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LACTOSE INDUCIBLE EXPRESSION OF TRANSCRIPTION FACTOR GENE *SEF1* INCREASES RIBOFLAVIN PRODUCTION IN THE YEAST *CANDIDA FAMATA*

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Riboflavin (vitamin B_2) is required for synthesis of the flavin coenzymes: riboflavin-5'-phosphate (flavin mononucleotide) and flavin adenine dinucleotide. Riboflavin is important biotechnological commodity with annual market around 250 million US dollars. It is mostly used as component of feed premixes for animals (80%), in food industry as food colorant, in medicine and component of multivitamin mixtures and as drug for treatment of some diseases. Over the past two decades, the microbial production of riboflavin by fermentation completely replaces the chemical synthetic route. The main producers of riboflavin in industry are engineered strains of the bacterium Bacillus subtilis and of the mycelial fungus Ashbya gossypii. Flavinogenic yeast Candida famata has great biosynthetic potential. Using combination of classical selection and metabolic engineering (overexpression of SEF1, RIB1 and RIB7 genes coding the positive regulator, the first and the last structural enzymes of riboflavin synthesis) resulted in the construction of genetically stable strain of C. famata that produces 16 gram of riboflavin per liter in bioreactor. However, the productivity of riboflavin biosynthesis remains still insufficient for industrial production of this vitamin. Studies of transcriptional regulation of genes involved in riboflavin synthesis and using of strong promoters of C. famata for construction of efficient producers of vitamin B_2 are areas of both scientific and industrial interest. **Aim.** *The aim of the current work was to improve riboflavin oversynthesis by the available C. famata strains in synthetic and natural lactose-containing media.* **Methods.** *The plasmid DNA isolation, restriction, ligation, electrophoresis in agarose gel, electrotransformation, and PCR were carried out by the standard methods. Riboflavin was assayed fluorometrically using solution of synthetic riboflavin as a standard. The cultivation of yeasts was carried out in YNB or YPD media containing different source of carbon and on whey.* **Results.** *The strains of C. famata expressed additional copy of central regulatory gene SEF1 under control of the promoter of LAC4 gene (coding for β–galactosidase) C. famata were constructed. The influence of SEF1 gene expression under control of lactose inducible promoter of CfLAC4 gene on riboflavin production was studied. It was shown that the C. famata strains containing "pLAC4_cf-SEF1_cf" expression cassette revealed 1.6-2.1-fold increase in riboflavin yield on lactose when compared to the parental strain. The riboflavin production constructed strains on whey reached 1.69 gram per liter in flask batch culture.* **Conclusions.** *The constructed strains containing additional copy of SEF1 gene under the control of LAC4 promoter is a perfect platform for development of industrial riboflavin production on by-product of dairy industry, whey.*

Keywords: Candida famata yeast, riboflavin, SEF1 gene, LAC4 promoter, whey.

Riboflavin (7,8-dimethyl-10-ribityl-isoalloxazine, vitamin B_2) serves as biosynthetic precursor of flavin mononucleotide and flavin adenine dinucleotide which participate in a range of redox reactions, some of which are indispensable to the function of aerobic cells. Flavins are involved in a large number of reactions in energetic metabolism, oxidative stress response, photosensitization and activation of other vitamins such as folate and pyridoxine. This vitamin can be synthesized by plants and microorganisms, but is essential for animals as they lack an endogenous

biosynthetic pathway. Riboflavin, which is exclusively synthesized biotechnologically using microorganisms, is mainly used as feed additive (about 80% of current market), whereas about 20% are used as food additive and for pharmaceutical applications, respectively [1, 2]. The two most important industrial riboflavin producers are *Bacillus subtilis* and *Ashbya gossypii* [1, 2, 3].

