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NEW STRAINS OF BASIDIOMYCETES FOR INDUSTRIAL CONVERSION OF LIGNOCELLULOSIC MATERIALS

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Dreval K. G., Boyko M. I. **New strains of basidiomycetes for industrial conversion of lignocellulosic materials. –** Here we characterize newly isolated basidiomycetes strains and describe biotechnological procedure of purification of lignocellulolytic enzyme complex from their liquid culture. Among 61 basidiomycetes strains spanning over 18 species of 17 generawe selected and explored 4 new cellulase-producing strains of Basidiomycetes. For the latter, determined optimal conditions for synthesis of cellulases (temperature and initial pH of medium), the dynamics of cellulolytic enzyme activity in the culture filtrates, optimal composition of the culture medium on sources of nitrogen and carbon.

As a result, we developed a method of purifying enzymatic preparations of cellulases from the cultural medium of basidiomycetes. A fundamentally new to this method is the elution of cellulases from the liquid medium of basidiomycetes. Applying our method allows purification of the enzymatic preparations with a high degree of purification within 3 stages (salting out the proteins, dialysis, and gel chromatography). With this method of obtaining cellulase preparations, we obtained original products of basidiomycetes strains Irpex lacteus K-1, A-Дон-02, Д-1 and Daedaleopsis confragosa f. confragosa AnSc-1. Further, we compared some basic physical and chemical properties (pH- and thermolability, pH- and thermostability) and associated enzymatic activities of cellulases derived from the cultural liquids of basidiomycetes with the lower fungi's one both commercial and laboratory origin was conducted.

Thus we proved that cellulolytic enzymes from basidiomycetes are more active than those from lower fungi. The cellulases from both basidiomycetes and lower fungi were found to exhibit a number of associated enzymatic activities, but cellulolytic enzymes from basidiomycetes have significantly higher activity of enzymes that act on starch, pectin and lignin.

Key words: lignocellulose bioutilisation, cellulases, ligninases, endoglucanases, cellobiases, basidiomycetes, plant biomass conversion.

Introduction

A persistently growing need for fossil resources, as well as a number of environmental problems caused a significant increase in interest in the world of science to the production of fuels and various chemical products from renewable sources using biotechnological processes [20, 24, 26]. Development of technologies exploiting enzymes that hydrolyze cellulose, can lead to the development of environmentally friendly means of production, thus reducing technogenic load on the environment [19]. One of the most promising technologies exploiting cellulases [8, 11] is processing of vegetable raw materials (including waste) to obtain clean biofuel [1, 7, 23] that is particularly relevant in current conditions in Ukraine. A further development of the technology of fuel ethanol production from plant biomass and its widespread adoption in industry has a significant economic value [31] and can be considered as one of the factors to ensure energy independence of Ukraine [9]. Transformation of raw materials containing cellulose is promising not only from the point of view creation of independent technologies but also from the point of view of the reduction of environmental risk of some enterprises which process vegetable raw materials [16]. The main prerequisite for the development of biotechnology-based industry of lignocellulose materials is finding bacteria or fungi that are capable of hydrolysing materials such as wood pulp, bagasse and other waste products, as well as understanding of the processes underlying the degradation of lignocelluloses by these organisms [20, 32]. It is out of doubt that the wood-destroying basidiomycetes play a significant role in decomposition of lignocelluloses [10, 29]. In the last decade a growing number of studies on the basidiomecetous fungi addressed their properties as producers of biologically active substances, including wood-destroying enzymes [2]. The potential of different strains to use cellulose or lignin varies [4, 15]. Moreover, the lack of highly active and economically effective producers is one of the main restrictions on the industrial exploitation of enzymes that capable to hydrolyze lignocellulose [6, 28, 30].

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The aim of this study was to find new strains of basidiomycetes for industrial conversion of lignocellulosic materials, to study their physiological and biochemical features, their ability to utilize lignocellulosic wastes, to purify their enzymatic preparations and to compare them with commercial one.

Materials and methods

Basidiomycetes were cultivated on the base of Čapek 's nutrient medium. We analyzed the cellulolytic activity of the cultural liquid of 61 strains of Basidiomycetes from 17 genera: *Schizophyllum, Trichaptum, Irpex, Fomes, Trametes* (=*Coriolus*), *Pleurotus, Daedaleopsis, Lepista, Inonotus, Stereum, Heterobasidion, Auricularia, Chondrostereum, Phellinus, Hirschioporus,* and *Flammulina*. We optimized the initial acidity of the medium and the cultivation temperature for the maximal production rate of cellulases. During the screening, the strains were cultivated at the temperatures optimal for the growth and on the medium with initial pH 5.0. During the optimization,the strains were cultivated at the temperatures from 24°C to 36°C on the medium with initial pH ranging from 3.0 to 9.0. Precipitation of proteins from the cultural liquid was performed by salting. The protein precipitate was dialyzed against cold (+6±1°C) distilled water. The protein solutions were subjected to gel chromatography on Sephadex G-75 (Sigma, Germany) columns.

