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НАЦІОНАЛЬНИЙ АВІАЦІЙНИЙ УНІВЕРСИТЕТ
ФАКУЛЬТЕТ ЕКОЛОГІЧНОЇ БЕЗПЕКИ, ІНЖЕНЕРІЇ ТА ТЕХНОЛОГІЙ
КАФЕДРА БІОТЕХНОЛОГІЇ

ДОПУСТИТИ ДО ЗАХИСТУ
Завідувач випускової кафедри
________ М.М. Барановський
«___» ______________2021 р.

ДИПЛОМНА РОБОТА

(ПОЯСНЮВАЛЬНА ЗАПИСКА)

ЗДОБУВАЧА ВИЩОЇ ОСВІТИ ОСВІТНЬОГО СТУПЕНЯ «БАКАЛАВР»
СПЕЦІАЛЬНІСТЬ 162 «БІОТЕХНОЛОГІЇ ТА БІОІНЖЕНЕРІЯ»
ОСВІТНЬО-ПРОФЕСІЙНА ПРОГРАМА «ФАРМАЦЕВТИЧНА БІОТЕХНОЛОГІЯ»

Тема: «Технології отримання протеаз з міцеліальних грибів»

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DEPARTMENT OF BIOTECHNOLOGY

ALLOWED TO DEFENCE
Head of the graduate department
____________ M.M. Baranovsky
«___»________________2021

BACHELOR THESIS

(EXPLANATORY NOTE)

OF GRADUATING STUDENT OF EDUCATIONAL DEGREE «BACHELOR»
SPECIALTY 162 «BIOTECHNOLOGY AND BIOENGINEERING»
EDUCATIONAL PROFESSIONAL PROGRAM «PHARMACEUTICAL BIOTECHNOLOGY»

Theme: « Technology of obtaining proteases from mycelial fungi »

Executor: student of group 404a, FESET Poliukh K.I.

Supervisor of studies: PhD in Biology, Assoc. Prof Andrianova T.V.

Standards inspector: Drazhnikova A.V.

KYIV 2021

НАЦІОНАЛЬНИЙ АВІАЦІЙНИЙ УНІВЕРСИТЕТ

Факультет екологічної безпеки, інженерії та технологій

Кафедра біотехнології

Спеціальність 162 «Біотехнології та біоінженерія»

Освітньо-професійна програма «Фармацевтична біотехнологія»

ЗАТВЕРДЖУЮ

Завідувач кафедри

__________ М.М. Барановський

«___»___________2021 р.

ЗАВДАННЯ

на виконання дипломної роботи

Полюх Катерини Ігорівни

1. Тема дипломної роботи: «Технології отримання протеаз з міцеліальних грибів» затверджена наказом ректора від «11» травня 2021 р. № 715/ст.

2. Термін виконання роботи: з 10 травня по 20 червня 2021 р.

3. Вихідні дані роботи: наукові публікації присвячені грибам, здатним до синтезу протеаз, дослідженню і впровадженню методик екстракції протеолітичних ферментів; власні експериментальні дані, одержані на базі навчальної лабораторії кафедри біотехнології факультету екологічний безпеки інженерії та технологій Національного авіаційного університету; зразки грибів та рослинний матеріал, що містив мікроміцети, зібрані власноруч та отримані з колекції KW Інституту ботаніки імені М. Г. Холодного НАН України.

4. Зміст пояснювальної записки: Вступ; Літературний огляд; Матеріали та методи досліджень; Результати досліджень продуцентів протеолітичних ферментів та технології отримання цих метаболітів; Висновки; Список бібліографічних посилань використаних джерел.

5. Перелік обов’язкового графічного (ілюстративного) матеріалу: 7 рис., 2 таблиці.

6. Календарний план-графік

№	Завдання	Термін виконання	Підпис керівника
1	Вибір теми дипломної роботи, узгодження змісту з дипломним керівником	10.05.2020 – 12.05.2020	
2	Літературний огляд та збір інформації за темою дипломної роботи: «Технології отримання протеаз з міцеліальних грибів».	12.05.2020 – 13.05.2020	
3	Складання схеми виконання бакалаврської дипломної роботи.	13.05.2020	
4	Ознайомлення з методами проведення експерименту, виконання експериментальної частини	13.05.2020 – 15.05.2020	
5	Аналіз та обробка отриманих даних.	15.05.2020 – 16.05.2020	
6	Оформлення практичної частини дипломної роботи на основі отриманих результатів.	16.05.2020 – 18.05.2020	
7	Формулювання висновків та рекомендацій.	18.05.2020	
8	Перевірка дипломної роботи керівником.	19.05.2020	
9	Попередній захист дипломної роботи.	02.06.2020	

10	Коригування, підготовка доповіді і презентації	21.05.2020 – 03.06.2020	
11	Захист дипломної роботи.	14.06.2020	

7. Дата видачі завдання: «10 травня 2021 р.»

Керівник дипломної роботи________________ /*Андріанова Т.В./*

Завдання прийняв до виконання________________ /*Полюх К.І.*/

NATIONAL AVIATION UNIVERSITY

Faculty Environmental Safety, Engineering and Technologies

Department of Biotechnology

Speciality: 162 «Biotechnology and bioengineering»

EPP: «Pharmaceutical Biotechnology»

APPROVED

Head of the Department

____________ M.M. Baranovsky

«___» _____________2021

TASK

From bachelor thesis of student

Poliukh Kateryna Igorivna

1. The theme of the thesis work: «Technology of obtaining proteases from mycelial fungi » approved by the Rector «11» of May 2021 № 715/art

2. The term of the work: from the 10th of May, 2021 to the 20th of June, 2021.

3. Output data of the work: scientific publications on fungi that can synthesize proteases, experimental study and implementation methods of proteolytic enzymes extraction, personal experimental data received on the basis of the educational laboratory of the Department of Biotechnology, Faculty of Environmental Safety, Engineering and Technology, National Aviation University; own collections of fungal samples in nature and the samples of micromycetes from the KW collection of the M.G. Kholodny Institute of Botany, NAS of Ukraine.

4. Content of the explanatory note: Introduction; Literary review; Materials and methods of research; Results of the proteolytic enzymes producers study and technology for this metabolites production; Conclusions; References.

5. List of compulsory (illustrative) materials: 2 tables, 7 figures.

6.Schedule

№	Task	Execution term	Signature of the head
1	The choice of the theme of the thesis, content conformation with the supervisor.	10.05.2020 – 12.05.2020	
2	Publications review and various information collection on the thesis topic: "Technology of obtaining proteases from mycelial fungi"	12.05.2020 – 13.05.2020	
3	Drawing up of the scheme of the bachelor's thesis fulfillment.	13.05.2020	
4	Acquaintance with some experimental methods, performance of experimental part	13.05.2020 – 15.05.2020	
5	Analysis and processing of the received data.	15.05.2020 – 16.05.2020	
6	Registration of the practical part of the thesis on the basis of the obtained results.	16.05.2020 – 18.05.2020	
7	Formulation of conclusions and recommendations.	18.05.2020	
8	Examination of the thesis by the supervisor.	19.05.2020	
9	Preliminary defence of the graduating work.	02.06.2020	
10	Editing of the report text and final presentation	21.05.2020 – 03.06.2020	
11	Defence of the graduating work.	14.06.2020	

7. Date of task receiving: «10» of May 2020

Supervisor of degree work_______________________ */Andrianova T.V./*

Task for execution was taken by__________________ */Poliukh K.I./*

ABSTRACT

Explanatory note to the thesis "Technology of obtaining proteases from mycelial fungi": 68 pages, 7 figures, 2 tables, 58 sources used.

Object of investigation - analysis of proteolitic enzymes as metabolites of mycelial fungi, their isolation and investigation for the pharmaceutical biotechnology.

Purpose of the work - to develop and improve biotechnological scheme of proteases production from mycelial fungi, to receive pure cultures of fungi which are proteases producers.

Proteases represent a large and diverse group of hydrolytic enzymes that are classified by their site of action, enzyme active site structure, and specific reaction mechanisms

The use of proteases in different industries has been prevalent for many decades and a number of microbial sources exist for the efficient production of this enzyme. Their vast diversity, specific range of action and property of being active over a very wide range of temperature and pH have attracted the attention of biotechnologists worldwide. Although they are widely distributed in nature, fungi are the preferred source of these enzymes in fermentation bioprocesses because of their fast growth rate and also because they can be genetically engineered to generate new enzymes with desirable abilities or simply for enzyme overproduction. The search for new microorganisms that can be used for protease production is a continuous process. Proteases have various applications in major areas of food processing, beverage production, animal nutrition, leather, paper and pulp, textiles, detergents, etc. and with the advent of new frontiers in biotechnology, the spectrum of protease applications has expanded into many new fields such as clinical, medicinal and analytical chemistry.

Some species of filamentous fungi, such as *Aspergillus, Penicillium* and *Paecylomices* have been identified as great producers of extracellular protease in submerged fermentation. Further studies on the optimized conditions will still be performed and the protease production will be conducted in bioreactors.

Methods of research – microbiological, mycological, analytical, statistical.

Subject of investigations - mycelial fungi of chesnut Castanea sativa.

PROTEOLYTIC ENZYMES, FUNGAL CULTURES, PHARMACEUTICAL SUBSTANCES, TECHNOLOGICAL SCHEME, ASCOMYCOTA, MYCELIAL FUNGI, SOLID-STATE FERMENTATION

РЕФЕРАТ

Пояснювальна записка до дипломної роботи "Технологія отримання протеаз з міцеліальних грибів": 68 сторінок, 7 малюнки, 2 таблиці, 58 використані джерела.

Об'єкт дослідження: аналіз протеолітичних ферментів як метаболітів міцеліальних грибів, їх виділення та дослідження для фармацевтичної біотехнології.

Мета роботи: запропонувати та вдосконалити біотехнологічні схеми отримання протеаз міцеліальних грибів, отримати чисті культури грибів, що є продуцентами протеаз.

Протеази представляють велику та різноманітну групу гідролітичних ферментів, які класифікуються за місцем їх дії, структурою активного сайту ферменту та специфічними механізмами реакції.

Застосування протеаз у різних галузях промисловості було поширеним протягом багатьох десятиліть, і існує ряд мікробних джерел для ефективного виробництва цього ферменту. Їх величезна різноманітність, специфічний діапазон дії та властивість бути активними в дуже широкому діапазоні температур та pH привернули увагу біотехнологів у всьому світі. Хоча вони широко поширені в природі, гриби є найкращим джерелом цих ферментів у ферментаційних біопроцесах через їх швидкий темп росту, а також тому, що вони можуть бути генетично розроблені для отримання нових ферментів з бажаними здібностями або просто для перепродукції ферментів. Пошук нових мікроорганізмів, які можуть бути використані для виробництва протеази, є безперервним процесом. Протеази мають різне застосування у основних галузях харчової промисловості, виробництва напоїв, харчування тварин, шкіри, паперу та целюлози, текстилю, миючих засобів тощо. такі як клінічна, лікарська та аналітична хімія.

Деякі види ниткоподібних грибів, такі як *Aspergillus, Penicillium* та *Paecylomices*, були визначені великими продуцентами позаклітинної протеази при зануреному бродінні. Подальші дослідження оптимізованих умов все ще будуть проводитись, а виробництво протеази проводитиметься в біореакторах.

Методи дослідження: мікробіологічні, мікологічні, аналітичні, статистичні.

Предмет дослідження: міцеліальні мікроскопічні гриби Castanea sativa.

ПРОТЕОЛІТИЧНІ ФЕРМЕНТИ, КУЛЬТУРИ ГРИБІВ, ФАРМАЦЕВТИЧНІ РЕЧОВИНИ, ТЕХНОЛОГІЧНА СХЕМА, ASCOMYCOTA, МІЦЕЛІАЛЬНІ ГРИБИ, ТВЕРДОФАЗНА ФЕРМЕНТАЦІЯ

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LIST OF SYMBOLS AND ABBREVIATIONS

TCA -trichloroacetic acid

DEAE- diethylaminoethanol

SDS-PAGE- sodium dodecyl-sulfate polyacrylamide gel electrophoresis

SSF- solid state fermentation

SmF- Submerged fermentation

GRAS- generally recognized as safe

INTRODUCTON

Actuality of theme. Proteases are present in all living organisms, participating in the hydrolysis of unwanted proteins as well as in the regulation of different physiological processes. Proteases are capable of breaking specific peptide bonds of targeted protein-producing peptides (limited proteolysis) and unable to reduce proteins to their constituent amino acids. There are nonspecific proteases which can reduce a complete protein to amino acids (unlimited proteolysis). The proteases represent the largest group of commercially available enzymes worldwide, accounting for 60% of the industrial enzymes market, due to their wide range of applications in food and beverage, cleaning products, animal feed, pharmacy, and cosmetics. Proteases constitute a broad and complex group of enzymes that differ in properties such as substrate specificity, nature of their active sites, catalytic mechanism, optimum pH, optimum temperature, and stability profile. Study of these properties is fundamental for proteases because knowledge of these enzymes' characteristics will dictate their application at the industrial level.

