EFFECT OF NANO-OXIDES TiO_2 AND Fe_3O_4 ON LIPASE BIOSYNTHESIS BY *ASPERGILLUS NIGER* CNMN-FD-01 MICROMYCETE

Bivol Cezara[1], Ciloci Alexandra[1], Tiurina Janeta[1], Labliuc Svetlana[1], Clapco Steliana[1], Dvornina Elena[1], Guţul Tatiana[2], Rusu Emil[2]

[1]*Institute of Microbiology and Biotechnology of Academy of Sciences of Moldova*
[2]*Institute of Electronic Engineering and Nanotechnologies„D. Ghitsu" of Academy of Sciences of Moldova*

Rezumat

Lucrarea prezintă rezultatele investigaţiei efectului nanoparticulelor TiO_2 şi Fe_3O_4 de diferite dimensiuni şi concentraţii asupra biosintezei lipazelor în dinamica zilelor 4-6 de cultivare la *Aspergillus niger* CNMN-FD-01. Nanoparticulele TiO_2 cu dimensiunea 40nm în concentraţia de 10mg/l au asigurat un spor semnificativ al activităţii lipolitice a producătorului, de 78,57% în ziua a 4-a şi de 57,49% în ziua a 5-a de cultivare. Nano-oxidul TiO_2 a determinat o accelerare a ritmului de biosinteză a lipazelor cu 24 h, probele suplimentate cu 10mg/l TiO_2 de dimensiunea 40nm prezentând în a 4-a zi de cultivare o creştere a activităţii lipolitice cu 25,00% faţă de indicele maximal al martorului înregistrat în a 5-a zi. În comparaţie, nanoparticulele Fe_3O_4 au manifestat influenţă neutră sau slab stimulatoare în ziua a 4-a de cultivare şi puternic inhibitoare în ziua a 5-a şi a 6-a de cultivare a micromicetei *A. niger* CNMN-FD-01.

Cuvinte cheie: Aspergillus niger, lipaze, nanoparticule, biosinteză.
Depus la redacţie 05 iunie 2017

Adresa pentru corespondenţă: Ciloci Alexandra, Institutul de Microbiologie şi Biotehnologie al Academiei de Ştiinţe a Moldovei, str. Academiei, 1, MD2028 Chişinău, Republica Moldova; E-mail: alexandra.ciloci@gmail.com; tel. (+373 22) 739824

Introduction

Lipases, members of the hydrolase family, are wide-spread enzymes that have been isolated from a variety of sources: animal, vegetal and microbial. Microbial lipases have a huge potential in food technologies, medical sciences and chemical industry, demonstrating broad substrate specificity and increased stability in organic solvents [9].

Mycelial fungi offer a number of obvious advantages in lipase production compared to other sources, including: the ability to grow on low-cost nutrient media prepared from the byproducts of food industry and to produce significant amounts of exocellular enzymes easily recoverable from the cultural liquid by simple and accessible

techniques; the adaptive metabolism that allows directing biosynthetic processes. Mycelial lipases are resistant and active in acid pH and demonstrated broad substrate specificity [5, 7, 13].

An innovative concept in the biotechnological production of microbial lipases is the use of nanoparticles as stimulating and regulating factors of biosynthetic processes of microorganisms [1, 3, 6, 8, 10, 12]. Nanoparticles present a unique tool for manipulating the biosynthetic activity of microorganisms, with proven effectiveness on biotechnological objects from various taxonomic groups. The literature presents the inhibiting, but lately, also stimulating effect of nanoparticles on the growth and development of mycelial fungi. This demonstrates the importance of studying the stimulating effect of nanoparticles that hides a huge biotechnological potential. Thus, the addition of ZnO nanoparticles to the culture medium of *Aspergillus terreus* and *Aspergillus flavus* induced an increase in the synthesis of alkaline and acidic phosphatases by 14-22%, of phytase - up to 144% [11]. The addition of $TiSiO_4$ microparticles to the culture medium of two strains of *Aspergillus niger* in submerged culture showed an increase in the activity of fructofuranosidase by 3.7 times and in the activity of glucoamylase by 9.5 times [4].

