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BIOSYNTHETIC ACTIVITY OF *Rhodotorula gracilis* CNMN-YS-03 AND *Rhodotorula glutinis* CNMN-YS-08 AFTER PRESERVATION AND LONG-TERM STORAGE

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Abstract

The conservation of microorganisms of biotechnological interest is a fundamental component of scientific progress and sustainable economic development. Pigmented yeasts belonging to the genus Rhodotorula represent a source of valuable carotenoid pigments. Strain stability and the maintenance of biosynthetic capacity during preservation are critical aspects for the development of reproducible industrial processes. To ensure a higher viability of Rhodotorula strains after long-term storage, it is necessary to identify the most effective biopreparations for use in protective and/or rehydration media. To optimize the biotechnological production of carotenoids, strains R. gracilis CNMN-YS-03 and R. glutinis CNMN-YS-08 were reactivated using different mannoprotein extracts from beer and wine production processes. The reactivation of Rhodotorula using mannoproteic extracts in the rehydration medium results in a significant increase in biomass productivity (by 10–40%) and in biosynthetic activity. These effects depend on the type and concentration of the extract used, as well as the characteristics of the analyzed strain. Under these conditions, carotenoid biosynthesis can increase by up to 45%. Furthermore, the carbohydrate content, particularly in biomass of the Rhodotorula gracilis CNMN-YS-03 strain, increased by 37.5%, and the protein content in biomass of the Rhodotorula glutinis CNMN-YS-08 strain increased by 29.4% compared to the control sample.

Keywords*: Conservation; Rhodotorula; Biosynthetic activity; Reactivation after lyophilization*

Introduction

The conservation of microorganisms of biotechnological interest is a fundamental component of scientific progress and sustainable economic development. These microorganisms, including bacteria, fungi, yeasts, and microalgae—play a crucial role in the food industry, pharmaceuticals, agriculture, and environmental protection. Their preservation in national and international culture collections ensures the long-term storage of microbial biodiversity and provides controlled access to valuable genetic resources [1].

First, the preservation of these microorganisms guarantees the reproducibility of scientific research. In the absence of well-characterized strains maintained under optimal conditions, experimental results could neither be verified nor reliably replicated. National culture collections function as true "living libraries," where each microorganism is systematically documented, accurately classified, and preserved in a stable state over the long term [2].

Secondly, these biological resources are of major economic importance. Microorganisms are widely used in the production of antibiotics, enzymes, vitamins, biofuels, and other compounds of industrial interest. By conserving, states protect their genetic heritage and ensure the sustainable

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exploitation of these resources, thereby contributing to innovation and economic competitiveness [1].

Another important aspect is the role of these collections in protecting biodiversity. In the context of climate change and ecosystem degradation, some microbial species may disappear from the natural environment. By conserving them *ex-situ* in specialized collections, a valuable biological reserve is established, which can be used for ecosystem restoration as well as for future scientific research.

National collections are also considered essential centers for the development of modern biotechnology, as they provide microorganisms used in agriculture, medicine, the food industry, and environmental protection, while also facilitating international collaboration. The exchange of microbial strains between research institutions across different countries contributes to scientific progress and supports the implementation of joint research projects. At the same time, these exchanges are regulated to ensure compliance with biosafety standards and international access to genetic resources [3], [4].

In conclusion, the conservation of microorganisms of biotechnological interest in national collections is fundamental for science, economic development, and environmental protection. It ensures the continuity and reproducibility of research, supports innovation, and contributes to the responsible management of global biodiversity. Within the National Collection of Non-Pathogenic Microorganisms of the Institute of Microbiology and Biotechnology, Technical University of Moldova, a wide range of microorganisms of biotechnological interest, such as bacteria, yeasts, fungi, cyanobacteria, and microalgae, are preserved using various conservation methods. Their long-term viability depends on the preservation techniques applied, as well as on the physical conditions and the composition of protection and reactivation media. To ensure a higher viability of yeast strains, especially those of the genus *Rhodotorula* - recognized as promising bioresources of carotenoid pigments and other bioactive compounds—it is necessary to identify the most effective biopreparations for use in protective and/or rehydration media.

