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REGULATION OF METABOLIC PATHWAYS TO ENHANCE RIBOFLAVIN BIOSYNTHESIS IN *Eremothecium ashbyi*

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Riboflavin is one of the most important water-soluble vitamins involved in numerous biochemical processes in living organisms. It serves as a precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are essential cofactors for enzymes involved in the electron transport chain, redox reactions, and the metabolism of amino acids, lipids, and carbohydrates. Currently, riboflavin is produced industrially using biotechnological approaches, including fermentation with the ascomycete *Eremothecium ashbyi*. However, the development of novel strategies to enhance riboflavin biosynthesis in natural producers remains an important research objective.

Aim. To investigate a novel approach to enhancing the riboflavin biosynthetic capacity of *E. ashbyi* by modulating its metabolic pathways using itaconic acid and ultraviolet (UV) radiation.

Methods. The *E. ashbyi* F-340 strain was modified by cultivation on GPDA medium supplemented with varying concentrations of itaconic acid (25–100 mM), followed by exposure to UV radiation. The cultures were subsequently cultivated on GPD medium supplemented with sunflower seed cake as a source of plant-derived oil. Riboflavin concentrations were determined spectrophotometrically.

Results. The experimental findings highlight the key role of isocitrate lyase in enhancing riboflavin biosynthesis under conditions of increased plant oil content in the culture medium. The addition of itaconate enabled the selection of colonies with enhanced riboflavin production. The most pronounced stimulation of riboflavin biosynthesis was achieved by combining treatment with 50 mM itaconate during strain selection with subsequent UV irradiation. Subsequent cultivation of the selected strain on medium supplemented with sunflower seed cake yielded a maximum riboflavin concentration of 511.73 mg/L, approximately 21-fold higher than the total riboflavin content of the unmodified strain cultivated on GPY medium (24.11 mg/L) and nearly twofold higher than that obtained on medium supplemented with sunflower seed cake (260.06 mg/L).

Conclusions. The obtained data demonstrate the effectiveness of combining agro-industrial waste utilization with stress-induced conditions to enhance the productivity of *E. ashbyi* for industrial riboflavin production.

Keywords: *Eremothecium ashbyi*, riboflavin, itaconate, ultraviolet radiation, glyoxylate cycle, ascomycete, biotechnology, cultivation.

The filamentous fungus *Eremothecium ashbyi* is known for its natural ability to produce high levels of riboflavin, a water-soluble vitamin B2 [1]. Riboflavin is essential for living organisms, including microorganisms, owing to its involvement in numerous metabolic and redox processes. It also contributes to cellular antioxidant defense by participating in the neutralization of reactive oxygen species generated under oxidative stress, including that induced by ultraviolet (UV) radiation. Through these

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antioxidant properties, riboflavin may help protect cellular components, including DNA, from oxidative damage.

Riboflavin plays a central role in a wide range of biochemical reactions, primarily through its conversion into the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). These flavin cofactors function as tightly bound prosthetic groups of numerous oxidoreductases, including dehydrogenases. During redox reactions, the isoalloxazine ring of the flavin moiety undergoes reversible oxidation–reduction, enabling the transfer of hydrogen atoms and electrons between substrates and electron acceptors.

Flavoproteins, i.e., enzymes that contain FMN or FAD as cofactors, are involved in diverse metabolic pathways, including lipid and protein metabolism, fatty acid desaturation, cholesterol metabolism in the endoplasmic reticulum, and ether lipid biosynthesis in peroxisomes [2, 3]. They also participate in the mitochondrial catabolism of amino acids and the oxidative folding of proteins in the endoplasmic reticulum [2, 4]. In addition, flavin-dependent enzymes are involved in fatty acid β-oxidation and amino acid deamination, thereby contributing to the regulation of lipid, carbohydrate, and protein metabolism. Riboflavin is also required for the conversion of tryptophan to niacin (vitamin B3) [5], further highlighting its essential role in cellular metabolism and the growth and development of eukaryotic organisms.