Until recently, industrial riboflavin production included the flavinogenic yeast, *Candida famata* (*Candida flareri*) [4]. Some years ago the industrial production of riboflavin using mutant strains of the yeast *C. famata* by ADM Company (USA) was stopped due to lack of profitability. One of the main reasons for the termination of this process was low stability of riboflavin producer used [5]. Several years ago, we have identified gene *SEF1* coding for Zn2-Cys6 transcription factor which acts as the central regulator of riboflavin oversynthesis. It was found that insertions or deletions in the gene *SEF1* block riboflavin oversynthesis in the *C. famata* wild-type strain and in the industrial riboflavin producer *C. famata* dep8. Inversely, introduction of the additional copies of *SEF1* enhanced capacity of riboflavin production and strongly elevated stability of the strain dep8 [6]. Stable riboflavin overproducing strain *C. famata* AF-4 was isolated using classic mutagenesis and selection [7]. Metabolic engineering of this strain involved introduction of the additional copies of positive regulator *SEF1*, gene *IMH3* coding for IMP dehydrogenase, and two structural genes of riboflavin synthesis *RIB1* and *RIB7* coding for GTP cyclohydrolase II and riboflavin synthase, respectively. Resulted strain accumulated 16 g of riboflavin per liter during fed-batch cultivation [8]. These researches made a great contribution to industrial production of riboflavin using this yeast strains. However, even in the best of the selected strains, the production of riboflavin per gram of consumed glucose does not exceed 50 mg, and the productivity of riboflavin biosynthesis remains still insufficient for industrial production of riboflavin. Further improvement of yeast strains, use of low-cost substrates and studies on industrial riboflavin processes are necessary to increase vitamin B_2 yield and reduce riboflavin production costs.

In this context, we studied effect of expression of *SEF1* gene coding for transcription factor under control of lactose inducible promoter of *LAC4* gene encoding β-galactosidase on riboflavin production by the best of the available *C. famata* overproducers and the use of whey as fermentation medium for the bioproduction of riboflavin.

Materials and Methods

Strains and growth conditions. *Candida famata* riboflavin overproducers AF-4 [7] and AF-4/*SEF1/RIB1/RIB7* (designated as BRP from the Best Riboflavin Producer) [8] strains were used throughout this work. Yeast cells were cultured at 30 ºC in YNB medium or YPD medium (0.5% yeast extract, 1% peptone and 2% glucose) or YPL medium (0.5% yeast extract, 1% peptone and 2% lactose). For selection of yeast transformants, 10 mg/L of nourseothricin (NTC) was added. In some experiments, whey with a lactose content of 5% was used. To estimate riboflavin synthesis the yeast cells from a fresh plate were grown in 20 mL of liquid media in 100 mL Erlenmeyer flasks.

The *Escherichia coli* DH5α strain (Φ80d-*lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17 (r_K^-, m_K^+), *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*) U169) was used as a host for plasmid propagation. The DH5α strain was grown at 37 °C in LB medium as described previously [9]. The transformed *E. coli* cells were maintained on a medium containing 100 mg/L of ampicillin.

Plasmid cloning and yeast transformation. Restriction endonucleases and DNA ligase (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) were used according to manufacturer's instructions. PCR-amplification of the fragments of interest was performed using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) according to the manufacturer specification. PCRs were performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). Promoter of *LAC4 C. famata* was amplified with primers Ko1 068:TTTGGGCCCTATATAGAGACAATAAGA CCAG and Ko1069: CGCGGATCCGTTGAGT ATATATCTATTACTTC from the genomic DNA of *C. famata* VKMY-9 (All-Russian Collection of Microorganisms, Pushchino, Russia). Fragment was combined by overleap PCR with primers, *Apa*I/*BamH*I double digested and cloned into corresponding sites of recipient plasmid already contained *SEF1 C. famata* and NTC genes [10]. The resulted recombinant plasmid was named pNTC/p*LAC4_cf-SEF1_cf* (Fig. 1). Transformation of the yeast *C. famata* by electroporation was carried out as described previously [11]. Integration of the "p*LAC4_cf-SEF1_cf*" expression cassette