Carotenoids represent an important class of natural compounds that are widely used in the chemical, pharmaceutical, and food industries due to their significant biological and functional properties [5], [6], [7]. These pigments are particularly known for their role as precursors of vitamin A, thereby contributing to the maintenance of essential physiological functions of the organism. In addition, carotenoids can enhance the immune response and exhibit pronounced antioxidant activity, being able to neutralize reactive oxygen species and reduce cellular oxidative stress [5], [8].

In the context of the increasing demand for natural and sustainable compounds, the biotechnological production of carotenoids has emerged as an attractive alternative to traditional chemical synthesis. In this regard, pigmented microorganisms, particularly yeasts, have received significant interest due to their ability to synthesize and accumulate carotenoids intracellularly. Among them, yeasts belonging to the genus *Rhodotorula* present promising microbial platforms for carotenoid biosynthesis and have been extensively studied in recent years [5], [9], [10]. These microorganisms are recognized as valuable sources of carotenoid pigments, such as β-carotene, γ-carotene, lycopene, torulene, and torularhodin, compounds with potential applications in various industries [5], [11], [12], [13].

The pigmentation of *Rhodotorula* colonies ranges from orange and salmon to pink and deep red, depending on the type and concentration of carotenoids produced. Among these, β-carotene is the predominant carotenoid, potentially accounting for up to 70% of the total cellular pigment content [14]. This characteristic makes yeasts of the genus *Rhodotorula* particularly attractive for the industrial exploitation of β-carotene.

The growing interest in the biotechnological use of these microorganisms is also supported by their metabolic robustness, high tolerance to various stress conditions (oxidative, osmotic, or thermal), and their ability to utilize low-cost substrates, including agro-industrial wastes [5], [9],

[15]-[19]. These advantages contribute to reducing production costs and support the development of sustainable and environmentally friendly processes.

However, despite the high potential of *Rhodotorula* yeasts, the mechanisms underlying carotenoid biosynthesis and the optimal fermentation conditions influencing their production remain insufficiently elucidated. For the implementation of stable and reproducible industrial processes, it is essential that the strains used not only maintain their viability during storage but also preserve their carotenoid biosynthetic capacity at levels comparable to the initial state, thus ensuring consistency and production efficiency.

The biochemical content of the biomass of pigmented yeasts of the genus *Rhodotorula*—especially proteins and carbohydrates—is strongly influenced by cultivation conditions and the composition of the fermentation medium. Several recent studies have demonstrated that variables such as carbon source, carbon-to-nitrogen ratio, nutrient type and concentration, and physicochemical environmental conditions regulate cellular metabolic responses, leading to significant changes in biomass composition. Thus, increasing carbon availability tends to stimulate the accumulation of carbohydrates and lipids at the expense of proteins, as excess carbon is redirected toward energy storage in the form of carbohydrates and triacylglycerols—a typical strategy of oleaginous yeasts under conditions of carbon surplus and nitrogen limitation [20].

Therefore, to evaluate the productive potential of the pigmented yeast strains *Rhodotorula gracilis* CNMN-YS-03 and *Rhodotorula glutinis* CNMN-YS-08 after preservation by lyophilization and long-term storage, this study aimed to determine its biosynthetic capacity following standard reactivation and reactivation using mannoprotein extracts obtained from residual yeast biomass derived from beer and wine production.

Experimental part

The main objective of this research is to evaluate the influence of mannan protein extracts obtained from residual yeast biomass remaining after the production of *Lager* beer and *Merlot* red wine on the survival capacity of *Rhodotorula* strains after long-term storage and their ability to regulate biosynthetic processes, with a particular focus on enhancing carotenoid accumulation in the biomass, given their importance.

