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CELLULOLYTIC ACTIVITY OF FUNGI ISOLATED FROM BIODETERIORATED POLYMERS OF SPACE TECHNICS

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The cellulolytic activity of 49 strains of micromycetes from genera *Penicillium, Aspergillus, Phoma, Cladosporium, Alternaria, Ulocladium* isolated from biodeteriorated polymeric samples of OC "Mir" has been studied. The biosynthesis of cellulases among the representatives of species of *Ulocladium botrytis, Alternaria alternata, Aspergillus fumigatus, A. versicolor, Penicillium aurantiogriseum, P.chrysogenum, Cladosporium macrocarpum, C.sphaerospermum, Phoma eupyrena* has been revealed during growth on Czapek-Dox medium with carboxymethylcellulose (CMC), amorphous cellulose and filter paper as the single carbon source. The enzymatic preparations of cellulase system of strains *U. botrytis* INMIA 12111 and *A. alternata* INMIA 12115 as active producers of cellulase have been isolated and characterized.

Ուսումնասիրվել է միկրոմիցետների *Penicillium, Aspergillus, Phoma, Cladosporium, Alternaria, Ulocladium* ցեղերի 49 շտամների ցելյուլոլիտիկ ակտիվությունը, անջատված «Միր» տիեզերական կայանի կենսավնասված պոլիմերային նմուշներից: Բացահայտվել է, որ ցելյուլազների կենսասինթեզը բնորոշ է *Ulocladium botrytis, Alternaria alternata, Aspergillus fumigatus, A. versicolor, Penicillium aurantiogriseum, P.chrysogenum, Cladosporium macrocarpum, C.sphaerospermum, Phoma eupyrena* տեսակների ներկայացուցիչներին Չապեկ Դոքս սննդամիջավայրի վրա աճեցնելիս, երբ որպես ածխածնի միակ աղբյուր օգտագործվել են կարբօքսիմեթիլցելյուլոզը (ԿՄՑ), ամորֆ ցելյուլոզը և ֆիլտրի քուղը: Անջատվել և բնութագրվել են *U. botrytis* ԻՆՄԻԱ 12111 և *A. alternata* ԻՆՄԻԱ 12115 շտամների ցելյուլազների ակտիվ արտադրիչների, ցելյուլազային ֆերմենտների պրեպարատները:

Изучена целлюлолитическая активность 49 штаммов микромицетов из родов *Penicillium, Aspergillus, Phoma, Cladosporium, Alternaria, Ulocladium*, выделенных из биоповрежденных образцов полимеров ОК "Мир". Выявлено, что биосинтез целлюлаз характерен для представителей видов *Ulocladium botrytis, Alternaria alternata, Aspergillus fumigatus, A. versicolor, Penicillium aurantiogriseum, P.chrysogenum, Cladosporium macrocarpum, C.sphaerospermum, Phoma eupyrena* при росте на среде Чапека-Докса с использованием в качестве источников углерода карбоксиметилцеллюлозы (КМЦ), аморфной целлюлозы и фильтровальной бумаги. Выделены и охарактеризованы ферментные препараты целлюлазного комплекса из культур *U. botrytis* ИНМИА 12111 и *A. alternata* ИНМИА 12115 – продуцентов целлюлаз.

Cellulases - fungi - cellulolytic activity

Cellulases represent system of enzymes - endoglucanase [1.4-(1.3; 1.4)-β-D glucan 4-glucano-hydrolase (EC 3.2.1.4)], exo-cellobiohydrolase (EC 3.2.1.91), exo-1.4-β-glucosidase (EC 3.2.1.74) and cellobiase (EC 3.2.1.21), hydrolyzing cellulose and cellulosic substrates to glucose [2,6].

Cellulose-hemicellulose system of enzymes is responsible for destruction and treatment of vegetable raw materials, for utilization of wood and agricultural wastes.

