Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Food Science and Technology
Open Access

New Method of Obtaining Proteolytic Enzymatic Preparation from Fungal Strain *Fusarium gibbosum* CNMN FD 12

Steliana CLAPCO*, Alexandra CILOCI*, Elena DVORNINA, Svetlana LABLIUC

Institute of Microbiology and Biotechnology, Technical University of Moldova, Academiei str. 1, Chisinau, MD2028, Republic of Moldova
** Corresponding author: S. Clapco e-mail: steliana.clapco@imb.utm.md; A. Ciloci e-mail: alexandra.ciloci@imb.utm.md*

RESEARCH ARTICLE

Abstract

Proteases, that are able to hydrolyze the peptide bonds in proteins and polypeptides, occupy a key position in diverse fields, such as food, agriculture, animal feed. Considering the increasing demand for microbial proteases, identification of new way for improving the productivity of producers is of great importance. The aim of the present study was to evaluate the effect of a new coordination compound $[Fe(H_2L)(H_2O)_2](NO_3)_3 \cdot 2.5H_2O$ (where H_2L = 2,6-diacetylpyridine bis(picolinoylhydrazone)) on the activity of proteolytic complex synthesized by the micromycete *Fusarium gibbosum* CNMN FD 12 under the submerged fermentations in shake flask, as well as in lab-scale fermenter. It was found that $[Fe(H_2L)(H_2O)_2](NO_3)_3 \cdot 2.5H_2O$ exerts a significant positive effect on proteolytic activity in *F. gibbosum* CNMN FD 12 both in flasks and bioreactor and can be used as stimulator and regulator of proteases activity. By varying the concentration of the coordination compound and the duration of cultivation, it is possible to obtain proteolytic complexes enriched in different types of proteases (acid, neutral or alkaline), depending on the practical needs.

Keywords: coordination compound, fungi, lab-scale fermenter, proteases.

Received: 6 September 2023
Accepted: 7 January 2024
Published: 15 May 2024

DOI:
10.15835/buasvmcn-fst:2023.0019

INTRODUCTION

Microbial enzymes have gained interest due to their ability to catalyze different biochemical processes in a cost-effective and eco-friendly manner with less time, energy, space and toxic chemicals requirements (Raveendran et al., 2018). They are widely used in various fields (agriculture, chemicals, pharmaceuticals, industries, research), the food and beverage application being dominant. Thus, in 2022 the global industrial enzymes market was reached at USD 6.95 billion, from which 20.9% was attributed to this filed of application (Industrial Enzymes Market). The food and beverage sector predominantly imply hydrolytic enzymes, such as proteases, lipases, pectinases, gluco-oxidases and amylases (Raveendran et al., 2018). Proteases, that are able to hydrolyze the peptide bonds in proteins and polypeptides, occupy a key position in diverse industries, such as detergent, leather, textiles, biofuels, waste management, agriculture, animal feed. They find application in food processing to improve the solubility and digestibility of food proteins, as well as the quality and nutritional value of foods (Solanki et al., 2021). Thus, proteases are employing in cheese making for milk coagulation, acceleration of cheese ripening and changing milk protein to decrease the allergenic effects of milk products (Khan and Selamoglu, 2020). In bakery, they are widely used for the production of bread, pastries, biscuits, crackers etc. Due to their capacity to modify the proteins of wheat flour, proteases decrease the gluten elasticity and dough consistency, assure the dough uniformity, reduce mixing time and improve the bread texture (Miguel et al., 2013). Proteases are

also exploited for soy protein, fish protein and gelatin hydrolysis, meat tenderization, as well as for degradation of the turbidity complex resulting from protein in fruit juices and alcoholic liquors (Singh et al., 2016a).

One of the major sources of industrial enzymes are micromycete that can grow on cheap substrates in a short period of time and secrete a large spectrum of extracellular enzymes, which could be ease purify (Hajji et al., 2010). Furthermore, fungal enzymes exhibit wide substrate specificity, catalytic activity and stability in different conditions (Singh et al., 2016b). Thus, filamentous fungi, especially those belonging to genus *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., produce a wide variety of proteolytic enzymes (acidic, neutral and alkaline), active in extended pH limits (2.5 - 9.0) (El-Gendi et al., 2021).

Considering the increasing demand for microbial proteases in industrial applications, identification of new way for improving the productivity of producers is of great importance. Some authors noted the efficiency of using coordination compounds of transition metals as regulators and stimulators of the bioactive substances' synthesis in different groups of microorganisms, including mycelial fungi (Efremova et al., 2013; Valuta et al., 2019; Personal communication). It is well known that transition metals are necessary for growth and survival of all organisms. Notably, the metals bound with different ligands are less toxic than the inorganic forms (Waldron et al., 2009). Also, it has to be note that various metal influenced the activity of proteases. They protect enzymes from conformational changes and can serve as inducers and stabilizers of enzymes (Zubi et al., 2022).

