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EXTRACELLULAR CELLULOLYTIC COMPLEXES PRODUCTION BY MICROSCOPIC FUNGI

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The aim of this work was to screen and to study the effect of inducers on the synthesis of the cellulolytic enzyme complexes by microscopic fungi. Cellulolytic and xylanolytic activities were determined by reducing sugar with DNS reagent, and β-glucosidase activity by pNPG hydrolysis. The enzyme preparations were obtained by ammonium sulphate precipitation. Among 32 studied strains of microscopic fungi 14 produced cellulo- and xylanolytic enzyme complexes. *Fusarium* sp. 5 and *Fennellia* sp. 2806 demonstrated the highest levels of all studied enzyme activities. Enzyme preparations with high endo-, exoglucanase, xylanase and β-glucosidase activities were obtained from these strains. *Fusarium* sp. 5 and *Fennellia* sp. 2806 were active producers of cellulase enzyme complexes during growth on natural substrates. It was shown that inductors of cellulolytic enzymes in *Fusarium* sp. 5 and *Fennellia* sp. 2806 differed from the ones in *Trichoderma reesei*.

Key words: microscopic fungi, endoglucanase, exoglucanase, xylanase, β-glucosidase.

Cellulases are widely used in the pulp and paper industry, textile industry and in fermentation of plant biomass for bioethanol production. Plant biomass, mainly represented by polymers of cell walls: cellulose and hemicellulose, is the most common source of food raw materials, fuels and chemical substances. Degradation of cellulose occurs due to the action of hydrolytic enzymes consisting mainly of cellulases and xylanases, and requires some additional components. Endoglucanases (EC 3.2.1.4) randomly hydrolyse internal glycoside bonds of the amorphous regions of cellulose, forming new ends in cellulose chains for the action of cellobiohydrolases. Exoglucanases or cellobiohydrolases (EC 3.2.1.91) split from two to four units from the ends of the chains, as a result forming tetra- or disaccharides, including cellobiose. Two exoglucanase types are classified: the first type preferentially hydrolyses cellulose from reducing end, while the second type from non-reducing one. β-Glucosidases or cellobiases (EC 3.2.1.21) degrade products of the exoglucanase hydrolysis to glucose. Xylanases (EC 3.2.1.8) hydrolyse hemicellulose, that is the second most abundant natural polysaccharide after cellulose, comprising mainly xylan, to xylose [1, 2].

In the last decade a large number of fungi — producers of cellulolytic enzymes were isolated and completely studied. They are the main sources of enzyme preparations of cellulases and xylanases [3]. Development of recombinant strains and enzyme products requires further improvement at the level of single activities, as well as in multi complexes for enhancement the enzyme synthesis and level of substrate hydrolysis [4]. Production of cellulolytic enzyme preparations is still quite expensive stage in the conversion technologies of lignocellulosic biomass. However, only a few articles on the study of mechanisms of induction and repression of these enzymes under cultivation of producers on complex lignocellulosic substrates were published [5].

The addition of cellulose in the culture medium induces the cellulase secretion by the most of studied fungi, in particular species

of *Trichoderma*, *Aspergillus* and *Penicillium* genera. The mechanism of the induction of cellulolytic enzyme synthesis by insoluble crystalline cellulose was developed only at the level of discussion models. Such soluble sugars, as cellobiose, sophrose, lactose, sorbose and galactose are able to induce the cellulase synthesis in *Trichoderma reesei*. Hemicellulases are induced by hemicellulose polymers, respectively. Xylans are the most effective inducers of xylanase synthesis. However, the peculiarities of induction of cellulases and hemicellulases in transformation process of complex natural substrates were not studied enough [6].