The purpose of the work is to develop and improve biotechnological scheme production of proteases from mycelial fungi

To achieve this goal, the following tasks were set:

1. To conduct a literature review of scientific works on the study of proteolytic enzymes.

2. To isolate the mycelial fungi into a pure culture and increase the mycelial mass for further experiments.

3. Describe the technological scheme of production of proteolytic enzymes from mycelial fungi.

Object of investigation: analysis of proteolitic enzymes as metabolites of mycelial fungi, their isolation and investigation for the pharmaceutical biotechnology.

Subject of investigations: mycelial fungi of chesnut Castanea sativa.

Research methods: microbiological, mycological, analytical and statistical.

Scientific novelty of the results. Novel theoretical and experimental investigations of mycelial fungi as potent alternative producers of proteolytic enzymes was carried out; pure cultures of fungi with capability to protease synthesis were isolated and accumulated; generalized schemes of isolation of these enzymes were constructed.

The practical significance of the results. Alternative ways of obtaining proteases are described. The materials of the work can be used during scientific research as well as in the practice of biotechnologists and pharmacists. The description of proteases and alternative ways of their isolation could be the basis for the development of a new group of drugs.

Graduate′s personal contribution. The entire volume of experimental research on the thesis, analysis of literature data, selection and description of mycelial fungi; the statistical processing of results, their description, analysis, improvement of extraction schemes of proteolytic enzymes have been performed by the graduate personally under the supervision of PhD Andrianova T.V.

Publications. It has been presented the abstract of the report for the conference "Polit. Challenges of science today" (April, 2021): Poliukh K.I. Modern state in the research and application of proteolytic enzymes of fungi. Book of Abstracts of the Conference "Polit. Challenges of science today" April, 2021.

ВСТУП

Актуальність теми. Протеази присутні у всіх живих організмах, беручи участь у гідролізі небажаних білків, а також у регуляції різних фізіологічних процесів. Протеази здатні розривати специфічні пептидні зв'язки цільових пептидів, що продукують білок (обмежений протеоліз), і не здатні відновити білки до складових амінокислот. Існують неспецифічні протеази, які можуть знижувати повноцінний білок до амінокислот (необмежений протеоліз). Протеази представляють найбільшу групу комерційно доступних ферментів у всьому світі, на які припадає 60% ринку промислових ферментів, завдяки їх широкому застосуванню в продуктах харчування та напоях, продуктах для чищення, кормах для тварин, фармацевтиці та косметиці. Протеази складають широку та складну групу ферментів, які відрізняються такими властивостями, як специфічність субстрату, природа їх активних центрів, каталітичний механізм, оптимальний pH, оптимальна температура та профіль стабільності. Вивчення цих властивостей є фундаментальним для протеаз, оскільки знання характеристик цих ферментів визначатиме їх застосування на промисловому рівні.

Метою роботи є розробка та вдосконалення біотехнологічної схеми виробництва протеаз від міцеліальних грибів

Для досягнення цієї мети були поставлені наступні завдання:

1. Провести огляд літератури наукових праць з вивчення протеолітичних ферментів.

2. Виділити міцеліальні гриби у чисту культуру та збільшити масу міцелію для подальших експериментів.

3. Охарактеризуйте технологічну схему виробництва протеолітичних ферментів з міцеліальних грибів.

Об'єкт дослідження: аналіз протеолітичних ферментів як метаболітів

міцеліальних грибів, їх виділення та дослідження для фармацевтичної біотехнології.

Предмет дослідження: міцеліальні мікроскопічні гриби Castanea sativa.

Методи дослідження: мікробіологічні, мікологічні, аналітичні, статистичні.

Наукова новизна результатів. Проведено нові теоретичні та експериментальні дослідження міцеліальних грибів як потужних альтернативних продуцентів протеолітичних ферментів; виділено та накопичено чисті культури грибів, здатних до синтезу протеаз; Були побудовані узагальнені схеми виділення цих ферментів.

Практичне значення результатів. Описані альтернативні способи отримання протеаз. Матеріали роботи можуть бути використані під час наукових досліджень, а також у практиці біотехнологів та фармацевтів. Опис протеаз та альтернативні шляхи їх виділення можуть стати основою для розробки нової групи препаратів.

Особистий внесок випускника. Весь обсяг експериментальних досліджень дипломної роботи, аналіз літературних даних, відбір та опис міцеліальних грибів; статистичну обробку результатів, їх опис, аналіз, вдосконалення схем екстракції протеолітичних ферментів випускник проводив особисто під керівництвом кандидата біологічних наук, доцента Т.В. Андріанової.

Публікації. Опубліковано тези конференції “Polit. Challenges of science today” (квітень, 2021): Poliukh K.I. Modern state in the research and application of proteolytic enzymes of fungi. Book of Abstracts of the Conference “Polit. Challenges of science today”, Kyiv, April, 2021.

CHAPTER 1
LITERATURE REVIEW

1.1. Description of proteolytic enzymes

Research on proteases has been going on for quite some time ever since Phoebus Aaron Theodore Levene reported his studies on “The Cleavage Products of Proteases” in the first issue of The Journal of Biological Chemistry published October 1, 1905. Today, after 116 years and hundreds of thousands articles on these enzymes in the scientific literature, proteases remain at the cutting edge of biological research.

A protease (sometimes it is possible to meet other names such as peptidase or proteinase) an enzyme belonging to the class of hydrolases. It has the ability to increase the rate, the proteolysis reaction. Proteolysis, process in which a protein molecules is broken down partially, into peptides, or completely, into amino acids, by proteolytic enzymes.

These enzymes are thought to have emerged in the early stages of the evolution of primitive organisms as essential destructive enzymes for protein catabolism and amino acid generation. Initial research on proteases focused on their original roles as aggressors associated with protein destruction. But, the realization that, beyond these nonspecific degradative functions, these ferments catalyze highly specific reactions of proteolytic processing, increased the scale of their significance. Recent research in this area has made considerable progress and a significant collection of findings, which indicates the importance of these enzymes in the control of a large number of biological processes in all living organisms. Proteases regulate the fate, localization, and activity of many proteins, modulate protein-protein interactions, create new bioactive molecules, regulation post-transcriptional modification, and generate, transduce, and amplify molecular signals. As a direct result of these multiple actions, proteases influence DNA replication and transcription, cell proliferation and differentiation, tissue morphogenesis and remodeling, heat shock and unfolded protein responses, angiogenesis, neurogenesis, ovulation, fertilization, wound repair, stem cell mobilization, hemostasis, blood coagulation,

inflammation, immunity, autophagy, senescence, necrosis, and apoptosis. Consistent with this fundamental role of proteases in cell behavior and in the survival and death of all organisms, changes in proteolytic systems lie in several pathological conditions such as cancer, neurodegenerative disorders, and inflammatory and cardiovascular diseases. Accordingly, many proteases are gaining attention in the pharmaceutical industry as drugs or as diagnostic and prognostic biomarkers. Proteases also play a key role in plants and contribute to the processing, maturation, or destruction of specific protein stores in response to signs of development or changes in environmental conditions. Similarly, many infectious microorganisms require proteases for replication or use proteases as virulence factors, which has facilitated the development of protease-targeted therapies for diseases of major importance to human life, such as acquired immunodeficiency syndrome.

The extraordinary diversity in protease functions is the result of the evolutionary invention of a multiplicity of enzymes that exhibit a variety shapes and sizes. Thus, the spatial design of proteases ranges from small enzymes made up of simple catalytic units (~20 kDa) to multimeric serine and threonine proteolytic complexes are have molecular masses of 0.7–9 MDa. Thus, some proteases are highly specific for the unique peptide binding of a single protein (e.g., angiotensin-converting enzyme); however, most proteases are relatively non-specific to substrates, and indiscriminately target multiple substrates (e.g., proteinase K). Proteases also follow different strategies, in most cases, operate in the context of complex networks comprising distinct substrates, proteases, cofactors, receptors, inhibitors, adaptors, and binding proteins, which provide an additional level of interest but also complexity to the study of proteolytic enzymes.

1.1.1. Classification of proteases

Three major criteria are used for the classification of the proteolytic enzymes: (1) the reaction catalyzed; (2) the chemical nature of the catalytic site; and (3) the evolutionary relationship, as revealed by structure. Proteases are classified in subgroup 4 of group 3 (hydrolases) (according to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology).

Proteases are divided into 14 subclasses according to the type of catalyzed reactions (Table 1.1), combined into two large groups: endopeptidases (EC 3.4.21–99), and exopeptidases (EC 3.4.11–19).

Table 1.1

Classification of proteases by the type of catalyzed reaction

EC index	Protease group	Mechanism of action
Exopeptidases		
3.4.11	Aminopeptidases	Act on the N-end polypeptide chain, releasing amino acid residue
3.4.13	Dipeptidases	Only act on dipeptides
3.4.14	Dipeptidyl-peptidases Tripeptidyl-peptidases	Act on the N-end polypeptide chain, releasing dipeptide and tripeptide
3.4.15	Peptidyl-dipeptidases	Act on the C-end polypeptide chain, releasing dipeptide
3.4.16	Serine-type carboxypeptidases	Act on the C-end polypeptide chain (Contains serine in the active site)
3.4.17	Metallocarboxypeptidases	Act on the C-end polypeptide chain (Proteases containing in active metal ions center)
3.4.18	Cysteine-type carboxypeptidases	Act on the C-end polypeptide chain (Contains cysteine in active center)
3.4.19	Omega peptidases	Releasing modified terminal residues

Continuation Table 1.1

Endopeptidases		
3.4.21	Serine endopeptidases	Acting on the connection inside polypeptide chain (Contains serine in the active site)
3.4.22	Cysteine endopeptidases	Acting on the connection inside polypeptide chain (Contains cysteine in the active site)
3.4.23	Aspartic endopeptidases	Acting on the connection inside polypeptide chain (Contains aspartic in the active site)
3.4.24	Metalloendopeptidases	Acting on the connection inside polypeptide chain (Proteases containing in active metal ions center)
3.4.25	Threonine endopeptidases	Acting on the connection inside polypeptide chain (Contains threonine in the active site)
3.4.99	Endopeptidases of unknown catalytic mechanism	-

There is an alternative classification of proteases according to the MEROPS database, which was founded in 1993 and created as a database on the Internet in 1996 [1]. The MEROPS classification has a hierarchical order. The MEROPS system groups the enzymes

according to their tertiary structure, considering the order of catalytic residues or "motif" sequences around these residues [2,3].

They can also be classified according to the pH range which they have a higher activity: acidic (pH 2.0 to 6.0), neutral (pH 6.0 to 8.0) and alkaline (pH 8.0 to 13.0).

1.1.2. Mechanism of action of proteases

Proteases are combined into two large groups: endopeptidases (EC 3.4.21–99), which are targeted on internal peptide bonds and exopeptidases (EC 3.4.11–19) (aminopeptidases and carboxypeptidases), the action of which is concentrated on the -NH2 and -COOH ends their respective substrates.

Exopeptidases are enzymes that sequentially cleave peptide bonds closest to the amino or carboxyl end of the substrate molecule (Fig. 1.1.).

Exopeptidases are mainly classified based on their mechanism of action on the substrate: aminopeptidases, carboxypeptidases, dipeptidases, and omegapeptidases.

Aminopeptidases are exopeptidases that selectively release N-terminal amino acid residues from polypeptides and proteins. Most aminopeptidases refers to metalloenzymes, for catalytic action, which the presence of cations of such metals as zinc, cobalt, manganese, magnesium or calcium in the active center is necessary. These enzymes are widely are common among fungi, bacteria, plants, and mammals [4]. They are specifically cleave the amino terminal residue of the polypeptide chain and are involved in the metabolism of biologically active peptides such as hormones, neurotransmitters, food peptides [5]. They are also involved in such biological processes such as activation and maturation of proteins, removal of abnormal proteins [6].

These enzymes are expressed as membrane or cytosolic, or can secreted from cells. The specificity of aminopeptidases is determined the ability to hydrolyze acidic, basic or neutral N-terminal residues [7].

One of the most well-known and widely used aminopeptidases is EC 3.4.11.1 leucyl aminopeptidase (LAP), which preferably catalyzes the hydrolysis of leucine residues from the amino terminus of protein or peptide substrates [8]. As a rule, microbial LAP are

intracellular, however extracellular enzymes have been found in filamentous fungi, in particular in *Aspergillus flavus*.

Carboxypeptidase (CP) cleaves the amino acid at the C terminal of a polypeptide chain to release individual amino acids or dipeptides. According to the structure of the active center, carboxypeptidases are divided into three main groups: Serine-type carboxypeptidases (EC 3.4.16), Metallocarboxypeptidases (EC 3.4.17), and Cysteine-type carboxypeptidases (EC 3.4.18). Serine carboxypeptidases isolated from *Penicillium spp*., *Saccharomyces spp*. and *Aspergillus spp*., are similar in substrate specificity, but slightly differ in other properties such as pH optimum, stability, molecular weight, and inhibitor response. For the activity of metallocarboxypeptidases from *Saccharomyces spp*. the presence of zinc and cobalt cations is necessary [9].