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The chemical synthesis of riboflavin begins with the condensation of D-ribose and 3,4-xylidine in methanol, resulting in the formation of a riboside [6]. The latter is subsequently hydrogenated to produce N-(3,4-dimethylphenyl)-D-1′-ribamine. This intermediate then reacts with a phenyldiazonium halide to form an azo compound, which subsequently undergoes cyclocondensation with barbituric acid, yielding riboflavin [7]. However, the chemical synthesis has several disadvantages. The maximum substrate yield is approximately 60%, resulting in the generation of substantial amounts of waste. Furthermore, the process requires organic solvents and consumes approximately 25% more energy than the fermentation-based production of riboflavin, making it less environmentally sustainable [6, 7].

Industrial riboflavin production commonly employs strains of *E. gossypii* and *Bacillus subtilis*. However, other microbial producers, including certain *Candida* species, have limitations that may hinder efficient riboflavin production. For example, *Candida famata* ATCC 20849 is highly sensitive to iron concentrations in the culture medium, which can complicate the fermentation process despite its high riboflavin biosynthetic potential [8]. Similarly, riboflavin biosynthesis in *Pichia guilliermondii* is induced under iron-limited conditions [9]. In *Escherichia coli* BL21, achieving high riboflavin yields requires additional genetic engineering, including the introduction of a plasmid carrying six genes involved in riboflavin biosynthesis [10].

However, commercial riboflavin production relies on natural flavinogenic ascomycetes, particularly *E. ashbyi* and *E. gossypii*. Both microorganisms synthesize and accumulate riboflavin in the mycelium toward the end of the growth phase, resulting in a characteristic intense yellow coloration caused by the accumulation of riboflavin crystals in the cellular vacuoles (Fig. 1) [11]. Among these biotechnological producers, *E. gossypii* is characterized by consistently high riboflavin productivity, making it a promising organism for large-scale production. In contrast, *E. ashbyi* exhibits a high capacity for FAD biosynthesis, which may increase its potential value for pharmaceutical applications [9, 12, 13]. Nevertheless, further improvement of riboflavin production technologies and strategies for increasing product yield remains an important research objective.

Isocitrate lyase EC 4.1.3.1 is an enzyme belonging to the class of lyases. It is responsible for the cleavage of the intermediate product of the tricarboxylic acid cycle (Krebs cycle), isocitrate, into succinate and glyoxylate (Fig. 2) [14].

Itaconate and oxalate, which act as antimetabolites of isocitrate lyase, can effectively inhibit the activity of this enzyme. However, mutant strains resistant to these compounds exhibit enhanced riboflavin production, which has been attributed to the redirection of metabolic carbon flux from the tricarboxylic acid (TCA) cycle toward the glyoxylate shunt [15]. Such metabolic adaptation is particularly relevant when plant oil is used as the sole carbon source. Isocitrate lyase catalyzes the cleavage of isocitrate into glyoxylate and succinate, thereby providing an alternative route for carbon metabolism and reducing the loss of carbon intermediates

within the TCA cycle. This bypass pathway may facilitate the conservation of key metabolic precursors required for riboflavin biosynthesis, including guanosine-5′-triphosphate (GTP). Thus, activation of the glyoxylate shunt may represent a strategic mechanism for enhancing riboflavin biosynthesis by redirecting metabolic carbon flux [14, 15].

The aim of this study was to develop a novel approach enhancing riboflavin biosynthesis in *E. ashbyi* through targeted modulation of metabolic pathways and to evaluate riboflavin production by strains selected following

