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# SELECTION OF ACTIVE STRAINS OF FILAMENTOUS FUNGI AS PRODUCERS OF HYDROLYTIC ENZYMES FOR STRUGGLE AGAINST PHYTOPATHOGENS

# ОТБОР АКТИВНЫХ ШТАММОВ МИЦЕЛИАЛЬНЫХ ГРИБОВ - ПРОДУЦЕНТОВ ГИДРОЛИТИЧЕСКИХ ФЕРМЕНТОВ ДЛЯ БОРЬБЫ С ФИТОПАТОГЕНАМИ

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*Abstract.* Enzymes of cellulase, hemicellulase, chitinase systems of local strains of mycelial and phytopathogenic, saprotrophic fungi isolated from various natural sources of the Republic of Uzbekistan were studied in a comparative aspect. According to the degree of hydrolysis of chitin and microcrystalline cellulose, added to the medium content as the only carbon source, and growth rate, 45 cultures were selected from the studied 151 representatives of microscopic fungi. Then 29 highly active cultures belonging to the genera *Fusarium, Aspergillus, Penicillium,* and also saprophytes *Ulocladium, Alternaria* were selected with deepening studies. Further among them, with the study of their action spectrum, 8 highly active cultures were collected. The activity of enzymes hydrolyzing natural polysaccharides (cellulose, chitin, xylan) was determined, as well as protein accumulation was observed in media containing chitin and microcrystalline cellulose as the sole source of carbon. It was shown that the activity of hydrolases (cellulases on the hydrolysis of cellulose–containing substrates — chitinase, xylanase) and the formation of protein depended on species affiliation of fungi, carbon sources in the culture medium and growth duration. The inhibitory effect of hydrolytically active culture fluids of fungi *Penicillium* sp. 18, *Penicillium* sp. 140 and *Aspergillus terreus* 461, *Aspergillus terreus* 499 on the phytopathogens *Rhizoctonia solani, Verticillium dahliae, Fusarium oxysporum* and *Fusarium solani* was defined. Selected fungi with high hydrolytic activity are the basis for the creation of highly effective ecologically safe biopreparations of microbial origin for protecting plants from phytopathogens and new biotechnologies in the field of crop cultivation, bioconversion of plant and industrial waste, environmental protection.

*Аннотация.* В сравнительном аспекте изучены ферменты целлюлолитической, гемицеллюлазной, хитиназной систем местных штаммов мицелиальных и фитопатогенных, сапротрофных грибов, выделенных из различных природных источников Республики. По степени гидролиза хитина и МКЦ, внесенных в состав среды в качестве единственного источника углерода, и скорости роста из исследованных 151 представителя микроскопических грибов были отобраны 48 культур, далее с углублением исследований были выбраны 29 высокоактивных культур, относящихся к родам *Fusarium, Aspergillus, Penicillium,* а также сапрофитам *Ulocladium, Alternaria, Acremonium.* В результате изучения их спектра действия были отобраны 8 высокоактивных культур. Определена активность ферментов, гидролизующих природные полисахариды (целлюлоза, хитин, ксилан), а также показано накопление белка на средах, содержащих хитин и микрокристаллическую целлюлозу в качестве единственного источника углерода. Показана зависимость активности гидролаз (целлюлазы по гидролизу целлюлозосодержащих субстратов — хитиназы, ксиланазы) и образования белка от видовой принадлежности грибов, источников углерода в среде культивирования и продолжительности роста. Показано ингибирующее действие гидролитически активных культуральных жидкостей грибов *Penicillium* sp. 18, *Penicillium* sp. 140 и *Aspergillus terreus* 461, *Aspergillus terreus* 499 на фитопатогены — *Rhizoctonia solani, Verticillium dahliae, Fusarium oxysporum* и *Fusarium solani.* Отобранные грибы, обладающие высокой гидролитической активностью, являются основой для создания высокоэффективных экологически безопасных биопрепаратов микробного происхождения для защиты растений от фитопатогенов и новых биотехнологий в области возделывания сельскохозяйственных культур, биоконверсии растительных и промышленных отходов, защиты окружающей среды.

*Ключевые слова:* микроскопические грибы, полисахариды, культивирование, гидролазы, белки, фитопатогены.

*Keywords:* microscopic fungi, polysaccharides, hydrolases, cultivation, proteins, phytopathogens.

*Introduction*

The study of hydrolytic enzymes of microorganisms, their components' composition and mechanism of action on various structural natural polysaccharides of cell walls of plants is increasingly becoming more relevant. The study of this problem is also necessary for the fact that many plant diseases caused by phytopathogens and other pests lead to a disruption of clearly, genetically coordinated metabolic processes and growth factors that adversely affect both the accumulation of phytomass and the quantity and quality of the crop [1]. Infection with phytopathogens leads to undesirable processes of damage to plants and contamination, and ultimately up to the death of the plant. The enzymes lysing the polysaccharides of the plant cell, which are not only structural elements, but also play the main role for the protection and maintenance of vital activity, as well as the productivity of plants, are actively participating in all these processes [2].

The composition of the cell membrane of fungi, including that of phytopathogens, is very diverse. For example, the cellulose–chitin complex predominates in the cell membranes of many species of zygomycetes, cellulose–glucan in oomycetes, chitosan–chitin in some species of oomycetes, chitino–glucan in chitridial, marsupials, basidial species of fungi and deuteromycetes. Polymers containing glucuronic acids (up to 20%), heteropolymers containing mannose, galactose, glucose, glucuronic acid have also been found in the shell of fungi [3]. The main components of the cell wall of the fungi are chitin, glucans, protein and lipids, which form a compound complex, the molecules of chitin are covered with a layer of glucanose molecules, connected by bridges. In such cases, chitinase without the involvement of cellulases does not hydrolyze the chitin contained in the cell membrane of the fungi [4, 5].