Iron is an essential micronutrient that plays a critical role in various metabolic processes, such as DNA synthesis, regulation of gene expression, respiration and photosynthesis. At the same time, iron is a component of the prosthetic group of many enzymes and regulatory proteins (Stanford and Voigt, 2020).

Therefore, the aim of the present study was to evaluate the effect of a new coordination compound $[Fe(H_2L)(H_2O)_2](NO_3)_3 \cdot 2.5H_2O$ (where H_2L = 2,6-diacetylpyridine bis(picolinoylhydrazone)) on the activity of the proteolytic complex synthesized by the micromycete *Fusarium gibbosum* CNMN FD 12 under the submerged fermentations in shake flask, as well as in lab-scale fermenter and to optimize the culture condition for better yield of the enzymes.

MATERIALS AND METHODS

The object of study was the fungal strain *Fusarium gibbosum* CNMN FD 12 – producer of proteases, stored in the National Collection of Nonpathogenic Microorganisms of the Institute of Microbiology and Biotechnology, Republic of Moldova. The micromycete was cultivated on a rotary shaker (180 rpm) at a temperature of 28–30°C in Erlenmeyer flasks with a capacity of 0.75 L, which contained 0.1 L nutritive medium with the following composition, g/L: wheat bran – 20.0; soy flour – 10.0; $CaCO_3$ – 2.0; $(NH_4)_2 SO_4$ – 1.0; the rest water up to 1.0 L, pH – 6.25.

Fermenter studies were carried out in Sartorius Biostat A plus Bioreactor System (Germany) with a capacity of 6.65 L and a working volume of 0.6-5 L, aeration speed (L/min) - 1.3-13, stirring speed (rpm) - 20-1300. The fermentation medium was the same with shake flask culture. Two series of experiments were set up, with the setting of the main technological parameters (aeration, agitation) as follow: I regime – volume of nutritive media - 1.5 L, stirrer speed - 180 rpm, the bioreactor was aerated with an air flow of 1.5 L/L/min (volume of air per volume of medium per minute); II regime – volume of nutritive media – 2.0 L, stirrer speed - 180 rpm, aeration rate – 2.0 L/L/min.

The spore suspension with a density of $2.5x10^6$ spores/mL, which were obtained by washing with sterile distilled water the culture grown on oblique malt-agar columns for 12 days, served as inoculation material. The inoculum concentration was 10% V/V nutrient medium. Concomitantly with the inoculum the coordinating compound $[Fe(H_2L)(H_2O)_2](NO_3)_3 \cdot 2.5H_2O$, wherein H_2L is 2,6-diacetylpyridine bis (picolinoylhydrazone), was added to the basic nutritive medium in the concentrations of 5.0, 10.0, 15.0 and 20 mg/L. A sample cultivated on nutrient medium without coordination compound served as a control. The cultivation was performing during 4-6 days.

After cultivation, the native liquid solution was separated from the biomass by filtration. In the culture filtrate the activity of neutral (pH-7.4), acid (pH-3.6) and alkaline (pH-9,0) proteases was essayed according to the Willstatter method. This method is based on determining the amount of released free carboxyl groups in ethanol solution of amino acids and polypeptides obtained at hydrolysis of 5% gelatin substrate, after 3 hours incubation at 40°C (Gracheva et al., 1982).

All experiments were performed at least three times and results are represented as the mean ± standard deviation (SD). The statistical significance (*$p \leq 0.01$ and ** $p \leq 0.05$) of differences between means of two samples was determined with a paired Student's *t*-test using Excel.

RESULTS AND DISCUSSIONS

The positive effect of different iron coordination compounds on microorganisms has been reported. As for example, iron compounds with Schiff bases increased the synthesis of phycobiliproteins in the biomass of the cyanobacteria

Nostoc linckia by 250% compared to the control (Valuta et al., 2019). The metallocomplex of iron (II) with thiocarbohydrazide and pyridine $[Fe_2(Py)_2(TCH)(SO_4)_2]\cdot 4H_2O$ exerted a stimulating action (36.5%) on the biosynthesis of neutral proteases in the micromycete *Trichoderma koningii* (Personal communication). Several compounds of iron showed a positive effect on biomass accumulation (increase by 57-53%) and catalases synthesis (108-120%) in the yeast *Saccharomyces cerevisiae* CNMN-Y-11 (Efremova et al., 2013).

Also, previously it was established the possibility to manipulate the biosynthetic activity in the micromycete *Fusarium gibbosum* CNMN FD 12 using different groups of coordination compounds as influencing factors. Thus, the compounds of Co(II), Cu(II) and Zn(II) with oxime ligands ensured increased yields of acid (approx. 20-80%, compared to the control) and neutral (approx. 40-95% *versus* control) proteases (Personal communication).

