REPUBLIC OF AZERBAIJAN

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ABSTRACT

of the dissertation for the degree of Doctor of Philosophy

ACTIVITY OF ACID PROTEASES IN FUNGI FEATURED BY GENERA *ASPERGILLUS* AND *PENICILLIUM* ISOLATED FROM THE SOILS OF AZERBAIJAN

Specialty: 2414.01 – Microbiology

Field of science: Biology

Applicant: **Safarova Aytan Khanlar**

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Dissertation work was performed at the department «Chemistry, biology and ecology» of Odlar Yurdu University and in the scientific research laboratory «Microbiology and Virology» of Baku State University.

Scientific supervisor: Doctor of Biological Sciences, Professor
Ganbarov Khudaverdi Ganbar

Official opponents: Doctor of Biological Sciences, Professor
Mirmusa Mirish Jafarov

Doctor of Biological Sciences, Professor
Farida Khosrov Gahramanova

Doctor of Philosophy in Biology
Mehriban Rauf Yusifova

Dissertation Council FD1.07 of Supreme Attestation Commission under the President of the Republic of Azerbaijan operating at the Institute Microbiology of MSEAR.

Chairman of the Dissertation Council:
Doctor of Biological Sciences, Professor,
correspondent member of ANAS
Panah Zulfugar Muradov

Scientific secretary of the Dissertation Council:
Doctor of Philosophy in Biology,
Associate Professor
Gunel Ali Gasimova

Chairman of the scientific seminar:
Doctor of Biological Sciences, Professor
Konul Farrukh Bakhshaliyeva

INTRODUCTION

Relevance and degree of development of the topic. Proteolytic enzymes play a crucial role in the regulation of various biological processes going on tissue, cellular and molecular level. Main function of these enzymes consists of hydrolyzing proteins and «*they have very exclusive significance in the lives of animals, plants and microorganisms*»[1].

Study of proteases takes a central place in enzymology. It is associated by both their physiological role they play in an organism and the wide application in agriculture and scientific-research works. Nowadays «*proteases account for 60% of enzymes applied*»[2] in the industry and medicine. Acid proteases in the food industry are used for baking bread, accelerating the cheese-making process, forming its taste and smell, producing fermented milk products, improving meat quality, and getting protein hydrolyzate.

Proteases have been transformed into irreplaceable medicaments for the purpose of treatment «*as activator of proteins of the hemostasis system in digestion improvement and organism*»[3]. Great perspectives are opened up in the direction of proteases' application *«for the treatment of neoplasms (different malignant tumors)»* [4] emerging in the organism. Proteolytic enzymes are proposed as an effective means in blood vessels *«in the treatment of thrombi, dissolution of fibrins and fibrinogens»*[5].

Obtaining proteases from animals and plants is limited due to

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[1] Jarocki, V.M., Tacehi, J.L., Djordjevic, S.P. Nonproteolytic functions of microbial proteases increase pathological complexity // Proteom, – 2015, – V.15 (5-6), – P.1075-1088.

[2] Tavano, O.L. Protein hydrolysis using proteases: An important tool for food Biotechnology // Jour. Molec. Catal. B Enzyme, – 2013, – V.90, – P.1-11.

[3] Mane, P., Tale, V. Overview of microbial therapektic enzymes // Inter. Jour. Current Microbiol Appl. Sciences, – 2015, – V.4, – P.17-26.

[4] Mane, P., Tale, V. Overview of microbial therapektic enzymes // Inter. Jour. Current Microbiol Appl. Sciences, – 2015, – V.4, – P.17-26.

[5] Mane, P., Tale, V. Overview of microbial therapektic enzymes // Inter. Jour. Current Microbiol Appl. Sciences, – 2015, – V.4, – P.17-26.

dependence on climate, lack of enzyme output, large amount of living material required, and ethical issues. Thus, proteases widely used in the industry are obtained from microorganisms (fungi and bacteria). This, first of all, is related to their relatively easy production, *«high growth rate and capability of continuous enzyme synthesis»*[6]. Protease medications obtained from animals and plants are now being replaced by those of bacteria and fungi. For example, proteases obtained from bacteria and fungi are used instead of the abomasum enzyme obtained from the stomach of calves, and used in the production of cheese made up milk. One of the most studied proteases' producers among bacterias is referred to as genus *Bacillus*. It must be noted that proteases synthesized by bacterias can reveal efficient activity in narrow acidity range (pH 7-9.5), except for small exceptional cases. Synthesized by fungi *«both proteases are sustainable and in broad acidity range (pH 2,5-9,0)»*[7] they operate effectively. Basidium fungi, yeast fungi and mold fungi have been studied as producers of proteases. Mold fungis have been widely studied as they are capable of synthesizing proteases in broad pH range (acid, neutral and alkaline). Of acid proteases *«maximum activity ranges pH 2,5-5,5»*[8] and widely applied in food industry. Acid proteases synthesized by mold fungi present in 2 types: pepsin-like and renin-like proteases. Pepsin is a protease found in digestive juices, whereas renin is a protease synthesized in the abomasum of ruminants. In the scale of industry pepsin-like proteases are obtained from various specimens of fungi of genus *Aspergillus*, while renin-like proteases from fungi specimens of genus *Mucor*. Fungi types which synthesize acid proteases, such as *Aspergillus oryzae, A. niger, A. terreus, A. hennebergii, A. tamari,*

[6]Madhu, P., Pallan, N. Production of Bacterial acid Protease by submerged fermentation using *Aeromonas caviae* from dairy effluent // Jour. Biotechnol. Bioenginering, – 2018, – V.2, – N1, – P.1-7.

[7]Madhu, P., Pallan, N. Production of Bacterial acid Protease by submerged fermentation using *Aeromonas caviae* from dairy effluent // Jour. Biotechnol. Bioenginering, – 2018, – V.2, – N1, – P.1-7.

[8]Madhu, P., Pallan, N. Production of Bacterial acid Protease by submerged fermentation using *Aeromonas caviae* from dairy effluent // Jour. Biotechnol. Bioenginering, – 2018, – V.2, – N1, – P.1-7.

Penicillium bilaiae, P. caseicolum, P. griseoroseum, Mucor circinellaides, Rhizopus oryzae have been thoroughly studied.

It follows to be noted that productivity of mold fungi used in the industry as producers of proteases is gradually reducing in the period of exploitation and even may drop to minimum. Therefore search and finding of new producers with high activity able to sythesize protease is always needed.

Object and subject of the research. The object of the research was fungal strains, like *Aspergillus clavatus* BDU-11, *A. clavatus* BDU-32, *A. clavatus* BDU-91, *Aspergillus flavus* BDU-12, *A. flavus* BDU-44, *Aspergillus fumigatus* BDU-2, *A. fumigatus* BDU-8, *A. fumigatus* BDU-48, *Aspergillus niger* BDU-6, *A. niger* BDU-15, *A. niger* BDU-28, *A. niger* BDU-42, *Aspergillus terreus* BDU-18, *A. terreus* BDU-38, *A. terreus* BDU-64, *Aspergillus versicolor* BDU-16, *A. versicolor* BDU-21, *A. versicolor* BDU-42, *Penicillium alboroseum* BDU-A2, *P. albo-roseum* BDU-16, *P. albo-roseum* BDU-LK22, *Penicillium chrysogenium* BDU-A22, *Penicillium corelueum* BDU-A18, *P. corelueum* BDU-L11, *Penicillium lividum* BDU-M6, *P. lividum* BDU-LK14, *Penicillium notatum* BDU-M5, *Penicillium olivaceum* BDU-A21, *P. olivaceum* BDU-LK-17, *P. olivaceum* BDU-M8, *Penicillium ramosum* BDU-A19, *P. ramosum* BDU-LK27, *P. ramosum* BDU-M5, *Penicillium spinolosum* BDU-L34, *P. spinolosum* BDU-M132, *Penicillium subcinereum* BDU-L16, *P. subcinereum* BDU-M21, *Penicillium turbatum* BDU-A117, *P. turbatum* BDU-M35, *Penicillium vinaceum* BDU-A13, *P. vinaceum* BDU-L15, *P. vinaceum* BDU-M20, which were isolated from the soils of Azerbaijan.

Subject of the research was to detect proteolytic activity of mold fungi and to characterize them as producers of acidic pH 2,5, acidic pH 5,5, neutral (pH 7,2) and alkaline (pH 9,5) proteases.