Dipeptidases (EC 3.4.13) specifically cleave peptide bonds into free amino acids only in dipeptides. To them crepe dipeptidase A and membrane dipeptidases.

Omegapeptidases (EC 3.4.19) form a group of exopeptidases that have no preference for the free N- or C-terminus in the substrate. Although there is no need for a charged end group, they often operate close to one end or the other, and are thus completely different from endopeptidase. Omegapeptidases remove terminal substituted, cyclic or linked by an isopeptide bond (different from the bond between α-carboxy and α-amino groups) amino acid residues. Examples of omegapeptidases are EC 3.4.19.12 ubiquitinyl hydrolase 1, EC 3.4.19.2 peptidyl-glycinamidase, EC 3.4.19.3 pyroglutamyl-peptidase I, EC 3.4.19.6 pyroglutamyl-peptidase II, EC 3.4.19.7 N-formylmethionyl-peptidase, and EC 3.4.19.13 glutathione γ-glutamate hydrolase [8,2].

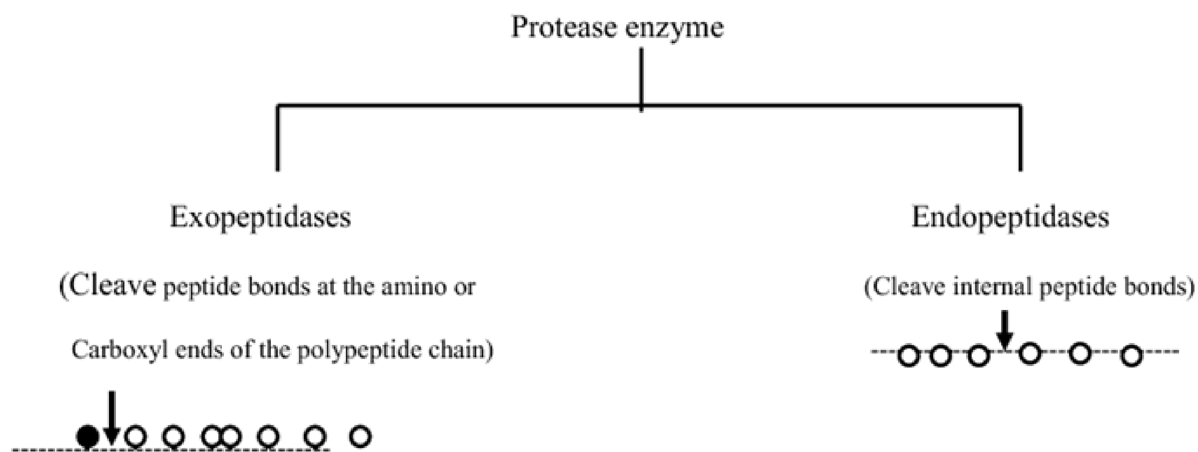

Fig. 1.1. Mechanism of action of proteases

Endopeptidases preferentially hydrolyze peptide bonds in the inner regions of the polypeptide chain (Fig. 1.1.) [8].

The division of endopeptidases into subgroups is primarily based on the structure of the catalytic center. On this basis, they are divided into five subgroups: serine, cysteine, aspartate, threonine and metalloendopeptidase.

Serine endopeptidases (EC 3.4.21). In most serine peptidases there is a catalytic triad consisting of a serine, which bears the nucleophilic hydroxyl group, a histidine, which acts as the general base, and an aspartate which is believed to orientate the imidazolium ring of the histidine so that the Ser and His are close and the hydroxyl group is activated [10,11].

Serine proteases catalyze a two-step hydrolysis reaction with the formation of covalently linked intermediate enzyme-peptide complexes, with the loss of amino acids or a fragment of the peptide [12]. This acylation step is followed by a deacylation process which occurs by a nucleophilic attack on the intermediate by water, resulting in hydrolysis of the peptide. Serine endopeptidases can be classified into three groups based mainly on their primary substrate preference: (i) trypsin-like, which cleave after positively charged residues; (ii) chymotrypsin-like, which cleave after large hydrophobic residues; and (iii) elastase-like, which cleave after small hydrophobic residues.

The subtilisin family is widespread- nonspecific endopeptidases, which include bacterial serine endopeptidase subtilisin (EC 3.4.21.62) and its homologues. They have a wide range of biological functions, in particular, are involved in nutrition and protein

processing [12]. Similar enzymes are produced by various *Bacillus subtilis* strains and other *Bacillus* species [13,14].

Cysteine endopeptidases (EC 3.4.22) catalyze the hydrolysis of carboxylic acid derivatives through a double-displacement pathway involving general acid-base formation and hydrolysis of an acyl-thiol intermediate. The mechanism of action of cysteine proteases is thus very similar to that of serine proteases.

This subclass includes a number of important proteases of plant origin, such as papain, ficain, chymopapain, and some microbial proteases [15].

Metalloendopeptidases (EC 3.4.24) contain divalent metal cations (Zn^{2+}, Mg^{2+}, Mn^{2+}, or Co^{2+}) in the active site. Metalloproteases include enzymes of various origins, such as of EC 3.4.24.3 microbial collagenase, hemorrhagic toxins of snake venom, thermolysin of bacteria, etc.

Threonine endopeptidases (EC 3.4.25) contain threonine residues in the active center. Representatives of this class are part of a relatively well-characterized proteolytic complex, the proteasome. The most widespread are threonine peptidases of the T1-3 families [2].

Aspartate endopeptidases (EC 3.4.23) contain asparagine residues in the active center of the dyad, one of which is in ionized form, the other in protonated form. Catalysis of aspartate proteases follows a common acid-base mechanism. The catalytic dyad, exhibiting basic properties, activates a water molecule on itself a proton. An activated water molecule acting as a nucleophile attacks the carbonyl carbon atom cleavable peptide bond. Aspartate proteases include a number of homologous proteases: pepsin A and B, chymosin, renin, cathepsin D, and related enzymes.

1.2. Sources of protease

Proteases are physiologically necessary for all living organisms, they are ubiquitous and are found in a variety of sources, such as animals, plants and microorganisms.

Plant Proteases. The use of plants as producers is influenced by several factors. Land availability and climatic conditions are essential. In addition, the production of plant

proteases is quite a laborious process. Well-known plant proteases include: bromelain, papain, keratinases, and ficin.

Animal Proteases. Trypsin of the pancreas, chymotrypsin, pepsin, and renin is the best-known proteases of animal origin [16]. They are prepared in pure form in large quantities. However, their production depends on the availability of livestock for slaughter, which, in turn, is governed by political and agricultural policies. Modern society is increasingly inclined to believe that such production is inhumane. For example, Animal rennet is a milk clotting enzyme isolated from calf stomachs. The major component of rennet is chymosin (EC 3.4.23.4)

Viruses. Proteases have been identified in a wide range of viruses, with no correlation to capsid complexity, presence of lipid envelope, or nature of their genomes. They can be found in nonenveloped ssRNA viruses (picornaviruses), enveloped ssRNA viruses (flaviviruses, retroviruses), nonenveloped dsDNA viruses (adenoviruses), and also in enveloped dsDNA viruses (herpesviruses).

Research in the field of the viral process is becoming increasingly important as they are functionally involved in the processing of proteins of viruses that cause diseases such as Covid-19, AIDS, and cancer.

Bacteria. Most proteases, especially neutral and alkaline, are produced by organisms of the genus Bacillus. Neutral bacterial proteases are active in a narrow pH range (pH 5 to 8) and have relatively low thermotolerance. Neutral protease, insensitive to natural plant proteinase inhibitors and therefore suitable for the brewing industry. Bacterial neutral proteases are characterized by a high affinity for hydrophobic amino acid pairs. Their low thermotolerance is advantageous for controlling their reactivity in the production of food hydrolysates with a low degree of hydrolysis.

Bacterial alkaline proteases are characterized by their high activity at alkaline pH, and their broad substrate specificity. Their optimum temperature is about 60 °C. Due to these properties of bacterial alkaline proteases are suitable for use in the washing industry.

Fungi. Mycelial fungi synthesize alkaline, acid and neutral proteases. Extracellular alkaline proteases are produced by *Aspergillus clavatus* [17,18], *A. Fumigatus* [18],

Penicillium chrysogenum, Purpureocillium lilacinum, Conidiobolus coronatus [19], *Fusarium culmorum* [20] etc.

Synthesis of acid proteases is typical for *Aspergillus brasiliensis* [21], *Sporotrichum pulverulentum* [22], *Penicillium griseoroseum* [23], *Trichoderma parasiticus* [24], *Trichoderma harzianum* [25], *Thermomyces lanuginosus* [26], etc.

Neutral proteases were detected in *Aspergillus carneus* [27], *A. sojae* [28], *Fusarium culmorum* [19], etc.

Fungal proteases are the enzymes referred to diverse families and subfamilies [29]. Thanks to proven schemes cultivation and separation of the synthesized product from the mycelial biomass, industrial mushroom strains are promising cultures for the production of proteolytic enzymes [30]. One of the earliest fungal proteases was proteinase K, an alkaline enzyme from *Parengyodontium album*. Proteases actively produce fungi belonging to the genera *Aspergillus*, *Penicillium*, *Rhizopus*, *Humicola*, *Mucor*, *Thermomyces*, *Trichoderma*, etc [31]. A. oryzae is the dominant source of fungal proteases [32].

Proteolytic enzymes of filamentous fungi differ in physicochemical and catalytic properties, which determines aspects of application protease preparations. Acid proteases are most active at pH 4.0-4.5, neutral - at pH 7.0, alkaline - at pH 7.0–11.0 [33].

Acid proteases of filamentous fungi are mainly represented by pepsin-like aspartate proteases [32]. Their molecular weight varies between 35 and 50 kDa. They are most active at pH 3.0-4.0. Fungal strains synthesize several types of aspartate proteases [34].

Fungi of the genus *Aspergillus* produce aspergillopepsin I and aspergillopepsin II.

Aspergillopepsin I (EC 3.4.23.18) does not have milk-clotting activity, hydrolyzes various protein substrates, is widely used to obtain protein hydrolysates without bitterness, protein-based flavoring components, for flavor modification in dairy production, for processing flour in a bakery. This enzyme is also called aspergillopepsin A (producer of *A. awamori*), aspergillopepsin F (*A. foetidus*), proteinase B, proctase B (*A. brasiliensis*), aspergillopeptidase A (*A. saitoi*). Aspergillopepsin II (EC 3.4.23.18) does not have milk-clotting activity, hydrolyzes the β-chain of insulin. Due to its narrow specificity, it is not used in the industry [35].

Penicillopepsin (EC 3.4.23.20), synthesized by fungi of the genus *Penicillium*, has a wide substrate specificity, preferably catalyzes the hydrolysis of hydrophobic residues at the P1 and P1 'positions, and also cleaves the Gly-Gly bonds in the β-chain of insulin. Ferment EC 3.4.23.20 is widely used to obtain peptides that do not form bitterness [36].

Rhizopuspepsin (from *Rhizopus sp.*) and mucorpepsin (from *Rhizomucor pusillus*, *R. miehei*) are also known. Enzymes have milk-clotting activity.

Alkaline serine proteases are widespread in nature and are present in all forms of cell life. Serine proteases are produced by bacteria including *Trichosporiella,* filamentous fungi including *Conidiobolus*, *Aspergillus*, *Neurospora*, yeast, including *S. cerevisiae* [37].

Serine proteases are produced by fungi of the genera *Aspergillus*, *Penicillium*, *Rhizopus*, *Trichoderma*, *Sarocladium*, etc [38]. Well studied and Serine proteases of filamentous fungi of the genus *Aspergillus* are widely used: *A. brasiliensis*, *A. flavus*, *A. fumigatus* [39], *A. clavatus* [40].

Among the neutral endopeptidases of filamentous fungi, the most common is zinc-dependent neutral metalloproteinase [32,41].

1.3. Applications of fungal proteases

Food industry: in brewing, alcohol production, winemaking proteases are used to remove various types of protein turbidity and accelerate filtration; in the bakery industry to reduce the duration of batches in the production of custard bread [41]; in the production of cheese and curd masses to accelerate ripening (Proteases *Cryphonectria parasitica*); to soften (tenderization) of meat, meat products, fish, which facilitates and accelerates the processing of intermediates and improves their quality (protein and pronase preparations). Alkaline and neutral proteases of fungi *Aspergillus flavus* and *Aspergillus parasiticus* play a key role in production of soya sauce [42]. Proteases are used in the food concentrate and canning industries for the preparation of concentrates from hard-to-boil cereals, peas, beans.

Light industry: in leather and fur production, proteinases, which are extracellular proteases of streptomycetes, is used to accelerate hair removal from skins and soften leather

raw materials [43]. In the textile industry, the process of tissue treatment with enzyme preparations of the class of proteases of fungal origin is accelerated 7-10 times; the same drugs are used for the removal sericin in unwinding cocoons of the mulberry silkworm in the production of natural silk [44].

Household chemicals: proteases are components of detergents and detergents. Some proteases together with glucose oxidase and catalase are added to toothpaste - they provide their antimicrobial action and prevent the occurrence of caries [43].