Microfibrils of chitin and cellulose in most fungi form a structure in which many other components of the cell membrane are connected. It is believed that protein and polysaccharides bind microfibrils of chitin and cellulose [6, 7].

Cellular membranes of *Pythium* and *Phytophthora* contain cellulose. When lytic enzymes exposed to the shells of *Fusarium* hyphae in lysates, glucose (14%), N–acetylhexosamine (47%), insoluble residue formed after hydrolysis of galactose, mannose, uronic acid [6, 7, 8].

The cell wall of fungi as a result of lysis can be destroyed in two ways: by the action of enzymes released by other cells or organisms and enzymes formed in the cell of the fungus itself. The most important enzymes catalyzing the decomposition of individual components of the cell membrane of fungi are α– and β–glucanases, proteases and peptidases, cellulases, lipases, chitinases, hexosaminidases, glucuronidases, glucosaminidases, cellobiasis. Thus, the destruction of the cell membrane of fungi is catalyzed by various enzymes' complex. This complex depends on components' composition of the cell membrane of different fungi species [9–11].

Specific cellulases and hemicellulases produced by plants themselves-participate in anabolic processes and build bricks of molecules–biopolymers, connect the molecules of the photosynthetic–glucose product to macromolecules of cellulose and other polysaccharides by their synthetase ability [12–17].

Still remains the question of the physiological role of xylanases in organisms synthesizing them, although it is known that they participate in the degradation of xylan to xylose, which is further used as a source of carbon and energy, moreover, they are part of the cell wall. *Fusarium* fungi (for example, *F. oxysporum*, which causes a tobacco wilt) absorb well xylose known to be specific for the cell wall of plants and some algae [18]. The physiological function of xylanolytic enzymes, as well as cellulases in microorganisms–phytopathogens, apparently, is the destruction of

the wall of a plant cell for penetration into the cytoplasm [19, 20]. Chitin is a part of the cell wall of many fungi, the composition of the external skeleton of invertebrate animals and is constantly present in the soil, reaching a tenth of a percent. For a number of physicochemical properties, chitin is similar to cellulose, but the presence of acetamide groups in the molecule gives it particularly valuable properties in practice [21]. Known bacterial chitinase has been used as a means of protecting plants from pathogens [22]. However, the problem of the decomposition of chitin in the soil remains insufficiently disclosed up to the present time.

The fungus *Trichoderma harzianum* lysing plant pathogenic fungi, secretes a complex of lytic enzymes containing endo– and exo– p-1,3-glucanases, protease and chitinase [22, 23]. The different components of *Trichoderma harzianum* lytic system probably include mutually complementary enzymes, with the full system required for maximum hydrolysis efficiency [23]. Since the cell walls of the fungi contain b–glucan microfibrils and chitin immersed in the protein matrix, it is likely that mainly p–glucanase, chitinase and protease participate in the lysis of their cells [24]. The formation of these enzymes is also observed in the parasitic interaction of certain microorganisms [25, 26]. Glucanases can have a direct lytic effect (such enzymes are often found in the group of extracellular b-1,3-glucanases) or play an important role in lysis of the cell wall of the microorganism with lytic enzymes' complex. They also have vital functions in the microbial cell's own metabolism [19].

The processes of plant infection with phytopathogens begin with the penetration of pathogens into both healthy and weakened plants that have fallen into unfavorable conditions, especially when there is a lack of macro and microelements. Saprophytic and parasitic (obligate–parasitic, conditional) microorganisms exert a different influence both on the host and the plant [18].

Higher, there was conducted the detailed comparative study of the enzymes of the cellulolytic, hemicellulase, chitinase systems of mycelial and phytopathogenic fungi, with high hydrolytic activity, providing full biological growth, directed regulation of growth and development of agricultural plants, increasing their yield and preservation. Creation of effective ecologically safe biologics and biotechnologies for their cultivation on their basis is one of the important and promising directions of modern science and production.

Proceeding from the foregoing, this work is devoted to the search and selection of fungi–active producers of hydrolytic enzymes, decomposing natural polysaccharides, the ultimate goal of which is the creation of enzymatically active biopreparations of microbial origin for bioconversion of waste, as well as the protection of plants from phytopathogens.

## *Materials and methods*

*Screening and methods of growing fungi on nutrient media.* For screening more than 151 strains of museum cultures of microscopic fungi, mainly belonging to the genera *Fusarium* (42 strains), *Aspergillus* (37), *Penicillium* (51) and some Saprophytic fungi (21) isolated from diseased leaves, plant stems were used. The museum cultures had been stored on wort agar and Czapek agar in the laboratory of the microorganisms' collection of institute of microbiology of Academy of Sciences.

For the initial selection and evaluation of the hydrolysing ability of the fungi, a surface method was conducted for cultivating fungi using polysaccharides — microcrystalline cellulose and chitin (crab) at concentrations of 2.0% added to Czapek synthetic medium as the sole source of carbon. The zones of hydrolysis of polysaccharides by test fungi were taken into account during 3–5 days of growth, estimating the periodic growth and development, the appearance of the fungal colonies and the formation of the substrate hydrolysis zone.

*Screening of fungi in liquid nutrient media.* To quantify the content of enzymes and proteins, the method of submerged cultivation of fungi on media of the following composition was used:

1. The nutrient medium no. 1 (g/l): colloidal chitin — 20.0; $KH_2PO_4$ —1,0; $MgSO_4 \times 7H_2O$ — 0,5; KCl — 0,5; $FeSO_4 \times 7H_2O$ — 0,01; $NaNO_3$ — 3,0. The initial pH of 5.5.

