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ORIGINAL RESEARCH PAPER

THE PROCESS OF ASSESSMENT OF THE TOXICITY OF NANOPARTICLES OF THE OXIDES OF METALS WITH THE USE OF THE YEAST *RHODOSPORIDIUM TORULOIDES*

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Abstract: This study provides new information directed to solving the objective of highlighting and assessing the degree of hazard of nanoparticles to the environment. The proposed procedure is to apply as a model in the practice of monitoring the toxicity of metal oxide nanoparticles of pigmented yeasts. The proposed procedure is to apply, as a model, the nanoparticles of pigmented yeast *Rhodosporidium toruloides* (synonymous *Rhodotorula gracilis*) CNMN-Y-30 in the practice of monitoring the toxicity of metal oxide. Based on the response of the yeast, the level of toxicity of nanoparticles is determined based on the concentrations decrease by 50 % of the content of *β*-carotene in the biomass of yeasts and catalase antioxidant enzyme activity. This information can be used by professionals in the food industry, microbiology, medicine, cosmetology, textiles, etc., where those nanomaterials have applications.

Keywords: *β-carotene, catalase, Fe_3O_4 nanoparticles, Rhodosporidium toruloides, TiO_2 nanoparticles*

INTRODUCTION

Nanoparticles have dimensions of 1-100 nm and present practical interest for various fields: electronic, medical industries, pharmaceutical, food, cosmetology and environment protection [1 – 3]. There are a number of risks associated with the production and use of nanoparticles. According to recent studies, it has been shown that nanoparticles, due to the smaller size compared to cells and cell organelles, are very mobile and can penetrate biological structures, thereby disrupting their normal operation [4, 5].

Worldwide and European human and environmental health and safety organizations are taking measures to provide regulations for risk assessment of nanomaterials. The particular interest of the community is focused on assessing the toxicity of nanoparticles. A major importance for determining the potential effects of nanomaterials on living organisms has research with the application of biological experimental models.

An important factor that determines the toxicity of a substance on biological models is the concentration. This parameter or its derivatives shall be used for the purpose of assessing the toxicity of substances and their toxicological classification. The variation of individual response to the administration of lethal doses of the substance with toxic effect requires the determination of the degree of toxicity. The establishment of quantitative relations concentration-effect provides the necessary information to characterize the safety of the chemical compound. To determine the toxicity, depending on the amount of test substance, certain conventional criteria are accepted.

According to EU Parliament's Regulation (EC) No 1272/2008, the LC50 (96 hours) for fish is used to assess the toxicity of chemicals, EC50 (48 hours) for crustaceans and EC50 (72 or 96 hours) for algae species representative of the aquatic environment [6]. Nowadays, several species of microalgae are used as models for determining the toxicity of different types of nanoparticles. Different physiological, biochemical and genetic-molecular indicators are assessed as benchmarks. One of the procedures for assessing the toxicity of nanoparticles for microalgae consists in the cultivation of *Porphyridium cruentum* for 6 hours on nutritional medium with the addition of more than one hour after the inoculation of the microalgae of nanoparticles in different concentrations, then determining the malondialdehyde content in algal biomass. The concentrations of nanoparticles which cause increase of malondialdehyde content in biomass [7] are considered toxic. This process presents general information on the toxicity of nanoparticles but does not contain information about maximum effective concentrations (EC50 % index), lethal (CL 50) or maximum tolerable (CMT).

For the determination of the toxicity of different substances, other normative acts are proposed, in particular, we mention "methodical recommendations for the study of the general toxicity of pharmacological substances", approved by the Pharmacological State Committee of Ministry of Health of the Russian Federation in 2005. According to the recommendations, the toxicity of pharmacological remedies is recommended to be assessed having as-test-experimental animal objects (rats, rabbits, other rodents) [8].

In the microbial biotechnology, as functional tests to determine the degree of inhibition or stimulation of various chemical compounds, it is proposed to use the terms "effective concentration" (EC50 %) or "inhibition concentration" (IC50 %) of Compound [9].

