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OPTIMIZATION OF THE CULTIVATION CONDITIONS AND THE BASIC MOLECULAR TOOLS FOR ROSEOFLAVIN PRODUCER *STREPTOMYCES DAVAWENSIS*

Abstract. *Roseoflavin (RoF), and its metabolic precursor 8-dimethylaminoriboflavin (AF), produced by Gram-positive bacteria Streptomyces davawensis and Streptomyces cinnabarinus, reveal a strong antibiotic effect against Staphyloccus aureus, Bacillus subtilis, Bacillus cereus, Micrococcus luteus and other Gram-positive bacteria and could be promising for developing the new effective antibacterial drugs. In this work, we present the optimization of basic molecular tools for S. davawensis including total DNA preparation, PCR amplification of target gene, cell transformation. In addition, the compositions of cultivation media were studied and the optimal cultivation conditions for increased of RoF production have been developed.*

K e y w o r d s: Streptomyces davawensis transformation, roseoflavin production, nutrient media compositions, total DNA isolation, rosA gene amplification.

Roseoflavin (RoF) is synthesized in bacterial cells from riboflavin (vit. B_2) by 8 consecutive reactions, get its name due to the specific bright pink color with absorption peaks at wavelengths of 219, 259 and 505 nm [2, 3] (Fig. 1).

RoF and AF are transported into the target cells through the riboflavin-transport systems. Inside the cell, RoF and AF could be converted to the analogs of flavin cofactors FMN and FAD which cannot substitute normal flavin coenzymes leading to the cell death. Additionally, RoF and AF are able to bind and block specific bacterial genetic element – FMN riboswitch, which leads to a repression of the expression of riboflavin operons and thus to the deficiency of riboflavin in the cell. Unfortunately, RoF is quite toxic to mammals. Fortunately, a human riboflavin-transport system, unlike bacterial, is unable to transport the AF, which makes it more promising as a medical drug, because of its selective toxicity on bacteria [4, 5].

Currently, commercially available RoF and AF are produced by chemical synthesis only, since, a natural microbial producers synthesize riboflavin analogs in a small quantities. In Ukraine, the production of these antibiotic compounds is absent. Large quantities of relatively cheap antibiotics in the industry are obtained by microbiological synthesis by recombinant strains of bacteria and mycelial fungi. Therefore, construction of the strains of *S. davawensis*, overproducing RoF and/or AF is the prerequisite for domestic production of these antibiotics at industrial scale.

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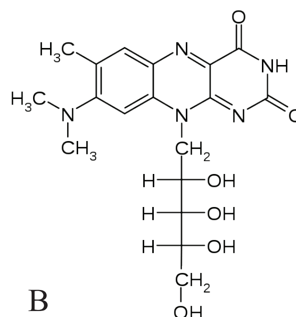


C

1	2	3	4	5	6	7	8
ribofla-vin	riboflavin-5-phosphate	8-demethyl-8-formyl-riboflavin-5-phosphate	8-demethyl-8-carboxy-riboflavin-5-phosphate	8-demetyl-8-amino-ribofla-vin-5-phosphate	8-de-methyl-8-amino-riboflavin	8-demetyl-8-methyl-amino-riboflavin	roseofla-vin

Fig. 1. *S. davawensis* and roseoflavin: A) *S. davawensis* colonies on agar oatmeal medium, B) structural formula of roseoflavin, C) pathway of RoF biosynthesis from riboflavin

In this work, cultivation conditions for RoF production by *S. davawensis* were optimized. In addition, basic molecular tools for *S. davawensis* were elaborated.

Materials and Methods

Strains and plasmids.

Strain of *S. davawensis* JCM 4913 was purchased from Japanese Collection of Microorganisms, strain *Escherichiacoli* WM6026 and plasmid pGUS were obtained from the microbial culture collection (I. Franko Lviv National University)

Cultural media.

S. davawensis cultivation was carried out using a number of classical nutrient media [1, 3, 6] (Table 1).

Single colony analysis of transformed *S. davawensis* performed on solid oatmeal agar medium (g/L: oats – 20.0; agar – 15.0).