Materials

Yeast Strains, Culture Media, and Experimental Conditions: The study was conducted using *Rhodotorula gracilis* CNMN-YS-03 and *Rhodotorula glutinis* CNMN-YS-08 strains, producers of carotenoids preserved in the National Collection of Non-Pathogenic Microorganisms at the Institute of Microbiology and Biotechnology, Technical University of Moldova.

The reactivation of these microorganisms, which were stored for 12 years in a lyophilized state, was carried out standardly by using 1 mL of distilled water or with the addition of 1, 5 and 10% mannoprotein extracts obtained from yeast biomass remaining after the production of *Lager* beer (MPB) and *Merlot* red wine (MPV). To obtain an active microbial culture, after reactivation, strains were subcultured in four consecutive passages for regeneration on YPD agar media. After IV passages, to prepare the suspension, the yeast strains were cultured on a beer wort medium with a sugar content of 6.0°Blg for 48 hours; the suspension was then inoculated at a concentration of 5% vol. onto the beer wort medium with a sugar content of 6.0°Blg and cultured at a temperature of 27...28°C, on a shaker at 200 rpm, and under continuous illumination for 120 hours.

Productivity and biosynthetic activity: the biomass productivity was determined gravimetrically according to the usual method by drying biomass in an oven at a temperature of 105ºC to constant mass and recalculating the dry weight (d.w.). Total protein concentration was

determined using the Lowry method, with bovine serum albumin (BSA) as the standard. An aliquot of the sample was mixed with Folin–Ciocalteu phenol reagent (Sigma-Aldrich, USA), and absorbance was measured at 750 nm after incubation at room temperature for 30 min. Protein concentration was calculated from the standard calibration curve and expressed as % of d.w. [21]. The total carbohydrate content was determined spectrophotometrically using the Anthrone reagent, standard-D-glucose [22], and the carotenoid content was extracted from biomass using the method described by Sharma [23]. Antioxidant activity was assessed using the ABTS and DPPH methods [24], [25]. Antioxidant activity was expressed as the percentage of radical inhibition.

All experiments were conducted in triplicate using three biologically independent replicates. Results are presented as mean ± standard deviation (SD). Statistical significance between control and treated groups was assessed using Student's t-test, with significance set at $p < 0.05$.

Results and discussion

The results obtained regarding the influence of mannoprotein extracts on the regeneration of the pigmented yeast cultures *Rhodotorula gracilis* CNMN-YS-03 and *Rhodotorula glutinis* CNMN-YS-08, after reactivation from a lyophilized form and long-term storage, revealed significant changes in biomass productivity and carotenoid biosynthesis, as well as in biochemical composition and antioxidant activity, depending on the type and concentration of used extract and the physiological characteristics of the strains.

Under these conditions, the *Rhodotorula glutinis* strain CNMN-YS-08, reactivated in the presence of the MPV mannoprotein extract, exhibited a 31–40% increase in productivity compared to the control across all tested concentrations. In contrast, the *Rhodotorula gracilis* strain CNMN-YS-03 showed a weaker response, with an approximate 11% increase in productivity observed only at the intermediate extract concentration (Table 1).

Table 1. The values of productivity, carotenoid content, and antioxidant activity in the biomass of *Rhodotorula gracilis* CNMN-YS-03 and *Rhodotorula glutinis* CNMN-YS-08 strains after reactivation from the lyophilized state using mannoprotein extracts were $p < 0.05$