The cheap natural substrates are the major advantage of technology with using micromycetes. For selection of culture medium components should be considered need not only the growth of fungi, but also induction of the processes of expression and secretion of cellulolytic enzymes. In the most cases, hydrolysis efficiency of lignocellulosic materials is caused by the specific characteristics of the substrates, especially natural, but not activity of complex preparations [7]. The presence of a wide spectrum of hydrolytic complexes of wild fungal strains and the synergy actions of different types of cellulases and xylanases in the process of enzymatic hydrolysis makes prospective studies of the wild strains with the ability to synthesize cellulase and xylanase complex [8].

The aim of this work was to carry out a screening and to study the effect of inducers on the synthesis of the cellulolytic enzyme complex by microscopic fungi.

Materials and Methods

As the objects of the study were used 32 strains from the culture collection of Institute of Microbiology and Virology NASU and 18 recently isolated strains of micromycetes belonging to genera: *Botrytis* (1), *Ceratocystis* (5), *Fennellia* (2), *Fusarium* (4), *Mucor* (1), *Penicillium* (4), *Rhizopus* (5), *Trichoderma* (10). The 19 strains were isolated using the traps method and from natural cellulose-containing substrates [9].

Inoculum was grown on stubble potato-glucose agar in tubes for 10–14 days at 24±2 °C. The cultivation of fungi was carried out under submerged conditions (210–230 rpm, t° 22–24 °C) in Erlenmeyer flasks (750 ml volume capacity) with 200 ml of liquid nutrient medium, g/l: $NaNO_3$ — 2, KH_2PO_4 — 1, KCl — 0.5, $MgSO_4$ — 0.5, $FeSO_4$ — 0.01 with addition of different carbon sources. Pre-selection cultivation of strains capable to synthesize enzymes of cellulolytic complex was carried out in a nutrient medium with 5 g/l of filter paper (FP) as a single carbon source. Assay of endoglucanase activity in the culture filtrate on 6^{th} day by viscometric method was analyzed [10]. For study of the induction of cellulolytic enzyme synthesis by fungi were used lactose, arabinose and sorbitol (5 g/l) with the addition of FP and Na-CMC (Sigma), and wheat straw (5 g/l) without FP. Cultivation of fungal strains was carried out under submerged conditions during 7 days. Sampling and determination of enzymatic activities were monitored daily.

Endoglucanase activity was determined by the reduction of viscosity of a 0.5% Na-CMC solution at a temperature of 50 °C after 10 min incubation with 1 ml of culture filtrate using Oswald's viscometer (d = 0.9 mm). The activity was assayed according to the relevant recommendations [10, 11]. Endoglucanase activity in enzyme preparations was estimated after 30 min incubation with 2.0% solution of Na-CMC at 50 °C [12], and exoglucanase activity was determined by the hydrolysis of filter paper (FPU). The reaction mixture contained 1 ml of culture filtrate and 0.05M citrate buffer (pH 4.5) with of 50 mg of FP. The incubation time was 1 h at 50 °C. Xylanase activity was determined by hydrolysis of 1% beechwood xylan solution (Sigma) in 0.05 M citrate buffer (pH 4.5) at 50 °C for 5 min [10]. Reducing substances were estimated using DNS method [13], the protein content by the method of Bradford [14].

The appropriate amount of buffer in the control was added instead of culture filtrate or respectively diluted partially purified enzyme preparation. All samples of the culture filtrates were measured in two ways: positive sample contained culture filtrate with native enzyme complex, and negative sample with enzymes inactivated by 10 min boiling. The enzymatic activity was determined as the difference between positive and negative samples.

As a unit of endo-, exoglucanase and xylanase activity was defined the amount of enzyme that formed 1 μmol of glucose or xylose, respectively, under specified conditions per 1 min per 1 ml of culture filtrate or 1 mg of protein.

β-Glucosidase activity was determined spectrophotometrically by the formation of *p*-nitrophenol in the hydrolysis of 10 mM

solution of *p*-nitrophenyl-β-D-glucopyranoside (pNPG, Sigma) for 30 min at 50 °C [15].

