ASPECTS OF DIRECTED SYNTHESIS OF PROTEASES IN MYCELIAL FUNGI USING METAL NANO-OXIDES

A. Ciloci[1], J. Tiurina[1], S. Labliuc[1], E. Dvornina[1], S. Clapco[1], C. Bivol[1], T. Guțul[2], E. Rusu[2], and D. Dvornicov[2]

[1]*Institute of Microbiology and Biotechnology, Academy of Sciences of Moldova, Academiei str. 1, Chisinau, MD-2028 Republic of Moldova*
[2]*Ghitu Institute of Electronic Engineering and Nanotechnologies, Academy of Sciences of Moldova, Academiei str. 3/3, Chisinau, MD-2028 Republic of Moldova*
E-mail: tatiana.g52@mail.ru

(Received October 16, 2017)

Abstract

Data on the effect of some metal nano-oxides on the biosynthesis of individual components of proteolytic enzymes complex of micromycetes *Trichoderma koningii* CNMN FD 15 and *Fusarium gibbosum* CNMN FD 12 have been described. For each strain, optimum conditions of cultivation in the presence of the studied nanoparticles, which provide an increased level of proteolytic activity, have been determined. It has been emphasized that the stimulating effect depends on the composition, size, and concentration of the nanomaterials and on the initial pH value of the medium.

1. Introduction

In the last few years, the use of extracellular enzymes produced by microorganisms has expanded with the intensive development of biotechnology. Thus, optimization of enzyme biosynthesis remains relevant. A particular interest is given to finding ways to stimulate biosynthesis with the use of different factors (physical, chemical, etc.).

In this context, the use of nanometer materials involved in the vital processes of organisms at the cellular and subcellular level is promising. The range of physicochemical properties of nanoparticles differs from that of larger particles (increased reactivity and catalytic activity, adsorption capacity, formation of a large number of chelated compounds, possibility of crossing biological membranes, etc.). Nanoparticles are used in various branches of industry owing to their unique properties. In recent years, it has been shown that they also exhibit biological activity and can be used in various fields of medicine, biology, and biotechnology to control the physiological and biochemical functions in cells [1–8].

The importance of studying the effect of nanoparticles on living organisms has significantly increased. Reports on the toxic [9–11] and stimulating effect of different types of nanoparticles on growth, development, and productivity (synthesis of biologically active substances) in microorganisms [12–14] have been published.

The aim of the paper was to study the effect of some metal nano-oxides on the biosynthesis of microorganisms *Trichoderma koningii Oudemans* CNMN FD 15 and *Fusarium gibbosum* CNMN FD 12, which are active producers of exocellular protease enzymes [15, 16].

2. Materials and Methods

The objects of study were two strains of micromycetes with a high level of hydrolase biosynthesis—*Trichoderma koningii Oudemans* CNMN FD 15 and *Fusarium gibbosum* CNMN FD 12—producers of proteases.

Cultivation of micromycetes was carried out in 1.0-L Erlenmeyer flasks with 200 mL of a nutritive medium at a temperature of 28–30°C under agitation at a rate of 180–200 rpm using a culture medium with the following composition (g/L): wheat bran, 20.0; soybean flour, 10.0; $(NH_4)_2SO_4$, 1.0; and $CaCO_3$, 2.0; a pH of 6.25.

The seed material was a spore suspension with a density of 2×10^6–3×10^6 spores/mL, which was prepared by washing a 15-day culture grown on malt-agar oblique columns with sterile distilled water. The amount of the seed material in each flask was 10% v/v.

Metal oxides ZnO and Fe_3O_4 selected in previous studies as a stimulator of proteolytic enzymes were used in the research. Nanoparticles with different sizes (10, 30, 65–70 nm; 1 μm) were added to the sterile culture medium in a concentration of 1, 5, 10, and 15 mg/L concomitantly with the inoculum.

