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BEȘLIU ALINA

THE EFFECTS OF METAL OXIDE NANOPARTICLES ON YEASTS OF THE GENUS *RHODOTORULA*

167.01 BIOTECHNOLOGY, BIONANOTECHNOLOGY

Summary of the doctoral thesis in biological sciences

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Thesis tutor:

USATII Agafia, Doctor habilitatus of Biological Sciences, research professor

Members of the Doctoral Committee:

Duca Maria, doctor habilitat, professor, academician - president (USDC)

Cepoi Liliana, doctor, associate professor - referent (ȘDȘB)

Sturza Rodica, doctor habilitat in technique, university professor, UTM – referent

Chirsanova Aurica, PhD in biology, associate professor, UTM – referent

Rudic Valeriu, doctor habilitat, university professor, academician - member

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The summary was sent _____.

Thesis tutor, Usatîi Agafia,

Doctor habilitatus in Biological Sciences, research professor

Author Beșliu Alina

CONTENTS

PURPOSE AND OBJECTIVES OF RESEARCH

The development of science and technology in the last decade is characterized by intensive studies on the properties of nanoparticles and elaboration of different ways of their practical application. Metal nanoparticles are among the most widely used in various fields [21, 25, 35]. An important category of nanoparticles are nanooxides, including ZnO and Fe_3O_4, which offer attractive possibilities for implementation in biomedicine, food, cosmetology, pharmaceuticals, environmental protection and biotechnology [1, 9]. An important solution for broadening the spectrum of use of ZnO and Fe_3O_4 nanoparticles is their coating with various polymers, including chitosan. They contribute to the efficient stabilization of metal oxide nanoparticles, giving them increased biocompatibility, chemical functionality, reducing their toxicity and oxidative capacity [11, 19].

The cytotoxicity of nanoparticles, including metal oxides and their interaction with biological systems is still unclear [20], so there is an urgent need to understand the influence and assess possible risks, so both the beneficial and adverse effects of these nanomaterials need to be evaluated using different organisms [18]. Pigmented yeasts of the genus *Rhodotorula* can serve as representative biotechnological objects and as suitable models that offer enormous possibilities in modeling the effects and establishing the mechanisms of action of nanoparticles on vital processes in the eukaryotic cell [11, 14]. Different microbiological and biochemical indicators are proposed as benchmarks. The research problem resulting from the analysis of the situation in the field, consists in the need to elucidate the degree of influence of metal oxide nanoparticles on yeast strains of the genus *Rhodotorula* in order to efficiently exploit the biotechnological potential of these nanomaterials.

The purpose of the work is to determine the degree of influence of nanoparticles of metal oxides ZnO and Fe_3O_4 on microbiological and biochemical indices in the yeasts of biotechnological interest in the genus *Rhodotorula* and to evaluate the prospects for use in bionanotechnologies.

Objectives of the work:

- Validation of microbiological and biochemical tests in the context of ensuring the qualification and quality of the methods used to evaluate the effects of nanoparticles of metal oxides;
- Establishing the particularities of the action of ZnO and Fe_3O_4 nanoparticles with different dimensions on yeasts of the genus *Rhodotorula*;
- Development of procedures for using ZnO and Fe_3O_4 nanoparticles in yeast cultivation biotechnology;

- Obtaining the chitosan-zinc and chitosan-iron nanocomposites and appreciating the perspectives of their use in the biotechnology of pigmented yeasts.

Research hypotheses:

1. ZnO nanoparticles can be used as stimulators of the biosynthesis of bioactive components of pigmented yeasts of the genus *Rhodotorula*.

2. Pigmented yeasts of the genus *Rhodotorula* can serve as model organisms for testing the harmfulness of metal oxide nonoparticles.

The main scientific results. For the first time, microbiological and biochemical tests have been validated and the cellular functional groups of pigmented yeasts of the genus *Rhodotorula* have been identified, involved in the response to the action of the nanoparticles. For the first time, the action character of ZnO and Fe_3O_4 nanoparticles on viability, biomass production, cellular and colonial morphological characteristics, protein, carbohydrate, carotenoid content, antioxidant enzymes in yeasts of the *Rhodotorula* genus depending on size and concentration is elucidated. It has been shown that ZnO nanoparticles, used in concentrations 1-20 mg/L in the cultivation of *Rhodotorula gracilis*, positively influence the processes of multiplication and biosynthesis of proteins, carbohydrates and carotenoid pigments. It has been shown that Fe_3O_4 nanoparticles induce *Rh. gracilis* significant changes in biosynthetic processes and antioxidant defense system, which is manifested by decreased carotenoid content and catalase enzyme. For the first time, the character of action of chitosan-zinc and chitosan-iron nanocomposites on yeasts of the genus *Rhodotorula* is elucidated. Two new procedures have been developed to increase the quantity and quality of proteins with the use of ZnO nanoparticles and to evaluate the toxicity of Fe_3O_4 nanoparticles with the use as a model of yeast *Rhodotorula gracilis*. The processes are confirmed by a patent and patent application.

The important scientific problem solved in the paper. The effects of nanoparticles of ZnO and Fe_3O_4 metal oxides on yeasts of biotechnological interest in the genus *Rhodotorula* have been established, which has contributed to the elucidation of some mechanisms of action, which has allowed to appreciate the prospects for use in bionanotechnologies.

Theoretical significance. The particularities of action of the ZnO and Fe_3O_4 nanoparticles, based on their size and concentration, and of the chitosan-zinc and chitosan-iron nanocomposites on the microbiological and biochemical indices in yeasts of the genus *Rhodotorula*, have been argued, which served as a basis for the elucidation of some of action and elaboration of the procedures for use in the biotechnology of yeast cultivation.

Applicative value. Proposed for recovery: a process for increasing the protein content of strains *Rh. gracilis* CNMN-Y-03 and *Rh. gracilis* CNMN-Y-30 using ZnO nanoparticles; a new

process for testing the harmfulness of Fe_3O_4 nanoparticles with the use as a model of pigmented yeasts of the genus *Rhodotorula*; a new process of making chitosan–zinc and chitosan-iron nanocomposites with biotechnological perspectives.

Implementation of scientific results. The results of the research "Establishing the effect of nanomaterials on the development and productivity of yeasts" are applied within the Institute of Electronic Engineering and Nanotechnologies "D. Ghițu "Act no. 121 of 29.11.2018. "The validated spectrophotometric method for determining the activity of the catalase enzyme" is used in the practical work in the soil Microbiology laboratory, the Institute of Microbiology and Biotechnology Act no. 96 of 07.11.2019.

Thesis publications. The results of the research on the doctoral thesis have been published in 46 scientific papers: 15 articles in reviewed journals (7 in international journals, 3 listed ISI and SCOPUS; 8 in national journals category B, 3 in monoauthor), 7 articles in collections (3 in mono-authoritative), 1 scientific-methodological work, 14 theses at international and national conferences (3 in mono-authoritative), 2 patents and 1 patent application, 7 materials at invention salons (5 gold medals, 1 bronze medal), 2 implementing acts.

KEYWORDS

ZnO, Fe_3O_4 nanoparticles, *Rhodotorula gracilis*, viability, morphological characters, carbohydrates, proteins, carotenoid pigments, catalase, superoxide dismutase.

RESEARCH METODOLOGY

According to the purpose and objectives presented, to demonstrate the research hypothesis, classical and modern methods were used to determine the influence of nanoparticles on yeast, including:

- methods of validation of microbiological and biochemical parameters for nanotoxicology studies;
- classical microbiological methods for yeast cultivation, assessment of viability and cellular and colonial morphological characteristics;
- biochemical methods for determining the content of proteins, carotenoid pigments, carbohydrates, antioxidant enzymes catalase and superoxide dismutase.
- sonochemical method of preparation of chitosan Zn and chitosan Fe_3O_4 nanocomposites;
- mathematical methods of research planning;
- methods of statistical processing of results.

