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CAROTENE SYNTHESIS CHANGES OF RHODOTORULA AURANTIACA INDICATOR YEASTS IN THE PRESENCE OF COPPER (II) SALTS

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Abstract. *Rhodotorula aurantiaca* yeasts are ubiquitous microorganisms found in water, soil, plants, etc. Since the main requirement of an indicator organism is the wide occurrence in nature, this species can be an informative bioindicator of the ecological state of the environment. The aim was to study the influence of Cu (II) compounds on the color of carotenoid pigments intensity of two *Rh. aurantiaca* strains (Y-1193 and Y-1195).

Rh. aurantiaca Y-1193 strain was more susceptible to the compounds of Cu (II) than *Rh. aurantiaca* Y-1195. Thus, due to the action of Copper (II) chloride in a concentration of 100 mg/dm³ and Copper (II) sulfate in a concentration of 200 mg/dm³, the synthesis of pigments in *Rh. aurantiaca* Y-1193 was inhibited, which was 2 and 2.5 times, respectively, lower than concentrations at which the *Rh. aurantiaca* Y-1195 strain lost its ability to form carotenoid pigments. The most toxic effect on *Rh. aurantiaca* yeast was caused by the compound Cu(NO₃)₂. There was no cell growth at a concentration of 200 mg/dm³ of Cu²⁺ ions, and the concentration interval between the loss of pigments and growth retardation was also not detected. dE (the difference in the intensity of pigment accumulation of microorganisms) yielded a greater value when the difference between control and experimental yeast samples was bigger. Thus, for intensively pigmented colonies, this index varied from 7.4 to 9.6 c. u., however, for non-pigmented colonies under the influence of Copper (II) compounds, dE was in the range from 19.8 to 21.5 c. u. To confirm the effectiveness of using the scale for visual assessment of pigment accumulation for studied microorganisms, the quantitative content of carotenoids in *Rh. aurantiaca* Y-1193 strain was determined by spectrophotometric methods. When comparing the visual assessment of pigment accumulation in yeast and the concentration of carotenoids in cells, a strong correlation was established (r = 0.9). The dependence is statistically significant (p<0.05). Therefore, the carotenogenic culture of *Rh. aurantiaca* Y-1193 can be recommended for use in bioindication studies of water contamination with Cu (II) compounds. The prospect of further research is to study the influence of other compounds of the I and II hazard classes on the intensity of pigment accumulation of yeasts in the *Rhodotorula* genus.

Keywords: bioindication, aquatic ecosystems, yeasts, pigments, carotenoids, *Rhodotorula aurantiaca*.

1. INTRODUCTION

Copper and its compounds are very widespread pollutants of the II hazard class. The main sources of Copper are non-ferrous metallurgy enterprises, transport, copper-containing fertilizers and pesticides, welding processes, galvanization, and burning of hydrogen fuel in various branches of industry. Wastewaters of metallurgical, machine-building, chemical, and pharmaceutical enterprises may contain up to 400-500 mg/dm³ of Copper ions (Izydorczyk et al., 2021; Mikoda et al., 2018). High concentrations of Copper are also found in some seafood, which can negatively affect the health of people who consume them (Asih, 2020). Based on this information, the search for sensitive contamination bioindicators of water and other biotopes with heavy metal (HM), including Cu (II), is an urgent and important problem today.

Microorganisms are effective bioindicators because they allow us to see the influence of pollutants at the cellular, organismal, and population levels simultaneously. Thus, the *Rhodotorula aurantiaca* yeasts live in different biotopes of the environment and synthesize several types of carotenoids (β - and γ -carotene) (Bhosale, 2004), therefore, due to the rapid accumulation of biomass and intensive carotenogenesis, the genus *Rhodotorula* contains yeast species that are used to produce food additives. Kolesnyk et al. (2022) with co-authors summarized modern aspects of a healthy lifestyle, as it was proven that changing food habits and including antioxidants (carotenoids, lycopene) in the diet plays an important role in reducing the risks of breast cancer, colorectal cancer, prostate cancer, and lung cancer (World Health Organization, 2022). We noticed that visual observation of the brightness changes of pigments under the influence of various concentrations of metal ions and other xenobiotics has a noticeable advantage over monitoring the state of the natural environment using physicochemical methods, given the high cost of the reagents and equipment used. Therefore, the study of the influence of HM and other pollutants on the intensity of pigment accumulation will allow expanding the range of indicative features in microorganisms with the aim of using them to indicate the ecological state of the environment.