For the measurement of the cellulolytic activities of the cultural liquids of the strains, we used a panel of substrates, namely, the filter paper (filter paper activity, FPA), Na-carboxymethylcellulose, hydroxyethylcellulose (endoglucanase activity), and cellobiose (cellobiase activity) solutions. One unit of enzyme activity was defined as the amount of enzyme, releasing 1 μmol of reducing sugars (for polymer substrates) or 1 μmol of glucose (for cellobiose) per minute. As substrates for the measurement of the lignolytic activities, we used Remazol brilliant blue R (general lignolytic activity, GLA), syringaldazine, guaiacol, and pyrocatechol (laccase activity, LcS, LcG, LcP). Pectinolytic activities were determined as ability to act on apple pectin by viscometric (endopolygalacturonase activity, EPG) and iodometric methods (pectinesterase activity). The composition of the reaction mixtures and conditions were set up according to IUPAC recommendations [22] and generally accepted methods [1, 12, 18, 25, 27].

In all assays, the release of reducing sugars was measured with the Shomogui-Nelson technique (glucose standard curve were used) [12]. The glucose concentration was measured using glocoseoxidase-peroxidase method according to the manufacturer's protocol (Dnipropetrovsk, Ukraine). Protein concentration was assayed spectrophotometrically on the SF46 supplier (Russia) [1]. The specific activity (U/mg protein) was calculated as general activity to protein concentration ratio.

The enzymatic preparations were purified according to standard techniques for enzyme purification, modified for cullulases of basidiomycetes. As a reference for the Basidiomycetous preparations we used following preparations: «Xybeten-Xi» and «Xybeten-Cel» (JSC «Biovet», Bulgaria), kindly given us by Prof. Dr. A. Sinitsyn (Lomonosov Moscow State University, Moscow, Russia), «Celluclast 1,5L» («Sigma», Germany) and laboratory preparation *Penicillium* sp., kindly given us by Dr. María Jesús Martínez (Centro de Investigaciones Biológicas, Madrid, Spain) and «Cellulase» (Ladyzhin enzymes factory, Ukraine).

All assays were performed at least three times. The data obtained were subject to ANOVA; comparison of arithmetic averages was conducted using Duncan method [13].

Results and discussion

Basidiomycetes are active producers of cellulases and can be perspective objects for the biotechnology of cellulolytic enzymes. It was found, that cellulases of strains *Irpex lacteus* (Fr.) Fr. К-1, А-Дон-02 and Д-1 and *Daedaleopsis confragosa* f. confragosa (Bolton) J. Schröt. AnSc-1 have the highest FPA between researched cultures of Basidiomycetes (fig. 1). The difference in activity of cellulases and in composition of cellulase complex between different strains of one species of basidiomycetous fungi was established.

