UDC 579.873.7:575.224.2:577.181.4

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ISOLATION OF *STREPTOMYCES GLOBISPORUS* AND *BLAKESLEA TRISPORA* MUTANTS WITH INCREASED CAROTENOID CONTENT

Hyperpigmented mutants of Streptomyces globisporus 1912-Hp7 and Blakeslea trispora 18(+), 184(-) were isolated by action of hydrogen peroxide and nitrosoguanidine, correspondingly, from initial strains S. globisporus 1912-4Lcp and B. trispora 72(-), 198(+). The carotenoids of dry biomass of obtained strains, rubbed thoroughly with glass powder by a pestle in porcelain mortar, were extracted by acetone and purified by TLC. Identification of the individual carotenoids was performed by means of HPLC and LC/MS spectrometry. It was shown that strain S. globisporus 1912-4Crt produced β-carotene/lycopene (6.91/3.24 mg/L), mutants 1912-4Lcp and 1912-7Hp synthesized only lycopene (26,05 and 50,9 mg/L, respectively), and strains B. trispora 18(+) and 184(-) - β-carotene (6.2 % in dry biomass or more 2.5 g/L) without illumination in shake flasks. It is the first example of high constitutive production of the carotenoids by the representative of genus Streptomyces without photoinduction or increased synthesis of sigma factor. The improved strains of B. trispora 18(+) and 184(-) can be used for biotechnological production of beta-carotene in industrial conditions.

K e y w o r d s: Streptomyces globisporus, Blakeslea trispora, mutagenesis, β-carotene , lycopene

Carotenoids represent the terpenes dissolved in the fats and synthesized by plants, some bacteria, streptomycetes and fungi. They play a very important role in the pharmaceutical, chemical, food and feed industries as a vitamin A substitute, coloring, antioxidant, bio-stimulant and tumor inhibiting natural pigments (1, 5, 14). Carotenoid carbohydrates belong to carotenes (β-carotene and its isomeres, lycopene, torulene) and oxygenated xanthophiles (lutein, zeaxanthin, astaxanthin, canthaxanthin). Fungus *Blakeslea trispora* and red yeasts *Rhodotorula glutinis, Rhodotorula rubra, Spoprobolomyces roseus,* and *Phaffia rhodozyma* are known to produce the different carotenoids (β-carotene, torulene, torularhodin, astaxanthin) and therefore present an important and perspective industrial group of microorganisms. Green alga *Haematococcus pluvialis* and heterothallic strains of *B. trispora* are used in industrial production of astaxanthin and beta-carotene/lycopene, respectively. Representatives of the genus *Streptomyces* contain in their genome one or several clusters of *crt* genes encoding the biosynthesis of carotenoids. Many of these *crt* genes are cryptic (4, 11) or require illumination with blue light for induction of their transcription (15). Transcription of cryptic genes of carotenogenesis in *Streptomyces setonii* (6) and *Streptomyces griseus* (9) can be induced by an increased copy number of a *crtS* gene that encodes a stress-responce sigma factor (15). Product of *litS* gene of *S. coelicolor* A3(2) has no homology with CrtS and acts as a light-induced sigma factor that directs transcription of the carotenoid biosynthesis gene cluster. Carotenogenesis in *Blakeslea, Rhodotorula* and *Phaffia* can be improved by different strategies including genetic engineering, induced mutagenesis, and optimization of media composition and conditions of fermentation.

The initial strain *Streptomyces globisporus* 1912, the weak producer of antitumor antibiotic landomycin E from angucycline family, and its derivative strains 3-1 and RSp2, the high active producers of this antibiotic, in spontaneous manner and by action of the mutagenic factors and protoplast formation form with low frequency the colonies of yellow, orange or pink color, synthesizing beta-carotene and lycopene (2, 3, 8, 10).

The heterothallic strains of *Blakeslea trispora* K1(+) and K1(-), obtained by means of nitrosoguanidine action (12), and their derivative strains K2(+) and K2(-), received by protoplasts fusion and regeneration (13), produced 1.57 g/L and 2.0 g/L of beta-carotene correspondingly during growth of mixture cultures in soy-bean medium in shake flasks.These strains earlier were used in plant conditions for production of beta-carotene in Ukraine.

The aim of this paper was the selection of improved strains of *S. globisporus* and *B. trispora* by means of mutagenesis, identification of individual carotenoids synthesized by more pigmented mutants, and examination of productivity of these strains during growth in shake flasks.