SUMMARY OF CHAPTERS

1. NANOPARTICLES OF METAL OXIZES - CHARACTERIZATION AND APPLICATIONS (LITERATURE REVIEW)

The chapter includes a comprehensive analysis of scientific achievements on the topic of research. The bibliographic study contains 361 relevant works published in research in the field of biotechnology, bionanotechnology, nanotechnology, microbiology, biomedicine and nutrition worldwide. Chapter 1 is dedicated to the classification, characterization and establishment of the physico-chemical properties of nanoparticles of metal oxides. The elucidation of the cellular mechanism of nanoparticle toxicity and highlighting the main responsible causes. Methods of preparation and stabilization of metallic nanoparticles such as co-precipitation, thermal decomposition of precursors, hydrothermal, micro emulsion, sonochemical, gel solution, bioabsorption, bioreduction and stabilization with natural polymers are analyzed. Particular attention is focused on the high potential for the implementation of metal nanoparticles in biomedicine, pharmaceuticals, food, industry, environmental protection and biotechnology. Next, the opportunity to involve yeasts of the genus *Rhodotorula* in nanobiotechnologies and to test nanotoxicity for different applications is exposed. The chapter ends with the exposition of the research problem and the directions for solving it, the purpose, objectives and hypotheses of the research are formulated.

2. RESEARCH MATERIALS AND METHODS

The chapter includes a description of the materials and methods used in the research. In the experiments, the effect of inorganic nanoparticles of metal oxides: ZnO with dimensions of <50 nm, <100 nm, Fe_3O_4 with dimensions of 10 nm, 30 nm [27], <50-100 nm, chitosan-zinc and chitosan-iron nanocomposites was studied.

As object of study were served the pigmented yeasts *Rh. gracilis* CNMN-Y-03 carotenoid pigment producer and *Rh. gracilis* CNMN-Y-30 carotenoid protein and pigment producer. The strains are stored in the National Collection of non-pathogenic microorganisms of the Institute of Microbiology and Biotechnology.

To carry out the research, classical and modern methods were used to determine the influence of nanoparticles on yeasts. Microbiological methods were used to determine cell viability by quantifying the number of colonies formed on agarized YPD plates. Changes in the cellular and colonial morphological characters induced by the tested nanoparticles were studied. In order to identify the influence of nanoparticles of metal oxides, a number of biochemical methods were used: to determine the content of proteins, carbohydrates, carotenoid pigments, the activity of

antioxidant enzymes catalase and superoxide dismutase. In order to reduce the toxicity of Fe_3O_4 and ZnO nanoparticles, various chitosan coating processes have been developed and tested.

Statistical processing of the results was performed using the set of MS Excel programs and Statistics 9.0. The results of 3-4 repetitions obtained were expressed by calculating the average, standard deviation and confidence interval for an average. All differences were considered statistically significant for $P \leq 0.05$.

3. THE EFFECTS OF ZnO NANOPARTICLES ON THE YEAST STRAIN *RHODOTORULA* GENUS

3.1. Validation of microbiological and biochemical tests

To achieve the purpose and to ensure the qualification and quality of the methods used in the research, the validation of the microbiological and biochemical tests was performed. The obtained results showed that the tested methods for determining the viability, protein content and activity of the antioxidant enzyme catalase in the yeast biomass *Rh. gracilis* CNMN-Y-03, grown in the presence of NPs ZnO (50 nm) within the limits of 0-20 mg/L, are specific, precise, linear, robust and valid and can be used to test the influence of nanoparticles of metal oxides on yeasts [23].

3.2. Viability, cell biomass production and morphological characteristics of *Rh. gracilis* yeasts under the influence of ZnO nanoparticles

Data from the study of the effects of ZnO nanoparticles by size and concentration revealed significant changes in the viability of *Rh. gracilis* yeast cells. According to the obtained results, an increase in cell viability was observed after 24 hours of cultivation compared with the first 6 hours for both types of nanoparticles and strains investigated when applying concentrations of 1-30 mg/L ZnO <50 nm (fig. 3.1).

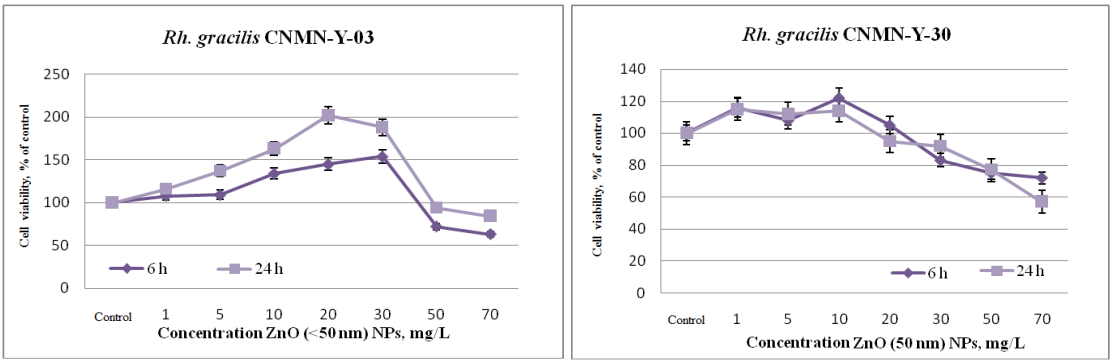


Fig.3.1. Percentage versus control values of cellular viability of *Rh. gracilis* upon contact with ZnO <50 nanoparticles nm depending on concentration and contact duration

The comparative analysis of the results of the action of the nanoparticles in the concentrations of 50-70 mg/L, shows cytotoxic effects on the studied strains both after 6 hours of contact and after 24 hours, the viability of the cells decreases by over 40%. Thus, the quantification of the number of colonies formed on agarized YPD plates showed that the minimum inhibitory concentration (IC50%) of ZnO nanoparticles with dimensions of <50 nm constitutes 70 mg/L on the studied yeasts strains [32].

Analyzing the data obtained regarding the ZnO NPs action with dimensions <100 on *Rh. gracilis* yeasts, we found some differences. Regarding the effect on *Rh. gracilis* CNMN-Y-03, it was established that cell viability after 6 and 24 hours of cultivation increases when applying concentrations of 1...30 mg / L by 16-41%, compared to the control sample [7].

The results obtained for strain *Rh. gracilis* CNMN-Y-30 indicate that both after 6 hours of contact with nanoparticles and after 24 hours, the viability of cells under the action of concentrations of 20-30 mg/L decreases by up to 24-32% (fig. 3.2). The degree of viability in the strain *Rh. gracilis* CNMN-Y-30 was found to be associated with the concentrations used in experiments.

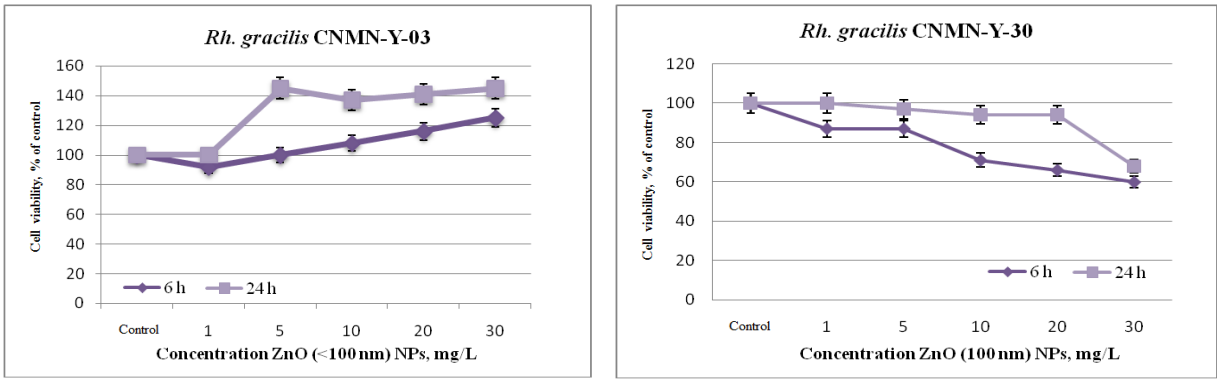


Fig.3.2. Percentage versus control values of cell viability of *Rh. gracilis* upon contact with ZnO <100 nanoparticles nm depending on concentration and contact duration

Analyzing the data obtained regarding the biomass production after the cultivation of the yeasts for 120 hours, some insignificant differences in the effects produced by the nanoparticles were found. According to studies, ZnO <50 nm nanoparticles in concentrations 1-70 mg/L and ZnO <100 nm in concentrations of 1-30 mg/L do not show substantial changes in cell biomass production in pigmented yeasts of the genus *Rhodotorula* [32].