The aim of the research was to evaluate the effect of TiO_2 and Fe_3O_4 metal nano-oxides on the biosynthesis of exocellular lipase in the *Aspergillus niger* CNMN-FD-01 micromycete strain.

Materials and methods

Object of study was the mycelial fungi *Aspergillus niger* CNMN-FD-01 with biotechnological significance, distinguished by high and stable synthesis of exocellular lipases [2].

The culture is stored at the National Collection of Nonpathogenic Microorganisms of the Institute of Microbiology and Biotechnology, Academy of Sciences of Moldova.

Cultivation of micromycete strain was carried out in Erlenmeyer flasks of 0.5 L volume, with 100 ml nutritive medium, under 180-200 rpm agitation at the temperature of 28 to 30°C. Cultivation lasted 4-6 days – period of maximum accumulation of lipases in this strain. The liquid fermentation media was inoculated with spore suspension with density of $2\text{-}3 \bullet 10^6$ spores/ml, obtained by washing with sterile distilled water a 14 days culture grown on malt-agar oblique columns. The amount of seed material in each flask constituted 5-10% v/v.

As the control medium was used the basic culture medium of the studied micromycete with the following composition (g/L): soybean flour - 35.0; KH_2PO_4 - 5.0; $(NH_4)_2SO_4$ - 1.0; pH – 7.0-7.2. As experimental media were used the basic media supplemented with nanoparticles with distinctive characteristics and determined concentrations.

Nanomaterials. Metal oxides TiO_2 (of 20nm, 40nm, 1μm) and Fe_3O_4 (of 10nm, 30-35nm, 70nm) were used in the research. The nanoparticles were supplemented in the culture medium in the concentration of 5-10 mg/L concomitantly with the inoculum.

The lipolytic activity was assayed by the modified Otto-Yamad method, according to the degree of hydrolysis of the olive oil in the solution of polyvinyl alcohol up to oleic acid. One unit of lipolytic activity was defined as the amount of enzyme (the amount of culture liquid), with determined the formation of 1μmol of oleic acid from the 40% olive oil emulsion in polyvinyl alcohol at a pH of 7.2 and a temperature of 37°C during 1 hour [14].

All the experiments were done in triplicate and the results are presented as the mean of three. The level of significance is $P<0.05$ [15].

Results and discussions

Recent researches performed in the Enzymology Laboratory from the Institute of Microbiology and Biotechnology of Academy of Sciences of Moldova to evaluate the influence of nanoparticles MgO, TiO_2, ZnO, Fe_3O_4 on biosynthesis of exocellular enzymes in mycelial fungi demonstrated their differentiated action, depending on the nature of the nanoparticles and their size and concentration [16].

On the same note, the effect of TiO_2 and Fe_3O_4 nanoparticles (NPs) of various sizes and concentrations on lipase biosynthesis in *A. niger* CNMN-FD-01 – the producer of lipolytic enzyme was studied in the dynamics (4^{th} to 6^{th} day of cultivation). The results, presented in Table 1 and Figures 1 and 2, demonstrated the stimulating effect of TiO_2 nanoparticles on lipolytic activity in the *A. niger* CNMN-FD-01 micromycete. TiO_2 nano-oxide increased considerable the enzymatic activity more than 1.5 times and accelerated the lipase biosynthesis by 24 h, with a concomitant shortening of the enzyme synthesis duration only on 2 days (4^{th} and 5^{th} day).

Table 1. The influence of TiO_2 and Fe_3O_4 nanoparticles of different sizes and concentrations on the dynamics of lypolitic activity in *A. niger* CNMN-FD-01 micromycete.

