosynthesis and the pentose phosphate pathway (PPP) (Bacher et al., 2000; Abbas and Sibirny, 2011; Schwechheimer et al., 2016).

The riboflavin biosynthetic pathway begins with GTP as the substrate, catalyzed by GTP cyclohydrolase II, which first isolated from *E. coli*, is encoded by the gene *ribA* (in yeast designated gene *RIB1*). GTP cyclohydrolase II opens the imidazole ring and hydrolytically releases inorganic pyrophosphate from the side chain of the ribose moiety of GTP, resulting in the formation of 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone-5'-phosphate (DARPP). Studies across multiple organisms, including *B. subtilis*, *Helicobacter pylori*, and the flavinogenic yeast *Pichia guilliermondii*, have provided insights into this enzyme's properties (Figure 1.2) (Bacher et al., 2000; Abbas and Sibirny, 2011).

There is also the enzyme GTP cyclohydrolase I, which is involved in the biosynthesis of folic acid and biopterin, while not involved in riboflavin biosynthesis (Bacher et al., 2000; Bacher and Mailänder, 1978). Additionally, GTP cyclohydrolase III, discovered in archaea and some eubacteria, also catalyzes the conversion of GTP to DARPP, but unlike GTP cyclohydrolase II, it opens the imidazole ring, but does not

remove the formyl group. Despite similarities in function, GTP cyclohydrolase III shares no homology with GTP cyclohydrolase II, highlighting the diverse evolutionary pathways leading to riboflavin biosynthesis.

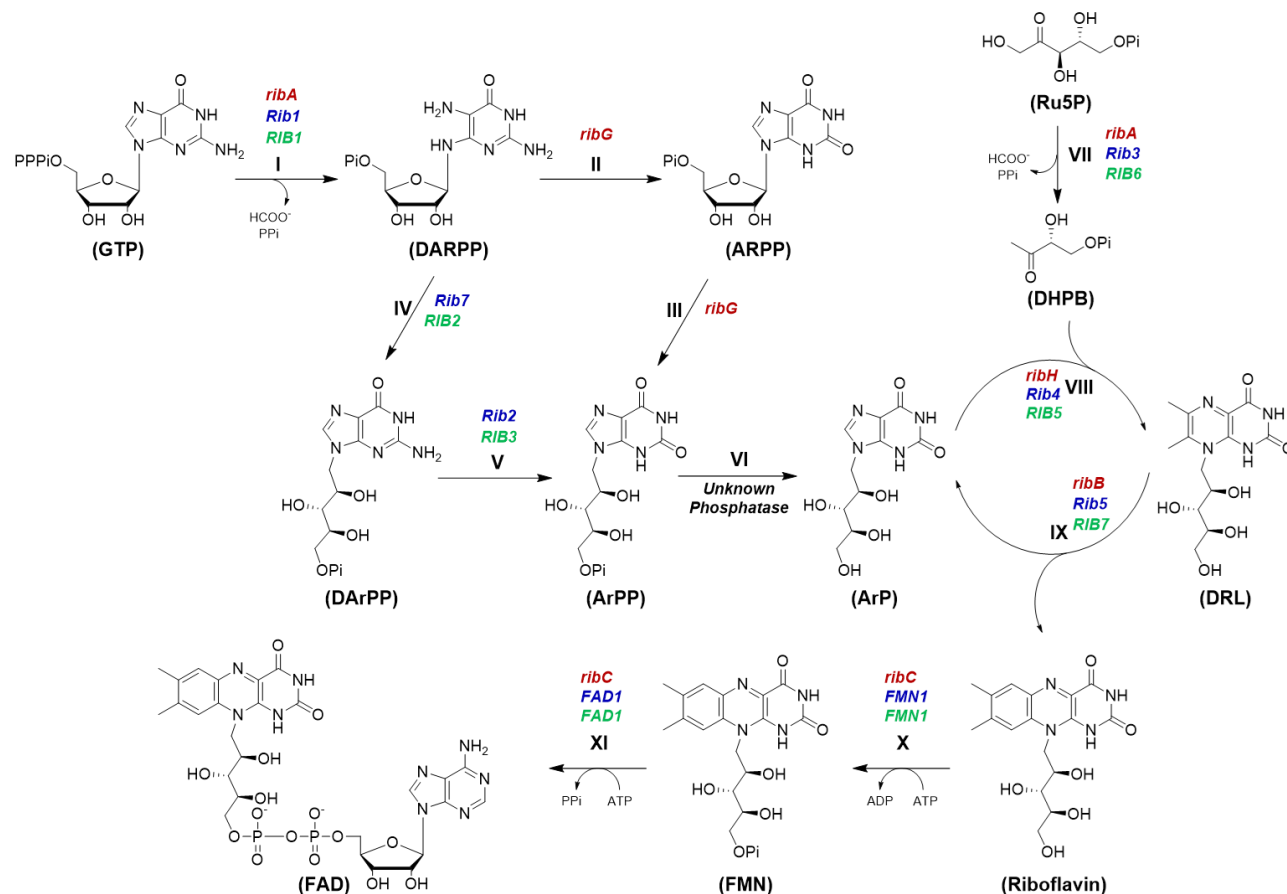


Figure 1.2 Schematic diagram of the riboflavin and flavocoenzymes biosynthesis pathways in *Bacillus subtilis*, *Ashbya gossypii*, and *Candida famata* reproduced from references (Bacher et al., 2000; Abbas and Sibirny, 2011; Patel and T.S., 2020; Sibirny, 2023). The enzymatic reaction steps (I–XI) encoded by the genes (*B. subtilis* in orange, *A. gossypii* in blue, and *C. famata* in green) are shown, which are responsible for the conversion of the precursors GTP and ribulose-5-phosphate into riboflavin, FMN, and FAD. I, GTP cyclohydrolase II; II, 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone-5'-phosphate deaminase; III, 5-amino-6-ribosylamino-2,4(1H,3H)-pyrimidinedione-5'-phosphate reductase; IV, 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone-5'-phosphate reductase; V, 2,5-diamino-6-

ribitylamino-4(3*H*)-pyrimidinone-5'-phosphate deaminase; VI, hypothetical phosphatase; VII, 3,4-dihydroxy-2-butanone-4-phosphate synthase; VIII, 6,7-dimethyl-8-ribityllumazine synthase; IX, riboflavin synthase; X, riboflavin kinase; XI, FAD synthetase; GTP, guanosine triphosphate; DARPP, 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone-5'-phosphate; ARRP, 5-amino-6-ribosylamino-2,4(1*H*,3*H*)-pyrimidinedione-5'-phosphate; DArPP, 2,5-diamino-6-ribitylamino-4(3*H*)-pyrimidinone-5'-phosphate; ArPP, 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione-5'-phosphate; ArP, 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione; Ru5P, ribulose-5-phosphate; DHBP, 3,4-dihydroxy-2-butanone-4-phosphate; DRL, 6,7-dimethyl-8-ribityllumazine; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.

In the next two reactions, DARPP is subsequently converted into 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione-5'-phosphate (ArPP) through two sequential reactions: reduction and deamination. In yeasts, fungi, and archaea, the DARPP reductase catalyzing the ribosyl side chain of DARPP is first reduced to 2,5-diamino-5-ribitylamino-4(3*H*)-pyrimidinone-5'-phosphate (DArPP) uses NADPH (reduced nicotinamide adenine dinucleotide phosphate). Then, the DArPP is deaminated by the enzyme DArPP deaminase with the formation of 5-amino-6-ribosylamino-2,4(1*H*,3*H*)-pyrimidinedione-5'-phosphate (ArPP) (Figure 1.2) (Burrows and Brown, 1978).

However, the order of these two reactions is reversed in eubacteria. In eubacteria and plants, DARPP is first deaminated to 5-amino-6-ribosylamino-2,4(1*H*,3*H*)-pyrimidinedione-5'-phosphate (ARPP), and the ribose moiety of the intermediate ARPP is then reductively ring-opened to generates ArPP (Figure 1.2).

In the fourth reaction, ArPP undergoes further dephosphorylation into 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (ArP) by the catalytic action of a non-specific phosphatase (Figure 1.2), however, the mechanism of dephosphorylation is not known (Fischer et al., 2005; Abbas and Sibirny, 2011). Furthermore, it was found that haloacid dehalogenase superfamily members catalyze the dephosphorylation of ArPP in *B. subtilis*, *E. coli*, and *Bacteroides thetaiotaomicron* (Burrows and Brown, 1978; Richter et al., 1997), serving the function of this phosphatase.

In the fifth reaction, the other initial precursor of riboflavin biosynthesis, Ru5P, is transformed into 3,4-dihydroxy-2-butanone-4-phosphate (DHBP) through a skeletal rearrangement catalyzed by the DHBP synthase (Figure 1.2). This reaction is characterized by extraordinary complexity and the DHBP synthase has now been isolated and identified from many microorganisms (Fassbinder et al., 2000).

In the sixth reaction, 6,7-Dimethyl-8-ribityllumazine synthase (or lumazine synthase), was first isolated from *B. subtilis* as a complex with RF synthase, catalyzes the condensation of ArP with DHBP to generate 6,7-dimethyl-8-ribityllumazine (DRL) (Figure 1.2) (Bacher et al., 1997).

In the final step in the riboflavin biosynthesis pathway, two molecules of DRL transformed into one riboflavin and the other one into ArPP via an unusual dismutation, which is catalyzed by the riboflavin synthase. The reproduced ArPP may be recycled for the biosynthesis of another DRL (Figure 1.2) (Bacher et al., 1980).

FMN, also known as riboflavin-5'-phosphate (Figure 1.1), is formed by phosphorylation of riboflavin in the C-5'-position of the ribityl chain catalyzed by riboflavin kinase. In total, two groups of riboflavin kinases were identified. One group is represented by monofunctional riboflavin kinase proteins in fungi, plants, animals,

archaea, and eubacteria (rarely) (Bacher, 1991). Other group is bifunctional riboflavin kinase/FAD synthetase, which was found to be the basic enzyme in eubacteria (Efimov et al., 1998; Mack et al., 1998). FAD synthetase catalyzes the transfer of adenylyl residues from ATP to FMN and generates FAD.

1.3. Riboflavin biosynthesis regulation and strain improvement

At present, industrial riboflavin production is performed using recombinant strains of *A. gossypii* and *B. subtilis* (Stahmann et al., 2000; Kato and Park, 2012). Companies such as Hubei Guangji Pharmaceutical Co. Ltd. (China) and DSM[®] (Netherlands) use engineered strains of *B. subtilis*, while riboflavin production by BASF[®] (Germany) performs based on *A. gossypii* (Abbas and Sibirny, 2011; Schwechheimer et al., 2016). Previously, the strain dep8 of *C. famata* was used by ADM company (USA) (Abbas and Sibirny, 2011).

It is reported that the industrial riboflavin synthesis processes using three microorganisms, *A. gossypii*, *B. subtilis*, and *C. famata*, have been improved, and the riboflavin titer can reach more than 20 g/L (Heefner et al., 1993; Lee et al., 2004; Schwechheimer et al., 2016), while other microorganisms have not shown even close potential on riboflavin production. Therefore, the following content mainly reviews the riboflavin biosynthesis regulation and improvement of these three microorganisms.

1.3.1. In *Bacillus subtilis*

The Gram-positive bacterium *B. subtilis* cannot naturally oversynthesize riboflavin. During the past two decades, it has become the main producer of riboflavin after mutagenesis and genetic engineering (Schallmeyer et al., 2004).

The European Food Safety Authority (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) concluded that the use of riboflavin

produced by *B. subtilis* KCCM 10445 (EFSA and FEEDAP et al., 2022) and *B. subtilis* CGMCC 13326 (EFSA and FEEDAP et al., 2023) are safe for the target species, the consumer and the environment. Additionally, through a comprehensive characterization of the enzymes involved in the riboflavin biosynthetic pathway, *B. subtilis* has been successfully engineered to serve as a cell factory for riboflavin production (Mack et al., 1998; Wang et al., 2014; You et al., 2021). Therefore, *B. subtilis* has been the species of an important choice for commercial riboflavin production (Averianova et al., 2020; You et al., 2021; Liu et al., 2023).

Many studies focused on precursor supply and regulation of the key enzymes, which were considered to be the two major limiting factors in riboflavin production (Hümbelin et al., 1999; Tännler et al., 2008).

In *B. subtilis*, Ru5P and GTP as precursors in riboflavin biosynthesis pathway. The main generation of Ru5P in *B. subtilis* are the pentose phosphate pathway (PPP) and gluconate pathway.

In the oxidative branch of PPP, glucose-6-phosphate (G6P) is first converted to 6-phosphogluconolactone by G6P dehydrogenase and then hydrolyzed to 6-phosphogluconic acid by 6-phosphogluconate dehydrogenase, which is further catalyzed to Ru5P through oxidative decarboxylation. In the non-oxidative pentose phosphate pathway, fructose-6-phosphate and glyceraldehyde-3-phosphate undergo transaldolase and transketolase reactions to produce Ru5P (Moritz et al., 2000; Rakitzis and Papandreou, 1998; Phégnon et al., 2024). In the gluconate pathway, glucose is catalyzed by glucose dehydrogenase to gluconate, which is then phosphorylated by gluconate kinase to G6P and enters the oxidative branch of PPP to produce Ru5P (Nogae and Johnston, 1990; Ohnishi et al., 2005; He et al., 2007).

Several metabolic engineering strategies were developed to modify the flux of the synthetic pathways and increase the availability of Ru5P in *B. subtilis* strains. To enhance the intracellular pool of Ru5P, gene *zwf* under the control of the inducible *P_{xyl}* promoter was overexpressed in *B. subtilis* PY leading to 25% increased riboflavin production (Duan et al., 2010). The individual overexpression of genes *zwf* and *gnd* from *Corynebacterium glutamicum* in *B. subtilis* RH33 led to approximately 18% and 22% increased riboflavin production, respectively, moreover, co-expression of the two genes led to a 31% increase in riboflavin production (Wang et al., 2011). In the first step of the non-oxidative branch of the PPP, ribulose 5-phosphate 3-epimerase (encoded by gene *RPE*) converts Ru5P into xylulose 5-phosphate. Yang et al. constructed the strain with inactivation of *RPE* in *B. subtilis* LY, resulting in a 5-fold increase of riboflavin production compared to the parental strain (Yang et al., 2021).

Additionally, Zhang et al. overexpressed genes *gntP* (encoding *gluconate permease*), *gntK* (encoding *gluconokinase*), and *gntZ* (encoding *phosphogluconate dehydrogenase*) in the gluconate pathway to redirect the carbon flux into the PPP, moreover, using sodium gluconate, which is a cheap bulk material, instead of glucose, resulting in a 56.3% increase of riboflavin titer.

A deeper understanding of central carbon metabolism is essential for designing effective metabolic engineering strategies aimed at enhancing riboflavin production (Abbas and Sibirny, 2011).

Gluconeogenesis is a biological process leading to the generation of glucose from non-sugar carbon substrates in central carbon metabolism. Its modification has been successfully applied to redirect the carbon flux toward the PPP in *Corynebacterium glutamicum* (Becker et al., 2005, 2007). Additionally, it also has

suggested that further improvement of riboflavin production by deregulating the key enzymes involved in gluconeogenesis could be feasible (Tännler et al., 2008).

Wang et al. investigated the effects of deregulation of gluconeogenesis on the improvement of riboflavin production by overexpression of the critical gluconeogenic genes in strain *B. subtilis* RH33. The resulting data showed that co-overexpression of the genes *gapB* (encodes NADPH-dependent glyceraldehyde 3-phosphate dehydrogenase) and *fbp* (encodes fructose-1,6-biphosphatase) led to a 21.9% increase in riboflavin titers up to 4.89 g/L in shake-flask fermentation. The study demonstrated that deregulation of gluconeogenesis is an effective strategy to enhance riboflavin production, as well as other metabolites directly from PPP and NADPH-dependent compounds using glucose as a carbon source (Wang et al., 2014).

GTP is produced in cells through the *de novo* purine synthesis pathway, which consists of ten different enzymatic reactions to generate inosine-5'-monophosphate (IMP) from 5-phosphoribosyl-1-pyrophosphate (PRPP) (Shi et al., 2014). Next, IMP is converted to GTP and ATP.

Early studies have shown *B. subtilis* mutants resistant to purine analogs 8-azaguanine, methionine sulfoxide, or decoyinine increased GTP generation and riboflavin production due to deregulation in the purine pathway (Ishii and Shiio, 1972; Matsui et al., 1977, 1979; Saxild and Nygaard, 1987).

In contrast to fungi, genes involved in the purine pathway are clustered as *pur* operon (*purEKBCSQLFMNHD*) in *B. subtilis*. The transcription of all genes starts with a δA type promoter upstream of the gene *purE*, in which no internal promoter has yet been identified (Ebbole and Zalkin, 1987).

Recent studies have focused on genetically engineering targeted modifications of purine operon regulatory elements to increase gene expression levels of the purine pathway. Expression of *pur* operon is subject to two types of regulation: transcriptional initiation and transcriptional weakening (Shi et al., 2009). Special DNA sequences called *Pur*-Boxes are present in the upstream control region of the purine operon transcription initiation site (Bera et al., 2003).

The *PurR-PurBox* system is involved in purine synthesis, transport, and metabolic function (Mironov et al., 1994). When cells contain high concentrations of PRPP, which binds to the repressor protein PurR (encoded by *purR* gene), preventing PurR from binding to *Pur*-Box and thereby allowing normal transcription of the purine operon (Bera et al., 2003; Weng et al., 1995). Besides regulating the expression of purine pathway genes, PRPP also competes with adenosine-5'-monophosphate (AMP) for the catalytic binding site of PRPP amidotransferase, encoded by *purF*, which is a key regulatory enzyme in the de novo purine biosynthesis pathway. (Smith et al., 1994).

Therefore, reducing the generation of the protein PurR is considered an effective strategy. Asahara et al. knocked out the gene *purR* in *B. subtilis* 168, which increased the β -galactosidase activity encoded by the reporter gene *lacZ* by 5-fold (Asahara et al., 2010).

Additionally, a study reported that genetic manipulation of deregulated purine biosynthetic pathway in the *B. subtilis* BS89, via elimination of transcription repression by deletion of the repressor PurR (encoded by gene *purR*) and the 5'-untranslated region of *pur* operon with a guanine sensing riboswitch. Subsequently, removal of the product-feedback inhibition of PRPP aminotransferase (encoded by gene *purF*) by site-directed mutagenesis was done (Shi et al., 2014). The data showed that the metabolic flux through the purine biosynthesis pathway was successfully improved, resulting in

a maximum riboflavin production of 827 mg/L in shake flask fermentation by the engineered strain BS110 (Shi et al., 2014).

GTP can also be synthesized from purine bases or purine nucleosides catalyzed by purine phosphoribosyl transferases in the purine salvage pathway. Knockout of adenine phosphoribosyl transferase (encoded by gene *apt*), xanthine phosphoribosyl transferase (encoded by gene *xpt*) and adenine deaminase (encoded by gene *adeC*) increased riboflavin production by 14.02%, 6.78%, and 41.50% in the background of strain BSRP (*purRΔ*), respectively (Sun et al., 2020).

In contrast to fungi, the regulation of riboflavin biosynthesis regulation in *B. subtilis* is controlled by feedback repression of the *rib* operon through the riboswitch FMN-specific element (RFN) (Jiménez et al., 2005; Ledesma-Amaro et al., 2015; Pedrolli et al., 2015) (Figure 1.3). This highly conserved RNA motif selectively binds to the coenzyme FMN and regulates the expression of FMN biosynthesis-associated genes (Meyer et al., 2015; Pedrolli et al., 2015). The *rib* operon has also been studied in *B. amyloliquefaciens*, *B. halodurans*, *B. abortus*, *Shewanella oneidensis*, and *C. glutamicum* etc. (Vitreschak, 2002; García-Angulo, 2017).

In *B. subtilis*, the total length of *rib* operon is nearly 4.3 kb, and it consists of five genes *ribG*, *ribB*, *ribA*, *ribH*, *ribT* (Figure 1.3). Among them, *ribA* and *ribG* encode bifunctional (2-domain-containing) enzymes.

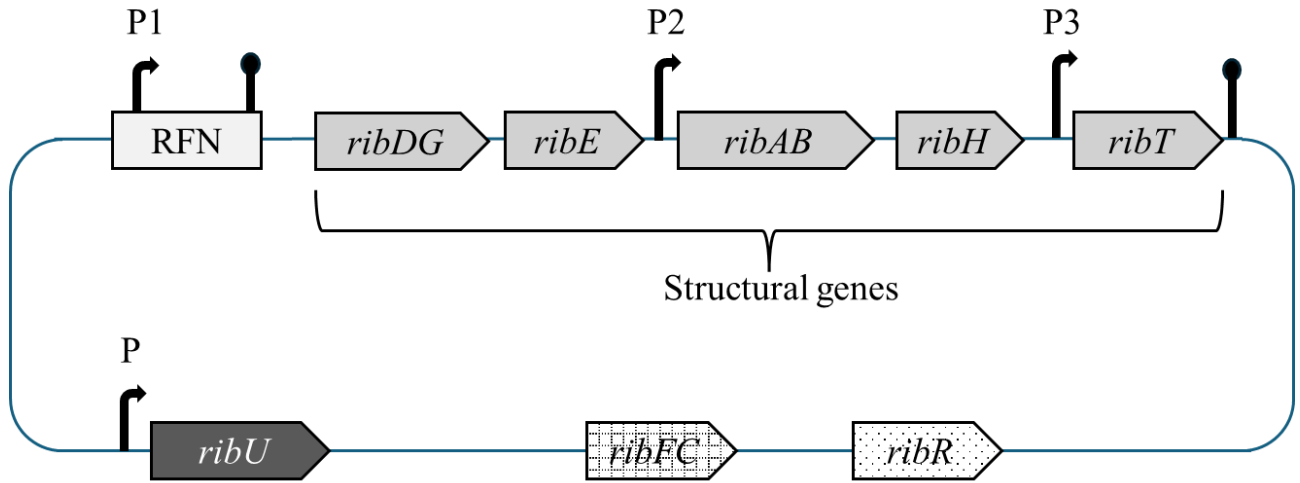


Figure 1.3 Scheme of *rib* operon in riboflavin biosynthesis regulation by *B. subtilis* reproduced from references (Averianova et al., 2020; Zhang et al., 2022). RFN, chromosomal FMN-specific element; *ribDG-E-AB-H-T*, *rib* operon; *ribU*, gene encoding riboflavin transporter; *ribFC*, gene encoding bifunctional flavokinase/FAD synthetase; *ribR*, gene encoding monofunctional flavokinase RibR; P1, P2, and P3 denote confirmed promoters (indicated by arrows); P – predicted promoter (indicated by arrows). The hairpins symbols denote confirmed transcription terminators.

The gene *ribA* encodes GTP cyclohydrolase II at its 3'-end and DHBP synthase at its 5'-end, which reacts with the precursors GTP and Ru5P, respectively (Bacher et al., 2000). Overexpression of truncated gene *ribA* decreased riboflavin production in *B. subtilis* RB50::[pRF69]_n::[pRF93]_m Ade⁺, while the introduction of intact gene *ribA* increased riboflavin production by 25% (Hümbelin et al., 1999). Hohmann et al. inserted the strong *VegI* promoter in the chromosome that drives the expression of gene *ribA*, and let to increase in riboflavin yield (Hohmann et al., 1998).

In addition, the genetic manipulation of the *rib* operon was performed in *B. subtilis* strain BS77, including overexpression of gene *ribA*, using the strong promoter *P43*, and deletion of gene *ribO*, which functions to regulate the transcription of

riboflavin biosynthesis pathway genes. As a result, constructed *B. subtilis* strain BS89 was 1.4-fold higher than that of parental strain (Shi et al., 2014).

The gene *ribG* encodes bifunctional DARPP deaminase and ARPP reductase activity, with a deaminase encoded at the 5' end and a reductase at the 3' end (Richter et al., 1997) (Figure 1.3). Both genes *ribB* and *ribH* encode riboflavin synthase, which is a complex enzyme consisting of a light enzyme (3 α -subunits) and a heavy enzyme (approximately 60 β -subunits) (Bacher et al., 2000). The gene *ribH* encodes the β -subunits of riboflavin synthase, also known as lumazine synthase, which catalyzes the reaction between the 5-position amino group of ArP and the carbonyl group of DHBP to eventually produce DRL (Bacher et al., 2000; Abbas and Sibirny, 2011). The gene *ribB* encodes the α -subunits of riboflavin synthase that catalyzes the dismutation of two molecules of DRL, the immediate precursor of riboflavin synthesis, yielding one molecule of riboflavin and one molecule of ArPP (Bacher et al., 2000; Abbas and Sibirny, 2011).

Recently, CRISPR-based genomic editing has been developed for multiplex gene editing in *B. subtilis* (Liu et al., 2019; Lu et al., 2019). The improved CRISPR-Cas9n mediated multiplexing system reached an efficiency of 65% for three-point mutations in genes *ribA*, *ribB*, and *ribH* (Figure 1.3) (Liu et al., 2019).

The gene *ribT* is located at the end of this operon and its function has not yet been elucidated (Figure 1.3) (Yakimov et al., 2014), until recent research revealed that its enzyme belongs to a member of GCN5-related N-acetyltransferase, which transfers the acetyl group from acetyl-CoA to a variety of substrates (Srivastava et al., 2018).

The genes *ribFC* and *ribR* play an indirect regulatory role in *rib* operon expression (Figure 1.3) (Solovieva et al., 1999). The gene *ribC* of bifunctional

flavokinase/FAD-synthetase is not linked to the riboflavin operon, but it is located elsewhere in the chromosome (Pedrolli et al., 2015). Moreover, *B. subtilis* mutant of gene *ribFC* led to an increase of riboflavin concentration up to 15 g/L (Karelov et al., 2011).

The gene *ribR* encodes an RNA-binding protein that is also not part of the *rib* operon (Figure 1.3), and is believed to act as a regulatory protein as it seems to intervene with the RFN function (Higashitsuji et al., 2007; Pedrolli et al., 2015).

Besides, the introduction of the gene *ribM* (encoding the energy-independent functional riboflavin transporter RibM) from *Streptomyces davawensis* into a high-performance *B. subtilis* riboflavin production strain increased riboflavin export (Hemberger et al., 2011). It was the first example suggesting that the improvement of riboflavin excretion is a useful strategy to increase the riboflavin yield in *B. subtilis*.