Solutions with a required concentration of nanoparticles were prepared from the solid or liquid initial preparations using deionized water at a pH of 6.25. Immediately before introduction into the nutrient medium, the prepared solutions were ultrasonically treated in an AOYU-9050 WilTec ultrasonic bath (Wildanger Technik GmbH, Germany, 30–50W, frequency of 40 kHz) for 5 min to provide the dispersion of the nanoparticles.

The basic culture medium without nanomaterials was used as a reference medium.

Proteolytic activity was assayed in the dynamics of cultivation on the 4^{th}–6^{th} day for the *Fusarium gibbosum* CNMN FD 12 and on the 8^{th}–10^{th} day for *Trichoderma koningii* Oudemans CNMN FD 15; this is the period of maximum accumulation of enzymes in the growth of this strain under conventional conditions.

The activity of exocellular protease was determined by the Willstatter method via measuring the amount of released free carboxyl groups in an ethanol solution of amino acids and polypeptides obtained by hydrolysis of a 5% gelatine substrate with a pH of 7.4 for neutral proteases and a pH of 3.6 for acid proteases after 3-h incubation at 40°C [17].

All the experiments were performed thrice; the results were averaged over three measurements. The level of significance was $P < 0.05$ [18].

3. Results and Discussion

Proteases represent a complex of enzymes that provide partial or complete hydrolysis of proteins to oligomers and monomers (amino acids) owing to their ability to hydrolyze the peptide bonds. Depending on the nature of the functional groups in the active center, proteases are classified into six large groups: serine proteases, cysteine proteases, threonine proteases, aspartic proteases, glutamic proteases, and metalloproteases [19], which determine their use for solving both theoretical problems (study of the metabolism of living organisms) and practical problems by applying them in various fields of industry, agriculture, medicine, etc.

As a result of the screening of nanomaterials reported in [20], it was found that the most effective way to stimulate protease biosynthesis in micromycetes *Trichoderma koningii* and *Fusarium gibbosum* is to use nanoparticles from composite ZnO/MgO (1 : 4), ZnO, and Fe_3O_4. The obtained results suggest that the effect of nanomaterials on the biosynthesis of exocellular hydrolases in micromycetes depends on the type and structure of the nanocompounds, the

physiological and biochemical properties of the strain, and the properties of the synthesized enzyme complex.

In this study, ZnO nanoparticles were selected for *Trichoderma koningii* strain and Fe_3O_4 nanoparticles were used for *Fusarium gibbosum*. An essential parameter of nanoparticles, which determines their physicochemical and biological properties, is their size. Another equally important parameter is their concentration in the culture medium that can affect the living organism. Thus, for a number of metals, a narrow concentration range is required to provide the vital activity of living organisms or express their toxicity [21]. The effect of different sizes and concentrations of ZnO and Fe_3O_4 nanoparticles on the proteolytic activity of *T. koningii* CNMN FD 15 and *F. gibbosum* CNMN FD 12, respectively, was estimated to determine the biologic properties of the tested nanoparticles.

To reveal the effect of ZnO nanoparticles on the activity of acid, neutral, and alkaline protease of *T. koningii* producer, ZnO nano-oxides with a size of 10 and 30 nm and 1 μm in a concentration of 5, 10, and 15 mg/L were studied (Fig. 1).

