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UVA RADIATION ENHANCEMENT OF B-CAROTENE YIELD IN *RHODOTORULA MUCILAGINOSA*

ZWIĘKSZENIE PRODUKCJI BETA-KAROTENU W DROŹDŻACH *RHODOTORULA MUCILAGINOSA* POD WPLYWEM PROMIENIOWANIA UVA

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Summary: The influence of UVA and VIS radiation on the β -carotene biosynthesis by yeast *Rhodotorula mucilaginosa* was studied. The impact of radiation characteristic on biomass growth, substrate consumption and β -carotene production in submerged batch culture in bioreactor was studied. Three different types of light sources were used in the experiment: VIS – 15 W, wavelength range from 400 to 800 nm, the largest emission at 420 nm; UVA – 18 W, blue light, wavelength range of 350 to 430 nm, the largest emission at 370 nm; UVA – 20 W, wavelength range from 350 to 575 nm, the largest emission at 350 nm. In cultures conducted with UVA – 18 W radiation the highest β -carotene and carotenoids cellular concentration were achieved, respectively $63.20 \mu\text{g/g}_{\text{d.w.}}$ and $0.97 \text{ mg/g}_{\text{d.w.}}$. In cultures subject to VIS radiation a lower β -carotene concentration at $43.60 \mu\text{g/g}_{\text{d.w.}}$ and the total carotenoids concentration at $0.83 \text{ mg/g}_{\text{d.w.}}$ was obtained. The ultraviolet radiation with the emission peak of radiation at 370 nm promoted the β -carotene and carotenoids production in yeast *Rhodotorula mucilaginosa*.

Keywords: β -carotene, carotenoids, *Rhodotorula mucilaginosa*, UVA, VIS, batch culture, bioreactor.

Streszczenie: Badano wpływ promieniowania UVA i VIS na biosyntezę β -karotenu przez drożdże *Rhodotorula mucilaginosa*. Określano wpływ promieniowania charakterystycznego na wzrost biomasy, zużycie substratu i produkcję β -karotenu we wglębnej hodowli okresowej prowadzonej w bioreaktorze. Zastosowano trzy różne rodzaje źródeł światła: VIS – 15 W, zakres długości fali od 400 do 800 nm, największa emisja przy 420 nm; UVA1 – 18 W, zakres długości fali od 350 do 430 nm, największa emisja przy 370 nm; UVA2 – 20 W, zakres długości fali od 350 do 575 nm, największa emisja przy 350 nm. W kulturach prowadzonych

za pomocą promieniowania UVA otrzymano najwyższe stężenie β -karotenu i karotenoidów, odpowiednio 63,20 $\mu\text{g/g.s.m.}$ i 0,97 mg/g.s.m. W hodowlach napromieniowanych lampą VIS uzyskano niższe stężenie β -karotenu (43,60 $\mu\text{g/g.s.m.}$) i karotenoidów (0,83 mg/g.s.m.). Promieniowanie ultrafioletowe ze szczytem emisji promieniowania przy 370 nm promowało produkcję β -karotenu i karotenoidów w komórkach drożdży *Rhodotorula mucilaginosa*.

Słowa kluczowe: β -karoten, karotenoidy, *Rhodotorula mucilaginosa*, UVA, VIS, hodowla okresowa, bioreaktor.

1. Introduction

β -carotene's health-promoting properties and its application as a food colorant generate strong interest in enlarging the methods of obtaining β -carotene [Zhang et al. 2016; Harasym and Oledzki 2014; Berman et al. 2014; Sandmann 2015; Sant'Anna et al. 2013]. The main source of β -carotene are fruit and vegetables while an alternative method of β -carotene production is biosynthesis using microorganisms, like yeast, bacteria and algae. Yeast in particular deserve intense attention, due to their naturally occurring carotenogenesis process as well as single-celled structure, high biomass and β -carotene production rate [Braunwald et al. 2013; Cutzu et al. 2013; Marova et al. 2012; Moliné et al. 2010].