Most efforts have been focused on modifying the regulation of the *B. subtilis rib* operon and overexpressing the structural genes *ribGBAHT* (Abbas and Sibirny, 2011; Liu et al., 2023) (Figure 1.3).

Highly efficient riboflavin-producing strains were developed by introducing additional copies of genes *ribGBAHT* (Figure 1.3), which were regulated by either strong native promoters or strength-optimized synthetic bacterial and phage promoters (Sepúlveda Cisternas et al., 2018; Han et al., 2019). However, the industrial production of riboflavin in *B. subtilis* still faces several unresolved challenges, including the *ribR*-mediated regulation of FMN riboswitches, which limits riboflavin production; the unidentified phosphatases involved in the riboflavin biosynthesis pathway; the reactivity of flavins, which can damage cells; and the absence of an efficient transport system for actively exporting flavins, unlike *A. gossypii* (Acevedo-Rocha et al., 2019).

In addition to the method of reviewed above, optimization of cultivation conditions was also as a viable method to increase riboflavin production in *B. subtilis* (Wu et al., 2007; Abd-Alla et al., 2016).

Oxygen dissolved in the culture medium is one of the most important factors in the fermentation process and is related to cell biomass accumulation and riboflavin production (Yan et al., 2005; Krishna Rao et al., 2008; Zafar et al., 2012).

Man et al. investigated the effects of the change of agitation speed on riboflavin production by engineered *B. subtilis* RF1 in fed-batch fermentation. A strategy of gradually increasing the agitation speed from 600 to 900 r/min was established, and led to a 21.4% increase in riboflavin yield compared to when using a single speed 600 r/min (Man et al., 2014).

Oraei et al. selected three minerals FeSO_4 , MgSO_4 , and K_2HPO_4 as the supplements of the medium, which significantly affected riboflavin production, from thirteen different minerals via Plackett–Burman design. Subsequently, an optimized medium containing fructose, yeast extract, FeSO_4 , MgSO_4 , and K_2HPO_4 were 38.10, 4.37, 0.02, 0.85, and 2.27 g/L, respectively, revealed an 11.73 g/L riboflavin titer in a 72-h shake-flask fermentation (Oraei et al., 2018).

The FMN riboswitch is an RNA regulatory element located in the 5' untranslated region (5' UTR) of mRNAs involved in riboflavin biosynthesis. It consists of two key regions: the aptamer domain, which specifically binds FMN, and the expression platform, which determines the regulatory outcome. The aptamer domain features a conserved three-dimensional structure with helices, loops, and a binding pocket that accommodates FMN through hydrogen bonding, π -stacking, and van der Waals interactions (Nudler, 2004; Cochrane and Strobel, 2008; Roth and Breaker, 2009).

Upon FMN binding, the riboswitch undergoes a conformational change that affects gene expression. At the transcriptional level, FMN binding induces the formation of a transcription terminator hairpin, halting RNA polymerase and preventing transcription. At the translational level, FMN binding sequesters the Shine-Dalgarno sequence within a stem-loop, blocking ribosome binding and translation. Additionally, FMN binding can destabilize the RNA, promote degradation, and further reduce gene expression. This ligand-specific, energy-efficient mechanism provides a feedback loop where elevated FMN levels directly repress riboflavin biosynthesis by regulating transcription, translation, or RNA stability without the need for protein factors (Barrick and Breaker, 2007; Roth and Breaker, 2009).

1.3.2. In *Ashbya gossypii*

The filamentous fungus *A. gossypii* was firstly isolated from diseased cotton plants of the genus *Gossypium* and was later discovered to naturally overproduce riboflavin (Aguiar et al., 2015; Goodman and Ferrera, 1954). After strain improvements, high specific productivity was achieved in 1990s when biotechnological riboflavin production using *A. gossypii* was established (Stahmann et al., 2001; Wendland and Walther, 2005).

Recently, optimizing the riboflavin production process using *A. gossypii* and developing high riboflavin-producing strains of *A. gossypii* remain key research goals (Nieland and Stahmann, 2013; Aguiar et al., 2017; Zhang et al., 2022).

The riboflavin production of *A. gossypii* can be increased by mutagenesis, the main methods of which include UV mutagenesis (Park et al., 2007), chemical mutagenesis (Tajima et al., 2009), and disparity mutagenesis (Kato and Park, 2012). Moreover, Park et al. obtained a mutant strain *A. gossypii* W122032 with the help of a

new genetic mutation technique, which used the mutation of DNA polymerase δ on the lagging chain, losing its DNA repair function. In an optimized medium containing waste plant oils, this strain produced 13.7 g/L of riboflavin in a 3 L fermenter for nine days, and this riboflavin yield revealed an increase of 9-fold compared to the parental strain (Park et al., 2011).

Till today, *A. gossypii* has been utilized in industrial riboflavin production for over twenty years (Schwechheimer et al., 2016; Liu et al., 2023). Current studies primarily focus on the molecular level (Kato et al., 2021, 2024).

To characterize the riboflavin biosynthetic pathway, Jeong et al. conducted ^{13}C -metabolic flux analysis in a wild-type strain, *A. gossypii* ATCC 10895, and a mutant strain, *A. gossypii* W122032, to analyze differences in their central carbon pathways. The study found that the metabolic flux into pentose-5-phosphate via the PPP was 9% higher in the mutant strain compared to the wild-type strain. Additionally, the metabolic flux of the purine synthetic pathway in the mutant strain was 16-fold that of the wild-type strain. These results indicate that riboflavin production in the mutant strain of *A. gossypii* was enhanced by an increased GTP flux through the PPP and purine synthetic pathway. This suggests that reinforcing the expression of genes related to the PPP and purine synthetic pathway could be a potential strategy for improving riboflavin production (Jeong et al., 2015).

Since GTP is one of the key precursors for riboflavin biosynthesis, the *de novo* purine biosynthesis pathway has attracted much attention (Abbas and Sibirny, 2011). Purine biosynthesis is a highly regulated pathway controlled at both the transcriptional and metabolic levels (Mateos et al., 2006). The PRPP is the central compound of cell metabolism and is directly involved in the *de novo* and *salvage* biosynthesis of GTP (Dmytruk et al., 2020).

Two key enzymes in the purine biosynthesis pathway, PRPP amidotransferase (encoded by gene *AgADE4* in *A. gossypii*) and PRPP synthetase (encoded by the genes *AgPRS1*, *AgPRS2,4*, *AgPRS3* and *AgPRS5* in *A. gossypii*), are regulated through feedback inhibition by their end products. Accordingly, *A. gossypii* strains were constructed by overexpressing inhibition-resistant forms of PRPP amidotransferase and PRPP synthetase, and riboflavin production of constructed strains revealed 10-fold and 2-fold increases, respectively (Jiménez et al., 2005, 2008).

A. gossypii as a flavinogenic strain, it should ensure a strong flux through the guanine nucleotide pathway to increase the bioavailability of GTP. The inosine-5'-monophosphate (IMP) is the common precursor of adenine and guanine nucleotides in the purine biosynthetic pathway, and its oxidative transformation into xanthosine-5'-monophosphate (XMP) catalyzes by inosine-5'-monophosphate dehydrogenase (IMPDH), a reaction step which is rate-limiting. Finally, XMP is converted into GMP and further to GTP in successive steps (Buey et al., 2015).

It was conducted a comprehensive functional and structural analysis of the enzyme IMPDH from *A. gossypii*. It revealed that overexpression of the gene *impdh* in *A. gossypii* ATCC 10895 led to an enhanced metabolic flux through the guanine biosynthetic pathway, ultimately resulting in a 1.4-fold increase in riboflavin production (Buey et al., 2015).

(Ledesma-Amaro et al., 2015) provided a scalable strategy to increase industrial riboflavin production. It was found that riboflavin production in *A. gossypii* A330 was improved 5.4-fold by combining the overexpression of all the *rib* genes and the inactivation of the gene *ADE12*, which encodes adenylosuccinate synthase and controls the formation of AMP from IMP (Figure 1.4).

Meanwhile, glycine as a precursor of purine nucleotides stimulates riboflavin biosynthesis in *A. gossypii*. To improve the supply of glycine, overexpression of the gene *GLY1* (encoding threonine aldolase) increased a 10-fold in the specific activity of threonine aldolase and 9-fold riboflavin production when the medium was supplemented with threonine (Figure 1.4) (Monschau et al., 1998). Similarly, a successful strategy for increasing the glycine precursor supply was the inactivation of hydroxymethyltransferase (encoded by gene *SHM2*), converting glycine into serine in *A. gossypii*, consequently, leading to the 10-fold increase in riboflavin production (Figure 1.4) (Schl pen et al., 2003).

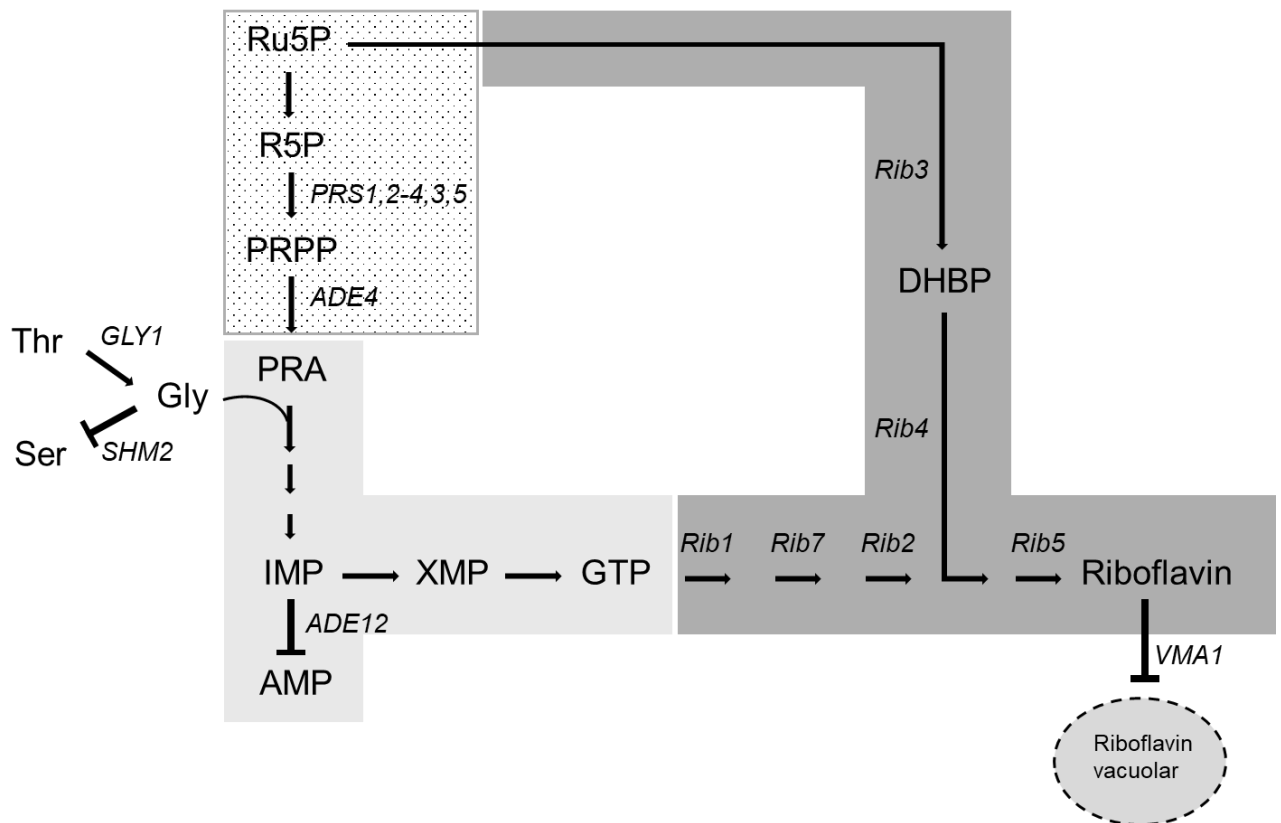


Figure 1.4 Metabolic engineering strategies for riboflavin overproduction in *A. gossypii*. The pentose phosphate pathway is indicated as a dot box, purine biosynthesis pathway is indicated as light gray box, and riboflavin biosynthesis pathway is indicated as dark box. AMP, adenosine-5'-monophosphate; DHBP, 3,4-dihydroxy-2-butanone-4-

phosphate; GTP, guanosine triphosphate; IMP, inosine monophosphate; PRA, 5-phosphoribosylamine; PRPP, 5-phosphoribosyl-1-pyrophosphate; R5P, ribose-5-phosphate; Ru5P, ribulose-5-phosphate; Gly, glycine; Ser, serine; Thr, threonine (Revuelta et al., 2017).

Schlösser et al. found that overexpression of the three genes *Rib3*, *Rib4*, and *Rib5* in *A. gossypii* involved in the riboflavin biosynthesis pathway resulted in 2-fold riboflavin accumulation (Figure 1.4) (Schlösser et al., 2007). Moreover, Ledesma-Amaro et al. performed the overexpression of all the *Rib* genes under the guide of computational metabolic modeling. Although genes *Rib1* and *Rib3* were the major limiting steps in riboflavin biosynthesis, the strain overexpressing all the *Rib* genes showed the highest riboflavin production (Ledesma-Amaro et al., 2015).

Silva et al. were the first who investigated the effect of blockage of the *de novo* pyrimidine biosynthetic pathway on riboflavin production by *A. gossypii* (Silva et al., 2015). The *A. gossypii* Agura3 is an uracil auxotrophic strain derived from *A. gossypii* ATCC 10895 (Aguiar et al., 2014). It was found that riboflavin production was increased in this strain on a standard solid complex medium. Furthermore, high concentrations of uracil repressed the riboflavin production and cell growth of this strain and its parent strain. However, the molecular mechanism underlying the extreme sensitivity of *A. gossypii* Agura3 to uracil remains unclear (Silva et al., 2015).

It is also important to note that the knockout of the gene *VMA1*, which encodes the structure of vacuolar ATPase in *A. gossypii*, prevents riboflavin from remaining in the vacuole compartment and directs riboflavin flux to the medium (Figure 1.4) (Förster et al., 1999, 2001).

Glucose, yeast extract, and vegetable oil are often used as carbon sources in the culture medium in *A. gossypii*. Yeast extract's primary role in microbial anabolism is to provide nitrogen and other essential growth factors required for biosynthetic processes, while vegetable oil is a carbon source in microbial fermentation, and primarily supplies lipids and fatty acids (Schwechheimer et al., 2016). In industrial fermentation, vegetable oil was preferred as the main carbon source in *A. gossypii*. However, an effective glyoxylate cycle is necessary to convert acetyl-CoA into the carbohydrate precursors required for riboflavin production (Averianova et al., 2020).

Overexpression of the gene *ICL1*, encoding isocitrate lyase, enhanced riboflavin production in a medium containing vegetable oil (Sugimoto et al., 2014). To improve the efficiency of vegetable oil consumption and riboflavin production was performed by overexpressing the malate synthase, which is the second enzyme of the glyoxylate pathway (Sugimoto et al., 2014).

Schwechheimer et al. successfully achieved for the first time the quantitative calculation of carbon fluxes in the growth phase of *A. gossypii* in vegetable oil and complex medium, as well as the quantitative calculation of the carbon fluxes during the riboflavin biosynthesis phase in an industrial riboflavin production set-up with the help of ^{13}C isotope experiments. These results highlighted the significant impact of vegetable oil as the main carbon source for riboflavin production in *A. gossypii*, even though yeast extract was used to activate the strain growth. These results brought some new insights into metabolism of *A. gossypii* under complex industrial cultivation conditions (Schwechheimer et al., 2018a, 2018b).

Jiménez et al. used two modern effective CRISPR-Cas9 (Jiménez et al., 2019) and CRISPR-Cas12a (Jiménez et al., 2020) mediated genome-editing systems that have been adapted for *A. gossypii*, enabling the efficient introduction of deletions,

insertions, and nucleotide substitutions. The two CRISPR-Cas systems suitable for metabolic engineering of *A. gossypii* will contribute to facilitating engineering approaches for riboflavin production.

Recently, the significance of branched-chain amino acids was investigated by overexpressing gene *AgILV2* (encoding acetohydroxy-acid synthase which catalyzes the first reaction of branched-chain amino acid biosynthesis) in *A. gossypii* MT. This resulted in increasing strain growth and riboflavin production in *A. gossypii*. Thus, it opens a new approach for the effective riboflavin overproduction in *A. gossypii* (Kato et al., 2023).

The disruption of genes *AgHST1* and *AgHST3* (sirtuins that are NAD⁺-dependent protein deacetylases) leads to the overproduction of riboflavin in *A. gossypii* (Kato et al., 2021). Furthermore, it found that the generation of reactive oxygen species in *AgHST1Δ* and *AgHST3Δ* mutants of *A. gossypii* was increased compared with the parental strain, which was caused by oxidative stress. These data established that oxidative stress is an important trigger for riboflavin overproduction in sirtuin gene-disrupted mutant strains of *A. gossypii* (Kato et al., 2024).

1.3.3. In *Candida famata*

C. famata (teleomorph *Debaryomyces subglobosus*), is the second natural riboflavin-producing strain. *C. famata* has the ability to assimilate multiple carbon sources and tolerate high salinity levels, grows in the presence of high concentrations of NaCl, up to 2.5 M (Dmytruk and Sibirny, 2012).

C. famata belongs to one of flavinogenic yeasts (Nguyen et al., 2009; Dmytruk and Sibirny, 2012), which overproduce riboflavin under iron starvation, and the exact reasons are not known until today. This flavinogenic yeasts also includes *Meyerozyma*

guilliermondii, *Schwanniomyces occidentalis*, *Candida albicans* and some others (Abbas and Sibirny, 2011; Dmytruk and Sibirny, 2012).

The industrial strain *C. famata* dep8 (ATCC 20849) was selected using conventional mutagenesis by Coors and was used for industrial production of riboflavin by ADM (Heefner et al., 1992, 1993; Stahmann et al., 2000). However, its industrial fermentation was terminated because of genetic instability (Abbas and Sibirny, 2011).

Dmytruk et al. has found that introduction to *C. famata* dep8 additional copy of gene *SEF1* (encoding a putative transcription factor) derived from the relative flavinogenic yeast *D. hansenii* (with sequenced genome) greatly stabilized the strain and increased riboflavin production.

Subsequently, the riboflavin-overproducing strain AF-4 was derived from the wild-type strain *C. famata* VKM Y-9 through classical selection methods. It showed to be genetically stable and exhibited comparable levels of riboflavin production compared to strain dep8. In shake flasks, it accumulated about 680 mg riboflavin/L in iron-sufficient medium, whereas strain VKM Y-9 accumulated riboflavin only 2 to 3 mg/L under the same condition (Dmytruk et al., 2011).

Besides, effective genetic transformation of *C. famata* was developed, reaching frequency of 10⁵ transformants/mg plasmid DNA (Voronovsky et al., 2002).

Notably, *C. famata* uses an alternative genetic code in which the CUG codon (leucine) codes for serine. Fortunately, the gene *ble* of *Staphylococcus aureus* does not contain a CUG codon within its open reading frame and encodes a protein that confers resistance to the antibiotic phleomycin (Gatignol et al., 1987; Semon et al., 1987). Therefore, it was used as a dominant selection marker for yeast transformant selection.

Moreover, modified genes *IMH3* and *ARO4* of *D. hansenii* conferring resistance to mycophenolic acid and fluorophenylalanine, respectively, were successfully used as dominant selection markers (Dmytruk et al., 2006, 2011, 2014; Bratiichuk et al., 2020).

A system for analysis of promoter activities was developed for the yeast *C. famata* and was found the strong promoter *TEF1* derived from *C. famata*. The gene *LAC4* (encoding β -galactosidase) of *Kluyveromyces lactis* as a reporter gene and mutant *C. famata lac4* unable for lactose utilization as a recipient strain were used in the system. The resulting constructed strain was able to utilize lactose as a single carbon source (Ishchuk et al., 2008). Moreover, the CRISPR-Cas9 genome editing methods have been adapted for use in *C. famata*. (Lyzak et al., 2017).

Besides, methods of insertion mutagenesis have been developed for *C. famata* and disruption of genes *SEF1* and *MET2* (homoserine *o*-acetyltransferase) led to the inability to oversynthesize riboflavin (Dmytruk et al., 2006).

In *C. famata*, the structural genes of riboflavin biosynthesis: *RIB1* (coding for GTP cyclohydrolase II), *RIB2* (coding for DARPP reductase), *RIB3* (coding for DArPP deaminase), *RIB5* (coding for lumazine synthase), *RIB6* (coding for DHBP synthase) and *RIB7* (coding for riboflavin synthase) were cloned from a gene library by complementation of the corresponding mutations in strains defective in riboflavin synthesis (Figure 1.2) (Dmytruk et al., 2004; Voronovsky et al., 2004).

Increasing the riboflavin biosynthesis in *C. famata* was done by overexpression of the native structural genes *RIB1* and *RIB7*, as well as the transcription activator gene *SEF1* based on the non-reverting strain AF-4. The resulting engineered strain is designated as BRP, which can overproduce riboflavin up to 16.4 g/L in a 7 L laboratory bioreactor during fed-batch fermentation in the optimized medium (Dmytruk et al.,

2014). Additionally, overexpression of the native structural gene *RIB6* also increased riboflavin production in strains AF-4 and BRP (Petrovska et al., 2022).

It was shown earlier that the disruption of the gene *VMAL* (coding for vacuolar ATPase) caused riboflavin oversynthesis in the *A. gossypii* and *M. guilliermondii* (Förster et al., 1999; Boretsky et al., 2011). Knock out of gene *VMAL* in the *C. famata* L2 (*leu*) also led to riboflavin oversynthesis (Andreieva et al., 2020a), still the mechanisms by which vacuolar ATPase regulates riboflavin synthesis remain unclear (Sibirny, 2023).

It was found that knock out or point mutations of the mentioned before regulatory gene *SEF1* led to the inability to overproduce riboflavin whereas its overexpression led to enhanced genetic stability and an increase in riboflavin production (Dmytruk et al., 2011, 2006). A study demonstrated that the *SEF1* promoters from flavinogenic yeasts (*C. famata* and *C. albicans*), as well as from *Candida tropicalis*, were capable of restoring riboflavin overproduction in mutant *sef1Δ* of *C. famata*. In contrast, *SEF1* promoters from non-flavinogenic yeasts, like *Scheffersomyces stipitis* and *S. cerevisiae*, could not do so (Andreieva et al., 2020b).

Additionally, another regulatory gene *SFUI* was identified in *C. famata*. In *C. albicans*, the suppression of gene *SEF1* by the GATA family transcription factor Sfu1 occurs due to physical binding with factor Sef1, thereby sequestration of factor Sef1 to the cytoplasm, where it is unable to perform transcriptional activation of iron acquisition genes (Chen and Noble, 2012; Lan et al., 2004). The mutant *sfu1Δ* of *C. famata* exhibited increased riboflavin production, in contrast to that of *SFUI* deletion or points mutation in *C. albicans*. This suggested that factor Sef1 plays a role of negative factor in regulation of riboflavin biosynthesis (Andreieva et al., 2020b).

The mammary gland is known to contain riboflavin pump that is responsible for the efflux of this vitamin into milk (van Herwaarden et al., 2003). Several homologs of the mammalian gene *BRCP* were identified from the flavinogenic yeast *D. hansenii* with sequenced genome (Mao et al., 2004). An additional factor involved in the regulation of riboflavin biosynthesis in the flavinogenic yeasts appeared to be the gene *RFE1* (homolog of gene *BCRP*), which encodes putative riboflavin efflux protein Rfe1 responsible for the excretion of riboflavin from the yeast cell (Tsyrlunyk et al., 2020). It was performed that overexpression of gene *RFE1* under control of strong constitutive promoter *C. famata TEF1* in the advanced riboflavin overproducing strain BRP. The results data showed that the constructed strain BRP/RFE1 had an increase in riboflavin production accumulation after 120 h of flask cultivation near 1.7 g/L (Tsyrlunyk et al., 2020). It is interesting to note that the expressed protein Rfe1 was localized in the yeast cell membranes but not in the nucleus.

The activation of the biosynthesis of GTP was achieved via overexpression of the modified genes *PRS3* (coding for phosphoribosyl pyrophosphate synthetase) and *ADE4* (coding for phosphoribosyl amidotransferase) to avoid the feedback inhibition of both enzymes of purine nucleotide biosynthesis *de novo*. Subsequently, the study by Dmytruk et al. showed that the advanced strain designed as BRPI via overexpression of the modified heterologous genes *PRS3* and *ADE4* from *D. hansenii* in the strain BRP, led to an increase of GTP pool and 2-fold enhanced riboflavin production (Dmytruk et al., 2020).

Also, overexpression of gene *GND1* coding for 6PG dehydrogenase in the *C. famata* strains L2, AF-4, and BRP led to increased riboflavin accumulation on cheese whey (Ruchala et al., 2022). Till now, the role of 6PG dehydrogenase in yeasts and filamentous fungi has not been studied whereas overexpression of gene *gnd* from *C.*

glutamicum was found to increase intracellular pool of Ru5P and riboflavin yield in bacteria *B. subtilis* and *E. coli* (Wang et al., 2011; Lin et al., 2014).

Using cheap and readily available substrates is a major goal in industrial fermentation. Previously, riboflavin producers were cultivated in the medium of glucose as carbon sources. However, *C. famata* grows on lactose and displays β -galactosidase activity (Ishchuk et al., 2008). Riboflavin synthesis on cheese whey was activated in *C. famata* strains, which overexpressed gene *SEF1* under the control of lactose-induced promoter of gene *LAC4* (encoding β -galactosidase) (Tsyrunyk et al., 2021). Recently, it was found that *C. famata* strains also grow and overproduce riboflavin in cheese whey supplemented only with nitrogen source like ammonium sulfate (Ruchala et al., 2022).

In a recent study, it was found that lignocellulosic hydrolysate, including xylose and L-arabinose, supports robust growth and riboflavin synthesis in *C. famata*. The engineered strain BRPI/XYL1, which overexpressed gene *XYL1* coding for xylose reductase, exhibited increased riboflavin production and reached 1.5 g/L during a bioreactor using bagasse hydrolysate as the carbon source (Dzanaeva et al., 2024).