2. The nutrient medium No. 2 (g / l): microcrystalline cellulose - 20.0; $KH_2PO_4$ - 1,0; $MgSO_4 \times 7H_2O$ - 0,5; KCl - 0,5; $FeSO_4 \times 7H_2O$ - 0,01; $NaNO_3$ - 3.0. The initial pH of 5.5.

Cultivation was carried out in Erlenmeyer flasks with a volume of 1 liter containing 200–300 ml of nutrient medium no. 1 and no. 2 at temperature of 28–32 °C and pH of 5.6–6.0 for 3–7 days depending on the nature of the fungi.

Samples were collected, filtered and analyzed every 12 hours for the determination of enzymatic activity, protein content, etc.

*Methods for the determination of enzyme activity in culture fluid.* Cellulase activity in culture fluid was determined by the method of Somogyi–Nelson in the modification of Feniksova R.V. Determination of the resulting reducing sugars during the hydrolysis of a 1.0% solution of microcrystalline cellulose of "Sigma" (USA). Specific cellulase activity was expressed per unit weight (mg) of total protein contained in culture fluid and cleaving from the substrate 1 μg of glucose, which is measured at 490 nm of FEC wave. The amount of glucose that formed under the action of cellulase is subtracted from the calibration schedule constructed using a standard glucose solution [27].

Xylanase activity was determined by the FEC–method of Somogyi–Nelson in the modification of Feniksova R. V, using 1.0% colloidal xylan oats solution Spelt from Sigma (USA). The xylanase activity is 1 μmol xylose, also the product of the reaction cleaved from the substrate under optimal conditions [28].

The activity of enzymes (cellobiohydrolase, endoglucanase, avicelase and xylanase), was determined by colorimetric method based on measuring the amount of reducing sugars cleaved from substrates under the action of enzymes under optimal conditions of fermentolysis [29].

To determine the endoglucanase activity, 1% solution of Na–microcrystalline cellulose in 0.1 M acetate buffer with pH 5.0 was used as the substrate [30]. The amount of enzyme which catalyzes the hydrolysis of Na–microcrystalline cellulose to form 1 μmol of glucose under standard conditions (pH 5.0, incubation temperature of 50 °C, hydrolysis duration of 30 min) was taken as the unit of activity.

To determine the avicelase activity, microcrystalline cellulose was used as a substrate. The reaction mixture was incubated at 50 °C for 24 hours. The resulting hydrolysates were filtered and the content of reducing sugars was determined using 3,5-dinitrosalicylic acid [31]. The amount of enzyme that catalyzes the hydrolysis of microcrystalline cellulose with the formation of 1 μmol of reducing sugars under standard conditions was taken as the unit of avicelase (exoglucanase) activity.

The activity of extracellular chitinase was determined by the amount of reducing sugars formed during the hydrolysis of colloidal chitin [31]. The reaction mixture contained 1.0 ml of 1,0% colloidal chitin in 0.1 M acetate buffer. The amount of enzyme resulting hydrolysis of colloidal chitin to reducing substances equivalent to 1 mg of N–acetyl–D–glucosamine at 40 °C and pH — 5,0 in 60 min was taken as the unit of activity. The amount of N–acetyl–D–glucosamine was determined from a standard curve composed of a calibration solution containing from 0.04 to 1.0 mg of the substance.

Protein content in the samples was determined by Lowry's method [32], bovine serum albumin (BSA) was used as the standard.

*Results and discussion*

At the first stage of the research primary selection of mycelial fungi isolated from various natural sources of the Republic of Uzbekistan related to different genera and species was carried out by means of a comparative evaluation of their hydrolyzing activity against natural hardly hydrolysing polysaccharides. Thus, for example, the degree of hydrolysis of chitin and microcrystalline cellulose added to Czapek synthetic medium as the sole carbon source at concentrations of 2.0%. It was found that 45 cultures (29,8%) of out 151 tested cultures of local soil fungi were relatively more active in terms of the cellulose and chitin hydrolysis zone while solid–state cultivation.