Medical practice [45]: The search of medicinal proteases producers should be done amongst the fungi as only the bacterium *Bacillus mesentericus* is applied for coating wounds nowadays. In medical practice, for treatment of burns is used the coating «Elastotherase immobilized» (as gauze napkins or bandages) based on the bacterial proteases. They fasten the healing of burns of II-IV degrees, trophic ulcers, bedsores and purulent wounds. In general, proteolytic enzymes of microorganisms can be used in medicine for treatment of liver diseases, frostbites, trophic ulcers and for acceleration of dead tissues rejection and for cleaning purulent-necrotic plaques. Proteolytic complexes are also essential in antibacterial chemicals for processing surgical instruments and cleaning medical surfaces [46,47].

Proteases are tools in struggle with several serious diseases. Fungi of the genus *Candida albicans* (SAPS) are secreting asparagine proteinases. It was found that inhibitors of this protease, directed against asparagine proteinase of the viral origin are used in the treatment of HIV infection, could also inhibit *Candida* SAPS and reduced the incidence of candidiasis in such patients [48]. So, the asparagine proteinases are an important target for development of new medicinal preparations based on protease inhibitors for the treatment of candidiasis. The other example is serine protease and its role in treatment of allergies. It is known that proteins from catalytic type of serine are allergens. The secreted alkaline serine protease of *A. fumigatus* has been shown to help avoid the host immune response by degrading human complement proteins and is, therefore, a good target for further development of anti-allergic drugs. Besides, associated with proteases metabolites of fungi could be used in cancer treatment. The thiol-protease specific inhibitor, E-64, originally isolated from the fungus *A. japonicus*, is being studied extensively as a potential antitumor

agent in cell culture. Proteolytic complexes are also indispensable in antibacterial agents for the treatment of surgical instruments and medical surfaces [41].

1.4. Novel drug development based on proteolytic enzymes

Proteolytic enzymes (proteases) are the active ingredient in many enzyme preparations used to correct the secretory dysfunction of the stomach and disrupt the digestive processes in the small intestine (Table 1.2).

Table 1.2

Preparations containing proteolytic enzymes

The drug	Dosage form	Content of proteolytic enzymes, FIP
Acidin-pepsinum	coated tablet	300
Digestal	coated tablet	300
Enzystal	pill	300
Festal	coated tablet	300
Kreon 8000	Microspheres in capsules	450
Kreon 25000	Microspheres in capsules	1000
Mezym Forte	pill	250
Mezym Forte 10000	pill	375
Pancreatin	coated tablet	200
Pancurmen	pill	63
Pankreoflat	pill	400
Panzinorm Forte	pill	900
Panzinorm 10000	capsule	400
Panzyrat 10000	Microtablets in capsules	500
Panzyrat 25000	Microtablets in capsules	1250

The first type of drugs containing proteolytic enzymes are extracts of the gastric mucosa, the main active ingredient of which is pepsin. It, as well as other peptidases contained in the gastric mucosa, break down almost all natural proteins. These drugs are used mainly in gastritis with low acidity and are not recommended in the treatment of diseases of the gastrointestinal tract with high acidity [4].

Proteolytic enzymes are also used in drugs for the treatment of burns, bedsores, trophic ulcers, purulent wounds, purulent otitis, sinusitis and bronchopulmonary diseases. An example of such a drug is the enzyme preparation Chymopsin, which contains a combined active ingredient containing trypsin and chemotrypsin.

1.5. Conclusions to Chapter

Some species of filamentous fungi, such as *Aspergillus, Penicillium* and *Paecylomices* have been identified as great producers of extracellular protease in submerged fermentation. Further studies on the optimized conditions will still be performed and the protease production will be conducted in bioreactors.

Proteolytic enzymes play a crucial role in the digestion of food proteins in the human stomach and intestines. Most proteolytic enzymes of the digestive system are produced in the form of proenzymes. The physiological meaning of this is that the act of production of the enzyme (proenzyme) is separated from the act of its activation - the transformation into an enzyme and, thus, the proteins of tissues that produce enzymes are not exposed to these same enzymes.

Proteolytic enzymes (proteases) are the active ingredient in many enzyme preparations used to correct the secretory dysfunction of the stomach and disrupt the digestive processes in the small intestine.

CHAPTER 2
MATERIALS AND METHODS

2.1. Methods of obtaining a culture of mycelial fungi capable of synthesizing proteolytic enzymes.

Organization of any microbiological synthesis in practice important substances begins with the study of the physiology of the producer. It is necessary to study the nature of the producer's diet, as well as the influence of external factors on the condition of cells and populations. These issues are clarified by optimizing the process under study. The basis for this is the cultivation of microorganisms in controlled and controlled conditions. Finally, establish the composition of the media and the mode of cultivation, the most economically acceptable, which can be further empirically improved in the process on an industrial scale.

The sterilization process is one of the most necessary measures in the practice of industrial biotechnology. Microbiological media, utensils, tools, some devices are subject to sterilization procedure. It is done in order to prevent the development of foreign microorganisms when working with the studied crops [47].

Sterilization (from the Latin sterilis - infertile) is a complete neutralization of the material from the vegetative cells of microorganisms and their forms of rest (spores, cysts, etc.).

The experiment on the isolation of micromycetes was carried out following all the requirements of the sterility of the laboratory, as well as under conditions of personal safety.

2.2. Sampled and studied material

Sampling. Of all the substrates of the environment, the most densely populated by microorganisms is the soil, in which they find all the necessary conditions for their life: nutrients, moisture, optimal pH and Eh, gas ratios, protection from sunlight. The largest

number of microorganisms that are used in industrial biotechnology as producers of industrially important substances are isolated from the soil.

Sampling of soil for analysis. Samples were taken from the upper soil layers.

From surfaces of the experimental plot, shifting plant debris and 0.5-1 cm of topsoil. At each point, 100-200 g of soil was selected in sterile bags or in sterile containers. The soil was sieved through a sieve with a hole diameter of 2 mm, then placed in a sterile container and stored at a moderate temperature.

Production of soil suspension for sowing. Before the analysis from the soil choose small roots, various foreign remains. Weigh 10 g of soil and pour into a flask with 100 ml of sterile water. The flask closed with a cotton swab is shaken on a rocking chair for 15 minutes, wait 30 seconds for the coarse soil particles to settle and make breeding.

To the flask to 100 ml of water was added 10 g of soil. Received a dilution of 1:10, or the first dilution. With a new sterile pipette take 10 ml from the first flask and transfer to the second with 100 ml of sterile water.

Get a dilution of 1:100 (or second), according to this principle prepared the third and fourth dilutions.

Enumeration techniques. According to Pitt and Hocking (1997), methods for quantification of filamentous fungi could be divided in two:

Direct plating. This method was used for detecting, counting, and isolating fungi *Cryphonectria parasitica* from surfaces of the bark of stems and branches, leaves and calybium (spiky cupules) chestnut. Particles are placed directly on solidified agar media. In most situations, particles should be surface disinfected before plating, as this removes the inevitable surface contamination arising from dust and other sources, and permits recovery of the fungi actually growing in the particles. Results from direct plating analyses are usually expressed as percentage of infected particles. This was the method used in most of the studies included in this thesis.

Serial dilution. Serial dilution, as the name suggests, is a series of sequential dilutions that are performed to convert a dense solution into a more usable concentration. In this case, this method was used to reduce the concentration of soil microbiota to simplify the operation.

As a result of the experiment, I received a dilution of soil microbiota in the value 10^{-4}, 10^{-5}, 10^{-6}. Next, the obtained microbiota material was distributed on the surface of solid coated media and incubated.

Isolation techniques. One of the problems in the isolation of fungi from infected material is that single species rarely occur alone. There will usually be a mixture of different species of yeasts as well as moulds and bacteria.

Isolation techniques can be divided into two broad categories: direct methods and selective methods. Both are routinely used in mycology laboratories and can be further divided into a number of subtypes.

Direct isolation. The term 'direct' is applied to techniques involving the simple transfer of a mould from its natural habitat to a pure culture in the laboratory. Isolation of filamentous fungi usually consists in picking a small sample of hyphae or spores and placing it on a fresh plate as a point inoculum, preferably near the centre of the plate as this will allow the best colony development and sporulation in most fungi.

This method was used to select a pure culture of fungi *Cryphonectria parasitica*.

Selective methods. In some instances, selective methods have been developed to enable the target species to be isolated from material where it is only a relatively minor component of the mycoflora present. This could be achieved by surface sterilisation of a particulate food, exposition to stress conditions, use of selective nutrients, selective temperatures, etc., techniques focussed on favouring the development of the target mould among the total mycoflora.

To identify fungi of the genus *Aspergillus*, a selective differential medium is used in which there is an chloramphenicol. The antibiotic chloramphenicol was added to the potato dextrose agar used to isolate the fungi to suppress the unwanted microflora.

2.3. Nutrient media for the cultivation process

In the laboratory, microorganisms are grown on nutrient media that must meet certain requirements: to be nutritious, they must meet all the necessary nutritional needs of microorganisms; contain the necessary nutrients in easily digestible form (nitrogen,

hydrocarbons, minerals, vitamins); contain the required amount of water; have certain viscosity, redox potential Eh, pH value; be isotonic with respect to the microbial cell; have buffering properties; be sterile and transparent.

The biomass of the original recipient *Aspergillus brasiliensis* strain for isolation and biomass growth was obtained with using Chapek's medium with the addition of an antibiotic. Chloramphenicol acts as a selective agent to inhibit bacterial overgrowth of competing microorganisms from mixed specimens, while permitting the selective isolation of fungi.

Potato dextrose agar medium was used to isolate *Cryphonectria parasitica*. Potato Dextrose Agar is composed of dehydrated Potato Infusion and Dextrose that encourage luxuriant fungal growth. Agar is added as the solidifying agent. Many standard procedures use a specified amount of sterile tartaric acid (10%) to lower the pH of this medium to 3.5 +/- 0.1, inhibiting bacterial growth.

Preparation of culture filtrate

Wheat bran under the conditions of solid-state fermentation Erlenmeyer corucal flask of 750 ml capacity was used containing 50 ml of liquid media without organic source and in tap water containing 1% wheat bran, 3% barley flour, KH2PO4, -0.5%. and sterilized at 0.08 МПа for 40 minutes, cooled to 30 °C inoculation spore suspension of organism preparation sterile distilled water and adjusted to (1×106) spores/ml.

Cultivation was carried out at 32˚C on a microbiological shaker at a speed of 260 rpm for 42-72 hours the contain of the flask were filtered through Whatman No. 3 filter paper disc. Culture filtrate was centrifuged at 5000 g for 10 mins/4˚C and supernatant was used as the crude enzyme.

The medium for the production of enzymes has the following composition: soybean meal, glucose, $NaNO_3$, Skim milk, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$.

A medium containing sources of carbohydrates, nitrogen and inorganic salts with a fungus of the species *Cryphonectria parasitica*, allowing aerobic fermentation to take place until a substantial amount of said enzyme is produced, and thereafter recovering the so produced enzyme from the fermentation medium.

2.4. Study of the proteolytic activity

Determination of enzymatic indices of micromycetes. To assess the proteolytic potential of micromycete cultures, we carried out Petri dishes on their surface cultivation in agar media of the following composition (g / L): KH_2PO_4 - 0.5, $MgSO_4$ - 0.25, peptone- 5.0, protein substrate (sodium caseinate, gelatin, fibrin, elastin) 10.0, agar- 15.0.

The inoculation was performed by injecting it into the center of the dish. Determination of enzymatic indices (EI) was carried out by measuring the diameters of the hydrolysis zones and micromycete colonies after 7 days and calculating the values using the following formula: El= (D + d) / D, where D is the diameter of the colony in mm, and d is the diameter of the hydrolysis zone in mm

Determination of gelatinolytic properties. Gelatin is poured into test tubes of 3-5 ml (strictly adhering to the selected dose) and sterilized with dry steam. To determine the measurement of pH during the growth of the microorganism in gelatin add an indicator - bromothymol blue (0.1% solution). In an acidic environment, gelatin turns yellow, in an alkaline environment - in blue.

- Sowing of the culture of the fungus from test tubes with beveled agar gelatin medium is carried out by injection of the loop. The media is placed in a thermostat with a certain temperature. At certain intervals (1, 2, etc. days) test tubes with the medium are placed in the refrigerator to solidify the gelatin. After 1 h, the tubes are removed, shaken slightly. If the microorganism produces proteolytic enzymes, the gelatin column thins. The amount of dilute gelatin (expressed in millimeters of gelatin column) is judged by the presence of enzymes and their relative activity. For example, 1 day after sowing, one strain of the fungus is diluted with 5 mm of a column of gelatin (the entire column of gelatin in a test tube is 30 or 50 mm), and the other at the same time - 15 mm; it is obvious that the second strain has a more active complex of proteolytic enzymes.
- As a substrate using ordinary photographic film or RF-3. The film is illuminated, detected, and fixed. In test tubes pour 3-5 ml of culture fluid filtrate or mycelial extract, place in each tube a strip of cut film (0.5 X 2 cm) and put in a thermostat at 30-35 ˚C, at certain intervals (hours, days) take into account the degree of hydrolysis of the

emulsion layer and determine the time for which the film becomes transparent. In the case of the high proteolytic activity of the culture fluid, the film may become transparent after 20-30 minutes.