Materials and Methods. *Strains. S. globisporus* 1912-4Lcp, the producer of lycopene, was received as the spontaneous mutant of *S. globisporus* 1912-4Crt synthesizing beta-carotene and lycopene (2). *B. trispora* strains 198(+) and 72(-) are the derivative of the strains K2(+) and K2(-), respectively, earlier used in the plant conditions for beta-carotene production in Ukraine (13).

Media and growth conditions. For carotenoid biosynthesis the streptomycetes were grown on a corn-soy medium (g/L): corn meal 20.0, soy meal 10.0, NaCl 5.0, agar 15.0, distilled water 1.0 L, pH 7.0; sterilization at 1.0 bar overpressure for 30 min. Cultures of *B. trispora* were stored on a malt agar and their productivity was examined in the fermentation medium (g/L): corn meal 50.0, soy meal 30.0, soluble starch 20.0, thiamine hydrochloride 0.002, KH_2PO_4 0.5, $MgSO_4.7H_2O$ 0.5, sun flower oil 20.0, distilled water 1.0 L, pH 6.4, sterilization at pressure 0.75 atmosphere for 30 min. Suspensions of spores and mycelium of streptomycetes and fungus prepared separately from slant cultures were inoculated into 80 ml of the corresponding media in 750-ml shake flasks and were grown for 2 days at 28 °C and 240 r.p.m. Received inoculum of streptomycetes was added to 80 ml of corn-soy medium at 10% (v/v). The heterothallic forms of fungus were inoculated at 10 % (v/v) in correlation 1(+):9(-) into 80 ml of fermentation medium in 750-ml shake flasks. All cultures were grown for 72 h and 240 r.p.m. at 28 °C.

Mutagenesis. Mutagenesis of *S. globisporus* 1912-4Lcp and *B. trispora* 72(-), 198(+) was attempted with hydrogen peroxide (2% for 60 min) and N-methyl-N`-nitro-N-nitrosoguanidine (1 mg/ml in 0.05 M tris-maleic buffer, pH 8.0 for 60 min), correspondingly. The mutagens after treatment were removed by cell washing in a buffer and centrifugation. Diluted cell suspension was then distributed on the agar medium in Petri dishes by glass spatula and incubated at 28 °C for a period of 7 days. Separate colonies with more orange or red color were chosen for further investigation.

Extraction, purification and analysis of carotenoids. The biomass of fungus and streptomycetes was harvested by centrifugation, washed in water and the resulting sediment was dried at 80 °C until no change in weight was observed. The dry biomass was reduced to powder using glass powder and a porcelain mortar with a pestle. This procedure was done 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. The absorption spectra of the acetone solutions of carotenoids were registered by means of a Beckman DU-8 spectrophotometer. The quantity of pure carotenoids obtained was determined by using the specific extinction coefficients E 1%.1 cm of lycopene (3200 at 502 nm), and β-carotene (2500 at 450 nm) in hexane.

HPLC and LC/MS of the carotenoids purified by TLC was performed by means of liquid chromatograph Agilent Technologies 1200 with a single quadrupole detector and the Multimode ions in APCI mode. The conditions of separation were: column Zorbax Hypersyl ODS 125 x 2.0 mm, 3 µm, with acetone/water + 0.1 % formic acid (95:5) as solvent at a flow rate 0.3 ml min^{-1}. Detection of carotenoids was performed at 540 nm, and the UV-vis absorption spectra were recorded online with the photodiode-array detection system. The chromatograms have been developed with Chemstation software.

Results and Discussion. The frequency of origin of colonies with more intensive pink or red-orange color after mutagens treatment was found to be 0.02 % for *S. globisporus* 1912-4Lcp and 0.2 % for *B. trispora*. Examination of these primary mutants during prolonged storage and repeated sowing showed the different mutability of the property of carotenoid production. The frequency of spontaneous mutability of the carotenogenesis (appearance of the colorless colonies) of the initial strain *S. globisporus* 1912-4Lcp and obtained from them more active mutant 1912-Hp7 consists of 10^{-6}. Only one stable mutant *B. trispora* 184(-) was chosen among 60 primary selected colonies after mutagenesis and repeat examination of *B. trispora* 72(-). By similar manner the more productive mutant strain 18(+) was chosen among the derivatives of *B. trisora* 198(+).