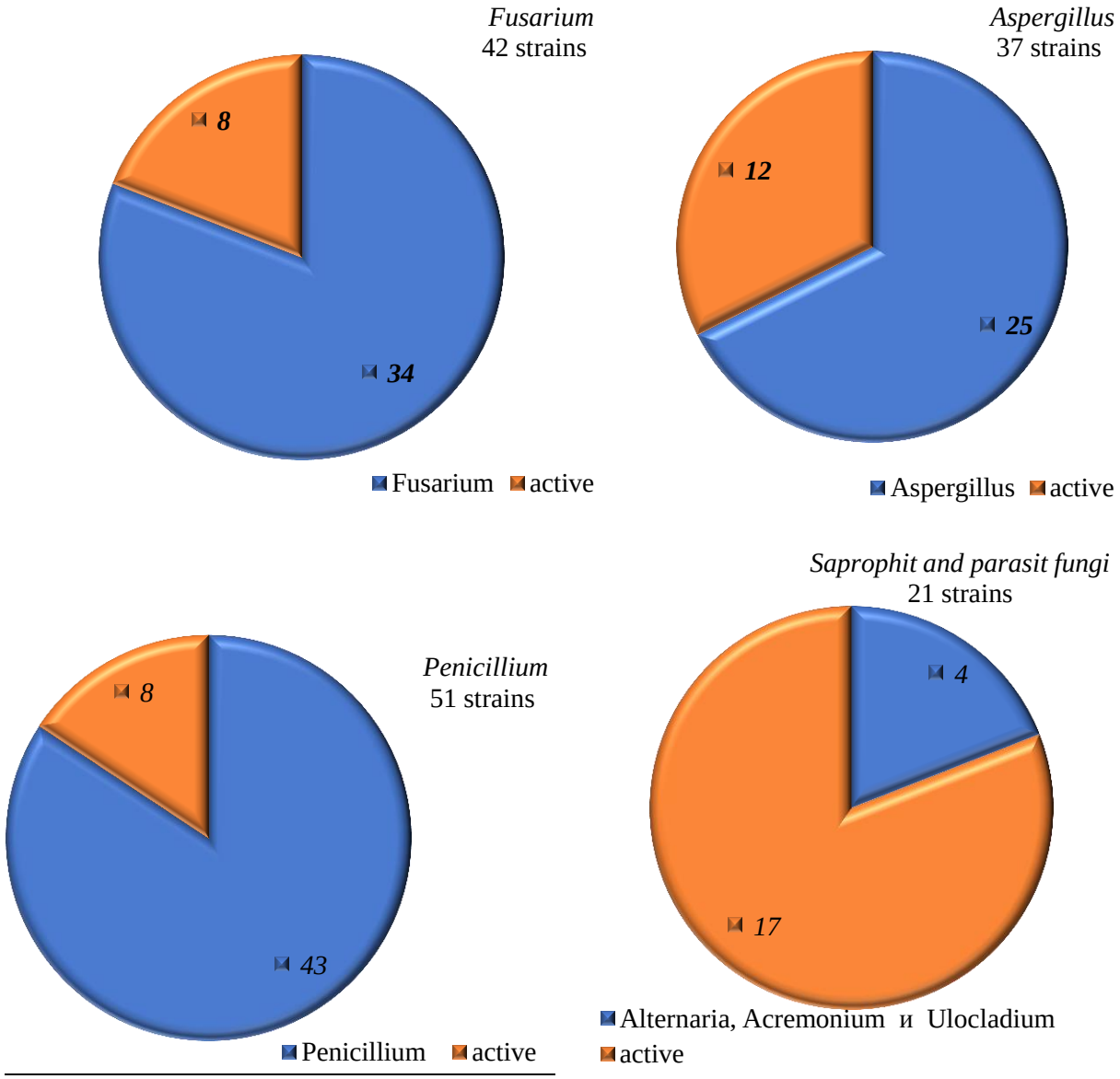



Figure 1. Distribution of activity of hydrolytic enzymes between genera of test fungi

Cellulolytic and chitinolytic enzymes were synthesized by 8 cultures of fungi (19%) out of 42 representatives of the genus *Fusarium*, 12 cultures of the genus *Aspergillus* (32,4%) were active out of the tested 37 ones, only 8 cultures (15,7%) out of 51 studied representatives of the genus *Penicillium* were highly active, whereas 17 cultures of isolated fungi (81%) out of 21

representatives of saprophytic and parasitic fungi isolated from diseased leaves, plant stems related to the genera *Alternaria*, *Acremonium* and *Ulocladium* were active.

While submerged cultivation in Czapek synthetic medium containing chitin and microcrystalline cellulose at concentrations of 2,0% introduced as the only carbon source, 29 fungi cultures out of 45 ones were selected from the genera *Fusarium*, *Aspergillus*, *Penicillium* and saprophytes, forming highly active hydrolytic enzymes of cellulolytic, xylanolytic, chitinolytic and proteolytic complexes (Table 1).

Table 1.

THE RESULTS OF SELECTION OF HYDROLYTICALLY ACTIVE FILAMENTOUS FUNGI

| *Representatives of the genus Fusarium and their strains* | *Representatives of the genus Penicillium* | *Representatives of the genus Aspergillus* | *Representatives of saprophytic fungi* |
|---|---|---|---|
| *Fusarium lateritium* 187 | *Penicillium* sp. 140 | *Aspergillus sp.* 466 | *Alternaria* sp.76 |
| *Fusarium oxysporum* 173 | *Penicillium* sp.18 | *Alternaria tenuis* 66 | *Acremonium* sp.3 |
| *Fusarium moniliforme* 191 | *Penicillium purpurogenium* 159 | *Aspergillus sp.* 499 | *Acremonium terricola* 2 |
| *Fusarium* sp. 552 | *Penicillium* sp. 678 | *Aspergillus flavus* 139 | *Alternaria* sp.136 |
| *Fusarium moniliforme* 183 | *Penicillium* sp. 374 | *Aspergillus sp.* 91 | *Alternaria* sp. 67 |
| *Fusarium* sp. 554 | *Penicillium* sp. 766 | *Aspergillus sp.* 7 | *Alternaria* sp. 62 |
| *Fusarium solani* 169 | *Penicillium* sp.86 | | *Alternaria malvae* 55 |
| *Fusarium sambusinum* 109 | *Penicillium* sp. 140 | | *Ulocladium* sp.134 |

The results of the conducted tertiary screening showed that 9 cultures out of the total tested 29 fungi–producers of chitinolytic and cellulolytic enzymes were active.

Further, the cellulolytic ability of the selected fungi was analyzed by the method of submerged cultivation in medium containing 2% microcrystalline cellulose in which the protein amounts and enzymatic activity were determined in the dynamics of their growth for 144 hours, taking samples for analysis every 12 hours.