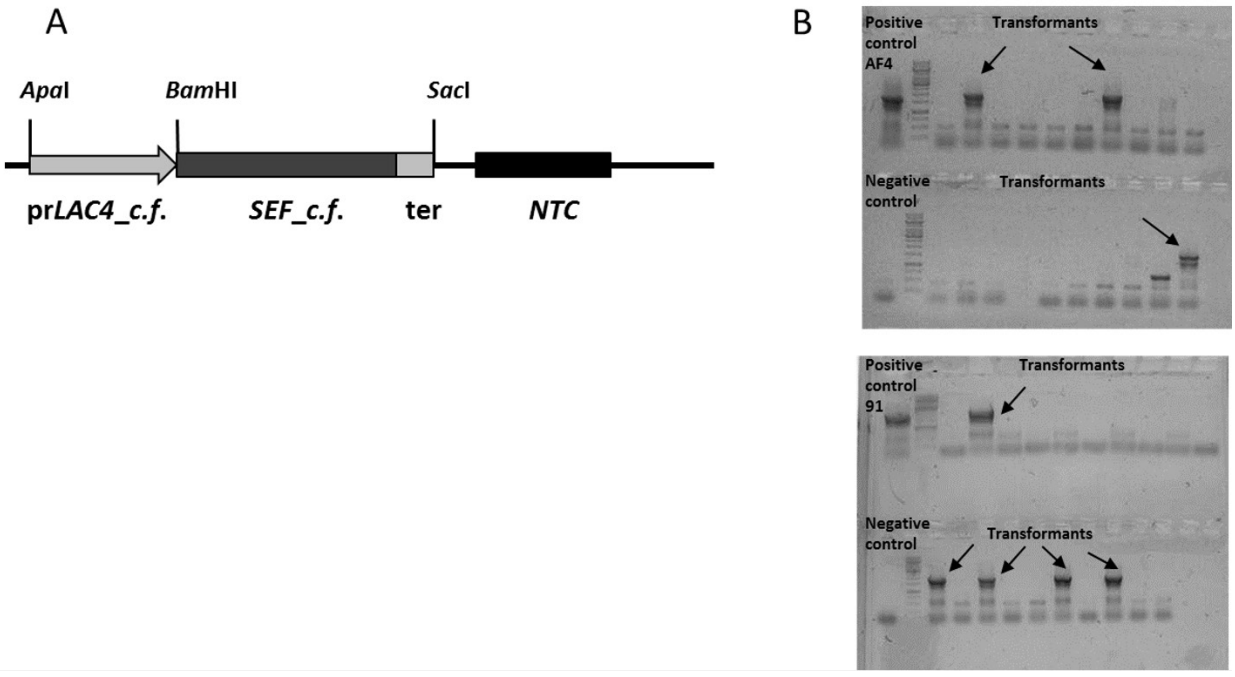
into genome of transformed cells was determined by PCR analysis.

Biochemical analyses. Cell biomass was determined turbidimetrically with a Helios Gamma spectrophotometer (OD, 590 nm; cuvette, 10 mm) with gravimetric calibration. Riboflavin concentration was determined by measuring fluorescence (Turner Quantech FM 109510-33 fluorometer, excitation maximum = 440 nm, emission maximum = 535 nm).

All the experimental data shown in this manuscript were collected from three independent samples. Each error bar indicates the standard deviation (SD) from the mean obtained from triplicate samples.

Results. Early it was demonstrated that integration of an additional copy of the positive regulator of riboflavin synthesis *SEF1* ortholog from *D. hansenii* under native promoter into the genome of riboflavin producer *C. famata* AF-4 resulted in enhancement of riboflavin production [7]. We hypothesized that the introduction of additional copies of own *SEF1 C. famata* into the genome of the best riboflavin overproducing strains of *C. famata* would further increase riboflavin production. We paid attention that *C. famata* grows in the media with lactose as sole carbon and energy source. Therefore we used promoter *LAC4* gene (coding for β –galactosidase) for expression of additional copy of *SEF1* gene in riboflavin overproducing strains AF-4 obtained by classical selection [7] and metabolic engineering strain #91 (AF-4/*SEF1/RIB1/RIB7*) [8]. The constructed plasmids harboring *SEF1* gene *C. famata* under the control of the *LAC4* promoter was used (Fig. 1a).

Corresponding transformants were selected on a solid rich medium, supplemented with NTC after five days of incubation. The selected transformants were stabilized by alternating cultivation on a non-selective followed by selective media. The transformation frequency was amounted to about 50 transformants/μg of DNA. After stabi-



F i g. 1. Scheme of *SEF1* gene contained plasmid and PCR screening of positively transformed yeast colonies

a) Scheme of plasmid "pNTC/p*LAC4_cf-SEF1_cf*" introduced in riboflavin overproducing (AF-4 and #91) *C. famata* yeast strains. *C. famata LAC4* promoter and terminator are shown as grey boxes, *C. famata SEF1* gene is shown as dark grey box, gene of nourseothricin (NTC) resistance – black box, thin line – bacterial plasmid pUC57. Sites of restriction endonucleases: *Apa*I, *Bam*HI, *Sac*I.