1.4. Concluding remarks

The literature review revealed the biosynthesis pathway of riboflavin and flavin coenzymes in bacteria, fungi, and yeasts. The main difference between the synthesis of riboflavin in bacteria, and, on the other hand, in yeasts and fungi is considered. It consists of changing the order of reactions of deamination and reduction of the ribosylated pyrimidine product of GTP cyclohydrolase II. Still the nature of phosphatase(s) in the riboflavin biosynthesis pathway is yet to be identified.

The literature review focused on the regulatory factors of the riboflavin biosynthesis production pathway in three microorganisms *B. subtilis*, *A. gossypii*, and *C. famata*, as well as the effective strategies for increasing the supply of riboflavin precursors GTP and Ru5P. The riboflavin production has been significantly improved via genetic manipulation, metabolic pathway regulation, optimization of culture conditions, etc.

B. subtilis, *A. gossypii*, and *C. famata* still have unknown modifications and are still far away from the theoretically possible maximal yield. The mechanisms for the role of riboflavin overproduction by *Candida* spp. under iron-deficient conditions are still unknown. The role and effect of various antimetabolites remain unknown in these microorganisms.

2. MATERIALS AND RESEARCH METHODS

2.1. Chemical and biological materials

Chemical compounds, reagents and enzymes produced by the companies "Sigma-Aldrich" (USA), Honeywell Fluka™ (Germany), "FIRMA CHEMPUR" (Poland), "Merck Millipore" (USA), "New England Biolabs" (USA), "Promega" (USA), "Roth" (Germany), "DIFCO" (USA), "REANAL" (Hungary), "Fermentas" (Lithuania), "ZYMO RESEARCH" (USA) were used for the research. Chemical reagents of Ukrainian production with the qualification "xx" and "очч" were also used.

2.2. Growth conditions

The yeast strains were used throughout the described work and were grown on 30 °C on rich YPD (1% yeast extract, 1% peptone, and 2% glucose), or mineral YNB (0.17% yeast nitrogen base, 0.5% ammonium sulfate, and 2% glucose) media. The antibiotics phleomycin or nourseothricin at a final concentration of 20 mg/L were added to the YPD medium for the selection of yeast transformants.

To characterize the riboflavin production, the constructed yeast strains were carried out in the shaker (200 rpm) at 30 °C in 10 mL of liquid medium in 100 mL Erlenmeyer flasks for 5 days. The following cultivation liquid media were used: YPD (rich medium); YNB (minimal medium) + 0.05% yeast extract (YE); cheese whey (The supernatant of high temperature deproteinization after centrifugation, with 5% lactose and 0.3% ammonium sulfate).

The *Escherichia coli* strain DH5 α was grown at 37 °C in LB (0.5% yeast extract, 1.5% peptone, and 0.5% NaCl) medium as described (Sambrook et al., 1989). The transformed *E. coli* cells were maintained in LB medium containing 100 mg/L of ampicillin.

2.3. Strains, primers, and plasmids

Yeast strains *Candida famata*, *Debaryomyces hansenii*, *Candida albicans*, *Candida tropicalis*, *Scheffersomyces (Picha) stipitis*, *Saccharomyces cerevisiae* and a bacterial host *Escherichia coli* DH5 α were used in this work.

Table 2.1

The list of strains used in this work.

Strains	Description of Relevant genotype	Source/reference
1	2	3
<i>C. famata</i> VKM Y-9	Wild-type strain	All-Russian Collection of Microorganisms, Russia
<i>C. famata</i> L2	leucine-deficient (<i>leu2</i> ⁻)	(Voronovsky et al., 2002)
<i>C. famata</i> AF-4	Isolated from VKM Y-9 by classical mutagenesis and selection.	(Dmytruk et al., 2011)
<i>C. famata</i> BRP	AF-4/SEF1-RIB1-RIB7	(Dmytruk et al., 2014)
L2/ZWF1	Derived from L2 by overexpressed gene <i>ZWF1</i> .	(Andreieva, 2022)
AF-4/ZWF1	Derived from AF-4 by overexpressed gene <i>ZWF1</i> .	(Andreieva, 2022)

1	2	3
BRP/ZWF1	Derived from BRP by overexpressed gene <i>ZWF1</i> .	(Andreieva, 2022)
L2/GND1	Derived from L2 by overexpressed gene <i>GND1</i> .	(Ruchala et al., 2022)
AF-4/GND1	Derived from AF-4 by overexpressed gene <i>GND1</i> .	(Ruchala et al., 2022)
BRP/GND1	Derived from BRP by overexpressed gene <i>GND1</i> .	(Ruchala et al., 2022)
L2/ZWF1-GND1	Derived from L2 by co-overexpressed genes <i>ZWF1</i> and <i>GND1</i> .	(Andreieva, 2022)
AF-4/ZWF1-GND1	Derived from AF-4 by co-overexpressed genes <i>ZWF1</i> and <i>GND1</i> .	(Andreieva, 2022)
BRP/ZWF1-GND1	Derived from BRP by co-overexpressed genes <i>ZWF1</i> and <i>GND1</i> .	(Andreieva, 2022)
V9/RFE1-RIB6	Derived from VKM Y-9 by co-overexpressed genes <i>RFF1</i> and <i>RIB6</i> .	Chapter 3.2
V9/RFE1-GND1	Derived from VKM Y-9 by co-overexpressed genes <i>RFF1</i> and <i>GND1</i> .	Chapter 3.2

1	2	3
V9/RIB6-GND1	Derived from VKM Y-9 by co-overexpressed genes <i>RIB6</i> and <i>GND1</i> .	Chapter 3.2
V9/RFE1-RIB6-GND1	Derived from VKM Y-9 by co-overexpressed genes <i>RFE1</i> , <i>RIB6</i> , and <i>GND1</i> .	Chapter 3.2
AF-4/RFE1-RIB6	Derived from AF-4 by co-overexpressed genes <i>RFE1</i> and <i>RIB6</i> .	Chapter 3.2
AF-4/RFE1-GND1	Derived from AF-4 by co-overexpressed genes <i>RFE1</i> and <i>GND1</i> .	Chapter 3.2
AF-4/RIB6-GND1	Derived from AF-4 by co-overexpressed genes <i>RIB6</i> and <i>GND1</i> .	Chapter 3.2
AF-4/RFE1-RIB6-GND1	Derived from AF-4 by co-overexpressed genes <i>RFE1</i> , <i>RIB6</i> , and <i>GND1</i> .	Chapter 3.2
<i>C. famata</i> L2 <i>vma1Δ</i>	Gene <i>VMA1</i> of L2 was knocked out.	(Andreieva et al., 2020b)
<i>C. famata</i> L2 <i>sef1Δ</i>	Gene <i>SEF1</i> of L2 was knocked out.	(Andreieva et al., 2020b)
L2 <i>sef1Δ</i> /pNTC/SEF1pr_Cf	L2 <i>sef1Δ</i> with expression of <i>SEF1</i> promoter from <i>C. famata</i> .	(Andreieva et al., 2020b)

1	2	3
L2 <i>sef1Δ</i> /pNTC/ SEF1pr_Ca	L2 <i>sef1Δ</i> with expression of <i>SEF1</i> promoter from <i>C. albicans</i> .	(Andreieva et al., 2020b)
L2 <i>sef1Δ</i> /pNTC/ SEF1pr_Ct	L2 <i>sef1Δ</i> with expression of <i>SEF1</i> promoter from <i>C. tropicalis</i> .	(Andreieva et al., 2020b)
L2 <i>sef1Δ</i> /pNTC/ SEF1pr_Ss	L2 <i>sef1Δ</i> with expression of <i>SEF1</i> promoter from <i>S. stipitis</i> .	(Andreieva et al., 2020b)
L2 <i>sef1Δ</i> /pNTC/ SEF1pr_Sc	L2 <i>sef1Δ</i> with expression of <i>SEF1</i> promoter from <i>S. cerevisiae</i> .	(Andreieva et al., 2020b)
<i>Debaryomyces hansenii</i> CBS767	—	(Voronovsky et al., 2002)
<i>Candida albicans</i> SC5314	—	(Gillum et al., 1984)
<i>Candida tropicalis</i> MYA-3404	—	(Butler et al., 2009)
<i>Scheffersomyce stipitis</i> CBS6054	—	American Type Culture Collection, USA
<i>Saccharomyces</i> <i>cerevisiae</i> S288C	—	(Fisk et al., 2006)
<i>Escherichia coli</i> DH5α	[Φ80dlacZΔM15, <i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>hsdR17</i> (r _K m ⁺ _K), <i>supE44</i> , <i>relA1</i> , <i>deoR</i> , Δ(<i>lacZYA</i> - <i>argF</i>) U169]	(Neidhardt, 1996)

In this work, plasmids were constructed using the method described earlier (Sambrook et al., 1989) and the instructions of the enzyme manufacturers based on the basic framework plasmids.

Table 2.2

The list of plasmids is used in this work.

Plasmids	Source/reference
1	2
pTb	(Dmytruk et al., 2006)
pUC57	(Fermentas, Vilnius, Lithuania)
pTTb	(Tsyurulnyk et al., 2020)
pZWF1	(Andreieva, 2022)
pGND1	(Ruchala et al., 2022)
pZWF1/GND1	(Andreieva, 2022)
pRFE1	(Tsyurulnyk et al, 2020)
pRFE1/GND1	Chapter 3.2
pZWF1/RIB6	Chapter 3.2
pGND1/RIB6	Chapter 3.2
pRFE1/GND1/RIB6	Chapter 3.2
p19L2	(Voronovsky et al., 2002)
pVMA1Δ_ble	(Andreieva et al., 2020a)
pSEF1Δ_LEU2	(Andreieva et al., 2020b)
pG-SAT-1	(Millerioux et al., 2011)
pNTC/SEF1pr_ <i>Cf</i>	(Andreieva et al., 2020b)
pNTC/SEF1pr_ <i>Ca</i>	(Andreieva et al., 2020b)

1	2
pNTC/SEF1pr_ <i>Ct</i>	(Andreieva et al., 2020b)
pNTC/SEF1pr_ <i>Ss</i>	(Andreieva et al., 2020b)
pNTC/SEF1pr_ <i>Sc</i>	(Andreieva et al., 2020b)

The primers used in this work were designed by our research group and then synthesized by "Fermentas".

Table 2.3

The sequences of primers used in this work.

Primer name	Primer sequence 5'-3' (restriction sites are underlined)
1	2
Ko1052	CGC <u>GGA TCC</u> ATG TCA GTA GAA CCA TTT GGT AAA CAC
Ko1053	GCA <u>CTG CAG</u> TTA CAT TTT ACC CTT GAC GTT TGG TG
Ko1054	GCA <u>CTG CAG</u> <u>GCG GCC</u> <u>GCA</u> TGT CTG CTC CAA CGT ATG TAT TCT TC
Ko1055	GCA <u>CTG CAG</u> CTA AGC ATC GTA AGT AGA GGC AGA AAC
Ko1056	CGG <u>GGT ACC</u> AAA TTG ACT GGT CTG AAA TAA TAG
Ko1057	CGG <u>GGT ACC</u> GAT TAT TGA CTC GAG ATG TTG CG
Ko1058	CAC GAA ACA ATC CAA ATA AAG CTG
Ko1059	GTT CAA TAA AAG CAT CAA CTG G
Ko817	CGC GGA TCC ATG ATA TCT ATA AGT AAC CCA ATG
Ko1352	CCG <u>CTC GAG</u> TCC TCG CAC CAA AAC CCA TCT C
Ko1353	CCG <u>CTC GAG</u> AAG TTA CAG TTA ACA TCA AAT CAA TTA AAG ATG

1	2
Ko1355	CTC TTG TGT CAT CAA TTC CG
Ko1356	ATT TTG AGT TCT TTT TCC TAC GG
Ko1357	CGG <u>GGT ACC</u> AAA TTG ACT GGT CTG AAA TAA TAG
Ko1358	CGG <u>GGT ACC</u> ATG TTG CGC CGA ACA ATC AC
Ko1359	GTT CAA TAA AAG CAT CAA CTG G
Ko1437	ATC ACC TCT GAA CCG CTT CGG
Ko1438	GTC AGA AGG CAT CGC AAG TG
Ko1439	ACT GTC ATC GAT TCA GCT GCA
Ko1440	TGC AGC TGA ATC GAT GAC AGT
ZWF1f	TTT CGT CAG AGA TGA TGA ATT AG
ZWF1r	TGC GCT GAT AAG AAA GGT CTT
GND1f	ACC CAT TCT TCA ACG ATG CTA
GND1r	CAC TTG GAA AGT GTG GGC
RFE1f	GTT CAT TGC CTC TGT TTT CCC
RFE1r	TCG CAG TCA AAT ATA CGT TGT TC
RIB6f	TTT GAT GAT GAG ATT GGA TGA TTG
RIB6r	AAA TTA CTG GTA AAA GAA AGG CC

2.4. Molecular biology techniques

2.4.1. Basic molecular genetic methods

Isolation and purification of plasmid DNA, preparation and transformation of competent *E. coli* cells, electrophoresis of DNA in agarose gel, elution of DNA fragments from agarose gel, digestion of DNA with restriction enzymes, ligation of

linearized DNA fragments, amplification of DNA fragments using polymerase chain reaction (PCR) were carried out as described in (Sambrook et al., 1989). DNA purification was performed on the columns of the company "Quiagen" (USA) (Quiagen PCR purification Kit). For amplification of DNA fragments by PCR, synthetic oligonucleotide primers from the companies "IDT Technologies" or "Sigma" (USA) were used. PCR amplification of fragments was carried out using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. PCR was performed using a GeneAmps PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA).

2.4.2. Isolation of plasmid DNA from *E. coli* cells

To isolate plasmid DNA, a set of reagents (midi-kit) Wizards Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA) was used.

E. coli transformant cells were grown in LB medium (volume 100 mL) supplemented with ampicillin on the shaker (180 rpm) at 37 °C, overnight. And sedimented by centrifugation (4,000 rpm, 10 min) in 50 mL of plastic tubes to completely remove the remains of the nutrient medium. The cell pellets were resuspended in 2 mL of TE buffer (10 mM Tris HCl, pH 8.0; 1 mM EDTA, pH 8) with stirring and added 2 ml of solution I (0.2 M NaOH solution and 1% sodium dodecyl sulfate (SDS)), then incubated at room temperature for 15 min. After that, add 3 mL of solution II (2 M potassium acetate), gently mixed, and incubated on ice for 15 min. The protein precipitate was removed by centrifugation at 4,000 rpm for 10 min at room temperature and the supernatant (approximately 7 mL) was collected in clean test tubes. One volume of isopropanol was added to the supernatant, mixed, and centrifuged at 4,000 rpm for 20 min at room temperature. The precipitate of DNA was washed with 70% ethanol, after drying, dissolved in 80 µL of TE buffer, then stored at -20 °C.

2.4.3. Isolation of total DNA from yeast cells

This method is a modification of the method developed for *S. cerevisiae* (Johnston, 1994) using lyticase for spheroplasting of cells, ribonuclease A for RNA cleavage, and DNA precipitation with alcohol.

Yeast cells were grown in 3 mL of liquid selective medium at 30 °C until the late logarithmic phase (OD_{600} 4–6; cuvette 1 cm). Biomass was precipitated by centrifugation and resuspended in 0.3 mL of 50 mM ethylenediaminetetraacetic acid (EDTA) solution (pH 8.0). Lyticase was added to the resuspended cells and incubated for 60 min at 37 °C. Cells were pelleted by centrifugation at 10,000 rpm for 2 min, the supernatant was drained, and 0.3 mL of lysis solution (0.2% SDS, 50 mM EDTA) was added to the pellets. After resuspending the pellets, the microtubes were heated at 65 °C for 15–30 min. The microtubes were cooled to room temperature, 0.1 mL of 3 M potassium acetate (pH 5.2) was added to the incubation mixture, mixed vigorously, and kept on ice for 5 min, then centrifuged at 10,000 rpm for 3 min. Supernatant transferred to clean microtubes containing 0.3 mL isopropanol each. The samples were kept for 10 min at room temperature, after which they were centrifuged at 10,000 rpm for 3 min, the supernatant was poured off, and the pellets were washed with 70% ethanol and dried. The resulting DNA was dissolved in 100 μ L of TE buffer, 5 μ L of ribonuclease A solution (10 mg/mL) was added, mixed and incubated for 20 min at 37 °C. 100 μ L of phenol was added to the incubation mixture, centrifuged at 12,000 rpm for 15 min, the aqueous phase was transferred to a fresh centrifuge tube, 0.1 volume of 3 M potassium acetate pH 5.2 and 2 volumes of 96% ethanol were added. It was kept on ice for 15 min, centrifuged at 12,000 rpm for 15 min, and the resulting precipitate was washed with 70% ethanol and dried. The obtained DNA was dissolved in 30 μ L of TE buffer and stored at -20 °C.

2.4.4. Construction of plasmids

The method of DNA cleavage by restriction enzymes is based on the ability of class II restriction endonucleases to cleave DNA at certain specific sites. In this work, we used the method described earlier (Sambrook et al., 1989), and the instructions of the enzyme manufacturers: "Fermentas" (Lithuania), "NEB" (USA), "Promega" (USA). T4 DNA polymerase was used to blunt the "sticky" (complementary) ends of linearized vector DNA (for the purpose of subsequent ligation of DNA fragments with "blunt" ends). Ligation of linearized DNA fragments was carried out using T4 DNA ligase. To isolate recombinant plasmids, the mixture after ligation was transformed into *E. coli* cells by electroporation (Sambrook et al., 1989).

2.4.5. Transformation of yeast cells

Transformation of *C. famata* cells was performed by electroporation as previously described (Voronovsky et al., 2002). Fresh colony cells of *C. famata* were inoculated in 3 mL YPD liquid medium and cultivated at 30 °C with shaking for approximately 24 h. Of the obtained culture 0.5 mL was added to 500 mL of YPD liquid medium and cultivated overnight at 30 °C to a final concentration of OD₆₀₀ ~ 1.8–2. The cells were harvested, suspended in 100 mL (0.2 volume) of 50 mM phosphate buffer, pH 7.5, containing 25 mM DTT (dithiothreitol) and incubated for 15 min at 30 °C. The cells were then spun down and washed twice with water (500 and 250 mL, respectively) and once with 20 mL of pre-cold of 1 M saccharose. The cells were resuspended in 3 mL of pre-cold sterile solution of 1 M saccharose. To 200 µL of cell suspension (approximately 9×10^8 cells) 0.1–0.4 µg of plasmid DNA was added. The mixture was tapped on the bottom of a pre-cold 2-mm electroporation cuvette and electroporation was carried out with the Electro Cell Manipulator 600 (ECM600) from BTX, USA. The following electroporation conditions were used: field strength 2.3 kV

cm⁻¹; capacitance 50 µF; resistance 100 Ω resulting in a pulse length of ± 4.5 ms. After electroporation, 0.8 mL of 1 M saccharose was quickly added to the cuvette. The suspension of transformed cells was spread onto the YPD plates with selective antibiotic, then the plates were incubated at 30 °C. Transformants were selected after 5–7 days.

2.5. Quantitative real-time PCR

Total RNA from yeast cells was isolated using the GeneMATRIX Universal RNA Purification Kit with DNase I (EURx Ltd., Poland). All RNA manipulations were performed under refrigeration. Yeast cells were pelleted at 4000 rpm for 5 min, resuspended in 1 mL of TRIzol. 0.2 mL of glass beads (diameter 0.45 mm) were added to the obtained suspension and the cells were destroyed by shaking on a "FastPrep" vortex at 6,000 rpm for 20 seconds. The homogeneous mixture was centrifuged at 12,000 rpm for 15 minutes. The supernatant was transferred to clean Eppendorf tubes, 0.2 mL of chloroform was added, incubated at room temperature for 5 min and centrifuged at 14,000 rpm for 15 min. An insoluble fraction of total RNA was obtained from the selected aqueous phase by precipitation with 0.5 mL of isopropanol, which was precipitated at 14,000 rpm for 15 min. The precipitate was washed with 70% chilled ethanol, dried and dissolved in water treated with diethylpyrocarbonate (DEPC).

The obtained samples served as matrices for the synthesis of single-stranded cDNA. RNA concentration was determined using a Picodrop Microliter UV/Vis Spectrophotometer and dissolved in water devoid of RNase activity. Real-time PCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR System using the SG OneStep qRT-PCR kit (EURx Ltd., Poland), RNA as a template and ROX as a dye to normalize the fluorescence intensity of intercalating Syber Green dyes according to manufacturer's instructions. 100 ng of RNA was used in 20 µL of a reaction mixture

containing 0.4 μM of the appropriate primers (Table 2.3). The following amplification parameters were used: 30 min at 50 °C for cDNA synthesis; 3 min at 95 °C preparation, 40 cycles of 15 s at 94 °C and 30 s at 60 °C. The fold change of the amplicon in the test sample compared to the control sample was determined in three replicates and described as RQ (Ruchala et al., 2017).

2.6. Biochemical analyses

2.6.1. Measurement of cell biomass and riboflavin production

The cell biomass was determined turbidimetrically with a Helios Gamma spectrophotometer (OD, 600 nm; cuvette, 10 mm) with gravimetric calibration. The riboflavin concentration was determined by measuring fluorescence (TurnerQuantechFM109510-33 fluorometer, excitation maximum = 445 nm, emission maximum = 535 nm) after cultivation in batch culture in flasks for 5 days.

2.6.2. Preparation of yeast cell-free extracts

Yeast cell-free extracts were prepared as described previously (Moritz et al., 2000). Yeast cells were precipitated by centrifugation (4,000 rpm) for 10 min at 4 °C and used distilled water wash twice, then centrifuged at 4,000 rpm for 10 min, used the lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM PMSF (phenylmethanesulfonyl fluoride), 2 mM MgCl_2 , and 1 mM DTT) wash twice more.

Cells were suspended in the same buffer to a final cell concentration of 50–100 mg/mL. The resulting suspension was transferred into 2 mL of plastic Eppendorf tubes and glass beads (diameter 0.45-0.5 mm) were added in the amount of 3/4 of the volume of the suspension and froze. Cells were destroyed by vibration at 4 °C for 2 min. After each vibration, the suspension was cooled on ice for two minutes. The destruction was repeated six times. The homogenate was centrifuged for 20 min at 12,000 rpm at 4 °C

in a microcentrifuge. The supernatant was used for protein and enzyme assays. Protein concentration was determined by the Lowry method (Lowry et al., 1951).

2.6.3. Measurement of enzyme G6PDH and 6PGDH activities

During the oxidative branch of PPP, G6PDH catalyzes the conversion of glucose 6-phosphate (G6P) into 6-phosphogluconolactone (6PGL), which is then rapidly hydrolyzed into 6-phosphogluconate (6PG) by 6PGDH and generates NADPH. Thus, measuring G6PDH activity accurately requires accounting for the fact that the NADPH generated includes contributions from total dehydrogenase activity (G6PDH + 6PGDH) when substrate G6P is only used (Zhang et al., 2000).

G6PDH and 6PGDH activities were measured in the cell-free extracts using a Helios Gamma spectrophotometer (Tian et al., 1994). Initially, cell-free extracts were added to the reaction buffer (50 mM Tris, 1 mM MgCl₂, pH 8.1) with NADP⁺ at a final concentration of 0.4 mM and substrates at a final concentration of 0.2 mM. The rate of change was determined over a 6-minute observation period at 340 nm and room temperature. Total dehydrogenase activity was determined by monitoring the conversion of NADP⁺ to NADPH in the presence of substrates G6P and 6PG. Subsequently, the activity of 6PGDH alone was measured by adding only 6PG as substrate. G6PDH activity was then calculated by subtracting 6PGDH activity from the total dehydrogenase activity. The enzyme activity value is expressed in concentration U/mL, where one unit corresponds to the conversion of 1 μ M NADP⁺ per minute (Zhang et al., 2000; Redhu and Bhat, 2020).

Enzyme activity was calculated in U/mL according to formula 2.1, where U/mL is the number of micromoles of substrate in 1 mL, the transformation of which is catalyzed by one enzyme unit per minute; $(A_{340\text{nm}}/\text{min test} - A_{340\text{nm}}/\text{min blank}) -$

difference in extinction between the tested sample and the blank sample; V – the volume of the reaction mixture; df – dilution factor; 6.22 – millimolar extinction coefficient of β -NADPH at 340 nm; 0.1 - the volume of the enzyme (protein) used in 1 mL. The specific activity of a unit of activity was determined as the activity of the enzyme in U/mL divided by the protein concentration in the sample in mg/mL.

Formula 2.1

$$\text{U/mL enzyme} = \frac{(A_{340\text{nm}}/\text{min test} - A_{340\text{nm}}/\text{min blank}) * V * df}{6.22 * 0.1}$$

2.7. Software tools

The work used databases of *D. hansenii* – ("*Debaryomyces hansenii* CBS 767", 2024) (Dujon et al., 2004; Sacerdot et al., 2008) and *S. cerevisiae* – ("*Saccharomyces* Genome Database", 2024). DNA nucleotide sequence analysis was performed using the following programs: NEBcutter 3.0 ("NEBcutter 3.0", 2023); Oligonucleotide Properties Calculator ("Oligonucleotide Properties Calculator", 2022) and a suite of programs available at ("The sequence manipulation suite", 2022). For comparative analysis of amino acid and nucleotide sequences, programs available at ("Multiple Sequence Alignment", 2022) were used. The Multalin algorithm version 5.4.1 ("Multalin interface page", 2000) was used to analyze amino acid sequences. The search for similarity of amino acid sequences was performed using the BLAST network service of the National Center for Biotechnology Information (Bethesda, MD, USA) ("BLAST: Basic Local Alignment Search Tool", 2022). SnapGene Version 6.0.2 was used to viewing, editing, and annotating DNA sequences ("SnapGene | Software for everyday molecular biology", 2023).

2.8. Statistical analysis

All the experimental data shown in this manuscript were collected from three independent samples to ensure reproducibility of the trends and relationships observed in the cultures. Each error bar indicates the standard deviation (SD) from the mean obtained from triplicate samples.

3. RESULTS

3.1. New findings on the role of genes *GND1*, *RIB6*, and *RFE1* in riboflavin oversynthesis of the yeast *C. famata*

3.1.1. Overexpression of genes involved in pentose phosphate pathway in the yeast *C. famata*

The pathway of riboflavin biosynthesis starts from two immediate precursors, GTP and Ru5P (Abbas and Sibirny, 2011). We assume that the accumulation of an increased pool of these precursors will contribute to the improvement of riboflavin biosynthesis. Attempts to increase the yield of riboflavin by increasing the level of synthesis of the purine precursor in *C. famata* have already been successfully carried out.