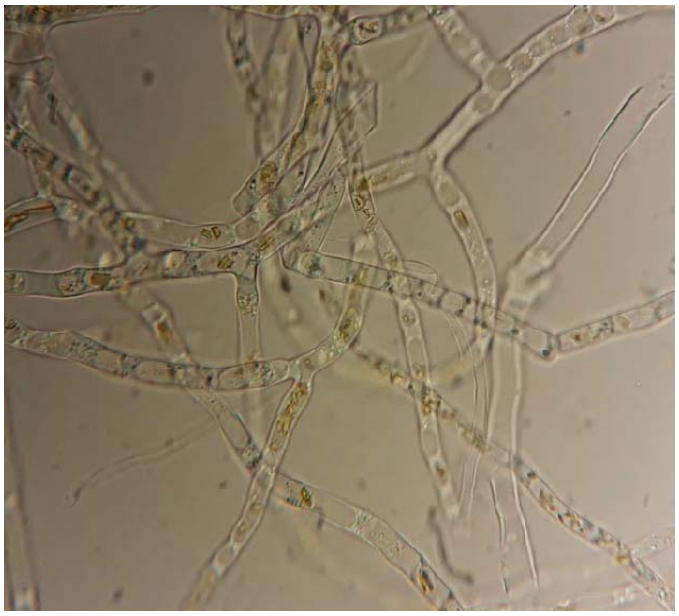

Fig. 1. **Microscopic image of the *E. ashbyi* mycelium with riboflavin inclusions (×1000)**

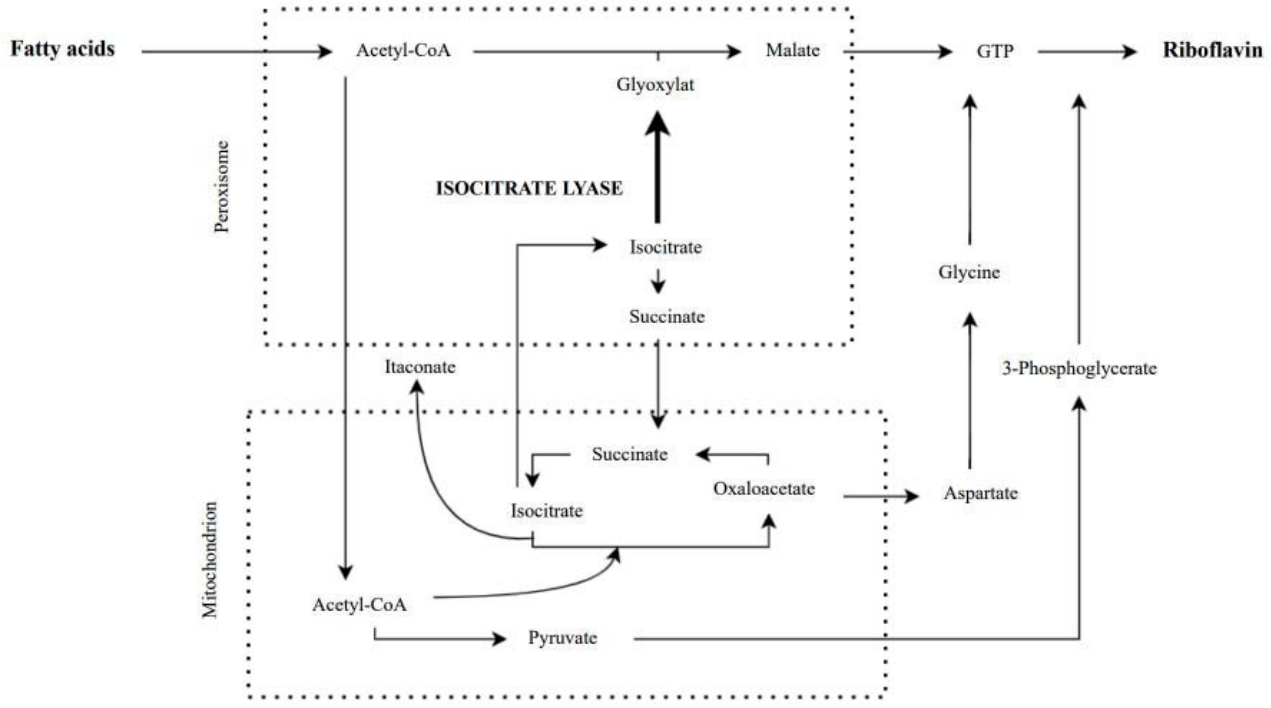

Fig. 2. **A proposed mechanism by which isocitrate lyase may influence riboflavin biosynthesis, adapted by the authors from [14]**

treatment with ultraviolet (UV) radiation and itaconic acid.

Materials and Methods

The object of the study was the *E. ashbyi* F-340 strain obtained from the Culture Collection of the National Technical University of Ukraine "Igor Sikorsky Kyiv Polytechnic Institute". The strain was maintained on standard glucose–peptone–yeast agar (GPYA) medium containing glucose (10 g/L), peptone (3 g/L), yeast extract (5 g/L), and agar (20 g/L) [16]. Prior to the experiments, the culture was reactivated on the same medium by incubation at 28 °C for 7 days.