Nanoparticles	Sizes	Concentration, mg/L	Lipolytic activity, U/ml		
			4^{th} day	5^{th} day	6^{th} day
TiO_2	20 nm	5	14437	13970	0
		10	15896	10937	0
	40 nm	5	16596	21729	0
		10	21875	27562	0
	1 µm	5	15690	5542	0
		10	14204	7000	0
Fe_3O_4	10 nm	5	12250	3937	0
		10	12979	3500	0
	30-35 nm	5	12250	5308	1250
		10	12250	7875	1250
	70 nm	5	14000	2042	0
		10	13125	2333	0
Control		0	12250	17500	4687

All sizes and concentrations of tested TiO_2 nanoparticles showed on day 4 of *A. niger* CNMN-FD-01 cultivation a stimulatory effect on lipase biosynthesis compared to the control sample. The most obvious increase was observed for the nanoparticles of 40 nm, which ensured a 35.47% increase at NPs concentration of 5mg/L and 78.57% increase at the concentration of 10mg/L (Figure 1). On the 5^{th} day of cultivation TiO_2 NPs of 40nm showed an increase in enzymatic activity by 24.65% and 57.49% at the concentration of 5mg/L and 10mg/L, respectively, compared to the control of the corresponding day (Figure 2).

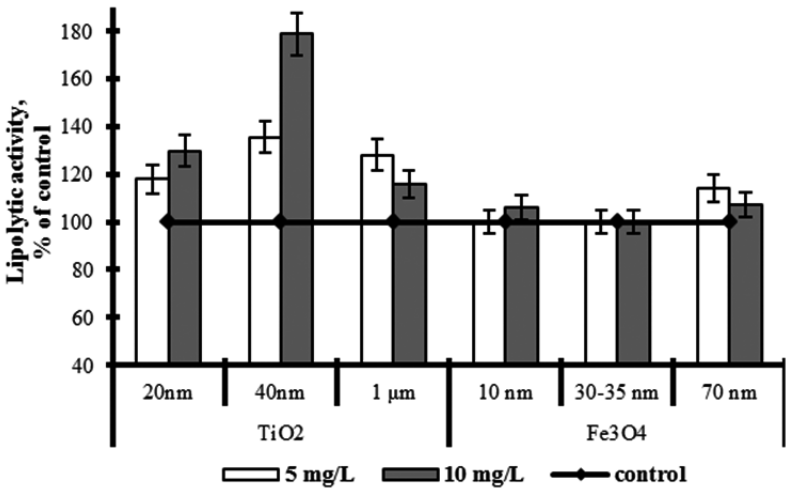

Figure 1. Influence of TiO_2 and Fe_3O_4 nanoparticles on the lipolytic activity of *A. niger* CNMN FD 01 micromycete on the 4th day of cultivation.

Titanium oxide NPs with dimension of 20nm stimulated less the lipolytic activity of the culture. The increase was observed only on the 4th day of cultivation. Lipolytic activity exceeded the control sample with 17.85% at the concentration of 5mg/L and with 29.76% at the concentration of 10mg/L. On the 5th day of cultivation, the indices of lipolytic activity in *A. niger* CNMN-FD-01 were negative. Enzymatic biosynthesis was reduced compared to the control by 20.18% at the concentration of 5mg/L and 37.51% at the concentration of 10 mg/L.

An identical effect has been seen, as well, at the addition of TiO_2 NPs with dimension of 1µm. On day 4 the lipase biosynthesis increased by 28.08% in the presence of 5mg/L TiO_2 and by 15.95% in the presence of 10mg/L TiO_2. On the 5th day of cultivation the lipolytic activity of the producer decreased by 68.31% at the concentration of 5mg/L TiO_2 and by 60.00% at 10mg/L compared to the control samples.