Introduction of the compounds $[Co(DH)_2(An)_2][PF_6]$ and $[Co(NioxH)_2(Thio)_2][PF_6]\cdot 0.5DMF\cdot 0.5H_2O$ (where DH= monoanion of dimethylglyoxime; An = aniline; NioxH = monoanion 1,2-cyclohexanedione dioxime and Thio = thiourea) into the nutrient medium of the producer determined the enhance of acid and neutral proteases activity by 63.6 and 92.5%, respectively (Bourosh et al., 2013). Also, as stimulators of the proteolytic activity in *F. gibbosum* can be used the Mn-containing compounds (Darii et al., 2022).

In the present study the effect of coordination compound of Fe (III) varied depending on the compound concentrations and the type of enzyme (Figure 1.).

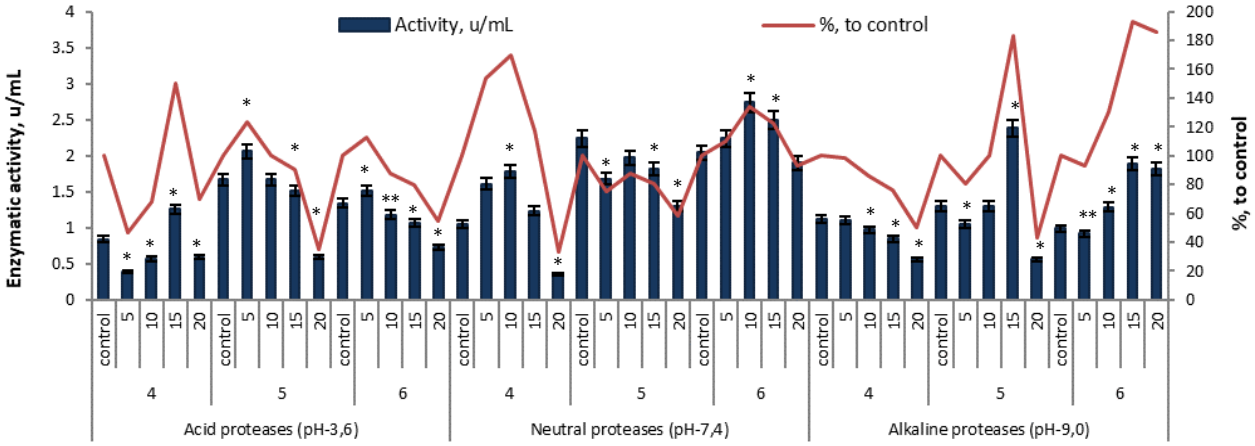

Figure 1. Influence of different concentrations (5-20 mg/L) of coordination compound of iron on the activity of proteolytic enzymatic complex synthesized by the fungal strain *Fusarium gibbosum* during submerged cultivation in the flasks in dynamic (4-6 days of cultivation). Note: * and ** indicates a statistically significant difference between samples cultivated in the presence of iron compound and control at the level $p \leq 0.01$ and $p \leq 0.05$, respectively.

At the shake flasks level, the concentration that determines the maximum increase (23%) of the acid proteases activity on the 5th day of cultivation, which is known as optimal duration for accumulation of proteases in *F. gibbosum*, is 5 mg/L. As the concentration increases, the effect decreased to neutral (at a concentration of 10-15 mg/L) and pronounced negative (at a concentration of 20 mg/L). It should be noted that in the concentration of 15 mg/L the compound provides a high level of acid protease activity 24 hours earlier. Thus, the activity on the 4th day is 1.26 u/mL, compared to 1.67 u/mL determined in the control sample on the 5th day, the difference being only about 20%.

In the case of neutral proteases, the positive effect of the compound compared to the control from the same day is found only on the 4th and 6th day of cultivation. Although the peak of proteolytic activity (2.24-2.74 u/mL at compound concentrations of 5-15 mg/L) was marked on the last day of cultivation. Considering the economic efficiency, the effect of the compound exerted on the 4th day of cultivation (24 h earlier than in control sample) is of particular interest. It was found that in the concentration of 10 mg/L the compound provides a level of proteolytic activity that exceeds the reference sample of the same day by about 70% and is reduced by about 20% compared to the maximum value of the control. The maximum increase in enzyme activity was found in the case of alkaline proteases, when the compound was added in a concentration of 15 mg/L, on the 5th day, the activity being 2.38 u/mL, which exceeded the control by 83%. In the majority of cases the differences between samples cultivated in the media including the metalocomplex of iron and control were significant.