The next stage of this work was identification of the main individual carotenoids in the extract of the dry cell biomass of microorganisms. TLC of the extracts showed the presence of two carotenoids produced by 1912-4Crt, and one pigment synthesized by 1912-4Lcp and 1912-7Hp strains and mixture culture of fungus (Fig. 1). Purified carotenoids were identified according to their absorp-

tion spectra. One pigment was identified as β-carotene (λmax 425, 452, 478 nm) in *S. globisporus* 1912-4Crt and both strains 18(+) and 184(-) of *B. trispora,* and the second one was lycopene (λmax 446, 472, 505 nm) in *S. globisporus* 1912-4Lcp, 1912-7Hp and 1912-4Crt (Fig. 2). The results of preliminary carotenoid identification were confirmed by HPLC and LC/MS spectrometry (Fig. 3, 4). The *m/z* values 536.4 for β-carotene and lycopene were obtained by APCI NI. The values λmax, *m/z* and R_t of both carotenoids agreed well with the literature data (16).

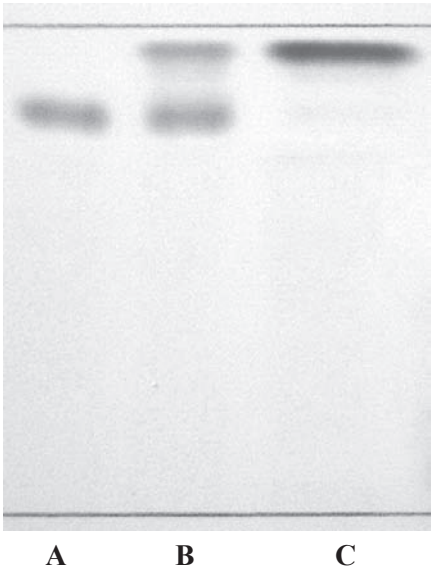

Fig. 1. Thin layer chromatogram of the carotenoids: A, lycopene (strain 1912-4Lcp); B, lycopene and beta-carotene (strain 1912-4Crt); C, beta-carotene (*B. trispora* 18(+) x 184(-). Solvent: 25 % acetone in hexane.

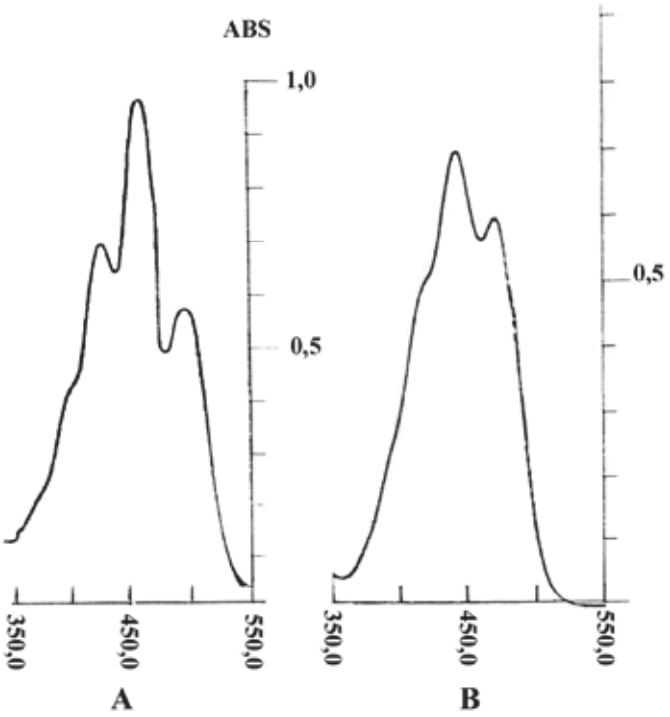

Fig. 2. Absorption spectra of carotenoids in acetone: A, β-carotene (strain 1912-4Crt); B, lycopene (strain 1912-4Lcp).

Biosynthetic activity of the selected strains was investigated by their growth in the shake flasks on the appropriate media. The mean output of the carotenoids produced by the strain *S. globisporus* 1912-4Crt was found to be 6.91 mg/L and 3.24 mg/L of β-carotene and lycopene, respectively (Table). The strains 1912-4Lcp and 1912-Hp7 produced only lycopene in the quantity of 26.3 and 50.9 mg/L, respectively. Hydrogen peroxide added to the culture of 1912-4Lcp after 24 h of growth at concentration 1.0 % increased production of lycopene to 48 % (38.09 mg/L) in comparison with control experiment. Selected mutant strain 1912-Hp7 produced 93% lycopene more than the initial strain 1912-4Lcp.