On the 6th day of *A. niger* CNMN-FD-01 cultivation, no lipolytic activity was detected in the presence of TiO_2. The control sample showed a minimum lipolytic activity (4687 U/ml), being 2.6 times lower than the control in the day 4 and 3.7 times lower than the value of the control in the day 5 (Table 1). Thus, the dynamic of exocellular lipase activity in *A. niger* CNMN-FD-01 showed the highest level of enzymatic activity on the 5th day of cultivation of the producer both in the control sample and in the experimental variants, and namely the samples that was supplemented with 10mg/L TiO_2 of 40 nm. However, although the enzymatic activity of the strain grown in the presence of titanium oxide was lower on day 4 of cultivation than on day 5, the samples supplemented with 10 mg/L TiO_2 of 40 nm showed an increase of the lipolytic activity of 25.00% compared to the control in the 5th day of cultivation. This phenomenon can be applied in order to reduce the cultivation time of the producer by 24 hours while keeping the stimulant effect of TiO_2 with 40 nm and concentration of 10mg/L.

The study of the influence of Fe_3O_4 inorganic nanoparticles on the lipase biosynthesis by *A. niger* CNMN-FD-01 demonstrated a neutral effect on the 4th day of micromycete growth and an inhibitory effect on days 5 and 6 of cultivation (Table 1).

Thus, the samples from the 4th day of cultivation of *A. niger* CNMN-FD-01 in the presence of Fe_3O_4 NPs with the dimensions of 10 nm and 30-35 nm in both 5 mg/L and 10 mg/L concentrations showed values at the level of control. The moderate stimulatory action was obtained in the presence of 5 mg/L iron oxide nanoparticles with dimension of 70nm, which demonstrated an increase in lipolytic activity by 14.28% (Figure 1).

On the 5th day of cultivation of the producer was observed a strong inhibitory effect of Fe_3O_4 NPs, practically indifferent of the tested sizes and concentrations. The lowest activity of lipolytic enzymes, of 88.31%, was observed in the presence of 5mg/L Fe_3O_4 with the dimension of 70nm. The lowest inhibitory effect of 55.00% was obtained by 30-35nm Fe_3O_4 in the concentration of 10mg/L (Figure 2).

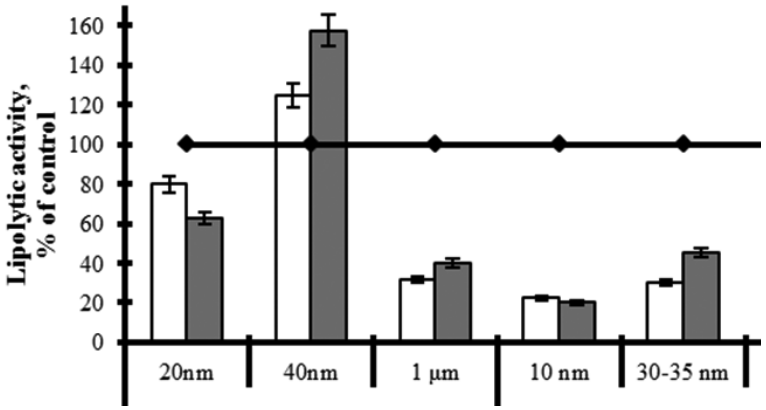

Figure 2. Influence of TiO_2 and Fe_3O_4 nanoparticles on the lipolytic activity of *A. niger* CNMN-FD-01 micromycete on the 5th day of cultivation.

On the 6th day of cultivation, only traces of lipase activity were identified in the samples grown in the presence of iron nano-oxide. Experimental variants supplemented with 5mg/L and 10mg/L NPs Fe_3O_4 with dimension of 30-35nm showed an enzymatic activity lower by 73.33% than the control (the same samples that showed the highest enzyme activity on day 5 of cultivation). The other samples grown in the presence of Fe_3O_4 NPs showed complete lack of lipolytic activity, thus having some tangent to the results obtained on the day 6 in the *A. niger* CNMN-FD-01 micromycete cultivated in the presence of TiO_2 NPs.

Summarizing the results we can conclude:

1. The TiO_2 nanoparticles with dimension of 40 nm supplemented in the concentration of 10mg/L in the culture medium of *A. niger* CNMN-FD-01 ensured the significant increase of the lipolytic activity of the producer, by 78.57% on 4th day of cultivation and by 57.49% on the 5th day of cultivation.