Environmental pH is a factor that affects the growth of microorganisms by modulating enzyme activity, protein conformation, and activity of functional group. In addition, environmental pH influences the adsorption of metal ions to microorganisms (Jiang et al., 2022). Optimal pH for the growth and synthesis of different bioactive

substances varies depending on the peculiarities of the microorganism and the metabolite of interest. Thus, the media with initial neutral pH was reported as optimal for the production of alkaline proteases in *P. aeruginosa* MCM B-327 (Zambare et al., 2011), while alkaline (pH 8.0–9.0) media was optimal for production of proteases in bacteria *Bacillus velezensis* HM49 (Mushtaq et al., 2023), *B. caseinilyticus* (Mothe et al., 2016), micromycete *Aspergillus brasiliensis* (Chimbekujwo et al., 2020) and, respectively, *Penicillium sp.* LCJ228 (Benluvankar et al., 2015). Maximum acid protease production by the isolate Z1BL1 of *Aspergillus* was recorded at initial media pH 4.5 (Usman et al., 2021).

Thus, another objective of the presented research consisted in revealing the optimum initial pH of the nutrient media that ensures the maximum biosynthesis of proteases in the micromycete *Fusarium gibbosum* CNMN FD 12 cultivated in presence of 15 mg/L of iron compound (Figure 2).

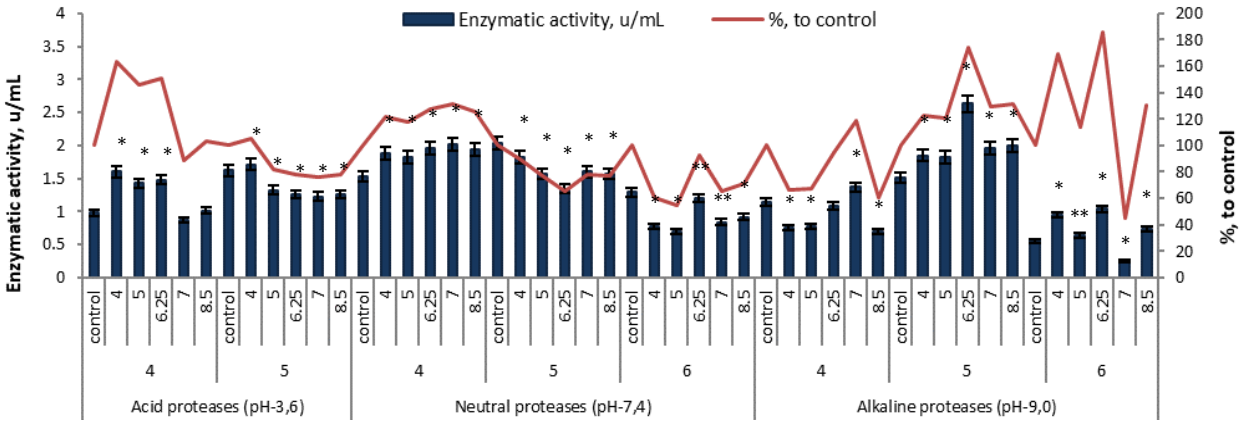

Figure 2. Influence of the coordination compound $[Fe(H_2L)(H_2O)_2](NO_3)_3 \cdot 2.5H_2O$, in the concentration of 15 mg/L, on the activity of the proteolytic complex synthesized by the micromycete *Fusarium gibbosum* CNMN FD 12 depending on the initial pH of the cultivation medium. Note: * and ** indicates a statistically significant difference between samples cultivated in the presence of iron compound and control at the level $p \leq 0.01$ and $p \leq 0.05$, respectively.

As control was used the value of proteolytic activity determined in the sample cultivated in the media with pH-6.25, previously established as optimal for fungal strain, which not include coordination compound.

The highest activity of acid proteases was found, in particular, in the acid range of the pH (4.0-6.25). Thus, at the 4th day of cultivation activity varied between 1.43-1.60 u/mL, exceeding the control by around 46-63%. It should be noted that at pH 4.0 and 6.25 the activity determined in the 4th day of cultivation was practically similar to maximal value of control established in the 5th day. In the next day the activity was slightly lower, excepting the sample cultivated in the media with pH 4.0.

Similar to acid proteases, in the case of neutral proteases the effect was preferentially manifested in the 4th day of cultivation. Thus, the activity exceeded the level of the control in all analyzed variants, the activity ranged from 1.82 to 2.02 u/mL, being 18.2-31.2% higher than control. The maximum value of the activity was established in the media with neutral pH, being practically equivalent to the value recorded in the control on the day of maximum biosynthesis. The level of activity in the environment with pH 6.25 and 8.5 is practically identical, being 27.3, respectively, 25.3% higher compared to the control. In the following days the activity was lower compared to the control.