Among the most popular microbial producers of β -carotene are yeast, belonging to *Rhodotorula* genus, characterized by high product yield. Yeast *Rhodotorula spp.*, known as oleaginous yeast, exhibit high yield of carotenoids which are their secondary metabolites. Regarding the complexity of the cultivation and pigment extraction, *Rhodotorula spp.* deserves particular attention.

The physiological regulation of yeast causes a significant increase of the carotenoids concentration in the cells of *Rhodotorula spp.* during the biosynthesis process. The environmental changes can induce physico-chemical changes of the metabolic pathways of the carotenogenesis process [Mahmoud et al. 2014; Čertík et al. 2009; Frengova, Beshkova 2009; Breierová et al. 2008; Malisorn, Suntornsuk 2008; Davoli et al. 2004; Simova et al. 2004].

The carotenogenesis process in many organisms is induced by light. Some microorganisms produce carotenoids to protect themselves from harmful ultraviolet radiation. [Marchal et al. 2013; Zhen et al. 2012; Mogedas et al. 2009; Mykolaiovych et al. 2008; Oswald 1988; Dodd 1986]. So far, the existing reports in the literature have focused on the effect of light intensity on the carotenogenesis process [Zhang et al. 2014; Moliné et al. 2010; Stachowiak, Czarnecki 2007; Garcia-Gonzalez et al. 2005; Bhosale, Gadre, 2002; del Campo et al. 2001]. For the research development on the process of β -carotene biosynthesis, of great importance is the selection of the wavelength proper for the carotenogenesis process.

This study aimed to determine the impact of visible light and ultraviolet radiation on the biosynthesis of carotenoids, including β -carotene in yeast *Rhodotorula*

mucilaginosa. The scope of the research included the analysis of biomass growth, substrate consumption and β -carotene production in submerged batch culture in a bioreactor.

To the best author's knowledge, this is the first research presenting the impact of visible light and ultraviolet radiation on the biosynthesis of β -carotene and other carotenoids by yeast *Rhodotorula mucilaginosa*.

2. Materials and methods

Biological material. *Rhodotorula mucilaginosa* KKP 350 was bought from the Culture Collection of Industrial Microorganisms of Agricultural and Food Biotechnology Institute (Warsaw, Poland).

Inoculum. Inoculum medium composition: 9% (m/v) malt extract. The inoculum was cultivated in four steps: **1.** Yeast were wiped from agar slants and yeast cells were pre-cultured in 30 ml malt extract solution and incubated for 24 hours at 28°C. **2.** The content of the flasks from step 1 was transferred into the round flat bottom flask of 500 ml volume, containing 100 ml of sterile medium and incubated at 28°C for 24 hrs on a rotary shaker with 80 rpm. **3.** 20 ml of sterile 25% (v/m) glucose solution was delivered into the culture and incubated at previous set conditions for 24 hours, but with a different speed of rotary shaker (200 rpm). **4.** Yeast were centrifuged (5000 rpm, MPW 251 Med. Instruments, Poland) for 30 mins and transferred into a sterile saline solution (0.9% (v/m) NaCl). To adjust the inoculum volume and concentration the Thoma chamber was used.

Biosynthesis medium. Medium composition was as follows (g/L): glucose – 60.0 (PPH Chempur Piekary Śląskie), glycerol – 30.0 (PPH Chempur Piekary Śląskie), $(\text{NH}_4)_2\text{SO}_4$ – 17.1 (PPH Chempur Piekary Śląskie), KH_2PO_4 – 4.9 (PPH Chempur Piekary Śląskie), yeast extract – 2.2 (PPH BTL Łódź), ZnCl_2 – 0.4 (PPH Chempur Piekary Śląskie). pH was maintained at 5.0 with 5N NaOH (PPH Chempur Piekary Śląskie) and 2N HCl (PPH Chempur Piekary Śląskie). Acepol 7287 (Lubrizol Deutschland GmbH) was used as an antifoam solution.