Purpose and duties of the research. Purpose of the dissertation work was to conduct screening of mold fungi having acid protease activity spreaded in the territory of Azerbaijan and to obtain producers with high activity.

The following duties have been set in order to reach the certain purpose:

1. Obtain pure cultures of mold fungi from soil and plant samples taken from the territory of Azerbaijan and evaluate them for their growth rate;

2. Evaluate high growth rate of mold fungi based on their common proteolytic activity and select active producers;

3. Evaluate selected active producers for the activity of acidic (pH 2.5 and pH 5.5), neutral and alkaline proteases and implement their identification;

4. Study the effect of medium factors on enzyme synthesis in fungal strains with high acid protease activity;

5. Obtain relatively purified acid protease medicaments from fungal strains with high activity;

6. Study some biotechnological properties of the obtained acid protease medicaments (temperature and pH optimum, thermostability, acid resistance).

Methods of the research. Separation of mold fungi from soil and plant samples, obtaining pure cultures, and their evaluation for growth rate were carried out by generally accepted microbiological methods. Identification of fungal strains was implemented on the basis of relevant determinants. The common proteolytic activity of the selected strains was determined by the viscometric method, an Ostwald viscometer with a capillary diameter of 0.8 mm and gelatin as a substrate was used in this process. The activity of acidic (pH 2.5 and pH 5.5), neutral (pH 7.2) and alkaline (pH 9.5) proteases in strains with high proteolytic activity was determined by the modified Anson method and casein was used as a substrate. Relatively pure protease medicaments were obtained from the fungus's culture liquid by precipitation via acetone, ethanol and $(NH_4)_2SO_4$. The optimum temperature and acidity, thermostability and acid resistance of acid protease medicaments were determined by generally accepted methods. All practices have been conducted in 4 repetitions and obtained actual numbers have been statistically worked.

The main provisions of the defense:

1. Mold fungal strains of high growth rate are featured with high proteolytic activity. Acid (pH 5.5) protease activity in fungi of the genera *Aspergillus* and *Penicillium* selected as active producers is

much higher than the activity of neutral and alkaline proteases;

2. Proteases are constitutively synthesized in the exponential phase as the primary metabolite in the studied fungal strains *Aspergillus flavus* BDU-44 and *Penicillium notatum* BDU-M5;

3. The optimal biosynthesis condition of acid protease is different in Aspergillus flavus BDU-44 and Penicillium notatum BDU-M5 strains with high proteolytic activity;

4. $(NH_4)_2SO_4$, is applied as an optimal precipitating agent in order to obtain relatively pure protease medicaments from the studied mold fungal strains;

5. Acid protease medicaments obtained from *Aspergillus flavus* BDU-44 and *Penicillium notatum* BDU-M5 strains vary in relation to temperature and acidity.

Scientific innovation of the research. Fungi of genera *Aspergillus* and *Penicillium* with high proteolytic activity collected from the territory of Azerbaijan were able to synthesize acid (pH 2.5 and pH 5.5), neutral (pH 7.2) and alkaline (pH 9.5) proteases. Activity of acid (pH 5,5) protease has been significantly much more than that of neutral and alkaline proteases.

It has been determined that proteases in the strains of *Aspergillus flavus* BDU-44 and *Penicillium notatum* BDU-M5 are constitutively synthesized in the exponential phase in an active manner as the primary metabolite.

It has been shown that optimal biosythesis temperature (35°C) of acid proteases in the strain of *Aspergillus flavus* BDU-44 is different from optimal growth temperature (30°C) of the fungus, however, optimal biosythesis temperature (30°C) of acid proteases in the strain of *Penicillium notatum* BDU-M5 overlaps with optimal growth temperature (30°C) of the fungus.

It has been determined that acid proteases obtained from active producers vary in relation to temperature and acidity. So, the maximum activity of acid (pH 5.5) protease obtained from *Aspergillus flavus* BDU-44 strain was recorded at 60°C temperature and pH 4.5 acidity, the enzyme was resistant to 40-60°C temperature and pH 3.5-6.5 acidity for 3 hours. The optimum activity of acid protease

obtained from *Penicillium notatum* BDU-M5 strain was observed at 55°C and pH 5.0, the enzyme was resistant to 40-50°C temperature and pH 3.5-5.5 acidity for 3 hours.

Thereotical and practical significance of the research. Conclusions made about proteolytic activity of fungi of genera *Aspergillus* and *Penicillium* enrichen know-how on enzymatic properties of these fungi. The obtained scientific results regarding the relationship of acid (pH 5.5) protease medicaments to temperature and acidity show that the biotechnological properties of proteolytic enzymes can be very different depending on its origin.

As a result of optimization of biosynthesis process of acid proteases special activity of enzyme in *Aspergillus flavus* BDU-44 strain rose from 115 units to 142 units, but in *Penicillium notatum* BDU-M5 strain from 226 units 277 ones. Obtained results allow these fungi to use as producers of acid proteases.

It was determined that during the precipitation of acid proteases from the culture medium with $(NH_4)_2SO_4$ and acetone, their activity increased by 2.2-2.3 and 1.7-1.8 times, respectively, compared to the culture medium, and ethanol became ineffective as a precipitating factor, even reduced the activity of enzymes by 1.3-1.5 times compared to the culture medium. These obtained results can be used for proper choice of precipitating agents in obtaining of acid protease medicaments from the mold fungi.

The study of the biotechnological properties of acid protease medicaments obtained from fungi, such as the optimum activity temperature, optimum acidity (pH), thermostability and resistance to acidity, gives rises for their application by various purposes.

Approval and application of the dissertation. 7 articles and 8 theses were published in connection of the dissertation topic. The materials of the dissertation were reported at the VII International scientific conference on "Innovation problems of modern biology" for young scientists and researchers dedicated to the 94th anniversary of the Great Leader Heydar Aliyev, the great son of the Azerbaijani people, (Baku, April 27-28, 2017), at the International scientific-practical conference on "Heydar Aliyev's legacy: islamic solidarity in the modern era" (Baku, May 10, 2017), at the III International

scientific conference on "Ecology: nature and society problems" dedicated to the 110th anniversary of Academician Hasan Aliyev (Baku, December 26-27, 2017), at the VIII International scientific conference on "Innovative approaches in modern biology" for young scientists and researchers dedicated to the 95th anniversary of the Great Leader Heydar Aliyev, the great son of the Azerbaijani people, (Baku, April 27-28, 2018), at the International scientific-practical conference on "Heydar Aliyev model of statehood in the modern era: realities and facts" (Baku, May 8, 2018), at the IX International scientific conference on "Innovative approaches in modern biology" dedicated to the 100th anniversary of Baku State University (Baku, May 24- May 25, 2019), at the International scientific-practical conference "Biotechnology of microorganisms" dedicated to professor Y.K.Fomichov (Minsk, November 27-29, 2019), at the I International scientific and practical conference on "Modern Science: Innovations and Prospects" (Stockholm, October 10-12, 2020).

Name of the institution where the dissertation work was fulfilled. «Chemistry, biology and ecology» department of Odlar Yurdu University, «Microbiology and Virology» scientific-research laboratory of Baku State University.

Structure of the dissertation and its total volume with characters. The dissertation work consists of introduction and 5 chapters, final analysis of the results, results and used literature list. The dissertation contains 160 pages including tables, figures and literature list which makes up 251937 characters in total.

BRIEF SUMMARY OF THE DISSERTATION

Chapter I. Proteolytic activity of microorganisms

In Section 1.1. of the dissertation general information is presented about functions of the proteolytic enzymes, classification and its importance in industry, biotechnology, medicine. Proteolytic activity of bacterias, characteristics of bacterias as main producers of neutral and alkaline proteases is researched in Section 1.2. of the dissertation; proteolytic activity of basidium fungi and characteristics

of proteases synthesized by them in Section 1.3.; proteolytic activity of mold fungi in Section 1.4. In Section 1.5 of the dissertation, the proteolytic activity of mold fungi, biotechnological characteristics of alkaline, neutral and acid proteases synthesized by them, the main producers of proteolytic enzyme medicaments, properties, such as thermostability and resistance to acidity of proteases are analyzed.

