STUDIES CONCERNING THE BIOSYNTHESIS CAPACITY OF CAROTENOID PIGMENTS IN RHODOTORULA SP.

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Abstract

The quantity of extracellular carotenoid pigments in Rhodotorula sp., determined by the absorbance observed at 520 nm, reached a maximum after 4 days, varying between 0,8729 µg/g of dry mass in A2 and 7,5365 µg/g of dry mass in B1.The accumulation of carotenoid pigments in the intra-cellular environment had values between 1,888µg/g of dry mass in B2E and 1.711µg/g of dry mass in A2E.

Key words: Rhodotorula sp., carotenoid pigments, cellular biomass, culturing medium

INTRODUCTION

Carotenoids are colored pigments (red, orange, yellow, purple) often found in plants along holoproteids and carbohydrates (carotenoproteids, carotenoid glycosides). Their quantity varies according to species and environmental conditions. In carrots, they accumulate in quantities between 6 and 24 mg/100g of product. From a chemical point of view, carotenoid pigments have a structure consisting of conjugated double bonds which confers their unsaturated character and the possibility for oxidation and antioxidation reactions in the presence of air, the capacity for absorbtion of certain light frequencies, etc. They are used as antioxidant substances, natural coloring agents for a large number of food products, in pharmaceutics and cosmetics, in animal feed, to improve the taste of certain food products etc.

Carotenoids synthesized by microorganisms represent an important alternative to those synthesized chemically with an identical structure, as well as to those extracted from plant prime material. Yeasts of the Rhodotorula genus (Buzzini et al., 2005, Malisorn and Suntornsuk, 2008; Malisorn and Suntornsuk, 2009), Rhodosporidium (Sperstad et al., 2006), Sporobolomyces (Maldonade et al., 2008) and Cryptococcus sp. (Libkind and Broock, 2006) have the ability to synthesize carotenoid pigments. The Dunaliella, Haematococcus pluvialis (Johnson; Conklin; Lewis, 1979, Johnson; Schroeder, 1995) microalgae can produce carotenoids with similar structures to those produces by yeasts, and the Mucor circinelloides (Papp et al., 2009), Blakeslea trispora (Filotheou et al., 2010) fungi have the ability to synthesize carotenoidic pigments.Carotenoids synthesized by

microorganisms contain β- caroten and ϒ- caroten, torulahodineandtorulene(Meenu Thakur,Wamik Azmi,2013).Torulene is a carotenoid typical for yeasts, due to its structure consisting of half b-carotene and half licopene, which is responsible for its antioxidant properties.

The role some phitohormones can have in stimulating biomass growth and carotenoid pigment synthesis in yeasts of the Rhodotorula genus has not been studied before. References regarding the important role small concentrations of IAA (indol-3-acetic acid) can have in the process of filamentation and adhesion in Saccharomyces cerevisiae was evidenced by Prusty R. et al, in 2004. The presence of yeasts with the ability to synthesize IAA in the philosphere of carnivorous plants (Drosera indica L.) was made public by Sun P-F et al., in 2014. The ability of some bacteria to synthesize IAA and to use it in their interaction with plants, for phitostimulation, colonization and to elude plant defense mechanisms was highlighted by Spaepen S. et al. in 2007. The effects of adding IAA in an attempt to induce root colonization, cell elongation and hyphae ramification of the mycrrhizal Tricholoma vaccinum fungus were reported by K. Krause et al. in 2015. This approach was prompted by the fact that often times Rhodotorula sp. Infections have been reported in synthetic culturing media (Nitche, MS, Gamborg) with various concentrations of phitohormones.

MATERIAL AND METHODS

The yeast was isolated in dishes with explants grown *in vitro* on the Murashige-Skoog medium. In order to achieve pure culture isolation, the Sabourad medium was used, with gentamicin (120g/L glucose, 90g /LNaCl,30g/L peptone,45g/L agar,1,5 ml/L gentamicin). Inoculation was performed in Petri dishes kept at 35^{0} C, with a photoperiod of 16 h of light and 8 h of darkness. After 6 days, an inoculation solution was made from the developed colonies, with a cellular density of 9,2 $x10^{7}$. The culturing medium variants used were: liquid MS medium and MYEA medium (3g/L malt extract, 5g/L peptone,3g/L yeast extract,10 g/L glucose). The experimental variants were:

1.1.MS medium without phitohormones –control
1.2. MS medium with added BA -0,4 mg/L (A1)
1.3. MS medium with added ANA -0,4 mg/L (A2)
1.4. MS medium with added 2,4 D -0,4 mg/L(A3)
2.1. MYEA medium without phitohormones –control
2.2. MYEA mediumwith added BA -0,4 mg/L (B1)
2.3. MYEA medium with added ANA -0,4 mg/L(B2)
2.4. MYEA mediumwith added 2,4 D -0,4 mg/L(B3)

250 ml Erlenmayer flasks were used, each containing 100 ml of medium, inoculated with 5 ml of the inoculation solution, with 3 repetitions per variant. Dishes were placed on an orbital agitator at 110 rotations/minute, at 80 lux. and 35^0 C.The dynamics of cellular growth were determined every 24 hours with a DEN-1 (550 nm) Densiometer. The intracellular and extracellular pigments were extracted based on the protocol proposed by K.Shivalkar and R. Prahba (2014). For each experimental variant were three rehearsals and the results were analyzed with ANOVA method.