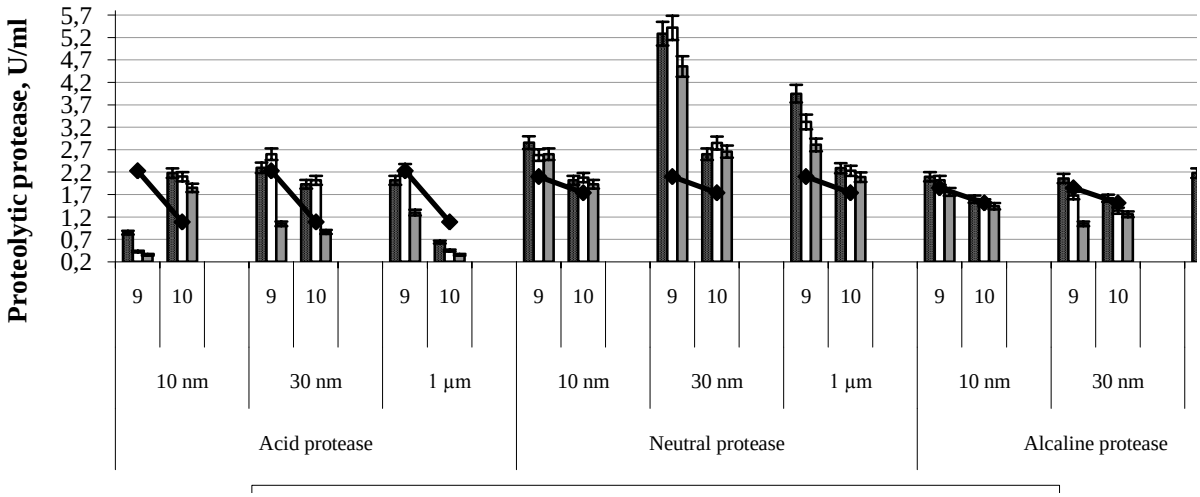

Fig. 1. Effect of ZnO nanoparticles with different sizes and concentrations on the proteolytic activity of fungal strain *Trichoderma koningii* CNMN FD 15.

The highest level of proteolytic activity in the reference samples was observed on the 9th day of cultivation of the strain, being 2.10 U/mL for neutral proteases, 2.23 U/mL for acid proteases, and 1.85 U/mL for alkaline proteases. In the optimized versions, the ZnO nanoparticles showed different effects on the activity of these three types of proteases synthesized by micromycete, depending on the particular size and concentration. Thus, for acid proteases, the 10-nm ZnO nanoparticles retained the biosynthesis for 24 h; the highest values were observed on the 10th day of the cultivation. The level of activity of acid proteases in the presence of ZnO nano-oxide on the 10th day was similar to the activity of the reference sample on the 9th day of cultivation. The 30-nm nanoparticles showed a moderate stimulating effect (16.6% compared with the reference sample at a concentration of 10 mg/L) on the 9th day of cultivation. The size of 1 μm showed a neutral effect.

In the case of neutral proteases, ZnO nanoparticles of all tested sizes showed a significant positive action depending on the applied concentration of 5–15 mg/L. The stimulating effect varied as follows: from 36.2 to 22.8% for a size of 10 nm; from 151.9 to 117.1% for 30 nm; and from 88.1 to 33.8% for 1 μm. The highest proteolytic activity was obtained in the case of 30 nm;

the activity increased by 2.5 times on the 9th day of the cultivation.

For alkaline protease, the use of ZnO nanoparticles of all sizes, except for the concentration of 5 mg/L, provided an increase in protease activity; however, the effect was less significant than that for neutral proteases: by 13.5% for 10 nm, by 11.3% for 30 nm, and by 17.8% for 1 μm compared with the reference sample on the 9th day of cultivation. For all types of proteases, an inhibitory effect of ZnO nanoparticles was observed with an increase in the concentration to 15 mg/L. Regardless of size, the proteolytic activity of *T. koningii* micromycete decreased.

On the 10th day of cultivation, the enzyme activity of neutral and alkaline proteases in the presence of ZnO nanoparticles was reduced; the samples with 10-nm ZnO in all tested concentrations for neutral proteases and all experimental samples of alkaline proteases showed values at the level of the reference sample. The moderate stimulating effect of some samples grown with 30-nm ZnO in the case of acid proteases and 30-nm and 1-μm ZnO in the case of neutral proteases did not exceed the level of the reference sample on the 9th day of cultivation.