The study of the influence of the coordination compound on the activity of alkaline proteases showed that in the 4th day of cultivation the activity was superior to the control only in the sample grown on media with neutral pH, the increase in activity constituting 19%. In the 5th day the activity was higher compared to control in all samples. The activity varied between 1.82-2.63 u/mL, with an increase of 20.5-74.3%. The peak of activity was revealed in the media with pH 6.25. Based on the obtained results, it is found that the pH of the media that ensures the synthesis of proteolytic enzymes, especially alkaline proteases, at high levels in the case of micromycete cultivation in the presence of the chemical biostimulator is 6.25, similar to the value established as optimal for the synthesis of proteases under classical cultivation conditions (in absence of metalocomplex) of the producer.

To obtain high yields of enzymes, the transfer of cultivation technology from shake flasks to a bioreactor is needed. Therefore, the next experiments were performed at lab-scale fermenter. Considering the results of similar studies including other fungal strains producers of hydrolases (Unpublished results) two regimes of cultivation were tested. The liquid volume was 1.5 L and 2.0 L, the stirrer speed was set to 180 rpm and the culture was aerated with an air flow of 1.5 and 2.0 L/ L/ min.

Excepting the 6th day of cultivation, significant differences were found between enzymatic activity of *F. gibbosum*

cultivated in the nutritive media without coordination compound (control) under the conditions of first and second regime of cultivation. The maximum activity of acid (1.28 u/mL), neutral (2.25 u/mL) and alkaline (2.34 u/mL) proteases was observed at the 5th day of cultivation according to second regime, which provides a higher rate of aeration (Figure 3).

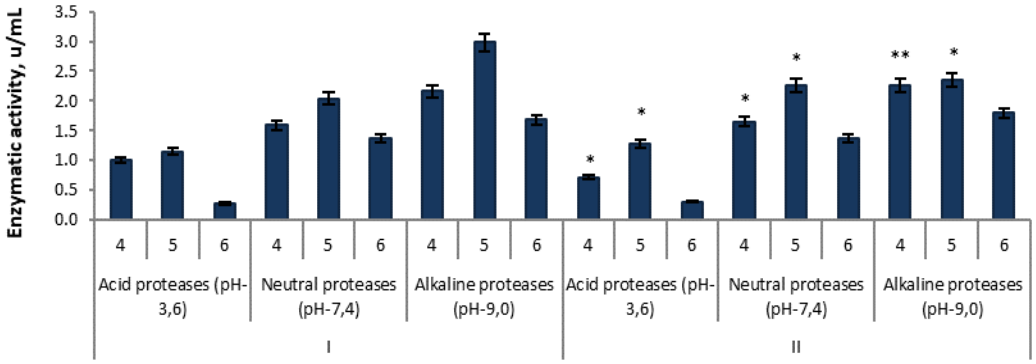

Figure 3. The influence of two regimes of cultivation in lab-scale fermenter (I – volume of nutritive media - 1.5 L, stirrer speed - 180 rpm, aeration rate - 1.5 L/L/min; II – volume of nutritive media – 2.0 L, stirrer speed - 180 rpm, aeration rate – 2.0 L/L/min) on the proteolytic activity of fungal strain *Fusarium gibbosum* CNMN FD 12. Note: * and ** indicates a statistically significant difference between samples cultivated under the conditions of first and second regime at the level $p \leq 0.01$ and $p \leq 0.05$, respectively.

It is known that aeration is a very important factor for aerobic fermentation processes in order to achieve maximal product accumulation in bioreactors (Radchenkova et al., 2020). Especially it is a key parameter when mechanical agitation speeds are relatively low as in the case of fungi, whose morphological characteristics, as well as productivity, could be affected by increased agitation speed (Zhou et al., 2018, Ibrahim et al., 2015).
In the experimental variants, containing stimulator coordination compound, the technological parameters (aeration, stirring) were set at the level of the optimal regime established for classical cultivation: nutrient medium volume - 2 L, agitation - 180 rpm, aeration volume - 2.0 L /L medium / min. The coordinating compound of iron was included in the cultivation medium in the concentrations of 5-15 mg/L (Figure 4).