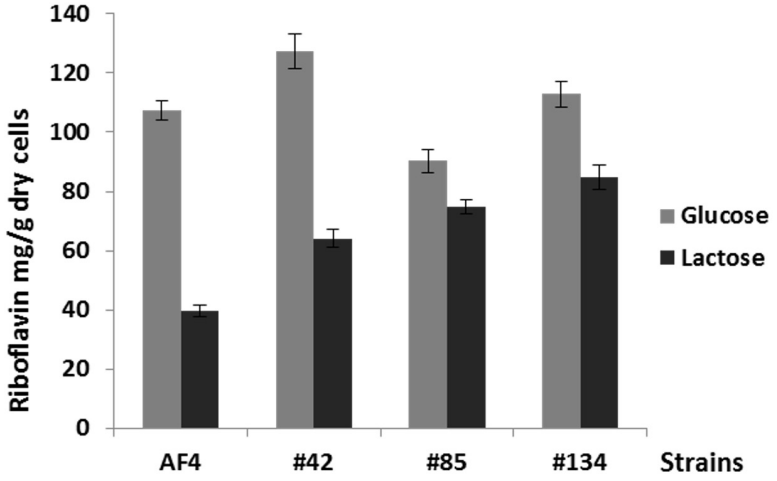
b) PCR verification of positively transformed AF-4/p*LAC4_cf – SEF1_cf* and #91/p*LAC4_cf – SEF1_cf* yeast colonies. Constructed plasmid was used for positive control. Primer pairs where specific to "p*LAC4_cf- SEF1_cf*" expression cassette.

lization, selected strains were verified by PCR (Fig. 1b).

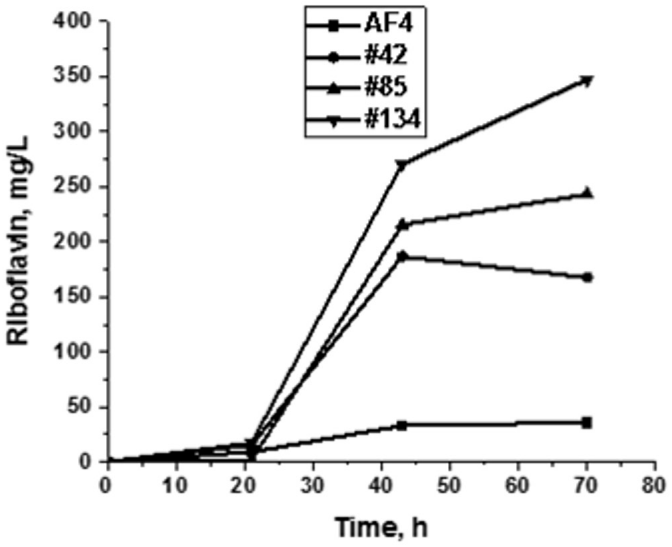
Initial characterization of riboflavin production was performed in the recombinant strains of *C. famata,* expressing *SEF1* under the control of *LAC4* promoter on 72 h of growth in YNB medium + 0.2% of yeast extract with different source of carbon. All tested recombinant strains were characterized by higher riboflavin yield in medium containing lactose as a carbon sources as compared to that of the parental strain AF-4 (Fig. 2).

Representative profiles of riboflavin yields for the strains AF4 and AF-4/ p*LAC4_cf-SEF1_cf* in YPL medium are shown in Fig. 3. Strains #42, #85 and #134 revealed an increase in riboflavin yields as compared to that of the parental strain AF-4.