Strains	Regenerated sample		Productivity [g/L]	Carotenoids [µg/g]	Carotenoid productivity [mg/L]	DPPH AA of carotenoids [% inhibition]
R. gracilis CNMN-YS-03	Control	H_2O	9,53±0,1	285,30±1,3	2.72	31.02±0.2
	MPB	1%	10,03±0,2	308,45±0,6	3.09	34.57±1.0
		5%	9,28±0,2	285,95±1,3	2.65	29.63±2.3
		10%	8,67±0,1	372,39±1,9	3.23	31.94±0.7
	MPV	1%	9,35±0,1	254,46±2,2	2.38	30.02±0.1
		5%	10,61±0,2	338,39±2,8	3.59	35.34±1.7
		10%	9,29±0,2	340,06±2,1	3.16	33.33±0.3
R. glutinis CNMN-YS-08	Control	H_2O	10,36±1,6	77,89±2,0	0.81	30.63±1.0
	MPB	1%	10,25±0,1	112,93±4,0	1.16	30.71±1.0
		5%	9,44±0,1	80,95±3,4	0.76	27.78±0.9
		10%	8,52±0,4	91,50±0,3	0.78	29.78±2.1
	MPV	1%	14,50±0,1	83,72±4,0	1.21	24.46±1.0
		5%	14,31±0,3	86,32±1,3	1.24	25.31±0.2
		10%	13,60±0,2	60,67±2,5	0.83	23.92±0.7

Although the productivity of the analyzed cultures varies within similar ranges, the carotenoid content in the biomass of the *Rhodotorula gracilis* strain CNMN-YS-03 is significantly higher than that of *Rhodotorula glutinis* CNMN-YS-08, being 3.66 times greater (285.30 µg/g compared to 77.89 µg/g).

Reactivation of the strains in the presence of mannoprotein extracts also stimulates carotenoid biosynthesis. Thus, in the presence of the MPB extract, carotenoid content exceeds control values by 1.30–1.45 times, depending on the strain. The optimal extract concentration

was 10% for *Rhodotorula gracilis* CNMN-YS-03 and 1% for *Rhodotorula glutinis* CNMN-YS-08, resulting in a 20–40% increase in carotenoid productivity.

In the case of the MPV extract, carotenoid content in the biomass was 1.10–1.20 times higher, with an optimal concentration of 5% for both analyzed strains. However, due to the increased biomass yield per liter of medium, a more pronounced increase in carotenoid productivity was observed, reaching values 40–53% higher than the control. Furthermore, higher carotenoid accumulation in the biomass is associated with increased antioxidant activity, as evidenced by a greater capacity to scavenge the DPPH radical.

Therefore, the use of MPB-type manoprotein extract has shown that as its concentration increases, it leads to a gradual decrease in biomass productivity compared to the control, with a maximum reduction up to 18%. Along with this, a significant enhancement of carotenoid biosynthesis was observed, carotenoid content in the biomass increasing up to 31–45%, suggesting a redirection of cellular metabolism toward the production of secondary metabolites with protective roles, which is consistent with recent data showing that this species tends to prioritize carotenoids under stress or in modified media [26], [27], [28].

Obtained results indicate that mannoprotein extracts differentially influence both the regeneration and growth of biomass and carotenoid biosynthesis in the *Rhodotorula* strains, with the effects being dependent on both the type and concentration of the used extract and physiological potential of the strain.

The analysis of the biochemical composition of the biomass highlighted differences between two types of mannoprotein extracts.

Reactivation and regeneration of the *Rhodotorula gracilis* CNMN-YS-03 culture in the presence of higher concentrations of MPB extract (5 and 10%) resulted in a significant increase in carbohydrate synthesis in the biomass, with values exceeding the control by 18.1–37.55%. In contrast, protein content remained approximately similar to that of the control. In cultures reactivated in the presence of MPV extract, biomass protein content decreased by 9.5–19.0%, whereas carbohydrate levels increased by up to 29.50% compared to the control across all tested concentrations (Fig. 1).