It was found that the fungi *Penicillium* sp. 18, *Penicillium purpurogenum* 159, *Aspergillus terreus* 461, *Fusarium moniliforme* 183 showed a high activity by 72 hours of growth, while fungi *Aspergillus sp.* 461, *Aspergillus sp.* 499, *Fusarium moniliforme* 183 were active by 120 hours. The fungi *Aspergillus sp.* 461, *Aspergillus sp.* 499, *Penicillium purpurogenum* 159 and *Fusarium moniliforme* were active by 144 hours, whereas *Aspergillus sp.* 461, *Aspergillus sp.* 499, *Penicillium* sp. 18, *Penicillium purpurogenum* 159 by 168 hours. *Aspergillus sp.* 461, *Aspergillus sp.* 499, *Penicillium* sp. 18 were more active within 192 hours of growth, whereas *Aspergillus sp.* 461, *Aspergillus sp.* 499, *Alternaria sp*. 9 within 240 hours.

Despite the fact that the activity of cellulase on the hydrolysis of cotton cellulose in all fungi began with 72 hours of growth, the activity of the xylotrophs, such as *Aspergillus sp.* 499, *Aspergillus sp.* 461 remained high up to 240 hours of growth. It should be noted that along with these fungi, strains of phytopathogens *Fusarium moniliforme* 183 and *Fusarium solani* 169, saprophytes *Ulocladium* sp. 134, as well as fungi *Penicillium* sp. 18, *Penicillium purpurogenum* 159 also exhibited an ability to actively hydrolyze crystalline and rigidly regulated cotton cellulose.

The obtained data showed that indeed cellulases play an important role in the penetration of phytopathogens through the cell walls of plants, which leads to their defeat, further to the disease.

When studying the chitinolytic activity of fungi on a cellulose–containing medium, it was found that their activity was different depending on the duration of cultivation and the species of

fungi. The fungi *Aspergillus sp.* 461, *Aspergillus sp.* 499, *Penicillium sp.* 18 were the most active, while the fungi *Alternaria sp.* 9 *Streptomyces sp.* 4 and *Ulocladium sp.* 134 were less active in the hydrolysis of chitin.

*Aspergillus sp.* 499, *Aspergillus sp.* 461, *Penicillium sp.* 18, *Fusarium solani* 169 and *Ulocladium sp.* 134 had high xylanolytic activity. The formation of protein in the culture medium during the growth of fungi on cellulose–containing and chitin–containing media showed that proteins begin to accumulate from 24 hours of growth, reaching a high level on the 7–10$^{th}$ day of growth.

The amount of formed proteins varies depending on the composition of the nutrient medium and the species of fungi. The most amount of protein was accumulated by *Alternaria* sp. 9, *Penicillium* sp. 18, *Fusarium moniliforme* 183 on cellulose containing medium and by *Aspergillus sp.* 461 and *Aspergillus sp.* 499 on both cellulose–containing and chitin–containing medium.

Further, the cellulolytic activity of culture fluid of highly active fungi selected during the active stage of fermentation on various cellulose substrates, such as Na–carboxymethylcellulose, wood pulp, microcrystalline cellulose was determined with the preparation of a reaction mixture with a specified concentration of these substrates.

The ability of fungi to hydrolyze Na–carboxymetylcellulose was different: *Aspergillus sp.* 499, *Aspergillus sp.* 461, *Penicillium* sp. 18 was active during the entire period of growth, *Penicillium purpurogenum* 159 in the initial stage of growth, whereas *Alternaria* sp. 9 on the 7$^{th}$ day of cultivation.

The rate of hydrolysis of wood pulp, taken as a substrate of the reaction mixture, showed that although this substrate is highly crystalline and difficult to hydrolyse, selected fungi formed such forms of the cellulolytic complex enzymes that lead to fermentolysis of the substrate, but in varying degrees. High fungal activity was demonstrated by fungi *Aspergillus sp.* 499, *Aspergillus sp.* 461, *Ulocladium* sp. 134, *Penicillium* sp. 18, followed by *Fusarium moniliforme* 183.

The similar pattern was also observed in the study of the activity of filtrates culture fluid while the hydrolysis of cotton cellulose, where the fungi *Penicillium* sp. 18, *Aspergillus sp.* 461, *Aspergillus sp.* 499, *Penicillium purpurogenum* 159 and *Fusarium moniliforme* 183 were active.

The study of the protein content formed during the growth and development of fungi showed that the maximum amount of protein in the culture fluid was found in the fungi *Penicillium purpurogenium* 159, *Acremonium* sp. 3, *Alternaria* sp. 62, *Alternaria* sp. 76, *Fusarium solani* 169, *Fusarium moniliforme* 183, *Penicillium* sp. 18, *Aspergillus sp.* 461, *Aspergillus sp.* 499, *Ulocladium* sp. 134. During the cultivation of fungi the pH of the nutrient medium was relatively increased.

Thus, fungi *Fusarium solani* 169, *Fusarium moniliforme* 183, *Penicillium purpurogenium* 159, *Penicillium* sp. 18, *Aspergillus sp.* 461, *Aspergillus sp.* 499, *Ulocladium* sp. 134, *Alternaria* sp. 9 showed high cellulase activity in medium with 2.0% microcrystalline cellulose.

It was found that 8 fungi cultures out of the selected 29 ones had the highest activity of hydrolase in comparison with other cultures.

After careful selection of 8 highly active cultures, the most active fungi *Penicillium sp*. 140, *Penicillium sp*. 18, *Aspergillus sp.* 461 and *Aspergillus sp.* 499 were taken as promising producers of chitinases and cellulases in deepening experiments.

The formation of proteins and enzymatic activities of xylanase, endoglucanase, cellobiohydrolase, avicelase, chitinase and protease during submerged cultivation with optimal concentrations of polysaccharides (chitin, cellulose) as the only carbon source in the nutrient medium were studied. The results of further secondary screening of 29 strains of filamentous fungi, producers of chitinolytic and cellulolytic enzymes, showed that the strains *Penicillium* sp. 140, *Penicillium* sp. 18, *Aspergillus sp.* 461 and *Aspergillus sp.* 499 had high activity and growth and they were further tested as promising producers of chitinases and cellulases (Figure 2).