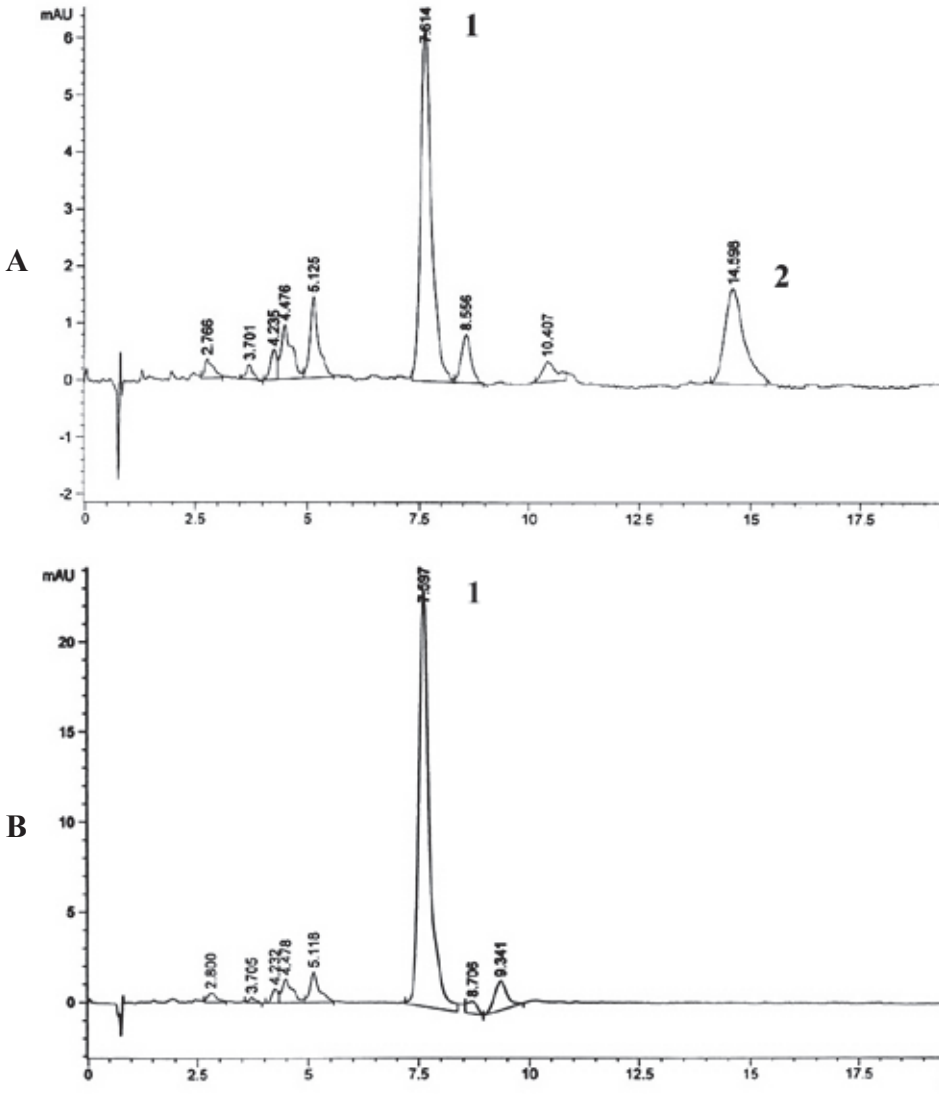

Fig. 3. HPLC of carotenoids: A, strain 1912-4Crt: 1 – lycopene; 2 - β-carotene; B, strain 1912-4Lcp: 1 – lycopene.