Bioreactor. The study was conducted in the laboratory bioreactor Biomer 10 with a total capacity of 7.0 L and a working volume of 5.0 L [Pietkiewicz et al. 1987]. The bioreactor was equipped with analysers for the continuous measuring of oxygen (VO_2) and carbon dioxide content (VCO_2) – Servomex Analyser 1400 (Servomex Ltd, England).

Radiation device. A radiation device (Figure 1) was used for the emission of visible and ultraviolet radiation during the submerged batch cultivation conducted in Biomer 10 to investigate the effect of wavelength on the β -carotene biosynthesis. Around the bioreactor was a set of two radiation devices. The bioreactor and radiation devices were covered with a reflecting blanket to prevent the light escaping. In the radiation device, three types of similar power lamps were used interchangeably. The lamps were characterized by different ranges of radiation emission, as follows:

- F1 – VIS – 15 W, wavelength range from 400 to 800 nm, the largest emission of radiation at a wavelength of 420 nm;
- F2 – UVA – 18 W, wavelength range of 350 to 430 nm, the largest emission of radiation at a wavelength of 370 nm;
- F3 – UVA – 20 W, wavelength range from 350 to 575 nm, the largest emission of radiation at a wavelength of 350 nm.

The lamp tubes emitting ultraviolet light were selected for the highest radiation at ultraviolet A (315 – 400 nm), which can pass through the transparent glass of a bioreactor.

Cultivation method. The calculated and measured volume of the inoculum was introduced into bioreactor Biomer 10, containing 5 L of sterile medium, so the concentration of yeast cells was 10^6 /ml. Cultivation was carried at 28°C, with a constant rotary speed of agitator at 550 rpm. The initial aeration rate was set on 50 L/h, after 24hrs the aeration rate was increased to 100 L/h in order to ensure an adequate level of pO_2 . The stop of respiratory activity was considered as the end of the cultivation, therefore at all trials the cultivation time was 80 hrs. Two trials were performed for each radiation source. The first sample was taken and that moment was set as the beginning of the cultivation. Then the samples were taken in four-hour intervals. After the analyzes of biomass was centrifuged (MPW 251, MPW Med. Instruments), suspended in saline solution and then frozen at -20°C ; before carotenoids extraction biomass was lyophilized (Lyophilizer CHRIST ALPHA 1-2, B. Braun Biotech International).

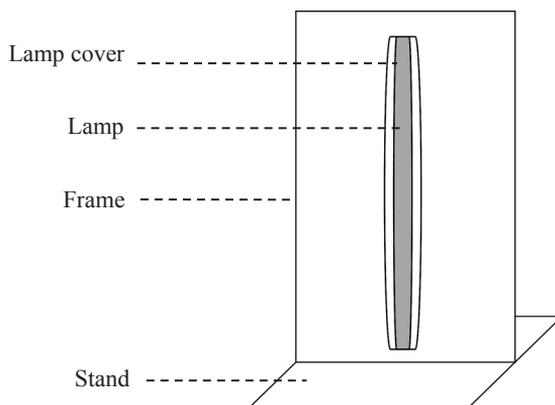


Fig. 1. Schematic diagram of the lighting device

Rys. 1. Schemat urządzenia oświetlającego

Source: author's own study.

Źródło: opracowanie własne

Carotenoids extraction. The lyophilized biomass of yeast (0.5 g) was inserted into a solution of 50 ml of C_3H_6O and 50 ml of 10% KOH in C_2H_5OH and thereafter heated at $90^\circ C$ for 30 mins with a reflux condenser cap. After this time the sample was heated at the same temperature for another 60 mins without the condenser. Next, from the cooled sample the pigments were extracted three times with 20 ml of petroleum ether (PPH Chempur Piekary Slaskie, Poland) in a separatory funnel. Next, to the separated layer containing carotenoids, 5 g of anhydrous sodium sulphate (BTL Sp. Z o.o.) were added, then covered with parafilm protecting from oxygen, and put into the refrigerator for 30 minutes at $5^\circ C$. Using SPE techniques, anhydrous sodium sulphate and lipids were separated from carotenoids as follows: the extract was filtered through the filter Bakerbond column (SPE Column, J.T. Baker) filled up