Chapter II. Material and methods of the dissertation work

2.1. Separation of pure cultures of mold fungi from nature and their identification methods. The main object of the research was mold fungi separated from soils and plant residues in decay taken from the territories of Azerbaijan. The standard liquid and agar malt juice nutrient medium was used for the separation of fungi from natural substrates, their extraction into pure culture and the study of their morpho-cultural properties. For identification of mold fungi their morphological signs *«shape, size, presence of septum in hyphae, form and size of spores, form, size and colour of spore carriers»* [9] were recorded by microscope. Culture features, like the color of the colony, the shape of its surface and edges, consistency and reverzum, the nature of growing in a liquid nutrient medium were studied in dry and liquid nutrient mediums.

2.2. Method of identification of growth rate of fungal cultures. Initial evaluation of fungal cultures has been implemented in accordance with their growth rate. For that *«growth rate»*[10] of fungi was identified *based on the determined formula* in modified agar Czapec-Dox nutrient medium.

2.3. Method of identification of general proteolytic activity of fungal cultures. General proteolytic activity of cultures was identified by viscometric method and used *«2,75% gelatin solution as enzyme substrate»*[11]. For this purpose, an Ostwald viscometer with a

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[9] Nagamani, A., Kanwar, I., Manoharachary, C. Hand book of soil fungi. – LK. International Pvt. Ltd, – 2006, – 386p.

[10] Бухало, А.С. Высшие съедобные бизидиомицеты в чистой культуре / А.С.Бухало. – Киев: Наукова Думка, – 1988. – 143с.

[11] Бухало, А.С. Высшие съедобные бизидиомицеты в чистой культуре / А.С.Бухало. – Киев: Наукова Думка, – 1988. – 143с.

capillary diameter of 0.8 mm was applied. The degree of hydrolysis of gelatin was found on the basis of the decrease in the viscosity of the reaction mixture in the experimental version. Activity unit of enzymes was expressed as %/min/mg protein (or V/mg protein).

2.4. Methods of identification of acid, neutral and alkaline proteases. Acid (pH 2,5 və pH 5,5), neutral (pH 7,2) and alkaline (pH 9,5) «proteases' activity by modified Anson method»[12] was identified. As a substrate of enzyme 2% casein solution (sodium caseinate) was used. Such the amount of enzyme was taken as activity unit that enables to transform casein to 1mcmol tyrosine (0,181 mg) at 37°C for 1 min. Activity of enzymes was expressed as mcmol/min/mg protein (or V/mg protein).

The biosynthesis process was optimized by studying the effect of temperature, medium acidity, carbon and nitrogen sources on the biosynthesis of proteases.

2.5. Methods of identification of some biotechnological properties of relatively purified proteases. Relatively purified enzyme was used in order to study some biotechnological properties of proteases (optimum temperature, thermostability, optimum pH and resistance to acidity(pH)). For that purpose, proteases were precipitated from culture liquid via ethyl alcohol, acetone and $(NH_4)_2SO_4$ solution.

All practices have been conducted in 4 repetitions and obtained outcomes have been «statistically»[13]worked.

Chapter III. Obtaining of pure cultures of mold fungi from the soils of Azerbaijan and evaluation of their proteolytic activity

3.1. Obtaining of pure cultures of mold fungi and evaluation for their growth rate. 135 soil and 25 decaying plant specimens were taken from different parts of Azerbaijan of which pure cultures

[12] Лабораторный практикум по технологии ферментных препаратов / И.В.Грачева, Ю.П.Грачев, М.С.Мосичев [и др.]. – Москва: Легкая и пищевая промышленность, – 1982. – 240с.

[13] Кобзарь, А.И. Прикладная математическая статистика / А.И.Кобзарь, – Москва: ФИЗМАТЛИТ, – 2019. – 816с.

of 142 mold fungi's strains were obtained and the cultures were evaluated for growth rate (factor). The genus and species composition of the strains with high growth rate was determined, 18 of them were determined to refer to genus *Aspergillus* (*A. clavatus* – 3 strains, *A. flavus* – 2 strains, *A. fumigatus* – 3 strains, *A. niger* – 4 strains, *A. terreus* – 3 strains, *A. versicolor* – 3 strains) 24 of them to genus *Penicillium* (*P. alba-roseum* – 3 strains, *P. chrysogenium* – 1 strains, *P. corelueum* – 2 strains, P. *lividum* – 2 strains, *P. notatum* – 1 strain, *P. olivaceum* – 3 strains, *P. ramosum* – 3 strains, *P. spinulosum* – 2 strains, *P. subcinereum* – 2 strains, *P. turbatum* – 2 strains, *P. vinaceum* – 3 strains). The upcoming experiments were contiued with these strains having growth rate more than 40.

3.2. Evaluation of proteolytic activity of fungi of genera *Aspergillus* and *Penicillium*. Screening of fungi of genera *Aspergillus* and *Penicillium* high growth rate was carried out for general proteolytic activity. General proteolytic (gelatinase) activity of 18 strains relating to 6 species of fungi of genus *Aspergillus* was studied, the maximum general proteolytic activity was found out in the strains of *Aspergillus niger* BDU-15 and *A. flavus* BDU-44.

General proteolytic activity of 24 strains relating to 11 species of fungi of genus *Penicillium* with high growth rate was studied, and the maximum activity was found out in the fungus of *Penicillium notatum* BDU-M5.

Biosynthesis dynamics of the extracellular acid (pH 2,5 and pH 5,5), neutral (pH 7,2) and alkaline (pH 9,5) proteases was studied in the fungi of *Aspergillus niger* BDU-15, *A. flavus* BDU-44 (figure 3.2.1) and *Penicillium notatum* BDU-M5 (figure 3.2.2) with both high growth rate and high general proteolytic activity. It has been determined that active biosynthesis of proteases in all strains takes place in the exponential phase of the fungus - the intensive development phase and maximum activity reveals itself in the 72nd hour of incubation. Growth of the fungus slows down after the 72nd hour of incubation and enters the stationary phase. Enzyme activity in the stationary phase is sharply decreasing.

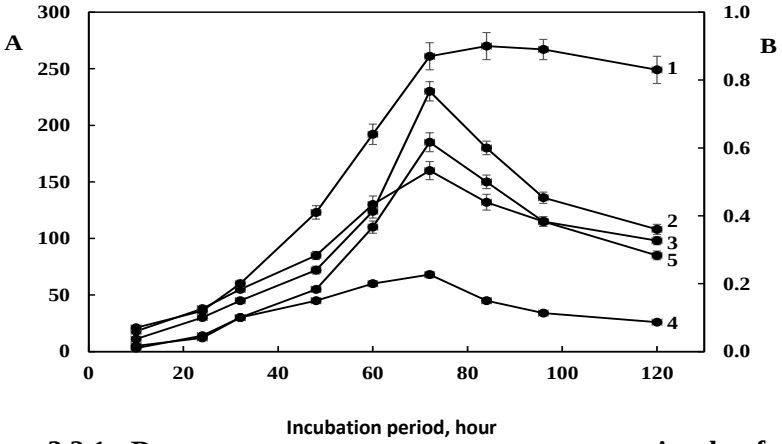

Figure 3.2.1. Dynamics of biosynthesis of proteases in the fungus *Aspergillus flavus* BDU-44. A – protease activity, V/mg protein; B – fungal biomass, g/l; 1 – growth of fungus; 2 – acid protease (pH 5,5); 3 – acid protease (pH 2,5); 4 – neutral protease (pH 7,2); 5 – alkaline protease (pH 9,5)