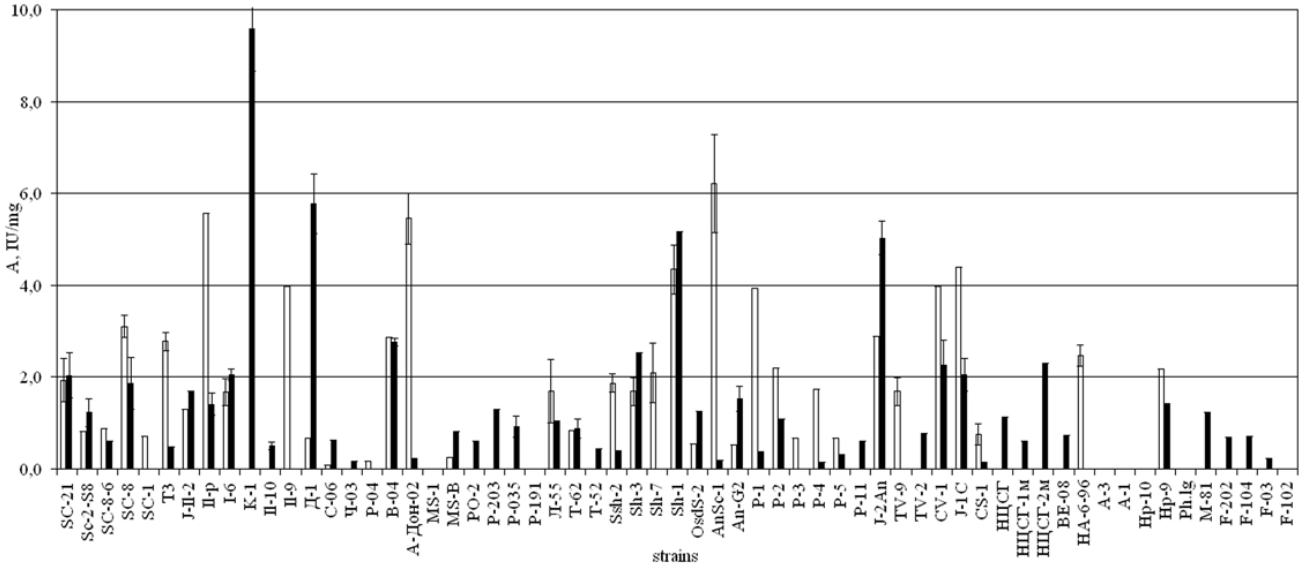

Fig. 1. Filter paper activity of basidiomycetes on 7th (□) and 14th (■) days of cultivation.

Selected strains show high endoglucanase and cellobiase activities. Additionally, the strains mentioned showed activity of lignin- and pectin-degrading enzymes (Table 1). It should be mentioned that activities of ligninases in their cultural liquids were significantly higher on the 7th day of cultivation compared to the 14th day. This fact means that selected strains are more promising as far as low time of enzyme synthesis is one of the most important parameter for biotechnological promissing organisms.

Afterselecting the best producers of cellulases, we induced the synthesis of the cellulases and tried to intensify their products to the culture medium. As the result of optimization the values of the total cellulolytic activity increased by 16-159%, and the specific one increased by 9-143% dependent on the strain. The optimal initial acidity of the medium for production of the cellulolytic enzymes was found to be pH 7.0 for all strains. The optimal cultivation temperature for strains *I. lacteus* К-1, А-Дон-02 and Д-1 is 34°С, and 32°С for the strain *D. confragosa* f. confragosa AnSc-1. On the 7th day of cultivation, the FPA of the strain K-1 displays a sharp peak (t=34 °С, pH 7.0), which is shifted (t=32°С, pH 5.0) by 14th day of the experiment (fig. 2).

Table 1

Activities of wood-destroying enzymes in cultural liquids of the studied basidiomycetes strains, U/mg protein

Strain	Enzymatic activity				
	EPG	GLA	LcS	LcG	LcP
7th day of cultivation					
Д-1	0,3	193,2	2,9	4166,7	60,1
А-Дон-02	0,5	59,7	1,0	630,3	55,6
AnSc-1	0,9	237,2	3,4	649,4	24,6
К-1	0,3	91,5	1,8	1253,1	19,0
14th day of cultivation					
Д-1	0,4	123,3	0,7	759,9	23,0
А-Дон-02	0,9	59,7	0,1	315,1	23,8
AnSc-1	0,1	50,4	0,1	155,3	11,8
К-1	0,3	59,5	0,1	549,5	27,7

Note. Mean are given in table, standart errors are less then 5%; $p<0,05$.

Fig. 2. Filter paper activity of strain *Irpex lacteus* K-1 on 7th (*a*) and 14th (*b*) days of cultivation depending from initial acidity of nutrient medium and temperature of cultivation.

Presented in table 2 received results are shows that enzymatic preparations Д-1 and К-1 have the highest activity of cellulase complex components but are uncapable to hydrolyze filter paper. Regarding that this activity has their initial cultural liquids it can be suggested that some linking component was removed during purifying process or its autolysis occurs.

Researching of pH influence on endoglucanase and cellobiase activities of basidiomycetes and lower fungi enzymatic preparations showed that optimum of endoglucanase action is varying between pH 4 (preparations А-Дон-02, Д-1 and К-1, synthesized by basidiomycetous fungi) and pH 5 (another enzymatic preparations), which agrees with literature data [14]. High pH values has negative effect on endoglucanase activity both basidiomycetes and lower fungi. At the same time, maximal cellobiase activity is in higher values of reaction mixture pH. Cellobiase in preparations «Xybeten-Xyl», «Xybeten-Cel», «Cellulase», Celluclast 1,5L, А-Дон-02, Д-1 and AnSc-1 have maximal activity at pH 5, and in preparations *Penicillium crude* and К-1 – at pH 6.