These parameters offer the possibility of conducting a complete study of the assessment of the degree of influence of chemicals on living objects.
The yeasts present convenient and representative objects that offer opportunities in shaping vital processes and establishing mechanisms for the action of various substances on cellular components. To determine the toxicity level of nanoparticles, it is proposed as a model for eukaryotes to use *Saccharomyces cerevisiae* yeasts. As tests to determine the effects of metallic nanoparticles are proposed the procedures for determining the oxygen consumption and the impact on the integrity of the microbial cell membrane [10]. Another procedure recommended for assessing the effects of nanoparticles is to determine the viability of the yeasts culture by determining the EC50 % index (maximum effective concentration) [11].
Another known process of assessing the toxicity of heavy metals is the use of pigmented yeasts in the genus *Rhodotorula*. This procedure involves assessing the color intensity of carotenoid pigments by photographing colonies and determining the difference between the colors of the model canals and, in parallel, determining the amount of malondialdehyde in the cell biomass [12]. This type of testing requires the application of sophisticated analytical techniques (determination of pigment color intensity with the application of specific programmes) and does not reveal the safe degree of toxicity of metals.
Those exposed have determined the purpose of the research that consists in developing a new process of assessing the toxicity of the nanoparticles of metal oxides with the use of pigmented yeast *Rhodosporidium toruloides*.

MATERIALS AND METHODS

Objects of study

Pigmented yeast *Rhodosporidium toruloides (*synonymous *Rhodotorula gracilis)* CNMN-Y-30 was selected for the research. The strain is preserved in the collection of Yeasts Biotechnology Laboratory and in the Collection of Nonpathogenic Microorganisms of Institute of Microbiology and Biotechnology of Moldova.

Nanoparticles

TiO_2 nanoparticles (30 nm) stabilized in polyvinylpyrrolidone (PVP) in the stock concentration of 1 $mg \cdot mL^{-1}$, obtained and characterized under laboratory conditions, made available for us with great kindness by researchers of the Institute of Electronic Engineering and Nanotechnologies “D. Ghițu” of the Academy of Science of Moldova [13].
Fe_3O_4 nanoparticles (dry powder 10 and 50-100 nm) were used (ALDRICH) the suspension was prepared according to the method specified [10].
The suspension of nanoparticles in deionized water is sonicated using a 130 W ultrasonic processor DADI DA 968 at 70 % amplitude for 5 minutes. The *p*H of the dispersions is adjusted to 6-7 using dilute 0.1 N NaOH and 0.1 N HCl as needed. The stock suspension is stored at 4 °C. Prior to use, the stock suspension is adapted to room temperature (23 ± 2 °C) and sonicated (70 % amplitude, 5 minutes).

Culture media

For inoculation and submerged cultivation of yeasts was used fermentation media specific to strains in YPD study and wort [14]. The submerged cultivation was carried out in-depth capacity 1 liter Erlenmeyer flasks on the shaker (200 rpm) at a temperature of 27…28 ºC, illumination 2000-3000 Lx, the duration of cultivation 72 hours. Yeast cells in the amount of 5 %, $2x10^6$ cells·mL^{-1} were inoculated in the liquid medium.

Models of equipments and purity of reagents

For carrying out the process are required:
Reference strain - yeasts of the genus *Rhodosporidium.*
Nanoparticles of metal oxides: TiO_2, Fe_3O_4, other nanoparticles.
Required equipment:

a) For the sterilization of microbiological utensils and nutritive medium - autoclave, oven with thermostatic control;
b) For sowing and cultivating yeasts - sterile box, 200 rpm stirrers, temperature 27...28 °C; illumination 2000 Lx;
c) Glass Dishes – Petri boxes, pipettes of 1.5, 10 mL, tubes, balloons of 0.5, 0.75 and 1 L, stands;
d) Other equipment – spectrophotometer, thermostat, ultrasound processor, microscope, centrifuge, balances, refrigerator, UV lamp, filter paper, cotton wool, gauze;

Nutritional and reactive environments:

a) Wort of 7 Balling, agar, peptone, yeasts extract, dextrose;
b) Hexane, potassium iodide, acetone, 3 % H_2O_2, 0.05 M phosphate buffer, 0.5 mM EDTA, H_2O_2 3 %, anhydrous sodium sulfate.

