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ISOLATION OF *Phaffia rhodozyma* YEASTS MUTANTS UNDER INCREASED CAROTENOID CONTENT

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The aim of the paper was the selection of the improved strains of *Phaffia rhodozyma* using chemical mutagenesis and the identification of individual carotenoids synthesized by isolated more pigmented mutants. Hyperpigmented mutants of *P. rhodozyma* NRRL Y-17268-1 and IMB Y-5021-15 were isolated from initial strains NRRL Y-17268 and IMB Y-5021 by nitrosoguanidine mutagenesis. Pigments were purified by TLC and identified using HPLC and liquid chromatography-mass spectrometry. It was shown that initial strains *P. rhodozyma* NRRL Y-17268 and IMB Y-5021 and obtained from them mutants NRRL Y-17268-1 and IMB Y-5021-15 produced torulene and torularhodin without illumination in shaking flasks at 20 °C. The content of torularhodin produced by the mutant strains Y-17268-1 (18.2 μg) and Y-5021-15 (16.5 μg) per 1.0 g of dry biomass was increased to 33.8 and 18.4%, respectively, in comparison with the content of this pigment in the initial parental strains. The obtained strains present interest for further selection of more active producers of carotenoids and examination of the action of reactive oxygen species as stimulators of carotenoid production in yeasts.

Key words: *Phaffia rhodozyma*, mutagenesis, torulene, torularhodin.

Natural carotenoid pegments play the important role in the life of the animals and humans as biostimulators, antioxydants, vitamin A substitutes, coloring and tumor inhibiting compounds. Red yeasts of the genus *Phaffia* are known to produce the different carotenoids presented by torulene, torularhodin and astaxanthin. The low carotenoid content in the biomass limited these yeasts in industrial exploitation. Carotenoid production by microorganisms can be increased using mutagenic treatment of the cells. Natural pigments carotenoids in most cases are presented by tetraterpenoids synthesized from eight isoprene pyrophosphate units [1]. Many red yeasts of the genera *Rhodotorula, Rhodospiridium, Sporobolomyces* and *Phaffia* are known to produce different carotenoids in natural conditions [2 4]. Carotenoids play the important role in the life of animals and humans as biostimulators, antioxidants, vitamin A substitutes, coloring and tumor inhibiting pigments.

Representatives of the genera *Rhodotorula* and *Phaffia* synthesize different commercially important carotenoids (beta-carotene, torulene, toriularhodin and astaxanthin) and therefore belong to potential industrial microorganisms. Industrial exploitation of these yeasts is limited by the low carotenoid content in their biomass.

The aim of the paper was the selection of the improved strains of *Phaffia rhodozyma* using chemical mutagenesis and the identification of individual carotenoids synthesized by isolated more pigmented mutants.

Materials and Methods

Strains. Wild type strain *Phaffia rhodozyma* NRRL Y-17268 (BKM Y-2059) was obtained from the Institute of Cell Biology, National Academy of Sciences of Ukraine, kindly presented by Prof. C. P. Kurtzman from Microbial Genomics and Bioprocessing Research Unit, Peoria, Illinois, USA [5]. The mutant *P. rhodozyma* IMB Y-5021 (stored in the Depository of IMV) was isolated in the Institute of Animal Biology, National Academy of Agrarian Sciences of Ukraine from the strain NRRL Y-10921 by mutagenesis.

Media. Yeasts strains were grown on the modified S medium (g/L): K_2HPO_4 — 2,0; $MgSO_4 \cdot 7H_2O$ — 0,5; yeast extract — 4,0; peptone — 4,0; malt — 2,0; glucose — 20,0; biotin — 1.10^{-6}; fountain water — 1,0 L; pH 6,0, sterilization at 1,0 bar overpressure for 30 min.

The yeast cultures were incubated in 750 mL conical shaker flasks containing 80 mL of suitable medium and rotating at 240 rpm at 28 °C or 20 °C for 72 h.

Mutagenesis. Mutagenesis of *P. rhodozyma* NRRL Y-17268 and IMB Y-5021 was performed with N-methyl-N′-nitro-N-nitrozoguanidine (SERWA, Heidelberg). The concentration of the mutagen in the Tris-maleic acid buffer (0.05 M Tris base and 0.05 M maleic acid), pH 8.0 was 300 μg/mL for 60 min. Nitrozoguanidine, after treatment, was removed by cells' washing in a buffer and centrifugation. Diluted cell suspension was then distributed on an agar medium in Petri dishes by glass spatula and incubated at 20 °C for a period of 7 days. Separate colonies with more orange or red color were chosen for further investigation.