Determination of caseinolytic properties. Properties of skim or separated milk (whole milk is centrifuged for 1 h at a speed of 3000 rpm) is decomposed into test tubes of 5 ml, sterilized at a pressure of 0.5 atm at a distance of 20-30 minutes Then the mushrooms are sifted from the wort-agar shoals into tubes with milk and set in a thermostat at a certain temperature. The accounting of results is carried out through 1, 2, etc. In this case, you can report the coagulation of milk or its peptonization, or first peptonization, and then coagulation. At coagulation the clot is created, and at peptonization milk becomes transparent. Sometimes both processes are carried out simultaneously. Fermentation activity is determined by the degree of coagulation or peptonization and the speed of these processes, over time.

Determination of albuminolytic activities. Ingredients: 1 liter of distilled water; 1- K_2HPO4; 0.5 - $MgSO_4$,; 0.5 - KCI; 0.01 - $FeSO_4$, two beaten egg whites. The thus obtained liquid (turbid) protein liquid is poured into 5 ml tubes and sterilized by steam. Sowing of mushrooms is done in the same way as in determining the caseinolytic properties of mushrooms. The tubes are placed in a thermostat with a certain temperature and after 1, 2, etc. days determine the activity as the enlightenment of the column of protein in the tube and the speed of this process. Instead of a liquid medium, dense ones are used, and the presence and size of enlightenment zones around the growing colony of the fungus determine the presence of enzymes.

Determination of geolytic properties. The culture fluid after cultivation of mushrooms under certain conditions is poured (0.1 to 0.2 ml) in special glass cylinders mounted in solidified blood agar in Petri dishes. The cups are kept in a thermostat for 1-4 days at different temperatures. The appearance of enlightenment zones around the cylinders indicates the ability of the filtrate to cause hemolysis.

Determination of fiblinolytic properties:

- Pour 1 volume of citrated blood into test tubes (16 mg of sodium citrate is added to 20 ml of blood to prevent coagulation) and 1 volume of thrombin; at the same time there

are dense cylindrical thrombi with a diameter of 8 mm and a length of 15 mm (the sizes of a thrombus depend on the quantity of blood and thrombin in a test tube). To the blood clots add culture fluid (2-3 ml), kept in a thermostat at 30-37 °C. The fibrinolytic activity is judged by the rate of dissolution of the blood clot.

- Fibrinolytic activity of fungi can be determined by the method of plates. In an Erlenmeyer flask with a capacity of 50 ml mix 10 ml of 0.3% solution of fibrinogen and 0.3 ml of 1% solution of thrombin (prepared in saline). A mixture of fibrinogen and thrombin is quickly poured into sterile Petri dishes with a diameter of 10 cm Under the influence of thrombin fibrinogen is converted into insoluble gelfibrin, on the surface of which is applied with a micropipette 0.03 ml of culture fluid. Three to four determinations can be made on each cup. The films are incubated at 38 °C; incubation time is different. Fibrinolytic activity is evaluated by the size of the fibrin lysis zone and expressed in conventional units per 1 ml of culture fluid (conventional unit corresponds to a lysis zone of 10 mm).

Determination of activity for milk making. A certain amount of skim milk (for example, 100 ml) is homogenized with 50 ml of 0.02 M $CaCl_2$, kept at a temperature of 25 or 37 °C, the pH is adjusted to 6.1. In standard test tubes with 10 ml of milk add 1-2 ml of culture fluid or enzyme solution, mix and place in an ultrathermostat or water bath at the specified temperatures. Mark on the stopwatch the time of introduction of the enzyme or culture fluid and the time of the beginning of coagulation of milk, which is determined by the occurrence of milk clots on the walls of the tubes during shaking. The activity of the enzyme is expressed in seconds.

Viscometric method of protein determination. The method is based on reducing the viscosity of protein solutions from the action of enzymes. A solution of any protein (usually 1-2% gelatin solution) is poured into an Ostwald viscometer and placed in a water thermostat at 30 °C. After 10 minutes, when the gelatin solution reaches this temperature, measure its viscosity. Then the test enzyme preparation is added to the solution and at certain intervals (3 5-7 min, etc.) the viscosity of the solution is measured. The more active the enzyme, the faster the viscosity will decrease. The activity of the enzyme preparation is expressed in seconds.

Method of formal titration. As a result of hydrolysis of proteins by proteolytic enzymes, peptide bonds are destroyed with the release of equivalent amounts of ammonia and carboxyl groups, which determine the amounts of ammonia by methods. However, the determination of ammonia and carboxyl groups in amphoteric electrolytes, etc. amino acids, polypeptides, are accompanied by great difficulties of various and carboxyl groups in amphoteric electrolytes, as free ammonia groups with alkaline properties are neutralized in an aqueous medium by free carboxyl groups having an acidic character. To prevent this process, a solution of formalin, which binds amine groups, is introduced into aqueous amino acid solutions; carboxyl groups are titrated with an ammonia base and indirectly determine the number of amine groups.

The degree of hydrolysis is determined by the difference between the amount of alkali spent on titration before and after hydrolysis. The concentration of hydrogen ions, at which the carboxyl groups are completely neutralized by alkali, is in the range of pH 9-9.5 with intense red staining with phenolphthalein.

Reagents: 0.5% solution of phenolphthalein in 50% alcohol solution; 0.2 N. NaOH solution; freshly prepared solution of formalin mixture (up to 50 ml of 40% (industrial) formalin add 2 ml of 0.5% solution of phenolphthalein and neutralize 0.2 N. NaOH to a pale pink color).

The sequence of determination. To a certain amount of protein hydrolyzate or liquefied under the action of proteolytic enzymes gelatin (for example, 5 ml) add two drops of phenolphthalein and titrate 0.2 N. NaOH to a faint pink color. The control is the same amount of distilled water (free of CO_2) or a solution of protein that has not been exposed to the enzyme. Then to the solutions add 2.5 ml of formalin mixture and titrate with 0.2 N. NaOH solution to a bright red color. From the amount of alkali spent on the titration of the test sample, subtract the amount of alkali spent on the titration of the control.

Calculation: 1 ml of 0.2 N. the NaOH solution used for the titration corresponds to 2.8 mg N. The content of amine nitrogen in the solution is determined by multiplying the amount spent on the titration of 0.2 N. NaOH solution (in milliliters) by 2.8.

Method for determining protase activity by amin nitrogen increase. The method is based on iodometric determination of the ability of amino acids and various peptides to form complex soluble compounds with copper.

To a certain amount of the test solution (eg, enzyme hydrolyzate) containing a mixture of amino acids and peptides, add an excess suspension of copper phosphate in copper salts in buffer. As a result, after shaking, the copper salts of most of the aa copper are filtered off, acetic acid is added to the clear solution, and copper is determined by the iodometric method.

Reagents: 1) copper chloride solution (27.3 g per 1 l of H_2O); 2) 64.5 g of $Na2HPO_4$, dissolved in 50 ml of distilled water without CO_2; 3) borate buffer (5.7 g of borax $Na_2B_4O_7$ - sodium tetraborate) is dissolved in 150 ml of water, add 10 ml of 1 N. HCl solution and bring the volume of the solution to 200 ml; 4) a suspension of copper phosphate (one volume of reagent is added to two volumes of reagent 2, mix and add two volumes of reagent 3; 5) a solution of 0.25 g of thymolphthalein 100 ml of 50% ethyl alcohol: 6) 80% acetic acid; 7) freshly prepared solution of 0.01 N. sodium hyposulfite solution ($Na2S_2O_3$); 8) 0.5 n. NaOH solution.

The sequence of determination. 1-2 ml of enzyme hydrolyzate is placed in a flask with a capacity of 25 ml, neutralized with 0.5 N. NaOH solution of thymolphthalein to a light blue color. To the flask add 10-12 ml of a mixture consisting of a suspended precipitate of copper phosphate (reagent 4), the volume of the solution is adjusted to the mark with water, shaken and centrifuged or filtered through a tight filter. 10-15 ml of the filtered solution is transferred into a flask, acidified with 0.25-0.5 ml of 80% acetic acid, add 0.2-0.4 g of KSI and titrate the released iodine to 0.01 N. $Na_2S_3O_3$ solution, adding 2-4 drops of 0.1% starch solution to the end of titration until the blue color disappears.

Calculation: the amount of 0.01 N. the $Na_2S_2O_3$ solution used for titration is multiplied by 0.28. The result corresponds to the content of amine nitrogen in the sample (in milligrams).

Spectrophotometric method for determining protease activity. The method is based on the ability of protein substrates and products of their enzymatic cleavage to absorb light in the ultraviolet part of the spectrum. The absorption of light is due to the presence of aromatic amino acids, mainly tyrosine and to a lesser extent tryptophan, and the maximum

absorption is observed at a wavelength of 275-280 nm. Casein or hemoglobin is used as a substrate.

Preparation of hemoglobin. A solution of sodium citrate (3 g per 1 liter of blood) is added to the vessel from bovine blood to prevent its coagulation. The blood is stirred and centrifuged. Blood plasma and white blood cells, which form a thin layer on the surface, remove the process of siphoning. The erythrocytes are mixed with an equal volume of cooled 1% NaCl solution until centrifuged again. The supernatant is removed and the precipitate is shaken and dialyzed in cellophane bags against cold distilled water. After 24 hours of dialysis, the contents of all bags are mixed and the hemoglobin is stored frozen. A 2.5% hemoglobin solution is usually taken for determination.

Preparation of casein solution. A portion of casein is suspended in 0.1 M citrate-phosphate buffer (pH 8) and kept in a water bath at 60-70 °C for 40 min (until complete dissolution of casein). Then 0.05 M citric acid solution adjust the pH of the solutions to the desired level. The casein solution can be stored in the cold for a week. usually 1% casein solution is used for determination.

The sequence of determination. In test tubes pour 1 ml of citrate-phosphate buffer, 1 ml of the test culture fluid or enzyme solution and place them in a thermostat at a certain temperature for 5-10 min to heat to this temperature. Then 1 ml of substrate (hemoglobin or casein) is added to the mixture, mixed and incubated. The reaction is stopped by adding an equal volume of TCA (Trichloroacetic acid). The control experiment is prepared in the same way, only before adding the substrate solution to the reaction mixture add a 10% solution of TCA. The solutions are filtered, and the amount of unprecipitated trichloroacetic acid reaction products is determined in the filtrate on a spectrophotometer. The unit of proteolytic activity is the amount of enzyme at which non-precipitated TCA hydrolysis products of the substrate are formed in 10 min at 40 °C, the optical density of which at 280 nm corresponds to the optical density of 1 μg / ml of tyrosine. When determining the amount of tyrosine use a standard curve. To build it, prepare a number of dilutions of tyrosine and determine their optical density, then on the abscissa axis put the amount of tyrosine, and on the ordinate axis - the readings of the device.

2.5. Purification of protease

There are several effective methods of protease purification:

Ammonium sulfate precipitation. The neutral protease was concentrated by adding ammonium sulphate (20–90%) at 4 °C, followed by overnight incubation. The sample was centrifuged at 4000g for 20 min at 4 °C. The enzyme activity and protein content of ammonium sulfate precipitation were determined. The precipitates were collected and then dialyzed with a Millipore 8–14 kDa dialysis bag against a 50 mM Tris–HCl buffer (pH 7.0) at 4 °C for 72 h to remove the ammonium sulphate.

DEAE-Sepharose Fast Flow chromatography. The sample was concentrated by polyethylene-glycol 20,000 (PEG-20M) at 4 °C, and the concentrated sample was loaded on DEAE-Sepharose Fast Flow chromatography (30 × 1.6 cm) and washed under the following conditions: Buffer A (50 mM Tris–HCl, pH 7.0); Buffer B (50 mM Tris–HCl, 2 M NaCl pH 7.0), with a flow rate of 0.5 mL/min. The sample was separated by use of the gradient elution method, and the protein elution surge was collected and assayed for protease activity and protein content. Active fractions were loaded by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and checked for their homogeneity.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis. The molecular mass of purified protease was determined by SDS-PAGE, with its activity demonstrated to be 12% of Native-PAGE. Denaturing gel was stained in Coomassie Brilliant Blue R-250 for clear visualization of the bands. For zymogram analysis, the enzyme samples were separated using regular SDS-PAGE containing 0.2% casein in the separating gel. The SDS was omitted in the buffer and samples were not boiled. After each run, SDS from the gel was removed by incubating the gel twice in 2.5% Triton X-100 for 1 h. The gel was washed thrice for 20 min in distilled water to remove excess Triton X-100 and then incubated for 3 h in 50 mM Tris–HCl buffer (pH 7.0) at 37 °C. Finally, the gel was stained with a Coomassie Brilliant Blue R-250 staining solution.

2.6. Conclusions to Chapter

Application of the latest methods of production according to the developed generalized schemes for the simultaneous isolation of proteolytic enzymes micromycetes allows significantly intensifying and increasing the efficiency of the process. Developed technological scheme application mycelium fungi as producers of proteases describe the new way of alkaloids production.