Method of preparing the inoculums

A perfectly isolated yeast colony is suspended in 50 mL beer wort. The cell suspension is cultured on a shaker (200 rpm) for 24-48 hours, at a temperature of 28 °C, with continuous illumination 2000 Lx. The final concentration of the inoculum is determined spectrophotometrically to Spectrophotometer T60 UV VIS at wavelength λ = 600 nm [15]. As a rule, native yeasts preparation is examined microscopically between the blade and the blade. Native preparations show the existence of yeasts cells, the shape and mobility of cells, the purity of the inoculum. The content of β-carotene in the yeast biomass was determined spectrophotometric techniques as described [16, 17]. Catalase activity was determined by the method [18, 19]. Statistical processing of results was done using statistical software kit 7, veracity compared to the control $p \leq 0.05$.

RESULTS AND DISCUSSION

In previous research, it was established that the magnitude of the degree of action of the TiO_2 and Fe_3O_4 nanoparticles on the *Rhodosporidium toruloides* strain (synonym *Rhodotorula gracilis*) CNMN-Y-30 is determined by the concentration and duration of

contact with yeast [20, 21]. Following the development cycle of the population of yeasts, the viability of the cells, the activity of antioxidant enzymes, and carotenoid pigment content, there is a decrease in these indicators for the development of the stem.
Since natural antioxidant components are the first to react to toxic factors in the environment with the function of stabilizing and neutralizing the negative effects on the cell, it has become necessary to elucidate the influence of nanoparticles on the antioxidant status of the strain of yeasts. In order to select the most favorable variants that would ensure the determination of nanoparticle toxicity, experiences have been made in which titanium dioxide and magnetite nanoparticles with different sizes and concentrations that have been added to the YPD culture environment. At 72 hours of contact with nanoparticles, yeast biomass was separated by centrifugation and the *β*-carotene content and catalase activity were determined, which showed high sensitivity to the action of nanoparticles. The results obtained are generalized in Figures 1-3.
The data in Figure 1 demonstrates that the response reaction of the *Rhodosporidium toruloides* CNMN-Y-30 to the action of TiO_2 nanoparticles (30 nm) is manifested by changes in the *β*-carotene content and catalase activity. At concentrations of 0.5-15.0 $mg \cdot L^{-1}$ of nanoparticles in the cultivation environment, the *β*-carotene content decreases essentially from the control, which can be considered that the tested nanoparticles present a harmful factor for the development of yeasts culture. The response reaction of the stem to the introduction of nanoparticles in concentrations of 10-15 $mg \cdot L^{-1}$ is also expressed by decreasing the catalase activity by 13-29 % compared to the control. Thus, according to the recorded results we can say that the toxicity index (IC 50 %) can be calculated by % = Blank-test/blank x 100 by % of the concentration inhibited by TiO_2 nanoparticles (30 nm), according to the test the amount of *β*-carotene is > 10 $mg \cdot L^{-1}$.

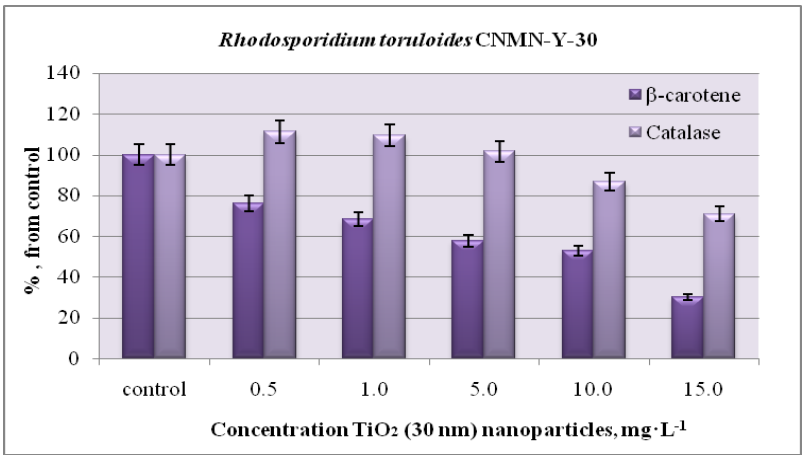

Figure 1. *The level of β-carotene and catalase activity of Rhodosporidium toruloides CNMN-Y-30 at different concentrations of TiO_2 (30 nm) nanoparticles*