2. RESEARCH METHODS

The objects of research were *Rh. aurantiaca* Y-1193 and *Rh. aurantiaca* Y-1195, provided by the D.K. Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine collection of museum cultures. These cultures were chosen because they proved to be sensitive and informative bioindicators in our previous studies (Krupiei, 2016). The following HM salts were used in the research: CuCl₂·2H₂O, CuSO₄·5H₂O, Cu(NO₃)₂, since these compounds are often found in wastewaters from industrial enterprises. According to the degree of purity and purpose of the salt, HM salts were labeled PFA (pure for analysis).

Sabouraud agar was prepared with certain concentrations of HM salts. Sabouraud's medium without the addition of HM served as a medium for control samples. After the solidification of the medium, 18-hour-old cultures (0.2 ml of inoculum per Petri dish) were streaked on the agar to form a lawn. The density of the suspension was 10⁷ cells/cm³. Cultures were incubated in a thermostat at a temperature of 28 °C. The results were calculated on the third day of cultivation.

The intensity of pigment accumulation was determined visually, by comparing test samples with the control sample, according to the 5-point system, where we used such terms as: growth – meaning the quality of culture growth on a medium (++++ - continuous, uniform layer, +++ - good, ++ - moderate, + - weak, - - absent); pigmentation – meaning the presence of colony pigmentation (++++ - intense, +++ - good, ++ - moderate, + - weak, - - absent, ± - presence of pigmented and non-pigmented colonies). Yeast cell morphology was studied by light microscopy and simple staining of smears with Ziel carbol fuchsin (oil immersion objective with ×90 magnification).

After visual assessment of the intensity of pigment accumulation of yeast colonies, Petri dishes with grown cultures were photographed at a distance of 25-30 cm from the camera objective in the "Macrofilming" mode without flash. A Nikon D3100 digital SLR camera was used. After that, the photos were loaded into the Adobe Photoshop CS8 graphic editor. A Gaussian Blur filter with a Radius of 20 pixels was used to remove minor digital noise in the image. After that, the "Lab" mode was set and the parameters of each of the three channels were obtained: L - brightness; a - the value of the red-green component; b - the value of the yellow-blue component (at 10 arbitrary points of the Petri dish; the number of petri dishes in the sample is 5 Petri dishes for each sample of Copper ions concentration) (Fig. 1) (Rylsky et al., 2010). Unlike the classic RGB model, it is the Lab color model that represents colors as they are seen by a person with normal eyesight. After that, the average arithmetic value was calculated for each of the Lab channels, the data was entered into the CIEDE2000 program, and the dE value was obtained (the difference in the intensity of pigment accumulation of the yeast culture between the control and experimental samples).



Fig. 1. Defining Lab color model parameters in Adobe Photoshop CS8

To determine the productivity of carotene-containing yeast, namely the concentration of carotenoids in biomass under the influence of HM, a well-known spectrophotometric method (without preliminary separation) was used (Musienko et al., 2001). Determining the concentration of pigments included the following standard procedures: weighing, grinding the material, extracting the pigments with a solvent (acetone) and measuring the optical density of the extracts using a KFC-2 photoelectrocolorimeter. The concentration of carotenoid pigments was determined at a wavelength of 450, as well as at 509 and 537 nm.

Determination of the concentration of carotenoids, namely β -carotene ($\mu\text{g}/\text{cm}^3$), was calculated according to the Formula 1:

$$C = 3,9 \cdot D_{450} + 1,8 \cdot D_{537} - 3,6 \cdot D_{509}, \quad (1)$$

Where: C – concentration of pigments, $\mu\text{g}/\text{cm}^3$;

D450 – optical density of the solution at 450 nm;

D509 – optical density of the solution at 509 nm;

D537 – optical density of the solution at 537 nm.