Partially purified enzyme preparations of *Fusarium* sp. 5 and *Fennellia* sp. 2806 were obtained by ammonium sulphate precipitation (85% saturation). The supernatant was carefully decanted, centrifuged for 10 min at 3000 rpm. The precipitate was dissolved in 50 mM acetate buffer (pH 4.7) and centrifuged for 10 min at 8000 rpm. The supernatant was used for assay of enzymatic activity.

The experiments were performed in triplicate. The results were carried out and statistically analyzed using the software Microsoft Excel. All tables and figures contain the average values and their standard deviations.

Results and Discussion

The preliminary stage of screening by the ability of micromycetes to grow on the FP and by the formation of clearing zones on a solid nutrient medium with Na-CMC was conducted. The 32 strains of micromycetes were selected; they were able to synthesize extracellular cellulolytic enzymes [16]. It was found that 21 among these strains showed endoglucanase activity under the specified conditions (Fig. 1), but for half of them the level of this activity did not exceed 5.0 U/ml. For further study were selected six strains with activity higher than 8.0 U/ml: *Fennellia* sp. 2806, of *Fusarium* sp. (strains 5, 25, 420), *Penicillium* sp. 2729 and *Trichoderma* sp. 17/1. The three the most active ones from different genera of microscopic fungi: *Fennellia* sp. 2806, *Fusarium* sp. 5 and *Trichoderma* sp. 17/1 among six strains were selected for further study of the peculiarities of the induction of cellulolytic enzyme complex.

Thus, as a result of a two-stage screening, six strains of micromycetes were selected; they are able to produce extracellular endoglucanase.

Two types of inducer of the cellulolytic enzyme synthesis in the main world producer of cellulases *Trichoderma reesei* were identified: specific (polymer substrates of sugars containing β-1,4-glycosidic bonds — straw, FP, Na-CMC) and nonspecific (lactose, sorbitol, arabinose) [17].

It was found that the cellulase activity was usually detected in medium in the presence of specific inducers. Addition of nonspecific inducers to the culture medium did not cause synthesis activation of the cellulolytic enzymes in studied fungi. However, addition of these inductors in the nutrient medium with FP repressed synthesis of cellulases almost completely. Unlike *Trichoderma reesei*, such mechanisms were observed for cellulases of *Penicillium echinulatum* 9A02S1, but with using lactose as a single carbon source [18].

The study of the dynamics of endoglucanase activity in the presence of specific inducers in nutrient medium was