Table 1. *S. davawensis* nutrient media compositions (g/L).

Casein-Starch	Yeast-Starch	YMG	TSB	Matsui
starch - 10.0 casein - 0.3 KNO_3 - 2.0 NaCl - 2.0 K_2HPO_4 - 2.0 $MgSO_4x7H_2O$ - 0.05 $CaCO_3$ - 0.02 $FeSO_4x7H_20$ - 0.01	starch* - 10.0 yeast extract - 2.0 KNO_3 - 2.0 NaCl - 2.0 K_2HPO_4 - 2.0 $MgSO_4x7H_2O$ - 0.05 $CaCO_3$ - 0.02 $FeSO_4x7H_20$ - 0.01	malt extract - 10.0 glucose - 4.0 yeast extract - 4.0	glucose - 2.5 soybean extract - 3.0 casein - 17.0 NaCl - 5.0 K_2HPO_4 - 2.5	Mannose -10.0 NaCl - 1.0 $MgSO_4x7H_2O$ - 1.0 K_2HPO_4 - 1.0 $CaCO_3$, - 2.0 $(NH_4)_2SO_4$ - 2.0 $FeSO_4x7H_2O$ - 1.0 $MnCl_2x4H_2O$ - 1.0 $ZnSO4x_7H_2O$ - 1.0

* YS medium with substitution of starch by malt extract, mannose, mannitol, sucrose and glucose by equivalent and two higher concentrations (1 % and 2 %, respectively), used for studies of media-depend RoF production.

LB-medium (g/L: NaCl – 5.0; peptone – 15.0; yeast extract – 5.0) used for *E.coli* WM6026 cultivation.

Biomass and roseoflavin production measurement.

Cellular wet weight (ww) was determined by using 10-ml culture aliquots that were centrifuged at 6,000 rpm for 20 min in pre-weighed tubes, washed once with water, and centrifuged again. Supernatant was discarded and wet cells were weighted. Spectrophotometer measurements of RoF optical density (OD) performed at 505 nm in 1 cm cuvette. Results presented as absolute values: cell wet weigh (g/L), the calculated amount of RoF (mmol, used the molar coefficient of extinction – 32.8 and their ratio (RoF/ww).

DNA manipulations.

The total DNA isolation was performed according to the standard protocol [1, 6]. *S. dawavensis* cells were cultivated in TSB, YS, YMannit, YSuc and YGlu media. 100 mg of cells were used for total DNA isolation.

The PCR was carried out by Applied Biosystems 7500 PCR System using the High Fidelity PCR Enzyme Mix (Thermo scientific) according to the manufacturer's instructions. In brief, 10–100 ng of total DNA was used in a total reaction volume of 20 μl with 1 μM of each primer. The following primer pairs were used: Frw.-atgcggccggaaccgaccgagc and Rew.- ccgcggcacggccggctga. The cycling parameters were 20 s at 50 °C and then 6 min at 96 °C, preparation step, followed by 10 cycles of 1.1 min at 96 °C, 40 s at 65 °C and 1 min at 72 °C, than 22 cycles of 1.0 min at 96 °C, 40 s at 68 °C and 1,1 min at 72 °C. Sequences of the *rosA* genes, coding N, N-8-amino-8-demethyl-D-riboflavin dimethyltransferase [8], was taken from NCBI-nucleotide database, [ref. 7]. The amplified DNA product was assessed visually using electrophoresis in 1 % agarose gel with BrEt.

Transformation of *S. davawensis*.

The transformation was performed as described elsewhere [1, 6].

The strain of *E.coli* WM6026 (auxotroph for diaminopimelic acid, DAP) with suicide plasmid pGUS was used. For the detection of transformed colonies, the 5-day plates were flooded with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) solution and incubated at 30 °C for 2–3 h. The final concentration of the X-Gluc dilution used for flooding plates was 200 mM. Glucuronidase activity provides the intensive blue color of the transformed colonies.

Results and Discussions

Roseoflavin production and biomass accumulation under different culture conditions.