Study and search of new producers of cellulases is conditioned by wide application of these enzymes for production of glucose and different glucosides, ethanol, for removing stains from surfaces of cellulosic materials as well as for application of cellulases in complex of cleansing agents, etc [1-3].

The present work reports the results of investigation of the cellulolytic activity of fungi isolated from biodeteriorated polymers of space technics.

Materials and Methods. 49 strains of micromycetes of genera *Penicillium, Aspergillus, Phoma, Cladosporium, Alternaria, Ulocladium* were obtained from Culture Collection of State Microbial Depository Centre, NAS of Armenia. The strains isolated from biodeteriorated polymeric samples of Orbital Complex (OC) "Mir" were represented by species *Ulocladium botrytis* (2 strains), *Alternaria alternata* (1 str.), *Aspergillus fumigatus* (6 str.), *A. versicolor* (4 str.), *Penicillium aurantiogriseum* (21 str.), *P.melinii* (9 str.), *P.chrysogenum* (3 str.), *Cladosporium macrocarpum* (1 str.), *C.sphaerospermum* (1 str.), *Phoma eupyrena* (1str.).

The cellulolytic activity was studied on Hutchinson medium, containing (grams/liter): K_2HPO_4 – 1.0, $CaCl_2$ – 0.1, NaCl – 0.1, $FeSO_4$ – 0.04, $NaNO_3$ – 2.5, pH 7.2 – 7.3. Filter paper was used as single carbon source. 250 ml Erlenmeyer flasks were filled with 30 ml of nutritious medium and were sterilized. The flasks with sterile medium were inoculated with fungal cultures. The filter paper folded conic form separately sterilized was put into flasks and incubated at 28° for 10-14 days.

Presence or lack of fungal growth on surface of filter paper is testified the cellulolytic activity.

Specific enzymatic assays were used to determine the cellulolytic activities: endoglucanase and exo-1,4-β-glucosidase [5,7,9].

Endoglucanase activity: reaction mixture containing 1ml of cultural liquid or a solution of enzyme, 2ml buffer pH 5.0, 50 mg filter paper was incubated at 50°C for 1 h. One unit of endoglucanase activity [1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase (EC-3.2.1.4)] was defined as the amount of the enzyme that produced 1 mg of glucose per h under standard conditions (pH5.0 and 50°).

Exo-1,4-β-glucosidase activity: reaction mixture containing 1ml of cultural liquid or a solution of enzyme, 2 ml of 1% solution Na-CMC in buffer pH 5.0 was incubated at 50° for 15 min. One unit of exo-1,4-β-glucosidase activity [1,4-β-D-glucan-glucohydrolase (EC-3.2.1.74)] was defined as the amount of the enzyme that produced 1 μmol of glucose per min under standard conditions (pH5.0 and 50°).

Reducing sugars were determined by method of Somogyi-Nelson [8].

Production of enzymatic preparations of cellulases: the fungal biomass of strains *U. botrytis* 12111 and A. alternata 12115 was separated by filtration and cell free cultural liquid was used for isolation of cellulases. Partial purification of cellulases was performed by precipitation of ammonium sulfate (40% saturation). The resulting complex was separated by centrifugation at 6000g for 10 min, washed with distilled water and eluted with 0.6 M NaCl solution for 1 h at 30°. The resulting eluate was dialyzed against distilled water at 5° for 24 h.

Concentration of protein was determined by method of Lowry et al [4].

Results and Discussion. Cellulolytic activity of 10 species of micromycetes isolated from biodeteriorated polymeric samples of space technics is represented in Table 1.

The results showed that the active producers were revealed among strains of *Ulocladium botrytis, Alternaria alternata, Aspergillus fumigatus, Penicillium aurantiogriseum, P.chrysogenum, Phoma eupyrena*. The cellulolytic activity is not revealed among strains of *P.melinii*.