Morphological study showed that the action of ZnO <50 nm nanoparticles on *Rh. gracilis* yeasts is confirmed by changes in cell size, in particular the effect is more evident when the cells are at a concentration of 70 mg/L. Another particularity of the action is the formation of cell

agglomerations, more pronounced effects are observed upon contact with concentrations of 20 and 70 mg/L (fig. 3.3) [15].

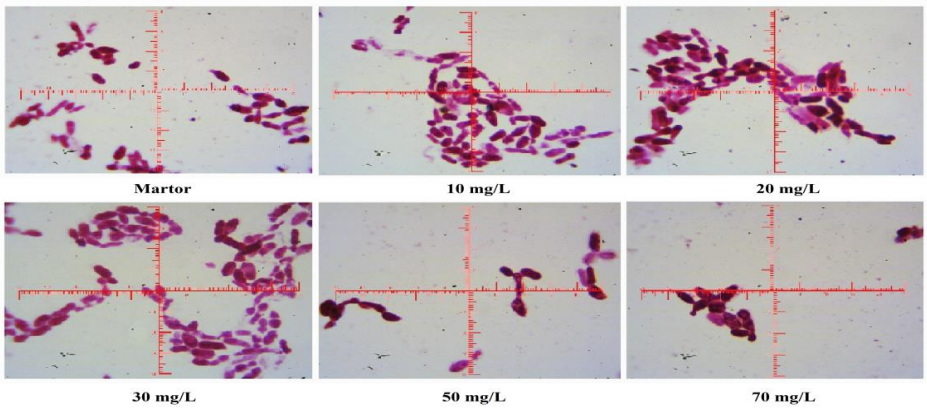


Fig. 3.3. The morphological appearance of *Rh. gracilis* CNMN-Y-30 cells after 24 hours contact with ZnO <50 nm nanoparticles, (ocular 15/100)

In the following experiments, assessments of the colonial morphological characters were carried out on pigmented yeasts of the genus *Rhodotorula*, grown on solid YPD medium for 120 hours. The results showed that the studied strains in contact with ZnO nanoparticles <50 nm, registered some changes when applying the concentrations of 30-70 mg/L on the color, diameter and edge of the colonies.

3.3. Protein synthesis in *Rh. gracilis* yeasts under the influence of ZnO nanoparticles

Further it was determined the character of action of ZnO nanoparticles on the process of protein biosynthesis at the *Rh. gracilis* yeasts. The results showed that their content in the cellular biomass of the yeast *Rh. gracilis* CNMN-Y-03, under the influence of concentrations from 5 to 30 mg/L, is increasing by up to 57% [7].

Regarding the effect of ZnO (<50 nm) NPs on another strain *Rh. gracilis* CNMN-Y-30, it was found that concentrations from 5 to 20 mg/L also increase the protein content in the yeast biomass by up to 38% (fig. 3.4). In the experimental samples in which the concentrations of the nanoparticles constituted 50-70 mg/L, a relatively moderate decrease of the amount of protein accumulated in the biomass of both studied yeasts is observed.

When applying the ZnO nanoparticles with dimensions <100 nm in the yeast cultivation process, increased values of the total protein content were found. In particular, the protein content of strain *Rh. gracilis* CNMN-Y-03 increased the application of concentrations of 1.0-20.0 mg/L by up to 47% (fig. 3.4). Comparative analysis on the protein biosynthesis process at strain *Rh. gracilis*

CNMN-Y-30 showed that their content in cellular biomass, under the influence of nanoparticle concentrations from 5 to 20 mg/L, is increasing by up to 28%.

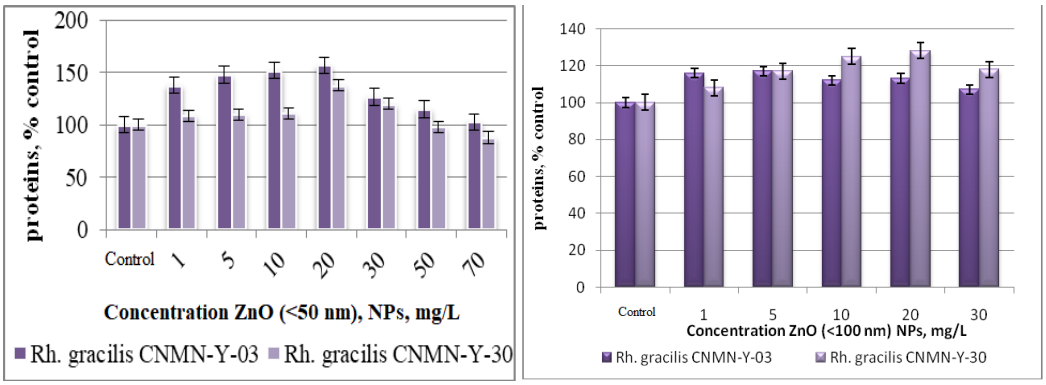


Fig. 3.4. Percentage versus control values of protein accumulation in *Rh. gracilis* yeast biomass under the action of ZnO nanoparticles depending on concentration and dimension, compared to control

Thus, it has been established that the effective concentrations of ZnO nanoparticles with dimensions <50 and <100 nm for stimulation of the protein biosynthesis process are in the range 10-20 mg/L.

3.4. Carbohydrate synthesis in *Rh. gracilis* yeasts under the influence of ZnO nanoparticles

ZnO nanoparticles <50 nm in concentrations of 5-20 mg / L contribute to the increase of up to 19% in the amount of carbohydrates in the *Rh gracilis* CNMN-Y-03 strain. [7]. Significant effect can also be seen in strain *Rh. gracilis* CNMN-Y-30, in which the amount of carbohydrates accumulated in the biomass increases up to 32%, compared to the control sample. In the experimental samples in which the nanoparticles concentration was 50-70 mg/L, a relatively moderate decrease of the amount of carbohydrate accumulated in the yeast biomass is observed.

Thus, we can deduce that the effective concentration of ZnO (<50 nm) and ZnO (<100 nm) nanoparticles for stimulating the carbohydrate biosynthesis process is at the level of 5-20 mg/L.

3.5. Carotenoid synthesis in *Rh. gracilis* yeasts under the influence of ZnO nanoparticles

The content of carotenoid pigments in the biomass of yeast *Rh. gracilis* is given in fig. 3.6. NP ZnO (<50 nm) applied at concentrations of 1...30 mg/L does not significantly alter the amount of carotenoids in the *Rh gracilis* CNMN-Y-03 strain and in the *Rh gracilis* CNMN-Y-30 strain, at concentrations 5.0-10.0 mg/L, the amount of pigments in yeast biomass increases by up to 29%. Increasing the concentrations of ZnO nanoparticles up to 30-70 mg/L, conditions leads to a decrease in the amount of carotenoids by up to 18%, compared to the control.

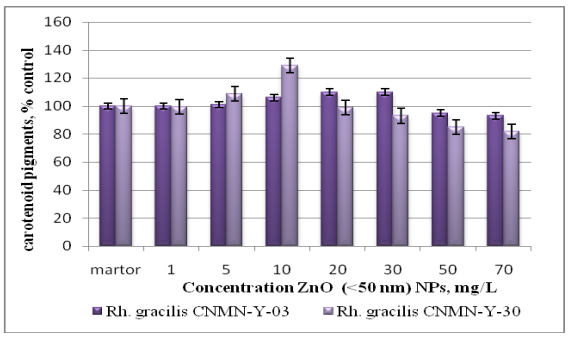


Fig. 3.6. The amount of carotenoids in *Rh. gracilis* under action ZnO nanoparticles <50 nm compared to control

Regarding the effect of ZnO nanoparticles with dimensions <100 nm, it was established that when applied to the culture medium 5-20 mg/L of nanoparticles, the amount of carotenoids changes insignificantly for both strains studied. However, supplementing the culture medium with the concentration of 30 mg/L of nanoparticles initiates an essential decrease by up to 36%.

Thus, from the experimental results we conclude that the large dimensions and high concentrations of 30 -70 mg/L of the examined nanoparticles affect the carotenoid pigment formation pathways [3].