For analysis of biomass and RoF production, the following panel of cultivation conditions was used: Casein-Starch (CS), Yeast-Starch (YS), YMG, TSB, Matsui media, at four values of pH (6.0, 6.5, 7.0, 7.5) and two temperatures (30 ^{0}C and 37 ^{0}C). The same volumes of spore suspension were used for inoculation of *S. davawensis* into flasks. To calculate the number of added spores, the suspension was titrated and plated onto agar oatmeal medium (Fig. 2).

Among the media used, only YS and to a lesser degree CS, were of clearly distinguished visible pink color (data not showed). The RoF synthesis in the

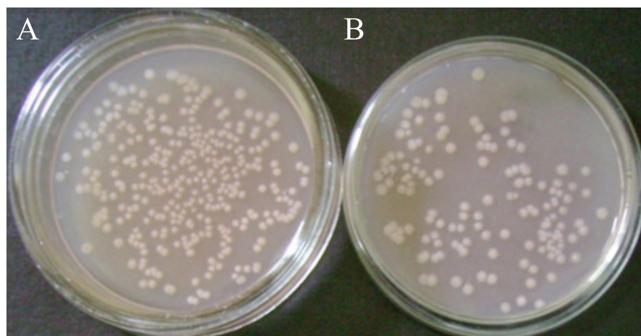


Fig. 2. Counting of the *S.davawensis* spore number: titrated and plated spore suspension onto agar oatmeal medium: A) 500 and B) 1000 fold diluted

cell required a riboflavin and thiamin as precursor and cofactor respectively [2]. Therefore, used the yeast extract as amino acid compound, which naturally reach by these vitamins, is much more efficient in comparison to the casein. The most intensity of the color was observed at pH 6.5, temperature 37 ^{0}C after 5–7 day cultivation (data not shown).

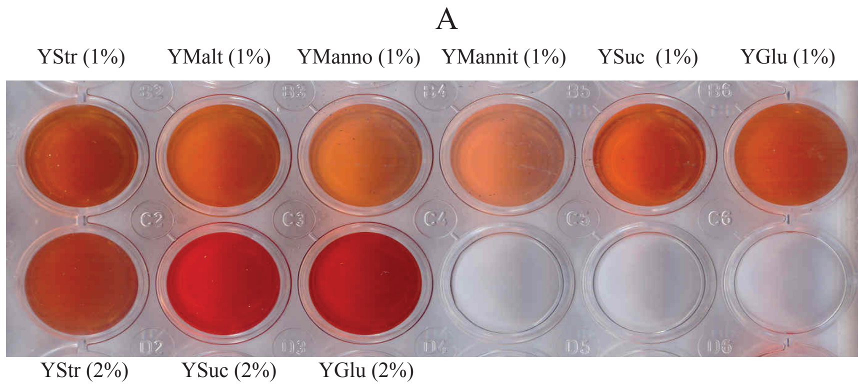
The modified YS medium (pH 6.5) was used for more detailed study of RoF production depending on the nature of carbon substrate. RoF production was assayed on 7th day of cultivation at 37 ^{0}C. The starch was replaced by malt extract, mannose, mannitol, sucrose or glucose at concentration 1 %. Additionally, 2 % of the cheapest substrates as starch, sucrose and glucose were used. It was shown that all used carbohydrate substrates (except sucrose and glucose) in concentration 1 % supported similar RoF production (0.023–0.027 mmol/L). RoF production on sucrose and glucose (both, 1 %), starch, sucrose as well as glucose (all, 2 %) was higher and reached from 0.036 to 0.045 mmol/L (Fig. 3).