Previously, an advanced riboflavin producer BRPI, was constructed by overexpression of modified genes *PRS3m* (encoding phosphoribosyl pyrophosphate synthetase) and *ADE4m* (encoding phosphoribosyl pyrophosphate amidotransferase) involved in *de novo* purine nucleotide biosynthesis in strain BRP (Dmytruk et al., 2020).

Our task was to increase the pool of another precursor Ru5P, which is the end-product of the oxidative branch of PPP. In PPP, glucose-6-phosphate dehydrogenase (G6PDH, encoded by gene *ZWF1*) and 6-phosphogluconate dehydrogenase (6GPDH, encoded by gene *GND1*) are two consecutive NADP⁺ dependent dehydrogenases, their reactions convert G6P into Ru5P, and at the same time generates NADPH, which serves as the major source of reducing power for biosynthetic reactions (Nogae and Johnston, 1990; He et al., 2007).

The motivation for engineering the PPP can be seen in two major aims: (i) rerouting the metabolism towards a specific metabolite of interest and (ii) increasing the NADPH concentration, due to PPP's central role in carbon metabolism (Masi et al.,

2021). Thus, it is a feasible strategy to increase the generation of NADPH and Ru5P, or/and reduce the consumption of Ru5P, enhancing the carbon flux toward the riboflavin biosynthesis in yeast *C. famata*.

Several metabolic engineering strategies were developed to modify the flux of the metabolic pathways and regulatory elements in riboflavin-producing strains. To enhance the intracellular pool of Ru5P, overexpressed gene *zwf* increased riboflavin production in strain *B. subtilis* PY (Duan et al., 2010). The overexpression genes *zwf* and *gnd* from *C. glutamicum* increased riboflavin production in *B. subtilis* (Wang et al., 2011). Yang et al. constructed mutant *rpeΔ* (disrupted ribulose-5-phosphate 3-epimerase), resulting in a 5-fold increase of riboflavin production in *B. subtilis* (Yang et al., 2021).

Until now, the regulatory mechanisms of the PPP of riboflavin biosynthesis in yeast *C. famata* are still unknown. In this present work, genes *ZWF1* (coding for G6PDH) and *GND1* (coding for 6PGDH) from *C. famata* were expressed on the background of strains L2 (*leu2*), AF-4, and BRP, respectively. Our task is to evaluate the expression level of both genes, G6PDH and 6PGDH activity, and riboflavin production of those recombinant strains.

3.1.1.1. Plasmid construction

For overexpression of the structural genes of the oxidative branch of PPP, three recombinant plasmids were constructed based on the vector pTb (Dmytruk et al., 2006), which contains the *C. famata* *TEF1* promoter and *D. hansenii* *TEF1* terminator, and a selective marker conferring resistance to phleomycin - gene *ble*. The recombinant plasmids used in this study have been described previously (Andreieva, 2022; Ruchala et al., 2022). Here is a brief description, genes *ZWF1* and *GND1* were amplified by

PCR from genomic DNA of *C. famata* VKM Y-9 using a pair of primers Ko1052/Ko1053 and Ko1054/Ko1055, respectively (Table 2.3). The PCR products were cloned into the restriction sites *Bam*HI/*Pst*I and *Bam*HI/*Not*I of the linearized vector pTb, respectively. As a result, the constructed plasmids were designated as pZWF1 and pGND1 (Figure 3.1) (Andreieva, 2022; Ruchala et al., 2022).

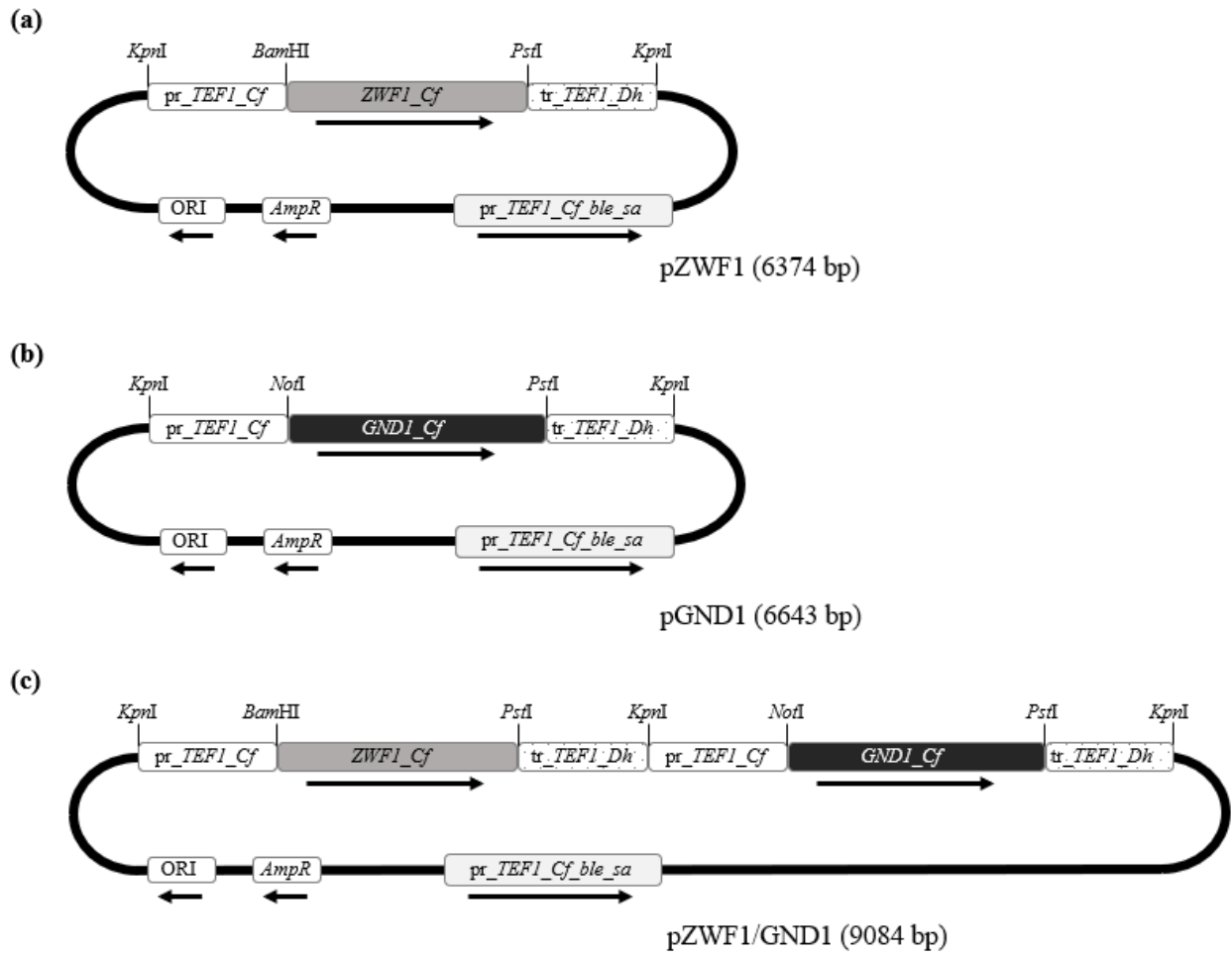


Figure 3.1 The schemes of circular plasmids pZWF1 (a), pGND1 (b), and pZWF1/GND1 (c). *C. famata* *TEF1* promoter and *D. hansenii* *TEF1* terminator indicated as an open box and a spotted box, respectively. Genes *ZWF1* and *GND1* from *C. famata* indicated as a dark grey box and a black box, respectively. The gene *ble* under control of *C. famata* *TEF1* promoter conferring resistance to phleomycin and

indicated as a as light gray box, pUC57 sequence—thin line. ORI—Origin of replication, *AmpR*—ampicillin resistance gene (Andreieva, 2022; Ruchala et al., 2022).

To construct plasmid containing a combination of genes *ZWF1* and *GND1*, the expression cassette *prTEF1Cf_GND1_trTEF1Dh* was amplified by PCR from the plasmid pGND1, using a pair of primers Ko1056/Ko1057 (Table 2.3) and cloned into the restriction site *KpnI* of the linearized plasmid pZWF1 to generate plasmid pZWF1/GND1 (Figure 3.1). All constructed plasmids were verified by restriction digest (Figure 3.2).

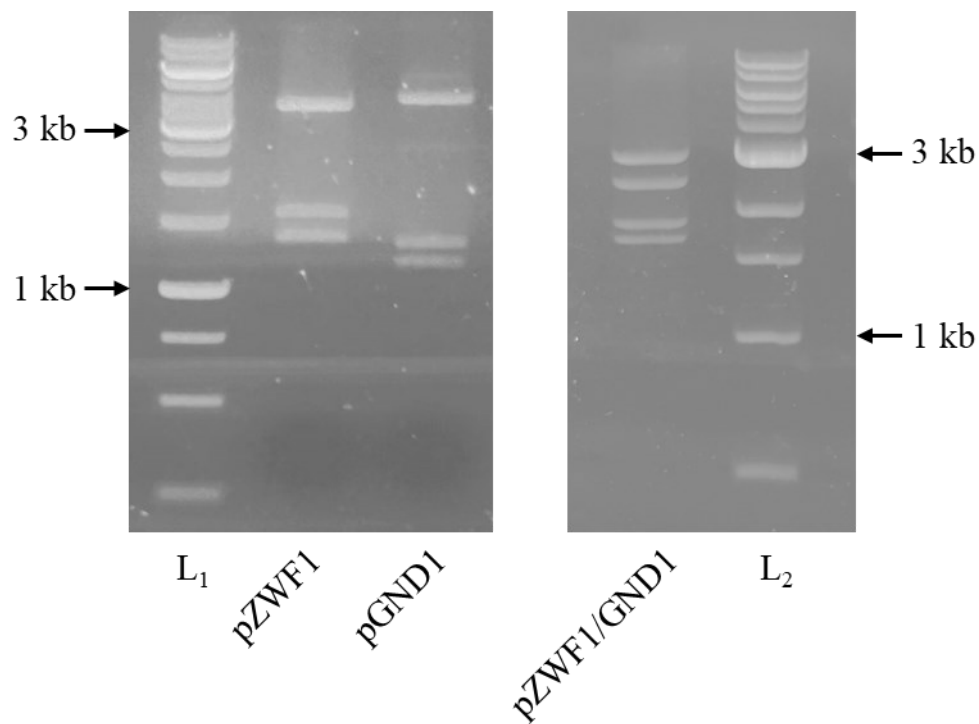


Figure 3.2 Gel electrophoresis of the constructed plasmids after restriction digestion with restriction endonucleases. Plasmid pZWF1 cut by *KpnI* + *HindIII* into 3912, 1300, and 1162 bp. Plasmid pGND1 cut by *PstI* + *EcoRI* into 3690, 1587, and 1366 bp. Plasmid pZWF1/GND1 cut by *BamHI* + *SacI* into 3024, 2458, 1880, and 1772 bp.

3.1.1.2. Construction of recombinant strains

The constructed plasmids pZWF1, pGND1, and pZWF1/GND1 (Figure 3.1) were linearized at the restriction site *AhdI* and used to transform in three parental *C. famata* strains L2, AF-4, and BRP by electroporation (Voronovsky et al., 2002). The plasmids were randomly integrated into the genome of those *C. famata* strains, respectively.

The yeast transformants were selected on the solid YPD medium, containing phleomycin at a final concentration of 20 mg/L after 5–7 days of incubation. As previously described, the selected yeast transformants were stabilized by alternating cultivation on a non-selective followed by selective media (Dmytruk et al., 2011).

The presence of expression cassette of plasmid pZWF1 in selected L2, AF-4, and BRP transformants was confirmed by diagnostic PCR, using a pair of primers Ko1056/Ko1058, respectively (Figure 3.3).

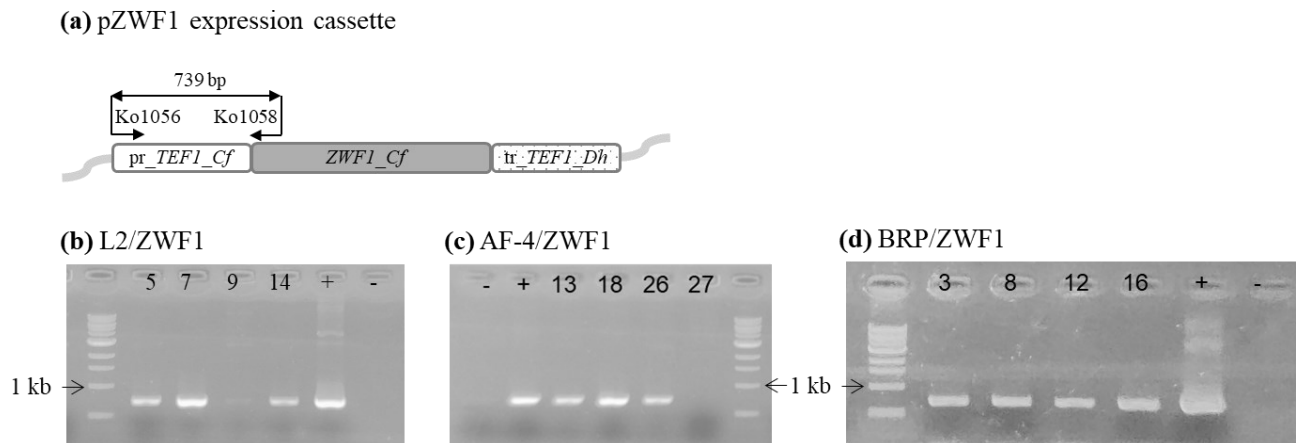
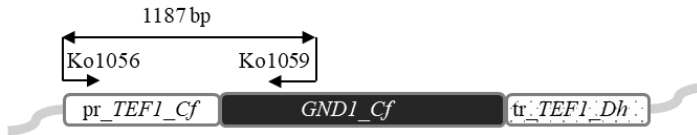


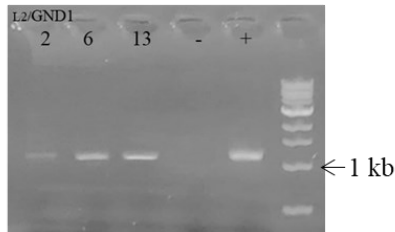
Figure 3.3 Scheme of plasmid pZWF1 expression cassette with primers for PCR verification indicated by arrows (a). PCR verification of strains L2/ZWF1 (b), AF-4/ZWF1 (c), and BRP/ZWF1 (d) with a pair of primers Ko1056/Ko1058, which amplify 739 bp fragment.

The presence of expression cassette of plasmid pGND1 in selected L2, AF-4, and BRP transformants was confirmed by diagnostic PCR, using a pair of primers Ko1056/Ko1059 (Table 2.3), respectively (Figure 3.4).

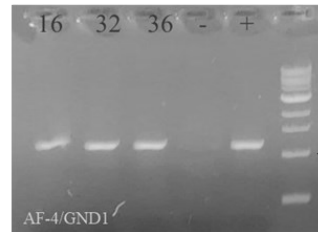
(a) pGND1 expression cassette



(b) L2/GND1



(c) AF-4/GND1



(d) BRP/GND1

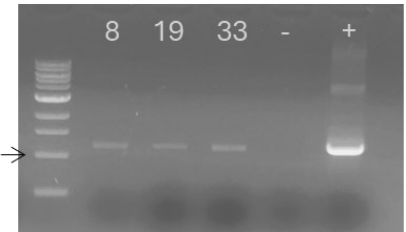


Figure 3.4 Scheme of plasmid pGND1 expression cassette with primers for PCR verification indicated by arrows (a). PCR verification of strains L2/GND1 (b), AF-4/GND1 (c), and BRP/GND1 (d) with a pair of primers Ko1056/Ko1059, which amplify 1187 bp fragment.

The presence of expression cassette of plasmid pZWF1/GND1 in selected L2, AF-4, and BRP transformants was confirmed by diagnostic PCR, using pairs of primers Ko1056/Ko1058 and Ko1056/Ko1059 (Table 2.3), respectively (Figure 3.5).

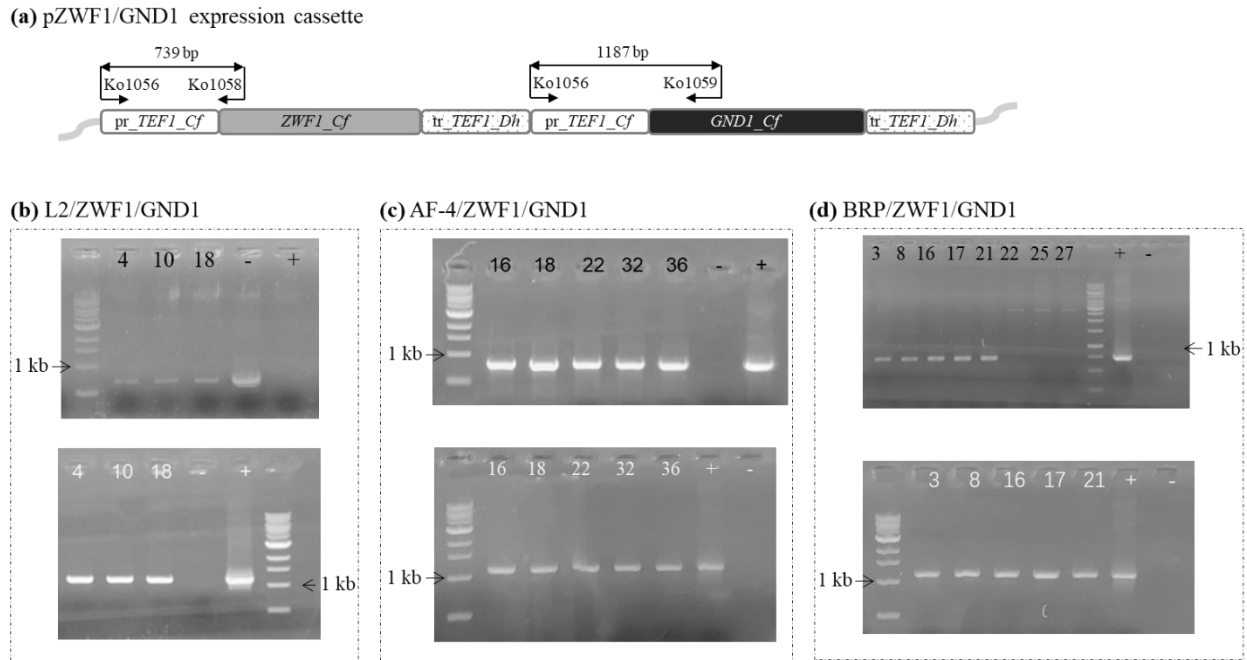


Figure 3.5 Scheme of plasmid pZWF1/GND1 expression cassette with primers for PCR verification indicated by arrows (a). PCR verification of strains L2/ZWF1-GND1 (b), AF-4/ZWF1-GND1 (c), and BRP/ZWF1-GND1 (d) with pair of primers Ko1056/Ko1058 and Ko1056/Ko1059, which amplify 739 bp and 1187 bp fragments.

Selected recombinant strains were named L2/ZWF1, AF-4/ZWF1, BRP/ZWF1, L2/GND1, AF-4/GND1, BRP/GND1, L2/ZWF1-GND1, AF-4/ZWF1-GND1, and BRP/ZWF1-GND1 (Andreieva, 2022; Ruchala et al., 2022).

The expression of genes *ZWF1* and *GND1* was analyzed by qRT-PCR in all recombinant strains of *C. famata*, respectively. The results showed that gene *ZWF1* of recombinant strains L2/ZWF1, AF-4/ZWF1, and BRP/ZWF1 had 5.0-, 22.6-, and 10.6-fold increase compared to corresponding parental strains, respectively (Figure 3.6). The gene *GND1* of recombinant *C. famata* strains L2/GND1, AF-4/GND1, and BRP/GND1 had 1.6-, 11.1-, and 3.74-fold increase compared to corresponding parental strains, respectively (Figure 3.6) (Andreieva, 2022). However, both genes *ZWF1* and

GND1 of constructed *C. famata* strains L2/ZWF1-*GND1*, AF-4/ZWF1-*GND1*, and BRP/ZWF1-*GND1* had no obvious improved (Figure 3.6) (Andreieva, 2022).

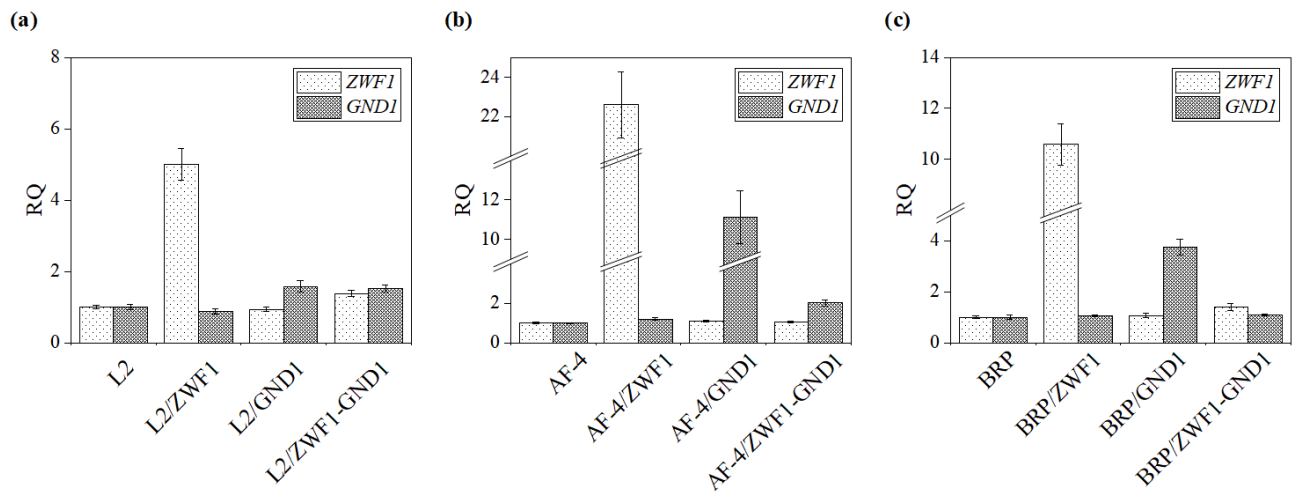


Figure 3.6 The relative quantification (RQ) of genes *ZWF1* and *GND1* expression in all recombinant strains of *C. famata*.

3.1.1.3. Biochemical characteristics of recombinant strains producing riboflavin

To reveal the role of genes *ZWF1* and *GND1* in riboflavin biosynthesis of yeast *C. famata*, the genes were overexpressed in three different strains of *C. famata* with significant differences in riboflavin production. Among, *C. famata* L2 (*leu2*), which lacks a gene homologous to the *S. cerevisiae* gene *LEU2* encoding L-isopropylmalate dehydrogenase, was isolated from *C. famata* VKM Y-9 (Voronovsky et al., 2002). The non-reverting riboflavin overproducing strain AF-4 was isolated from *C. famata* VKM Y-9 through several stages of mutagenesis and subsequent selection (Dmytruk et al., 2011). Strain BRP harboring additional copies of genes *RIB1*, *RIB7*, and *SEF1*, which accumulated a 45% increase in riboflavin production compared to parental strain AF-4 (Dmytruk et al., 2014).

Thus, initial characterization of riboflavin production was performed in the recombinant strains L2/ZWF1, AF-4/ZWF1, BRP/ZWF1, L2/GND1, AF-4/GND1,

BRP/GND1, L2/ZWF1-GND1, AF-4/ZWF1-GND1, and BRP/ZWF1-GND1 in YNB medium supplemented with 0.05% yeast extract for 5 days of growth.

As a result, stains L2/GND1, AF-4/GND1, and BRP/GND1 accumulated nearly the amount of biomass compared to the corresponding parental strain, while revealed 97%, 26%, and 30% increase in riboflavin production, respectively (Table 3.1) (Andreieva, 2022). On the contrary, stains L2/ZWF1, AF-4/ZWF1, and BRP/ZWF1 accumulated around 39%, 16%, and 52% less biomass, as well as revealed 11%, 26%, and 57% decrease in riboflavin production compared to their parental strain, respectively (Table 3.1) (Andreieva, 2022).

Interestingly, stains L2/ZWF1-GND1, AF-4/ZWF1-GND1, and BRP/ZWF1-GND1 accumulated 7%, 19%, and 12% increase in riboflavin production, respectively (Table 3.1) (Andreieva, 2022). In contrast, L2/ZWF1-GND1 and AF-4/ZWF1-GND1 revealed 21% and 6% decrease in biomass accumulation compared to their parental strain, respectively (Table 3.1) (Andreieva, 2022).

Table 3.1

Cell biomass, riboflavin production, and riboflavin yield of recombinant strains of *C. famata* in YNB + 0.05% yeast extract medium.

Strain	Biomass (g/L)	Riboflavin (mg/L)	Riboflavin yield (mg/g of CDW)
L2	2.38 ± 0.03	3.48 ± 0.02	1.46 ± 0.03
L2/ZWF1	1.46 ± 0.03	3.08 ± 0.05	2.11 ± 0.04
L2/GND1	2.49 ± 0.07	6.85 ± 0.06	2.75 ± 0.11
L2/ZWF1-GND1	1.88 ± 0.02	3.74 ± 0.08	1.98 ± 0.08
AF-4	2.71 ± 0.19	318.10 ± 8.05	117.38 ± 7.83
AF-4/ZWF1	2.28 ± 0.06	236.90 ± 2.71	103.90 ± 2.45
AF-4/GND1	2.65 ± 0.15	400.45 ± 9.25	151.11 ± 6.38
AF-4/ZWF1-GND1	2.56 ± 0.09	380.09 ± 7.89	148.47 ± 8.11
BRP	2.40 ± 0.12	510.56 ± 9.47	212.73 ± 9.94
BRP/ZWF1	1.15 ± 0.06	220.83 ± 6.64	192.02 ± 12.38
BRP/GND1	2.57 ± 0.08	662.96 ± 10.48	257.96 ± 18.54
BRP/ZWF1-GND1	2.44 ± 0.07	570.66 ± 11.78	233.87 ± 12.92

Besides, stains L2/GND1, AF-4/GND1, BRP/GND1, and their parental strains were cultivated in cheese whey, which is an abundant waste of the dairy industry. It is interesting to note that all strains produce more riboflavin in cheese whey relative to that in YNB. Strain L2/GND1 showed a 2-fold increase of riboflavin production in

YNB with 5% glucose as well as in cheese whey relative to L2 (Ruchala et al., 2022). Despite the significant increase in *GND1* expression, strain AF-4/*GND1* produced only 15% more riboflavin in cheese whey than parental strain AF-4. Strain BRP/*GND1* accumulated 1.3-fold more riboflavin in cheese whey as compared to BRP. The highest titer of riboflavin was produced by BRP/*GND1* in cheese whey reaching 1094 mg/L (Ruchala et al., 2022).