With reference to the effects of another type of nanoparticles, we can affirm that during the experiments it was observed the same mode of action on yeast. From the Figure 2, it can be seen that the content of *β*-carotene in the biomass of yeasts micro is decreasing direct proportional with the concentrations of nanoparticles Fe_3O_4, with sizes of 10 nm, applied in the cultivation medium YPD. Under the influence of concentrations of

10-15 mg·L^{-1}, a substantial decrease is observed, with 66.7 % respectively by 73.4 % of the *β*-carotene quantity compared to the control. The determination of the degree of between the amount of carotenoid pigments in the yeast biomass and the concentrations of Fe_3O_4 (10 nm) nanoparticles used in the experiments demonstrated a strong association $R^2 = 0.849$.

The activity of the antioxidant enzyme catalase, at the cultivation of the strain in the presence of Fe_3O_4 nanoparticles (10 nm), is also severely affected. The response reaction of the yeasts strain in the introduction of Fe_3O_4 nanoparticles (10 nm) is expressed by decreasing the catalase activity by up to 52.3 % compared to the control sample. These disturbances can lead to a slowing of metabolic processes in the cell due to the accumulation of hydrogen peroxide in large quantities. Regression analysis that has predictive value and shows the mathematical relationship of dependence has demonstrated that its equation is y = -0,1550x + 81.14. The correlation report confirms an average dependence on the values of the catalase activity with those of the concentrations of nanoparticles, the coefficient of determination being moderate ($R^2 = 0.722$). The data in Figure 2 demonstrates that the toxicity index (IC50 %) of Fe_3O_4 nanoparticles (10 nm), according to the tests of the *β*-carotene content and the catalase activity, constitutes 10 mg·L^{-1}.

It is evident the negative influence of Fe_3O_4 nanoparticles with dimensions of 50-100 nm, present in the cultivation medium at concentrations from 0.5 mg·L^{-1} to 30 mg·L^{-1}, on biosynthesis processes of *β*-carotene and catalase activity at *Rhodosporidium toruloides* CNMN-Y-30.

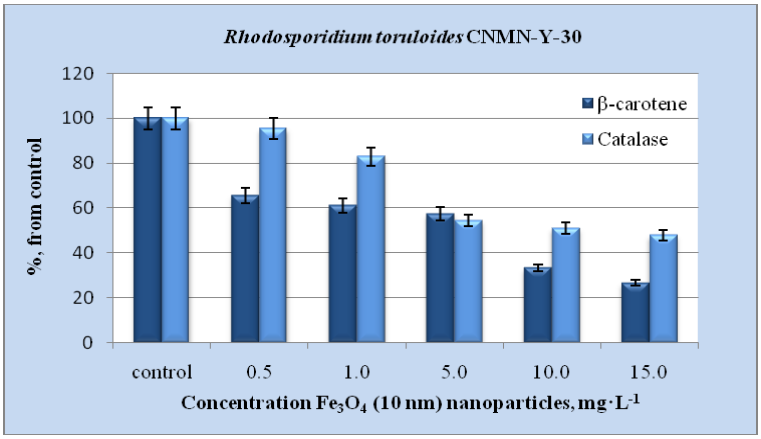

Figure 2. *Level of β-carotene and catalase activity of Rhodosporidium toruloides CNMN-Y-30 at various concentrations of Fe_3O_4 (10 nm) nanoparticles*

For all concentrations of nanoparticles used to cultivate the stem, smaller amounts of carotenoids were recorded compared with the control. The pronounced toxic effect expressed concentrations of 20, 25 and 30 mg·L^{-1}, in these variants the content of carotenoids constituted only 53.3 %, respectively 51.4 % and 30.4 % against the control (Figure 3). The determination of the correlation from contents on *β*-carotene and the concentrations of nanoparticles Fe_3O_4 (50-100 nm) used in experiments demonstrated a strong association $R^2 = 0.8391$.