Carotenoid extraction and analysis. The yeast cells were harvested by centrifugation, washed in the water and the resulting sediment was dried at 80 °C until no change in weight was observed. The dry biomass was reduced to a powder using glass powder and a porcelain mortar with a pestle on the ice to prevent the degradation of the carotenoids which were then extracted twice with acetone. The obtained extract was cleared by centrifugation at 11,000 rpm, dried in a vacuum rotor evaporator and the pigments were dissolved in acetone. The carotenoids were separated by means of thin layer chromatography on Silica gel 60 F254 (Merck) with 25% acetone or 5% ethyl acetate in hexane. The absorption spectra of the acetone solutions of carotenoids were registered by a Beckman DU-8 spectrophotometer. The content of carotenoids was determined using the specific extinction coefficients E1%, 1 cm for torulene (3200 at 484 nm) and torularhodin (1932 at 495 nm) in hexane.

HPLC and LC/MS (liquid chromatography–mass spectrometry) of the carotenoids purified by TLC were performed using a liquid chromatography system Agilent Technologies 1200 with a single quadrupole detector and the Multimode ions in APCI mode. The conditions of separation were the following: column Zorbax Hypersyl ODS 120 x 2.1 mm, 3 μm, with acetone/water + 0.1% formic acid (95:5) as a solvent at a flow rate 0.3 ml min^{-1}. Detection was performed at 540 nm, and the UV-VIS absorption spectra were recorded online with the photodiode-array detection system. The chromatograms have been analyzed with Chemstation software.

Results and Discussion

The frequency of origin of stable colonies with more intensive red-orange color after nitrosoguanidine treatment was found to be 0.07–0.08% among surviving colonies of both *P. rhodozyma* strains (Fig. 1). Four and three more pigmented mutants were isolated among 4600 and 4200 tested colonies of the strains Y-17268 and Y-5021, respectively. Observation of these mutants during prolonged storage and repeated sowing approved their stability. The next stage of this work was identification of the main individual carotenoids in the extract of the dry cell biomass of the yeasts. TLC of the extracts showed the presence of two carotenoids produced by the strains Y-17268, Y-17268-1, Y-5021, and Y-5021-15 (Fig. 2). Purified carotenoids were identified according to their absorption spectra. Torulene (λ_{max} = 460, 485, 528 nm) and torularhodin (λ_{max} = 485, 495, 524 nm) were identified in all strains of *P. rhodozyma* (Fig. 3).

The results of preliminary carotenoid identification were confirmed by HPLC and LC/MS spectrometry (Fig. 4). The following m/z values of the carotenoids were obtained by APCI NI: 534.4 for torulene and 564.4 for torularhodin. Index R_t was equal to 1.480 and 2.206 for torulene and torularhodin, respectively. The obtained values λ_{max}, m/z and R_t of the identified carotenoids are in good agreement with walues reported in literature [6].

Biosynthetic activity of the selected yeast strains was investigated by their growth in the shaking flasks on the appropriate media. Among 4 strains of *P. rhodozyma* the most active producers of carotenoids were the mutants Y-17268-1 and Y-5021-15 synthesizing in a dark environment 18.2 μg and 16.6 μg of torularhodin per 1.0 g of dry biomass, respectively (Table). These data are statistically reliable ($P < 0.001$). The contents of torularhodin produced by the mutant strains Y-17268-1 and Y-5021-15 were increased to 33.8% and 18.2%, respectively, in comparison with the output of this pigment in the initial parental strains.

According to our results the strains *P. rhodozuma* NRRL Y-17268 and IMB Y-5021, and isolated from them mutants Y-17268-1 and

Y-5021-15 produce torulene and torularhodin like the red yeasts *Rhodotorula*. Accurate identification of carotenoids produced by the strain Y-17268 was not found in the published papers [7–11]. Torulene (30%) and torularhodin (60–65%) were produced by 10 different *P. rhodozyma* strains of BKM collection, as well as by the strain NRRL Y-17268 (BKM Y-2059), during growth at 30 °C, whereas they synthesized astaxanthin at 20 °C [12]. Despite of our attempts the growth of above mentioned strains at 18–20 °C did not leaded to production of astaxanthin instead of torulene and torularhodin.

In comparison with representatives of *Rhodotorula*, the mutant strain Y-17268-1 produces similar quantities of torulene and torularhodin, 575.2 μg/L and 684.3 μg/L, respectively without illumination [13–15]. This strain presents an interest for further selection of better producers of carotenoids and study of possible stimulating influence of reactive oxygen species (H_2O_2, O_2^-, OH) on carotenoids production in yeasts [16].