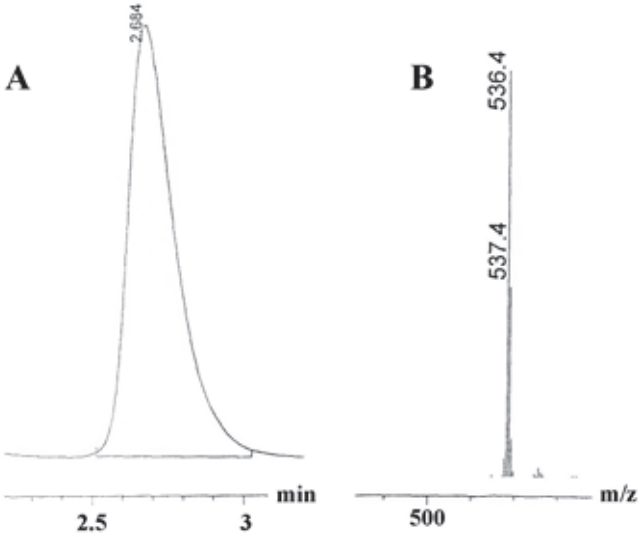

Fig. 4. LC/MS of β-carotene (strains 18(+) x 184(-): A – R_t; B – *m/z*.

Table

Production of carotenoids by strains of *S. globisporus* and *B. trispora* in shake flasks (three independent experiments)

Strain	Dry biomass, g/L	β-carotene, mg/g dry biomass (mg/L)	Lycopene, mg/g dry biomass (mg/L)
S. globisporus 1912-4Crt	12.61	0.58 (7.31)	0.25 (3.15)
	12.98	0.54 (7.01)	0.26 (3.37)
	13.40	0.48 (6.43)	0.24 (3.22)
S. globisporus 1912-4Lcp	15.25	0	1.69 (25.80)*
	15.51	0	1.68 (26.05)
	16.56	0	1.64 (27.16)
S. globisporus 1912-4Lcp + H_2O_2 (1%)	16.42	0	2.32 (38.09)*
	16.56	0	2.30 (38.08)
	16.38	0	2.33 (38.16)
S. globisporus 1912-Hp7	15.2	0	3.30 (50.16)
	14.8	0	3.50 (51.80)
	15.0	0	3.40 (51.00)
B. trispora 18(+)x184(-)	41.39	61.2 (2533.0)	0
	40.24	65.6 (2640.0)	0
	39.60	60.0 (2376.0)	0
B. trispors 72(-)x198(+)	40.99	45.1 (1848.6)	0
	39.49	47.1 (1859.9)	0
	40.16	44.3 (1779.0)	0

* $P<0.05$

Initial strain *S. globisporus* 1912 produced red-orange antibiotic landomycin E which may mask the yellow carotenoid pigments of the colonies. All landomycin E deficient mutants obtained by mutagenesis or spontaneously became white color and do not produce the carotenoids. Blue-light illumunation of their mycelium during growth on the surface of corn-soy medium in Petri dishes or in liquid medium in shake flasks did not induce the biosynthesis of carotenoids. *S. globisporus* 1912 spontaneously, by induced mutagenesis or protoplasts formation and regeneration, formed with low frequency the red or pink colonies containing β-carotene and/or lycopene. These mutants are not stable and dissociated to colorless derivatives with high frequency. We isolated one more stable strain 1912-4Lcp, producer of 26.3 mg/L lycopene, mutability of which consists of 10^{-6}. The output of lycopene can be increased to 38.0 mg/L by adding 1.0 % of hydrogen peroxide to the medium after 24 h of culture growth. The obtained more active strain 1912-Hp7 may be of biotechnological interest as the first streptomycete producing high lycopene quantity without illumination. Carotenogenesis of this strain was not increased by illumination. So, the carotenoid production in strains

of *S. globisporus* 1912-4Crt, 1912-4Lcp and 1912-Hp7 as against in *S. coelicolor* A3(2) is not light induced. We can suppose that these mutants inherited genetic changes in the regulatory system of carotenogenesis. One of the possible explanations for this may be the activation of the *crtS* gene encoding sigma factor or inactivation of anti-sigma factor. Strains 1912-4Lcp and 1912-Hp7 contain genetic information for the production of lycopene but lacks the active form of the enzyme lycopene cyclase for the transformation of lycopene into β-carotene. The carotenoid biosynthesis gene clusters are localized near the end of the linear chromosome of *S. coelicolor* A3(2) (7), *S. griseus* (11), and *S. avermitilis* (4), in the vicinity of terminal inverted repeats (TIR) which are the origin of frequent DNA rearrangement. The cause of the high spontaneous mutability of the strain 1912-4Crt can be explained by the localization of the *crt* gene cluster near the TIR element. Production of the carotenoids by strains 1912-4Crt, 1912-4Lcp and 1912-Hp7 is constitutive and does not depend on blue or white illumination as opposed to other streptomycetes (15). The dry red or pink biomass of the mycelium of the strains 1912-4Crt and 1912-Hp7 was preliminary and successfully used in poultry farming to increase the chicken productivity and intensify the yellow egg yolk color (not published)

The champion of the carotenoid production among all known microorganisms is the industrial fungus *B. trispora*. The isolated heterothallic strains 18(+) and 184(-) during combined growth produced 2.5 g/L of β-carotene in shake flasks that is 36% higher in comparison with initial strains 72(+) and 198(-) (Table 1). The high active strains 18(+) and 184(-) may already be put to use in large-scale fermentation process for biotechnological production of β-carotene.