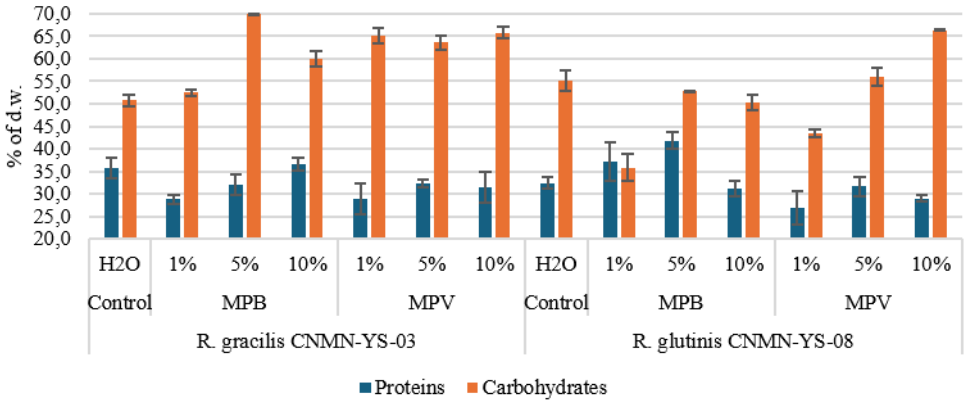

Fig. 1. Protein and carbohydrate content in the biomass of *Rhodotorula gracilis* CNMN-YS-03 and *Rhodotorula glutinis* CNMN-YS-08 after reactivation with mannoprotein extracts

If we compare the data obtained with those from the *Rhodotorula glutinis* CNMN-YS-08, we can see that during culture reactivation with MPB, the use of low concentrations resulted in a significant increase of protein content by 29.4% compared to the control, concomitantly with a decrease in carbohydrate content by 35.3%. Whereas reactivation with MPV produced the opposite effect, characterized by a reduction in protein content by 17.1% and an increase in carbohydrate content up to 20.2% as the extract concentration was increased.

Thus, the effects of the extracts are strain-specific and concentration-dependent. MPB stimulates protein synthesis in *Rhodotorula glutinis* and carbohydrate accumulation in *Rhodotorula gracilis*, whereas MPV promotes carbohydrate accumulation in both strains while reducing protein content. This suggests a redistribution of metabolic resources between storage compounds (carbohydrates) and structural components (proteins) [29].

The antioxidant activity of the biomass evaluated by ABTS and DPPH methods was more pronounced in the strain *Rhodotorula glutinis* CNMN-YS-08 reactivated with MPB compared to the one reactivated with MPV. The ABTS radical scavenging capacity ranged between 39.1% and 50.0%, while the DPPH radical scavenging capacity ranged between 9.1% and 14.3%, correlating with the higher carotenoid content and the enhancement of cellular antioxidant mechanisms induced by the MPB extract (Fig. 2).

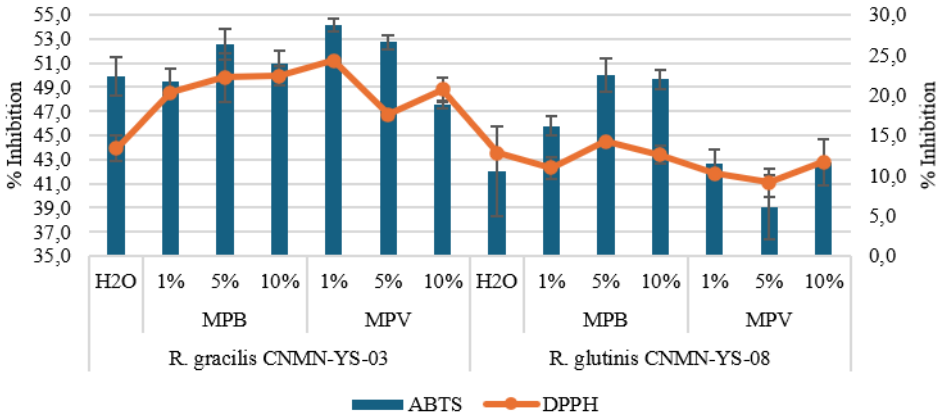

Fig. 2. Antioxidant activity of the biomass of *Rhodotorula gracilis* CNMN-YS-03 and *Rhodotorula glutinis* CNMN-YS-08 after reactivation with mannoprotein extracts

In *Rhodotorula gracilis* CNMN-YS-03 strains, the antioxidant activity of the biomass determined by the ABTS method remained largely constant, varying within the range of the control sample, while the DPPH radical scavenging capacity increased at reactivation with mannoprotein extracts, reaching 20.3–24.2% inhibition compared to 13.4% in the control.