Table 1. Calculation of cultivation parameters**Tabela 1.** Obliczanie parametrów hodowli

| Symbol | Cultivation parameters | Unit | Batch cultivation |
|-----------|--|-----------------|---------------------------|
| Q_p | β -carotene volumetric rate | $\mu g/L h$ | $\frac{dP}{dt}$ |
| q_p | β -carotene cellular rate | $\mu g/g h$ | $\frac{dP}{dt \cdot X}$ |
| $Y_{P/S}$ | Product yield (β -carotene efficiency acc. to carbon substrate input) | % (m/m) | $\frac{P}{S_0} \cdot 100$ |
| Q_s | Substrate volumetric consumption rate | $g/L h$ | $\frac{dS}{dt}$ |
| q_s | Substrate cellular consumption rate | $g/g h$ | $\frac{dS}{dt \cdot X}$ |
| Q_x | Biomass volumetric growth rate | $g/L h$ | $\frac{dX}{dt}$ |
| $Y_{X/S}$ | Biomass yield (Biomass efficiency acc. to carbon substrate input) | % (m/m) | $\frac{X}{S_0} \cdot 100$ |
| X_K | Final biomass concentration | g/L | - |
| P_{KB} | β -carotene cellular concentration | $\mu g/g_{d.w}$ | - |
| P_{KC} | Carotenoids cellular concentration | $mg/g_{d.w}$ | - |
| P_{KVB} | β -carotene volumetric concentration | $\mu g/L$ | - |

where: d – Change, delta,

P – β -carotene concentration, $\mu g/L$,

S – Substrate concentration in culture medium, g/L ,

S_0 – Substrate concentration in culture medium at cultivation start, g/L ,

S_z – Substrate input during feeding the bioreactor medium, g/L ,

t – Cultivation time, h,

X – Biomass concentration in medium, g/L .

Source: author's own study.

Źródło: opracowanie własne.

with 6 ml MgO: Celite in 1:1 ratio. As an eluent the solution of petroleum ether and acetone in 8:2 ratio was used. Petroleum ether was evaporated (Büchi Rotavapor R, Germany) using an amber flask. The procedure was performed in dark conditions.

Carotenoids determination. The total carotenoids content was determined spectrophotometrically (Media Marcel, Marcel Sp. z o.o. Poland) at a wavelength of 453 nm. The identification and determination of β -carotene and carotenoids was done by HPLC (chromatograph Beckman System Gold, Programmable Solvent Module 126 equipped with a UV/VIS CE Detector, USA) at the wavelength of 453 nm on the column C18 (Bionacom Velocity, England), 5 microns 4.6 x 250 mm using the pre-column with filter C18 (Bionacom Velocity, England), 5 microns, 3.0 x 4.6 mm. The injection loop volume was 50 μ L. The flow rate was 0.7 ml/min and eluent composition was acetonitrile, propanol and ethyl acetate in the ratio 4:4:2. Analysis time: 30 mins. The sample was diluted according to the concentrations of the carotenoids. Each sample was filtered through a nylon syringe filter having a pore size 0.2 μ m. The injection was repeated three times in volume of 100 μ L.

Analytical methods. The following parameters were measured with intervals of 4 hours: agitation speed (V_r), air flow rate (V_G), temperature (T), pH and dissolved oxygen concentration (pO_2), oxygen concentration (VO_2) and carbon dioxide concentration (VCO_2) in the exhaust gas.

Cultivation parameters calculation. Cultivation parameters were calculated as presented in Table 1.