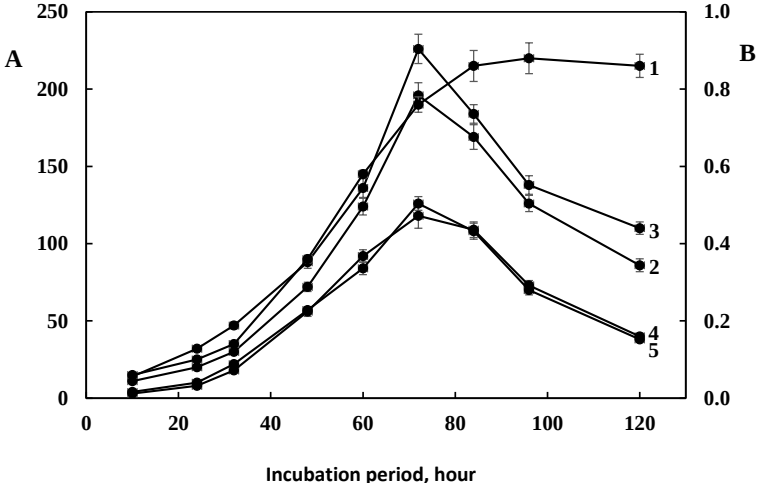

Figure 3.2.2. Dynamics of biosynthesis of proteases in the fungus *Penicillium notatum* BDU-M5. A – protease activity, V/mg protein; B – fungal biomass, g/l; 1 – growth of fungus; 2 – acid protease (pH 5,5); 3 – acid protease (pH 2,5); 4 – neutral protease (pH 7,2); 5 – alkaline protease (pH 9,5)

It must be noted that activity of acid proteases was higher than that of neutral and alkaline proteases in the studied strains. For instance, activity of acid proteases was higher than that of neutral and alkaline proteases in *Aspergillus flavus* BDU-44 strain. For example, activity of acid proteases was higher than that of neutral and alkaline proteases, correspondingly 3,4 and 1,5 times in *Aspergillus flavus* BDU-44 strain, 1,8 and 1,3 times in *A. niger* BDU-15 strain, 1,8 and 1,9 times in *Penicillium notatum* BDU-M5 strain. Thus, activity of acid proteases was significantly higher than that of neutral and alkaline proteases in the selected strains. Activity of proteases in *Aspergillus flavus* BDU-44 and *Penicillium notatum* BDU-M5 strains was 1,7-3,2 times more than activity of proteases in *Aspergillus niger* BDU-15 strain.

Regarding this, further studies were continued with strains of *A. flavus* BDU-44 and *P. notatum* BDU-M5.

Chapter IV. Effect of medium factors on the biosynthesis of acid proteases in the fungi with high proteolytic activity

The results presented in the third chapter showed that although fungi with high proteolytic activity are capable of synthesizing acid, neutral and alkaline proteases, the activity of acid proteases is significantly higher than the activity of neutral and alkaline proteases. Therefore, the effect of medium factors (incubation period, temperature, medium acidity, carbon sources, organic and inorganic nitrogen sources) on the biosynthesis of acid proteases was studied in the *Aspergillus flavus* BDU-44 and *Penicillium notatum* BDU-M5 strains.

It should be noted that the growth rate of *Aspergillus flavus* BDU-44 and *Penicillium notatum* BDU-M5 strains was sufficiently higher than the growth rates of the other studied strains.

4.1. Effect of medium factors on the biosynthesis of acid proteases in the fungus *Aspergillus flavus* BDU-44. The effect of the incubation period on the biosynthesis of acid proteases was studied in the fungus *Aspergillus flavus* BDU-44 and determined that the intensive biosynthesis of both pH 2.5 and pH 5.5 acid proteases begins in the 24th hour of incubation and reaches its maximum in the 72nd hour. In this case, the activity increases by 6.8 times. The stationary development phase begins after the 72nd hour of incubation,

the biosynthesis of enzymes gradually slows down, and the activity of enzymes decreases by 1.4-1.6 times in the 120th hour. In the 24th hour of incubation, the activity of acid pH 5.5 protease was 1.4 times higher than the activity of acid pH 2.5 protease. Most propably, the active biosynthesis of the first enzyme begins earlier than the synthesis of the second enzyme.

The study of the effect of temperature on the biosynthesis of acid proteases in *Aspergillus flavus* BDU-44 strain showed that the maximum biosynthesis of enzymes occurs at 35°C temperature, despite the fact that the maximum fungus growth is connected to the temperature of 30°C (figure 4.1.1). As the temperature rises above 35°C, the activity of enzymes decreases sharply. Thus, the activity of acid pH 2.5 and pH 5.5 proteases at 45°C temperature was respectively 6.8 and 5.8 times less than that at 35°C temperature. So, the maximum biosynthesis of acid proteases in *A. flavus* BDU-44 strain is observed at 35°C temperature and does not overlap with the optimum growth temperature of the fungus.

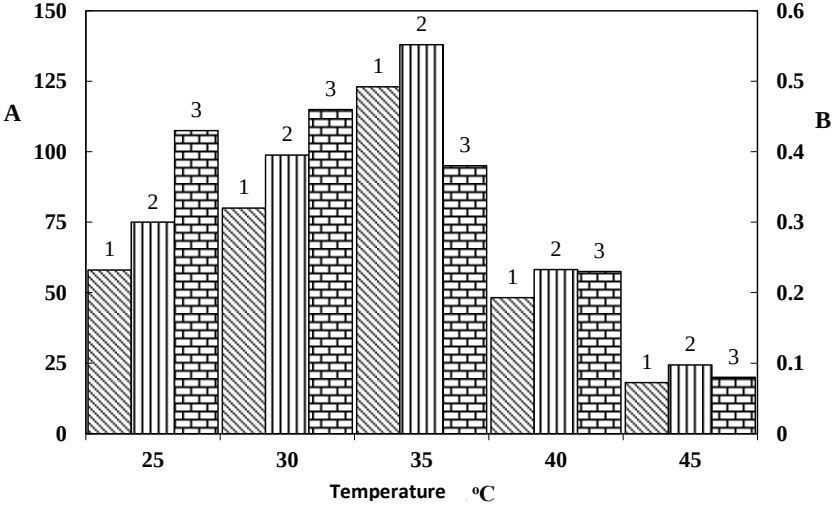

Figure 4.1.1. Effect of temperature on the biosynthesis of acid proteases in the fungus *Aspergillus flavus* BDU-44. A – protease activity, V/mg protein; B – fungal biomass, g/l; 1 – acid protease (pH 2,5); 2 – acid protease (pH 5,5); 3 – growth of fungus.

The study of the effect of medium acidity on the growth of *Aspergillus flavus* BDU-44 strain and the synthesis of acid proteases showed that the high activity of enzymes was in the range of pH 4.0-5.0, and the maximum activity indicated pH 5.0 (figure 4.1.2). The maximum activity of acid pH 5,5 protease was 1,3 times higher than that of pH 2,5 protease.

The effect of sugars, gelatin and casein was studied as a carbon source on the synthesis of acid proteases of Aspergillus flavus BDU-44 strain. High activity of enzymes was observed in sucrose and glucose containing mediums from sugars, but the maximum activity was recorded in the sucrose containing medium (figure 4.1.3). Casein and gelatin were capable to increase enzyme activity by 1.4-4.0 and 1.3-3.6 times, respectively, compared to sugars as the main substrates of proteases.

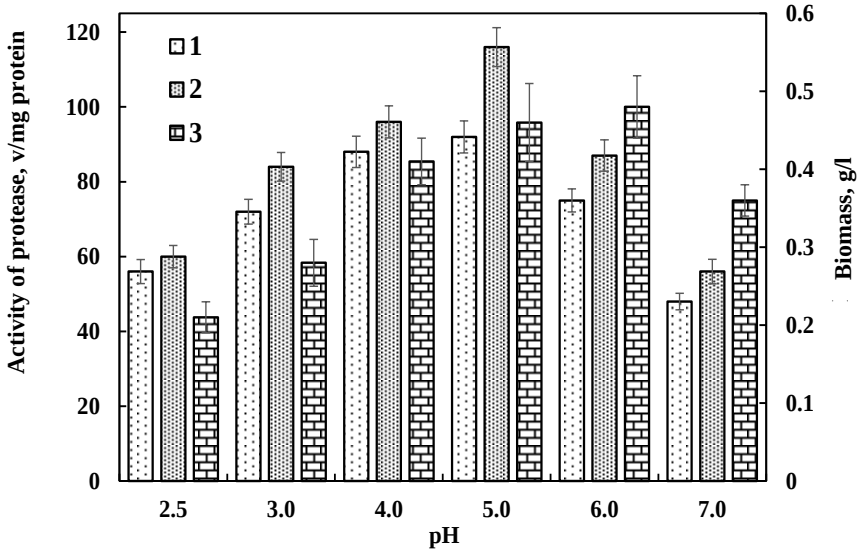

Figure 4.1.2. Effect of medium acidity (pH) on the biosynthesis of acid proteases in the fungus *Aspergillus flavus* BDU-44. 1 – pH 2,5 acid protease, 2 – pH 5,5 acid protease, 3 – growth of fungus.