The observed differences in biomass productivity, carotenoid biosynthesis, biochemical composition, and antioxidant activity of the *Rhodotorula glutinis* CNMN-YS-08 strain can be explained by the influence of MPV and MPB mannoprotein extracts on the balance between primary and secondary metabolism. The structural and bioactive components of mannoproteins may act as nutrient sources and as metabolic sensors for physiological adaptations.

Enhancing biomass productivity and increasing carbohydrate content in the presence of the MPV extract suggest an activation of primary metabolism oriented toward the synthesis of structural polysaccharides and accumulation of cellular reserves. Under these favorable growth conditions, cells do not experience significant metabolic stress, leading to a reduction in the synthesis of secondary metabolites, including carotenoids, as well as a relative decrease in protein content [30], [31], [32].

However, the MPB extract can potentially contribute to a different adaptive response, characterized by partial limitation of cell growth, increased protein content, and enhanced carotenoid biosynthesis. This redirection of metabolic flux may be associated with the activation of cellular protective mechanisms and synthesis of enzymes involved in stress response. Increased antioxidant activity assessed by the ABTS and DPPH methods supports the hypothesis that MPB induces moderate stress, which stimulates endogenous antioxidant systems, with carotenoids playing a vital role in neutralizing reactive oxygen species.

The correlation between the increased carotenoid content, higher protein content, and enhanced antioxidant activity in the biomass reactivated with MPB suggests the presence of a compensatory mechanism, whereby *Rhodotorula glutinis* cells redirect metabolic resources from biomass growth toward the synthesis of protective compounds. Thus, both types and concentrations of mannan protein extract influence the metabolic strategy of the strain, resulting in a trade-off between cellular growth, biochemical composition, and antioxidant capacity of biomass.

Conclusions

Mannoprotein extracts (MPV and MPB) significantly influence strain regeneration and metabolic activity of *Rhodotorula gracilis* CNMN-YS-03 and *Rhodotorula glutinis* CNMN-YS-08 strains, with effects depending on the extract type, its concentration, and the physiological characteristics of each strain.

MPV extract predominantly stimulates primary metabolism, resulting in increased biomass productivity (up to 40% for *Rhodotorula glutinis* CNMN-YS-08) and carbohydrate accumulation, while concurrently reducing protein content and moderately stimulating carotenoid biosynthesis.

MPB extract induces an adaptive metabolic response associated with moderate stress, characterized by a decrease in biomass productivity (up to 18%), alongside a significant increase in carotenoid biosynthesis (up to 45%) and, in certain strains, protein content.

Species-specific responses to mannoprotein extracts were observed: *Rhodotorula gracilis* CNMN-YS-03 demonstrated higher carotenoid content (up to 3.66 times), whereas *Rhodotorula glutinis* CNMN-YS-08 showed a more pronounced response in terms of biomass production and antioxidant activity.

Changes in carotenoid biosynthesis are directly correlated with enhanced antioxidant activity (ABTS and DPPH), indicating the presence of a compensatory metabolic mechanism in which cellular resources are reallocated toward the synthesis of protective compounds rather than biomass accumulation.

Overall, the results indicate the presence of a metabolic trade-off between biomass growth and carotenoid biosynthesis, regulated by the type and concentration of mannoprotein extract, as well as the cell's adaptation to stress conditions. These observations provide valuable insights for optimizing cultivation conditions of *Rhodotorula* strains to obtain biomass with specialized characteristics, including productivity, biochemical composition, and antioxidant activity.

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