In the presence of Fe_3O_4 nanoparticles (50-100 nm) the same is severely affected and the activity of catalase antioxidant enzyme. The response reaction of yeasts

Rhodosporidium toruloides CNMN-Y-30 to the introduction of nanoparticles in concentrations of 20 mg·L^{-1} is expressed by decreasing the catalase activity by 25 %, and the concentration of nanoparticles of 30 mg·L^{-1} reduces the activity of catalase with 50 % compared to the control sample. The regression analysis showing the mathematical relationship of the dependency of these two factors demonstrated that its equation is y = -9.4167x + 142.08.

The correlation report confirms an average dependence on the values of the catalase activity with those of the concentrations of nanoparticles, the coefficient of determination being moderate (R^2 = 0.6821).

Thus, the results showed that the toxicity index (IC50 %) of Fe_3O_4 nanoparticles (50-100 nm), according to the tests of the *β*-carotene content and the catalase activity, constitute 30 mg·L^{-1}.

Thus, the presented results confirm the possibility of assessing the toxicity levels of nanoparticles of metal oxides based on tests to determine the content of *β*-carotene and catalase activity at pigmented yeast *Rhodosporidium toruloides* CNMN-Y-30. The methodology for assessing the impact of nanoparticles of metal oxides provides for the research of biological indicators, in particular, the *β*-carotene content and the catalase activity at pigmented yeasts, and can be used to complement the tests estimation of the level of harmfulness and monitoring of the harmlessness of the processes for the use of nanoparticles.

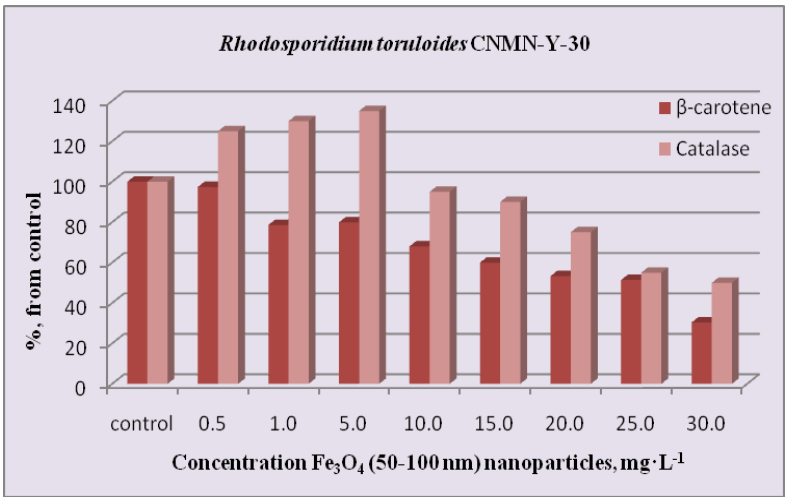

Figure 3. *Level of β-carotene and catalase activity of Rhodosporidium toruloides CNMN-Y-30 at different concentrations of Fe_3O_4 (50 -100 nm) nanoparticles*

The method for assessing the toxicity of nanoparticles provides the inoculation of pigmented yeast *Rhodosporidium toruloides* on a nutritional medium, addition of nanoparticles of oxides of metals in different concentrations, submerged cultivation within 72 hours, separation of biomass, biomass determination of *β*-carotene content and catalase activity. Concentrations of nanoparticles which cause the reduction of *β*-carotene content or catalase activity by 50 % compared to samples in which nanoparticles have not been added are considered toxic [22].

The exposed results have allowed highlighting the following parameters which ensure efficacy in the process of establishing the impact of nanoparticles on yeast. As a result

of this analysis, a new process is proposed to evaluate the toxicity of metal oxide nanoparticles with the use of pigmented yeast *Rhodosporidium toruloides*.

Protocol of the procedure for the determination of the toxicity of metal oxide nanoparticles

Prepare the sterile YPD nutrient medium with the following composition ($g \cdot L^{-1}$): peptone - 20.0; glucose - 20.0; yeast extract - 10 mL; drinking water - 1000 mL. Nanoparticles in different concentrations are added to the prepared medium. In 200 mL nutritional medium supplemented with nanoparticles add inoculum of *Rhodosporidium toruloides* ($2x10^6$ cells$\cdot mL^{-1}$), in a quantity of 5 % in volumetric basis. Cultivation is carried out in Erlenmeyer balloons with a capacity of 1.0 L, in continuous shaking conditions (200 rpm), at 28 ºC temperature, permanent illumination 2000 Lx, and duration of cultivation - 72 hours. As a control, it serves as a nutrient medium without application of nanoparticles.