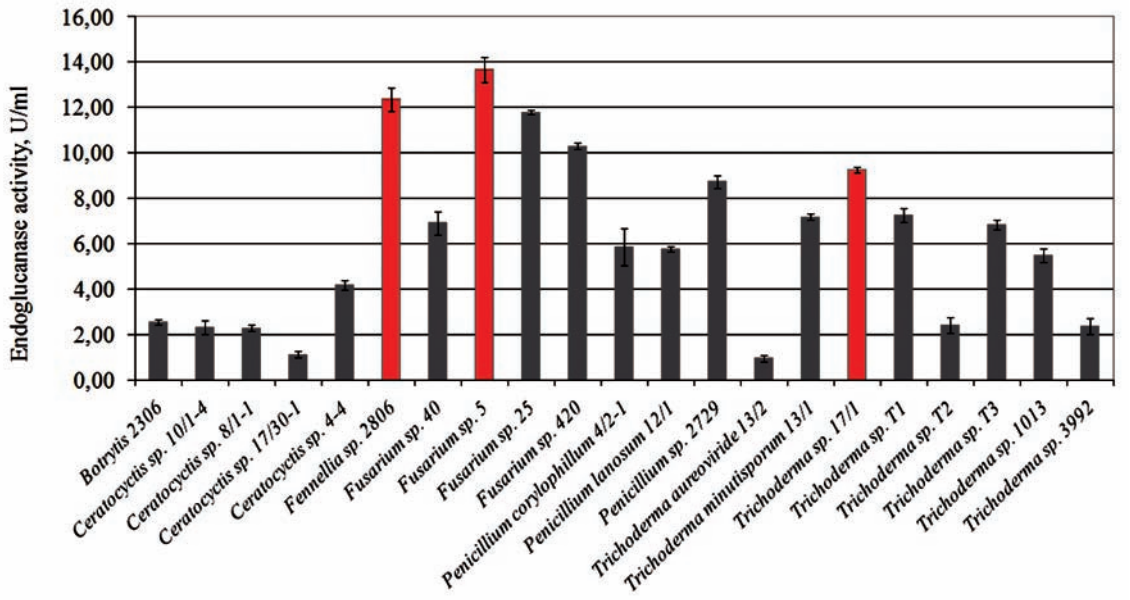

Fig. 1. **Endoglucanase activity of microscopic fungi ($P \leq 0.05$)**

carried out. It was shown that endoglucanase activity of studied fungi was the highest in the medium with wheat straw and FP (except *Fennellia* sp. 2806) and the lowest — with Na-CMC. We did not observe significant differences between *Fusarium* sp. 5 and *Fennellia* sp. 2806 strains in nutrient media with Na-CMC and wheat straw. However, in *Trichoderma* sp. 17/1 endoglucanase activity was 3–4 times lower.

In the medium with the FP endoglucanase activity of *Fusarium* sp. 5 was by 2.2 and 6.7 times higher than ones of *Trichoderma* sp. 17/1 and *Fennellia* sp. 2806, respectively. Maximum of enzyme activity on 5–7th day of cultivation was observed. Dynamics of endoglucanase activity was characterized by long-term (2–3 days) phase of adaptation to substrate. Lactose completely inhibited endoglucanase activity in *Fusarium* sp. 5 and *Fennellia* sp. 2806 strains, and in *Trichoderma* sp. 17/1 inhibited this activity by 2.6–4 times (Fig. 2).

High values of exoglucanase activity (from 0.3 to 0.4 U/ml) were established only for *Fennellia* sp. 2806 in the nutrient medium with wheat straw. In the nutrient medium with FP it was by 5–6 times higher in *Trichoderma* sp. 17/1 than the ones in *Fusarium* sp. 5 and *Fennellia* sp. 2806, and 2.2 times lower than in *Fennellia* sp. 2806 in the medium with wheat straw. In all other cases exoglucanase activity did not exceed 0.05 U/ml. As in case of endoglucanase activity, a long phase of adaptation was established, though the maximum activity of studied strains was observed earlier, generally on 4–5 days of cultivation, but in the medium with wheat straw — on 6th day. Unlike endoglucanase activity, exoglucanase activity of *Trichoderma* sp. 17/1, *Fennellia* sp. 2806 and *Fusarium* sp. 5 only on specific substrates was detected (Fig. 3).

Xylanase activity, as above mentioned two cellulase activities, was the highest on nutrient medium with wheat straw. Xylanase activity of *Fusarium* sp. 5 on other substrates was not detected. It could be explained by the fact that the FP, as well as Na-CMC is almost pure cellulose and do not contain residues of hemicellulose: arabinose and xylose that are inducers of this enzyme group. Unlike *Fusarium* sp. 5, growing on these substrates *Fennellia* sp. 2806 was characterized by the ability to xylanase synthesis (up to 5 U/ml), that may indicate the different mechanisms of induction in these two strains.

In the nutrient medium with wheat straw xylanase activity, as well as exoglucanase one was detected from the third day of fungal growth. It reached maximum on 7–8th day of cultivation and was 1.4 times higher in *Fennellia* sp. 2806 than in *Fusarium* sp. 5 (Fig. 4).