The results showed that ZnO nanoparticles with a size of 30 nm taken in a concentration of 5–10 mg/L are the most promising for stimulating the protease biosynthesis of fungal strain *T. koningii* CNMN FD 15. These parameters of ZnO nanoparticles provide an increase in the activity of neutral proteases—the basic enzymes of the strain—by 2.5 times compared with the maximal value obtained for the reference sample, with an increase in the activity of acid and alkaline proteases by 16.6 and 11.3%, respectively, without any modification of the period of maximal synthesis of proteases (on the 9th day of cultivation).

A similar study was conducted for the *F. gibbosum* CNMN FD 12 micromycete—the producer of acid (pH of 3.6) and neutral proteases (pH of 7.4)—using Fe_3O_4 nanoparticles of different sizes (10, 30, 65–70 nm) taken in three concentrations (5, 10, 15 mg/L) (Fig. 2). The most significant level of proteolytic activity in the reference samples was observed on the 5th day of cultivation: 2.04 U/mL for acid proteases and 2.52 U/mL for neutral proteases.

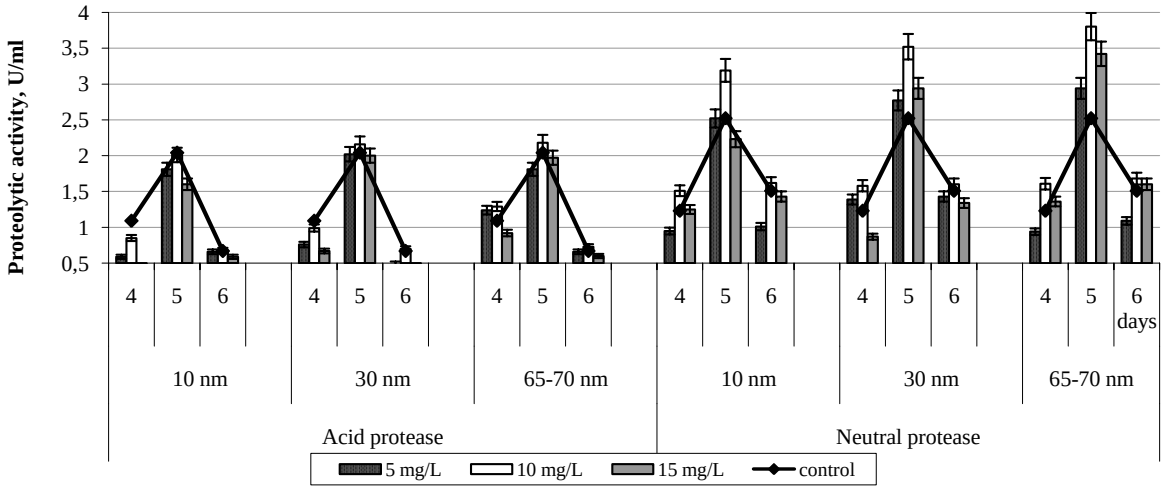

Fig. 2. Effect of Fe_3O_4 nanoparticles with different sizes and concentrations on the proteolytic activity of fungal strain *Fusarium gibbosum* CNMN FD 12.

In the optimized samples, the addition of Fe_3O_4 nanoparticles in the nutrient medium of the strain, regardless of size, had hardly any effect on the activity of acid proteases; their value remained at the control level of 98.5% (for 10 nm), 105.9% (for 30 nm), and 106.8%

(for 65–70 nm). Iron oxide was more effective for the production of neutral proteases than for acid proteases. The optimum concentration was 10 mg/L, regardless of size. This concentration stimulated the activity of neutral proteases as early as the 4th day of cultivation of the micromycete; it was 22.7% (for 10 nm), 28.5% (for 30 nm), and 30.9% (for 65–70 nm) higher than that of the reference sample in the same day (1.23 U/mL). On the 5th day of cultivation, the stimulating effect increased to 26.6% (for 10 nm), 39.7% (for 30 nm), and 50.8% (for 65–70 nm) compared with the maximum of the reference sample (2.52 U/mL) observed in the same day.