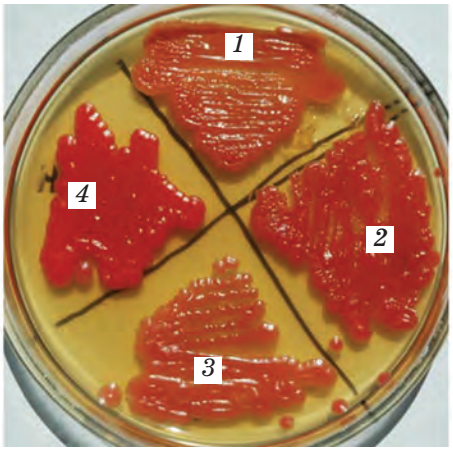

Fig. 1. **Growth of the initial strains and their more pigmented mutants on solid medium:**
1 — *P. rhodozyma* Y-5021; *2* — Y-5021-15; *3* — Y-17268; *4* — Y-17268-1

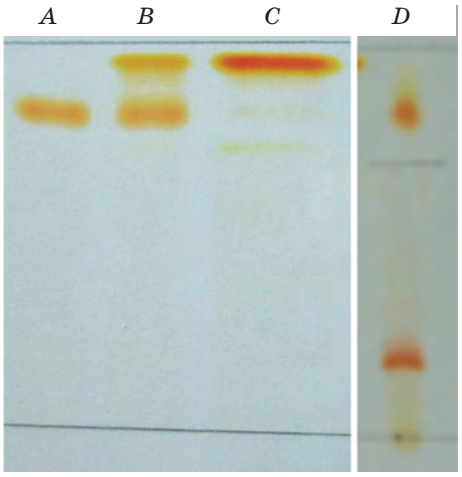

Fig. 2. **Thin layer chromatograms of the carotenoids:**
A — lycopene (control) [17]; *B* — lycopene and beta-carotene (control) [17]; *C* — beta-carotene (control) [17]; *D* — torulene (top spot): 25% acetone in hexane; torularhodin (bottom spot): 5% ethylacetate in hexane

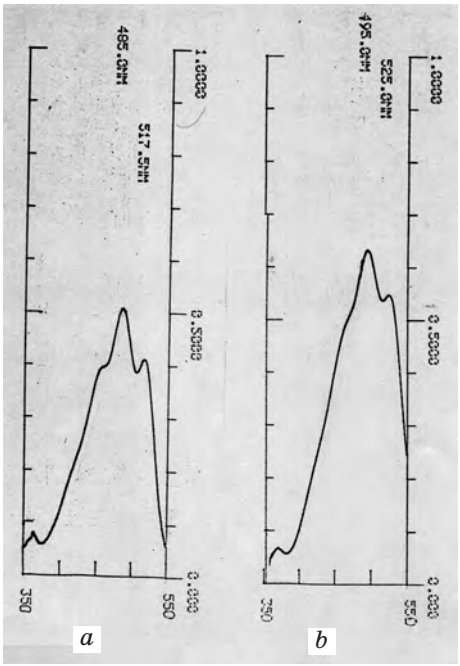

Fig. 3. **Absorption spectra of carotenoids in acetone:**
a —torulene; *b* — torularhodin

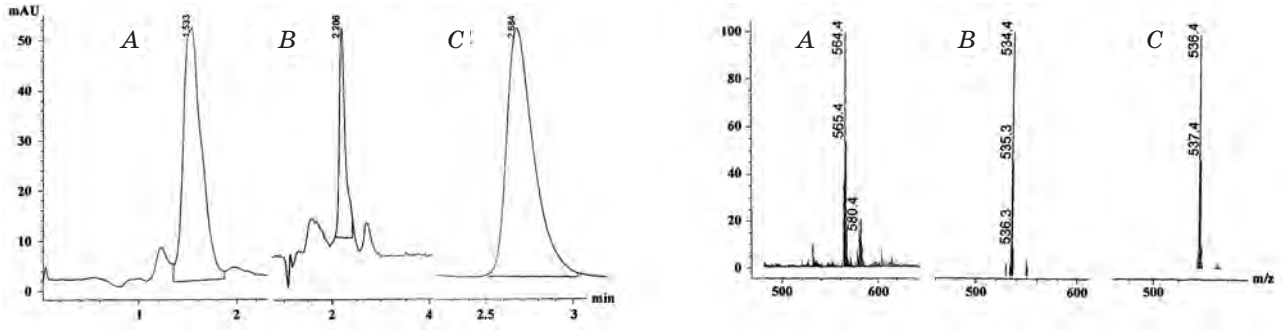

Fig. 4. **HPLC (top) and LC/MS (bottom) of carotenoids:**
A — torulathodin; *B* — torulene; *C* — β-carotene