Acid proteases' biosynthesis in all studied substrates and little rise in activity of the medium having their possible inducers (casein and gelatin) indicate that the biosynthesis of these enzymes carries constitutive nature.

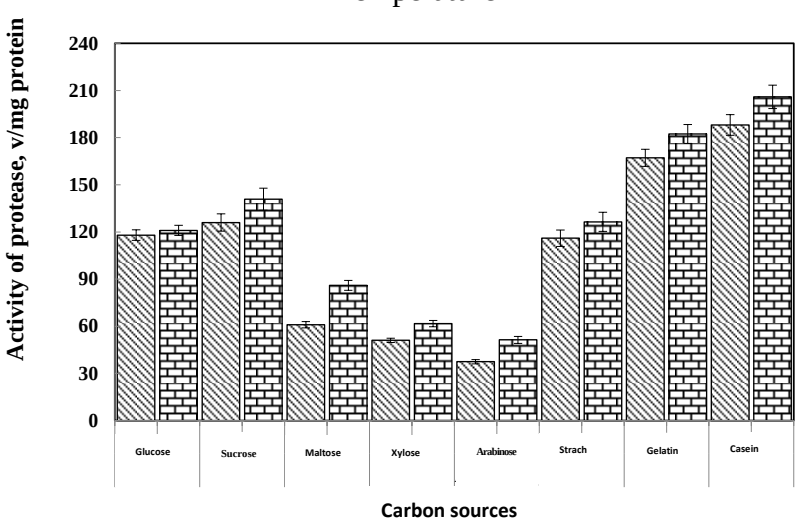

Figure 4.1.3. Effect of carbon sources on the biosynthesis of acid proteases in the fungus *Aspergillus flavus* BDU-44: 1 – pH 2,5 acid protease, 2 – pH 5,5 acid protease.

The study of the effect of nitrogen sources in *Aspergillus* BDU-44 strain on the biosynthesis of acid proteases detected that enzyme activity was high in the medium with organic nitrogen sources (asparagine, peptone and urea), but the maximum activity revealed itself in the medium with peptone. Ammonium salts from inorganic nitrogen sources led to high activity and the maximum activity was observed in the medium consisting of $(NH_4)_2HPO_4$. Activity of enzymes in mediums of KNO_3 and $NaNO_3$ was 2.3 and 3.4 times less than ammonium salts, respectively (figure 4.1.4).

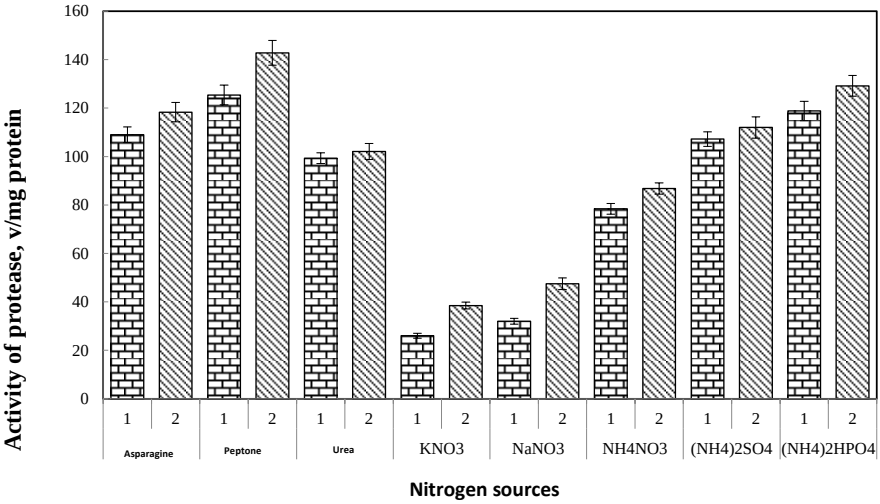

Figure 4.1.4. Effect of nitrogen sources on the biosynthesis of acid proteases in the fungus *Aspergillus flavus* BDU-44: 1 – pH 2,5 acid protease; 2 – pH 5,5 acid protease.

4.2. Effect of medium factors on the biosynthesis of acid proteases in the fungus *Penicillium notatum* BDU-M5. The effect of incubation period on the biosynthesis of acid proteases in *Penicillium notatum* BDU-M5 strain was studied along the growth dynamics of the fungus. It was determined that the intensive biosynthesis of acid proteases began in the 36th hour of incubation and reached its maximum in the 72nd hour. Nonetheless, the intensive development (exponential) phase of the fungus continued until the 84th hour, the biosynthesis process of enzymes decreased sharply and decreased by 2.1 times in the 120th hour (figure 4.2.1).

The study of the effect of temperature on the biosynthesis of acid proteases in *Penicillium notatum* BDU-M5 strain showed that the maximum biosynthesis of enzymes occurs at 30°C temperature, so that is, the maximum growth temperature of the fungus.

The effect of acidity indicators, such as pH 2,5; 3,0; 4,0; 5,0; 6,0 and 7,0 was studied on the biosynthesis of acid proteases and fungal growth in the fungus *Penicillium notatum* BDU-M5. In all indicators of acidity, the fungus developed and synthesized enzymes.

The maximum biomass of the fungus was recorded at pH 5.0, and the maximum biosynthesis of enzymes at pH 6.0.

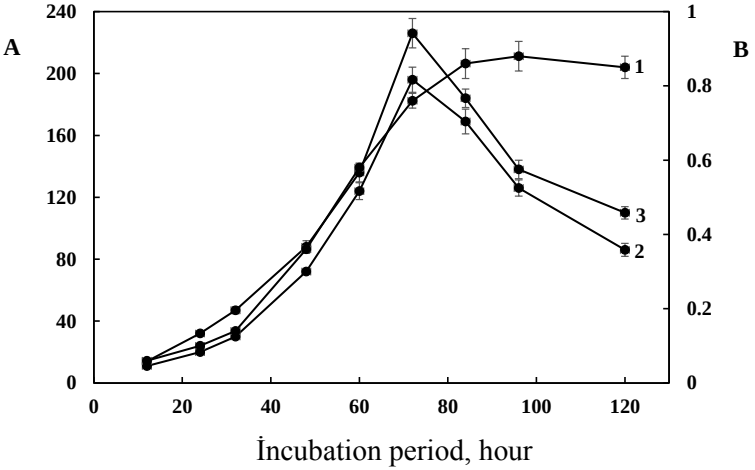

Figure 4.2.1. Biosynthesis dynamics of acid proteases in the fungus *Penicillium notatum* BDU-M5: A – protease activity, V/mg protein; B – fungal biomass, g/l; 1. growth curve of fungus; 2. pH 2,5 acid protease; 3. pH 5,5 acid protease.

The effect of carbon sources on the biosynthesis of acid proteases in *Penicillium notatum* BDU-M5 strain was studied, the maximum activity revealed in the glucose medium, though the high activity of the enzyme was recorded in the glucose and sucrose containing mediums from sugars. Although the activity of enzymes in casein and gelatin - substrates of proteases, is high, it has not significantly distinguished from sugars. Therefore, the biosynthesis of acid proteases in *P. notatum* BDU-M5 strain goes constitutively.

Of organic nitrogen sources in the peptone containing medium, the enzyme activity was 2 times more than in asparagine and urea. The impact character of inorganic nitrogen sources on the biosynthesis of acid proteases in *Penicillium notatum* strain was alike to the effect in *Aspergillus flavus* BDU-44 strain (figure 4.1.4). Thus, the biosynthesis of enzymes was high in the medium containing ammonium salts, and the maximum activity was recorded in the medium of

$(NH_4)_2HPO_4$.

So, it was determined that the maximum biosynthesis of acid proteases takes place in the 72nd hour of incubation, at of 35°C temperature, in the indicator of pH 5.0 acidity, in sucrose as a carbon source and in peptone as a nitrogen source in *Aspergillus flavus* BDU-44 strain, and in the 72nd hour of incubation, at 30°C temperature, in the indicator of pH 6.0 acidity, in glucose as a carbon source and in peptone as a nitrogen source. Therefore, referring to the optimal parameters of the biosynthesis of acid protease, *Aspergillus flavus* BDU-44 and *Penicillium notatum* BDU-M5 strains were similar in two parameters (incubation period and nitrogen source), but differed in optimal temperature, medium acidity and carbon source.