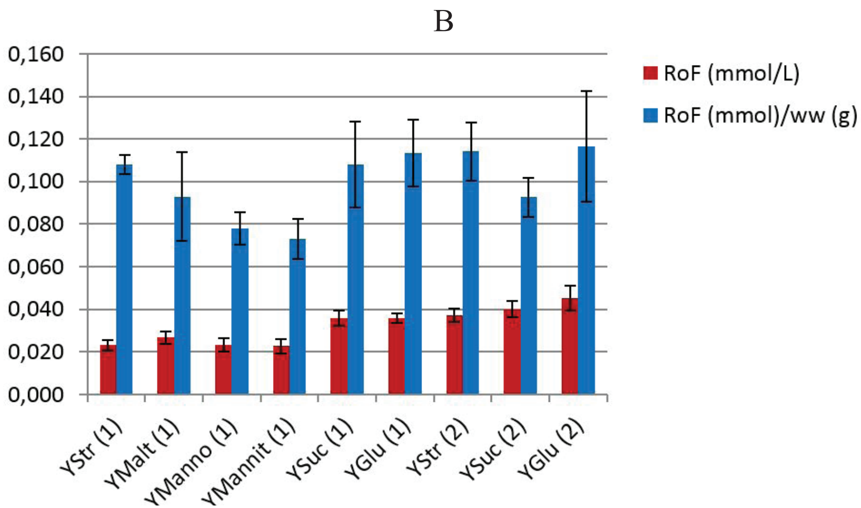
Our data showed that RoF productions in 1 % carbohydrate media YStr, YMalt, YManno and YMannit are in the similar level of published 0.021 mmol/L by Schwarz et al. [2]. Moreover, the 1 % of YSuc, YGlu and increased of carbohydrate concentration up to 2 % in the YS, YSuc, YGlu media, give nearly 2 times increased amount of RoF.

In conclusion, three compounds of the nutrient media (vit.B_1, vit.B_2 reach yeast extract, and 2 % sucrose/glucose) as well as 37 ^{0}C of temperature cultivation provided maximal RoF production by the wild strain of *S. davawensis*.

Isolation of total DNA and PCR amplification of the gene *rosA S. davawensis.*

Construction of new RoF and AF overproducers requires modification of the genes and/or promoters, coding the key enzymes for synthesis of these compounds. Introduction or deletion of DNA fragments to/from microbial genome widely used approaches. Therefore, selection of efficient protocols for total genomic DNA isolation and PCR amplification of DNA fragments are of great important. Strains of *Streptomyces* genus are characterized by pronounced mycelial growth and a rigid peptidoglycans wall [1, 6]. These features significantly complicate the process of cell lysis, transformation and selection of high-quality DNA. Growth as mycelium also makes it impossible to count the number of cells, plating and freezing in vegetative phase. Just





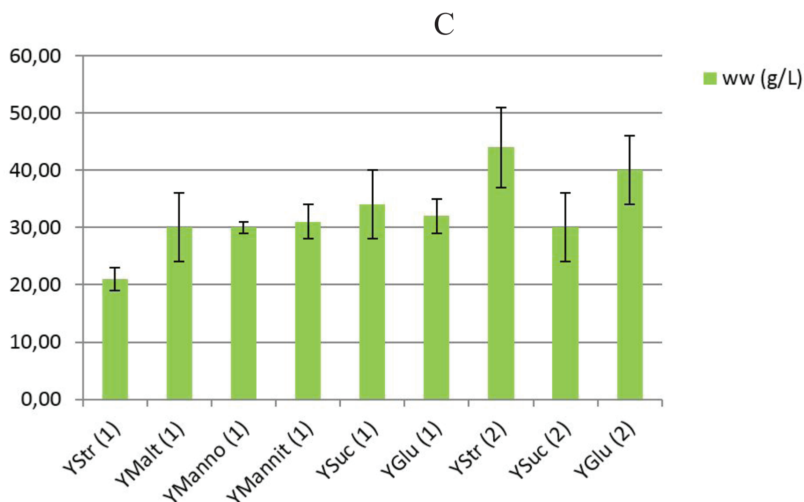


Fig. 3. RoF production and biomass accumulation of *S. davawensis* in liquid nutrient media (7 days cultivation, 37 ^{0}C, 1 % and 2 % of carbohydrate substrates): A) culture media after cell removing, B) concentrations of RoF in culture media (mmol/L) (columns № 1), RoF yield (mmol/g of cells) (columns № 2), respectively. C) wet weight (ww) of biomass (g/L)

therefore in practice the spores of these microorganisms are widely used. In addition, different species of *Streptomyces* show a differences regarding applicability of the standard methods of total DNA isolation, gene amplification

and transformation. Therefore, testing and modifications of classical methods, if necessary, are needed.