The activities of G6PDH and 6PGDH of recombinant strains, as well as parental strains L2, AF-4, and BRP were measured. It was found that 6PGDH activity of L2/*ZWF1*, AF-4/*ZWF1*, and BRP/*ZWF1* showed slight increase compared to the corresponding parental strain, and G6PDH activity of these strains showed 21.7-, 8.6-, and 11.5-fold increase (6.95, 17.36, and 15.24 U/mL), respectively. The results demonstrate a positive correlation with expression levels of the additional gene *ZWF1* in recombinant strains L2/*ZWF1*, AF-4/*ZWF1*, and BRP/*ZWF1* (Andreieva, 2022).

The 6PGDH activity of L2/*GND1*, AF-4/*GND1*, and BRP/*GND1* was 1.25, 2.01, and 1.99 U/mL, respectively, increased 1.7-, 3.0-, and 2.7-fold compared to the corresponding parental strain (Figure 3.7) (Andreieva, 2022). However, except for L2/*GND1*, whose G6PDH activity was comparable to that of the parent strain, AF-4/*GND1* and BRP/*GND1* were decreased by 83% and 98%, respectively (Figure 3.7) (Andreieva, 2022). The results indicated also a positive correlation with expression levels of the additional gene *GND1* in L2/*GND1*, AF-4/*GND1*, and BRP/*GND1*.

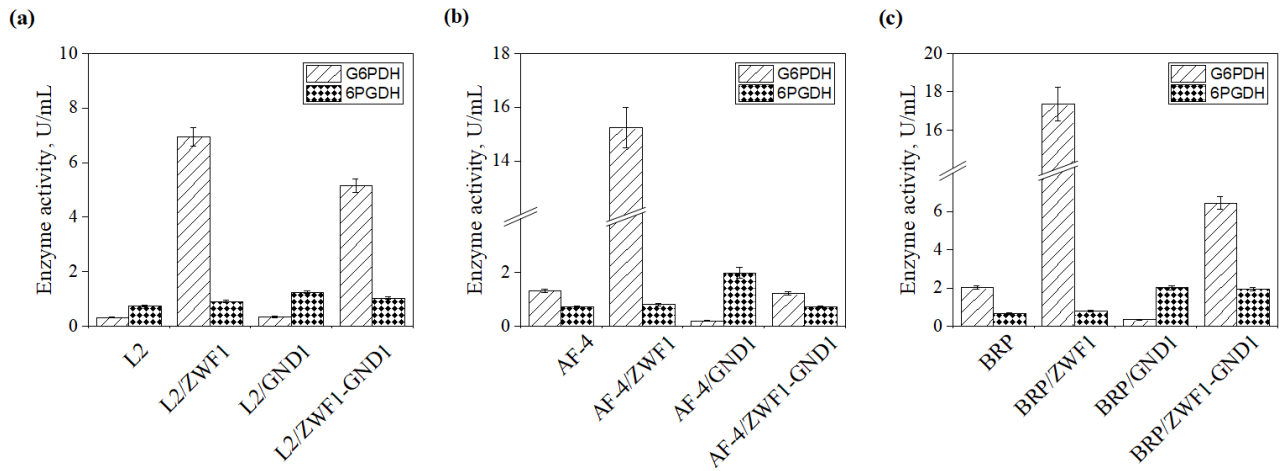


Figure 3.7 Enzymatic activity of G6PDH and 6PGDH of recombinant and parental strains of *C. famata*.

The three recombinant strains containing a combination of genes *ZWF1* and *GND1*, L2/ZWF1-GND1 showed 16.1- and 1.4-fold increase in G6PDH and 6PGDH activity compared to parental strain, respectively, and BRP/ZWF1-GND1 showed 3.2- and 2.9-fold increase in G6PDH and 6PGDH activity, whereas in AF-4/ZWF1-GND1 did not show obvious changes (Figure 3.7) (Andreieva, 2022).

In our present results, the expression level of gene *ZWF1* and G6PDH activity of recombinant strains L2/ZWF1, AF-4/ZWF1, and BRP/ZWF1 showed increased significantly compared to corresponding parental strains, however, the riboflavin production and biomass accumulation decreased. Recombinant strains L2/GND1, AF-4/GND1, and BRP/GND1 revealed increased in 6PGDH activity and riboflavin production relative to corresponding parental strains.

The riboflavin accumulation of the strains co-expressing genes *ZWF1* and *GND1* was not significantly changed, being slightly lower than that of the strains expressing the single gene *GND1*, but also a little higher than that of the parental strains.

The results of this subsection are presented as part of the work in the article: Ruchala, J., Andreieva, Y., Tsyurulnyk, A., Sobchuk, S., Najdecka, A., **Liu, W.**, Kang, Y., Dmytruk, O., Dmytruk, K., Fedorovych, D., and Sibirny, A (2022). Cheese whey supports high riboflavin synthesis by the engineered strains of the flavinogenic yeast *Candida famata*. *Microbial Cell Factories*, 21(1), 161-169. <https://doi.org/10.1186/s12934-022-01888-0>.

3.1.2. Co-overexpression of genes *RFE1*, *GND1*, and *RIB6* increases riboflavin production in the yeast *C. famata*

3.1.2.1. Co-overexpression of genes *RFE1*, *GND1*, and *RIB6* in wild-type strain

Our long-term objective is to develop riboflavin overproducing strains based on the flavinogenic yeast *C. famata*. Recently, the riboflavin excretase (encoding by gene *RFE1*), the homolog of the mammalian riboflavin efflux protein, was overexpressed in the background of strain BRP. The resulting strain BRP/*RFE1* produced 1.4- to 1.8-fold more riboflavin compared to the strain BRP (Tsyurulnyk et al., 2020). Strain BRPI/*RIB6* contains additional overexpression of gene *RIB6* involved in the conversion of Ru5P to DHBP accumulated during flask cultivation by 13–28% more riboflavin relative to the parental strain BRPI (Petrovska et al., 2022).

Therefore, we suggest that additional overexpression of gene *RFE1* and improved supply of Ru5P due to overexpression of gene *GND1*, as well as overexpression of gene *RIB6* will lead to the significant improvement of recombinant strains of *C. famata* able to produce a high level of riboflavin.

To test the efficiency of cloned plasmids with the mentioned genes, we decided to introduce them into the wild-type strain VKM Y-9 of *C. famata*. The low level of

riboflavin production in this native strain allowed for a clearer assessment of the specific influence of this gene combination on the riboflavin biosynthesis pathway in yeast *C. famata*. Additionally, the impact of different growth media on riboflavin production was evaluated.

For overexpression of genes *RFE1*, *GND1*, and *RIB6*, a number of plasmids were constructed based on the vector pRFE1 (Tsyrlunyk et al., 2020), which contains a gene *RFE1* from *D. hansenii* under the control of *D. hansenii TEF1* promoter, as well as a selective marker conferring resistance to phleomycin - gene *ble*. The gene *RIB6* was amplified by PCR from the genomic DNA of strain VKM Y-9 using a pair of primers Ko1352/Ko1053 (Table 2.3).

The expression cassette of gene *GND1*, prTEF1Cf_GND1_trTEF1Dh, which contains a *C. famata TEF1* promoter, was amplified by PCR from plasmid pGND1 (Ruchala et al., 2022), using a pair of primers Ko1357/Ko1358 (Table 2.3). Restriction sites were introduced into the primers to facilitate cloning.

The amplified gene *RIB6* and expression cassette of gene *GND1* were cloned into the *XhoI* and *KpnI* restriction sites of the linearized vector pRFE1 to generate plasmids pRFE1/RIB6 and pRFE1/GND1, respectively (Figure 3.8). To construct the plasmid containing a combination of three genes *RFE1*, *GND1*, and *RIB6*, the expression cassette of gene *GND1* was cloned into the *KpnI* restriction site of the linearized plasmid pRFE1/RIB6, resulting in a plasmid pRFE1/RIB6/GND1 (Figure 3.8).

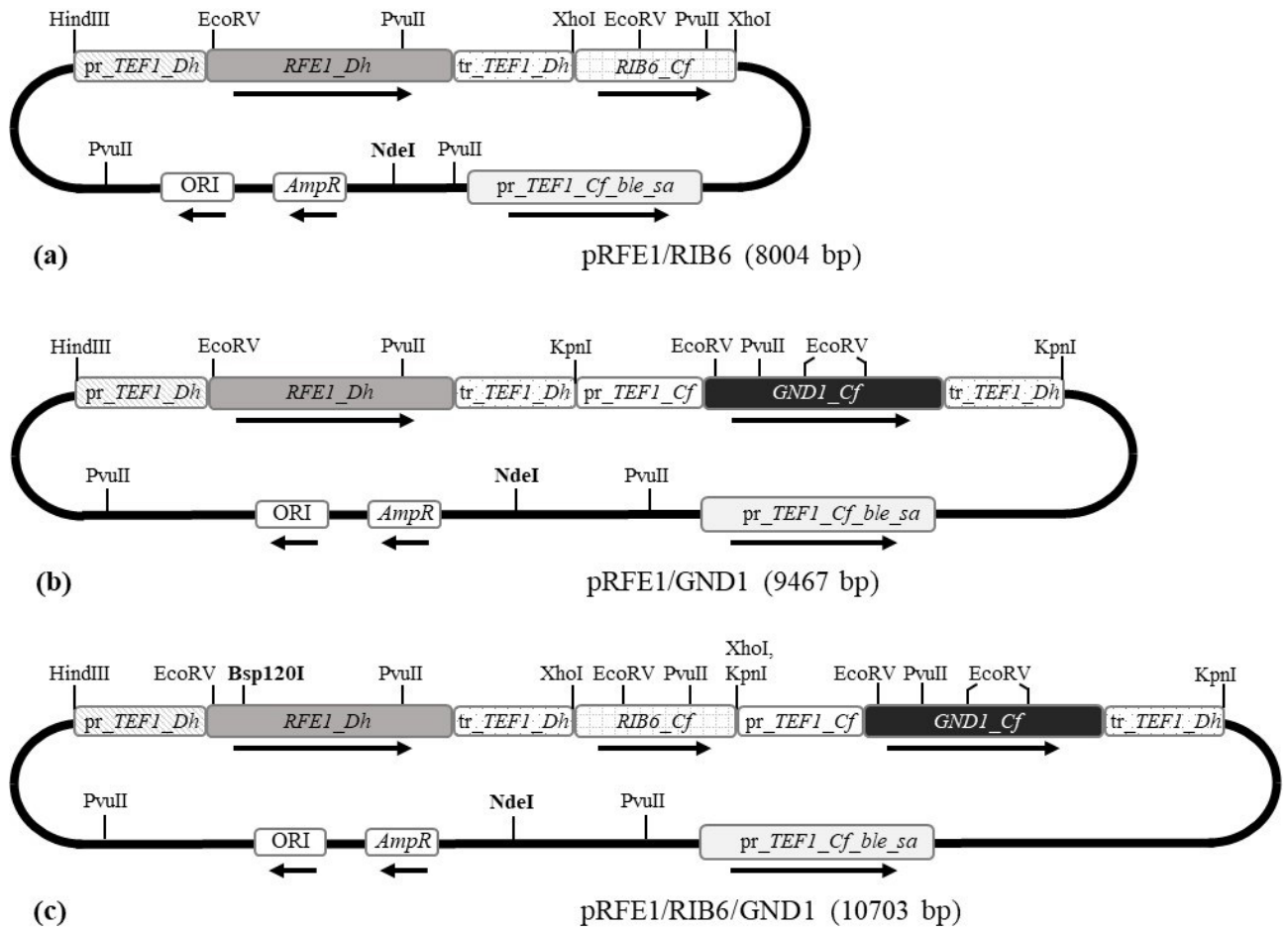


Figure 3.8 The schemes of circular plasmids pRFE1/RIB6 (a), pRFE1/GND1 (b), and pRFE1/RIB6/GND1 (c). *D. hansenii* *TEF1* promoter, *C. famata* *TEF1* promoter, and *D. hansenii* *TEF1* terminator are indicated as a slash box, an open box, and a spotted box, respectively. Genes *D. hansenii* *RFE1*, *C. famata* *RIB6*, and *C. famata* *GND1* are indicated as a dark grey box, a checkered box, and a black box, respectively. The gene *ble* under the control of *C. famata* *TEF1* promoter conferring resistance to phleomycin and is indicated as a light gray box, pUC57 sequence – thin line. ORI – Origin of replication, *AmpR* – ampicillin resistance gene.

All constructed plasmids were verified by restriction digestion with endonucleases which recognized restriction sites in the inserted genes as well as the vector backbone (Figure 3.9).

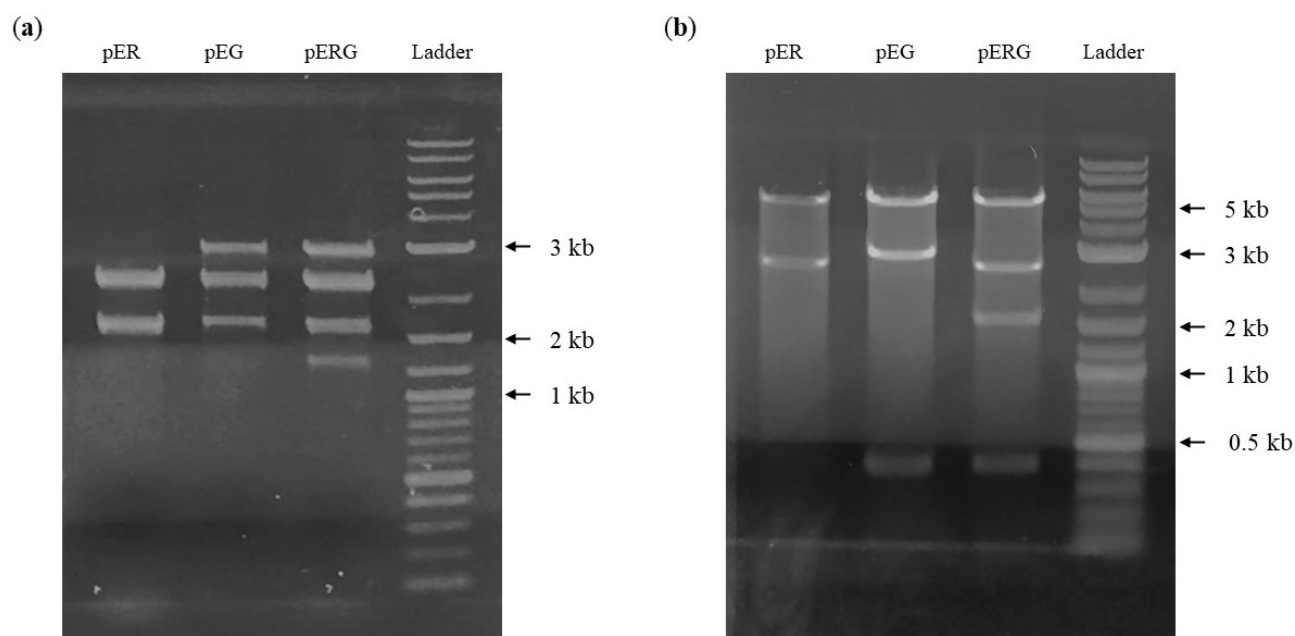


Figure 3.9 Gel electrophoresis of the constructed plasmids after restriction digestion with restriction endonucleases *PvuII* (a) and *EcoRV* (b), respectively. pER – pRFE1/RIB6 (cut by *PvuII* into 2364, 2312, 1684, and 1644 bp; cut by *EcoRV* into 5382 and 2622 bp). pEG – pRFE1/GND1 (cut by *PvuII* into 3067, 2364, 2312, and 1724 bp; cut by *EcoRV* into 5630, 2981, 433, and 423 bp). pERG – pRFE1/RIB6/GND1 (cut by *PvuII* into 3067, 2364, 2312, 1644, and 1317 bp; cut by *EcoRV* into 5630, 2622, 1596, 433, and 423 bp).

Constructed plasmids pRFE1/RIB6, pRFE1/GND1, and pRFE1/RIB6/GND1 were linearized by endonuclease *NdeI* and introduced into the genome of *C. famata* VKM Y-9 by electroporation (Voronovsky et al., 2002). To obtain an expression module with combined genes *RIB6* and *GND1* and without gene *RFE1*, the plasmid pRFE1/RIB6/GND1 was linearized by *Bsp120I* and used for transformation of strain VKM Y-9. These plasmids were randomly integrated into the genome of strain VKM Y-9.

Corresponding transformants were selected on a solid YPD medium supplemented with phleomycin after 5–7 days of incubation. The selected

transformants were stabilized by alternating cultivation on a nonselective followed selective media. After stabilization, the presence of the plasmid pRFE1/RIB6 in the stabilized transformants was confirmed by diagnostic PCR, using pairs of primers Ko817/Ko1358 and Ko1356/Ko1355 (Table 2.3) for expression cassette of genes *RFE1* and *RIB6* (Figure 3.10).

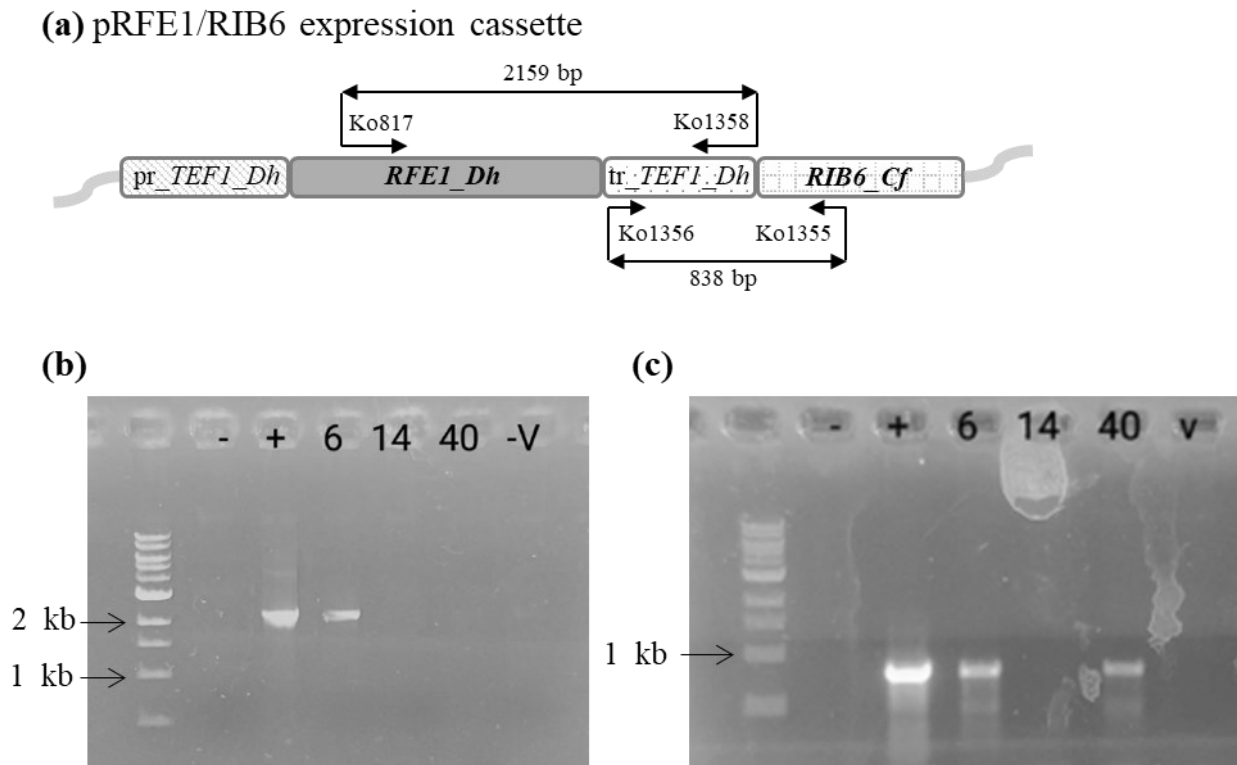


Figure 3.10 Scheme of expression cassette of plasmid pRFE1/RIB6 integrated into the genome of *C. famata* VKM Y-9, with primers for PCR verification indicated by arrows (a). PCR products of the correct integration of the genes *RFE1* (b) and *RIB6* (c).

Similarly, the presence of the plasmid pRFE1/GND1 in the stabilized transformants was confirmed by diagnostic PCR, using pairs of primers Ko817/Ko1358 and Ko1056/Ko1059 (Table 2.3) for expression cassette of genes *RFE1* and *GND1* (Figure 3.11).

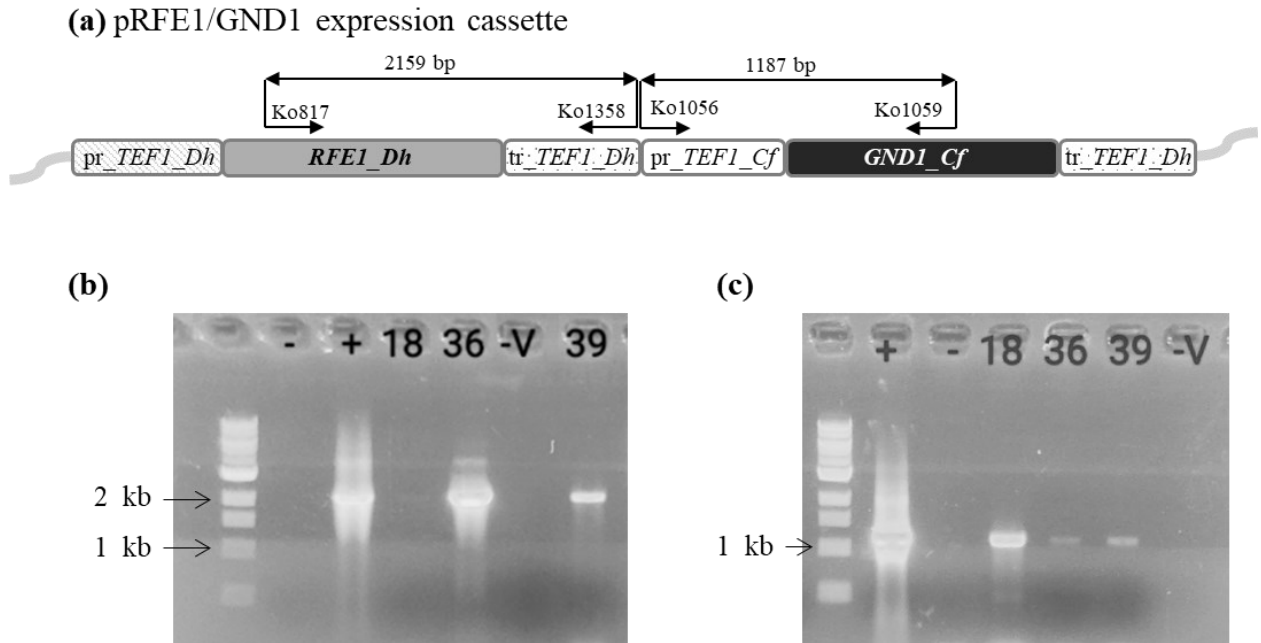


Figure 3.11 Scheme of expression cassette of plasmid pRFE1/GND1 integrated into the genome of *C. famata* VKM Y-9, with primers for PCR verification indicated by arrows (a). PCR products of the correct integration of the genes *RFE1* (b) and *GND1* (c).

Moreover, the presence of the plasmid pRIB6/GND1 in the stabilized transformants was confirmed by diagnostic PCR, using pairs of primers Ko1356/Ko1355 and Ko1056/Ko1059 (Table 2.3) for expression cassette of genes *RIB6* and *GND1* (Figure 3.12).

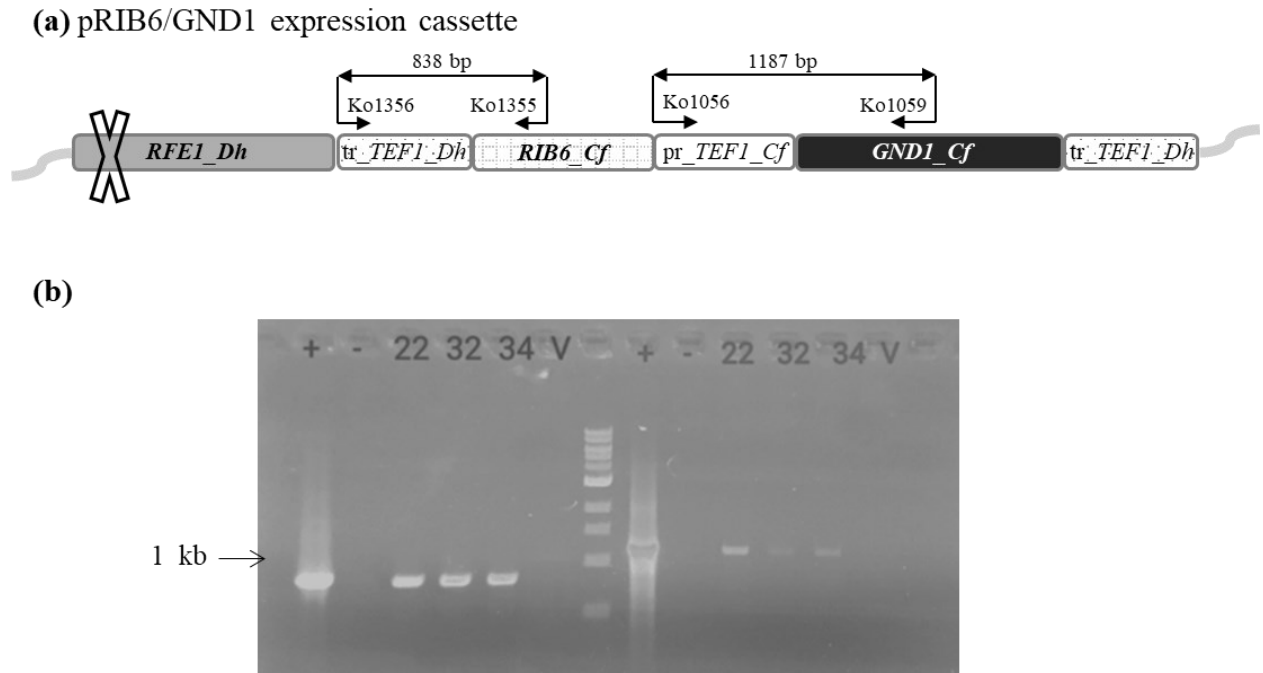


Figure 3.12 Scheme of expression cassette of plasmid pRIB6/GND1 integrated into the genome of *C. famata* VKM Y-9, with primers for PCR verification indicated by arrows (a). PCR products of the correct integration of the genes *RIB6* (left of b) and *GND1* (right of b).