It was established that xylanase activity of *Trichoderma* sp. 17/1 cultivated on medium with FP and FP with lactose, reached the maximum on the 6th day of cultivation and was 8–15 times higher than in *Fusarium* sp. 5

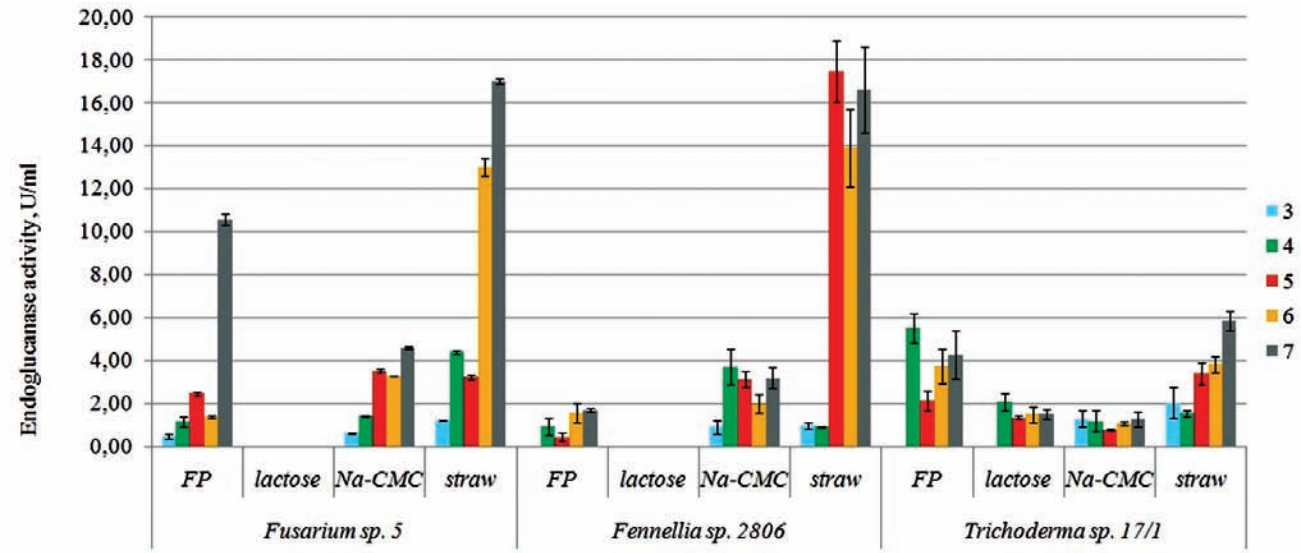

Fig. 2. **Dynamics of endoglucanase activity of *Fennellia* sp. 2806, *Trichoderma* sp. 17/1 and *Fusarium* sp. 5 on media with different substrates:**
3 — third day of cultivation; *4* — forth; *5* — firth; *6* — sixth; *7* — seventh ($P \leq 0.05$)