CHAPTER 3
RESULTS OF THE PROTEOLYTIC ENZYMES PRODUCERS STUDY AND TECHNOLOGY FOR THIS METABOLITES PRODUCTION

3.1. Investigation of micromycete isolates

In most modern classifications, fungi are ranked, like plants and animals, as a separate kingdom. A cluster of related species is grouped in a genus, of related genera in families, of families in orders, orders in classes, and classes in subkingdoms. *Zygomycotina, Ascomycotina*, and *Deuteromycotina* are the three subkingdoms of the kingdom Fungi that include the most significant genera in food spoilage [7]. Fungi from each of these subkingdoms have quite distinct properties, shared with other genera and species from the same subkingdom.

The name applied to any fungus is binomial: first appears a capitalised genus name followed by a lower case species name, both written in italics or underlined. The classification of organisms in genera and species was a concept introduced by Linneaus in 1753 and it is the keystone of biological science.

Usually, fungal identification is done on the basis of morphological characteristics of the colony, conidia and conidiogenous cells. Moulds are characterized by the development of hyphae, which result in the colony characteristics seen in the laboratory.

Characteristics of *Aspergillus brasiliensis*

Domain: Eukaryotes

Kingdom: Fungi

Phylum: Ascomycota

Subphylum: Pezizomycotina

Class: Eurocyomycetes

Subclass: Eurotiomycetidae

Order: Eurocium

Family: Aspergillaceae

Genus: *Aspergillus*

Section: Nigri

Species: *Aspergillus brasiliensis*

Aspergillus is a genus of fungi that consists of about 300 identified species of mold. *Aspergillus* can be found in a variety of environments throughout the world given that their growth is largely determined by the availability of water.

The rate at which they grow is largely determined by the temperature range in the environment they grow in. Regardless, studies have shown Aspergillus to be able to tolerate extreme conditions only if all of the other conditions are ideal.

Aspergillus is a filamentous, cosmopolitan and ubiquitous fungi found in nature. It is commonly isolated from soil, plant debris, and indoor air environment. While a teleomorphic state has been described only for some of the Aspergillus spp., others are accepted to be mitosporic, without any known sexual spore production.

Species from *Aspergillus* genus section Nigri present a thin stalk with a round black conidial head made up of spores of a characteristic shape, which bud from the organism's body as part of asexual reproduction. Its name is derived from this appearance since it resembles the holy water sprinkler called aspergillum, used by priests during the Asperges ceremony.

Mushroom colonies grow rapidly and are easily recognizable by their characteristic dusty appearance. At first, the mycelium is white, then it becomes dark and finally they acquire different colors, ranging from black to dark brown (Fig. 3.1.).

Given that a majority reproduce asexually, they are often described as conidial fungi. However, studies are yet to determine how a good number of others reproduce while some have been shown to reproduce sexually.

The fungal metabolites produced by *Aspergillus* were penicillin, citric acid, koji acid, L-malic acid, amylase, catalase, cellulase, galactosidase, glucanase, glucosidase, hemicellulase, lipase, pectinase and protease reported that within the genus *Aspergillus*, comprising a group of filamentous fungi with a large number of species, the most important for industrial applications are some members of the group of black Aspergilli (*Aspergillus brasiliensis* and *Aspergillus tubingensis*). Reclassification using molecular and biochemical

techniques resulted in clear distinctions being made between eight groups of black Aspergilli: *A. brasiliensis, A. tubingensis* and *Aspergillus foetidus*. Products of several of these species have attained a generally recognized as safe (GRAS) status, which allows them to be used in food and feed applications. Black Aspergilli have a number of characteristics that make them ideal organisms for industrial applications, such as good fermentation capabilities and high levels in protein secretion.

Fig. 3.1. Obtaining primary cultures of *Aspergillus brasiliensis* from soil samples

Characteristics of *Cryphonectria parasitica*

Domain: Eukaryota

Kingdom: Fungi

Phylum: Ascomycota

Subphylum: Pezizomycotina

Class: Sordariomycetes

Subclass: Sordariomycetidae

Order: Diaporthales

Family: Valsaceae

Genus: *Cryphonectria*

Species: *Cryphonectria parasitica*

Virulent *C. parasitica* strains are readily isolated from diseased tissues with conventional surface disinfestation and tissue plating on acidified, fungal-isolation media, such as acidified, potato-dextrose agar. Colonies should be yellow-orange and radially symetric.

Hyovirulent *C. parasitica* strains, infected with dsRNA hypoviruses, often have an abnormal colony appearance or atypical yellow-orange pigmentation and asexual sporulation (Fig. 3.2.). For European hypovirulent strains, colonies typically have a predominantly white colony appearance; single-conidium isolation can be used to recover the normal, yellow-orange pigmented, hypovirus-free colony type.

Fig. 3.2. Two fungal cultures of Cryphonectria parasitica were grown on potato dextrose agar [Sarah F Bryner, 2012]

Cryphonectria parasitica is a Sordariomycete (ascomycete) fungus in the family Cryphonectriaceae (Order Diaporthales). Closely related species that can also be found on chestnut include *Cryphonectria radicalis*, *Cryphonectria naterciae* and *Cryphonectria japonica*.

About 5 days after subculturing, the primordial conidiomata are seen in culture initially appearing as a dense, globose mat. Only conidiomata are formed in culture; ascomata are not produced. In virulent isolates, conidiomata are produced abundantly in

diurnal concentric rings. Hypovirulent isolates are characterized by a decreased ability to form conidiomata and by irregular distribution over the surface of the medium (Fig. 3.3).

Fig. 3.3. Attempt to isolate the fungus *Cryphonectria parasitica* from surfaces of bark and branches, leaves and calybium (spiky cupules) of chestnut

3.2. Development of the optimal scheme for the production of proteases from mycelial fungi

Modern lines for the production of deeply purified highly concentrated enzyme preparations (ultra-concentrates) along with fermentation (cultivation, microbiosynthesis) include the following stages (Fig. 3.4.):

- cultivation of fungi ;
- purification and sterilization of the culture liquid;
- the actual concentration of enzymes;
- processing (disposal) of waste: biomass of the producer strain and permeate from the concentration stage;

* sterilization and packaging of finished forms.

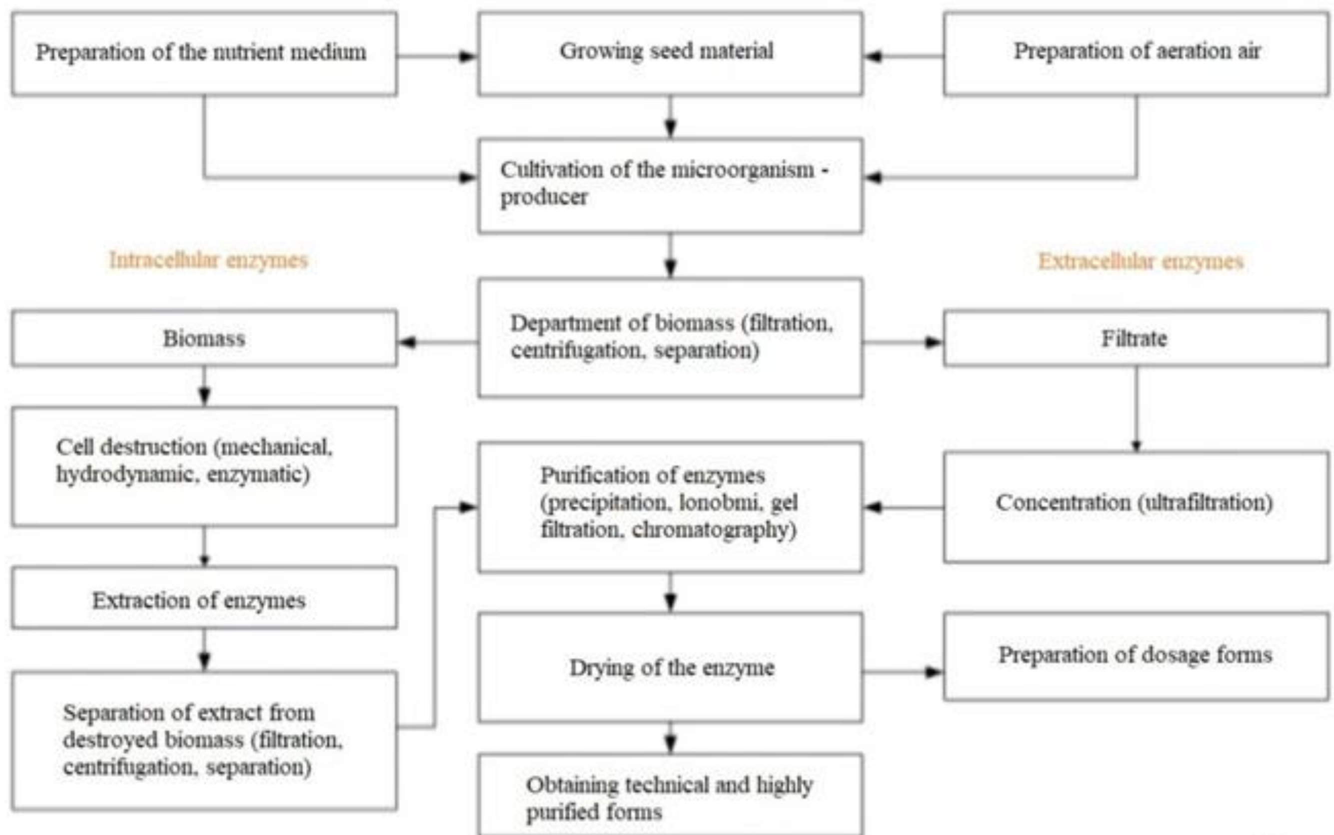

Fig. 3.4. General scheme for obtaining fungal proteolytic enzymes from mycelial fungi

Proteases can be cultured in large quantities in a relatively short time by established methods of fermentation and they also produce an abundant, regular supply of the desired product. In general, microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals [34].

Microorganisms elaborate a large array of proteases, which are intracellular and extracellular. Intracellular proteases are important for various cellular and metabolic processes, such as sporulation and differentiation, protein turnover, maturation of enzymes and hormones and maintenance of the cellular protein pool. Extracellular proteases are important for the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolytic products [52].

On an industrial scale, exoproteases are produced in complex media containing glucose and other costly substrates. Cultivation conditions are essential in successful enzyme production, that's why optimization of parameters such as pH, temperature and media composition must be controlled in process development. Specifically, the protease production is mainly influenced by the variation in C/N ratio, presence of some easily metabolizable sugars, such as glucose, and metal ion, besides several other physical factors, such as aeration, inoculum density, pH, temperature and incubation time. Protease synthesis is also affected by rapidly metabolizable nitrogen sources, such as amino acids in the medium [52]. For improving protease production, have been used screening for hyper-producing strains, cloning and over-expression, controlled batch and fed-batch fermentations using simultaneous control of glucose, ammonium ion concentration, oxygen tension, pH and salt availability, chemostat fermentations, and optimization of the fermentation medium through a statistical approach, such as response surface methodology [52].

Filamentous fungi are used in many industrial processes for the production of enzymes and metabolites. Among the many advantages offered by the production of enzymes by fungi are low material costs coupled with high productivity, faster production, and the ease with which the enzymes can be modified. Further, the enzymes, being normally extracellular, are easily recoverable from the media [15]. Proteases production of fungal origin have an advantage over bacterial protease as mycelium can be easily removed by filtration. Besides, the use of fungi as enzyme producer is safer than the use of bacteria, since they are normally recognised as GRAS (generally regarded as safe).

Proteases have been produced in submerged (SmF) and solid-state fermentations (SSF), and each technique has particular advantages the other unable to match [53]. SSF has certain advantages over the conventional SmF, like low production cost, uses raw materials as substrates, requires less energy and space, encounters less problems in downstream processing, stability of the product due to less dilution in the medium, and manufactures with higher productivity [54]. SmF has advantages in process control and easy recovery of extracellular enzymes, mycelia or spores. However, the products are dilute and enzymatic extracts might be less stable than those from SSF. The major problems in large-scale SSF

for fungal growth are the limited water and heat removal. In SmF, water is abundantly present and variations on temperature, oxygen concentration and nutrients are small [55]. In addition, the minimal amount of water allows the production of metabolites in a more concentrated form, making the downstream processing less time consuming and less expensive. However, the conditions in SSF, especially the low moisture content in the system, lead to several potential advantages for the production of fungal enzymes. Firstly, these conditions favour the growth of filamentous fungi, which typically grow in nature on solid substrates, such as pieces of wood, leaves and roots of plants and other organic natural materials. Secondly, the low moisture content can minimize problems with bacterial contamination during the fermentation. Finally, the environmental conditions in solid-state fermentation can stimulate the microorganism to produce enzymes with different properties than those of enzymes produced by the same organism under the conditions experienced in submerged fermentation.