Carotenoid production by *P. rhodozyma* strains in shaking flasks

Strain	Dry cell mass, g/L	Torularhodin, μg/g	Torulene, μg/g	Relation torularhodin/torulene	Sum of carotenoids, μg/g
Y-17268 (control)	38.0 38,6 37,4	13.6 13,4 13,8	14.9 14,7 15,1	0.91	28.5
Y-17268-1	37.6 37,4 37,8	18.2* 18,4 18,0	15.3 15,5 15,1	1.19	33.5
Y-5021 (control)	36.5 36,3 37,0	13.9 14,0 13,7	16.9 17,2 16,6	0.82	30.8
Y-5021-15	36.1 37,0 35,3	16.6* 16,2 17,0	13.4 13,0 13,8	1.24	30.0

Note: * $P < 0.001$ (as compared with control).

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ВИДІЛЕННЯ МУТАНТІВ ДРІЖДЖІВ *Phaffia rhodozyma* З ПІДВИЩЕНИМ ВМІСТОМ КАРОТИНОЇДІВ

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Метою роботи був добір поліпшених штамів *Phaffia rhodozyma*, одержаних за допомогою хімічного мутагенезу, та ідентифікація окремих каротиноїдів, синтезованих ізольованими гіперпігментованими мутантами. Мутанти з підвищеною пігментацією *P. rhodozyma* NRRL Y-17268-1 та IMB Y-5021-15 виділено з вихідних штамів NRRL Y-17268 і IMB Y-5021 за допомогою нітрозогуанідинового мутагенезу. Пігменти очищали тонкошаровою хроматографією й ідентифікували за допомогою високоефективної рідинної хроматографії та рідинної хроматографії/мас-спектрометрії. Показано, що вихідні штами *P. rhodozyma* NRRL Y-17268 і IMB Y-5021, а також одержані від них мутанти NRRL Y-17268-1 та IMB Y-5021-15 продукували торулін і торулародин без освітлення в колбах на качалці при 20 °С. Вміст торулародину в мутантних штамахY-17268-1 (18,2 мкг) і Y-5021-15 (16,5 мкг) в 1,0 г сухої біомаси збільшився до 33,8 і 18,4% відповідно порівняно зі вмістом цього пігменту у вихідних батьківських штамах. Одержані штами є перспективними для подальшої селекції активніших продуцентів каротиноїдів і дослідження дії реактивних сполук кисню для стимуляції утворення цих сполук дріжджами.

Ключові слова: *Phaffia rhodozyma*, мутагенез, торулін, торулародин.

ВЫДЕЛЕНИЕ МУТАНТОВ ДРОЖЖЕЙ *Phaffia rhodozyma* С ПОВЫШЕННЫМ СОДЕРЖАНИЕМ КАРОТИНОИДОВ

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Целью работы был отбор улучшенных штаммов *Phaffia rhodozyma*, полученных с помощью химического мутагенеза, и идентификация отдельных каротиноидов, синтезированных изолированными гиперпигментированными мутантами. Мутанты с повышенной пигментацией *P. rhodozyma* NRRL Y-17268-1 и ИМВ Y-5021-15 выделены из исходных штаммов NRRL Y-17268 и IMB Y-5021 нитрозогуанидиновым мутагенезом. Пигменты очищали тонкослойной хроматографией и идентифицировали с помощью высокоэффективной жидкостной хроматографии и жидкостной хроматографии/масс-спектрометрии. Показано, что исходные штаммы *P. rhodozyma* NRRL Y-17268 и ИМВ Y-5021, а также полученные от них мутанты NRRL Y-17268-1 и ИМВ Y-5021-15 образуют торулин и торулародин без освещения в колбах на качалке при 20 °С. Содержание торулародина в мутантных штаммах Y-17268-1 (18,2 мкг) и Y-5021-15 (16,5 мкг) в 1,0 г сухой биомассы увеличилось до 33,8 и 18,4% соответственно по сравнению с содержанием этого пигмента в исходных родительских штаммах. Полученные штаммы являются перспективными для последующей селекции более активных продуцентов каротиноидов и исследования действия реактивных соединений кислорода для стимуляции образования этих соединений дрожжами.

Ключевые слова: *Phaffia rhodozyma*, мутагенез, торулин, торулародин.