Thus, the aim of the experiment was to investigate the influence of Cu (II) salts on the intensity of pigment accumulation in *Rh. aurantiaca* Y-1193 and *Rh. aurantiaca* Y-1195.

3. RESULTS AND DISCUSSION

Rh. aurantiaca Y-1193 was found to be most sensitive to the action of Copper ions (Tab. 1, Fig. 2). This strain began to lose the color intensity of carotenoid pigments at $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ concentrations of 100 and 200 mg/dm^3 , respectively, which are 2 and 2,5 times lower than those concentrations for which *Rh. aurantiaca* Y-1195 lost its ability to accumulate pigments. Concentration interval between pigment loss and growth retardation between *Rh. aurantiaca* Y-1193 and *Rh. aurantiaca* Y-1195 was observed under the influence of the $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ concentration series and was equal to 60 and 40 %, respectively. Under the action of the $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ compound, these intervals were 60 and 9 %, respectively, for each yeast strain. In addition to weak oligodynamic effect, the most toxic effect on the pigment formation of yeast was caused by $\text{Cu}(\text{NO}_3)_2$. Despite the fact, that neither of the strains lost the color intensity of its pigments, at 200 mg/dm^3 of Cu^{2+} they were unable to grow.

Tab. 1

Visual evaluation of the pigment accumulation intensity of *Rh. aurantiaca* strains under the action of Cu (II) compounds

Copper salts (II)	Copper (II) ion concentration, mg/dm^3	Anion concentration, mg/dm^3	<i>Rh. aurantiaca</i> Y-1193		<i>Rh. aurantiaca</i> Y-1195	
			*growth	**pigment	*growth	**pigment
Control			++++	++++	++++	++++
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	25	28,0	+++	++	++++	++++
	50	55,9	++	++	++++	+++
	100	111,8	++	-	+++	+++
	150	167,7	++	-	+++	++
	200	223,6	+	-	+	-
	250	279,5	-	-	+	-
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	25	37,8	++++	++++	++++	++++
	50	75,6	++++	++++	++++	++++
	100	151,3	++++	++++	++++	++++
	150	226,9	+++	+++	++++	++++
	200	302,5	+++	-	+++	++
	250	378,2	++	-	+++	++
	400	605,1	+	-	+	±
500	756,4	-	-	+	-	
$\text{Cu}(\text{NO}_3)_2$	25	48,8	+++	+	+++	++
	50	97,6	++	±	+++	++
	100	195,2	++	±	++	+
	150	292,8	++	±	++	+
	200	390,4	-	-	-	-

Note (here and below):

- *Growth: ++++ – continuous, uniform layer, +++ – good, ++ – moderate, +- weak, -- absent.
- **Pigment accumulation: ++++ – intensive, +++ – good, ++ – moderate, +- weak, -- absent, ± – presence of weakly pigmented and non-pigmented colonies.

The greater was the difference between the color of the pigments (dE) in the control and test sample, the greater was the value of dE (Tab. 2). Thus, dE benchmarks for *Rh. aurantiaca* Y-1193 and *Rh. aurantiaca* Y-1195 were 8.0 ± 0.3 and 7.4 ± 0.01 c. u., respectively. The range of difference in color intensity for pigmented colonies grown on Sabouraud's medium under the influence of

Copper (II) salts varied from 19.8 to 21.5 c. u. Under the action of the $\text{Cu}(\text{NO}_3)_2$ on *Rh. aurantiaca* Y-1193 weakly pigmented colonies grew only at a Cu^{2+} concentration of 25 mg/dm^3 , while at 50-150 mg/dm^3 concentrations of Copper (II) ions, there were also single non-pigmented colonies (dE ranged from 20.8 to 21.3 c. u.) in addition to weakly colored ones.