The extraction of pigments from the culturing medium consisted of centrifuging the medium at 6.000rotations/minute (10 min.) and filtering the supernatant through a sterile filter, after which the pigment is extracted from the supernatant by using acetone (a 4/1 ratio was used – 2 ml acetone to 0.5 ml supernatant). For the intracellular pigments collected, the pellets per placed in HCl 0,1 N (1/10 ratio), at 90^0 C, for 10 minutes, then cooled in ice water for 10 minute, at room temperature, then centrifuged at 6000 rot./min. Pigments were extracted from the supernatant using the same ratio as above. Absorbance was measured at 520 nm using FTIR. The calculation was performed using the following relation - Carotenoid yield (µg/g of dry cell mass) = A520 (asorption at 520 nm)x volume of the acetone/volume of the sample x17 (Shivalkar and Prahba, 2014).

Establishing that the isolated yeast belongs to the Rhodotorula genus was performed through the comparative analysis of morphological and physiological characteristics. The observations were then compared with data on these yeasts, compiled in specialty literature. (Seifi et al. 2013, Gomez-Lopez Aet al. 2005)

Morphology: colonies are coral pink, smooth, moist and shiny. Yeast cells are spherical, and then ovoidal during bloom.(Fig. 1 a.b,c).

In order to characterize the yeast from a morphological perspective, the yeast was inoculated on the Sabouraud medium (2.0% glucose, 0.5% yeast extract, 0.2% monobasic sodium phosphate, 1.0% neopeptone) for 24-48 h, at 27°C;


a


b

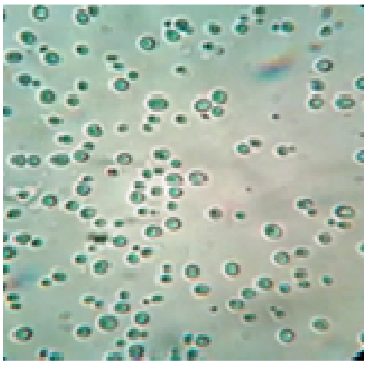

c

Fig. 1. a – medium for yeast isolation b – pure yeast culture c –isolated yeast cells

The ability to metabolise various types of carbohydrates (Tab. 1) was tested by culturing the yeast in Durham tubes, poured in 2 ml of medium (45% yeast extract, 0.75% peptone and bromtimol blue solution), to which 1 ml of the carbohydrate tested solution was added. Inoculation was performed with 1 ml of yeast suspension on Sabouraud medium. For incubation, incocules were kept at27 ^{0}C for 24 h.

In order to evaluate the capacity for assymilation of certain carbon compounds, the yeast was inoculated on Petri dishes, on YNB agar medium, with 0.5% final carbohydrate concentration. (Iriani Maldonade et al. 2007).

Tab.1

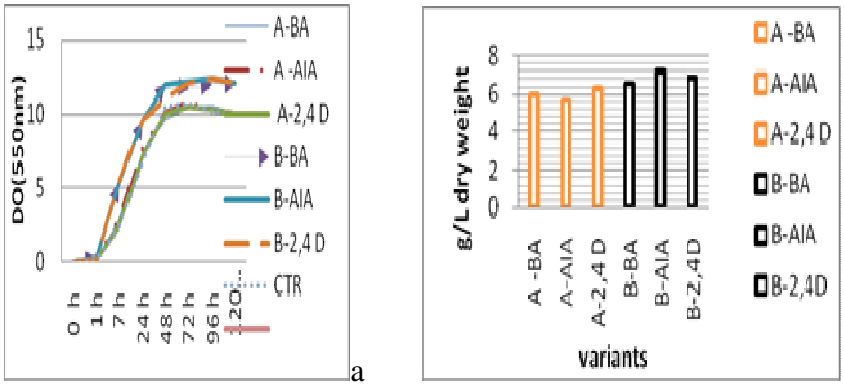
The ability of isolated yeast to metabolise certain carbohydrates

Fermentation	Physiological test
Glucose	+
Galactose	+
Sucrose	+
Maltose	+
Lactose	-
Arabinose	-
Glycerol	-
Myo-inositol	-

In order to test for urease, the yeasts were inoculated in test tubes with medium comprised of 1.17% YCB, 0.02 % acidic fuchsine, 2.0% agar and 2.0% urea, and incubated at 37 °C for 72 h. Evaluation was performed every hour, monitoring the change in color, which indicates the presence of urease.

