

Method for Torularhodin Separation and Analysis in the Yeast *Rhodotorula Rubra* Aerobically Cultivated in Lab Bioreactor

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The paper presents a procedure realized for the extraction, separation, and quantitative determination of the carotenoids, mainly beta-carotene and torularhodin, with antioxidant possible application, from the yeast *Rhodotorula rubra* ICCF 209. The procedure was applied after the aerobic bioprocess completion. Wet biomass was separated by centrifugation at 8000 rpm and the total carotenoid pigments was carried out by extraction with acetone-water and n-hexane, with all carotenoid pigments in the upper hexane layer. The pigmented hexane layer was then washed with distilled water. The optical density of the pigmented hexane filtrate was determined on a UV-VIS spectrophotometer (Jenway Spectrophotometer) at 500 nm against a hexane blank to give the total pigment concentration. The pigment torularhodin was separated from the hexane by extraction with methanol potash (0.1 N) and the visible spectrum at 380-700 nm was drawn to obtain the torularhodin concentration. A final concentration of the total carotenoids as beta-carotene of 250-450 µg/L medium and a concentration of torularhodin of 140-350 µg/L was determined in case of the aerobic bioprocess in the 3.7 L Bioengineering AG lab bioreactor with mechanical stirring with a duration of 72 hours. In the first 24 hours the beta-carotene was the major component of carotenoids, while after 48 hours it seems that more than 50% of this pigment is transformed into torularhodin via the metabolic pathway described in the literature.

1. Introduction

Carotenoids are important natural pigments, displaying yellow, orange and red colours, widely found in microorganisms and plants. Industrial carotenoid pigments such as β-carotene, astaxanthin, and recently torularhodin, are used as natural food colorants or food additives like pigments from *Monascus purpureus* mold (Ungureanu and Ferdes, 2010). Several studies have shown that carotenoids can be used as therapeutic agents against various type of cancer and other diseases due to their antioxidant and/or provitamin A properties (Frengova and Beshkova, 2009).

Carotenoid biosynthesis is a specific feature of the *Rhodotorula* or *Sporobolomyces* yeasts and *Phaffia* genera (Razavi and Marc, 2006).

Facing the growing economic significance of carotenoids, much interest has been dedicated to new supplies of this class of pigments. In particular, the development of carotenoid-preparation biotechnologies is considered a competitive solution, as it can provide important quantities of pigments such as torularhodin and β -carotene formed by *Rhodotorula* species or astaxanthin from *Phaffia rhodozyma* without facing the typical problems generated by the weather dependency of the agriculture production.

The major carotenoid pigments obtained by biotechnological methods are torularhodin, β -carotene, and torulene produced in various concentrations by *Rhodotorula* yeasts and astaxanthin from *Phaffia rhodozyma* or the green alga *Haematococcus pluvialis* (Dong and Zhao, 2004).

The paper presents a procedure realized for the carotenoids extraction, separation, and quantitative determination, mainly total carotenoids as beta-carotene and the torularhodin ratio in the pigments mixture, from the yeast *Rhodotorula rubra* ICCF 209.

2. Experimental Set-up

2.1 The microbial transformation

The experiments were carried out in 3.7 L (2 L working volume) bioreactor Bioengineering AG, with computer-controlled and recorded parameters.

The bioreactor has mechanical stirring (Rushton impellers) and the main parameters (temperature, pH, mixing speed, air flow rate, pO_2 , and foam level) are controlled, but the turbidity is monitored. The operating parameters were 30 °C, 500-600 rpm and air flow rate of 150-200 L/h.

The medium composition, defined as MS3, was obtained with the formula: 40 g/L glucose, 1.5 g/L yeast extract, 5 g/L NH_4NO_3 , 1 g/L KH_2PO_4 , 0.4 g/L $MgSO_4 \cdot 7H_2O$ and 0.4 g/L NaCl. Trace elements are assumed to be taken from the tap water.

The bioprocess was operated in discontinuous fed-batch mode with the glucose introduced at the beginning (1.5-2% concentration) and several additions to increase medium concentration with 0.5-1% during the first 30 hours. Three fed-batch cultivations designed as A, B, C were performed.

A suspension of the yeast cells in sterile water was used for the inoculum preparation. Inoculum was analyzed in terms of number of cells/mL. The cells growth was quantified by: Optical Density determination at $\lambda = 600$ nm, evolution of pH and dried biomass concentration. Dry matter concentration determination was done after the biomass separation from the culture medium by centrifugation. The biomass drying was achieved in the oven at 105 °C until constant mass.

2.2 Extraction of the total carotenoid pigments and torularhodin separation

After cells' separation by centrifugation three freeze-thaw cycles were performed. After this cellular walls permeability' increase the pigments extraction was done with / without previous cells disintegration by high pressure homogenizer - IKA WERKE.