Chapter V. Biotechnological properties of acid proteases obtained by the precipitation method from fungis' culture medium

5.1. Effect of precipitating agents on the acitivity of protease. Relatively purified enzyme medicaments were used in order to study the biotechnological properties of acid proteases and proteases were precipitated by acetone, ethanol and $(NH_4)_2SO_4$ from culture liquid of the fungus for this purpose. Precipitated enzymes were separated from the culture liquid and used as enzym medicaments.

The activity of acid, neutral and alkaline proteases was determined in the enzyme preparation precipitated from the culture liquid of *Aspergillus flavus* BDU-44 strain and compared with the activity in the culture liquid (figure 5.1.1). The activity of acid proteases in the enzyme medicament precipitated by acetone was 1,7-1,8 times, but the activity of neutral and alkaline proteases was 1,6-1,7 times more than the activity in culture liquid. The activity of acid proteases in the enzyme medicament precipitated by ammonium sulfate was 2,2-2,3 times, but the activity of neutral and alkaline proteases was 1,8 times higher than the activity in culture liquid. At the same time the activity of acid proteases in this medicament was 1,2-1,4 times more than the activity in medicament precipitated by acetone. Precipitation by ethanol affected enzymes negatively and the activity of proteases in the obtained drug was 1,2-1,5 times less than that in

culture liquid. It means that $(NH_4)_2SO_4$ was considered to become the most optimal precipitator for obtaining relatively purified proteases from the culture liquid of *Aspergillus flavus* BDU-44 strain. In all options the activity of pH 5,5 acid proteases was 1,5-2,5 times more than the activity of acid pH 2,5, neutral and alkaline proteases.

Table 5.1.1. Activity of proteases obtained from *Aspergillus flavus* BDU-44 fungus by precipitation method

Proteases	Activity of proteases, V/mg protein			
	Culture liquid	Precipitation with acetone	Precipitation with ethanol	Precipitation with $(NH_4)_2SO_4$
pH 2,5 acid protease	56±2,2	98±4,2	38±2,6	122±8,2
pH 5,5 acid protease	86±3,3	146±56	68±2,0	198±9,4
Neutral protease (pH 7,2)	43±2,1	68±3,2	38±1,5	78±3,3
Alkaline protease (pH 9,5)	56±2,4	96±4,3	52±1,1	103±4,5

It should be noted that the activity of proteases during the precipitation with acetone from the culture liquid of *Penicillium notatum* BDU-M5 strain was 1.9-2.3 times, during precipitation with ethanol 1.5-1.7 times, during precipitation with $(NH_4)_2SO_4$ 1.2-1.3 times more than that in culture liquid. Therefore, $(NH_4)_2SO_4$ was the most efficient protease precipitator, whereas ethanol did not negatively affect the activity of enzymes as a precipitating factor.

Thus, $(NH_4)_2SO_4$ was the best precipitator for obtaining the relatively purified proteases from the culture liquid of *Aspergillus flavus* BDU-44 and *Penicillium notatum* BDU-M5 strains. Etanol affected the activity of proteases negatively as a precipitating agent in *A. flavus* BDU-44 strain.

5.2. Biotechnological properties of pH 5.5 acid protease obtained from *Aspergillus flavus* BDU-44 strain. Some technological properties (opt. temp., thermostability, opt. acidity and acid resist.) of relatively pure acid (pH 5.5) protease obtained from *Aspergillus flavus* BDU-44 strain were studied. It was determined that high activity of the enzyme appears at 50-60°C, and its maximum activity at 60°C. When the temp. rises above 60°C, activity of the enzyme decreases sharply (fig. 5.2.1). Thermostability of acid protease was high and able to keep activity of enzyme at 40-60°C for 3 hours (fig. 5.2.2).

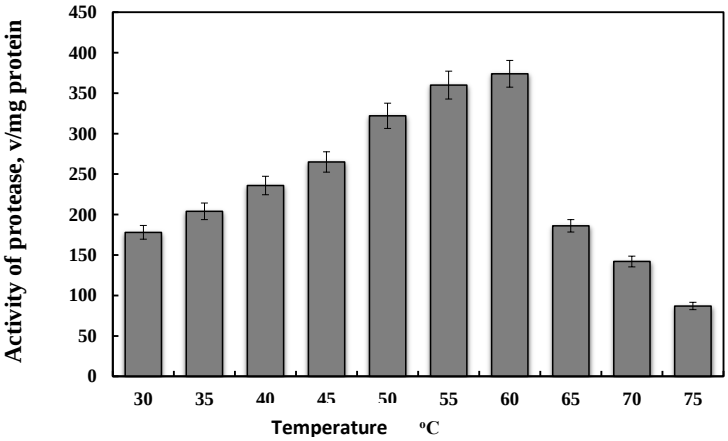

Figure 5.2.1. Effect of temperature on activity of acid protease obtained from *Aspergillus flavus* BDU-44 strain.

Effect of medium acidity (pH) on activity of acid protease was studied in pH 2.5-8.0 acidity. It was determined that high activity of enzyme is revealed in the range of pH 4.0-5.5, and maximum activity in the indicators of pH 4.5-5.0 (fig. 5.2.3). Enzyme was able to keep its activity the range of pH 3,5-6,5 for 3 hours (fig. 5.2.4).

In this case, it was determined that acid protease obtained from *Aspergillus flavus* BDU-44 strain has maximum activity at 60°C and in pH 4.5-5.0 acidity. The enzyme is able to keep its stability at 40-60°C temperature and in pH 3.5-6.5 acidity.

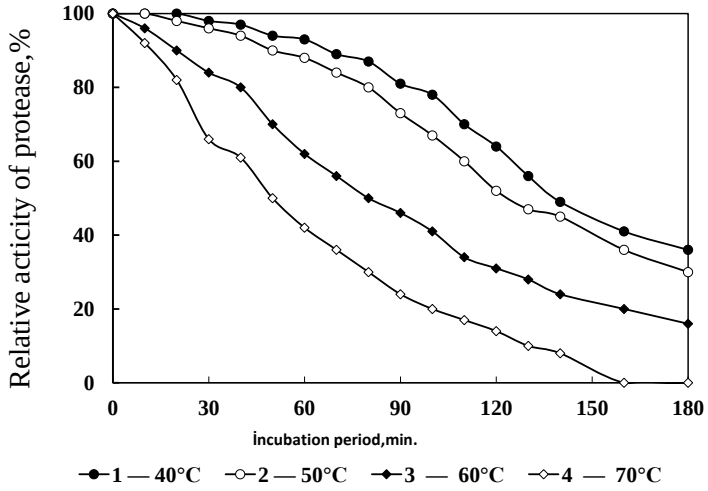

Figure 5.2.2. Thermostability of acid protease obtained from *Aspergillus flavus* BDU-44 strain. 1 – 40°C, 2 – 50°C, 3 – 60°C, 4 – 70°C

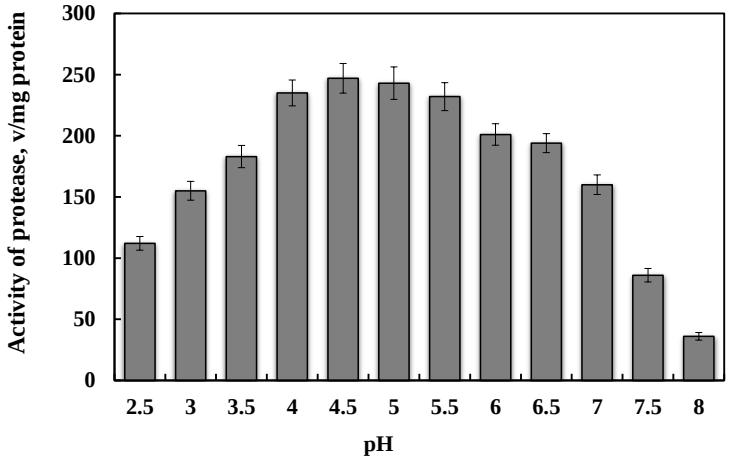

Figure 5.2.3. Effect of acidity (pH) on activity of acid protease obtained from *Aspergillus flavus* BDU-44 (pH) strain