RESULTS AND DISCUSSION

Figure 2 shows that the culturing medium which ensures the greatest dynamics in cell growth is the MYEA medium, in all three experimental variants. In both culturing mediums, cell number growth ceases after 96 hours. The accumulation of dry biomass reaches smaller values in the case of the MS medium (between 5.76 and 6.3 g/L d.s.) when compared to the MYEA medium (6.6 to 7.2 g/L). Also remarkable is the fact that on the MS medium, the most effective phitohormone in cell number growth stimulation and dry substance production is 2,4 D, whereas on the MYEA medium, it is IAA (indol-3-acetic acid).



a b

Fig. 2 Cellular biomass growth dynamics in McFarland units, at 550 nm (a) and yeast dry substance g/L (b)

The quantity of extracellular carotenoid pigments collected from the culturing medium and determined at a 520 nm absorbtion reached its peak after 4 days, varying between 0.8729 µg/g of dry mass in A2(IAA) and 7.5365 µg/g of dry mass in B1 (BA). Besides, the MYEA medium, with more complex composition (fatty acids, aminoacids, vitamins etc.) ensures superior biosynthesis of pigments on all three variants, compared to the A. medium (Fig. 3).

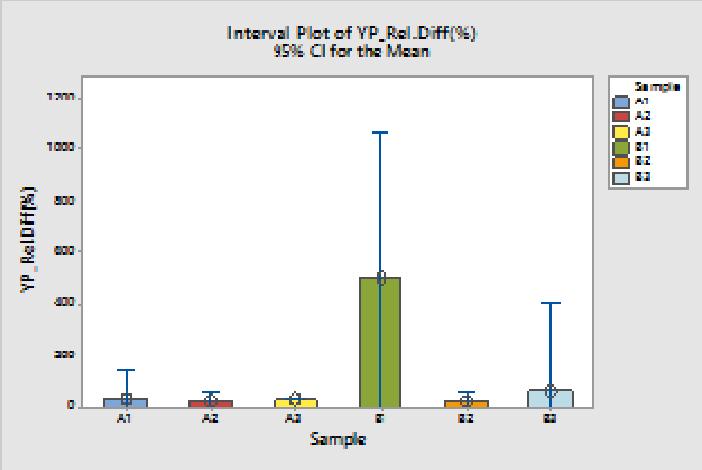


Fig. 3. Extracellular carotenoid pigments

The accumulation of intracellular carotenoid pigments (Fig.4) has values between 1.888µg/g of dry mass in B2E and 1.711µg/g of dry massin A2E.

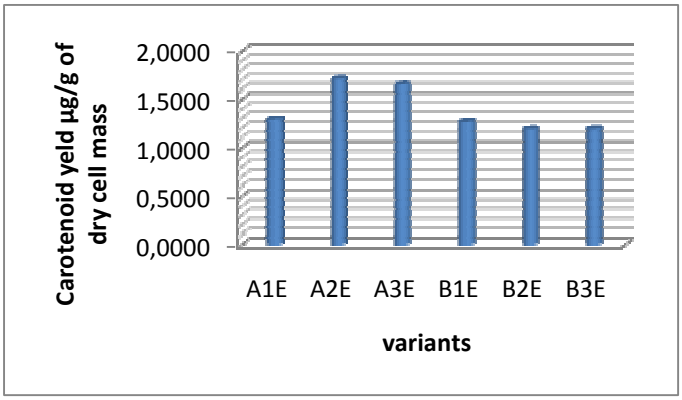


Fig. 4. Intracellular carotenoid pigment accumulation

The MS culturing medium, which is a synthetic medium less rich in nutrients, stimulates the intracellular fixation of pigments, an aspect which was observed in all 3 experimental variants of the medium. It is also noteworthy that both auxine variants favorize the intracellular accumulation of carotenoids to a greater degree than the BA variant.

CONCLUSION

1. The isolated Rhodotorula sp. exhibits the ability to release carotenoid pigments to the extracellular medium. After 96 h, 7,5365 µg/g of

dry mass were determined in B1, compared to only 1,888 μg/g of dry mass in the the B 2E intracellular.

2. Auxines added to the medium have a favourable effect both on stimulating yeast biomass and on the intracellular accumulation of carotenoids. However, this effect is influenced by the medium's nutritional ability.

3. Added benzyl-adenine within a nutrient rich medium favours the release of carotenoid pigments in the extracellular medium.

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