2. TiO_2 nano-oxide determined an acceleration of the lipase biosynthesis by 24 h in the *A. niger* CNMN-FD-01 micromycete; the experimental samples supplemented with 10mg/L TiO_2 of 40nm on the 4th day of cultivation demonstrated an increase in lipolytic activity by 25.00% compared to the control sample from the 5th day of cultivation. This phenomenon can be applied in order to reduce the cultivation time of the producer by 24 hours while keeping the stimulating effect of TiO_2 of 40 nm in concentration of 10mg/L.

3. Fe_3O_4 nanoparticles showed neutral or weakly stimulating effect (14.28% in the presence of 5mg/L Fe_3O_4 of 70nm) on the 4th day of cultivation, or strongly inhibitory effect on 5th and 6th days of cultivation on the biosynthesis of lipolytic enzymes in *A. niger* CNMN-FD-01 micromycete.

References

1. Deependra, K.B., Subhankar, P. Zinc oxide nanoparticles modulates the production of β-glucosidase and protects its functional state under alcoholic condition in *Saccharomyces cerevisiae*.//Appl. Biochem. Biotechnol., 2014, 173, p. 155–166.

2. Deseatnic-Ciloci, A., Sîrbu, T., Tiurina, J., Labliuc, S. Tulpină de fungi *Aspergillus niger* producătoare de enzime lipolitice. Brevet de invenţie MD 2362, BOPI 2004.01.31.

3. Dobias, J. Nanoparticles and microorganisms: from synthesis to toxicity. These nr. 5614 pour obtetion du grade de docteur es sciences. Ecole Polytechnique Federale de Lausanne. Suisse, 2013. 139 p.

4. Driouch, H. et al. Filamentous fungi in good shape: Microparticles for tailor-made fungal morphology and enhanced enzyme production.//Bioeng. Bugs., 2011, 2(2), p. 100-104.

5. Driouch, H., Sommer, B., Wittmann, C. Morphology engineering of *Aspergillus niger* for improved enzyme production.//Biotechnol. Bioeng., 2010, 105(6), p. 1058-1068.

6. Komkrit, S. et al. Role of surface area, primary particle size, and crystal phase on titanium dioxide nanoparticle dispersion properties.//Nanoscale Res. Lett., 2011, 6, p. 27.

7. Maiorano, E.A. et al. Microbial production of fructosyltransferases for synthesis of pre-biotics.//Biotechnol. Lett., 2008, 30, p. 1867-1877.

8. Rai, M., Duran, N. Metal nanoparticles in microbiology. Berlin; Heidelberg; New York: Springer, 2011, 305 p.

9. Singh, A.K., Mukhopadhyay, M. Overview of fungal lipase: A review.//Appl. Biochem. Biotechnol., 2012, 166, p. 486-520.

10. Suman, V. et al. Role of nanomaterials in symbiotic fungus growth enhancement. //Current Science, 2010, 99(9), p.1189-1191.

11. Tarafdar, J.C. et al. ZnO Nanoparticles induced synthesis of polysaccharides and phosphatases by *Aspergillus* fungi.//Advanced Science, Engineering and Medicine, 2012, 4, p. 1-5.

12. Vertegel, A.A., Reukov, V., Maximov, V. Enzyme-nanoparticle conjugates for biomedical application.//Methods Mol. Biol., 2011. 679, p. 165-182.

13. Wucherpfennig, T. et al. Customization of *Aspergillus niger* morphology through addition of talc micro particles.//J. Vis. Exp., 2012, 61, p. 4023.

14. Грачёва, И.М. и др. Лабораторный практикум по технологии ферментных препаратов. - М.: Легкая и пищ. пром., 1982. - с. 75-76.

15. Доспехов, Б. Планирование полевого опыта и статистическая обработка данных. - М.: Колос, 1985. - с. 192-196.

16. Чилочи, А. и др. Влияние нано окислов некоторых металлов на биосинтез внеклеточных гидролаз микромицетов.//Buletinul AŞM. Ştiinţele vieţii, 2016, 3(330), с. 164-171.