Data on study of cellulolytic activity of the active producers of cellulases *U. botrytis* 12111 and *A. alternata* 12115 showed that in comparison with reference

strain the high cellulolytic activity is produced during growth on Czapek-Dox medium with carboxymethylcellulose (CMC), amorphous cellulose and filter paper as the sole carbon source at 28° for 10 days (Table 2).

Table 1. Characterization of cellulolytic activity of different species of micromycetes isolated from biodeteriorated polymeric samples of space technics

Species of fungi	All tested strains cellulolytic activity	Strains possessed of cellulases	Active producers
Alternaria alternata	1	1	1
Aspergillus fumigatus	6	6	6
A. versicolor	4	4	0
Penicillium chrysogenum	3	3	3
Penicillium aurantiogriseum	21	8	3
P. melinii	9	0	0
Cladosporium macrocarpum	1	1	0
C. sphaerospermum	1	1	0
Phoma eupyrena	1	1	1
Ulocladium botrytis	2	2	2

Table 2. Cellulolytic activity of active producers of micromycetes with different cellulosic substrates

Fungal strains	Endoglucanase activity (units/ml cultural liquid)			Exo-1,4-β-glucosidase activity (units/ml cultural liquid)		
	CMC	Amorphous cellulose	Filter paper	CMC	Amorphous cellulose	Filter paper
Alternaria alternata 12145	0.01	0.007	0.005	0.15	0.02	0.03
Ulocladium botrytis 12111	0.04	0.02	0.003	0.2	0.05	0.053
Trichoderma longibrachiatum 10060 (Reference strain)	0.003	0.003	0.003	0.04	0.021	0.02

Czapek-Dox medium, incubation 10 days, 28°, substrates: CMC, amorphous cellulose and filter paper.

The enzyme preparation of cellulase system of *U. botrytis* 12111 with specific activity 0.75 units/mg protein for endoglucanase activity and 2.5 units/mg protein for exo-1,4-β-glucosidase activity and the enzyme preparation of cellulase system of *A. alternata* 12115 with specific activity 0.3 units/mg protein for endoglucanase activity and 2.0 units/mg protein for exo-1,4-β-glucosidase activity was obtained (Tables 3 and 4).

Study of the temperature dependence of the enzymatic preparation of *U. botrytis* INMIA 12111 showed that maximal hydrolysis of the substrate proceeds in the temperature range of 40-60°, the optimum being at 50° (Fig.1).

Study of the pH dependence of the enzymatic preparation of *U. botrytis* 12111 showed that the optimum pH is in two ranges at pH 5.0 and 7.0, maximum at 5.0, although the enzyme is active in wide range of pH at 4.0-8.0 (Fig.2).

Table 3. Characteristics of production of enzymatic preparation of cellulases from *U. botrytis* 12111

Fractions	Total volume, ml	Total protein,mg	Total activity, units		Specific activity, units / mg protein		Degree of purification	
			Endo-glucanase	Exo-1,4-β-glucosidase	Endo-glucanase	Exo-1,4-β-glucosidase	Endo-glucanase	Exo-1,4-β-glucosidase
Cell free filtrate	100	10	0.5	2	0.05	0.2	1	1
$(NH_4)_2SO_4$ precipitation (40% saturation)	2	5.04	3.78	13.6	0.75	2.5	15	13

Table 4. Characteristics of enzymatic preparation of cellulases from *A. alternata* 12115

Fractions	Total volume, ml	Total protein,mg	Total activity, units		Specific activity, units / mg protein		Degree of purification	
			Endo-glucanase	Exo-1,4-β-glucosidase	Endo-glucanase	Exo-1,4-β-glucosidase	Endo-glucanase	Exo-1,4-β-glucosidase
Cell free filtrate	100	7	0.28	0.91	0.04	0.13	1	1
$(NH_4)_2SO_4$ precipitation (40% saturation)	2	4.2	1.26	8.4	0.3	2.0	7.5	15.1

Study of the temperature dependence of the enzymatic preparation of *A. alternata* 12115 showed that maximal hydrolysis of the substrate proceeds in the temperature range of 45-60°, the optimum being at 50° (Fig.3).