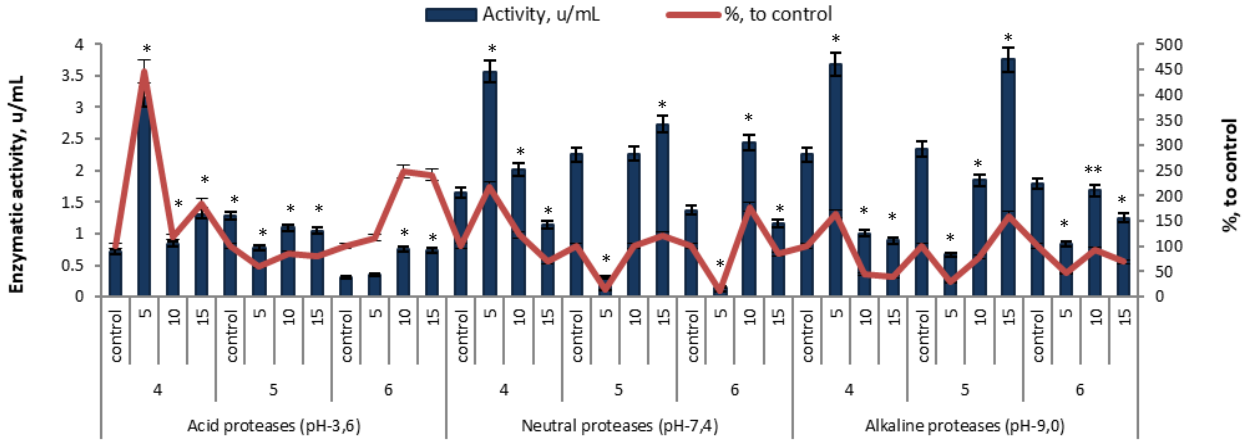

Figure 4. Influence of different concentrations (5-15 mg/L) of coordination compound of iron on the activity of proteolytic enzymatic complex synthesized by the fungal strain *Fusarium gibbosum* CNMN FD 12 during submerged cultivation in the lab-scale fermenter in dynamic (4-6 days of cultivation) Note: * and ** indicates a statistically significant difference between samples cultivated in the presence of iron compound and control at the level $p \leq 0.01$ and $p \leq 0.05$, respectively.

Different from the flasks level, the highest increasing of proteolytic activity in the bioreactor was found in first experimental variant (5 mg/L of compound). Thus, at 4th day of cultivation, the activity was 3.16, 3.57 and 3.68 u/mL for acid, neutral and alkaline proteases, respectively, being 146.2, 58.5 and 56.9% higher than the maximum value of the control established on the 5th day. In the following days of cultivation activity decreased significantly.

A less pronounced positive effect was observed in the samples cultivated in the media containing 10 or 15 mg/L of coordination compound of iron. Thus, in the variant with 10 mg/L a positive effect was determined for neutral proteases. Activity ranged from 2.016 to 2.436 u/mL, exceeding the control by 22.4 and 77.6% at 4th and, respectively, 6th day of cultivation.

In experimental variant including 15 mg/L of metalocomplex the proteolytic activity, on the 5th day of cultivation, was 1.05, 2.74 and 3.75 u/mL, respectively for acid, neutral and alkaline proteases, the activity of last two types of enzymes exceeding the control with 21.6 and 60.1%, respectively. The maximum activity of acid proteases (1.31 u/mL) was recorded on the 4th day of cultivation, this value being 84.9% higher than the control from the same day and similar to the reference sample from the 5th day. Thus, by varying the duration of cultivation, enzymatic complexes enriched in neutral and alkaline or acid proteases can be obtained.

The differences between experimental variants and control, with minor exceptions, were statistically significant at a level of $p \leq 0.01$.

Generalizing the obtained results, we concluded that the new Fe(III) coordination compound with 2,6-diacetylpyridine-bis(picolinoylhydrazone) exerts a significant positive effect on proteolytic activity in fungal strain *Fusarium gibbosum* CNMN FD 12 cultivated in flasks or lab-scale fermenter and can be used for increasing and manipulation of proteases activity.

By varying the concentration of the metalocomplex and the duration of cultivation, it is possible to obtain proteolytic complexes enriched in different types of proteases (acid, neutral or alkaline), depending on the practical needs. Thus, it is known that acid proteases are frequently used in soy sauce, protein hydrolysate obtaining, as well as in clearing beer and fruit juice, meat tenderization (Song et al., 2023). Neutral proteases, which are active from weakly acidic to weakly alkaline pH are very important in the food industry, while alkaline proteases, active in a neutral to alkaline pH range are widely used in detergent, pharmaceutical and leather industries (Razzaq et al., 2019). In current study the cultivation of the producer in flasks for 4 days into media containing 15 mg/L of Fe(III)) compound, ensured the obtaining (in a shorter period of time compared to control) of a proteases enzymatic complex predominantly active in the acid range. In the case of the concentration of 10 mg/L, keeping the other conditions unchanged, an enzymatic complex enriched in neutral proteases can be obtained. An enzyme preparation with increased content of alkaline proteases, against the background of maintaining the activity of acid and neutral proteases, can be obtained at the cultivation during 5 days in the presence of 15 mg/L compound.