Biomass and substrate determination. The concentration of yeast biomass dry matter (X) was determined by the gravimetric method using a moisture analyser (Sartorius MA 30, Germany) to the constant weight. To determine the concentration of glucose (SGL), and glycerol (SGC) the HPLC techniques were used as follows: Perkin Elmer chromatograph, RI detector Perkin Elmer Series 200, column Knauer Eurokat H size 8 x 300 mm, at temperature of 60°C in the thermostat (COBRABID Type KB-5506, Poland); flow rate: 0.6 ml/min; eluent: water for HPLC (PPH Chempur Piekary Slaskie, Poland); analysis time: 30 mins. Each sample was filtered through a nylon syringe filter having a pore size of 0.45 μ m. The injection was repeated three times in volume of 50 μ L. The injection loop volume: 20 μ L.

3. Results and discussion

The submerged batch cultivation of yeast *Rhodotorula mucilaginosa* KKP 350 were conducted for about 80 hours. The ultraviolet radiation (F2) with the largest emission of radiation at a wavelength of 370 nm promoted the β -carotene and carotenoids concentration (Figure 2). In cultures conducted with UVA radiation with lamp F2 the highest β -carotene and carotenoids cellular concentration was achieved, respectively 63.20 μ g/ $g_{d.w.}$ and 0.97 mg/ $g_{d.w.}$. Cultures of *Rhodotorula mucilaginosa* irradiated by the lamp F1 with VIS light obtained lower the β -carotene concentration

($P_{KB} = 43.60 \mu\text{g/g}_{d.w.}$) and the carotenoids concentration ($P_{KC} = 0.83 \text{ mg/g}_{d.w.}$). The application of the UVA lamp with the largest emission of radiation at a wavelength of 350 nm caused the increase of the β -carotene and carotenoids concentration, however the results did not surpass the results achieved during irradiation by light source F2. In cultures radiated by UV lamp F3, the final content of β -carotene was $58.90 \mu\text{g/g}_{d.w.}$ and was also higher than in cultures radiated with light in the visible range ($P_{KB} = 43.60 \mu\text{g/g}_{d.w.}$).

Kinetic parameters of β -carotene biosynthesis by yeast of *Rhodotorula mucilaginosa* in submerged batch cultures are presented in Figure 3. The highest β -carotene volumetric biosynthesis rate ($Q_p = 21.33 \mu\text{g/L h}$) and β -carotene cellular biosynthesis rate ($q_p = 0.79 \mu\text{g/g h}$), as well as β -carotene yield according to carbon substrate input ($Y_{p/S} = 18.96 \cdot 10^{-4}\%$ (m/m)) was achieved in cultures radiated by lamp F2. In the *R. mucilaginosa* cultivations radiated by lamp F1, the lowest β -carotene kinetics parameters were obtained.

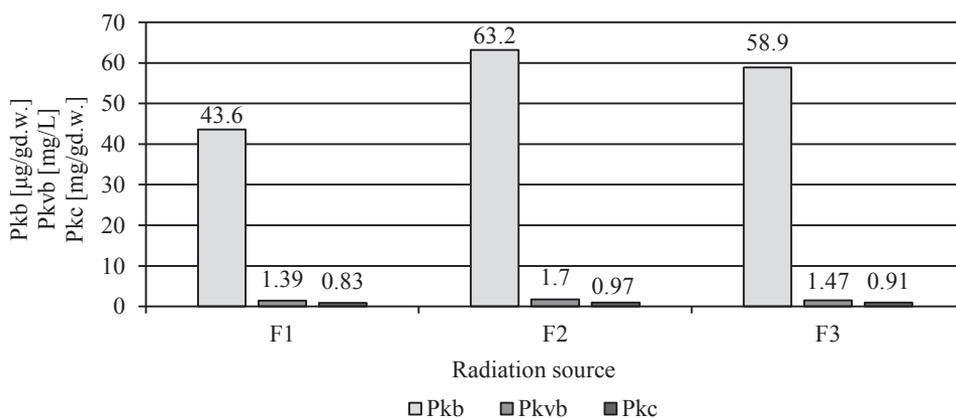


Fig. 2. β -carotene and carotenoids concentration in batch culture of *Rhodotorula mucilaginosa* irradiated with different radiation types (F1-VIS, F2-UVA (18W), F3-UVA (20W))

Rys. 2. Stężenie β -karotenu i karotenoidów w hodowlach okresowych drożdży *Rhodotorula mucilaginosa* naświetlanych różnymi rodzajami światła (F1-VIS, F2-UVA (18W), F3-UVA (20W))

Source: author's own study.