Different mechanisms have been described to regulate the synthesis and secretion of extracellular protease. The presence of a substrate can induce protease secretion. High levels of end products, such as amino acids, NH_4^+ and easily metabolizable sources of carbon may repress production. On the other hand, protease production may be increased when insufficient levels of carbon, nitrogen or sulfur are available. Finally, extracellular enzymes may be secreted constitutively at low levels regardless of the availability of a substrate [56]. The production of proteases by microorganisms is known to be influenced by the quality of the nitrogen source. Although complex nitrogen sources are usually used for protease production, the requirement for a specific nitrogen supplement differs from organism to organism. Generally, the fungi produce more proteolytic enzyme on a more complex proteinaceous nitrogen sources than on low molecular weight or inorganic nitrogen sources. Enzyme synthesis was found to be repressed by rapidly metabolizable nitrogen sources such as amino acids or ammonium ion concentrations in the medium.

3.3. Bioreactor design and implementation strategies for the cultivation of filamentous fungi and the production of fungal proteases

A bioreactor can be defined as mechanically stirred vessel in which organisms are cultivated in a controlled manner and/or materials are converted or transformed through specific reactions (Fig. 1.2.). Indeed, traditional methods for solid state fermentation include tray, drum and packed bed bioreactors with problems regarding the control of different parameters; while, for submerged fermentation they include continuous stirred-bioreactors, continuous flow stirred-tank reactors, plug-flow reactor, and fluidized-bed reactors. For biofilm processes, the material supporting the microorganism can be used in reactors applied for submerged fermentation and adapted for used in order to cultivate immobilized cells or biofilms with respect to the production of value-added molecules.

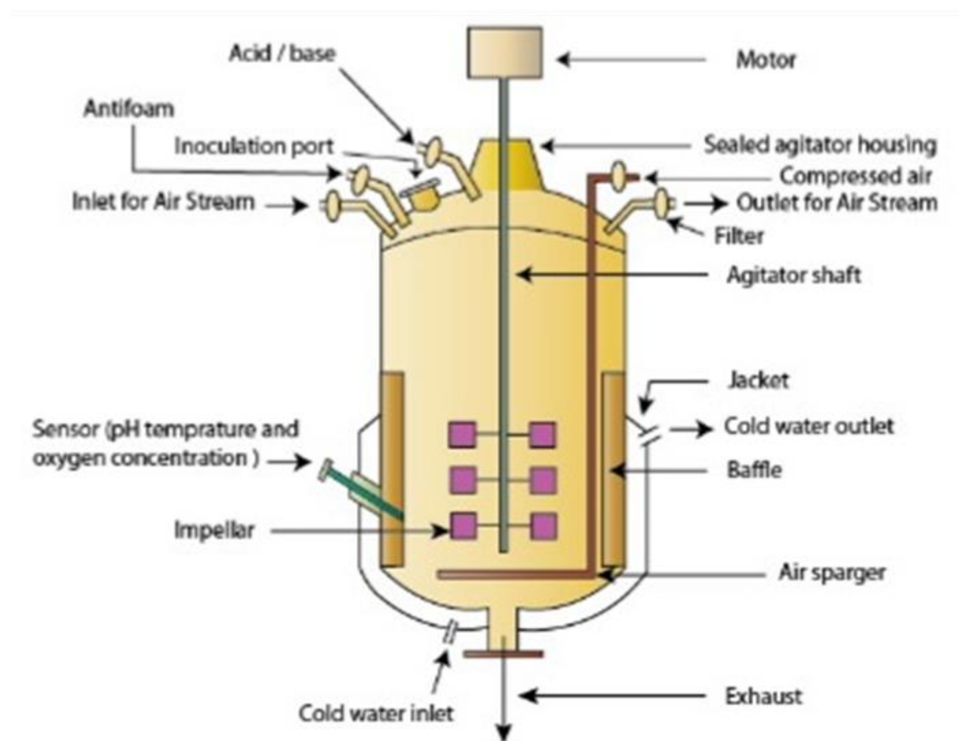

Fig. 3.5. Diagram of a typical bioreactor

The production of fungal metabolites and conidia at an industrial scale requires an adequate yield at relatively low cost. To this end, many factors are examined and the design of the bioreactor to be used for the selected product takes a predominant place in the analysis. One approach to addressing the issue is to integrate the scaling-up procedure according to the biological characteristics of the microorganism considered, i.e. in our case filamentous fungi. Indeed, the scaling-up procedure is considered as one of the major bottlenecks in fermentation technology, mainly due to the near impossibility of reproducing

the ideal conditions obtained in small reactors designed for research purposes when transposing them to a much larger production scale.

Three fungal genera are widely used for biotechnological applications: *Aspergillus sp., Penicillium sp.,* and to a lesser extent, Trichoderma sp. Filamentous fungi have the capability to produce extremely large quantities of homologous proteins, including several enzymes that can be used as industrial biocatalysts. Several volatile metabolites (MVOC) can also be produced. However, the production of these metabolites relies on the culture mode at both the quantitative and qualitative levels. Indeed, these fungi are able to grow in different forms: from free mycelium and pellet in liquid phase, to pellet and mycelium to conidia at a solid-air interface.

Among these three strategies, submerged fermentation is the most widely used mode of fermentation. However, the last two strategies lead to the enhancement of the development of aerial hyphae with the production of conidia on conidiophore position of filaments depending on the development stage of the microorganism. They will be able to generate fungal products of biotechnological interest, since the excretion capacity is increased when fungal biomass is attached on a given support.

3.3.1. Solid-state fermentation processes

Solid-state fermentation (SSF) is one of the methods for cultivating microorganisms, which has recently found wide application in many countries and is of significant interest for science and industry. Solid phase cultivation is defined as cultivation on solid wet particles of various nature in the absence of free water [57].

It is important to note that the conditions of solid-phase cultivation are close to the soil conditions natural for micromycetes. The key features of this method are that in the "microorganism-substrate" system, water is exclusively in a droplet form, which allows fungal hyphae to develop freely.

For the first time, a similar method for the cultivation of microorganisms was mentioned in ancient times, but in its modern form the method of solid-phase cultivation

was formed in 1965-70, when many works appeared on the use of SSF for enriching animal feed with proteins, obtaining various mycotoxins and antibiotics. It is worth mentioning that this method was used earlier, but, due to the rather narrow focus of science in the period between 1940-1950, caused by the Second World War, it did not receive proper development. Most of the work of that time was aimed at obtaining, isolating purification of antibiotics by classical cultivation methods such as submerged cultivation and surface cultivation [58].

As a rule, among the micromycetes most often used in solid-phase cultivation, there are most representatives of Ascomycota, such as, for example, *Aspergillus* or *Penicillim.*

With the growth of mycelial forms in the solid-phase cultivation system, some differentiation of hyphae occurs: some hyphae penetrate deeply into the substrate and form a conglomerate of anchored hyphae, the other part remains on the surface, forming aerial hyphae capable of sporulation.

The solid-phase cultivation method is of interest, first of all, due to the high quantitative yield of various products. Experimental data show that in the case of solid-phase cultivation, the yield of the target product is several times (from 2 to 14) higher than in the case of submerged cultivation of the same microorganism.

Thus, the solid-phase cultivation method is one of the most profitable methods for obtaining various products, such as enzymes , antibiotics, statins, some mycotoxins. In addition, some of these products can only be obtained by solid phase culture.

3.3.2. Model of the growth of mycelial microorganisms under solid-phase cultivation

The mycelium formed by the fungus can be divided into several types - aerial mycelium above the substrate surface, surface (wet) substrate mycelium, and internal, anchored into the substrate. Each type of hyphae performs a different function in a solid phase culture system. The air layer is responsible for the supply and transport of oxygen. In the surface mycelium, a supply of all nutrients transported from the internal mycelium, which actively hydrolyzes the substrate, is carried out. This system is more balanced than the submerged cultivation system (Fig. 3.6.).

The surface layer is a denser structure compared to aerial mycelium. In the space between the hyphae of this layer, in most cases, there is liquid. The structure of the surface layer resembles surface growth on an agar medium, but in this model it performs a specific function, namely, the accumulation of various nutrients, the transport of oxygen to the inner layer of hyphae, and nutrition of the aerial mycelium. As a rule, various polyols and sugar alcohols accumulate in the surface mycelium.

The deepest inner layer of hyphae, anchored into the carrier, hydrolyzes the surrounding substrate, which is often difficult to access, as, for example, in the case of natural carriers rich in cellulose. In the area of apical hyphae, micromycetes release a complex of corresponding enzymes into the environment, thus hydrolyzing the subetrate to available monomers.

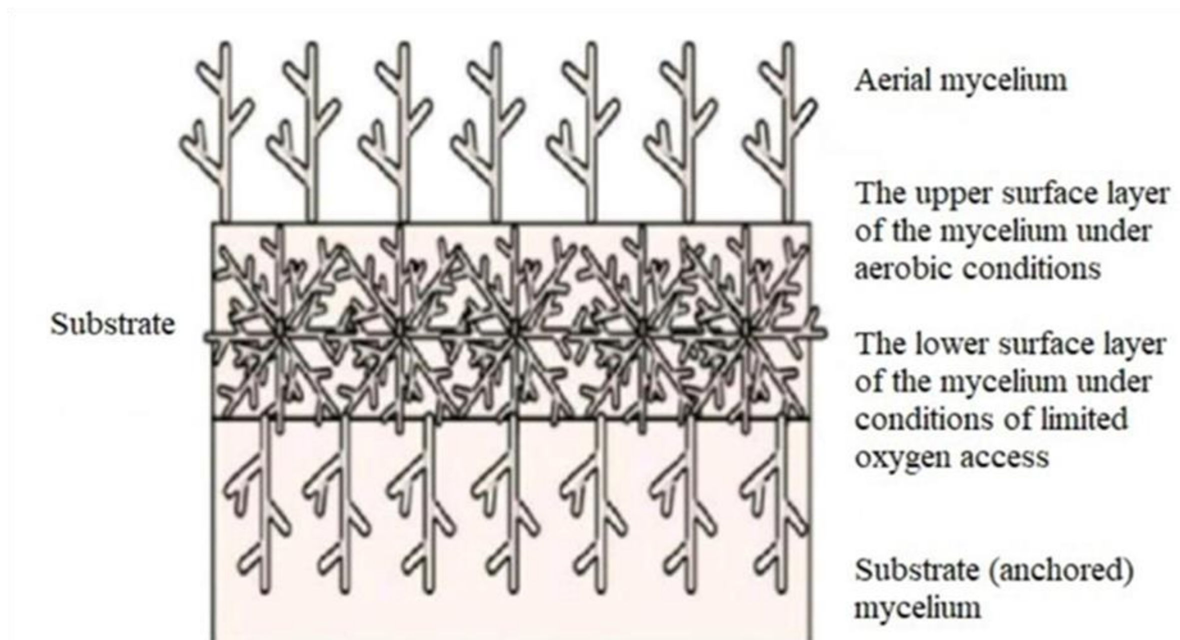

Fig. 3.6. Growth pattern of mycelial microorganism

3.4. Conclusions to chapter

As filamentous fungi are naturally adapted to grow on surfaces, under which conditions they show a particular physiological behaviour different from that observed in submerged fermentation, another bioreactor process named “biofilm” that can enhance the

production of fungal metabolites using a synthetic support within a liquid environment should also be used in order to enhance the production of biomolecules needed. This biofilm reactor can be considered as a promising technology combining the advantages of submerged fermentation in terms of process control and the advantages of solid-state fermentation in terms of fungal biology and development.

CONCLUSIONS

1. The use of proteases in different industries has been prevalent for many decades and a number of microbial sources exist for the efficient production of this enzyme. Their vast diversity, specific range of action and property of being active over a very wide range of temperature and pH have attracted the attention of biotechnologists worldwide. Although they are widely distributed in nature, microorganisms are the preferred source of these enzymes in fermentation bioprocesses because of their fast growth rate and also because they can be genetically engineered to generate new enzymes with desirable abilities or simply for enzyme overproduction. The search for new microorganisms that can be used for protease production is a continuous process. Proteases have various applications in major areas of food processing, beverage production, animal nutrition, leather, paper and pulp, textiles, detergents, etc. and with the advent of new frontiers in biotechnology, the spectrum of protease applications has expanded into many new fields such as clinical, medicinal and analytical chemistry.

2. Isolated mycelial fungi in pure culture and increased mycelial mass for further experiments.

Method of direct plating was used for detecting, counting, and isolating fungi *Cryphonectria parasitica* from surfaces of the bark of stems and branches, leaves and calybium (spiky cupules) chestnut. Particles are placed directly on a potato dextrose agar agar media.

Aspergillus brasiliensis fungi were isolated from soil samples by serial dilutions in the Czapek's medium for mushrooms medium using antibiotics to suppress the bacterial microflora.

3. Traditional methods used for the production of enzymes and secondary metabolites have been focused on solid-state fermentation without controlling factors essential for microorganism growth. However, submerged fermentation is more widely used, considering the possibility to implement robust control loops allowing to ensure the reproducibility of the process. The cost of secondary metabolite is one of the main factors

determining the economics of process. In some cases, solid-state fermentation offers advantages over submerged fermentation, but in general submerged cultures are used most often since the parameters can be controlled efficiently.