The optimal regime for submerged cultivation of the fungal strain *Fusarium gibbosum* CNMN FD 12 in lab-scale fermenter was established as follow: volume of nutritive media – 2.0 L, stirrer speed - 180 rpm, aeration rate – 2.0 L/L/min (volume of air per volume of medium per minute). It has to be mentioned that the increase of proteolytic enzymes ensured by the application of $[Fe(H_2L)(H_2O)_2](NO_3)_3 \cdot 2.5H_2O$ as stimulator is preserved inclusive at fermenter scale, the activity of all components of proteolytic enzymatic complex being significantly higher than the maximal values of control. Also, the shortening of the cultivation cycle by 24 hours, which is of great economical interest, was revealed.

Author Contributions: A.C. Conceived and designed the analysis; E.D. and S.L. Performed the analysis and collected the data; S.C. Contributed data or analysis tools; S.C. and A.C. Wrote the paper.

Funding Source: This study was supported by the research project 20.80009.5007.28 with funding from National Agency for Research and Development, Republic of Moldova.

Acknowledgments

The coordinating compounds were synthesized and offered for investigations, according to the project's objectives, by the partner team from the Institute of Chemistry of the Moldova State University, coordinated by dr. hab. Bulhac Ion.

Conflicts of Interest

The authors declare that they do not have any conflict of interest.

REFERENCES

1. Benluvankar V, Roseline G, Jebapriya VR, Gnanadoss JJ. Protease production by *Penicillium* sp. ICJ228 under solid state fermentation using groundnut oil cake as substrate. Int. J. Life Sci. Pharma Res. 2015;5:12-19.
2. Bourosh P, Coropceanu E, Ciloci A, Clapco S, Bologa O, Bivol C, et al. New Co(III) dioximates with hexafluorophosphate ion as stimulators of the proteolytic activity of the micromycete *Fusarium gibbosum*

CNMN FD 12. Russian Journal of Coordination Chemistry. 2013;39. https://doi.org/10.1134/S107032841311002X.
3. Chimbekujwo KI, Ja'afaru MI, Adeyemo OM. Purification, characterization and optimization conditions of protease produced by *Aspergillus brasiliensis* strain BCW2. Sci Afric. 2020;8:00398. https://doi.org/10.1016/j.sciaf.2020.e00398.
4. Darii M, Mikosch A, van Leusen J, Kravtsov VC, Dvornina EG, Clapco ST, et al. FeII/III and MnII complexes based on 2,4,6-tris(2-pyridyl)-triazine: synthesis, structures, magnetic and biological properties. RSC Adv. 2022;12(45):29034-29047. https://doi.org/10.1039/d2ra04868j.
5. Efremova N, Molodoi E, Usatîi A, Fulga L. The utilization of some iron and zinc compounds as regulators of catalase activity at Saccharomyces cerevisiae. An Univ Oradea Fasc Biol. 2013;2:80-3.
6. El-Gendi H, Saleh AK, Badierah R, Redwan EM, El-Maradny YA, El-Fakharany EM. A Comprehensive Insight into Fungal Enzymes: Structure, Classification, and Their Role in Mankind's Challenges. J Fungi (Basel). 2021;28;8(1):23. https://doi.org/ 10.3390/jof8010023.
7. Gracheva IM, Grachev YuM, Mosichev MS. Laboratory practicum in enzyme preparations technology. Moscow; Light and Food Industry; 1982.
8. Hajji M, Hmidet N, Jellouli K, Vallaeys T, Nasri M, Sellami-Kamoun A. Gene cloning and expression of a detergent stable alkaline protease from *Aspergillus clavatus* ES1. Process Biochem. 2010; 45:1746–1752. https://doi.org/10.1016/j.procbio.2010.07.005.
9. Ibrahim D, Weloosamy H, Lim SH. Effect of agitation speed on the morphology of *Aspergillus niger* HFD5A-1 hyphae and its pectinase production in submerged fermentation. World J Biol Chem. 2015;26;6(3):265-71. https://doi.org/10.4331/wjbc.v6.i3.265.
10. Industrial Enzymes Market Size, Share & Trends Analysis Report By Product, By Source (Plants, Animals), By Application (Food & Beverages, Detergents, Animal Feed), By Region, And Segment Forecasts, 2023 – 2030, https://www.grandviewresearch.com/industry-analysis/industrial-enzymes-market
11. Jiang N, Feng Y, Huang Q, Liu X, Guo Y, Yang Z, Peng C, Li S, Hao L. Effect of Environmental pH on Mineralization of Anaerobic Iron-Oxidizing Bacteria. Front Microbiol. 2022;12;13:885098. https://doi.org/10.3389/fmicb.2022.885098.
12. Khan U, Selamoglu Z. Use of Enzymes in Dairy Industry: A Review of Current Progress. Arch Razi Inst. 2020;75(1):131-136. https://doi.org/10.22092/ari.2019.126286.1341.
13. Miguel ÂSM, Martins-Meyer TS, Veríssimo da Costa Figueiredo E, Lobo BWP, Dellamora-Ortiz GM. Enzymes in bakery: Current and future trends. In: Muzzalupo I, editor. Food Industry. Rijeka, Croatia: InTech; 2013. https://doi.org/ 10.5772/53168
14. Mothe T, Sultanpuram VR. Production, purification and characterization of a thermotolerant alkaline serine protease from a novel species *Bacillus caseinilyticus*. Biotech. 2016;6(1):1–0. https://doi.org/10.1007/s13205-016-0377-y P.
15. Mushtaq H, Ganai SA, Jehangir A, Ganai BA, Dar R. Molecular and functional characterization of protease from psychrotrophic *Bacillus* sp. HM49 in North-western Himalaya. PLoS ONE. 2023;18(3):e0283677. https://doi.org/ 10.1371/journal.pone.0283677.
16. Radchenkova N, Boyadzhieva I, Hasköylü ME, Atanasova N, Yıldız SY, Kuncheva MJ, Panchev I, Kisov H, Vassilev S, Oner ET, Kambourova MS. High bioreactor production and emulsifying activity of an unusual exopolymer by *Chromohalobacter* canadensis 28. Eng Life Sci. 2020; 2;20(8):357-367. https://doi.org/10.1002/elsc.202000012.
17. Raveendran S, Parameswaran B, Ummalyma SB, Abraham A, Mathew AK, Madhavan A, Rebello S, Pandey A. Applications of Microbial Enzymes in Food Industry. Food Technol Biotechnol. 2018;56(1):16-30. https://doi.org/10.17113/ftb.56.01.18.5491.
18. Razzaq A, Shamsi S, Ali A, Ali Q, Sajjad M, Malik A, Ashraf M. Microbial Proteases Applications. Front Bioeng Biotechnol. 2019;12(7):110. https://doi.org/10.3389/fbioe.2019.00110.
19. Singh R, Kumar M, Mittal A, Mehta PK. Microbial enzymes: industrial progress in 21st century. 3 Biotech. b2016;6(2):174. https://doi.org 10.1007/s13205-016-0485-8.
20. Singh R, Kumar M, Mittal A, Mehta PK. Microbial Proteases in Commercial Applications, J Pharm Chem Biol Sci. a2016; 4(3):365-374.
21. Solanki P, Putatunda C, Kumar A, Bhatia R, Walia A. Microbial proteases: ubiquitous enzymes with innumerable uses. 3 Biotech. 2021;11(10):428. https://doi.org/ 10.1007/s13205-021-02928-z.