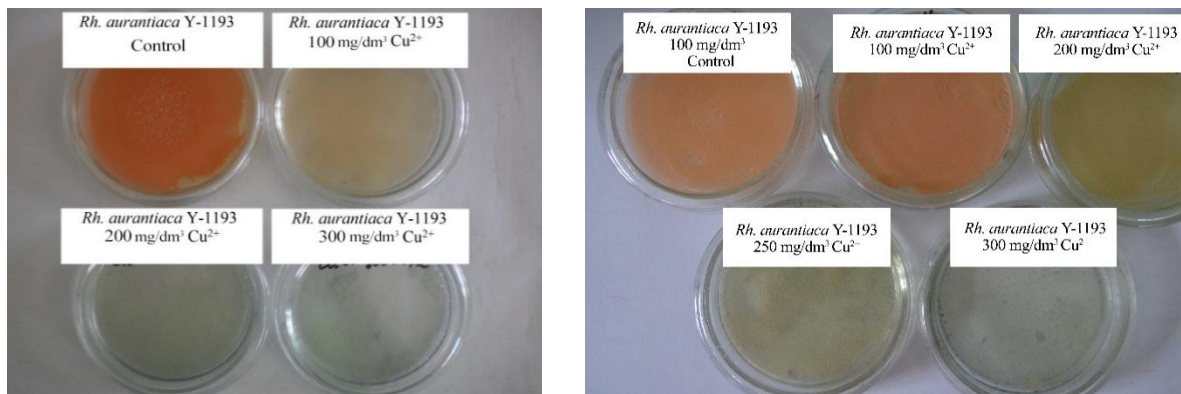


Fig. 2. The influence of Copper salts (II) in terms of cation on the intensity of pigment accumulation in *Rh. aurantiaca* Y-1193 yeast strain (left photo – $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, right photo – $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)

The least toxic compound was found to be $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ salt. *Rh. aurantiaca* Y-1193 and *Rh. aurantiaca* Y-1195 growth was noted for concentrations of Cu (II) ions in the composition of this salt of 400 and 500 mg/dm^3 , respectively.

Tab. 2

The influence of the concentration series of Copper (II) salts on the intensity of pigment accumulation of *Rh. aurantiaca* strains

Salt	Concentration of Cu^{2+} , mg/dm^3	<i>Rh. aurantiaca</i> Y-1193				<i>Rh. aurantiaca</i> Y-1195			
		L	a	b	dE	L	a	b	dE
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	25	44	22	33	$8,0 \pm 0,3$	45	16	26	$7,4 \pm 0,01$
	50	46	15	41	$13,9 \pm 0,7$	50	28	21	$10,7 \pm 0,3$
	100	53	10	31	$19,8 \pm 0,9$	57	28	26	$16,3 \pm 0,04$
	150	56	16	18	$21,0 \pm 1,2$	53	9	25	$16,4 \pm 0,6$
	200	56	18	15	$21,5 \pm 1,1$	56	7	29	$20,3 \pm 1,0$
	250	-	-	-	-	57	8	30	$20,7 \pm 0,5$
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	25	59	24	29	$7,2 \pm 0,02$	56	19	28	$7,9 \pm 0,01$
	50	58	23	29	$7,9 \pm 0,04$	56	20	28	$7,9 \pm 0,02$
	100	58	23	30	$7,8 \pm 0,1$	55	18	27	$8,9 \pm 0,05$
	150	56	20	29	$7,9 \pm 0,04$	55	17	22	$9,6 \pm 0,6$
	200	48	9	24	$19,2 \pm 0,7$	55	7	29	$16,6 \pm 1,1$
	250	47	8	20	$20,5 \pm 1,1$	49	7	29	$17,5 \pm 0,1$
	400	47	3	29	$21,1 \pm 0,2$	48	4	25	$19,5 \pm 0,8$
500	-	-	-	-	47	3	29	$21,1 \pm 0,02$	
$\text{Cu}(\text{NO}_3)_2$	25	53	10	28	$17,0 \pm 0,7$	50	8	25	$15,3 \pm 0,2$
	50	51	3	40	$20,8 \pm 0,5$	52	9	28	$16,6 \pm 0,2$
	100	51	4	39	$20,0 \pm 0,2$	51	5	35	$18,8 \pm 0,6$
	150	49	2	39	$20,1 \pm 0,7$	49	4	44	$19,7 \pm 0,5$
	200	54	4	33	$21,3 \pm 0,9$	56	10	14	$21,1 \pm 0,9$

Note:

1. L, a, b – indicators of CIE Lab color model channels.
2. dE – difference in color intensity between control and test sample calculated using the CIEDE 2000 computer program.