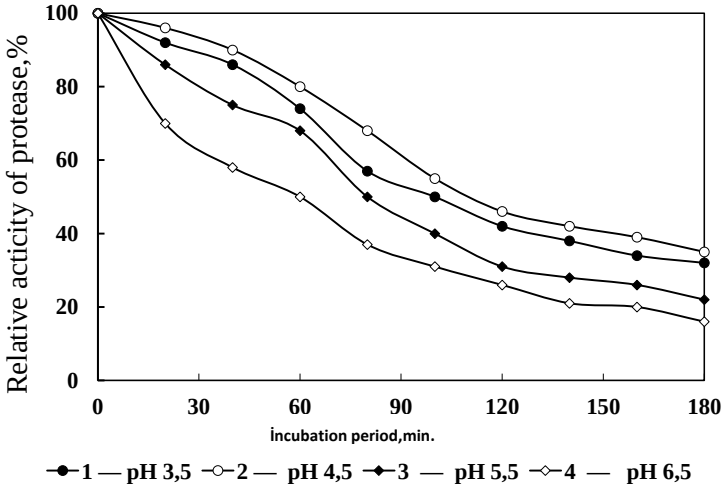

Figure 5.2.4. Resistance of acidity of acid protease obtained from *Aspergillus flavus* BDU-44 strain. 1 – pH 3,5; 2 – pH 4,5; 3 – pH 5,5; 4 – pH 6,5

5.3. Biotechnological properties of acid protease obtained from *Penicillium notatum* BDU-M5 fungus. Relation of acid protease (pH 5.5) obtained from *Penicillium notatum* BDU-M5 strain to temp. was studied in the range of 30-75°C. The enzyme had high activity in the range of 50-60°C, and maximum activity at 55°C (fig. 5.3.1), and was able to maintain its stability at 40-50°C for 3 hours (fig. 5.3.2).

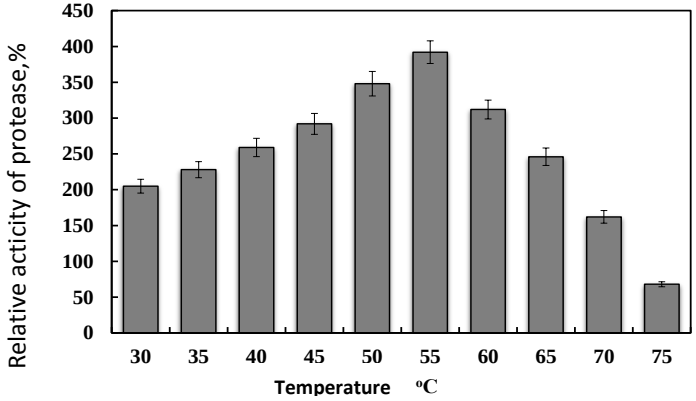

Figure 5.3.1. Effect of temperature on activity of acid protease (pH 5,5) obtained from *Penicillium notatum* BDU-M5 strain

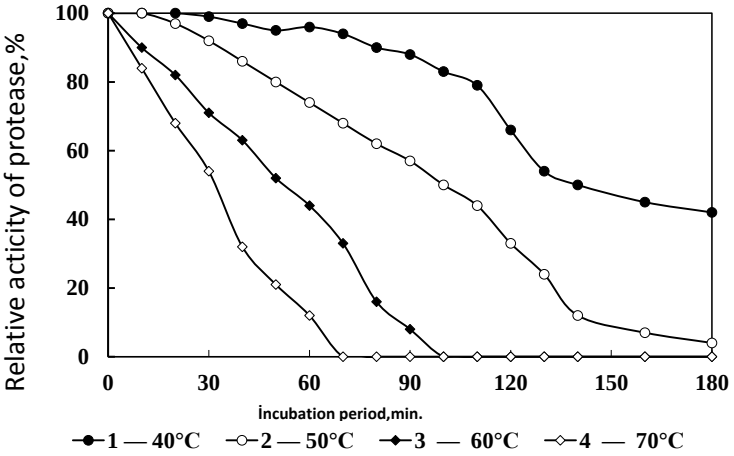

Figure 5.3.2. Thermostability of acid protease obtained from *Penicillium notatum* BDU-M5 strain. 1 – 40°C, 2 – 50°C, 3 – 60°C, 4 – 70°C

As it comes to its relation to acidity, it was determined that enzyme has high activity in the indicators of pH 3,5-5,5, and maximum activity in the indicator of pH 5,0 (figure 5.3.3). Enzyme was able to keep its activity in the indicators of acidity pH 3,5; 4,5 and 5,5.

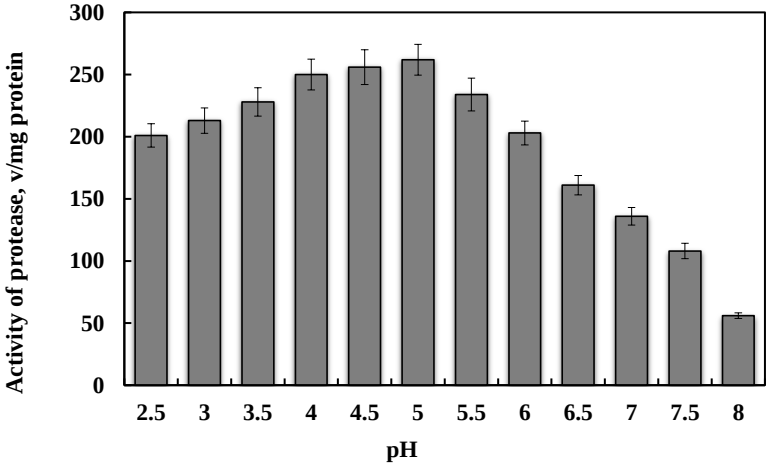

Figure 5.3.3. Effect of acidity on activity of acid protease obtained from *Penicillium notatum* BDU-M5 strain

So, acid protease obtained from *Penicillium notatum* BDU-M5 strain had maximum activity at 55°C temperature and in pH 5,0 acidity, kept its stability at 40-50°C temperature and in pH 3,5-5,5 acidity.

It must be noted that the protease obtained from *Aspergillus flavus* BDU-44 strain differed from the protease obtained from *Penicillium notatum* BDU-M5 strain in some properties. Therefore, ethanol as a precipitating agent decreases the activity by 1,2-1,5 times negatively affecting the proteases of *A. flavus* BDU-44 strain. The maximum activity of acid protease obtained from *A. flavus* BDU-44 strain is observed at 60°C temperature and in pH 4,5 acidity, and the maximum activity of acid protease obtained from *P. notatum* BDU-M5 strain at 55°C temperature and in pH 5,0 acidity. Acid protease of *A. flavus* BDU-44 strain shows its stability at 40-60°C temperature and in pH 3,5-6,5 acidity, but acid protease of *P. notatum* BDU-M5 strain at 40-50°C temperature and in pH 3,5-5,5 acidity.

FINAL ANALYSIS OF THE RESEARCH

135 soil and 25 plant specimens, separated 142 mold fungus's strains which were taken from the different parts of Azerbaijan as object of the research.

The growth rate of the strains obtained in the form of pure culture was determined according to the growth factor, and 42 strains with a high growth rate were selected. 18 of the strains were referred to the genus *Aspergillus,* and 24 to the genus *Penicillium*. Genus *Aspergillus* was represented with 6 samples (*A. clavus, A. flavus, A. fumigatus, A. niger, A. terreus* and *A. versicolor*), genus *Penicillium* with 11 samples (*P. alboroseum, P. chrysogenium, P. corebueum, P. Lividum, P. notatum, P. subcinereum, P. turbatum* and *P. vinaceum*).

Screening of fungal strains with high growth rate was conducted according to general proteolytic activity. *A. flavus* BDU-44 and *A. niger* BDU-15 strains with high proteolytic activity were selected among the fungi of genus *Aspergillus,* and *Penicillium notatum* BDU-M5 strain from the fungi of genus *Penicillium*.