Media supplemented with itaconate were prepared using GPYA medium as the base, with itaconic acid added at final concentrations of 25, 50, 75, and 100 mM [19]. After all components had been dissolved, the pH of the medium was adjusted to 6.0 using 50% NaOH solution.

For UV irradiation treatment, a spore suspension was prepared in sterile 0.9% sodium chloride solution. The suspension was collected using a sterile pipette and transferred to sterile Petri dishes, which were then exposed to UV radiation for 30 s at a distance of 20 cm from the UV lamps. Following irradiation, 0.5 mL of the suspension was plated onto GPYA medium and GPYA medium supplemented with itaconic acid.

For submerged cultivation, two media based on GPYA were used. The first medium contained glucose (40 g/L), peptone (3 g/L), and yeast extract (5 g/L), whereas the second contained glucose (10 g/L), peptone (3 g/L), yeast extract (5 g/L), and sunflower meal (30 g/L). Cultivation was performed in 250 mL Erlenmeyer flasks containing 50 mL of working medium for 7 days at 28 °C on a rotary shaker at 150 rpm. The inoculum was added at a concentration corresponding to 1% of the working medium volume. *The presence of riboflavin was initially assessed visually based on its characteristic fluorescence under UV illumination, whereas riboflavin concentration was subsequently determined spectrophotometrically.*

Riboflavin concentration after submerged cultivation was determined spectrophotometrically at 450 nm using a 1 cm path-length cuvette. After 7 days of cultivation, the culture liquid was filtered through a paper filter. An aliquot of 1 mL of the filtrate was transferred to a 5 mL volumetric tube, and 1 mL of 20% trichloroacetic acid (TCA) solution was added to precipitate proteins and hydrolyze FAD to FMN. The resulting solution was incubated overnight at 37 °C. For culture liquids obtained from protein-rich media (i.e., medium supplemented with sunflower meal), an additional protein precipitation step was performed by centrifugation at 3000 rpm for 5–7 min.

To determine the riboflavin content in the biomass, the filter paper used for filtration was previously weighed in weighing bottles, dried at 105 °C to a constant weight, and reweighed. *The filter paper containing the retained biomass was then transferred* to heat-resistant vessels and treated with 25 mL of 0.02 N HCl solution. The samples were incubated in a drying oven at 121 °C for 20 min. Sampling and riboflavin determination were performed using the same procedure as that applied to the culture liquid filtrate [17, 18].

To remove interfering yellow pigments, potassium permanganate solution was added dropwise until a stable violet coloration was obtained. Excess permanganate was then neutralized by adding hydrogen peroxide solution until the violet color disappeared. The volume was adjusted to 5 mL with distilled water, and further dilution with distilled water was performed when necessary. Riboflavin content was determined using a calibration curve [18]. The data are presented as means ± SE from three independent experiments.

Results and Discussion

A spore suspension obtained from *E. ashbyi* colonies was inoculated onto GPYA media supplemented with different concentrations of itaconic acid, with a medium without itaconic acid serving as the control. A portion of the spore suspension was subjected to UV irradiation and subsequently inoculated onto the corresponding media containing itaconic acid and onto the control medium. Growth was observed on all plates; however, differences in colony morphology were evident. As the concentration of itaconate increased, colony size decreased, whereas the intensity of colony pigmentation remained relatively unchanged. The most intensely yellow-pigmented colonies were selected for further cultivation in liquid GPY medium containing 20 g/L glucose to prepare the liquid inoculum.