3.6. The activity of antioxidant enzymes catalase and superoxide dismutase at *Rh. gracilis* under the influence of ZnO nanoparticles

The effects of ZnO nanoparticles <50 nm and <100 nm on the activity of the antioxidant enzyme catalase on yeasts of the genus *Rhodotorula* are shown in fig. 3.7. NP ZnO <50 nm, in concentrations of 10 ... 50 mg/L increase the catalase activity in the *Rh gracilis* CNMN-Y-03 strain by up to 50%, and in the *Rh gracilis* CNMN-Y-30 strain - up to 36% [3]. NPs ZnO <100 nm, in concentrations of 10 ... 30 mg / L increase the activity of the enzyme catalase in the *Rh gracilis* CNMN-Y-03 strain by 14-35% compared to the control sample. An equivalent effect can be observed in the *Rh gracilis* CNMN-Y-30 strain when applying concentrations of 10 ... 20 mg / L, but the concentration of 30 mg / L initiates a sudden decrease in enzyme activity by 30%.

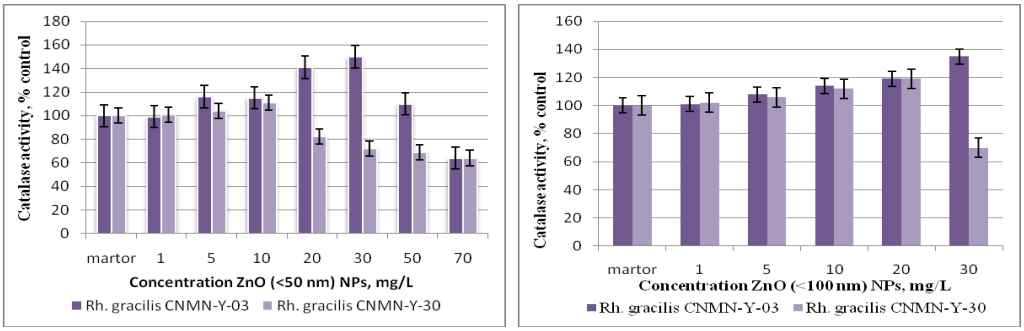


Fig. 3.7. Catalase activity *in Rh. gracilis* in the presence of ZnO NPs

ZnO <50 nm in concentrations from 5 mg/L to 70 mg/L increase SOD activity [3]. When applying NP ZnO <100 nm the activity of the SOD enzyme increases. A significant effect was recorded for the *Rh gracilis* CNMN-Y-30 strain - at the concentration of 20 mg/L of NP SOD activity increases by 85%. The *Rh gracilis* CNMN-Y-03 strain at concentrations of 10-20 mg / L NP SOD activity increases by 68% compared to the control [3, 11].

Thus, it can be concluded that the adaptive response mechanism of yeasts depends on the size and concentration of NP ZnO. Visible effects are manifested by small nanoparticles applied in high concentrations [3].

3.7. *Rh. gracilis* yeast cultivation process using ZnO nanoparticles

The results of the experiments carried out, in which it was shown that ZnO nanoparticles play an important role in the process of protein synthesis, have allowed proposing a process for increasing the protein content having as biotechnological object the strains of *Rh. gracilis* yeasts.

The proposed process ensures the protein content of the yeast biomass *Rh. gracilis* CNMN-Y-30 of 57.89%, being 23.1% higher than the control variant and for *Rh. gracilis* CNMN-Y-03 of 61.95 with 17%....32% is superior to the control variant.

Examination of the quantitative and qualitative composition of *Rh. gracilis* CNMN-Y-30 yeast proteins, when cultivated in the presence of ZnO <50 NPs, showed a high content of essential, immunoactive and proteinogenic amino acids that increased significantly by up to 236%. The process of cultivating *Rh. gracilis* yeasts using ZnO nanoparticles is confirmed by patent MD-4690 [22].

4. THE EFFECTS OF FE_3O_4 NANOPARTICLES ON *RH. GRACILIS* CNMN-Y-30 STRAIN

4.1. Viability, biomass production and morphological characteristics of *Rh. gracilis* strain CNMN-Y-30 under the influence of Fe_3O_4 nanoparticles with different dimensions

In the following researches were studied the effects of Fe_3O_4 nanoparticles in terms of concentrations and dimensions. The results obtained on the cell viability of the yeast *Rh. gracilis* CNMN-Y-30 are shown in Figure 4.1. The data analysis shows that the viability of the yeast cells is affected by Fe_3O_4 NPs with the dimensions of 10 nm and 30 nm, and the nanoparticles with the dimensions of 50-100 nm initiate a significant stimulation of the viability [10].

In the case of the productivity assessment in the experimental variants in which Fe_3O_4 NPs 10 nm and 30 nm were tested, no significant changes were noted. At the same time, incubation of

yeast with Fe_3O_4 (50-100 nm) NPs in concentrations with a range from 5 to 30 mg/L, induces a substantial increase of productivity by up to 24%, compared to the control.

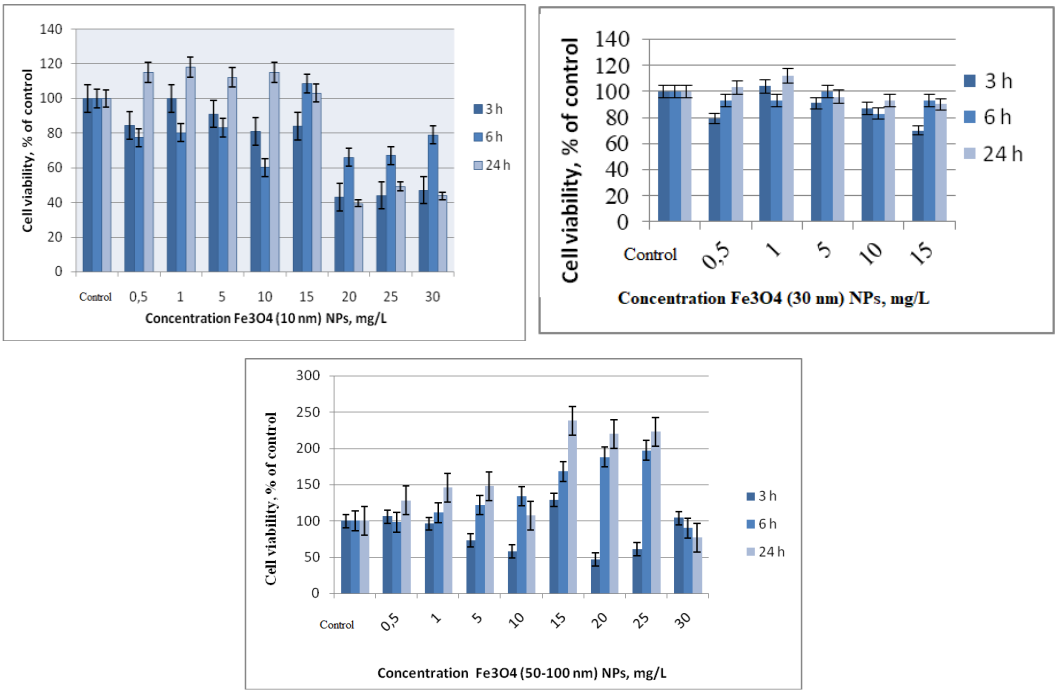
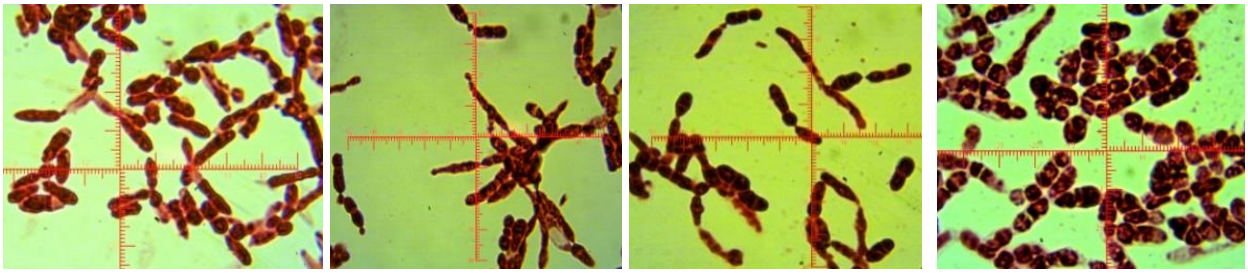


Fig. 4.1. Percentage values of *Rh. gracilis* CNMN-Y-30 viability in cultivation in the presence of Fe_3O_4 nanoparticles with different dimensions

Fe_3O_4 (10 nm) nanoparticles produced a series of morphological changes in the *Rh gracilis* CNMN-Y-30, strain which is manifested by partial or total overflow of cell contents due to damage to the cell wall and membrane. In the images in figure 4.2. fragments of cells completely or partially devoid of content are observed at concentrations of 10 and 15 mg/L. Another peculiarity of the action of Fe_3O_4 nanoparticles is the formation of cell agglomerations, especially at concentrations of 10 and 15 mg/L culture medium. Fe_3O_4 NPs with the size of 30 nm and 50-100 nm, does not change the cell morphology [4, 12]. Also Fe_3O_4 NPs at high concentrations induce changes in the shape, color, size and profile of the colonies [12].