Acknowledgement

This work was supported by the National Academy of Sciences of Ukraine (0111U000606).

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ВИДІЛЕННЯ МУТАНТІВ *STREPTOMYCES GLOBISPORUS* І *BLAKESLEA TRISPORA* З ПІДВИЩЕНИМ ВМІСТОМ КАРОТИНОЇДІВ

Р е з ю м е

Більш пігментовані мутанти *Streptomyces globisporus* Hp7 і *Blakeslea trispora* 187(+), 184(-) виділені під впливом перекису водню і нітрозогуанідину відповідно на вихідні штами *S. globisporus* 1912-4Lcp і *B. trispora* 72(-), 198(+). Каротиноїди із сухої біомаси одержаних штамів, розтертої за допомогою скляного порошку пестиком у фарфоровій ступці на льоду, екстрагували ацетоном і очищали тонкошаровою хроматографією.

Ідентифікацію індивідуальних каротиноїдів проводили за допомогою ВЕРХ і РХ/МС спектрометрії. Показано, що штам *S. globisporus* 1912-4Crt синтезує бета-каротин і лікопін (6,91 і 3,24 мг/л відповідно), мутанти 1912-4Lcp і 1912-Hp7 утворюють лише лікопін (26,05 і 50,9 мг/л відповідно), а штами *B. trispora* 18(+) x 184(-) – бета-каротин (6,2 % в сухій біомасі або більше 2,5 г/л) без освітлення в колбах на качалці. Це перший випадок конститутивного синтезу великої кількості каротиноїдів представниками роду *Streptomyces* у відсутності фотоіндукції. Покращені штами *B. trispora* 18(+) і 184(-) можуть використовуватися для біотехнологічного одержання бета-каротину в промислових умовах.

К л ю ч о в і с л о в а: *Streptomyces globisporus*, *Blakeslea trispora*, мутагенез, бета-каротин, лікопін.

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ПОЛУЧЕНИЕ МУТАНТОВ *STREPTOMYCES GLOBISPORUS* И *BLAKESLEA TRISPORA* С ПОВЫШЕННЫМ СОДЕРЖАНИЕМ КАРОТИНОИДОВ

Р е з ю м е

Более пигментированные мутанты *Streptomyces globisporus* Hp7 и *Blakeslea trispora* 187(+), 184(-) выделены под влиянием перекиси водорода и нитрозогуанидина соответственно на исходные штаммы

S. globisporus 1912-4Lcp и *B. trispora* 72(-), 198(+). Каротиноиды из сухой биомассы полученных штаммов, растертой с помощью стекляного песка пестиком в фарфоровой ступке на льду, экстрагировали ацетоном и очищали тонкослойной хроматографией. Идентификацию индивидуальных каротиноидов проводили с помощью ВЭЖХ и ЖХ/МС спектрометрии. Показано, что штамм *S. globisporus* 1912-4Crt синтезирует бета-каротин и ликопин (6,91 и 3,24 мг/л соответственно), мутанты 1912-4Lcp и 1912-Hp7 образуют только ликопин (26,05 и 50,9 мг/л соответственно), а штаммы *B. trispora* 18(+) x 184(-) – бета-каротин (6,2 % в сухой биомассе или больше 2,5 г/л) без освещения в колбах на качалке. Это первый случай конститутивного синтеза большого количества каротиноидов представителями рода *Streptomyces* в отсутствии фотоиндукции. Улучшенные штаммы *B. trispora* 18(+) і 184(-) могут использоваться для биотехнологического получения бета-каротина в промышленных условиях.

К л ю ч е в ы е с л о в а: *Streptomyces globisporus*, *Blakeslea trispora*, мутагенез, бета-каротин, ликопин.

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Отримано 10.09.2012