It was established, that endoglucanase in preparation Celluclast 1,5L shows maximal activity at temperature 40 °C, in preparations «Xybeten-Xyl», «Xybeten-Cel» and Д-1 – at 45°C, in preparations «Cellulase», А-Дон-02, К-1 and AnSc-1 – at 50°C, and in preparation *Penicillium crude* – at 55°C. Endoglucanases in all preparations did not inactivate at 50% under reaction temperatures 30°C or 80°C.

Table 2

Activity of cellulase complex of enzymatic preparations, U/mg protein

Preparation	Producer	Protein, mg/ml	Substrate		
			FP	Na-CMC	Cellobiose
Xybeten-Xyl	*Trichoderma longibrachiatum*	0,38	19,5	276,1	617,6
Xybeten-Cel		0,54	18,0	224,8	506,5
Cellulase	*Trichoderma viride*	0,20	9,9	455,9	301,6
Penicillium crude	*Penicillium sp.*	0,26	17,2	353,1	303,9
Celluclast 1,5L	*Trichoderma reesei*	0,24	76,0	534,8	406,8
А-Дон-02	*Irpex lacteus*	0,04	4,19	590,0	912,1
Д-1		0,03	0	1030,0	1531,9
К-1		0,03	0	1327,9	1807,2
AnSc-1	*Daedaleopsis confragosa f. confragosa*	0,16	5,6	174,9	481,53

Note. FP – filter paper, Na-CMC – Na-carboxymethyl cellulose; the mean value are given in table, errors are less then 5%; $p<0,05$.

Cellobiase optimum varied in a broader temperature range. Activity of this enzyme in preparations «Xybeten-Xyl», «Xybeten-Cel», «Cellulase» and Д-1 were maximal at 40°C; in preparations *Penicillium crude*, Celluclast 1,5L and AnSc-1 it was at 45°C, in preparation К-1 at

55°C, and in preparation А-Дон-02 at 60°C. Thus the optimal temperatures of enzymes produced by basidiomycetes lied in higher temperatures what might allow intensification of industrial processes of their exploitation.

The associated activities are known to be the important characteristics of cellulase preparations [1, 6, 17, 21]. It was established that researched preparations are capable to hydrolyze related to cellulose compounds such as lignin, pectin and starch (Table 3). It should be noted that basidiomycetous preparations showed higher lignolytic activity than preparations from lower fungi. Moreover, activity of cellulolytic preparations from basidiomycetes showed significantly higher activity of pectin and starch convertion. One can argue that enzymatic preparations from basidiomycetous fungi are more prospective for using in biotechnologies, where complex conversion of plant substances is required [16].

Thus we have shown that the best cellulolytic preparation is the А-Дон-02, synthesized by basidiomycetous fungi *Irpex lacteus*, as it contain the most stable endoglucanase and cellobiase which have the least lose of activity depending from holding time at optimal temperature at pH. The study of the associated activities showed that preparations from both basidiomycetes and lower fungi exhibit a number of associated enzymatic activities, but cellulolytic enzymes from basidiomycetes have significantly higher activity of enzymes that hydrolyse lignin, starch and pectin.

In order to study the ability of this preparation for lignocellulose conversion and measuring its prospectives in industrial conversion of lignocellulosic materials was measured transformation of several plant wastes by А-Дон-02 preparation. As one can see on the fig. 3, theconversion of different lignocellulosic materials is rising after 24 hours of incubation comparing with 0,5 h with almost all kinds of lignocellulosic wastes are being degraded by new strain of basidiomycetes.

Table 3

Associated activities of basidiomycete and lower fungus cellulases preparations (U/mg protein)