Total DNA from *S. davawensis* was isolated by standard protocol of total DNA preparation for *Streptomyces*. The quantity and quality of DNA, obtained from the same number of cells varies, depended on the mycelium formation in culture during growth (data not shown). It was revealed that DNA isolation was most efficient in the case mannitol or sucrose were used as carbon source. However, the DNA extracted from the cells grown in YGlu media shows the low quality (Fig. 4A). In comparison to *E.coli* and eukaryotic cells, the amplification of *Streptomyces* DNA fragments is usually more complicated due to the high content of GC-pairs (up to 70 %), which significantly increases the melting temperature of DNA and therefore require to optimize the PCR settings. For this reason, the 3 % DMSO was supplemented. The PCR data showed the high qualities of amplified *rosA* gene from all used DNA samples as PCR matrix (Fig. 4B).

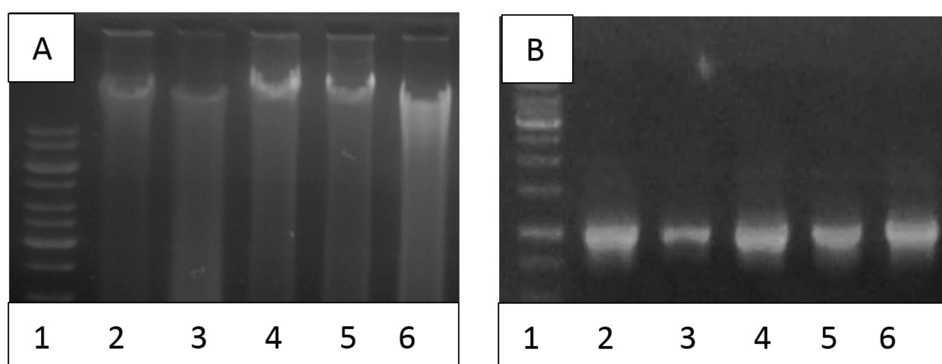


Fig. 4. Electrophoregrams of total DNA preparation and PCR amplification of *rosA* gene *S. davawensis*: A) 1kb DNA marker (1); DNA *S. davawensis* grow up on TSB; YS; YM; YSuc; YGlu (2–6), respectively, B) 1kb DNA marker (1); PCR-amplified RosA gene total DNA isolated from the cells grow up on TSB; YS; YM; YSuc; YGlu (2–6), respectively

Our data suggest, that using of sucrose in cultivation media and 3 % DMSO in PCR reaction mix, increase the effectivity of classic DNA isolation and gene amplification methods respectively.

Transformation of *S. davawensis* by conjugation.

For *Streptomyces* genome modification widely used method of conjugation with gram-negative bacteria *E.coli*, containing plasmid with factor F and the transgene DNA fragment [1, 6]. The strain of E.coli WM6026 with plasmid pGUS was used. Plasmid carried the apramycin resistance gene and marker gene of glucuronidase. Therefore, the selections of transformants, were performed by both – antibiotic and glucuronidase activity detections. With this regards, transformed colonies of the 5-day plates were flooded with X-Gluc as substrate and supplemented without/with apramicine (0, and 25 µg/ml, respectively), incubated at 30 °C for 3 h. Due to the glucuronidase activity the intensive blue color of the transformed colonies was detected (Fig. 5).

The high efficiency of conjugation and consequently transformation of *S. davawensis* was confirmed.