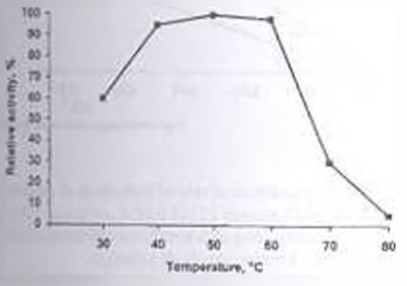

Fig. 1. Influence of temperature on cellulase activity of enzymatic preparation of *U. botrytis* 12111

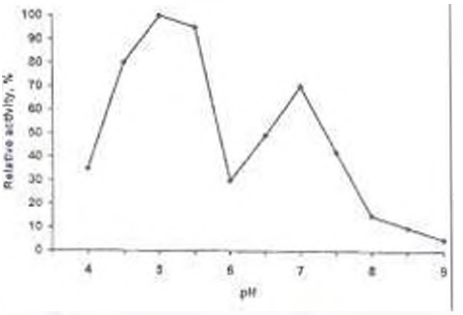

Fig. 2. Influence of pH on cellulase activity of enzymatic preparation of *U. botrytis* 12111

Buffers used: acetate (pH 4-6), phosphate (pH 6-8), glycine – NaOH (pH 8-10).

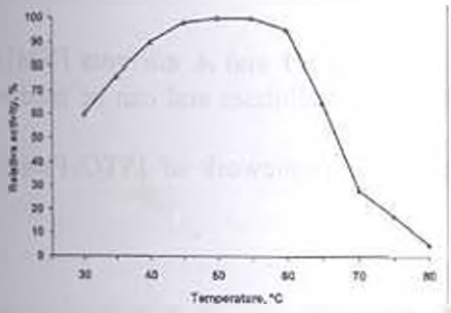

Fig. 3. Influence of temperature on cellulase activity of enzymatic preparation of *A. alternata* 12115

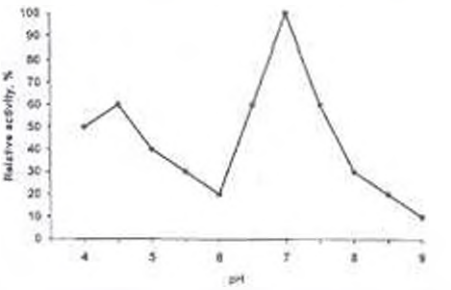

Fig. 4. Influence of pH on cellulase activity of enzymatic preparation of *A. alternata* 12115

Buffers used: acetate (pH 4-6), phosphate (pH 6-8), glycine – NaOH (pH 8-10).

Study of the pH dependence of the enzymatic preparation of *A. alternata* 12115 showed that the optimum pH is in two ranges: at pH 4.5 and 7.0, maximum at 7.0, although the enzyme is active in wide range of pH at 4.0-8.5 (Fig.4).

The kinetics of hydrolysis catalyzed by enzymatic preparations of *U. botrytis* 12111 and *A. alternata* 12115 at various substrate concentrations were studied (according to the Michaelis-Menten and Lineweaver-Berk equations (Fig.5 and Fig. 6).

The constant of Michaelis (Km) at different concentrations of Na-CMC and maximum rate (Vmax) are: for enzymatic preparation of *U. botrytis* 12111 Km = 9.1 mg/ml Na-CMC and Vmax = 0.8 mg/h glucose, for enzymatic preparation of *A. alternata* 12115 Km = 8.3 mg/ml Na-CMC, Vmax = 0.7 mg/h glucose (Fig.5).