Lozovaya et al. (2004) also noted that *Rh. aurantiaca* Y-1195 can grow at a concentration of Copper (II) ions in the nutrient medium of 500 mg/dm³. The pigment color of the yeast cells had the ability to renew itself on the 6th and 9th day of cultivation, along with an increase in the number of colonies. Microscopy of the colonies with restored carotenoid synthesis (objective with $\times 40$ magnification) revealed a faint pink coloration of their apex and colorless base. The pigmented tip of the colonies can be explained by the secondary growth of yeast cells that were not in contact with the toxic nutrient medium. Oil immersion microscopy of smears stained with carbol fuchsin allowed us to study the differences in the sizes of yeast cells. Non-pigmented cells of both strains of microorganisms were 1.5 to 3 times smaller in size compared to pigmented yeasts.

To confirm the effectiveness of using the scale for visual assessment of pigment accumulation in microorganisms, a study was conducted on the spectrophotometric determination of the quantitative content of carotenoids in *Rh. aurantiaca* Y-1193, which turned out to be a more sensitive bioindicator than *Rh. aurantiaca* Y-1195 (Tab. 3).

Tab. 3

Productivity of carotene-containing yeast Rh. aurantiaca Y-1193 under the influence of the concentration series of Copper (II) salts

Salt	Concentration of Copper (II) ions	Visual assessment of pigment accumulation in yeasts, %	β - carotene, $\mu\text{g}/\text{cm}^3$
Control (no Copper salts)		++++ (100 %)	0,182 \pm 0,001
CuCl ₂ ·2H ₂ O	25	++ (50 %)	0,096 \pm 0,0012
	50	++ (50 %)	0,074 \pm 0,0009
	100	- (0 %)	0,0097 \pm 0,0002
	150	- (0 %)	0,0017 \pm 0,0005
	200	- (0 %)	0,0011 \pm 0,000
CuSO ₄ ·5H ₂ O	25	++++ (100 %)	0,173 \pm 0,0044
	50	++++ (100 %)	0,152 \pm 0,0086
	100	++++ (100 %)	0,139 \pm 0,0032
	150	+++ (75 %)	0,101 \pm 0,0018
	200	- (0 %)	0,0032 \pm 0,0003
	250	- (0 %)	0,0026 \pm 0,0004
Cu(NO ₃) ₂	400	- (0 %)	0,0015 \pm 0,0005
	25	+ (25 %)	0,043 \pm 0,003
	50	± (20 %)	0,032 \pm 0,0044
	100	± (20 %)	0,030 \pm 0,0047
	150	± (20 %)	0,022 \pm 0,0051

When comparing the visual assessment of pigment accumulation in yeast and the concentration of carotenoids in cells, a strong correlation ($r = 0.9$) was established. The dependence of the signs is statistically significant ($p < 0.05$). Thus, the minimum concentrations of Copper (II) ions in the composition of CuCl₂·2H₂O and CuSO₄·5H₂O salts, of which *Rh. aurantiaca* Y-1193 and *Rh. aurantiaca* Y-1195 began to completely lose pigment, were 100 and 200 mg/dm³, respectively. The concentration of β -carotene in these non-pigmented colonies was, respectively, 18.7 and 56.9 times lower compared to the control indicators. The moderate intensity of pigment formation on a visual scale was at Cu²⁺ concentrations of 25 and 50 mg/dm³ (CuCl₂·2H₂O salt), and the concentration of carotenoids at these values were 1.9 and 2.5 times lower than the control sample, respectively.

So, the *Rh. aurantiaca* Y-1193 strain was more sensitive to the effects of the concentration series of Copper (II) ions than *Rh. aurantiaca* Y-1195 strain. With a concentration of Cu²⁺ of 25 mg/dm³,

the culture began to lose its ability to accumulate pigments, so it can be recommended as an informative bioindicator of water contamination with Copper (II) salts.