On the 6th day of cultivation, the activity of both neutral and acid proteases decreased in both the reference and optimized samples.

The optimum parameters of the Fe_3O_4 nanoparticles that provide the stimulating effect on the biosynthesis of proteases in micromycete *Fusarium gibbosum* are the nanoparticle size of 65–70 nm and the concentration of 10 mg/L. These conditions provide a 6.8 and 50.8% increase in the biosynthesis of acid proteases and neutral proteases, respectively, while maintaining the period of maximum synthesis of proteases (on the 5th day).

The next stage of the research was the submerged cultivation of *T. koningii* and *F. gibbosum* strains on a nutrient media supplemented with selected ZnO and Fe_3O_4 nano-oxides and different values of initial pH of the media, namely, acidic (pH of 4.0), neutral (pH of 6.25), and alkaline (pH of 8.5). The aim of the study was the estimation of the effect of the initial pH of the medium on the capacity of the strains to produce enzymes and on the biological properties of the nanoparticles.

For micromycete *T. koningii*, the experiment was conducted in the presence of ZnO nanoparticles with an optimum selected size of 30 nm in a wider concentration range—1, 5, 10, and 15 mg/L—and at the three above mentioned initial pH values of the nutrient medium (Fig. 3).

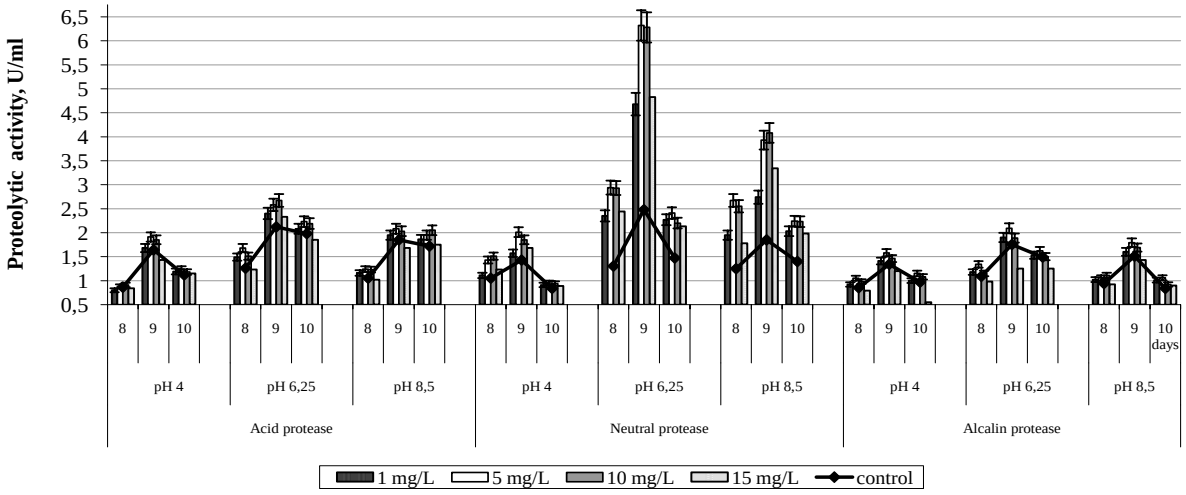

Fig. 3. Effect of ZnO nanoparticles with a size of 30 nm on the proteolytic activity of *Trichoderma koningii* CNMN FD 15 as a function of initial pH values of the nutrient medium.

The results showed that, in the reference samples containing no ZnO nanoparticles, the maximum activity of all the three types of proteases was observed on the 9th day of the cultivation of the strain, regardless of the modification of the pH of the medium. The use of the media with an initial pH of 6.25 was the most favorable for biosynthesis. The activity of acid proteases was 2.12 U/mL; neutral proteases, 2.48 U/mL; and alkaline proteases, 1.75 U/mL.