Preparation	Enzymatic activity											
	General lignolytic activity	Laccase (EC 1.10.3.2) (substrate – syringaldasine)	Laccase (EC 1.10.3.2) (substrate – guaiacol)	Laccase (EC 1.10.3.2) (substrate – pyrocatechol)	Endoglucanase (EC 3.2.1.4) (substrate – HEC)	Pectynesterase (EC 3.1.1.11)	Endopolygalacturonase (EC 3.2.1.1)	Maltase (EC 3.2.1.20)	Invertase (EC 3.2.1.26)	α-Amylase (EC 3.2.1.1)	β-Amylase (EC 3.2.1.2)	β-Galactosidase (EC 3.2.1.23)
Celluclast 1,5L	24,2	4,1	5654,8	0,0	81,8	0,0	0,0	1,6	0,0	0,0	0,1	0,0
Penicillium crude	111,5	2,7	5769,2	0,0	7,1	0,0	12,0	1,5	9,0	0,0	0,0	0,0
Cellulase	0,0	1,5	4642,9	27,0	31,4	0,0	0,0	2,9	11,8	0,0	0,1	0,5
Xybeten-Xyl	15,3	8,1	2255,6	28,4	22,4	0,7	19,1	1	8,7	0,0	0,0	0,0
Xybeten-Cel	0,0	2,7	0,0	0,0	28,1	0,2	12,8	0,7	4,4	0,0	0,0	0,0
А-Дон-02	724,6	171,5	23214,3	0,0	643,3	5,0	233,6	14,7	58,8	0,5	0,4	2,4
Д-1	386,5	65,6	40476,2	360,4	80,0	4,4	1049,6	19,6	47,1	0,4	0,1	16,2
К-1	772,9	137,4	47619,0	540,5	431,9	8,9	853,5	26,1	62,7	0,3	0,4	9,7
AnSc-1	72,5	1,0	8928,6	33,8	42,8	2,1	36,8	4,9	29,4	0,1	0,1	1,5

Note:

1) protein concentrations are given in table 1;

2) mean values are given in the table, errors are less then 5%; $p<0,05$.

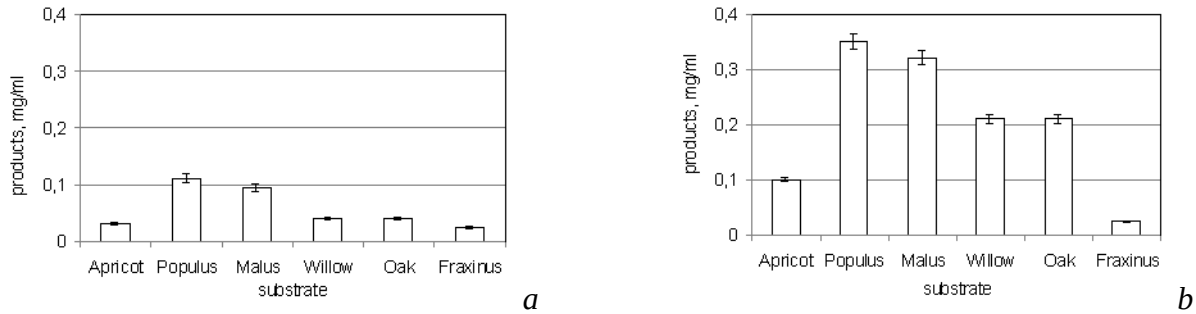

Fig. 3. Degradation of lignocellulosic substrates after 0,5 (*a*) and 24 h (*b*) by preparation of a new strain А-Дон-02 of *Irpex lacteus.*

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Древаль К. Г., Бойко М. И. **Новые штаммы базидиомицетов для промышленной конверсии лигноцеллюлозных материалов.** – В данной статье приводится характеристика новых штаммов базидиомицетов и описывается процедура биотехнологии получения ферментных препаратов лигноцеллюлазного действия из их культуральной жидкости. Среди 61 штамма базидиомицетов, которые относятся к 18 видам 17 родов, отобраны и изучены 4 новых культуры, способные к активному синтезу целлюлозолитических ферментов. Для полученных штаммов определены оптимальные условия культивирования. Разработан способ получения ферментных препаратов целлюлаз из культуральной среды базидиомицетов.

Ключевые слова: биоутилизация лигноцеллюлозы, целлюлазы, лигниназы, эндоглюканаза, целлобиаза, базидиомицеты, конверсия растительного сырья.

Древаль К. Г., Бойко М. І. **Нові штами базидіоміцетів для промислової конверсії лігноцелюлозних матеріалів.** – У поданій статті наводиться характеристика нових штамів базидіоміцетів та описується процедура біотехнології отримання ферментних препаратів лігноцелюлозної дії з їх культуральної рідини. Серед 61 штаму базидіоміцетів, які відносяться до 18 видів 17 родів, відібрано та вивчено нові культури, здатні до активного синтезу целюлозолітичних ферментів. Для отриманих штамів визначено оптимальні умови культивування. Розроблено спосіб отримання ферментних препаратів целюлаз із культурального середовища базидіоміцетів.

Ключові слова: біоутилізація лігноцелюлози, целюлози, лігніназа, ендоглюканаза, целобіоза, базидіоміцети, конверсія рослинної сировини.

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