The biosynthesis dynamics of extracellular acid (pH 2.5 and 5.5), neutral (pH 7.2) and alkaline (pH 9.5) proteases was studied in *Aspergillus niger* BDU-15, *A. flavus* BDU-44 and *Penicillium notatum* BDU-M5 strains with high proteolytic activity. It was determined that the active biosynthesis of proteases in the studied strains occurs in the exponential development phase of the fungus. The biosynthesis of proteases in *Aspergillus flavus* BDU-44 and *Penicillium notatum* BDU-M5 strains begins in the 10th hour of incubation, and in the 32nd hour of it in *A. niger* BDU-15 strain. On the other hand, the activity of acid proteases in *A. flavus* BDU-44 and *P. notatum* BDU-M5 strains was 3.2-3.5 times more than the activity of acid proteases of *A. niger* BDU-15 strain. In each of these three strains the activity of acid proteases was much higher that of neutral and alkaline proteases. Thus, acid proteases in the fungi are more actively sythesized relative to neutral and alkaline proteases.

As acid proteases in *Aspergillus flavus* BDU-44 and *Penicillium notatum* BDU-M5 strains with high proteolytic activity are more actively synthesized relative to neutral and alkaline proteases, the effect of medium factors (incubation period, temperature, medium acidity, carbon and nitrogen sources) was studied on the biosynthesis of acid proteases in these strains. It was determined that the active biosynthesis of acid proteases in both strains occurs in the exponential phase, and the maximum activity is revealed in the 72nd hour of incubation. However, active biosynthesis of protease in *Aspergillus flavus* BDU-44 strain begins in the 24th hour of incubation, and in *Penicillium notatum* BDU-M5 strain in the 36th hour of incubation.

By comparig the studied *Aspergillus flavus* BDU-44 and *Penicillium notatum* BDU-M5 strains, it gets apparent that the maximum biosynthesis of acid proteases in *A. flavus* BDU-44 strain occurs at a temperature of 35°C and does not overlap with the optimum temperature for the growth of the fungus. However, the maximum biosynthesis of the enzyme in *P. notatum* BDU-M5 strain occurs at 30°C temperature and overlaps with the maximum growth temperature of the fungus. Generally, the maximum activity of acid proteases occurs in *A. flavus* BDU-44 strain in the 72nd hour of incubation, at 35°C temperature, in the indicator of pH 5.0 acidity, in sucrose as

carbon source and peptone as nitrogen source, but in *P. notatum* BDU-M5 strain in the 72nd hour of incubation, at 30°C temperature, in the indicator of pH 6.0 acidity, in glucose as a carbon source, and peptone as a nitrogen source. So, in accordance with optimal parameters of the biosynthesis of acid proteases, *A. flavus* BDU-44 and *P. notatum* BDU-M5 strains are similar for two traits (incubation period and nitrogen source), while they are different for their optimum temperature, acidity and carbon sources. Acid proteases in both fungal strains are synthesized as the first metabolite in the intensive growth phase of the fungus and this synthesis carries constitutive character.

As a result of optimization of the biosynthesis condition of the acid proteases the enzyme activity increased from 115 units to 142 units in *Aspergillus flavus* BDU-44 strain, and from 226 units to 277 units in *Penicillium notatum* strain.

Relatively purified enzyme drugs were obtained from culture liquid by the precipitation method in order to study the biotechnological properties of acid pH 5,5 proteases. Optimal precipitating factor was $(NH_4)_2SO_4$ for precipitating the acid proteases. Maximum activity of the acid protease obtained from *Aspergillus flavus* BDU-44 strain was recorded at 60°C temperature and in pH 4,5-5,5 acidity, thermostability of the enzyme was at 40-60°C temperature, acid resistance in the indicators of pH 3,5-6,5. Maximum activity of the protease obtained from *Penicillium notatum* BDU-M5 strain was observed at 55°C temperature and in the indicator of pH 6,0, thermostability of the enzyme was at 40-50°C temperature, acid resistance in the indicators of pH 3,5-5,5.

RESULT

1. Proteolytic activity of 142 mold fungi' strains was studied which were separated from samples taken from the different parts of Azerbaijan and *Aspergillus flavus* BDU-44 and *Penicillium notatum* BDU-M5 strains with high activity were selected. Activity of the acid protease in both strains was 1,5-3,4 more than the activity of neutral and alkaline proteases.

2. It has been determined that the acid protease (pH 5,5) in Aspergillus flavus BDU-44 and *Penicillium notatum* BDU-M5 strains are synthesized as the first metabolite and constitutively. Maximum activity of the enzyme revealed itself in peptone medium as organic nitrogen source in the 72nd hour of culture growth, and in $(NH_4)_2HPO_4$ medium as inorganic nitrogen source. Maximum activity of the protease in *A. flavus* BDU-44 strain was observed at 35°C temperature, in pH 5,0 acidity and in sucrose medium, but in *P. notatum* BDU-M5 strain at 30°C temperature, in pH 6,0 acidity and in glucose medium.
3. As a result of optimization of the biosynthesis condition of the acid protease the enzyme activity increased from 115 V/mg protein to 142 V/mg protein in *Aspergillus flavus* BDU-44 strain, 226 V/mg protein to 277 V/mg protein in *Penicillium notatum* BDU-M5 strain. Activity of the acid protease (pH 5,5) in *P. notatum* BDU-M5 strain was double more than that of the acid protease in *A. flavus* BDU-44 strain.
4. It was determined that $(NH_4)_2SO_4$ and acetone are effective precipitating agents for obtaining relatively pure acid protease drugs in both *Aspergillus flavus* BDU-44 and *Penicillium notatum* BDU-M5 fungi. Nevertheless, the activity of acid protease in the drug precipitated with $(NH_4)_2SO_4$ was 1.4 times more than the activity in the drug precipitated with acetone. Ethanol as a precipitating agent negatively affected the activity of acid protease in *A. flavus* BDU-44 strain.
5. Maximum activity of acid protease obtained from *Aspergillus flavus* BDU-44 strain was observed at 60°C temperature and in pH 4,5 acidity and the enzyme was at 40-60°C temperature and resistant to pH 3,5-6,5 acidity for 3 hours. Maximum activity of acid protease obtained from *Penicillium notatum* BDU-M5 strain was observed at 55°C temperature and in pH 5,0 acidity and the enzyme was at 40-50°C temperature and resistant to pH 3,5-5,5 acidity for 3 hours.

List of published scientific works on the topic of dissertation

1. Səfərova, A.X. Mikroorqanizmlərin proteolitik aktivliyinin öyrənilməsi barədə / Azərbaycan xalqının böyük oğlu, Ulu öndər Heydər Əliyevin anadan olmasının 94-cü ildönümünə həsr olunmuş gənc alimlərin və tədqiqatçıların «Müasir Biologiyanın İnnovasiya Problemləri» mövzusunda VII Beynəlxalq elmi konfransın materialları. Bakı – 2017, – s.134.
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3. Səfərova, A.X., Şəfiyeva, S.M., Qənbərov, X.Q. Müxtəlif biotoplazdan ayrılmış kif göbələklərinin jelatinaza aktivliyi / Akademik Həsən Əliyevin 110 illik yubileyinə həsr olunmuş «Ekologiya: təbiət və cəmiyyət problemləri» III Beynəlxalq elmi konfransın materialları. Bakı – 2017, – s.222-223.
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5. Səfərova, A.X., Şəfiyeva, S.M., Babayeva, İ.T. *Aspergillus niger*-44 kif göbələyinin turş proteazaları / «Müasir dövrdə Heydər Əliyev dövlətçilik modeli: reallıqlar və faktlar» mövzusunda Beynəlxalq elmi-praktiki konfransın materialları. Bakı – 2018, – s.406.
6. Səfərova, A.X. *Aspergillus* cinsli kif göbələklərin proteolitik aktivliyinin böyümə əmsalına görə təyin edilməsi / Azərbaycan xalqının böyük oğlu, Ulu öndər Heydər Əliyevin anadan olmasının 95-ci ildönümünə həsr olunmuş gənc alimlərin və tədqiqatçıların «Müasir biologiyada innovativ yanaşmaları» mövzusunda VIII Beynəlxalq elmi konfransın materiallar. Bakı – 2018, – s.99.
7. Səfərova, A.X., Şəfiyeva, S.M., Ağayeva, N.A., Qənbərov, X.Q. *Penicillium* cinsli göbələklərin proteolitik aktivliyi // AMEA-nın Mikrobiologiya İnstitutunun elmi əsərləri, – Bakı, – 2018, – c.16, №1, – s.48-53.
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