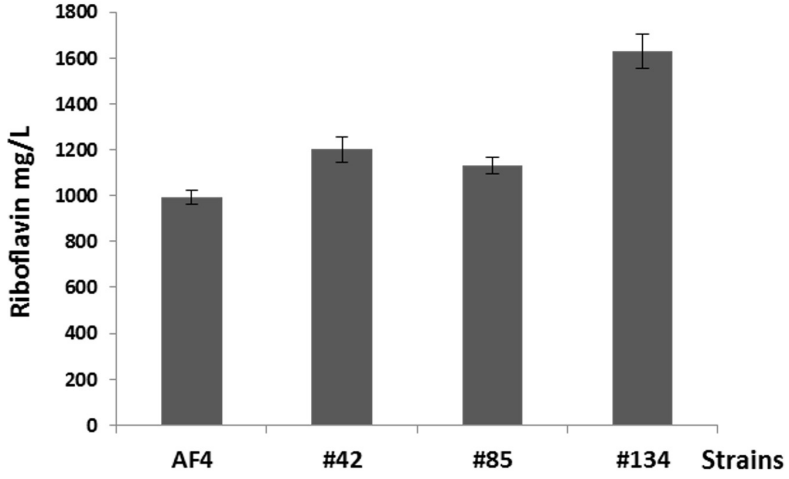
Early it was demonstrated that the yeast *C. famata* can assimilate lactose and synthesize riboflavin on whey supplemented with ammonium sulphate as a source of nitrogen [12]. Riboflavin yield of recombinant strains of *C. famata,* expressing *SEF1* gene under the control of *LAC4* promoter on 90 h of growth on whey supplemented with ammonium sulphate is shown in Fig. 4. The riboflavin production by the AF-4 strain, amounted to 1.03 g/L. Strains AF-4/p*LAC4_cf-SEF1_cf* demonstrated increase in riboflavin production up to 1.69 g/L. Similarly, it was found the increase in riboflavin yield in the recombinant strains #16, and #76, containing additional copy of *SEF1* gene under the control of *LAC4* promoter, relative to the parental strain *C. famata* #91 (Fig. 5).



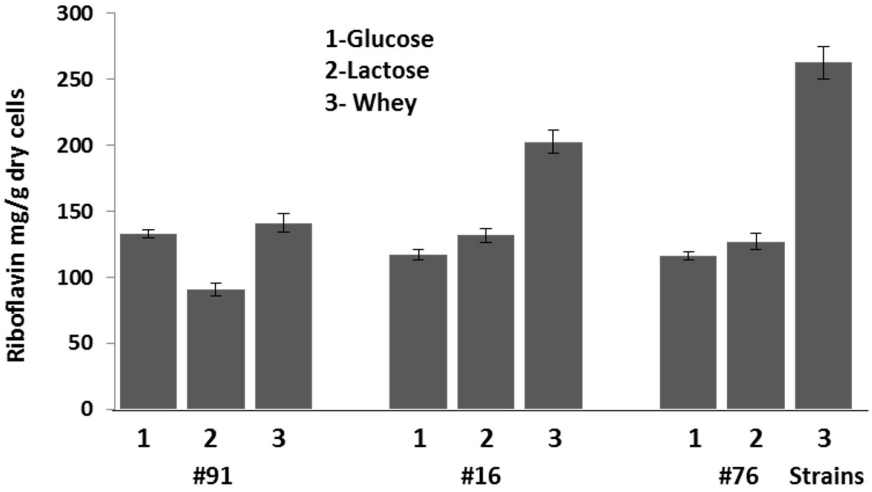
F i g. 2. Riboflavin yields of recombinant *C. famata* strains expressing *SEF1* under the control of *LAC4* promoter and parental strain AF-4 on 72 h of growth in YNB medium+ 0.2% of yeast extract with glucose or lactose (2%)



F i g. 3. Riboflavin production of recombinant strains of *C. famata* AF-4 and AF-4/ pr*LAC4_cf – SEF1_cf* expressing *SEF1* under the control of *LAC4* promoter. Cells grown in YPD medium for 24 h, transferred to YPL medium (100 µg/mL). Time of incubation – 70 h



F i g. 4. Riboflavin production by parental strain AF-4 and the recombinant strains #42, #85 and #134, containing additional copy of *SEF1* gene under the control of *LAC4* promoter grown on whey for 90 hours



F i g. 5. Riboflavin yields of recombinant strains of *C. famata* expressing *SEF1* under the control of *LAC4* promoter and parental strain 91 on 72 h of growth in YNB medium + 0.2% of yeast extract with different source of carbon and in whey (1 –glucose, 2 – lactose, 3 –whey)