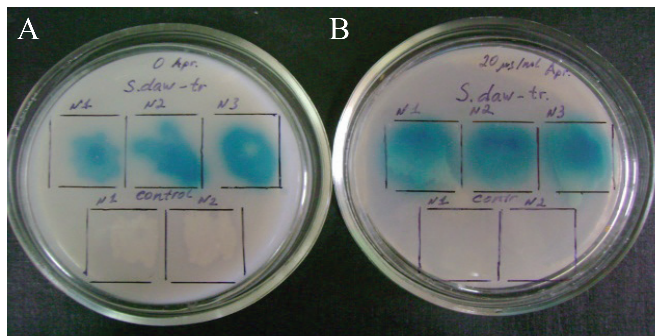


Fig. 5. *S. davawensis* transformation by plasmid pGUS used E.coli WM6026 conjugation technology. A) 3 colonies transformed *S. davawensis* (top row) and 2 control colonies (bottom row) on oatmeal agar medium without antibiotics. For direct detection of glucuronidase activity, 5-day plates were flooded with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc, final conc. 200 mM) solution and incubated at 30 °C for 3 h., B) 3 colonies transformed *S. davawensis* (top row) and 2 control colonies (bottom row) on oatmeal agar medium with apramycin 25 µg/ml with X-Gluc

Obtained data supposed to conclude that approbated technologies for total DNA isolation, PCR gene amplification and cell transformation can be applied for the complex *S. davawensis* genomic manipulation procedures. For the first time showed, that medium contained 2 % of sucrose/glucose and vitB_1, vitB_2 reach yeast extract compounds as well as increased temperature of cultivation up to 37 ^{0}C gives nearly two times higher level of RoF production.

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ОПТИМІЗАЦІЯ УМОВ КУЛЬТИВУВАННЯ ТА МОЛЕКУЛЯРНИХ МЕТОДІВ ДОСЛІДЖЕНЬ ПРОДУЦЕНТА РОЗЕОФЛАВІНУ *STREPTOMYCES DAVAWENSIS.*

Резюме

Розеофлавін (РоФ) та його метаболічний попередник 8-диметиламінорибофлавін продукуються грампозитивними бактеріями *Streptomyces davawensis* і *Streptomyces cinnabarinus.* Вони виявляють виражену антибіотичну активність щодо *Staphyloccus aureus, Bacillus subtilis, Bacillus cereus, Micrococcus luteus* та інших грампозитивних бактерій і можуть бути використані як нові ефективні антибактерійні препарати. У даній роботі представлено результати оптимізації базових молекулярних методів для роботи з *S. davawensis,* а саме: виділення геномної ДНК, ПЛР ампліфікація гена

rosA та трансформація клітин. Також проведено оптимізацію складу культурального середовища та умов культивування для підвищення продукції РоФ.

К л ю ч о в і с л о в а: трансформація *Streptomyces davawensis,* продукція розеофлавіну, склад культуральних середовищ, виділення геномної ДНК, ампліфікація гена *rosA*.

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ОПТИМИЗАЦИЯ УСЛОВИЙ КУЛЬТИВИРОВАНИЯ И МОЛЕКУЛЯРНЫХ МЕТОДОВ ИССЛЕДОВАНИЙ ПРОДУЦЕНТА РОЗЕОФЛАВИНА *STREPTOMYCES DAVAWENSIS.*

Резюме

Розеофлавин (РоФ) и его метаболический предшественник 8-диметиламинорибофлавин синтезируются грамположительными бактериями *Streptomyces davawensis* и *Streptomyces cinnabarinus.* Они проявляют выраженную антибиотическую активность по отношению к *Staphyloccus aureus, Bacillus subtilis, Bacillus cereus, Micrococcus luteus* и другим грамположительным бактериям и могут быть использованы в качестве новых эффективных антибактериальных препаратов. В данной работе представлены результаты оптимизации базовых молекулярных методов для работы с *S. davawensis*, а именно: выделение геномной ДНК, ПЦР амплификация гена *rosA* и трансформация клеток. Также проведена оптимизация состава культуральной среды и условий культивирования для повышения продукции РоФ.

К л ю ч е в ы е с л о в а: трансформация *Streptomyces davawensis*, продукция розеофлавина, состав культуральных сред, выделение геномной ДНК, амплификация гена *rosA*.

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