Additionally, the presence of the plasmid pRFE1/RIB6/GND1 in the stabilized transformants was confirmed by diagnostic PCR, using pairs of primers Ko1440/Ko1358, Ko1356/Ko1355, and Ko1354/Ko1059 (Table 2.3) for expression cassette of genes *RFE1*, *RIB6*, and *GND1* (Figure 3.13). Selected and verified transformants of *C. famata* VKM Y-9 were named strains V9/RFE1-RIB6, V9/RFE1-GND1, V9/RIB6-GND1, and V9/RFE1-RIB6-GND1.

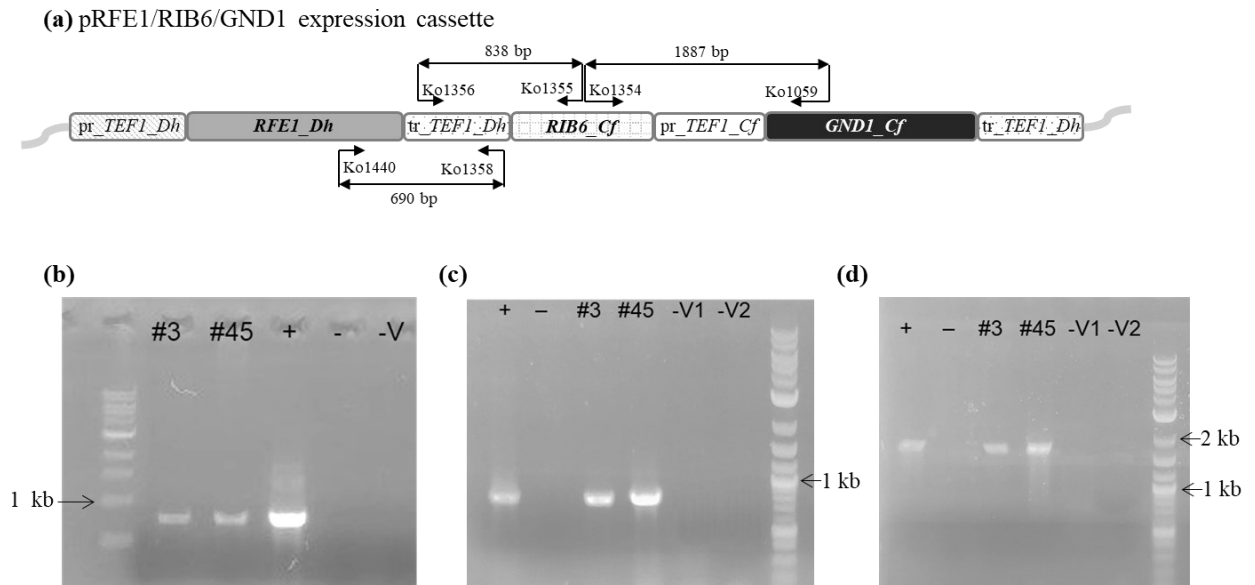


Figure 3.13 Scheme of expression cassette of plasmid pRFE1/RIB6/GND1 integrated into the genome of *C. famata* VKM Y-9, with primers for PCR verification indicated by arrows (a). PCR products of the correct integration of the genes *RFE1* (b), *RIB6* (c), and *GND1* (d).

Initial characterization of riboflavin production was performed in the strain VKM Y-9, and its derivatives V9/RFE1-RIB6, V9/RFE1-GND1, V9/RIB6-GND1, and V9/RFE1-RIB6-GND1 on five days of cultivation in the three different media: (i) YPD; (ii) YNB supplemented with 0.05% yeast extract; and (iii) cheese whey supplemented with 0.3% ammonium sulfate.

Strains V9/RFE1-RIB6, V9/RFE1-GND1, and V9/RIB6-GND1 demonstrated approximately a 10% decrease in biomass accumulation, while V9/RFE1-RIB6-GND1 showed a 19% decrease compared to VKM Y-9 during cultivation in YPD medium (Figure 3.14). At the same time, strains V9/RFE1-RIB6, V9/RFE1-GND1, V9/RIB6-GND1, and V9/RFE1-RIB6-GND1 revealed 1.22-, 1.10-, 1.19-, and 1.32-fold increase in riboflavin production compared to the parental strain (Figure 3.14).

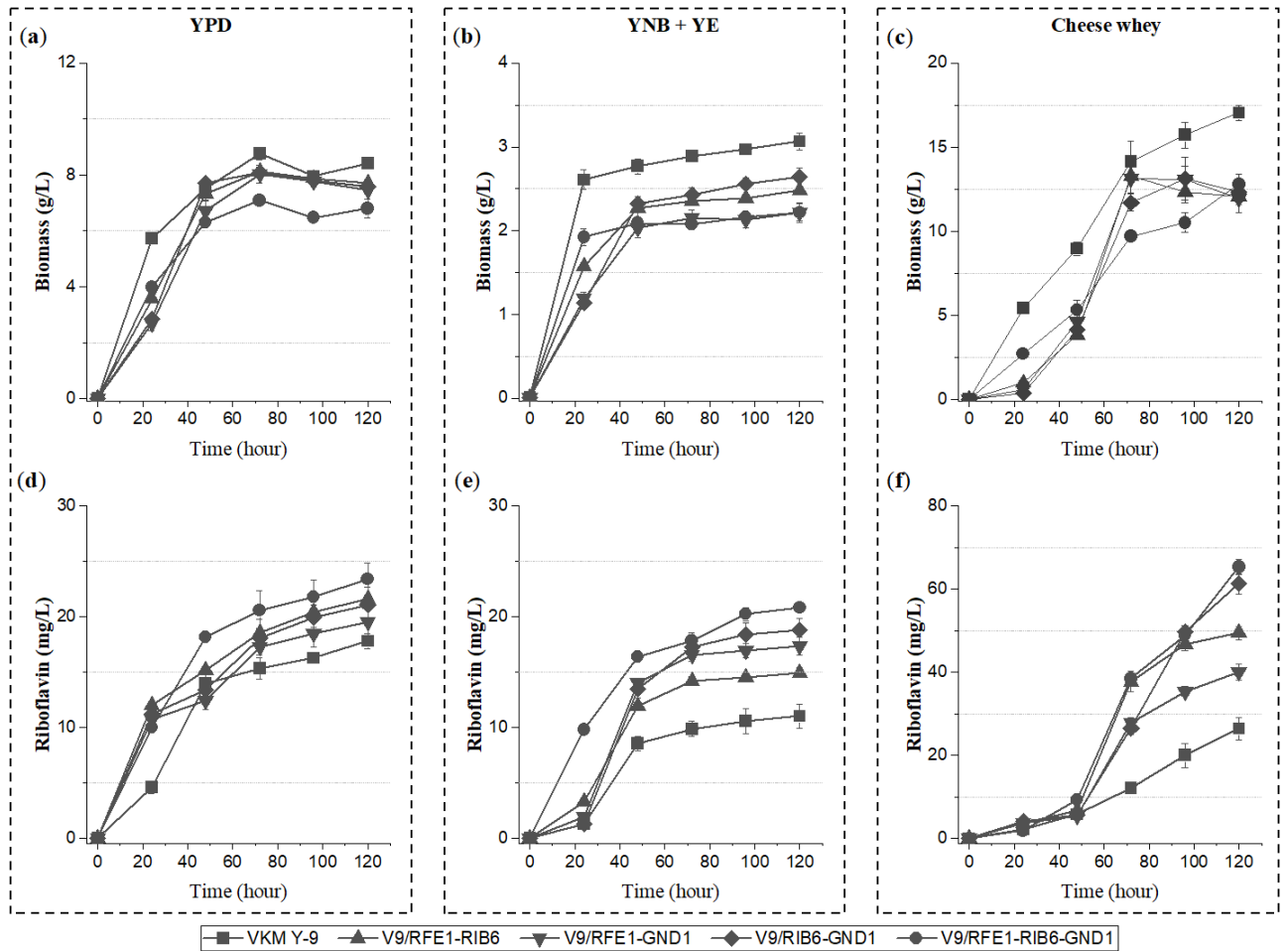


Figure 3.14 Time profiles of biomass accumulation and riboflavin production of strains VKM Y-9, V9/RFE1-GND1, V9/RFE1-RIB6, V9/RIB6-GND1, and V9/RFE1-RIB6-GND1 in different media in flasks: Biomass accumulation in YPD (a), YNB + YE (b), and cheese whey (c); Riboflavin production in YPD (d), YNB + YE (e), and cheese whey (f).

In YNB + YE medium, the biomass accumulation of the constructed strains was decreased by approximately 28% relative to parental strain VKM Y-9 (Figure 3.14). In contrast, the riboflavin production or riboflavin yield in V9/RFE1-RIB6, V9/RFE1-GND1, V9/RIB6-GND1, and V9/RFE1-RIB6-GND1 increased up to 1.36-, 1.58-, 1.71-, and 1.89-fold or 1.67-, 2.18-, 1.98-, and 2.61-fold, respectively (Figure 3.14).

In parallel, the biomass accumulation of the constructed strains decreased by approximately 25% (Figure 3.14), compared to the parental strain VKM Y-9 in cheese

whey. Moreover, the recombinant strains V9/RFE1-RIB6, V9/RFE1-GND1, V9/RIB6-GND1, and V9/RFE1-RIB6-GND1 demonstrated 1.87-, 1.52-, 2.32-, and 2.47-fold increase in riboflavin production, respectively (Figure 3.14).

Additionally, V9/RFE1-RIB6, V9/RFE1-GND1, V9/RIB6-GND1, and V9/RFE1-RIB6-GND1 showed a 1.33-, 1.24-, 1.32-, and 1.63-fold or 2.64-, 2.16-, 3.20-, and 3.29-fold increase in riboflavin yield (mg of riboflavin per gram of CDW) compared to the parental strain, during cultivation in YPD or cheese whey medium, respectively (Figure 3.15).

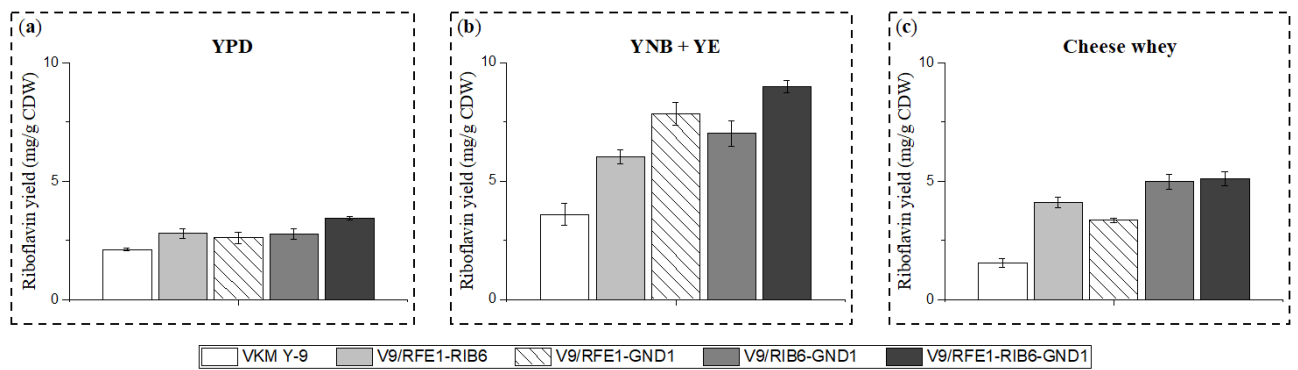


Figure 3.15 Riboflavin yield (mg/g CDW) of strains VKM Y-9, V9/RFE1-GND1, V9/RFE1-RIB6, V9/RIB6-GND1, and V9/RFE1-RIB6-GND1 in different media: YPD (a), YNB + YE (b), and cheese whey (c) in the fifth day of cultivation.

These results showed that the riboflavin production of all constructed strains in three different media was increased, with the highest production observed in cheese whey. The strains V9/RFE1-RIB6, V9/RFE1-GND1, V9/RIB6-GND1, and V9/RFE1-RIB6-GND1 amounted to 49, 41, 61, and 65 mg/L of riboflavin cheese whey, respectively (Figure 3.14).

The results of this subsection are presented as part of the work in the article: **Liu, W., Tsyrlunyk, A., Dmytruk, K., Fedorovych, D., Kang, Y., and Sibirny, A. (2025). Co-overexpression of genes *RFE1*, *GND1*, and *RIB6* enhances riboflavin production in**

yeast *Candida famata*. *Cytology and Genetics*, 59(1), 63–70.
<https://doi.org/10.3103/S0095452725010074>.

3.1.2.2. Co-overexpression of genes *RFE1*, *GND1*, and *RIB6* in riboflavin overproducing strain

The results presented above in section 3.2.1 showed that the introduction of plasmids increased riboflavin production in the wild-type strain. This aroused our interest and confidence that expressing these plasmids in riboflavin overproducing strains could improve them.

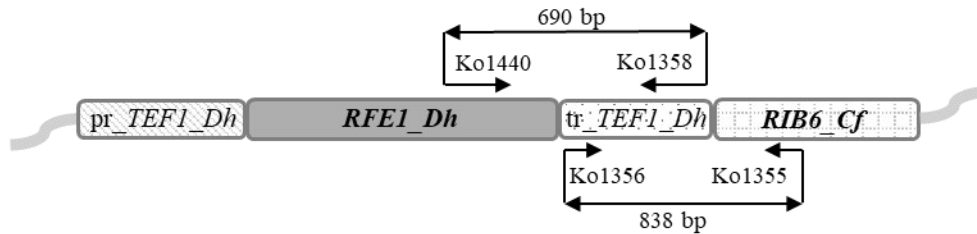
The riboflavin overproducing strain AF-4 was isolated from the wild-type strain of *C. famata* via several stages of mutagenesis and subsequent selection (Dmytruk et al., 2011). Strain AF-4 has some advantages, such as absence of manipulations with recombinant DNA before, genetic stability, robust cell growth, and good ability of riboflavin production. It has been shown to have excellent potential as a template strain (Dmytruk et al., 2014; Sibirny, 2023). The advanced riboflavin overproducer, BRP, was previously constructed by co-overexpressing riboflavin biosynthesis structural genes *RIB1*, *RIB7*, and the transcription activator gene *SEF1* based on strain AF-4 (Dmytruk et al., 2014). Subsequently, the advanced riboflavin overproducing strain designed as BRPI (Best Riboflavin Producer Improved) via overexpression of the modified heterologous genes *PRS3* and *ADE4* in strain BRP, led to 2-fold enhanced riboflavin production (Dmytruk et al., 2020).

Therefore, we decided to introduce these plasmids described in section 3.2.1.1 into strains into the non-reverting strain AF-4.

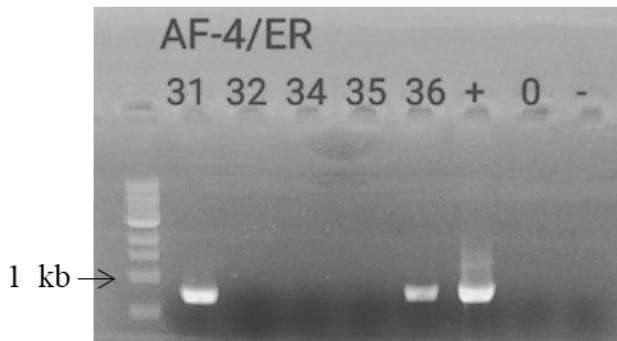
In the same way, we introduced *Nde*I-linearized plasmids pRFE1/RIB6, pRFE1/GND1, and pRFE1/RIB6/GND1, as well as *Bsp*120I-linearized plasmid pRFE1/RIB6/GND1 into the non-reverting strain AF-4 by electroporation.

Furthermore, the presence of the plasmid pRFE1/RIB6 in AF-4 derived transformants was confirmed by diagnostic PCR, using pairs of primers Ko1440/Ko1358 and Ko1356/Ko1355 (Table 2.3) for expression cassette of genes *RFE1* and *RIB6* (Figure 3.16).

(a) pRFE1/RIB6 expression cassette



(b)



(c)

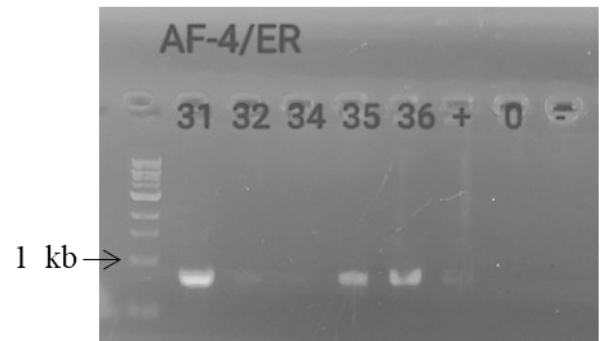


Figure 3.16 Scheme of expression cassette of plasmid pRFE1/RIB6 integrated into the genome of strain AF-4, with primers for PCR verification indicated by arrows (a). PCR products of the correct integration of the genes *RFE1* (b) and *RIB6* (c).

Furthermore, the presence of the plasmid pRFE1/GND1 in AF-4 derived transformants was confirmed by diagnostic PCR, using pairs of primers Ko1440/Ko1358 and Ko1056/Ko1059 (Table 2.3) for expression cassette of genes *RFE1* and *GND1* (Figure 3.17).

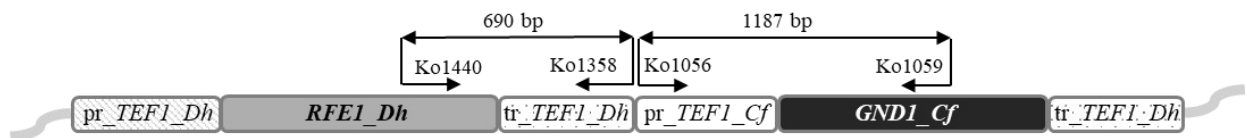
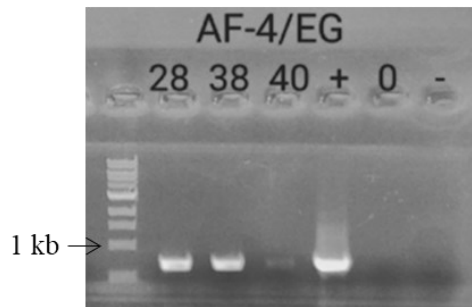
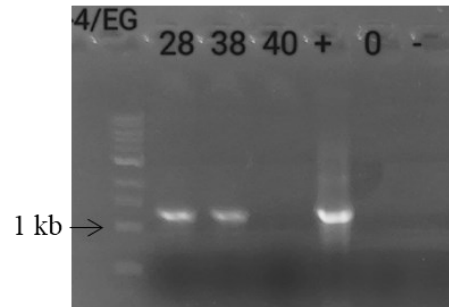
(a) pRFE1/GND1 expression cassette**(b)****(c)**

Figure 3.17 Scheme of expression cassette of plasmid pRFE1/GND1 integrated into the genome of strain AF-4, with primers for PCR verification indicated by arrows **(a)**. PCR products of the correct integration of the genes *RFE1* **(b)** and *GND1* **(c)**.

Furthermore, the presence of the plasmid pRIB6/GND1 in AF-4 derived transformants was confirmed by diagnostic PCR, using pairs of primers Ko1356/Ko1355 and Ko1056/Ko1059 (Table 2.3) for expression cassette of genes *RIB6* and *GND1* (Figure 3.18).

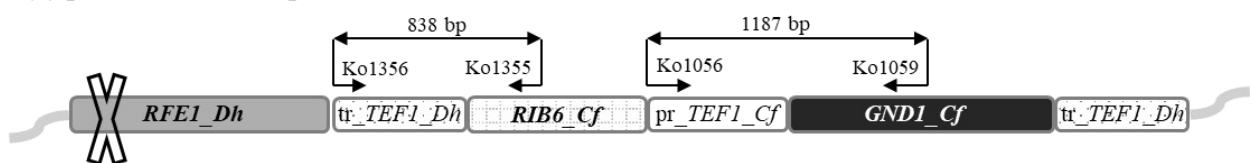
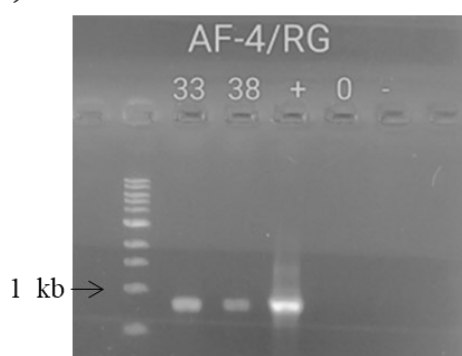
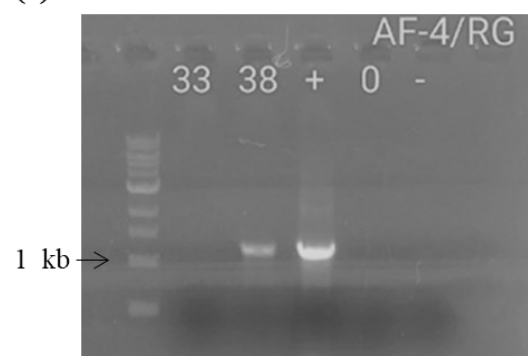
(a) pRIB6/GND1 expression cassette**(b)****(c)**

Figure 3.18 Scheme of expression cassette of plasmid pRIB6/GND1 integrated into the genome of strain AF-4, with primers for PCR verification indicated by arrows (a). PCR products of the correct integration of the genes *RIB6* (b) and *GND1* (c).

Moreover, the presence of the plasmid pRFE1/RIB6/GND1 in AF-4 derived transformants was confirmed by diagnostic PCR, using pairs of primers Ko1356/Ko1355 and Ko1056/Ko1059 (Table 2.3) for expression cassette of genes *RIB6* and *GND1* (Figure 3.19). Selected and verified transformants of *C. famata* AF-4 were named strains AF-4/RFE1-RIB6, AF-4/RFE1-GND1, AF-4/RIB6-GND1, and AF-4/RFE1-RIB6-GND1, respectively.

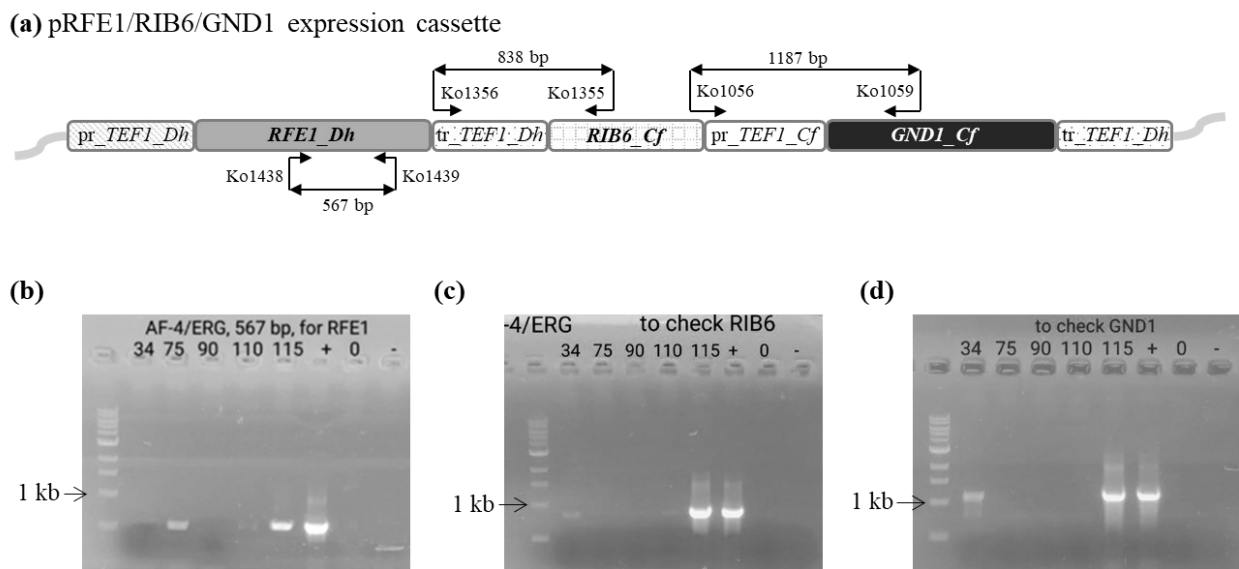


Figure 3.19 Scheme of expression cassette of plasmid pRFE1/RIB6/GND1 integrated into the genome of strain AF-4, with primers for PCR verification indicated by arrows (a). PCR products of the correct integration of the genes *RFE1* (b), *RIB6* (c), and *GND1* (d).

Initial characterization of riboflavin production was performed in the strain AF-4, and its derivatives AF-4/RFE1-GND1, AF-4/RFE1-RIB6, AF-4/RIB6-GND1, and

AF-4/RFE1-RIB6-GND1 on five days of cultivation in media: YPD, YNB, and cheese whey.

As a result, in YPD medium, except for strain AF-4/RFE1-GND1, which had no change in biomass accumulation, the biomass accumulation of the other three strains increased by 5–11% compared to parental strain AF-4 (Figure 3.20). Moreover, strains AF-4/RFE1-RIB6, AF-4/RFE1-GND1, AF-4/RIB6-GND1, and AF-4/RFE1-RIB6-GND1 demonstrated 9%, 45%, 49%, and 66% increase in riboflavin production, respectively (Figure 3.20).