Discussion. Some species of yeasts named ''flavinogenic'' overproduce riboflavin under conditions of iron deficiency. The highest flavinogenic potential among these organisms displays *C. famata* [5]. Molecular mechanisms of iron-dependent regulation of gene expression in flavinogenic yeasts are not known. Studies of transcriptional regulation of genes involved in riboflavin synthesis and using of strong promoters of *C. famata* for construction of efficient producers of vitamin B_2 are areas of both scientific and industrial interest. In general, metabolic engineering for enhanced riboflavin production was achieved by overexpressing the biosynthetic pathways of riboflavin or its precursors, which was accomplished by the combination of direct gene duplication, replacement of the native promoter with a strong one, disruption of competing pathways, or modification of regulatory genes [13].

Some mutants of *C. famata* are the most flavinogenic organisms known [4, 5, 14]. To obtain riboflavin overprodusers co-expression of the three genes *SEF1, RIB1* and *RIB7* coding for putative transcription factor, GTP cyclohydrolase II and riboflavin synthetase, respectively, on the background of non-reverting riboflavin producing mutant AF-4 isolated by classical selection were used [7, 8]. However, the productivity of riboflavin biosynthesis by available constructed strains of *C. famata* remains insufficient for industrial production of riboflavin and requires further metabolic engineering. We studied the effects of introduction

additional copy of *SEF1* gene under control of *LAC*4 promoter on riboflavin production by the best of the available *C. famata* overproducers. It was shown that, insertions or deletions in the gene *SEF1* block riboflavin oversynthesis in the *C. famata* [6], while introduction of the additional copies of *SEF1* from *D. hansenii* enhanced capacity of riboflavin production and strongly elevated stability of the riboflavin producer dep8. Homolog of transcriptional factor Sef1p *C. famata* were identified, cloned and deleted in other flavinogenic yeast *Meyerozyma* (*Pichia) guilliermondii* [15]. Deletion of a homologue of Sef1p transcriptional factor in *M.(P.) guilliermondii* completely blocked riboflavin oversynthesis under conditions of iron deficiency. The corresponding homologs of Sef1p might be universally involved in regulation of riboflavin biosynthesis in most flavinogenic yeasts that overproduce riboflavin under conditions of iron starvation. Recently it was shown that the *SEF1* promoters from other flavinogenic (*Candida albicans*) and non-flavinogenic (*Candida tropicalis*) yeasts fused with ORF of *SEF1* gene from *C. famata* are able to restore riboflavin oversynthesis in *sef1Δ* mutant whereas *SEF1* promoters from other non-flavinogenic yeasts did not [10, 16].

In this work we showed that overexpression of own *SEF1* gene under control of *LAC4* promoter in previously constructed *C. famata* overproducers of riboflavin led to significant increased riboflavin production on media with lactose (Fig. 2, 5). Moreover, constructed strains can synthesize riboflavin on cheap carbon source – whey, containing 5% lactose. Riboflavin yields of recombinant strains of *C. famata* expressing *SEF1* gene under the control of *LAC4* promoter on whey is higher than that on glucose. It is worth to mention that whey more significantly stimulates riboflavin synthesis by the construction strains as compared to lactose (Fig. 5). Quite possibly that whey contains some substances which additionally activate *LAC4* promoter. This is especially important as whey is cheap by-product of dairy industry.

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The influence of *SEF1* gene expression under control of lactose inducible promoter of *CfLAC4* gene encoding β-galactosidase on riboflavin production was studied. It was shown that the *C. famata* strains containing p*LAC4_cf-SEF1_cf* expression cassette revealed 1.6-2.1-fold increase in riboflavin yields on lactose as a soil carbon source when compared to the best riboflavin producers described in our previous work. The riboflavin production constructed strains on whey reached 1.69 g/L. The constructed strains containing additional copy of *SEF1* gene under the control of *LAC4* promoter is a perfect platform for development of industrial riboflavin production on by-product of dairy industry, whey.