22. Song P, Zhang X, Wang S, Xu W, Wang F, Fu R, Wei F. Microbial proteases and their applications. Front Microbiol. 2023;14:1236368. https://doi.org/10.3389/fmicb.2023.1236368.
23. Stanford FA, Voigt K. Iron Assimilation during Emerging Infections Caused by Opportunistic Fungi with emphasis on Mucorales and the Development of Antifungal Resistance. Genes. 2020;11:1296. https://doi.org/10.3390/genes11111296
24. Usman A, Mohammed S, Mamo J. Production, Optimization, and Characterization of an Acid Protease from a Filamentous Fungus by Solid-State Fermentation. Int J Microbiol. 2021:6685963. https://doi.org/10.1155/2021/6685963.
25. Valuta A, Codreanu L, Cepoi L, Rudi L, Codreanu S. Metal complexes with different ligands in cultivation of cyanobacterium *Nostoc linckia*. In: Life sciences in the dialogue of generations: connections between universities, academia and business community. Chisinau: Biotehdesign; 2019.
26. Waldron KJ, Rutherford JC, Ford D, Robinson NJ. Metalloproteins and metal sensing. Nature. 2009;460:823–830. https://doi.org/10.1038/nature08300
27. Zambare VS, Nilegaonkar S, Kanekar P. A novel extracellular protease from *Pseudomonas aeruginosa* MCM B-327: Enzyme production and its partial characterization. New Biotechnol. 2011;28:173-181. https://doi.org/10.1016/j.nbt.2010.10.002
28. Zhou Y, Han LR, He HW, Sang B, Yu DL, Feng JT, Zhang X. Effects of Agitation, Aeration and Temperature on Production of a Novel Glycoprotein GP-1 by *Streptomyces kanasenisi* ZX01 and Scale-Up Based on Volumetric Oxygen Transfer Coefficient. Molecules. 2018;11;23(1):125. https://doi.org/10.3390/molecules23010125.
29. Zubi YS, Seki K, Li Y et al. Metal-responsive regulation of enzyme catalysis using genetically encoded chemical switches. Nat Commun. 2022;13:1864. https://doi.org/10.1038/s41467-022-29239-y