It should be noted that both itaconic acid and UV irradiation were used exclusively during the strain selection stage on solid GPYA medium and were not applied

Table 1. **Riboflavin production by treated strains cultivated on GPY medium**

Strain	Absolutely dry biomass, g/L	Riboflavin concentration in the culture liquid, mg/L	Riboflavin concentration in the biomass, mg/L	Total riboflavin content, mg/L	Strain productivity per biomass, mg/g
0	4.537±0.127	21.34±1.07	2.77±0.13	24.11±1.08	5.31±0.19
25	5.717±0.229	13.88±0.56	3.21±0.12	17.08±0.57	2.99±0.11
50	6.042±0.278	14.84±0.45	3.30±0.13	18.15±0.47	3.00±0.12
75	5.636±0.197	22.40±0.90	5.63±0.24	28.03±0.93	4.97±0.20
100	5.643±0.271	18.53±0.56	4.76±0.21	23.28±0.60	4.13±0.19
0 UV	5.138±0.221	23.66±0.95	3.30±0.17	26.97±0.97	5.25±0.26
25 UV	5.953±0.232	24.34±0.73	3.84±0.17	28.18±0.75	4.73±0.24
50 UV	5.186±0.166	27.73±1.25	3.98±0.16	31.72±1.26	6.12±0.29
75 UV	5.192±0.177	32.48±1.14	4.13±0.13	36.61±1.15	7.05±0.27
100 UV	5.120±0.189	33.16±1.43	4.52±0.15	37.67±1.44	7.36±0.30

during subsequent submerged cultivation. Consequently, the final fermentation process does not require the addition of itaconic acid or exposure to UV radiation, thereby minimizing potential safety concerns associated with these agents at the production scale and simplifying the technological implementation of the proposed approach.

The liquid inoculum (0.5 mL) was inoculated into Erlenmeyer flasks containing GPY medium and GPY medium supplemented with sunflower meal as a source of plant-derived oil. During cultivation on the latter medium, aggregation of the mycelium and sunflower meal into large clumps was observed. The culture medium simultaneously developed a dark green coloration.

The riboflavin production levels of the investigated strains are presented in Table 1. The strain designations used in the table are as follows: the number indicates the concentration of itaconate in the medium from which the respective isolate was obtained, whereas "UV" denotes isolates subjected to ultraviolet radiation treatment.

Analysis of the data presented in Table 1 demonstrates that selection on media supplemented with itaconic acid at different concentrations, combined with UV irradiation of the producer spores, significantly enhanced riboflavin production by *E. ashbyi* during subsequent submerged cultivation compared with the untreated control. Notably, the combined treatment with 100 mM itaconic acid and UV irradiation increased the riboflavin concentration to 37.67 mg/L, representing a 56% increase compared with the untreated control (24.11 mg/L). A pronounced increase in specific riboflavin productivity was also observed, reaching 7.36 mg/g biomass compared with 5.31 mg/g biomass in the control, corresponding to a 39% increase.

The observed increase in riboflavin production may be associated with the biochemical effects of itaconic acid on microbial metabolism. Itaconate acts as an antimetabolite of isocitrate lyase, a key enzyme of the glyoxylate shunt involved in carbon metabolism. Alteration of isocitrate lyase activity may affect the distribution of metabolic carbon flux between the tricarboxylic acid (TCA) cycle and the glyoxylate shunt, thereby influencing the availability of metabolites required for riboflavin biosynthesis, including GTP [12, 20]. At the same time, UV irradiation may stimulate riboflavin biosynthesis as part of the cellular stress response to UV-induced oxidative stress [21]. Thus, the combined application of itaconic acid during strain selection and UV irradiation may produce a synergistic effect, resulting in increased riboflavin production and higher biomass-specific riboflavin content.

However, in flavinogenic fungi, including *E. ashbyi*, plant oils can serve as an important carbon source for riboflavin biosynthesis. Following hydrolysis by extracellular lipases, triacylglycerols are converted into fatty acids and glycerol. Fatty acids are subsequently degraded to acetyl-CoA through β-oxidation. Acetyl-CoA can then enter the glyoxylate shunt and the tricarboxylic acid (TCA) cycle, providing carbon skeletons for

Table 2. **Riboflavin production by treated strains cultivated on GPY medium supplemented with sunflower meal**