4. CONCLUSIONS

It was established that the *Rh. aurantiaca* Y-1193 and *Rh. aurantiaca* Y-1195 yeasts lose the ability to form pigments at certain concentration levels of heavy metal ions (Cu²⁺) in the composition of Copper chloride, Copper sulfate and Copper nitrate; there is a certain concentration interval between the loss of pigments and the blocking of growth, which varies significantly for each of the yeast strains and for the actions of certain compounds of Cu (II).

Based on the findings of the research, we recommend using the *Rh. aurantiaca* Y-1193 yeast strain in bioindication studies of water contamination with Copper (II) salts. The prospect of further research is to study the influence of other heavy metal compounds on the intensity of *Rh. aurantiaca* Y-1193 pigment accumulation, as well as researching other possible species of carotenogenic yeast as bioindicators of water contamination.

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Крупей Христина, Кравченко Гліб. Зміни каротиногенезу індикаторних дріжджів *Rhodotorula aurantiaca* в присутності солей Купрум (II). Журнал Прикарпатського університету імені Василя Стефаника, 9 (4) (2022), 25–32.

Дріжджі *Rh. aurantiaca* є убіквітарними мікроорганізмами та зустрічаються у воді, ґрунті, наземних частинах рослин тощо. Оскільки головною вимогою індикаторних організмів є їх широка зустрічальність в природі, цей вид може бути інформативним біоіндикатором екологічного стану навколишнього середовища. Метою роботи було вивчення впливу сполук Купруму (II) на інтенсивність накопичення каротиноїдних пігментів двома штамами дріжджів *Rh. aurantiaca* (Y-1193 та Y-1195).

Штам *Rh. aurantiaca* Y-1193 був уразливіше до дії сполук Купруму (II) ніж дріжджі штаму *Rh. aurantiaca* Y-1195. Так, за дії Купрум (II) хлориду та Купрум (II) сульфату синтез пігментів у *Rh. aurantiaca* Y-1193 пригнічувався з концентрації 100 та 200 мг/дм³, відповідно, що у 2 та 2,5 рази нижче тих концентрацій, за яких штам *Rh. aurantiaca* Y-1195 втрачав здатність до каротиноутворення. Найбільш токсичну дію на дріжджі *Rh. aurantiaca* спричинила сполука $\text{Cu}(\text{NO}_3)_2$, росту клітин не було за концентрації 200 мг/дм³ йонів Cu^{2+} , концентраційного інтервалу між втратою пігментів та затримкою росту також не виявлено. Помічено, чим більше була різниця в кольорі пігментів між контролем та дослідними зразками дріжджів, тим більшим було значення dE – різниці в інтенсивності пігментонакопичення мікроорганізмів. Так, для інтенсивно пігментованих колоній цей показник варіював від 7,4 до 9,6 ум. од., проте для апігментних колоній за дії сполук Купруму (II) dE був у межах від 19,8 до 21,5 ум. од. Для підтвердження ефективності використання шкали для візуальної оцінки пігментонакопичення мікроорганізмів визначали кількісний вміст каротиноїдів у дріжджах *Rh. aurantiaca* Y-1193 спектрофотометричними методами. При порівнянні візуальної оцінки пігментонакопичення дріжджів та концентрації каротиноїдів в клітинах було встановлено наявність сильного кореляційного зв'язку ($r = 0,9$). Залежність ознак статистично значуща ($p < 0,05$). Отже, каротиносинтезувальна культура *Rh. aurantiaca* Y-1193 може бути рекомендована для використання в біоіндикаційних дослідженнях при забрудненні води сполуками Купруму (II). Перспективою подальших досліджень є вивчення впливу інших сполук I та II класу небезпеки та інтенсивність пігментонакопичення дріжджів роду *Rhodotorula*.

Ключові слова: біоіндикація, водні екосистеми, дріжджі, пігменти, каротиноїди, *Rhodotorula aurantiaca*.