The most optimum concentrations of ZnO nanoparticles (30 nm) were concentrations of 5–10 mg/L; they provided an increase in all the three types of proteases. The maximum activity (similar to that of the reference sample) was observed on the 9th day of the cultivation of the strain; however, the proteolytic activity was modified as a function of the initial pH of the medium. The most significant enzyme activity was observed at an initial pH of the medium of 6.25 in the case of an optimum concentration of nanoparticles of 5 and 10 mg/L. Thus, the activity of acid proteases was 2.58 and 2.67 U/mL, respectively; it was higher than the activity in the reference sample by21.7 and 25.9%, respectively. In the case of neutral proteases, the activity was 6.32 and 6.28 U/mL, respectively, compared with 2.48 U/mL in the reference sample. The stimulating effect was higher by 154.8 and 153.2%, respectively. The activity of alkaline proteases was 2.09 and 1.88 U/mL, respectively, and the stimulating effect was higher by 19.4 and 8.0%, respectively.

For *F. gibbosum* micromycete, the experiment was conducted in the presence of Fe_3O_4 nanoparticles with an optimum selected size of 65–70 nm taken in a concentration of 5, 10, and 15 mg/L at three values of the initial pH of the nutrient medium (Fig. 4).

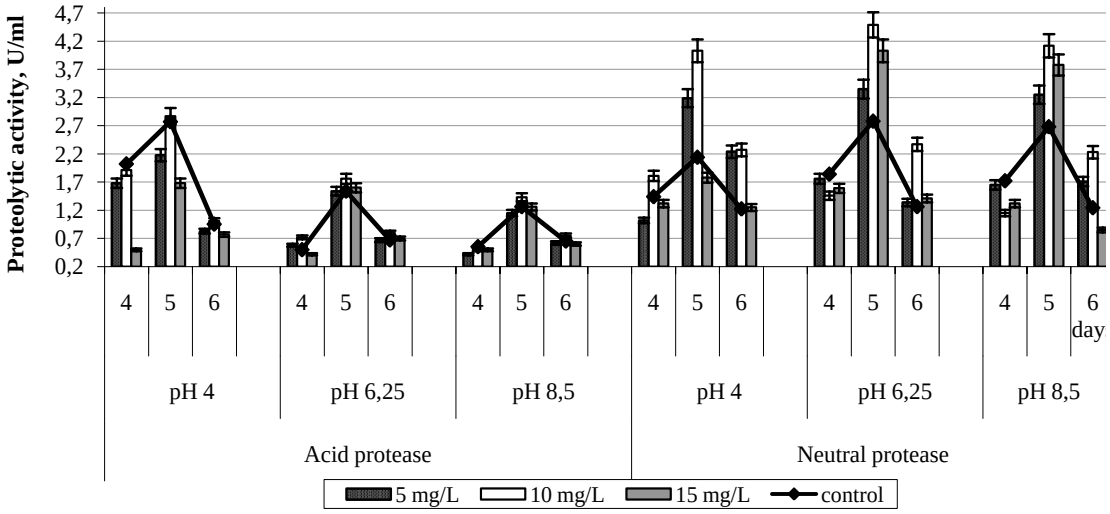

Fig. 4. Effect of Fe_3O_4 nanoparticles with a size of 65–70 nm on the proteolytic activity of *Fusarium gibbosum* CNMN FD 12 as a function of the initial pH values of the nutrient medium.

The results showed that the reference samples had the maximal activity of both acid and neutral proteases on the 5th day of cultivation, regardless of the initial pH of the medium. The use of acidic pH of the medium was the most effective for the biosynthesis of acid proteases; in this case, the enzyme activity was 2.77 U/mL. An increase in the pH value to 6.25 and 8.5 led to a decrease in the activity of acid proteases to 1.54 and 1.25 U/mL, respectively. The use of the initial pH of the medium that was close to the neutral pH (pH of 6.25 and (less significant) pH of 8.5) was the most promising for the biosynthesis of neutral proteases. The determined proteolytic activity was 2.78 and 2.58 U/mL, respectively.