Strain	Riboflavin concentration in the culture liquid, mg/L	Riboflavin concentration in the biomass, mg/L	Total riboflavin content, mg/L
0	245.95±6.89	14.11±0.54	260.06±12.48
25	213.29±8.53	43.98±2.20	257.27±10.03
50	227.54±10.47	49.91±2.35	277.45±11.93
75	304.77±10.67	126.07±4.29	430.84±17.02
100	223.47±10.73	72.81±2.67	296.28±10.96
0 UV	269.88±11.61	14.98±0.55	284.86±13.10
25 UV	306.51±11.95	16.92±0.54	323.43±11.32
50 UV	491.86±15.74	19.87±0.95	511.73±19.96
75 UV	282.19±9.59	17.89±0.82	300.08±10.80
100 UV	264.55±9.79	17.31±0.61	281.86±8.17

gluconeogenesis and the formation of central metabolic intermediates. These intermediates contribute to the biosynthesis of riboflavin precursors through interconnected pathways, including the pentose phosphate and purine biosynthetic pathways. In particular, the glyoxylate shunt enables the conversion of acetyl-CoA into four-carbon intermediates, including oxaloacetate, which can contribute to the formation of precursors required for purine biosynthesis [22].

Therefore, the use of plant oils or oil-rich agro-industrial by-products as carbon sources may be advantageous for riboflavin production by *E. ashbyi*. One such substrate is sunflower meal, a by-product of the oil industry that contains residual lipids and can serve as an additional nutrient source. The riboflavin production by the investigated strains cultivated on medium supplemented with sunflower meal is presented in Table 2.

The results of cultivating *E. ashbyi* on medium supplemented with sunflower meal demonstrated a substantial increase in riboflavin production compared with that obtained on conventional GPY medium. The highest riboflavin concentration, 511.73 mg/L, was observed in the strain selected following treatment with 50 mM itaconic acid and UV irradiation. This value was approximately 21-fold higher than that of the untreated strain cultivated on basal GPY medium (24.11 mg/L) and nearly twofold higher than that of the untreated strain cultivated on GPY medium supplemented with sunflower meal (260.06 mg/L). These findings indicate a pronounced stimulatory effect of the combined itaconic acid and UV treatment under cultivation conditions involving a plant-derived substrate.

Notably, among the strains selected using itaconic acid without UV irradiation, the highest riboflavin concentration was obtained at an itaconate concentration of 75 mM (430.84 mg/L). This value was approximately 18-fold higher than that of the untreated strain on basal GPY medium and 1.66-fold higher than that of the untreated strain cultivated on the same medium supplemented with sunflower meal. These results further support the potential role of itaconate as a metabolic regulator affecting carbon flux through pathways associated with riboflavin biosynthesis.

Under combined UV irradiation and the highest itaconate concentration tested (100 mM), riboflavin production decreased to 281.86 mg/L, whereas the maximum value of 511.73 mg/L was observed at 50 mM itaconate. This concentration-dependent response may indicate the development of metabolic stress or an excessive physiological burden at higher itaconate concentrations when combined with UV-induced stress during strain selection.

It is also noteworthy that itaconate treatment altered the distribution of riboflavin between the culture liquid and the biomass. For example, strain 75 accumulated 126.07 mg/L of riboflavin in the biomass, which was nearly ninefold higher than the corresponding value for the untreated control cultivated on medium supplemented with sunflower meal (14.11 mg/L). This increase may reflect enhanced intracellular accumulation of

riboflavin in response to metabolic stress and/or changes in the regulation of the riboflavin biosynthetic pathway.

In contrast, following UV treatment, the amount of riboflavin accumulated in the biomass was substantially lower than that observed under the corresponding conditions without UV irradiation. This pattern may indicate a shift in riboflavin distribution toward extracellular accumulation following UV treatment. Such redistribution could be associated with an adaptive response to UV-induced stress; however, the underlying mechanism requires further experimental investigation.

Overall, the combination of itaconic acid treatment with UV irradiation, particularly at moderate itaconate concentrations (50–75 mM), in the presence of sunflower meal resulted in an approximately 21-fold increase in riboflavin production compared with the untreated strain cultivated on basal GPY medium. These findings highlight the potential of combining metabolic regulation during strain selection with the use of oil-rich agro-industrial by-products as substrates for improving the biotechnological production of riboflavin.