From measurements performed previously been shown that cellular disintegration is enough by freeze-thaw cycles. Cell destruction has not been done with high pressure

homogenizer - IKA WERKE due to electricity consumption; the results are comparable with those obtained by other methods.

The pigments extraction procedure was done in accordance with the dedicated literature (Ananda and Vadlani, 2010), comprising acetone extraction of the total pigments mixture including water soluble species, followed by n-hexane extraction to separate the total carotenoids content; another extraction with alkaline methanol allows the torularhodin (the only pigment with acid structure) component isolation.

The total carotenoids concentration and the torularhodin concentration were determined based on the spectrometric recording of the extracts on the UV-VIS spectrophotometer. For each extract corresponding to the indicated 3 stages extractions in specific solvents: acetone, n-hexane and basic methanol, adsorption spectra were drawn in 380-800 nm domain and the peaks were determined.

To calculate the torularhodin concentration, the specific absorption coefficient $E^{1\%}_{1\text{cm}}$ 1932 was applied to the difference between the absorbance of the hexane extract before and after methanol phase extraction, at 515 nm (Peterson et al., 1954).

3. Results and Discussion

In order to quantify the carotenoid pigments concentration, the cell dry biomass concentration was determined for the final samples of the above mentioned experimental cultivations.

The results are presented in the Table 1 for 72 hours yeast growth in fed-batch bioprocess, MS3 medium composition.

Table 1: Determination of the cellular dry weight values for A-C bioprocesses, *Rhodotorula rubra* ICCF 209 strain

Experimental variant	Dry weight (g%)	Wet biomass in 100 mL medium (g)	Dry biomass in 1000 mL medium (g)
A	32.83	23.15	7.6
B	36.82	19.51	3.99
C	34.70	17.86	3.44

The pigment extraction was carried out in 3 stages in the mentioned specific solvents. For each extract absorption spectra were drawn in 380-800 nm domain and the peaks were determined.

All the three spectra show the same characteristic peaks for torularhodin extracts at 490 and 524 nm. The extraction was achieved for about 1 g wet biomass obtained by centrifugation of 72 hours old culture. The torularhodin concentration was then calculated for 1 liter culture.

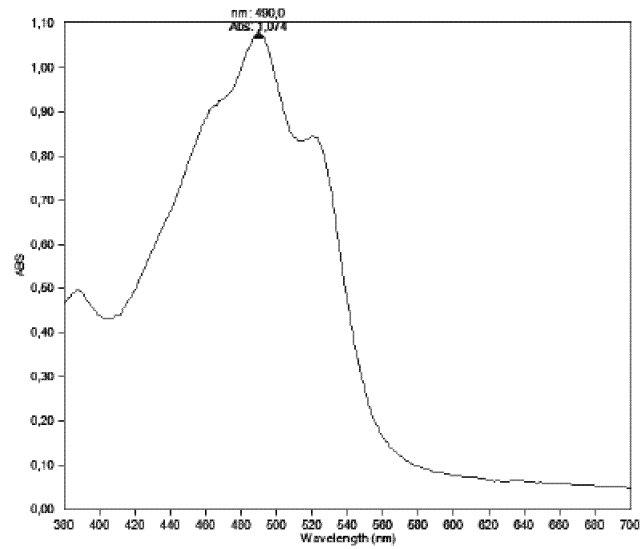


Figure 1: Absorption spectrum of the methanol extract of the biomass from the fed-batch bioprocess - A variant

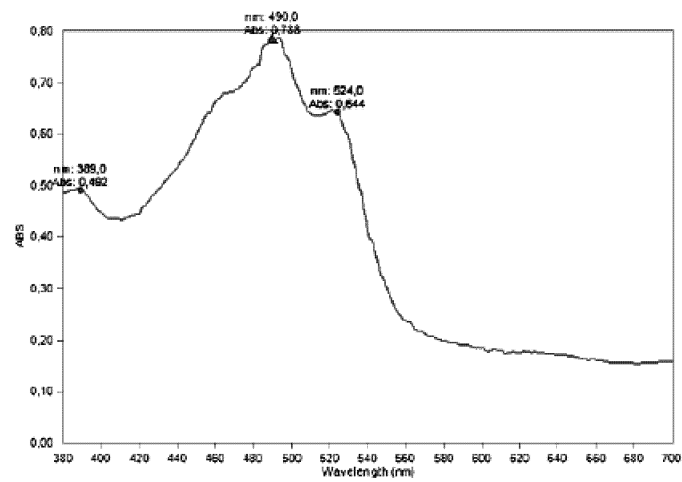


Figure 2: Absorption spectrum of the methanol extract of the biomass from the fed-batch bioprocess - B variant culture