Control **5 mg/L** **10 mg/L** **15 mg/L**

Fig. 4.2. Morphological characters of *Rh. gracilis* CNMN-Y-30 cells, under the action of Fe_3O_4 (10 nm) nanoparticles, contact time 120 hours, (ocular 15/100)

4.2. Protein synthesis at *Rh. gracilis* strain CNMN-Y-30 under the influence of Fe3O4 nanoparticles

In the presence of Fe_3O_4 (10 nm) NPs in a concentration of 15 to 30 mg/L protein content in the *Rh gracilis* CNMN-Y-30 strain was up to 22%. Fe_3O_4 (30 nm) NPs produced an increase in the amount of protein in all variants tested. In the presence of Fe_3O_4 nanoparticles of 50-100 nm, at concentrations of 0.5-30 mg/L, the protein content does not change [9, 29, 30, 34].

The analysis of the correlational ratio confirms the dependence of the values of the protein content on the concentrations of the nanoparticles Fe_3O_4 (10 nm) and Fe_3O_4 (30 nm), the coefficient of determination being 0.840 and 0.955 respectively. An average correlation between these two factors was observed in the case of Fe_3O_4 of 50-100 nm ($R^2 = 0.425$).

4.3. Carbohydrate synthesis at *Rh. gracilis* CNMN-Y-30 strain under the influence of Fe_3O_4 nanoparticles

Fe_3O_4 nanoparticles, with a size of 30 nm and 50-100 nm in concentrations of 0.5-15 mg/L lead to a moderate increase in the amount of carbohydrates, and those of 10 nm to the application of concentrations 20-30 mg / L - to the decrease by up to 49% [6, 9, 30, 31].

Thus, the synthesis of carbohydrates in the *Rh gracilis* CNMN-Y-30 strain in the presence of Fe_3O_4 nanoparticles is influenced by the size and concentrations of the nanoparticles.

4.4. Synthesis of carotenoids in *Rh. gracilis* CNMN-Y-30 strain under the influence of Fe_3O_4 nanoparticles

In the case of application to the culture medium of Fe_3O_4 nanoparticles with the size of 10 nm, a decrease of 32-73% of the carotenoid content in biomass is observed (fig. 4.3). In the case of Fe_3O_4 of 30 nm NPs, the results indicate a directly proportional decrease (by up to 82%) of the amount of carotenoids due to the increase of the nanoparticle concentration, and of those of 50-100 nm there is a reduction of the amount of carotenoids by up to 79% [17, 30, 31, 34].

There is a close inverse correlation between the amount of carotenoids and the concentration of nanoparticles, the value of the coefficient of determination being between 0.8555 and 0.9723 depending on the size of the NPs.

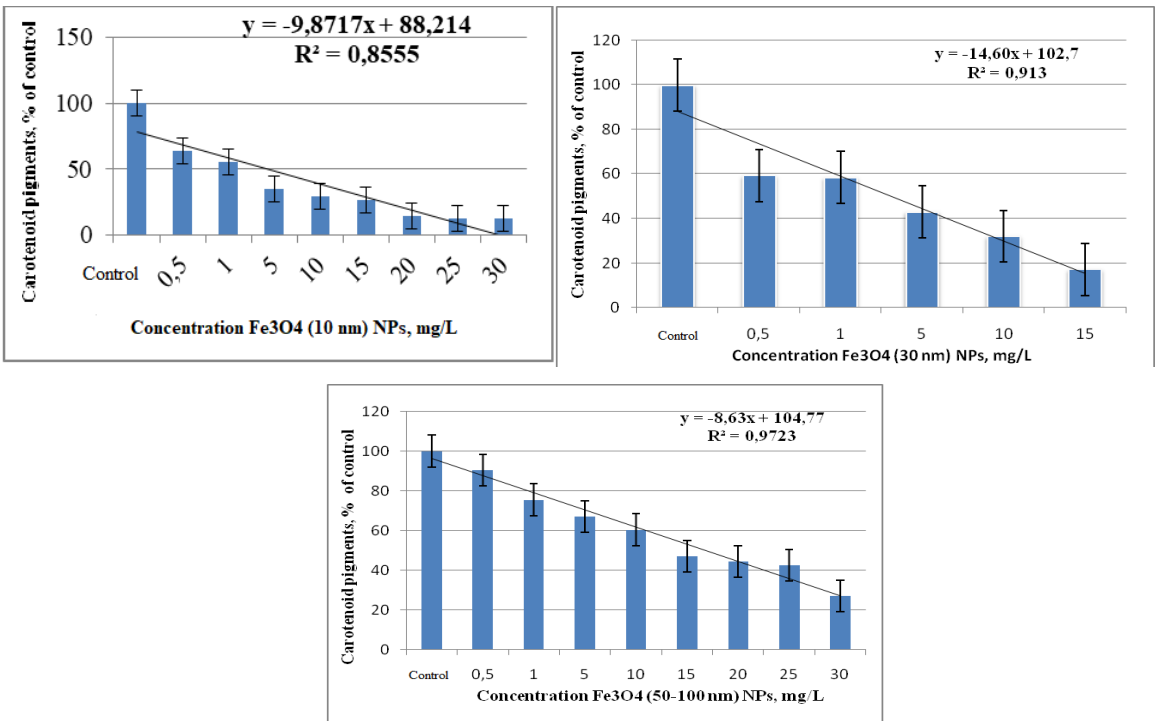


Fig. 4.3. Percentage values relative to the control of carotenoid content in *Rh. gracilis* CNMN-Y-30 biomass under the influence of Fe_3O_4 nanoparticles, with different dimensions

Thus, Fe_3O_4 nanoparticles negatively influence the accumulation of carotenoids in biomass *Rh gracilis* CNMN-Y-30.

4.5. Antioxidant enzyme activity of catalase and superoxide dismutase in *Rh. gracilis* CNMN-Y-30 strain under the influence of Fe_3O_4 nanoparticles

The impact of Fe_3O_4 nanoparticles on the concentrations and dimensions investigated on the activity of the antioxidant enzyme catalase was also evaluated, the results are presented in Figure 4.4. Response strain *Rh. gracilis* CNMN-Y-30 at the introduction of 10 nm Fe_3O_4 nanoparticles in concentrations from 1.0 to 30 mg / L is expressed by a significant decrease in catalase activity, by up to 64.4%. In the case of Fe_3O_4 of 30 NPs nm the catalase activity decreases at the application of concentrations 0.5-15 mg/L by up to 77%, and of Fe_3O_4 of 50-100 nm NPs - by 35-50% [8]. The correlational analysis confirms a strong inverse dependence of the catalase activity on the concentrations of Fe_3O_4 nanoparticles, the coefficients of determination being 0, 906 and 0.959 in the case of the dimensions of 10 and 30 nm respectively.