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ЛАКТОЗО-ЗАЛЕЖНА ІНДУКЦІЯ ГЕНА *SEF1*, ЯКИЙ КОДУЄ ТРАНСКРИПЦІЙНИЙ ФАКТОР, ПІДВИЩУЄ ПРОДУКЦІЮ РИБОФЛАВІНУ У ДРІЖДЖІВ *CANDIDA FAMATA*

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Резюме

Рибофлавін (7,8-диметил-10-рибітил-ізоалоксазин, вітамін B_2) є біосинтетичним попередником флавінмононуклеотиду і флавінаденіндинуклеотиду, які беруть участь у низці відновних реакцій, деякі з яких є необхідними для функціонування аеробних клітин. Флавіни залучені в низку реакцій енергетичного метаболізму, відповідь на оксидативний стрес, фотосенсибілізацію і активацію інших вітамінів, зокрема фолату і піридоксину. Рибофлавін є важливим біотехнологічним продуктом, річний ринок якого становить близько 250 мільйонів доларів США. Здебільшого він використовується як компонент кормових преміксів для тварин (80% від загального обсягу виробництва), у харчовій промисловості як барвник, у медицині як компонент полівітамінних сумішей і як препарат для лікування деяких захворювань. За останні два десятиліття виробництво рибофлавіну шляхом мікробної ферментації повністю замінило хімічний синтез. Головними промисловими продуцентами рибофлавіну є сконструйовані штами бактерії *Bacillus subtilis* і міцеліального гриба *Ashbya gossypii*. Низка видів дріжджів за умов дефіциту феруму здатна

до надсинтезу рибофлавіну. За допомогою методів класичної селекції отримано штами, здатні до надсинтезу рибофлавіну при високому вмісті заліза. Однак механізми регуляції синтезу рибофлавіну за участю заліза до цього часу не з'ясовані. Високий флавіногенний потенціал мають дріжджі *Candida famata***.** Використовуючи класичні методи селекції та методи генетичної інженерії (надекспресія генів *SEF1, RIB1* і *RIB7*, які кодують позитивний регулятор, перший і останній фермент синтезу рибофлавіну відповідно), сконструйовано генетично стабільний штам *C. famata*, який у біореакторі продукував 16 г рибофлавіну на літр культурального середовища. Однак продуктивність синтезу рибофлавіну ще залишається недостатньою для промислового виробництва цього вітаміну. Дослідження транскрипційної регуляції генів, залучених у синтез рибофлавіну, і використання сильних промоторів *C. famata* для конструювання ефективних продуцентів вітаміну B_2 мають науковий і практичний інтерес. **Мета**. Метою даної роботи було покращити надсинтез рибофлавіну у наявних штамах *C. famata* у синтетичному і природному лактозо-вмісному середовищах. **Методи.** Виділення плазмідної ДНК, рестрикцію, електрофорез в агарозному гелі, електротрансформацію і ПЛР проводили за стандартними методиками. Вміст рибофлавіну визначали флюориметрично, використовуючи синтетичний рибофлавін як стандарт. Дріжджі культивували у стандартних середовищах YPD і YNB, які містили глюкозу або лактозу як джерела карбону, або на молочній сироватці. **Результати.** Сконструйовано штами дріжджів *C. famata* з додатковою копією центрального регуляторного гена *SEF1* під контролем промотора гена *LAC*4 (кодує β–галактозидазу) *C. famata*. Досліджено вплив експресії гена *SEF1* під контролем лактозо індуцибельного промотора гена *LAC4* на продукцію рибофлавіну за різних умов вирощування. Встановлено, що рекомбінантні штами *C. famata*, які містять експресійну касету "р*LAC4_cf-SEF1_cf",* мають в 1,6–2,1 рази вищий вихід рибофлавіну у середовищі з лактозою як єдиним джерелом карбону у порівнянні з батьківським штамом AF-4. Продукція рибофлавіну сконструйованими штамами при вирощуванні в колбах досягала 1,69 г/л. **Висновки.** Сконструйовані штами дріжджів *C. famata*, що містять додаткову копію гена *SEF1* під контролем лактозо індуцибельного промотора *LAC4,* є ідеальною платформою для розвитку промислового виробництва рибофлавіну на побічному продукті молочної промисловості – сироватці.

Ключові слова: дріжджі *Candida famata*, рибофлавін, ген *SEF1,* промотор гена *LAC4*, молочна сироватка.

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