Źródło: opracowanie własne.

These results are contrary to reports of Bhosale and Gadre 2002 [Bhosale, Gadre 2002]. The authors investigated the submerged culture of the yeast *Rhodotorula glutinis* under continuous irradiation of light of 1000 lux. The authors found the inhibitory effect of radiation conditions on the carotenogenesis process. The results showed the decrease of carotenoids concentration from 125 mg/L in dark conditions to 83.0 mg/L. Bhosale and Gadre noted that irradiation at the late phase of logarithmic yeast growth resulted in the increase in carotenoids concentration up to 217.6 mg/L and in β -carotene content (91%) in all carotenoids.

The results of the study confirmed the positive relation between light and the carotenogenesis process claimed by Stachowiak and Czarnecki [Stachowiak, Czarnecki 2007] and Zhang and co-authors [Zhang et al. 2014]. The Stachowiak team examined the impact of the radiation intensity on the carotenogenesis process by yeast *Phaffia rhodozyma*. The authors noted higher carotenoids concentration in cultures radiated by light of 400 lux. Total carotenoids concentration exceeded 0.55 mg/g_{d.w.} at the end of the culture (192 hours). The researcher noted the lowest levels of carotenoids in cultures conducted in dark conditions in comparison to culture irradiated light with 400 lux. Zhang and others studied the effects of radiant energy flux density in the range from 800 to 2400 $\mu\text{mol}/\text{m}^2 \text{ s}$ emitted by the LED lamp on yeast *Rhodotorula glutinis*. The results of Zhang's study confirmed the results obtained by Stachowiak and Czarnecki.

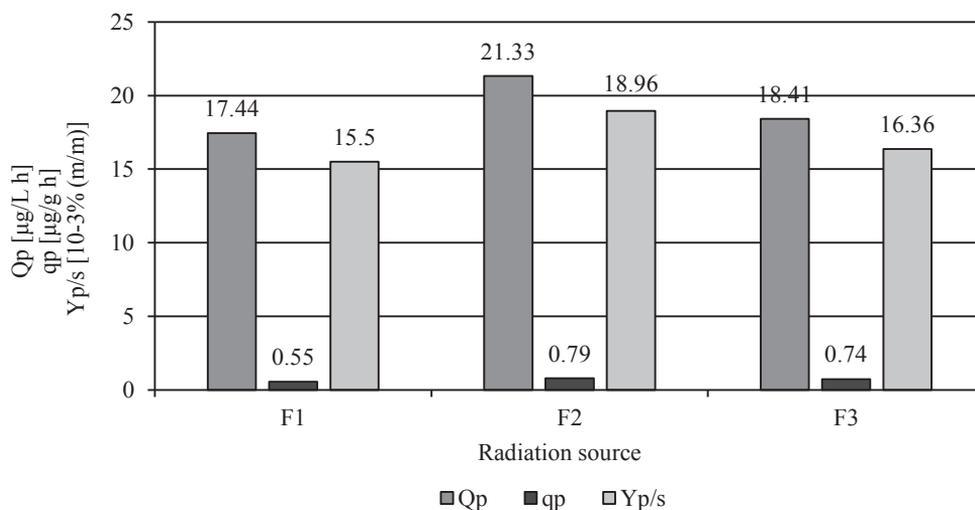


Figure 3. Kinetic parameters' comparison of *Rhodotorula mucilaginosa* batch culture with different radiation types (F1-VIS, F2-UVA (18W), F3-UVA (20W))

Rys. 3. Porównanie kinetycznych parametrów hodowli okresowych drożdży *Rhodotorula mucilaginosa* naświetlanych różnymi źródłami światła

Source: author's own study.