The most optimum concentration of the Fe_3O_4 nanocompound (65–70 nm) was 10 mg/L. The maximum activity of both acid and neutral proteases was observed on the 5th day of the cultivation of the producer (similar to the reference sample). Thus, the use of nano-oxide did not change the period of maximum synthesis of proteases; however, it led to an increase in the

enzyme activity. In addition, as the enzymatic activity of the reference was lower, the stimulatory effect in the optimized samples was higher; it varied as a function of the initial pH of the medium.

The highest activity of acid proteases was observed at a pH of 4.0; it was 2.77 U/mL in the reference samples and 2.87 U/mL in the optimized samples. The stimulatory effect was insignificant: at a level of 3.6%. The highest activity of neutral proteases was observed at a pH of 6.25; it was 2.78 U/mL in the reference samples and 4.49 U/mL in the optimized samples. The stimulatory effect was 67.5%.

Comparative analysis of the results obtained for both *T. koningii* and *F. gibbosum* strains showed that the initial pH value significantly affects the genesis of proteases and the biological properties of ZnO and Fe_3O_4 nano-oxides. For *T. koningii* micromycete, the most optimum value of pH is 6.25; it provided the biosynthesis of all the three types of proteases and determined the highest stimulating effect.

For *F. gibbosum*, the highest activity of the acid proteases was obtained at a pH of 4.0; however, the stimulating effect was hardly observed. The alkaline proteases of the strain were less significantly affected by changes in the pH of the medium. The highest activity of alkaline proteases and the most significant stimulating effect were observed at a pH of the medium of 6.25.

A decrease in the stimulating effect with a variation in the initial pH of the medium from an optimum value (pH of 6.25) can indicate changes in the biological properties of the ZnO and Fe_3O_4 nanoparticles, which can be attributed to a change in another important parameter, which is referred to as zeta potential; it is no less important as the particle size and, according to some authors, depends on the pH of the medium [11, 22].

4. Conclusions

It has been found that the biological effect of the nanoparticles varies as a function of the taxonomy of micromycetes and their physiological and biochemical properties and as a function of parameters of nanoparticles (size, concentration, and zeta potential).

ZnO and Fe_3O_4 nanoparticles can be used as potential stimulators of the biosynthesis of all the three types of proteases of micromycete *Trichoderma koningii* Oudemans CNMN FD 15. The highest stimulating effect has been achieved in the case of using ZnO nanoparticles with a size of 30 nm taken in a concentration of 5–10 mg/L for the cultivation of the strain on a nutrient medium with an initial pH value of 6.25. These conditions provide an increase in the activity of neutral proteases—the basic component of the proteolytic complex in this strain—by 2.5 times, with an increase in the activity of acid and alkaline proteases by 16.6 and 11.3%, respectively.

For strain *Fusarium gibbosum* CNMN FD 12, the most significant stimulating effect has been achieved in the case of using Fe_3O_4 nanoparticles with a size of 65–70 nm taken in a concentration 10 mg/L for the cultivation of the micromycete on a nutrient medium with an initial pH value of 6.25. These conditions provide an increase in the activity of neutral proteases by 61.5% compared with the reference sample and a less significant increase in the activity of acid proteases—by 14.3%.

The tested nanoparticles did not affect the duration of the life cycle of the micromycetes. In both cases, the period of maximum synthesis of exocellular proteases coincided with the optimum period of cultivation under conventional conditions: on the 5^{th} and 9^{th} day of the cultivation of *Fusarium gibbosum* CNMN FD 12 and *Trichoderma koningii* Oudemans CNMN FD 15, respectively.