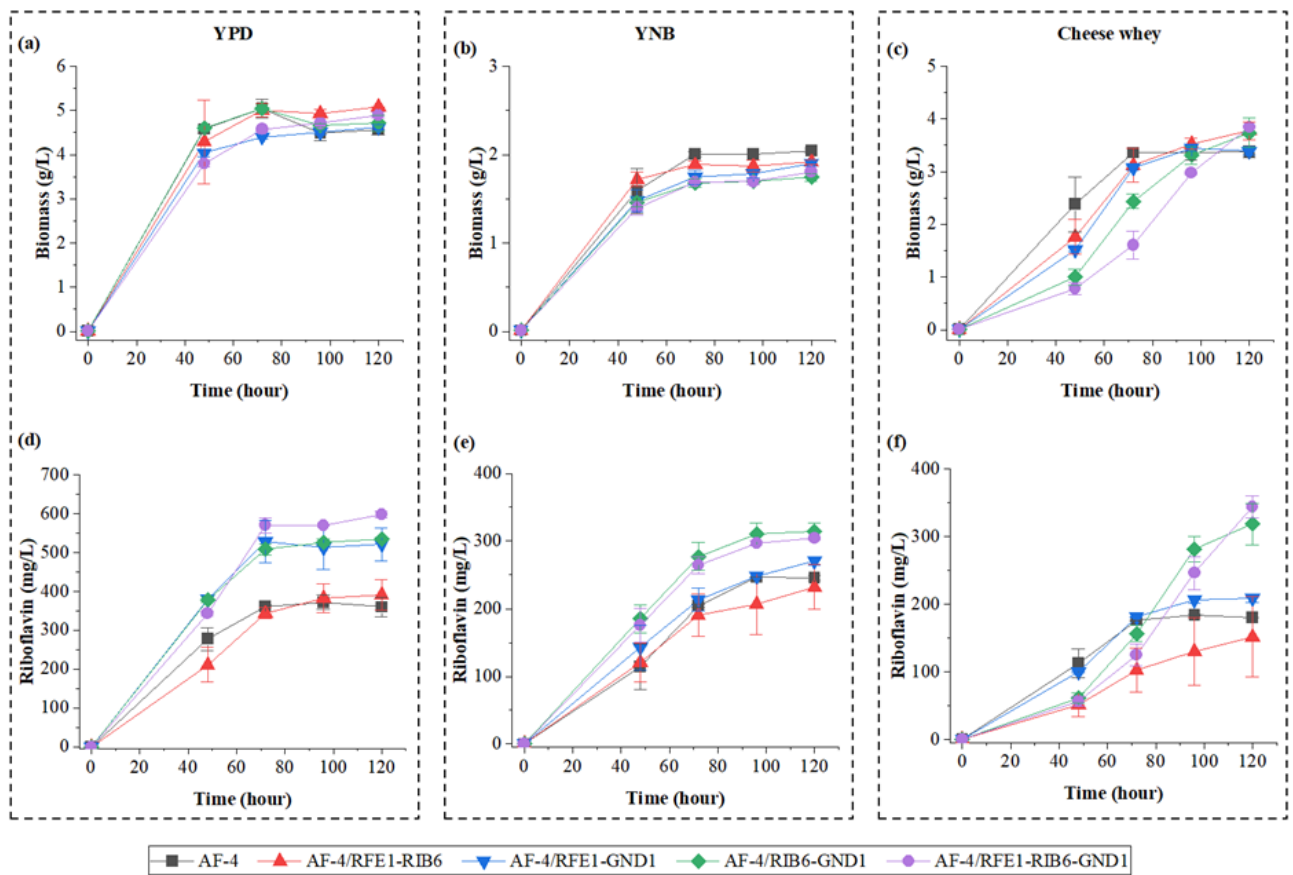


Figure 3.20 Time profiles of biomass accumulation and riboflavin production of strains AF-4, AF-4/RFE1-GND1, AF-4/RFE1-RIB6, AF-4/RIB6-GND1, and AF-4/RFE1-RIB6-GND1 in different media in flasks: Biomass accumulation in YPD (a), YNB (b), and cheese whey (c); Riboflavin production in YPD (d), YNB (e), and cheese whey (f).

In YNB medium, four recombinant strains showed decreased biomass accumulation by 6–15% compared to AF-4. In contrast, AF-4/RFE1-GND1, AF-4/RIB6-GND1, and AF-4/RFE1-RIB6-GND1 demonstrated 10%, 28%, and 24% increase in riboflavin production, respectively, but AF-4/RFE1-RIB6 had not changed (Figure 3.20).

In cheese whey medium, four recombinant strains showed similar results in biomass accumulation as in YPD. AF-4/RFE1-GND1 had not changed, while the other three strains increased biomass accumulation by 11–14% compared to AF-4 (Figure 3.20). At the same time, AF-4/RFE1-GND1, AF-4/RIB6-GND1, and AF-4/RFE1-RIB6-GND1 revealed 16%, 77%, and 91% increase in riboflavin production compared to AF-4, respectively, but AF-4/RFE1-RIB6 had decreased by 16% (Figure 3.20).

Additionally, strain AF-4/RFE1-GND1 showed a 43%, 18%, and 15%, increase in riboflavin yield (mg of riboflavin per gram of CDW) compared to AF-4, during cultivation in YPD, YNB, and cheese whey, respectively (Figure 3.21).

Strain AF-4/RIB6-GND1 showed a 44%, 50%, and 59% increase in riboflavin yield compared to AF-4, during cultivation in YPD, YNB, and cheese whey, respectively (Figure 3.21).

In parallel, strain AF-4/RFE1-RIB6-GND1 revealed a 55%, 40%, and 67% increase in riboflavin yield compared to AF-4, during cultivation in YPD, YNB, and cheese whey, respectively (Figure 3.21).

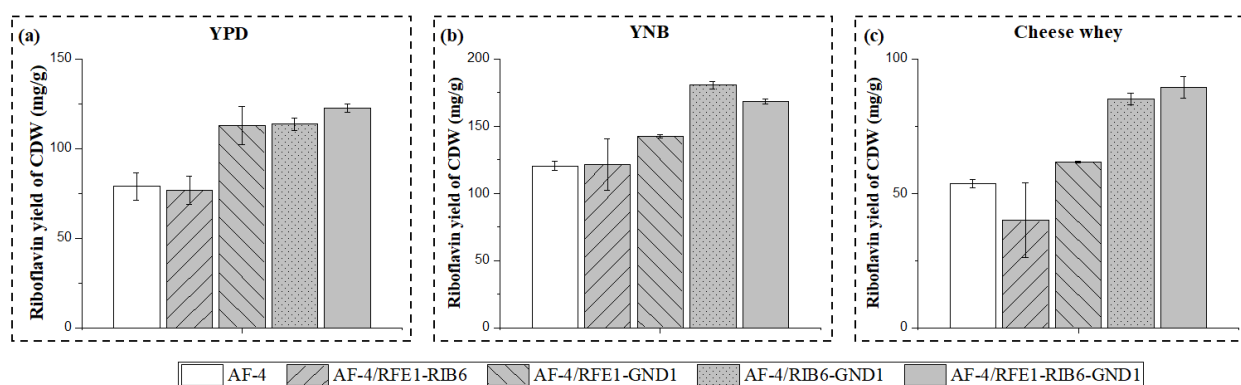


Figure 3.21 Riboflavin yield (mg/g CDW) of strains AF-4, AF-4/RFE1-RIB6, AF-4/RFE1-GND1, AF-4/RIB6-GND1, and AF-4/RFE1-RIB6-GND1 in different media: YPD (a), YNB (b), and cheese whey (c) in the fifth day of cultivation.

These results showed that the riboflavin production of constructed strains AF-4/RFE1-GND1, AF-4/RIB6-GND1, and AF-4/RFE1-RIB6-GND1 in three different media was increased, with the highest riboflavin production reaching up 605 mg/L observed in YPD by AF-4/RFE1-RIB6-GND1. Additionally, in cheese whey, AF-4/RFE1-RIB6-GND1 is the strain with the highest increase in riboflavin yield among all strains, 67%, compared to AF-4 (Figure 3.21).

We proved that *C. famata* wild-type strain VKM Y-9 and non-reverting riboflavin producer AF-4 showed the best ability of riboflavin production compared to strains overexpressing plasmid containing two genes by introducing the plasmid pRFE1/RIB6/GND1.

In addition, it performed that constructed strain AF-4/RFE1-RIB6-GND1 was cultivated in industrial media: YNB + molasses, beer wort, and YNB + hydrolysate, respectively.

Strain AF-4/RFE1-RIB6-GND1 revealed a light decrease in cell biomass accumulation compared to AF-4 in three industrial media. In the same time, AF-

4/RFE1-RIB6-GND1 revealed a 48.4%, 24.4%, and 16.8% increase in riboflavin production compared to AF-4, during cultivation in YNB + molasses, beer wort, and YNB + hydrolysate, respectively (Figure 3.22).

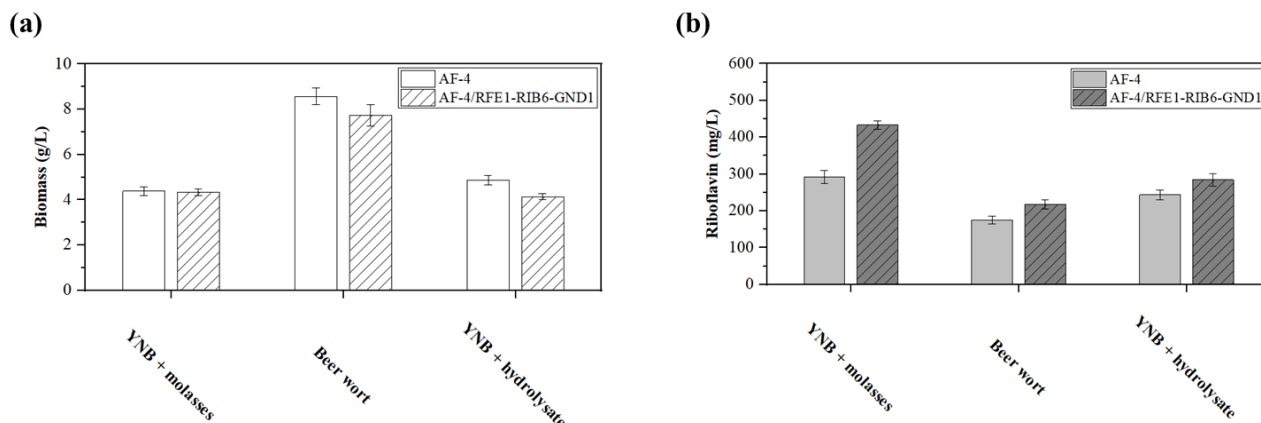


Figure 3.22 Cell biomass (a) and riboflavin production (b) by *C. famata* strains AF-4 and AF-4/RFE1-RIB6-GND1 during growth in YNB + molasses, beer wort, and YNB + hydrolysate at 96 hours.

Besides, we transformed plasmid pRFE1/RIB6/GND1 into the advanced riboflavin overproducer BRPI to generate strain BRPI/RFE1-RIB6-GND1, which accumulated 1.15-fold more riboflavin production in YNB with 0.2% yeast extract, reaching 1247 mg/L. However, this improvement is not particularly obvious.

Additionally, strain BRPI/RFE1-RIB6-GND1 was performed high-density fermentation (4 mg/L of cells) in industrial waste media in 72 hours. High-density fermentation allows for greater cell concentration, enhancing production of target compounds.

As a result, BRPI/RFE1-RIB6-GND1 revealed a 1.8-, 1.4-, and 1.3-fold increase in riboflavin production compared to BRPI, during cultivation in cheese whey, YNB + molasses, and YNB + hydrolysate, respectively (Figure 3.23). BRPI/RFE1-RIB6-

GND1 accumulated riboflavin up to 1716 mg/L in cheese whey in 72 hours (Figure 3.23).

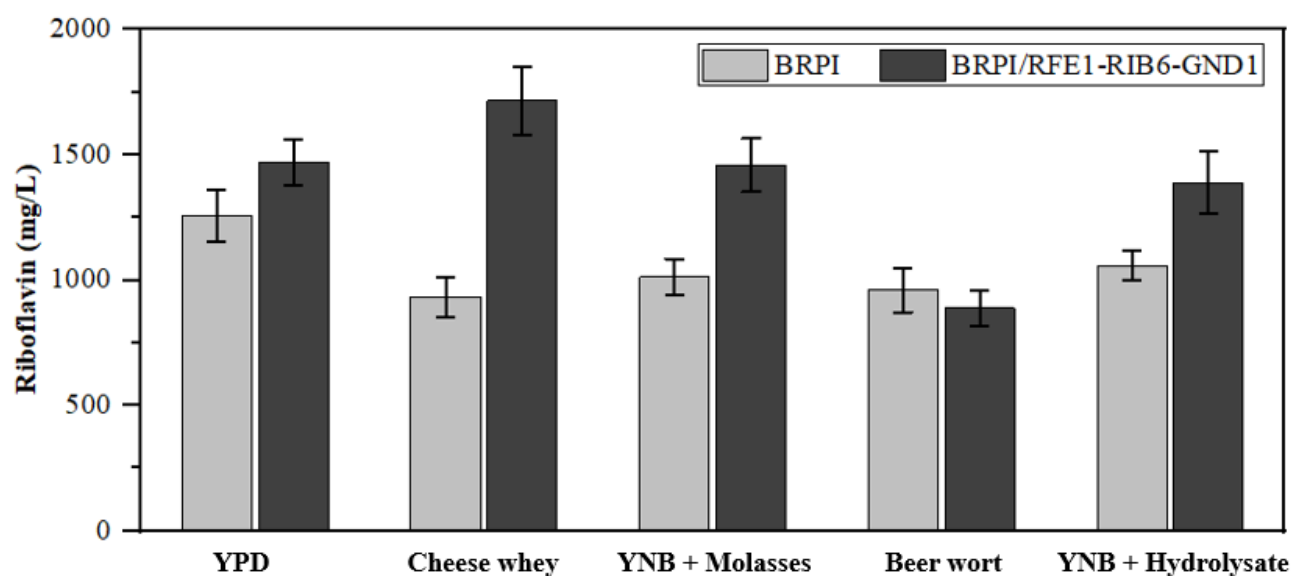


Figure 3.23 Riboflavin production by *C. famata* strains BRPI and BRPI/RFE1-RIB6-GND1 during growth in YPD, cheese whey, YNB + molasses, beer wort, and YNB + hydrolysate at 72 hours.

3.3. Effects of promoters of gene *SEF1* derived from various yeasts on riboflavin production in the yeast *C. famata*

Transcription factor Sef1, which is encoded by gene *SEF1*, is one of the positive regulators of riboflavin biosynthesis in *C. famata* (Dmytruk et al., 2006). This gene was found in the genomes of non-flavinogenic yeasts, however it did not participate in the regulation of riboflavin biosynthesis (Groom et al., 1998). The role of factor Sef1 in non-flavinogenic yeasts has not yet been fully elucidated. But it was found that factor Sef1 is involved in mitochondrial tRNA maturation in *Kluyveromyces lactis* (Groom et al., 1998), in addition, it can activate genes for the assembly of iron-sulfur clusters in

Candida glabrata (Gerwien et al., 2016; Ror and Panwar, 2019). Sef1 also activated the expression of several TCA cycle enzymes (Gupta and Outten, 2020).

In this work, the *SEF1* gene of *C. famata* was placed under the control of *SEF1* promoters from various flavinogenic and non-flavinogenic yeasts to assess their impact on transcriptional activator expression and riboflavin biosynthesis.

3.3.1. Plasmid construction

To analyze the function of promoters from different yeasts, A deletion cassette was created for inactivate gene *SEF1* in the genome of *C. famata*. The constructed cassette was have been described previously (Andreieva et al., 2020b). Here is a brief description, the 5'-part of gene *SEF1* was amplified by PCR from the genome of *C. famata* L2. The 5'-part of gene *SEF1* was digested with restriction endonuclease *Pst*I and cloned into *Pst*I-linearized plasmid p19L2. Further, 3'-part of gene *SEF1* was amplified and subcloned into the restriction sites *Sal*I and *Bam*HI of plasmid p19L2 containing 5'-part of gene *SEF1*. Constructed plasmid was designated as pSEF1Δ/LEU2 (Figure 3.24) (Andreieva et al., 2020b).

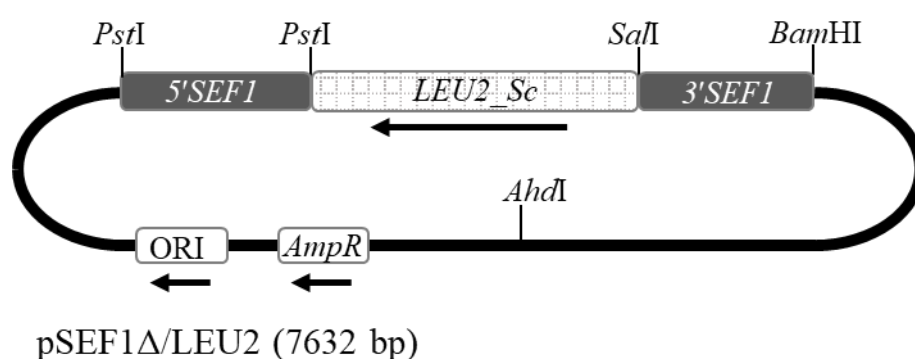


Figure 3.24 Circular scheme of plasmid pSEF1Δ/LEU2. Gene *LEU2_Sc* from *S. cerevisiae* indicated as a dashed square fill box; 5'- and 3'-parts of gene *SEF1* indicated as grey boxes; ori – Origin of replication; *AmpR* – ampicillin resistance gene; *Pst*I, *Sal*I, and *Bam*HI are restriction sites of endonucleases (Andreieva et al., 2020b).

The *AhdI*-linearized plasmid pSEF1 Δ _LEU2 was introduced in genome of the recipient strain *C. famata* L2 by electroporation (Voronovsky et al., 2002). Selected strain was named L2 *sef1* Δ , which lost the ability to overproduce riboflavin similar to that *C. famata* strain was inserted with truncated gene *SEF1* (Dmytruk et al., 2006).

To investigate possible species-specific transcriptional regulation of gene *SEF1*, a set of expression cassettes of gene *SEF1* were constructed. The plasmids were designated as pNTC/prSEF1_*Cf*, pNTC/prSEF1_*Ca*, pNTC/prSEF1_*Ct*, pNTC/prSEF1_*Ss*, pNTC/prSEF1_*Sc* (Figure 3.25) (Andreieva et al., 2020b).

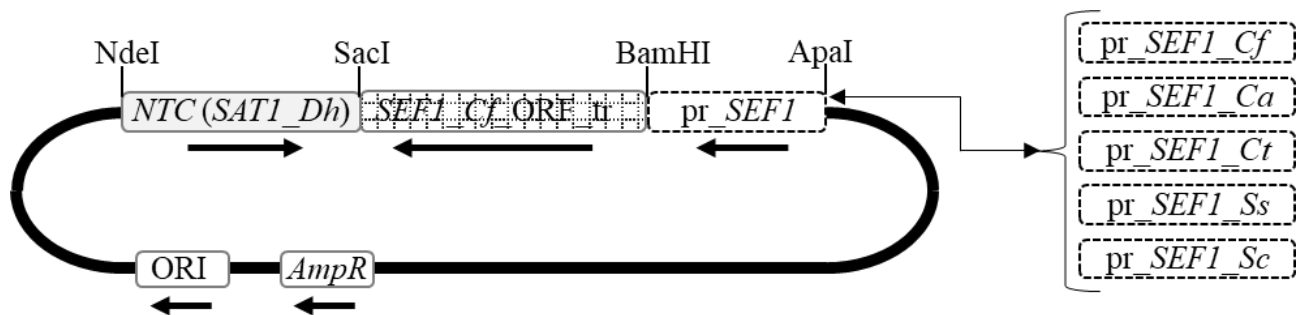


Figure 3.25 Circular scheme of the plasmids pNTC/prSEF1_*Cf*/*Ca*/*Ct*/*Ps*/*Sc*. *NTC* (*Dh_SAT1*) is a gene providing resistance to the antibiotic norzeothricin; *C. famata* gene *SEF1* promoter-driven RPs (pr_*SEF1*) from different yeasts are indicated by a long dashed rectangles and rectangles with a dashed outline; ori – the point of the start of replication; *AmpR* – ampicillin resistance gene; *NdeI*, *SacI*, *BamHI*, *ApaI* are hydrolysis sites of restriction endonucleases (Andreieva et al., 2020b).

In order to study the effects of *SEF1* promoters from different flavinogenic and non-flavinogenic yeast species on *SEF1* expression. Each of linearized plasmids pNTC/prSEF1_*Cf*, pNTC/prSEF1_*Ca*, pNTC/prSEF1_*Ct*, pNTC/prSEF1_*Ps*, and pNTC/prSEF1_*Sc* was introduced in *C. famata* L2 *sef1* Δ by electroporation. After stabilization, selected corresponding transformants were verified by diagnostic PCR, respectively (Andreieva et al., 2020b).

The confirmed transformants of *C. famata* were named as strains L2 *sef1Δ*/prSEF1_*Cf*, L2 *sef1Δ*/prSEF1_*Ca*, L2 *sef1Δ*/prSEF1_*Ct*, L2 *sef1Δ*/prSEF1_*Ss*, L2 *sef1Δ*/prSEF1_*Ss*, and L2 *sef1Δ*/prSEF1_*Sc* (Andreieva et al., 2020b).

3.3.2. Biochemical characteristics of constructed strains producing riboflavin

To initial characterization of the effect of each constructed promoter on riboflavin production, these strains L2 *sef1Δ*/prSEF1_*Cf_Ca_Ct_Ss_Sc* were cultured for five days on YNB supplemented with leucine in flasks. It was shown that the *SEF1* promoters from *C. albicans* and *C. tropicalis* are able to restore riboflavin overproduction in strain L2 *sef1Δ* (Figure 3.26 and Figure 3.27) (Andreieva et al., 2020b).

The constructed strains L2 *sef1Δ*/prSEF1_*Cf_Ca_Ct_Ss_Sc* demonstrated approximately 40% increased biomass accumulation compared to parental strain L2 *sef1Δ*, in contrast, comparable with wild-type strain L2 (Figure 3.26) (Andreieva et al., 2020b).

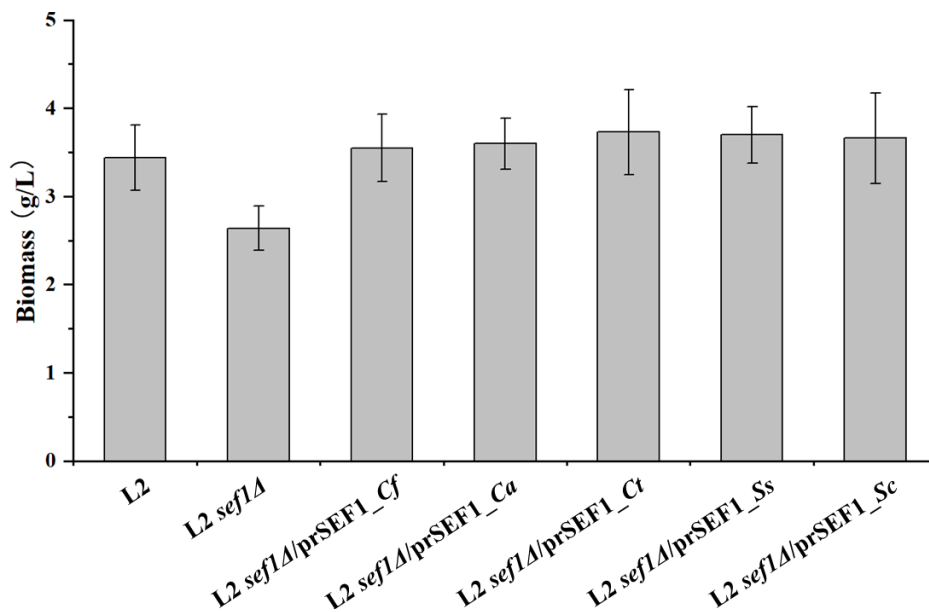


Figure 3.26 Biomass accumulation by *C. famata* strains L2, L2 *sef1Δ*, and L2 *sef1Δ*/prSEF1_*Cf_Ca_Ct_Ss_Sc* on the fifth day of cultivation in YNB medium supplemented with leucine in flasks.

Additionally, strains in which gene *SEF1* expression occurs under the control of promoters from the yeasts *C. famata*, *C. albicans*, and *C. tropicalis* increased 18.8-, 19.4-, and 13.5-fold on riboflavin production compared to strain L2, respectively (Figure 3.27) (Andreieva et al., 2020b).

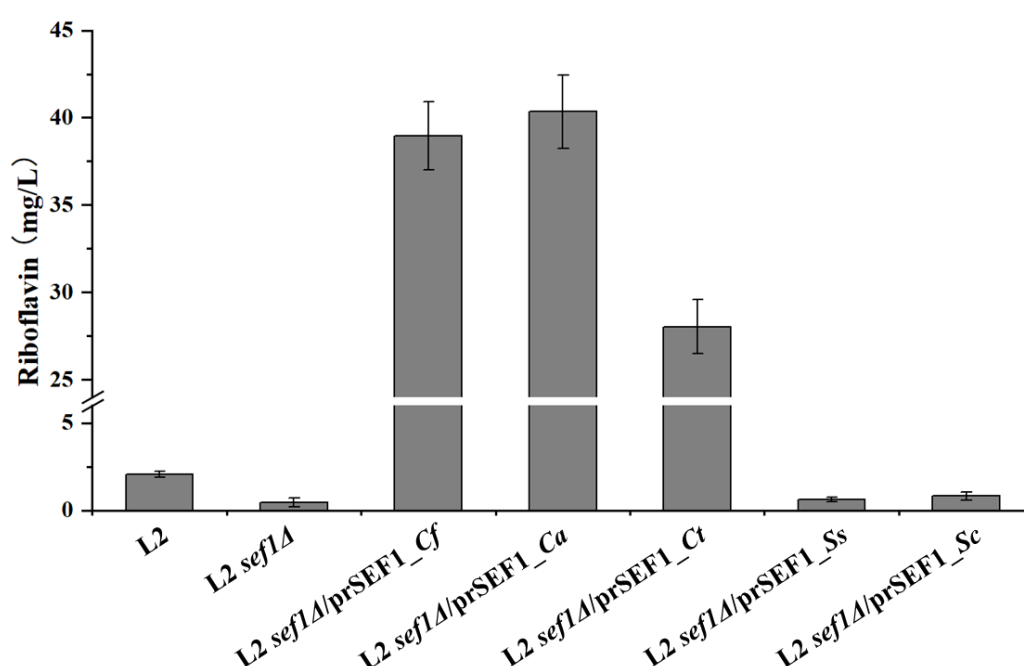


Figure 3.27 Riboflavin production by *C. famata* strains L2, L2 *sef1Δ*, and L2 *sef1Δ*/prSEF1_*Cf_Ca_Ct_Ss_Sc* on the fifth day of cultivation in YNB medium supplemented with leucine in flasks.