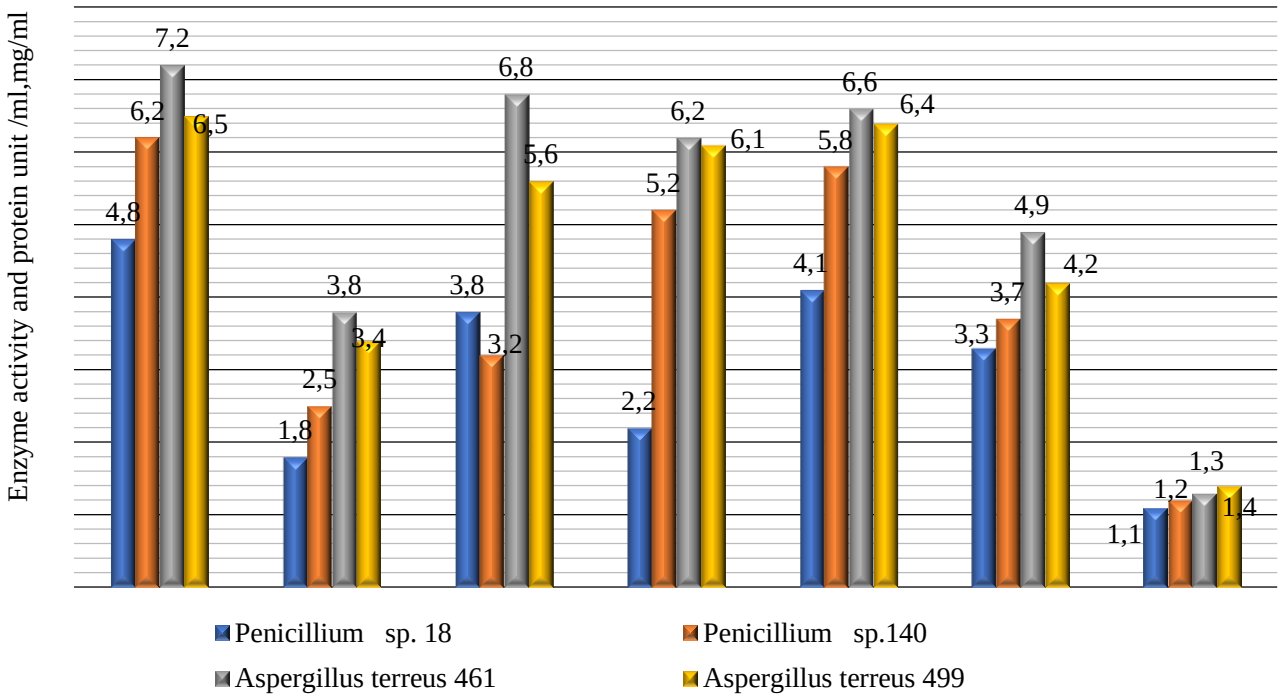



Figure 2. The activity of hydrolytic enzymes and the formation of protein by fungi while submerged cultivation in medium containing chitin

The data in Figure 2 showed that the fungi *Aspergillus* sp. 461 and *Aspergillus* sp. 499 had higher chitnase activity on chitin medium then *Penicillium* sp. 18 and *Penicillium* sp. 140. Cellobiohydrolase activity of fungi *Aspergillus sp.* 461 and *Aspergillus sp.* 499 was almost identical. The lowest value for this enzyme showed the fungus *Penicillium* sp. 18. The endogluconase activity was relatively high in all fungi, whereas avicelase activity was moderately high compared to other enzymes of the cellulase complex. Almost all the fungi had protease activity, which was accompanied by enzymes, as well as xylanase during the cultivation in chitin containing medium.

Enzyme activity and protein accumulation of tested strains *Aspergillus terreus* 461 and *Aspergillus terreus* 499 in the nutrient medium containing cellulose was higher than in the medium with chitin (Figure 3). The choice of an appropriate substrate is important for the effective synthesis of cellulases. This is due to the fact that the substrates serve not only as a source of carbon in the nutrient medium, but also as indispensable inducing compounds for microorganisms.

The formation of protein in the culture medium during the growth of fungi in cellulose and chitin containing media showed that proteins begin to accumulate in the medium within 24 hours of growth, reaching a high level on the 7–10$^{th}$ day of growth (Table 2).

The amount of formed proteins varies depending on the composition of the nutrient medium, the species of fungi and the growth duration. The fungi *Penicillium* sp. 140, *Aspergillus terreus* 499 formed large quantity of protein on cellulose medium, whereas fungi *Aspergillus terreus* 461 and *Penicillium* sp. 18 both on chitin and cellulose containing madia.

As a result of the conducted experiments, it was found out that the studied strains of fungi exhibit hydrolytic activity to a sufficient extent on media containing hardly hydrolysable polysaccharides such as chitin crab and microcrystalline cellulose. Cellulase activities were detected in the hydrolysis of carboxymethyl cellulose, wood and cotton cellulose.