Conclusions

The *Eremothecium ashbyi* F-340 strain demonstrated enhanced riboflavin production following selection with itaconic acid and UV irradiation. The maximum riboflavin concentration (511.73 mg/L) was achieved during cultivation on GPY medium supplemented with sunflower meal after strain selection on GPYA medium containing 50 mM itaconate combined with UV irradiation. This value was approximately 21-fold higher than that obtained for the untreated strain cultivated on basal GPY medium (24.11 mg/L) and nearly twofold higher than that of the untreated strain cultivated on GPY medium supplemented with sunflower meal (260.06 mg/L).

The results indicate that itaconic acid treatment during strain selection may alter metabolic regulation and carbon flux in pathways associated with riboflavin biosynthesis. UV irradiation may additionally contribute to enhanced extracellular accumulation of riboflavin, potentially as part of an adaptive response to UV-induced stress. However, the specific mechanisms underlying these effects require further investigation.

The stability of the enhanced riboflavin-producing phenotype was not evaluated during long-term cultivation. Preliminary data indicated retention of increased productivity over three passages; however, the stability of the phenotype during long-term storage and cultivation requires further investigation using genomic and metabolomic approaches.

The results demonstrate the potential of using agro-industrial by-products and stress-induced conditions to enhance the biosynthetic activity of *E. ashbyi* strains, providing a basis for further optimization of biotechnological approaches to industrial vitamin B_2 production.

Authors' contribution

Shopova, Z. S. — conducting an experiment, writing; Polishchuk, V. Yu. — supervision, reviewing, editing.

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Conflict

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РЕГУЛЯЦІЯ МЕТАБОЛІЧНИХ ШЛЯХІВ ДЛЯ ПІДВИЩЕННЯ БІОСИНТЕЗУ РИБОФЛАВІНУ У EREMOTHECIUM ASHBYI

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Рибофлавін — один з найважливіших водорозчинних вітамінів, що бере участь у численних біохімічних реакціях живих організмів як прекурсор коферментів флавінмононуклеотиду (ФМН) та флавінаденіндинуклеотиду (ФАД), що є незамінними компонентами ферментів у ланцюгах електронного транспорту, окисно-відновних реакціях, метаболізмі амінокислот, ліпідів і вуглеводів. Наразі промислове виробництво рибофлавіну здійснюється біотехнологічним шляхом, в тому числі за участю аскоміцета *Eremothecium ashbyi*. Проте пошук нових шляхів до підвищеного біосинтезу вітаміну B_2 у природних продуцентів залишається актуальним.

Мета. Пошук нового підходу до підвищення здатності *E. ashbyi* до біосинтезу рибофлавіну шляхом регуляції метаболічних шляхів із залученням ітаконової кислоти та ультрафіолетового випромінювання.

Методи. Штами *E. ashbyi* F-340 модифікували шляхом вирощування на середовищі ГПДА з різними концентраціями ітаконової кислоти (25–100 мМ) і додатковою обробкою УФ-випромінюванням. Культивування культури для визначення рівня рибофлавіну проводили на середовищах ГПД з додаванням соняшникової макухи як джерела рослинної олії. Для оцінювання синтезу рибофлавіну використовували спектрофотометрію.

Результати. Отримані експериментальні результати свідчать про ключову роль ізоцитратліази у збільшенні біосинтезу рибофлавіну при використанні середовищ з високим вмістом рослинних олій — внесення ітаконату до складу поживного середовища дозволяє виділяти колонії мікроорганізмів з підвищеною здатністю продукувати рибофлавін. Встановлено, що оптимальними умовами для стимуляції рибофлавіногенезу є комбіноване застосування 50 мМ ітаконату та УФ-опромінення при селекції штамів, подальше культивування яких на середовищі з додаванням соняшникової макухи забезпечило максимальний вихід рибофлавіну — 511,73 мг/л, що приблизно у 21 раз перевищує загальний вміст рибофлавіну у необробленому штамі на середовищі ГПД (24,11 мг/л) та майже у 2 рази — на середовищі з макухою (260,06 мг/л).

Висновки. Отримані дані свідчать про ефективність використання агропромислових відходів та стрес-індукованих умов для підвищення продуктивності E. ashbyi у промисловому виробництві рибофлавіну.

Ключові слова: *Eremothecium ashbyi*, рибофлавін, ітаконат, ультрафіолетове випромінювання, гліоксилатний шунт, *аскоміцет, біотехнологія, культивування.*

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