As expected, *C. famata* strains carrying *SEF1* promoters from flavinogenic yeasts overproduced riboflavin. Strains L2 *sef1Δ*/prSEF1_*Cf* and L2 *sef1Δ*/prSEF1_*Ca* accumulated approximately 40 mg of riboflavin, whereas L2 *sef1Δ*/prSEF1_*Ct* showed no significant increase. Reports suggest that *C. tropicalis* includes both flavinogenic and non-flavinogenic strains, positioning it between these groups (Amornrattanapan, 2013; Buzzini and Rossi, 1998).

Since riboflavin overproduction involves multiple genes and regulatory factors, it is likely that only some relevant genes, including *SEF1*, are active in the *C. tropicalis*. Consequently, its *SEF1* promoter was introduced into *C. famata* resulted in a riboflavin overproducing phenotype, with lower production than when using the *SEF1* promoter from the flavinogenic yeast *C. albicans*.

The results of the research presented in this section were published by the doctoral student in an experimental article: Andreieva, Y., Petrovska, Y., Lyzak, O., **Liu, W.**, Kang, Y., Dmytruk, K., and Sibirny, A. (2020). Role of the regulatory genes *SEF1*, *VMA1* and *SFU1* in riboflavin synthesis in the flavinogenic yeast *Candida famata* (*Candida flareri*). *Yeast*, 37(9–10), 497–504. <https://doi.org/10.1002/yea.3503>.

3.4. Effects of vacuolar ATPase disruption on riboflavin production in the yeast

C. famata

Previously, the engineered strain *C. famata* dep8 can accumulate about 20 g/L of riboflavin (Abbas and Sibirny, 2011; Stahmann et al., 2000), however the production process was stopped, due to the low genetic stability of engineered strain (Abbas and Sibirny, 2011). Therefore, *C. famata* remains less efficient than the established commercial producers *B. subtilis* and *A. gossypii*.

Disruption of the gene *VMA1*, which encodes the vacuolar ATPase in *A. gossypii*, prevents riboflavin from remaining in the vacuole chamber and transports riboflavin flux to the medium (Förster et al., 1999, 2001). Also, a similar effect is manifested in the flavinogenic yeast *P. guilliermondii* (Boretsky et al., 2011). Moreover, it was found the mutants of *P. guilliermondii* sensitive to elevated temperature, like the corresponding insertion mutants of *S. cerevisiae* (Kabani et al., 2002). These data suggested that the introduction of appropriate mutations in *C. famata* may lead to a positive effect on riboflavin synthesis.

3.4.1. Plasmid construction

A deletion cassette of gene *VMA1* was constructed as described previously (Andreieva et al., 2020a). Here is a brief description, 5'-part and 3'-part of gene *VMA1* were amplified by PCR from genomic DNA of *C. famata* VKMY-9, respectively. The two PCR products were fused by overlap-extension PCR, further, they were cloned into the restriction sites *SacI* and *SalI* of the vector pTb (Dmytruk et al., 2006), to generate the final plasmid pVMA1Δ/ble (Figure 3.28) (Andreieva et al., 2020a).

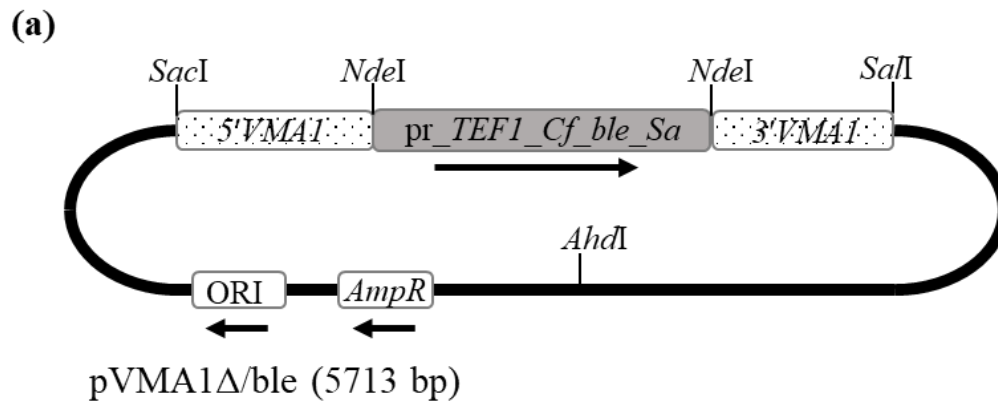


Figure 3.28 Circular scheme of plasmid pVMA1Δ/ble. Gene *ble* under control of promoter *TEF1* from *C. famata* indicated as dark grey box; 5'- and 3'-parts of gene *VMA1* are indicated as open boxes; ori – Origin of replication; *AmpR* – ampicillin resistance gene; *SacI*, *NdeI*, *SalI*, and *AhdI* are restriction sites of endonucleases (Andreieva et al., 2020a).

Cloned plasmid pVMA1Δ/ble was linearized by endonuclease *AhdI* and introduced in genome of the recipient strain *C. famata* L2 by electroporation (Voronovsky et al., 2002). Transformants were selected on a solid YPD medium supplemented with phleomycin after 5 days of incubation.

The selected transformants were stabilized by alternating cultivation on a nonselective followed selective media. After stabilization, selected transformants were verified by PCR for deletion cassettes of 5'- and 3'-parts of gene *VMA1* (Figure 3.29). Selected strain of *C. famata* was named L2 *vma1Δ* (Andreieva et al., 2020a).

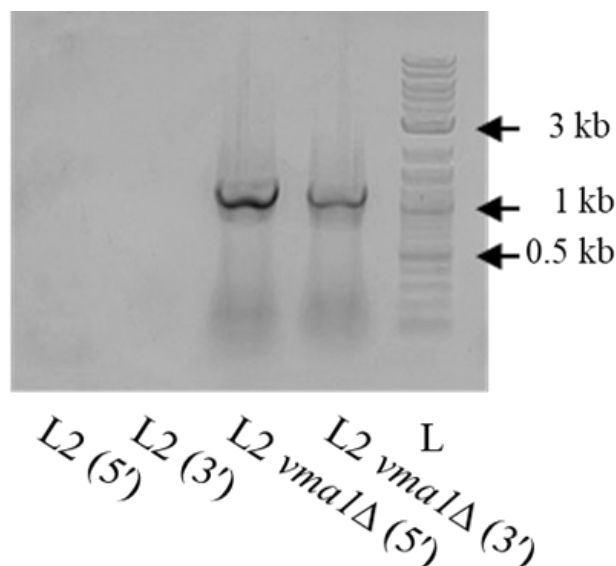


Figure 3.29 PCR verification of strains L2 and L2 *vma1Δ* to confirm the integration of 5'- and 3'-parts of gene *VMAL*; L – Ladder (Andreieva et al., 2020a).

3.4.2. Biochemical characteristics of mutant L2 *vma1Δ* producing riboflavin

Initial characterization of riboflavin production was performed in the strain L2 and its mutant L2 *vma1Δ* on five days of cultivation in the YNB medium supplemented with leucine in flasks.

On the fifth day, strain L2 *vma1Δ* decreased by approximately 3 times on biomass accumulation compared to the recipient strain L2 (Figure 3.30). At the same time, strain L2 *vma1Δ* showed approximately 9 times increased compared to the recipient strain L2 on riboflavin production, amounting to 20 mg/L (Figure 3.30). Additionally, 27 times increased on riboflavin yield in strain L2 *vma1Δ* was revealed compared to strain L2 (Figure 3.30) (Andreieva et al., 2020a).

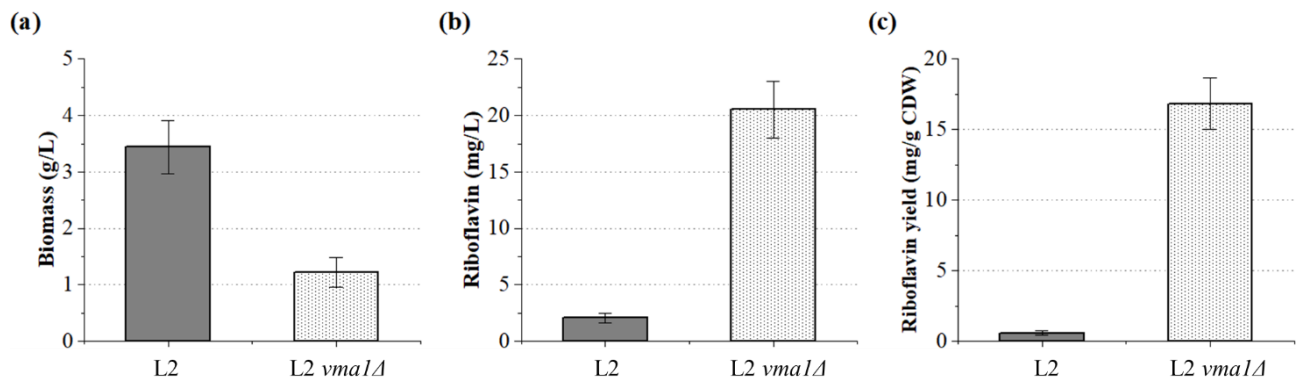


Figure 3.30 Cell biomass **(a)**, riboflavin production **(b)**, and riboflavin yield **(c)** by strains L2 and L2 *vma1Δ* on the fifth day of cultivation in YNB supplemented with leucine in flasks.

The results of the research presented in this section were published by the dissertation student in an experimental article: Andreieva, Y., Lyzak, O., **Liu, W.**, Kang, Y., Dmytruk, K., and Sibirny, A. (2020). *SEF1* and *VMA1* genes regulate riboflavin biosynthesis in the flavinogenic yeast *Candida famata*. *Cytology and Genetics*, 54(5), 379–385. <https://doi.org/10.3103/s0095452720050023>.

4. DISCUSSION

The vital role of riboflavin in life is obvious and requires no further elaboration. Our long-term goal is to develop riboflavin overproducers based on *C. famata* using some proven strategies (Voronovsky et al., 2002; Dmytruk et al., 2011, 2014, 2020; Tsyurulnyk et al., 2020; Andreieva et al., 2020b; Petrovska et al., 2022), and finding the suitable substrate for fermentation (Ruchala et al., 2022; Dzanaeva et al., 2024).

The riboflavin biosynthesis pathway has been basically established after years of research. It comprises seven enzymatic steps starting from the precursors GTP and Ru5P (Fischer et al., 2005; Abbas and Sibirny, 2011).

GTP is generated through the *de novo* purine nucleotide biosynthetic pathway, which starts with the synthesis of PRPP. Later, the two modified genes *PRS3* and *ADE4* coding for PRPP synthetase and PRPP amidotransferase, were co-overexpressed on the background of strain BRP to create the engineered strain BRPI, which showed a 2-fold increase in riboflavin production in shake flask relative to the parental strain (Dmytruk et al., 2020). Thus, we proved the essential role of GTP supply for riboflavin overproduction. However, the role of Ru5P in riboflavin overproduction in *C. famata* remains unknown (Dmytruk and Sibirny, 2012; Sibirny, 2023). The genetic manipulation led to an increase in Ru5P activated riboflavin accumulation in *B. subtilis* (Duan et al., 2010; Wang et al., 2011). Likely, increased activity of G6PDH and 6GPDH can significantly impact the PPP and contribute to an increased supply of Ru5P availability for riboflavin biosynthesis. Both G6PDH and 6GPDH generate intracellular NADPH, a crucial reducing agent that, when increased, can enhance cellular processes requiring reduction power in various biosynthetic pathways.

In our present results, the gene *ZWF1* was overexpressed in three different strains L2, AF-4, and BRP. The expression level of gene *ZWF1* and G6PDH activity of recombinant strains L2/*ZWF1*, AF-4/*ZWF1*, and BRP/*ZWF1* showed increased significantly compared to the parental strains, however, the riboflavin production and biomass accumulation decreased, which was contrary to our expectations. Recombinant strains L2/*GND1*, AF-4/*GND1*, and BRP/*GND1* revealed increased in G6PDH activity and riboflavin production relative to corresponding parental strains.

It was noted that overexpression of gene *zwf* slightly increased riboflavin production by about 20% in *B. subtilis*. We assume that the reason for the different results might be that increased NADPH generation in *C. famata* could disrupt metabolic homeostasis by causing feedback inhibition of key enzymes, thereby limiting riboflavin synthesis. Additionally, *C. famata* features a more complex regulatory network than bacteria, and overexpression of G6PDH might inadvertently repress riboflavin biosynthetic genes or enzymes through unknown regulatory pathways.

The riboflavin accumulation of the strains co-expressing genes *ZWF1* and *GND1* was not significantly changed, being slightly lower than that of the strains expressing the single gene *GND1*, but also a little higher than that of the parental strains. We speculate that the reason for these results may be due to the additional G6PDH, which catalyzes the conversion of G6P to more 6PGL, although the next step reaction 6PGL can occur spontaneously converted to 6PG slowly through non-enzymatic hydrolysis, while 6-phosphogluconolactonase is available to enhance the reaction rate greatly.

6GPL is a cyclic lactone, has high chemical reactivity, and can easily react with proteins, nucleic acids, and lipids in cells, which may lead to damage to the functions of these biological molecules (Rakitzis and Papandreou, 1998). Lactone is usually

harmless at low concentrations, but at high concentrations, it may be toxic to cells, causing cell membrane damage, protein denaturation, and DNA damage (Reyes et al., 2024). Therefore, the quick conversion of 6PGL prevents its accumulation, which ensures an efficient and smooth metabolic flux through the PPP while maintaining cellular homeostasis. Overexpression of G6PDH did not affect bacterial growth and riboflavin accumulation, which may be related to the permeability and transport proteins of bacterial cell membranes, which may more effectively exclude or transport 6PGL (Dong et al., 2007; Phégnon et al., 2024).

Our results showed that riboflavin production and biomass accumulation were improved when two genes *ZWF1* and *GND1* were co-expressed compared with single gene *ZWF1* expression in *C. famata* strains, which should support our speculation.

In future studies, it may be necessary to overexpress the gene *SOL3* in *C. famata* to increase the rapid conversion of 6GPL into 6PG and then enhance the accumulation of Ru5P (Nocon et al., 2016). An apart of Ru5P can enter the non-oxidative PPP to generate other sugar phosphates, such as erythrose 4-phosphate and xylulose 5-phosphate, which are precursors for the biosynthesis of different compounds (Masi et al., 2021). Inactivation of the corresponding enzymes can reduce the consumption of Ru5P, and enhancing the carbon flux toward riboflavin biosynthesis is a useful strategy (Yang et al., 2021). Furthermore, overexpression of the gene *GND1* increases the carbon flux to Ru5P, and overexpression of the gene *RIB6* allows more Ru5P to convert to the generation of DHPB (Petrovska et al., 2022), which should be able to effectively improve the riboflavin oversynthesis of *C. famata* strains.

Commercial riboflavin production is currently based on industrial fermentation using overproducing strains of genetically engineered microorganisms. Over the last few decades, several groups of researchers have reported successful achievements in

the construction of genetically modified strains of species, such as *B subtilis*, *A. gossypii*, *Corynebacterium ammoniagenes*, and *Candida* spp., by applying metabolic engineering strategies (Stahmann et al., 2000; Abbas and Sibirny, 2011; Schwechheimer et al., 2016; Wang et al., 2021; You et al., 2021). More frequently such strategies have led to the overexpression of structural and regulatory genes involved in the biosynthesis of riboflavin or that of its precursors; consequently, this has improved strain productivity and yield of the industrial fermentation product. The application of methods of classical selection and metabolic engineering, including overexpression of structural and regulatory genes of riboflavin biosynthesis and excretion, genes involved in GTP biosynthesis, and deletion of the genes of negative regulators of vitamin B₂ biosynthesis, resulted in the construction of highly effective riboflavin producers based on yeast *C. famata* (Dmytruk et al., 2011, 2014, 2020).

Another part of our work reports a strong positive impact of genes *RFE1*, *GND1*, and *RIB6* on activation of riboflavin production in strain VKM Y-9 in three different media. The expression of various combinations of two genes, and co-expression of all three genes, led to an increased riboflavin production in strain VKM Y-9. It was shown that strain co-expressing three genes, V9/RFE1-RIB6-GND1, significantly increased by a 2.61- and 1.63-fold riboflavin yield as compared to parental strain in YNB+YE, and YPD media, respectively. Strain V9/RFE1-RIB6-GND1 revealed the strongest growth and highest riboflavin accumulation (by 3.3-fold) in cheese whey compared to the other two media.

Global production of cheese whey is approximately 160 million tons per year (Vincenzi et al., 2014). Usually, part of the cheese whey is used to produce whey powder, whey proteins, and powdered lactose in industry, and the other part is discarded (Ruchala et al., 2022).

Riboflavin biosynthesis in cheese whey was activated in strains AF-4 and BRP by overexpressing transcription activator gene *SEF1* under the control of the lactose-induced promoter *LAC4* of the homologous β -galactosidase gene (Tsyrlunyk et al., 2021; Sibirny, 2023). The dairy industry waste, cheese whey, supports robust growth and riboflavin overproduction by *C. famata* comparable to glucose (Ruchala et al., 2022; Sibirny, 2023). Therefore, our studies suggest that cheese whey could serve as a promising substrate for industrial production of riboflavin.

This part of the work demonstrates an effective genetic approach to improve riboflavin production by co-expression of genes *GND1*, *RIB6*, and *RFE1* in strain VKM Y-9. In the foreseeable future, the introduction of these genes into the advanced riboflavin producer BRPI, together with the expression of the gene *SEF1* under the control of the *LAC4* promoter, as well as cultivation in cheese whey should greatly increase riboflavin production.

Additionally, this dissertation involved further study of the role of transcription factor Sef1 in riboflavin biosynthesis and the role played by the promoter of gene *SEF1* in this process. As we discussed above, factor Sef1 plays the central role in riboflavin biosynthesis and iron metabolism in *C. famata* and *C. albicans* (Abbas and Sibirny, 2011; Ror and Panwar, 2019). It was found that expressing the *SEF1* promoters from the flavinogenic yeasts *C. famata*, *C. albicans*, and *C. tropicalis* increased 18.8-, 19.4-, and 13.5-fold on riboflavin production compared to strain L2, respectively.

Understanding of function and regulation of factor Sef1 provides a powerful tool to enhance riboflavin production. However, the maximal potential of *SEF1* expression is still unknown. Discovery of the role of *SEF1* in riboflavin oversynthesis gave a unique possibility to engineer *C. famata* by shaping regulatory network in favor of riboflavin overproduction. The ability to oversynthesis riboflavin in industrial strains

probably is not tied to single gene mutations and can also depend not only on genes that are directly involved in riboflavin biosynthesis. This has been clearly demonstrated previously by insertion mutagenesis of gene *MET2* (Dmytruk et al., 2006) and now by disruption of gene *VMA1*.

CONCLUSION

This work investigated the genes *GND1*, *RIB6*, *RFE1*, *ZWF1*, *SEF1*, and *VMA1* involved in regulating riboflavin biosynthesis in the yeast *C. famata*. Based on our results, these approaches can be further applied to improve the competitiveness of riboflavin advanced overproducers. The main scientific and practical tasks of the work are outlined in the following:

1) To study the role of genes encoding key enzymes of the oxidative branch of the pentose phosphate pathway in riboflavin biosynthesis by *C. famata* strains L2, AF-4, and BRP.

2) To construct strains via co-overexpression of regulation genes *RFE1*, *GND1*, and *RIB6* in *C. famata* strains VKM Y-9 and AF-4 and analyze the level of riboflavin production in recombinant strains.

Main conclusions of the current work are as follows:

1. The effects of overexpressing genes *ZWF1* and *GND1*, which encode key enzymes of the oxidative branch of the PPP in riboflavin biosynthesis by yeast *C. famata* were investigated. It was shown that overexpression of gene *ZWF1* led to a negative effect in *C. famata*, especially in riboflavin overproducer BRP. In contrast, overexpression of the gene *GND1* elevated riboflavin production up to 1.9-fold compared to the parental strain.

2. The set of plasmids of various combinations with genes *GND1* (encodes 6PGDH), *RIB6* (encodes DHBP synthase) and *RFE1* (encodes riboflavin excretase) were constructed. The strain engineered for the co-overexpression of all three genes exhibited up to a 3.3-fold increase in riboflavin production in cheese whey, compared to the parental strain VKM Y-9.

3. It was revealed that expressing the promoters of gene *SEF1* from the flavinogenic yeasts *C. famata*, *C. albicans*, and *C. tropicalis* improved cell growth and increased significantly riboflavin production in mutant L2 *sef1Δ*.

4. It was indicated that disrupted vacuolar ATPase led to approximately 27 times increased riboflavin yield by yeast *C. famata* L2.

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APPENDIX

LIST OF PUBLICATIONS ON THE THESIS TOPIC:

1. **Liu, W.**, Tsyurulnyk, A., Dmytruk, K., Fedorovych, D., Kang, Y., and Sibirny, A. (2025). Co-overexpression of genes *RFE1*, *GND1*, and *RIB6* enhances riboflavin production in yeast *Candida famata*. *Cytology and Genetics*, 59(1), 63–70. <https://doi.org/10.3103/S0095452725010074>. Q4. Scopus and WoS. (*The author, together with co-authors, conducted research, analyzed and summarized the obtained data, took part in the writing, and design of the publication*).
2. Ruchala, J., Andreieva, Y., Tsyurulnyk, A., Sobchuk, S., Najdecka, A., **Liu, W.**, Kang, Y., Dmytruk, O., Dmytruk, K., Fedorovych, D., and Sibirny, A (2022). Cheese whey supports high riboflavin synthesis by the engineered strains of the flavinogenic yeast *Candida famata*. *Microbial Cell Factories*, 21(1), 161-169. <https://doi.org/10.1186/s12934-022-01888-0>. Q1. Scopus and WoS. (*The author, together with co-authors, conducted research, analyzed and summarized the obtained data, took part in the writing, and design of the publication*).
3. Andreieva, Y., Petrovska, Y., Lyzak, O., **Liu, W.**, Kang, Y., Dmytruk, K., and Sibirny, A. (2020). Role of the regulatory genes *SEF1*, *VMA1* and *SFU1* in riboflavin synthesis in the flavinogenic yeast *Candida famata* (*Candida flareri*). *Yeast*, 37(9–10), 497–504. <https://doi.org/10.1002/yea.3503>. Q2. Scopus and WoS. (*The author, together with co-authors, conducted research, analyzed and summarized the obtained data, took part in the writing, and design of the publication*).
4. Andreieva, Y., Lyzak, O., **Liu, W.**, Kang, Y., Dmytruk, K., and Sibirny, A. (2020). *SEF1* and *VMA1* genes regulate riboflavin biosynthesis in the flavinogenic yeast *Candida famata*. *Cytology and Genetics*, 54(5), 379–385.

<https://doi.org/10.3103/s0095452720050023>. Q4. Scopus and WoS. (*The author, together with co-authors, conducted research, analyzed and summarized the obtained data, took part in the writing, and design of the publication*).

Abstracts of reports at domestic and international conferences, scientific congresses and congresses:

5. **Liu, W.** Exploration of Role of Overexpressed Genes *ZWF1* and *GND1* in Riboflavin Synthesis by *Candida famata* (*Candida flareri*) // 1st International Conference of Young Scientists of the Institute of Cell Biology and the University of Rzeszów "Current Issues in Cell Biology and Biotechnology", June 02, Lviv, Ukraine. – 2021. – P. 25.

6. **Liu, W.** Role of the pentose phosphate pathway in riboflavin oversynthesis of the flavinogenic yeast *Candida famata* (*Candida flareri*) // Conference of Young Scientists of Institute of Cell Biology, June 08, Lviv, Ukraine. – 2021. – P. 21.

7. Andreieva, Y., **Liu, W.**, Dmytruk, K., Sibirny, A. Evaluation of the effect of overexpressed genes *ZWF1* and *GND1* on riboflavin synthesis by flavinogenic yeast *Candida famata* (*Candida flarerii*) // 8th International Conference "Human – Nutrition – Environment", October 13-14, Rzeszow, Poland. – 2021. – P. 66.

8. **Liu, W.**, Tsyurulnyk, A., Dmytruk, K., Fedorovych, D., Sibirny, A. Development of platform for constructing of riboflavin overproducers based on the flavinogenic yeast *Candida famata* // XX International Scientific Conference for Students and PhD Students "Youth and Progress of Biology", April 18-20, Lviv, Ukraine. – 2024. – P. 183-184.

9. **Liu, W.**, Tsyurulnyk, A., Dmytruk, K., Fedorovych, D., Sibirny, A. Combination of using positive regulator genes for riboflavin overproduction in the

flavinogenic yeast *Candida famata* // Conference of Young Scientists of Institute of Cell Biology, May 20, Lviv, Ukraine. – 2024. – P. 2.

10. **Liu, W.**, Tsyurulnyk, A., Dmytruk, K., Fedorovych, D., Sibirny, A. Construction of riboflavin overproducers by introduction of genes *RFE1*, *RIB6*, and *GND1* into the yeast *Candida famata* // 7th Congress for All-Ukrainian Public Organization Ukrainian Society of Cell Biology with International Representation, September 11-13, Lviv, Ukraine. – 2024. – P. 105.

11. Fedorovych, D., Tsyurulnyk, A., Dzanajeva L., **Liu, W.**, Ruchala, J., Wojdyla D., Dmytruk, K., Sibirny, A. *Candida famata* cell factory for production of vitamin B₂ // 7th Congress for All-Ukrainian Public Organization Ukrainian Society of Cell Biology with International Representation, September 11-13, Lviv, Ukraine. – 2024. – P. 93.