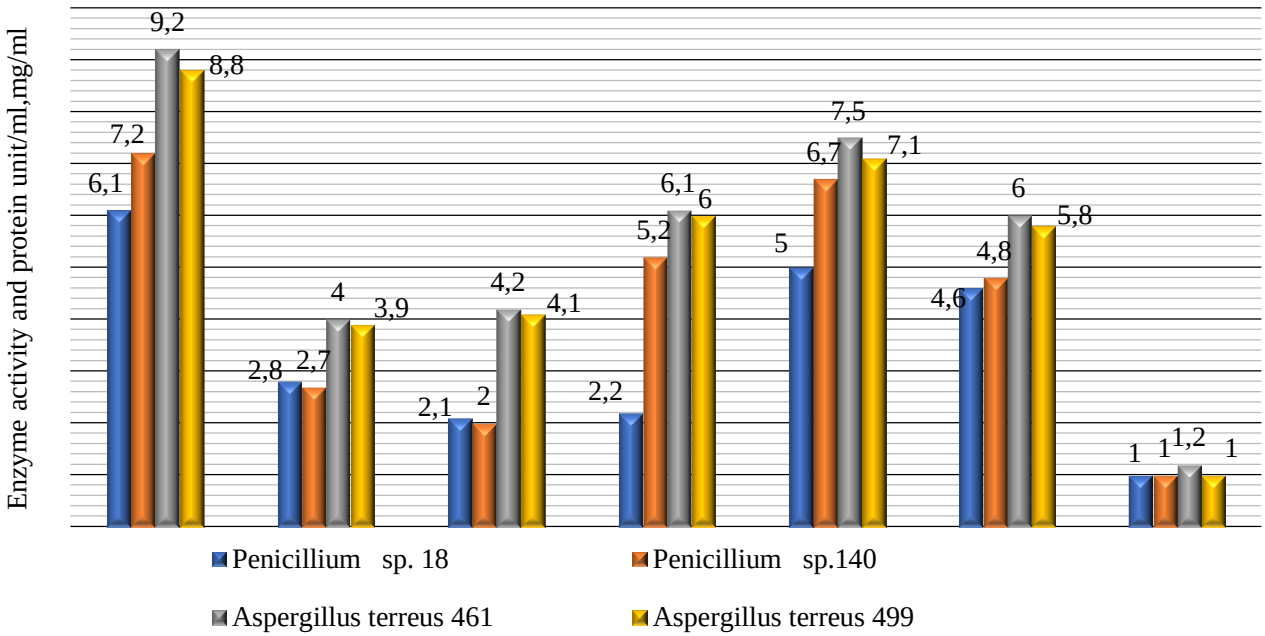



Figure 3. The activity of hydrolytic enzymes and the formation of protein by fungi while submerged cultivation in medium containing microcrystalline cellulose

Table 2.

FORMATION OF PROTEIN IN THE CULTURE LIQUID OF FUNGI, mg/ml

| *Fungi strains/medium* | *Cultivation duration, days* | | | | | |
|---|---|---|---|---|---|---|
| | *3* | *5* | *6* | *7* | *8* | *10* |
| *Penicillium* sp. 18 (chitin) | 0,28 | 0,57 | 1,14 | 2,15 | 2,23 | 3,19 |
| *Penicillium* sp. 18 (cellulose) | 0,32 | 0,49 | 1,27 | 2,18 | 2,16 | 3,13 |
| *Penicillium* sp. 140 (chitin) | 0,42 | 0,73 | 1,09 | 2,11 | 2,07 | 2,47 |
| *Penicillium* sp. 140 (cellulose) | 0,91 | 0,76 | 0,42 | 2,39 | 2,35 | 3,33 |
| *Aspergillus terreus* 461 (chitin) | 0,74 | 0,85 | 0,90 | 2,18 | 2,19 | 2,38 |
| *Aspergillus terreus* 461 (cellulose) | 0,63 | 0,94 | 1,21 | 2,47 | 2,44 | 2,74 |
| *Aspergillus terreus* 499 (chitin) | 0,13 | 0,67 | 0,72 | 2,11 | 2,11 | 3,01 |
| *Aspergillus terreus* 499 (cellulose) | 0,67 | 1,93 | 2,23 | 2,21 | 2,25 | 3,45 |

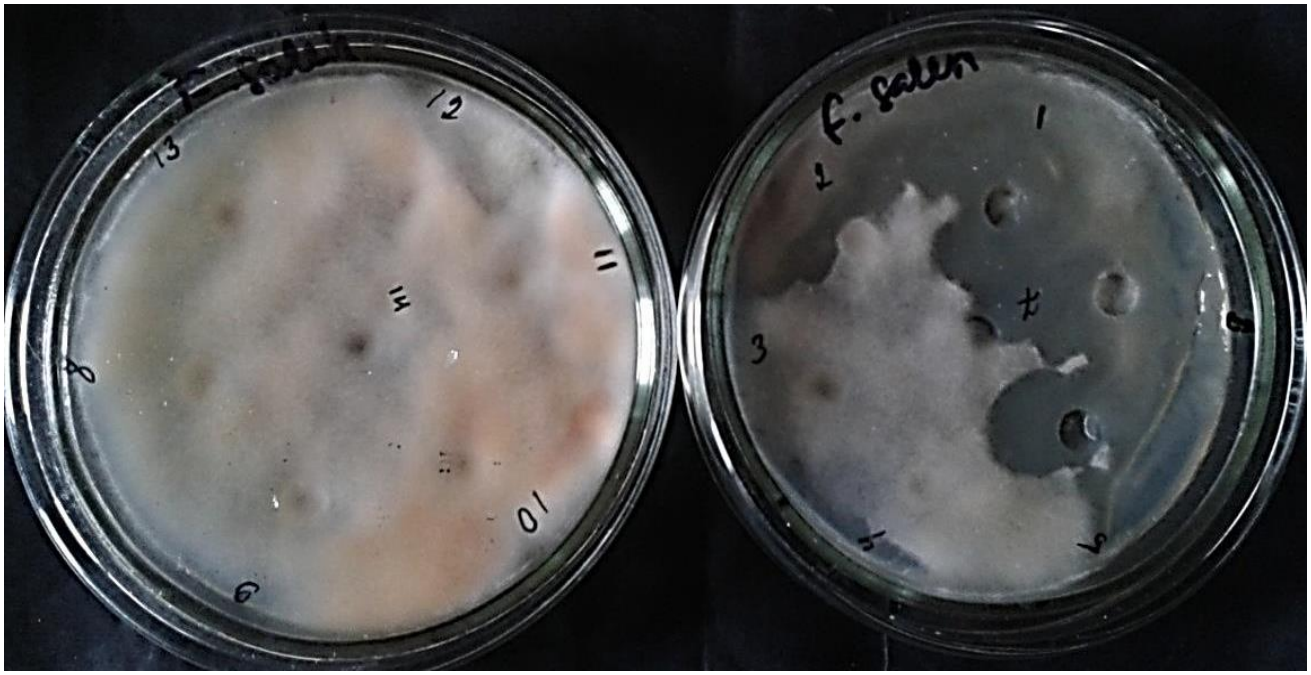
Further, in order to evaluate the effect of hydrolytic enzymes of fungi, the enzymes of culture fluids, as well as hydrolase preparations isolated from culture fluid were tested via laboratory experiments for the growth and development of some phytopathogens (Table 3).