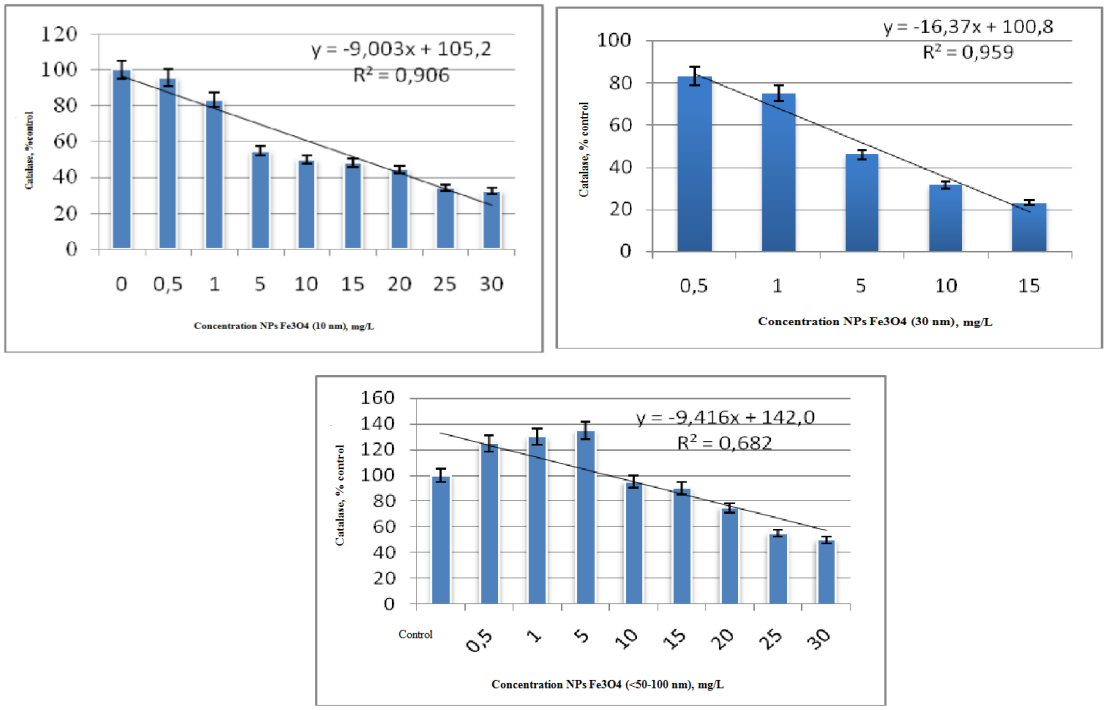


Fig. 4.4. Percentage values compared to control of catalase activity in *Rh. gracilis* CNMN-Y-30 yeast strain, in cultivation in the presence of Fe_3O_4 nanoparticles of different dimensions

Thus, Fe_3O_4 nanoparticles significantly alter the activity of the antioxidant enzyme catalase in the *Rh. gracilis* CNMN-Y-30 strain, which confirms the presence of the toxic potential of nanoparticles.

4.6. The process of evaluating the impact of Fe_3O_4 nanoparticles with the use as a model of the yeast *Rhodotorula gracilis*

In accordance with the results obtained, in which different concentrations and sizes of Fe_3O_4 nanoparticles were experienced, an impact assessment process with the use of pigmented yeasts of the genus *Rhodotorula* is proposed.

The level of inhibition is determined based on the concentrations of nanoparticles that cause the statistically reliable (IC50%) decrease of the β-carotene content in biomass or catalase activity. The results show that the toxicity index (50% CI) of Fe_3O_4 nanoparticles of different sizes ranges from 10 mg/L (Fe_3O_4 10 nm and Fe_3O_4 30 nm) to 20-30 mg/L (Fe_3O_4 50-100 nm) [2, 33]. The process for assessing the toxicity of Fe_3O_4 nanoparticles with the use of *Rh. gracilis* pigmented yeast is patented [28].

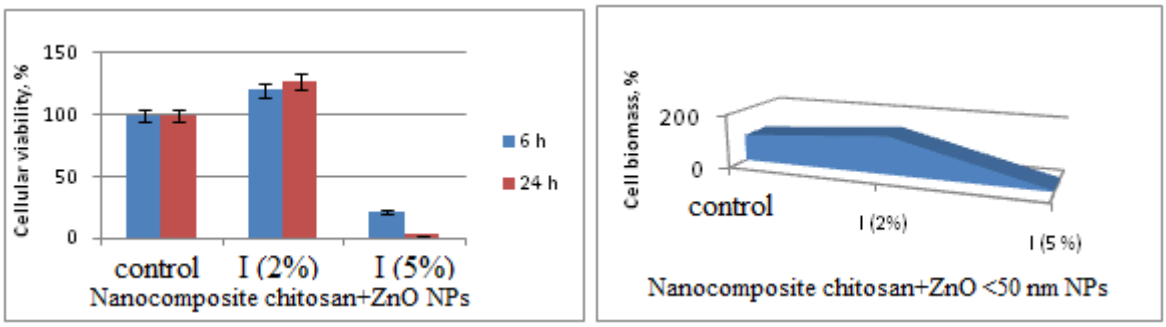
5. PRODUCTION OF CHITOSAN-ZINC AND CHITOSAN-IRON NANOCOMPOSITES WITH BIOTECHNOLOGICAL PERSPECTIVES

5.1. Evaluation of the action of chitosan-zinc nanocomposite on *Rh. gracilis* biosynthetic activity

The results of the experiments have shown that the nanoparticles of metal oxides due to their high toxicity and low stability present impediments for use in the field of yeast biotechnology [2, 15]. Due to their small size and large surface/volume ratio, nanoparticles tend to form agglomerations, they also have the ability to easily oxidize in the air. Therefore, it is necessary to change the surface to stabilize the nanoparticles of metal oxides and avoid oxidation processes. These shortcomings can be reduced by covering with biopolymers, in particular chitosan.

Initially, the optimal parameters for the preparation of chitosan-zinc and chitosan-iron nanocomposites were determined, in which the ratio between the amount of chitosan and the nanoparticles of zinc oxide or iron oxide varied. Subsequently, the action of the chitosan-zinc and chitosan-iron nanocomposite, applied in the cultivation medium in volumes of 2% and 5%, on the cell viability and biomass production in the yeast strain *Rh. gracilis* CNMN-Y-30 was evaluated. 4 types of new nanocomposites were made. As a witness, they served Kumar et. al., [24] and Mohammadi-Samani et. al. [26].

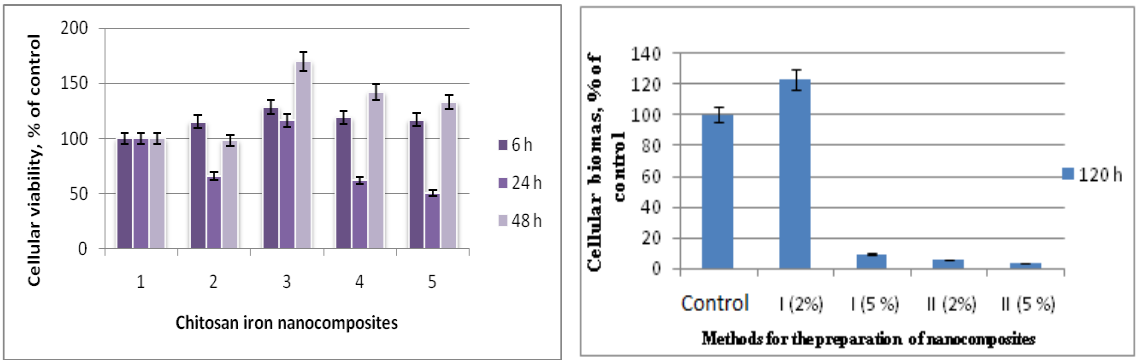
Subsequently, the action of chitosan-zinc and chitosan-iron nanocomposites, in the amount of 2% and 5% of the nutrient volume, on the cell viability and biomass production of the *Rh. gracilis* CNMN-Y-30 strain was evaluated. The results regarding the influence of the chitosan-zinc nanocomposite on the viability and biomass are presented in figure 5.1. The maximum values were recorded in the case of application of the nanocomposite prepared according to experimental procedure I (25 mg chitosan+ 50 and 70 mg/L NPs)applied in a volume of 2%. The nanocomposite applied in a volume of 5% shows inhibitory effect.