After 72 hours of cultivation, the yeast biomass is separated from the nutrient medium by centrifugation at 3000 rpm for 15 minutes. The biomass obtained determines the amount of *β*-carotene and the activity of the catalase.

The calculated values for experimental samples are reported to the blank values (where no nanoparticles have been introduced) and expressed in % M.

The level of toxicity of nanoparticles is determined from the concentrations of nanoparticles that cause truthful inhibition in statistical point (IC50 %) of the *β*-carotene content in biomass or catalase activity. The integrated scheme of the process is shown in Figure 4.

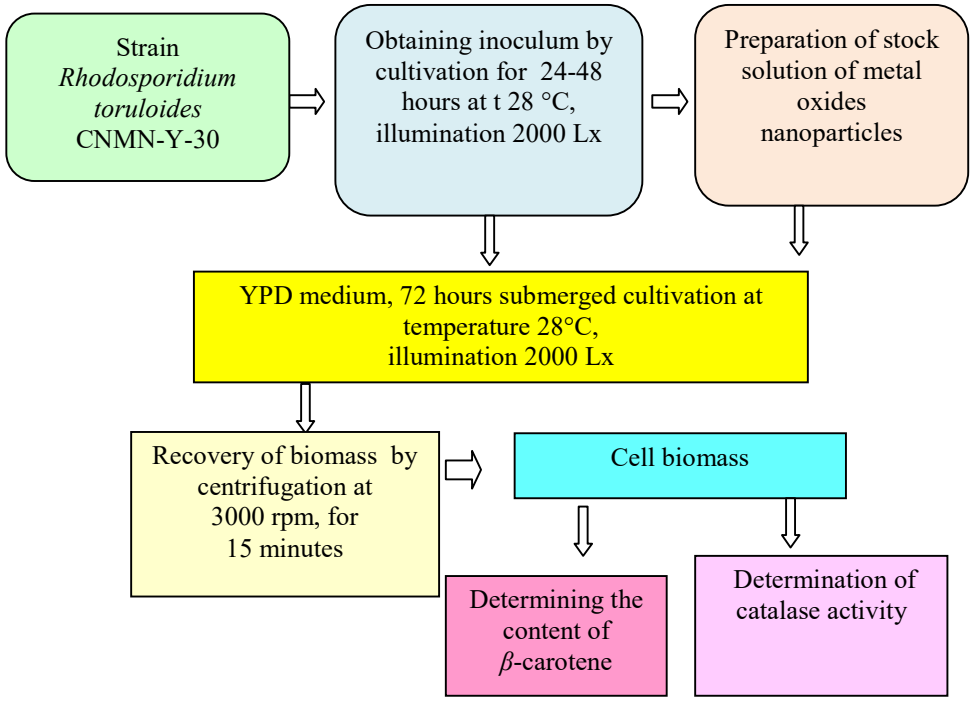

Figure 4. *Integrated scheme for the determination of nanoparticles toxicity*

CONCLUSIONS

For TiO_2 and Fe_3O_4 nanoparticles, in range of concentrations from 0.5 to 30 $mg \cdot L^{-1}$, the low level of β-carotene in the yeast biomass and catalase activity is demonstrated; a more pronounced effect is observed in variants with the application of nanoparticles with smaller dimensions. The toxicity index (IC 50 %) of TiO_2 nanoparticles (30 nm), according to the β-carotene indicator is > 10 $mg \cdot L^{-1}$, of Fe_3O_4 nanoparticles (10 nm), according to the β-carotene content indicators and the catalase activity constitutes 10 $mg \cdot L^{-1}$, and of Fe_3O_4 nanoparticles (50-100 nm), according to the β-carotene content and catalase activity constitutes 30 $mg \cdot L^{-1}$.
Experiments carried out allow proposing the alignment of pigmented yeast *Rhodosporidium toruloides* as a biological model for the purpose of solving the risks related to the use of nanotechnologies and products that contain nanoparticles.

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