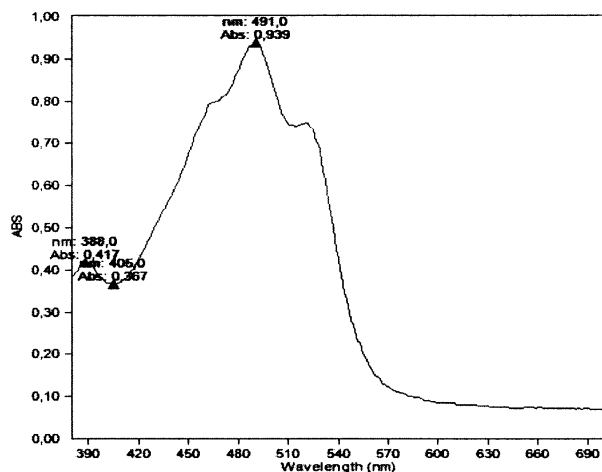


Figure 3: Absorption spectrum of the methanol extract of the biomass from the fed-batch bioprocess – C variant

The torularhodin concentration evolution for the 3 experimental variants in aerobic fed-batch bioprocess is also represented in the Figure 4.

The final results indicate that the highest torularhodin yield was obtained for the C experimental variant (322.6 $\mu\text{g/L}$ medium). The determined torularhodin concentrations are in agreement with the values presented in the specific literature demonstrating that both the yeast *Rhodotorula rubra* ICCF 209 and the cultivation medium with the composition MS3 can be considered as valuable solutions for the torularhodin preparation.

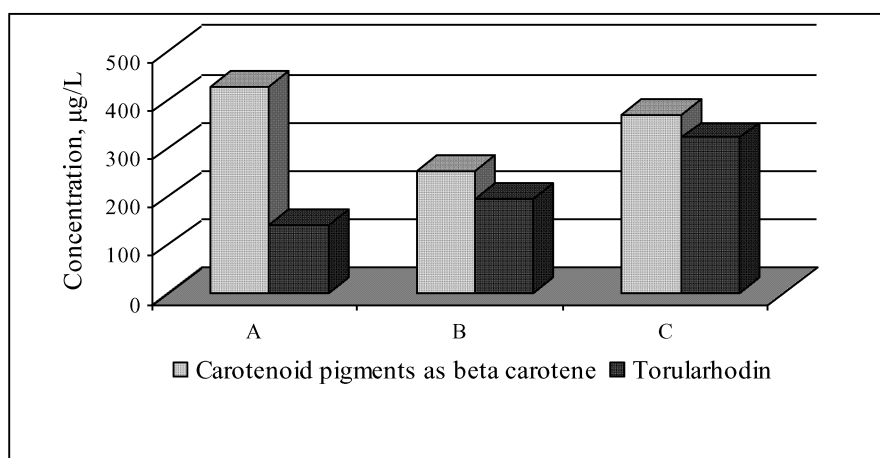


Figure 4: The torularhodin concentration function of the experimental variants

Conclusions

The study on the cells disintegration and pigments extraction demonstrated that three freeze-thaw cycles were needed before pigments extraction with appropriate solvents; the cells disintegration with high homogenizer was not necessary to semi quantitatively differentiate the experimental cultivation variants. The pigments extraction was achieved in n-hexane to determine the total carotenoids content and in basic methanol for the torularhodin concentration evaluation.

For the tested *Rhodotorula* strain the highest pigments yield was determined in case of C experimental variant: the total carotenoids concentration was 369.8 µg/L, but the torularhodin concentration represented 87% from the carotenoids mixture; the results validated the fed-batch mode of operation applied in case of the C model, meaning 1.5% glucose at start and three additions to determine medium concentrations of 0.5% each during the first 24 hours of cultivation.

Acknowledgments

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References

- Ananda N. and Vadlani P.V., 2010, Production and optimization of carotenoid-enriched dried distillers grains with solubles by *Phaffia rhodozyma* and *Sporobolomyces roseus* fermentation of whole stillage, *Journal of Industrial Microbiology and Biotechnology*, 37, 1183-1192
- Dong Q.L. and Zhao X.M., 2004, In situ carbon dioxide fixation in the process of natural astaxanthin production by a mixed culture of *Haematococcus pluvialis* and *Phaffia rhodozyma*, *Catalysis Today*, 98, 537-544
- Frengova G. and Beshkova D., 2009, Carotenoids from *Rhodotorula* and *Phaffia*: yeasts of biotechnological importance, *Journal of Industrial Microbiology & Biotechnology*, 36(2), 163-180
- Peterson W. J., Bell T.A., Etchells J.L. and Smart W.W.G., 1954, A procedure for demonstrating the presence of carotenoid pigments in yeasts, *Journal of Bacteriology*, 67(6), 708-713
- Razavi S.H. and Marc I., 2006, Effect of temperature and pH on the growth kinetics and carotenoid production by *Sporobolomyces ruberrimus* H110 using technical glycerol as carbon source, *Iranian Journal of Chemistry & Chemical Engineering*, 25, 59-64
- Ungureanu C. and Ferdeş M., 2010, Antibacterial and antifungal activity of red rice obtained from *Monascus purpureus*, *Chemical Engineering Transactions*, 20, 223-228