Legend: Witness - proceed [24]; I (2%) - experimental procedure I, nanocomposite applied in a volume of 2%; I (5%) - experimental procedure I, nanocomposite applied in a volume of 5%.

Fig. 5.1. Effects of chitosan-zinc nanocomposite on cell viability and biomass production in yeast strain *Rh. gracilis* CNMN-Y-30

Effects of chitosan-iron nanocomposite on viability and biomass in the yeast *Rh. gracilis* CNMN-Y-30 strain can be traced in figure 5.2. Thus, it was found that in the case of application of the nanocomposite prepared according to experimental procedure I (25 mg chitosan+ 50 and 70 mg/L NPs), introduced into the culture medium for yeasts in a volume of 2%, increases the cell viability by up to 16 , 5% and initiates the increase of the biomass quantity. Pronounced toxic effect on the viability and production of cellular biomass, showed the nanocomposite prepared according to experimental procedure II (50 mg chitosan, 25 ml acetic acid 1% and 50-70 mg/L Fe_3O_4 NPs) used in volume of 2% and 5%.



Legend: 1 - Control [26]; 2 - Control procedure I; 3,4,5 - experimental procedure I, with the content of Fe_3O_4 nanoparticles in concentration of 30, 50 and 70 mg/L, respectively.

Fig. 5.2. Effects of chitosan-iron nanocomposite on cell viability and biomass production in yeast strain *Rh. gracilis* CNMN-Y-30

However, the test results showed that the most effective nanocomposite is the one made according to the experimental procedure I, which consists of the following phases: to 50 mg of chitosan add 50 ml of 1% acetic acid and stirred for 10 minutes at 200 r.p.m. Subsequently, the neutral pH is established with NaOH, 1% of 96% ethyl alcohol is added, stirred for 10 minutes at 200 r.p.m. To the chitosan solution, add the Fe_3O_4 nanoparticles (50-100 nm) in a concentration of 70 mg/L and sonify for 10 minutes. During this process the chitosan molecules are absorbed on the surface of the metal nanoparticles.

5.2. Evaluation of the action of chitosan-iron nanocomposite on *Rhodotorula gracilis* biosynthetic activity

The chitosan-iron nanocomposite, in which the content of Fe_3O_4 nanoparticles was 30, 50 and 70 mg/L, prepared according to the proposed experimental procedure, ensures the increase of the protein content in the biomass of the yeast *Rh. gracilis* CNMN-Y-30 strain by up to 33%

compared to control. The maximum amount of protein was observed at the concentration of 70 mg / L nanoparticles. The chitosan-iron nanocomposite with 30-70 mg/L NPs provided an increase in the content of carbohydrates in biomass by up to 13% and carotenoids - by up to 11%.

The application of the chitosan-iron nanocomposite with the content of Fe_3O_4 nanoparticles (50-100 nm) in a concentration of 30 mg/L, led to a significant increase, by 26%, of the SOD activity. For the nanocomposite variants with nanoparticle concentrations of 50 and 70 mg/L, the SOD activity is decreasing compared to the control. In case of catalase activity, values up to 35% higher were recorded compared to the control variant.

GENERAL CONCLUSIONS

1. The effects of nanoparticles ZnO <50 nm, ZnO <100 nm, Fe_3O_4 10 nm, Fe_3O_4 30 nm, Fe_3O_4 50-100 nm, established on pigmented yeast strains of the genus *Rhodotorula*, which develop according to size and concentration, contribute to elucidating the pathways of action and identifying the cellular functional groups involved in the response to the action of nanoparticles [70, 71, 72, 110, 111, 112, 301, 302, 305], [chap. 3, 4].
2. Validated microbiological and biochemical indices demonstrate that cell viability, protein content and antioxidant enzyme catalase activity are accurate, robust tests and can be used to determine the degree of influence of nanoparticles of metal oxides in yeast [23], [chap. 3].
3. ZnO nanoparticles of <50 nm and <100 nm in concentrations 1-70 mg/L intensify the biosynthesis processes of proteins, carbohydrates, carotenoids, superoxide dismutase and catalase enzymes in *Rh. gracilis* yeast CNMN-Y-03 and *Rh. gracilis* CNMN-Y-30, presenting opportunity of application in the biotechnology of yeast cultivation from the point of view of stimulating the biosynthesis of cellular components [3, 7, 11, 13, 16], [chap. 3].
4. The elaborated cultivation process of *Rh. gracilis* yeasts with the application of ZnO nanoparticles with dimensions of <50 nm, at a concentration of 20 mg/L, allows to increase the protein content in the cellular biomass by 23-34% more than the witness and contributes to improving the quality of amino acids [22], [chap. 3].
5. Fe_3O_4 nanoparticles, with dimensions 10 nm, Fe_3O_4 30 nm, Fe_3O_4 50-100 nm, depending on the applied concentrations 0,5-30 mg/L, act at the level of biochemical systems of synthesis of the cellular components of the yeast *Rh. gracilis* CNMN-Y -30, expressed by decreasing the content of proteins, carotenoids, catalase activity. The determination of the degree of correlation between the concentrations of the nanoparticles and the quantitative values of the cellular components demonstrates a strong association, the coefficient of determination

being R^2 = 0.840 (protein), R^2 = 0.849 (carotenoid), R^2 = 0.722 (catalase) [4, 5, 6, 8, 10, 12, 17, 29, 30], [chap.4].

6. The hazard assessment process for Fe_3O_4 nanoparticles using pigmented yeast *Rhodotorula gracilis*, which provides for the determination of concentrations that cause a 50% decrease of the β-carotene content and the activity of catalase, allows the establishment of quantitative concentration-effect relationships and provides the necessary information for characterization of the toxicity of the nanoparticles. Inhibition concentrations (IC50%), according to the tests of catalase activity and β-carotene content, constitute 10 mg L-1 specific for Fe_3O_4 nanoparticles (10 nm) and 25-30 mg L^{-1} for Fe_3O_4 nanoparticles (50-100 nm) [28], [chap. 4].
7. The chitosan-zinc and chitosan-iron nanocomposites, developed according to the new experimental procedure, represent perspective products for bionanotechnologies, characterized by the increased ability to initiate the biosynthesis of cellular bioactive principles in yeasts of the *Rhodotorula* genus. Chitosan-iron nanocomposite exhibits biological activity, expressed by increasing *Rh. gracilis* CNMN-Y-30 protein content by 15-35%, carbohydrates by 13%, carotenoid pigments by 9-11%, CAT activity by up to 35%, SOD up to 26%, compared to the witness, [chap. 5].

Personal contribution: Materials presented in the patents MD-4522, "Process for evaluating the toxicity of nanoparticles of metal oxides with the use of yeasts" (Annex 1), MD-4690 "Process for growing yeasts of the genus *Rhodotorula*", and patent application (No. 5823 from 2020.02.18) the author has a share according to the list of authors. The results obtained during the elaboration of the PhD thesis, the analysis, generalizations and conclusions belong to the author.

PRACTICAL RECOMMENDATIONS

1. Methods for determining cell viability, protein content and activity of antioxidant enzyme catalase for determining the degree of influence of nanoparticles of metal oxides on microorganisms.
2. The process of cultivating *Rh. gracilis* yeasts with the application of ZnO nanoparticles with dimensions of <50 nm, to increase the protein content and to improve the quality of the amino acids in the cellular biomass.
3. The process of hazard assessment of nanoparticles of metal oxides using *Rh. gracilis* pigmented yeast.
4. Two processes for obtaining chitosan-zinc and chitosan-iron nanocomposites for use in bionanotechnologies.

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ADNOTARE

Beşliu Alina „Efectele nanoparticulelor oxizilor metalici asupra levurilor din genul *Rhodotorula''*. Teză de doctor în ştiinţe biologice, Chişinău, 2020.

Teza conţine introducere, cinci capitole, concluzii şi recomandări, bibliografie cu 361 titluri, 12 anexe, volumul capitolelor 129 pagini, 55 figuri, 19 tabele. Rezultatele obţinute sunt reflectate în 46 lucrări ştiinţifice publicate la tema tezei.

Domeniul de studiu: Științe ale naturii.

Cuvintele cheie: nanoparticule ZnO, Fe_3O_4, *Rhodotorula gracilis*, viabilitate, caractere morfologice, carbohidraţi, proteine, pigmenți carotenoizi, catalaza, superoxid dismutaza.