Table 3.

INHIBITING EFFECT OF CULTURE FLUID
OF SELECTED FUNGI ON SOME PHYTOPATHOGENS

| *Culture fluids of fungi* | *Phytopathogens, zone of suppression, mm* | | | |
|---|---|---|---|---|
| | *Rhizoctonia solani* | *Vert. dahliae* | *Fusarium oxysporum* | *Fusarium solani* |
| *Penicillium* sp. 18 | 20–22 | 24–22 | 18–24 | 16–18 |
| *Aspergillus terreus* 461 | 18–20 | 20–19 | 17–16 | 15–16 |
| *Aspergillus terreus* 499 | 18–16 | 20–18 | 15–13 | 14–15 |
| *Penicillium sp.* 140 | 17–18 | 15–17 | 14–16 | 13–15 |

Enzymatically active culture fluids of fungi also had the ability to suppress the growth and development of phytopathogens widely spread in the Republic of Uzbekistan, such as *Rhizoctonia solani* and *Fusarium oxysporum*, *Fusarium solani*, *Verticillium dahliae*, forming phytopathogen's growth inhibition zones in sizes from 20 to 24 mm in diameter.



A B

Figure 4. Phytopathogens of fungi of the genus *Fusarium* (A) after 3 days growth and the effect of culture fluids' mixture of *Penicillium* sp. 18 and *Aspergillus terreus* (B) on the growth of *Fusarium oxysporium* after 12 hours

Further, to evaluate the cooperative fungicidal activity of the hydrolytically active culture fluid of *Penicillium* sp. 18 and *Aspergillus terreus* 461, their influence on the growth and development of the most common phytopathogen *Fusarium oxysporum* in the Republic of Uzbekistan was determined (Figure 4**)**. Thus, a mixture of culture fluid of both fungi was applied pointwise to the surface of lawn of *Fusarium oxysporium* grown on Czapek medium for 3 days. After 12 hours of incubation at 30°C, the growth and development of the phytopathogen was suppressed.

The obtained results on the influence of fungi and their culture fluids on phytopathogens showed that the selected fungi have a suppressive effect.

Thus, the enzymes of the cellulase, hemicellulase, chitinase systems of local strains of mycelial and phytopathogenic, saprotrophic fungi were studied in a comparative aspect. 45 cultures were selected from the studied 151 representatives of microscopic fungi according to the degree of hydrolysis of chitin and microcrystalline cellulose, added to the medium as the only carbon source and growth rate. Then, 8 highly active cultures were selected from 29 cultures belonging to the genera *Fusarium, Aspergillus, Penicillium,* also saprophytes *Ulocladium, Alternaria, Acremonium* with deepening studies of the spectrum of their action.

The selected fungi possessed a high activity of hydrolytic enzymes, the formation of which depended on the polysaccharides used in the nutrient medium, the time of cultivation and the genera and species of fungi.

The obtained data show that selected fungi with high hydrolytic activity are the basis for the creation of highly effective ecologically safe biopreparations of microbial origin for protecting plants from phytopathogens and biotechnologies for cultivating agricultural plants, for bioconversion of plant and industrial waste.

*Conclusion*

The study of the formation of hydrolytic enzymes and proteins in the culture medium of some filamentous fungi showed that the substrates used for their cultivation (chitin and microcrystalline cellulose) added to Czapek synthetic medium as the sole carbon source contributed to the active growth of fungi, accumulation of protein and the activities of cellulolytic and hemicellulase enzymes' complexes (xylanase), chitinase in the medium. The highest enzymatic activity was possessed by *Aspergillus terreus* 461 and *Aspergillus terreus* 499, *Penicillium sp.* 140, *Penicillium* sp. 18 which synthesized enzymes in both chitin and cellulose containing medium. Enzymatically active culture fluids also had the ability to suppress the growth and development of phytopathogens widely spread in the Republic, agents of root rot, fusariosis and cotton leaf witches. The obtained data show that local strains of filamentous fungi belonging to the genera *Penicillium* and *Aspergillus* have the greatest ability to form enzymes that hydrolyse difficult–metabolizable and water–insoluble natural polysaccharides such as chitin and cellulose.

In assessing the cooperative fungicidal activity of hydrolytically active culture fluids of fungi *Penicillium* sp. 18 and *Aspergillus terreus* the suppression of the growth and development of the phytopathogen *Fusarium oxysporium* has been established, which can be used in the preparation of biologics against plant pathogens in the field of plant protection.

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