References

[1] I. N. Andrushina, I. A. Golub, and G.G. Didikin, Biotekhnologiya 4 (6), 51 ((2011).
[2] Metal Nanoparticles in Microbiology, edited by M. Rai, and N. Duran (Springer-Verlag, Berlin Heidelberg, 2011).
[3] R. Walisko, R. Krull, J. Schrader, and C. Wittmann, Biotechnol. Lett. Nov. 34 (11), 1975 (2012).
[4] G. I. Churilov, A. A. Nazarova, S. D. Polishchuk, S. D. Ivanycheva, and T.V. Zheglova, Vestn. Ryazan Gos. Agrotekh. Univ. 2, 30 (2009).
[5] K. G. Seghal, L. A. Nishanth, S. Priyadharshini, K. Anitha, and J. Selvin, BMC Biotechnol. 14, 48 (2014).
[6] Razrabotka, issledovanie i marketing novoi farmatsevticheskoi produktsi: sbornik nauchykh trudov, edited by M.V. Gavrilina (Pyatigorsk Gos. Farmatsevt. Akad., Pyatigorsk, issue 64, 2009).
[7] G. V. Pavlov and G. E. Folmanis, Biologicheskaya aktivnost' ul'tradispersnykh poroshkov (Issled. Tsentr Probl. Kachestva Podgotovki Spetsialistov, Moscow, 1999).
[8] D. V. Vinogradov and L. V. Potapova, Mezhdunarodn. Tekh.-Ekon. Zh. 3, 40 (2009).
[9] G. B. Zavil'gel'skii, V.Yu. Kotova, S.A. Khrul'nova, and I.V. Manukhov, Biotekhnologiya, 6, 8 (2013).
[10] Yu. P. Mukha, A. M. Eremenko, and N. P. Smirnova, Prikl. Biokhim. Mikrobiol. 49 (2), 215 (2013).
[11] R. T. Minullina, Otsenka toksichnosti nanomaterialov s ispol'zovaniem mikroorganizmov, Extended Abstract of Doctoral Dissertation, Kazan, 2014.
[12] R. Mahendra, E. Nelson Duran, Metal Particles in Microbiology (Springer, Berlin–Heidelberg–New York, 2011).
[13] K. B. Deependra and P. Subhankar, Appl. Biochem. Biotechnol. 173 (1), 155 (2014).
[14] H. Driouch, B. Sommer, and C. Wittmann, Biotechnol. Bioeng. 105 (6), 1058 (2010).
[15] A. Deseatnic-Ciloci, J. Tiurina, C. Bivol, S. Clapco, S. Labliuc, E. Dvornina, and M. Stratan, MD patent 4285, 2014.
[16] A. Deseatnic-Ciloci, J. Tiurina, G. Lupaşcu, S. Clapco, S. Labliuc, M. Stratan, E. Dvornina, and E. Sasco, MD patent 4186, BOPI 11/2012.
[17] I. M. Gracheva, Yu. P. Grachev, et al., Laboratornyi praktikum po tekhnologii fermentnykh preparatov (Legk. Pishch. Prom-st, Moscow, 1982).
[18] B. Dospekhov, Planirovanie polevogo opyta i statisticheskaya obrabotka dannykh (Kolos, Moscow, 1985).
[19] N. N. Kudryavceva, A. V. Sof'in, T. A. Revina, E. L. Gvozdeva, E.V. Ievleva, and T. A. Valueva, Prikl. Biokhim. Mikrobiol. 49 (5), 513 (2013).
[20] A. Chilochi, Zh. Tyurina, C. Lablyuk, E. Dvornina, C. Klapko, Ch. Bivol, T. Gutsul, E. Russu, and A. Nikorich, Bull. Acad. Stiinte Mold., Stiintele Viatii 3 (330), 164 (2016).
[21] D. A. Lemenovskii, Soros. Obraz. Zh. 9, 48 (1997).
[22] I. A. Gretskii, N. M. Zholobak, A. B. Shcherbakov, V. K. Ivanov, and E. N. Gromozova, Zhiv. Biokosn. Sist. 15 (2016).