Scopul lucrării constă în stabilirea gradului de influență a nanoparticulelor oxizilor de metale ZnO și Fe_3O_4 asupra indicilor microbiologici și biochimici la levurile de interes biotehnologic din genul *Rhodotorula* și aprecierea perspectivelor de utilizare în bionanotehnologii.

Obiectivele lucrării: Validarea testelor microbiologice și biochimice în contextul asigurării calificării și calității metodelor utilizate pentru evaluarea efectelor nanoparticulelor oxizilor metalici; Stabilirea particularităților acțiunii nanoparticulelor ZnO și Fe_3O_4 cu diferite dimensiuni asupra levurilor din genul *Rhodotorula*; Elaborarea procedeelor de utilizare a nanoparticulelor ZnO și Fe_3O_4 în biotehnologia cultivării levurilor; Obținerea nanocompozitelor chitosan–zinc și chitosan-fier și aprecierea perspective utilizării lor în biotehnologia levurilor pigmentate.

Noutatea și originalitatea științifică. În premieră au fost validate teste microbiologice și biochimice și identificate componentele celulare ale levurilor pigmentate din genul *Rhodotorula*, implicate în răspunsul la acțiunea nanoparticulelor; elucidat caracterul acțiunii nanoparticulelor ZnO și Fe_3O_4 asupra viabilității, producției de biomasă, caracterelor morfologice, conținutului de proteine, carbohidrați, carotenoide, enzimelor antioxidante la levurile din genul *Rhodotorula* în dependență de dimensiuni și concentrație. S-a demonstrat că nanoparticulele ZnO, utilizate în concentrații 1-20 mg/L, influențează pozitiv procesele de multiplicare și biosinteză a proteinelor, carbohidraților și pigmenților carotenoidici. S-a demonstrat că nanoparticulele Fe_3O_4 induc la *Rh. gracilis* modificări semnificative în sistemul de apărare antioxidant, care se manifestă prin scăderea conținutului de carotenoide și enzimei catalaza. În premieră este elucidat caracterul de acțiune a nanocompozitelor chitosan-zinc și chitosan-fier asupra levurilor din genul *Rhodotorula*. Au fost elaborate două procedee noi de sporire a cantității și calității proteinelor cu utilizarea nanoparticulelor ZnO și de evaluare a toxicității nanoparticulelor Fe_3O_4 cu utilizarea în calitate de model a levurii *Rh. gracilis.* Originalitatea procedeelor elaborate sunt confirmate de 2 brevete de invenţie și o cerere de brevet de invenţie.

Problema științifică importantă soluționată în lucrare. Au fost stabilite efectele nanoparticulelor ZnO și Fe_3O_4 asupra levurilor de interes biotehnologic din genul *Rhodotorula,* ceea ce a contribuit la elucidarea unor mecanisme de acțiune a nanoparticulelor, fapt ce a permis aprecierea perspectivelor de utilizare a lor în bionanotehnologii.

Semnificația teoretică. Sunt argumentate științific particularitățile de acțiune a nanoparticulelor ZnO și Fe_3O_4, în funcție de dimensiuni și concentrație, și a nanocompozitelor chitosan-zinc și chitosan-fier asupra indicilor microbiologici și biochimici la levurile din genul *Rhodotorula*, ceea ce a servit ca bază pentru elucidarea unor căi de acțiune și elaborarea procedeelor de utilizare în biotehnologia cultivării levurilor.

Valoarea aplicativă. Se propun spre valorificare: un procedeu de sporire a conţinutului de proteine la *Rh. gracilis* cu utilizarea nanoparticulelor ZnO; un procedeu nou de testare a gradului de nocivitate a nonoparticulelor Fe_3O_4 cu utilizarea în calitate de model a levurilor pigmentate din genul *Rhodotorula*; un procedeu nou de realizare a nanocompozitelor chitosan–zinc și chitosan-fier cu perspective biotehnologice.

Implementarea rezultatelor. Rezultatele cercetării sunt aplicate în cadrul Institutului de Inginerie Electronică și Nanotehnologii "D. Ghițu", și în laboratorul Microbiologia solului, Institutul de Microbiologie și Biotehnologie.

ANNOTATION

Beșliu Alina „The effects of metal oxide nanoparticles on yeasts of the genus *Rhodotorula*'', PhD thesis in biological sciences, Chisinau, 2020.

The thesis consists of an introduction, five chapters, general conclusions and recommendations, 361 of bibliographic sources, 12 appendices, main text 129 pages, 55 figures, 19 tables. The results were published in 46 scientific papers.

Field of study: Natural sciences.

Keywords: ZnO, Fe_3O_4 nanoparticles, *Rhodotorula gracilis*, viability, morphological characters, carbohydrates, proteins, carotenoid pigments, catalase, superoxide dismutase.

Objectives of the paper: Validation of microbiological and biochemical tests in the context of ensuring the qualification and quality of the methods used to evaluate the effects of nanoparticles of metal oxides; Establishing the particularities of the action of ZnO and Fe_3O_4 nanoparticles with different dimensions on yeasts of the genus *Rhodotorula;* Elaboration of the procedures for the use of ZnO and Fe_3O_4 nanoparticles in the biotechnology of yeast cultivation; Obtaining the chitosan-zinc and chitosan-iron nanocomposites and appreciating the perspectives of their use in the biotechnology of pigmented yeasts.

Scientific novelty and originality. For the first time, microbiological and biochemical tests are validated and the cellular functional groups of pigmented yeasts of the genus *Rhodotorula* have been identified, involved in the response to the action of the nanoparticles. For the first time is elucidated the action character of ZnO and Fe_3O_4 nanoparticles on viability, biomass production, cellular and colonial morphological characteristics, protein content, carbohydrates, carotenoids, antioxidant enzymes in yeasts of the *Rhodotorula* genus depending on size and concentration. It has been shown that ZnO nanoparticles, used in concentrations 1-20 mg/L in *Rh. gracilis* cultivation, positively influence the processes of protein multiplication and biosynthesis, carbohydrates and carotenoid pigments. It has been shown that Fe_3O_4 nanoparticles induce in *Rh.gracilis* significant changes in biosynthetic processes and antioxidant defense system, which is manifested by decreased carotenoid content and catalase enzyme. For the first time, the character of action of chitosan-zinc and chitosan-iron nanocomposites on yeasts of the genus *Rhodotorula* is elucidated. Two new procedures have been developed to increase the quantity and quality of proteins with the use of ZnO nanoparticles and to evaluate the toxicity of Fe_3O_4 nanoparticles with the use of *Rh. gracilis* as a model. The processes are confirmed by a 2 patents and patent application.

The important scientific problem solved in the paper. The effects of nanoparticles of ZnO and Fe_3O_4 metal oxides on yeasts of biotechnological interest in the genus *Rhodotorula* have been established, which has contributed to the elucidation of some mechanisms of action, which has allowed to appreciate the prospects for use in bionanotechnologies.

Theoretical significance. The particularities of action of the ZnO and Fe_3O_4 nanoparticles, based on their size and concentration, and of the chitosan-zinc and chitosan-iron nanocomposites on the microbiological and biochemical indices in yeasts of the genus *Rhodotorula*, have been argued, which served as a basis for the elucidation of some of action and elaboration of the procedures for use in the biotechnology of yeast cultivation.

The applicative value. It is proposed for recovery: a process for increasing the protein content of *Rh. gracilis* strains with the use of ZnO nanoparticles; a new process for testing the harmfulness of Fe_3O_4 nanoparticles with the use as a model of pigmented yeasts of the genus *Rhodotorula*; a new process for making chitosan-zinc and chitosan-iron nanocomposites with biotechnological perspectives.

Implementation of results. The results of the research are applied within the Institute of Electronic Engineering and Nanotechnologies "D. Ghițu", and the practical work in the soil microbiology laboratory, the Institute of Microbiology and Biotechnology.

BEŞLIU ALINA

THE EFFECTS OF METAL OXIDE NANOPARTICLES ON YEASTS OF THE GENUS *RHODOTORULA*

167.01 BIOTECHNOLOGY, BIONANOTECHNOLOGY

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