Źródło: opracowanie własne.

The results obtained in cultures radiated with the F3 source might be explained by the largest radiation emission at a wavelength of 350 nm, which was closer to the UVB range than other radiation sources. Moline and others studied the effects of UVB radiation on carotenoids production by yeast *Rhodotorula mucilaginosa*, where the yeast was maintained in quartz tubes from Spectroline XX15-emitting UVB at the wavelength range 280 to 320 nm. The results showed the survival decrease of

radiated yeast. However, the strains with a survival rate of up to 44.5% produced a higher carotenoids content and the strains with lower survival rate of 7.6% made two and a half times lower carotenoids concentration [Moliné et al. 2010].

The biomass concentration (Table 2) in *Rhodotorula mucilaginosa* cultures irradiated with the F3 source was lower than in cultivations using lamp F1 and F2 as the radiation source. The wavelength range of lamp F3 caused a decrease of biomass concentration, however the carotenoids concentration was still higher than in cultivations irradiated with visible light F1 (Figure 2). The highest biomass concentration ($X_k = 32$ g/L), biomass volumetric growth rate ($Q_x = 0.40$ g/L h) and biomass efficiency according to carbon substrate input ($Y_{x/s} = 35.56\%$ (m/m)) was achieved in cultivations radiated by F1 source. Considering that the carotenoids belong to the secondary metabolites, their carotenoids yield is not in direct proportion to the biomass yield. Carotenoids biosynthesis follows in nitrogen limitation conditions unlike the biomass biosynthesis process.

Table 2. Parameters of biomass biosynthesis in *Rhodotorula mucilaginosa* batch cultivations radiated with different light wavelengths. (X_k – Final biomass concentration)

Tabela 2. Parametry biosyntezy biomasy w hodowlach okresowych drożdży *Rhodotorula mucilaginosa* naświetlanych światłem o różnej długości fal (X_k – końcowe stężenie biomasy)

| Cultivation parameters | Radiation source | | |
|------------------------|------------------|-------|-------|
| | F1 | F2 | F3 |
| Q_x , g/L h | 0.40 | 0.34 | 0.31 |
| $Y_{x/s}$, % (m/m) | 35.56 | 30.00 | 27.78 |
| X_k , g/L | 32.00 | 27.00 | 25.00 |

Source: author's own study.

Źródło: opracowanie własne.

The study confirms the results obtained by Petrik, according to the application of glycerol combined with glucose in β -carotene biosynthesis [Petrik et al. 2013]. In a previous study, optimal ratio of glycerol and glucose (1:2) was established with a total concentration of 90 g/L (Data not shown). In all cultivations radiated with F1, F2 and F3, carbon substrates were depleted at the end of the batch culture.

The application of glycerol in the carotenogenesis process was confirmed by Cardoso in *Sporobolomyces ruberrimus* cultures [Cardoso et al. 2016] and by Lee and the other authors in *Rhodospiridium toruloides* bi-phasic cultures conducted toward carotenoids and lipids production [Lee et al. 2016].

Glycerol, due its properties and low cost, is used as the carbon source in the biosynthesis of carotenoids, microbial biomass, lipids and organic acids [Mirończuk et al. 2015; Rywińska et al. 2013; Almeida et al. 2012; Taccari et al. 2012]. The β -carotene biosynthesis by yeast *Rhodotorula spp.* using glycerol combined with glucose can be an alternative method of its production.

4. Conclusion

The β -carotene biosynthesis, due to many possibilities of its application in the food industry, medicine and cosmetics, is still a wide field for scientific research. Enhancing the efficiency of carotenogenesis process is the challenge for scientists. Influencing the determination of UVA and VIS radiation on β -carotene biosynthesis enables to find the optimal conditions. The presented results indicate the promoting properties UVA radiation on β -carotene and carotenoids production by yeast *Rhodotorula mucilaginosa*. However, in the next steps the optimal wavelength needs to be found.

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