The constant of Michaelis (Km) at different concentrations of amorphous cellulose and maximum rate (Vmax) are: for enzymatic preparation of *U. botrytis* INMIA12111 Km = 40 mg/ml amorphous cellulose, Vmax = 0.15 mg/h glucose, for enzymatic preparation of A. alternata INMIA12115 Km = 33.3 mg/ml amorphous cellulose, Vmax = 0.06 mg/h glucose (Fig.6).

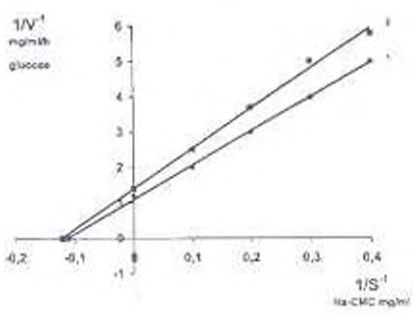

Fig. 5. Dependence of rate of hydrolysis of enzymatic preparations of *U. botrytis* 12111 and *A. alternata* 12115 at different concentrations of Na-CMC substrate according to the Lineweaver-Burk equation.

Enzymatic reaction conditions: substrate Na-CMC, pH 5.0, 50°, concentration of protein about 0.2 mg/ml.

1. Enzymatic preparation of U. botrytis 12111,

2. Enzymatic preparation of A. alternata 12115.

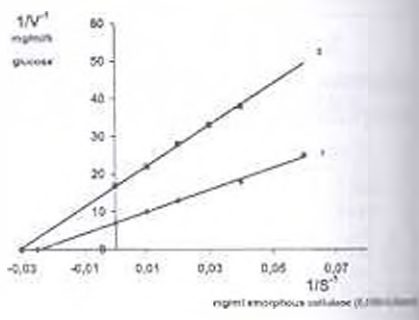

Fig. 6. Dependence of rate of hydrolysis of enzymatic preparations of *U. botrytis* 12111 and *A. alternata* 12115 at different concentrations of amorphous cellulose substrate according to the Lineweaver-Burk equation.

Enzymatic reaction conditions: substrate amorphous cellulose (MN-300, 0.002-0.02 mm, Serva), pH 5.0, 50°, concentration of protein about 0.2 mg/ml.

1. Enzymatic preparation of U. botrytis 12111,

2. Enzymatic preparation of A. alternata 12115.

Thus, the strains of *U. botrytis* INMIA 12111 and *A. alternata* INMIA 12115 are characterized by unique complex of cellulases and can be used in biotechnological processes.

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REFERENCES

1. *Ankudimova N.V., Baraznenok V.A., Bekker E.G., Okunev O.N.* Biokhimiya, *64*, 9, 1267-1273, 1999 (in russ.).

2. *Kastelyanos O.F., Ermolova O.V., Sinitsin A.P., Popova N.N., Okunev O.N.*

Kerns G., Kude E. Biokhimiya, *60*, 6, 925-943, 1995 (in russ.).
3. *Kastelyanos O., Sinitsin A.P., Ermolova O.V., Popova N.N., Guterres B., Antonova V.A., Berlin A., Kerns G.* Biokhimiya, *60*, 10, 1609-1617, 1995 (in russ.).
4. *Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J.* J.Biol.Chem., *193*, 265-280, 1951.
5. *Mandels M., Hontz L., Nystron J.* Biotechnol. Bioeng., *16*, 11, 1471-1493, 1974.
6. *Rabinovich M.L., Melnik M.S., Bolobova A.V.* Biokhimiya, *67*, 8, 1026-1050, 2002 (in russ.).
7. *Rukhlyadeva A.P., Poligalina G.V.* Methods for determination of activities of hydrolytic enzymes, M., 287, 1981 (in russ.).
8. *Somogyi M.A.* J. Biol. Chem., *195*, 1, 19-23, 1952 (in russ.).
9. *Velikaya E.I., Sukhadol V.I.* Laboratorial practics on course of general technologies of fermentative industries, M., 310, 1983 (in russ.).

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