REFERENCES

1. Ежова Г.П. Биоинформационные аспекты протеомики и деградации белка / Г.П. Ежова, А.А. Бабаев, В.В. Новиков // Нижний Новгород, Изд-во ННГУ – 2007 – 2007. – 86 с.
2. The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in the PANTHER database / N.D. Rawlings, A.J. Barrett, P.D. Thomas et al. // Nucleic Acids Res. – 2018. – Vol. 46. – P. 624–632.
3. Лаврентье ва Е.В. Основы молекулярных механизмов регуляции экспрессии генов пептидаз в прокариотной клетке / Е.В. Лаврентьева, Т.Г. Банзаракцаева, А.А. Раднагуруева // – Улан-Удэ, – 2012. – 66 с.
4. Gonzales T. Bacterial amino peptidase: properties and functions / T. Gonzales, J. Robert-Baudouy // FEMS Microbiol Rev. – 1996. – Vol. 18. – P. 319–344.
5. Leucyl aminopeptidase (animal and plant) / N. Strater, W.N. Lipscomb, A. Barret, N.D. // Handbook of Proteolytic Enzymes. – NY: Academic press. – 1998. – Vol. 473. – P. 1384–1389.
6. Lazdunski A. Peptidases and proteases of *Escherichia coli* and *Salmonella typhimurium* / A. Lazdunski // FEMS Microbiol Rev. – 1989. – Vol. 63. – P. 265–276.
7. Proteases, production / Encyclopedia of Microbiology // O.P. Ward, M.B. Rao, A. Kulkarni et al. – Third edition. – 2009. – P. 495–511.
8. Rawlings N.D. Introduction: metallopeptidases and their clans / Handbook of Proteolytic Enzymes // N.D. Rawlings, A.J. Barrett, J.F. Woesner. – Second edition. – San Diego, USA: Elsevier/Academic Press. - 2004. – P. 231–263.
9. Rawlings N. D. Families of cysteine peptidases. Methods Enzymol / Rawlings N. D., Barrett A. J. // Proteolytic Enzymes: Serine and Cysteine Peptidases. – 1994. – Vol. 244. – P 461-486.
10. Mikhailova R.V. Proteolytic enzymes of mycelial fungi / R.V. Mikhailova // Microbiology and Biotechnology. – 2011. – Vol. 3. – P. 47–62.

11. Kräusslich H. G. Viral proteinases / Kräusslich H. G., Wimmer E. // Annu Rev Biochem. – 1988. – Vol.57. – P. 701-754.

12. Modeling and structural analysis of evolutionarily diverse S8 family serine proteases / A. Laskar, E.J. Rodger, A. Chatterjee et al. // Bioinformation. – 2011. – Vol 7(5). – P. 239-245.

13. Markland F. S. Subtilisins: Primary structure, chemical and physical properties / Markland F. S., Boyer D. // The Enzymes. – 1971. – Vol. III. – P. 561–608.

14. Philipp M. Kinetics of subtilisin and thiolsubtilisin / Philipp M., Bender M. L. // Mol Cell Biochem. – 1983. – Vol. 51. – P. 5–32.

15. Molecular and biotechnological aspects of microbial proteases / M.B. Rao, A.M. Tanksale, M.S. Ghatge et al. // Microbiol. and Mol. Biol. Rev. – 1998. – Vol. 62(3). – P. 597–635.

16. Boyer P. D. The Enzymes / Boyer P. D. // Basic Food Chemistry. – 1971. – NewYork, N.Y: Academic Press. – P. 177-198.

17. Optimization of alkaline protease production by *Aspergillus clavatus* ES1 in *Mirabilis jalapa* tuber powder using statistical experimental design / Hajji M., Rebai A, Gharsallah N et al. // Appl Microbiol Biotechnol. – 2008. – Vol.79 (6). – P. 915-923.

18. Tremacoldi C.R. Production of extracellular alkaline proteases by *Aspergillus clavatus* / Tremacoldi C.R. // World J. Microbiol. Biotechnol. – 2005. – Vol. 21. – P. 169–172.

19. Phadatare S.U. High activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20): enzyme production and compatibility with commercial detergents / Phadatare S.U., Deshpande V.V., Srinivasan M.C // Enzyme Microb. Biotechnol. – 1993. – Vol. 15. – P. 72–76.

20. Pekkarinen A.I. Purification and properties of an alkaline protease of *Fusarium culmorum* / Pekkarinen A.I., Jones B.L., Niku-Paavola M.L // Eur. J. Biochem. – 2002. – Vol. 269. – P. 798–807.

21. Jarai G. Nitrogen, carbon, and pH regulation of extracellular acidic proteases of *Aspergillus niger* / Jarai G., Buxton F // Curr. Genet. – 1994. – Vol. 26. – P. 238–244

22. Eriksson K.E. Acid proteases from *Sporotrichum pulverulentum* / Eriksson K.E., Pettersson B // Methods Enzymol. – 1988. – Vol.160. – P.500–508.

23. Protease biosynthesis by mutant strain of *Penicillium griseoroseum* and cheese formation / Haq I., Mukhtar H. // Pak. J. Biol. Sci. – 2004. – Vol. 7. – P. 1473–1476.

24. Formation of the extracellular proteases from *Trichoderma reesei* QM9414 involved in cellulose degradation / Haab D., Hagspiel K., Szakmary K. et al. // J. Biotechnol. – 1990. – Vol. 16. – P. 187–188.

25. Delgado-Jarana J. Overproduction of b-1, 6-glucanase in *Trichoderma harzianum* is controlled by extracellular acidic proteases and pH / Delgado-Jarana J., Pintor-Toro J.A., Benitez T // Biochim. Biophys. Acta. – 2000. – Vol. 1481. – P. 289–296.

26. Li D.-Ch. Protease production by the thermophilic fungus *Thermomyces lanuginosus* / Li D.-Ch., Yang Y.-J., Shen Ch.-Y. // Mycol. Res. – 1997. – Vol.101. – P.18–22.

27. Extracellular proteolytic enzymes of microscopic fungi from thermal springs of Barguzin valley (Northern Baikal region) / Bazarzhapov B.B., Lavrent'eva E.V., Dunaevskii Ya. et al. // Appl. Biochem. Microbiol. – 2006. – Vol. 42. – P.186–190.

28. Sekine H. Neutral proteinases II of *Aspergillus sojae* and I: some enzymatic properties / Sekine H. // Agric. Biol. Chem. – 1972. – Vol. 36. – P. 207–216.

29. Гришин Д.В. Биоактивные белки и пептиды: современное состояние и новые тенденции практического применения в пищевой промышленности и кормопроизводстве / Д.В. Гришин, О.В. Подобед, Ю.А. Гладилина // Вопр. Питания. – 2017. – Т.86. – Р. 19–31.

30. Biotechnological applications of proteases in food technology / O.L. Tavano, A. Berenguer-Murcia, F. Secundo et al. // Comprehensive Reviews in Food Science and Food Safety. – 2018. – Vol. 17. – P. 412–436.

31. Kumar N.S. A review on microbial proteases / N.S. Kumar, Sreeja Devi P.S., A.S. Nair // International Journal of Advanced Research. – 2016. – Vol. 4 (7). – P. 2048–2053.

32. Sumantha A. Microbiology and industrial biotechnology of food-grade proteases: A perspective / A. Sumantha, C. Larroche, A. Pandey // Food Technol. Biotechnol. – 2006. – Vol. 44(2). – P. 211–220.

33. Reid V.J. Extracellular acid proteases of wine microorganisms: gene identification, activity characterization and impact on wine: MS dissertation / V.J. Reid. – Stellenbosch University, South Africa. – 2012. – 94 p.

34. Fungal proteolytic enzymes: features of the extracellular proteases of xylotrophic basidiomycetes / O.A. Kudryavtseva, Ya.E. Dunaevsky, O.V. Kamzolkina et al. // Mikrobiologiya. – 2008. – Vol. 77(6). – P. 725–737.

35. Ottesen M. The subtilisins / Ottesen M. // Methods Enzymol. – 1970. – Vol. 19. – P. 199-221.

36. BRENDA in 2019: a European ELIXIR core data resource / L. Jeske, S. Placzek, I. Schomburg et al. // Nucleic Acids Research. – 2019. – Vol. 47. – P. 542–549.

37. An overview of microbial proteases for industrial applications / B.S. Mienda, A. Yahya, I.A. Galadima et al. // Research Journal of Pharmaceutical, Biological and Chemical Sciences. – 2014. – Vol. 5(1). – P. 388–396.

38. A biotechnology perspective of fungal proteases / P.M. Souza, M.L. Assis Bittencourt, C.C. Caprar et al. // Brazilian Journal of Microbiology. – 2015. – Vol. 46(2). – P. 337–346.

39. Sharma N. Production, purification and crystallization of an alkaline protease from *Aspergillus tamarii* [EF661565.1] / N. Sharma, K. De // Agriculture And Biology Journal Of North America. – 2011. – V. 2(7). – P. 1135–1142.

40. Singhal P. Studies on production, characterization and applications of microbial alkaline proteases / P. Singhal, V.K. Nigam, A.S. Vidyarthi // International Journal of Advanced Biotechnology and Research. – 2012. – V. 3(3). – P.653– 669.

41. North M.J. Comparative biochemistry of the proteinases of eucariotic microorganisms / North M.J. // Microbiol. Rev. – 1982. – Vol.46. – P.308–340.

42. Priest F. Extracellular enzymes / F. Priest. – Moscow, Mir Publ. – 1987. – 117 p.

43. Antipova L.V. Methods of analyzing meat and meat products / Antipova L.V., Glotova I.A., Rogov I.A. – Moscow, Kolos Press. – 2001. – 376 p.

44. Grishin, D. Heterogeneous expression and characterization of a new mutant DNA-binding protein from the *Thermotoga naphthophila* hyperthermophilic microorganism / Grishin D., Zhdanov D. Gladilina J., Pokrovskaya M. // Proceedings of universities Applied chemistry and biotechnology. – 2019. – Vol.9. – P. 288-301.

45. Braga-Silva L. A. Aspartic Protease Inhibitors as Potential Anti-*Candida albicans* Drugs: Impacts on Fungal Biology, Virulence and Pathogenesis / L. A. Braga-Silva, A. L.S. Santos // Current Medicinal Chemistry. – 2011. – Vol. 18. – P. 2401 – 2419.

46. Al-mohanna, Moshtaq. Methods for fungal enumeration, isolation and identification / Al-mohanna, Moshtaq //

47. Nirmal N.P. Fungal Proteases: An Overview / N.P. Nirmal, S. Shankar, R.S. Laxman // International Journal of Biotechnology and Biosciences. – 2011. – Vol. 1(1). – P. 1–40.

48. Давиденко Т. И. Иммобилизация ферментных препаратов / Т. И. Давиденко // Вісник ОНУ. – 2003. – № 4. – С. 135–147.

49. Ephrem Guchi. Aflatoxin Contamination in Groundnut (Arachis hypogaea L.) caused by *Aspergillus Species* in Ethiopia. / Ephrem Guchi. // Journal of Applied & Environmental Microbiology. – 2015. – P. 11-24.

50. Pitt, J.I. Fungi and food spoilage / Pitt, J.I. Hocking, A.D. – 2nd Edition, Blackie Academic and Professional, London. – 1997. – 593 p.

51. Savitha S. Fungal protease: Production, purification and compatibility with laundry detergents and their wash performance / Sivam S, Swaminathan // Journal of the Taiwan Institute of Chemical Engineers. – 2011. – Vol.42. – P. 298-304.

52. Gupta R. Bacterial alkaline proteases: molecular approaches and industrial applications / Gupta R // Applied microbiology and biotechnology. – 2002. – Vol. 59. – P. 15-32.

53. Sandhya C. Comparative evaluation of neutral protease production *by Aspergillus oryzae* in submerged and solid-state fermentation / Sandhya C. // Process Biochemistry. – 2010. – Vol. 40. – P. 2689-2694.

54. Das Kishore. Comparison of lipopeptide biosurfactants production by *Bacillus subtilis* strains in submerged and solid-state fermentation systems using a cheap carbon source: Some industrial applications of biosurfactants / Das Kishore // Process Biochemistry. – 2010. – Vol. 42. – P. 1191-1199.

55. R. te Biesebeke. *Aspergillus oryzae* in solid-state and submerged fermentations / R. te Biesebeke // Progress report on a multi-disciplinary project of Yeast Research. – 2002. – Vol. 2. – P. 245-248.

56. Geisseler D. Regulation of extracellular protease activity in soil in response to different sources and concentrations of nitrogen and carbon Soil Biology & Biochemistry / Geisseler D. // Soil Biology & Biochemistry. – 2008. – P. 3040-3048

57. J.S. White. Source Book of Enzymes / J.S. White, D.C. White. – N.Y.: Taylor Francis Inc. – 1997. – 1328 p.

58. Vishwanatha K. S. Acid protease production by solid-state fermentation using *Aspergillus oryzae* MTCC 5341: optimization of process parameters / Vishwanatha K. S. // Journal of industrial microbiology & biotechnology. – 2010. – Vol. 37(2). – P. 129-38.