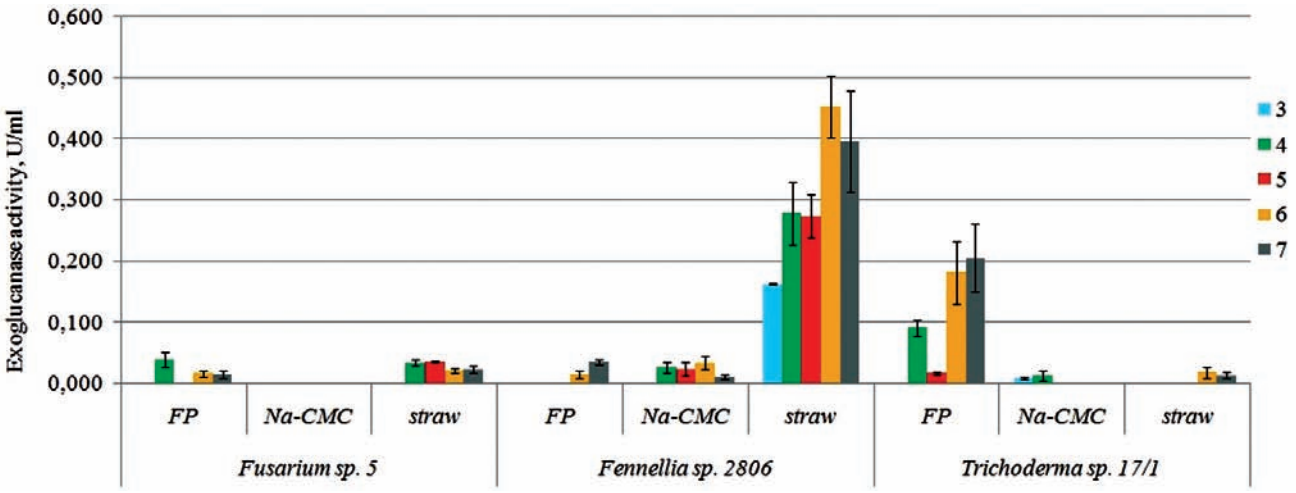

Fig. 3. **Dynamics of exoglucanase activity of *Fennellia* sp. 2806, *Trichoderma* sp. 17/1 and *Fusarium* sp. 5 on media with different substrates:**
3 — third day of cultivation; *4* — forth; *5* — firth; *6* — sixth; *7* — seventh ($P \leq 0.05$)

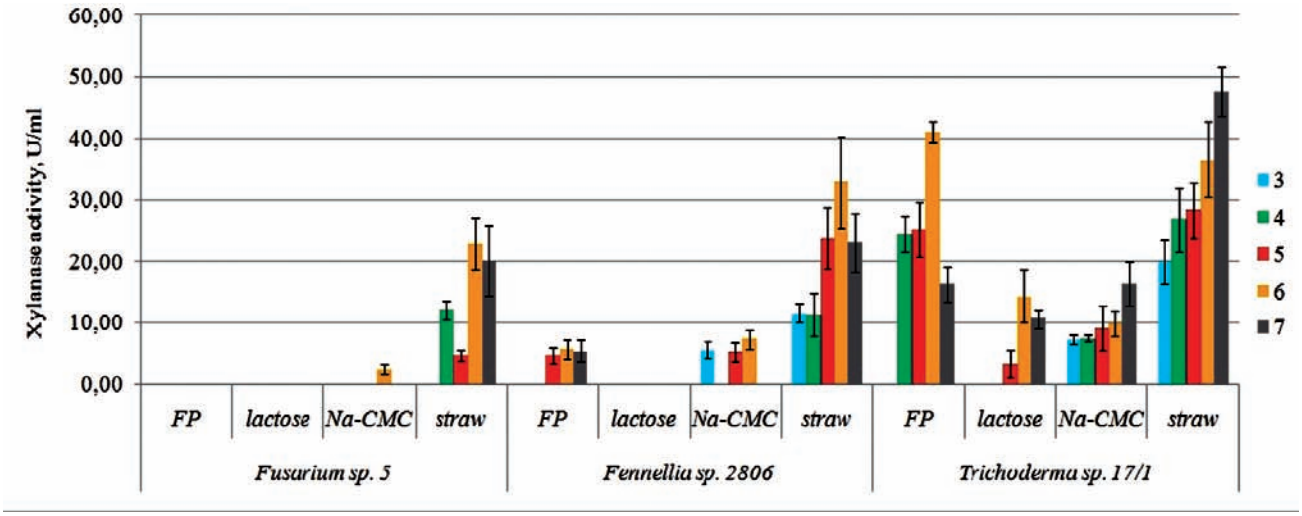

Fig. 4. **Dynamics of xylanase activity of *Fennellia* sp. 2806, *Trichoderma* sp. 17/1 and *Fusarium* sp. 5 on media with different substrates:**
3 — third day of cultivation; *4* — forth; *5* — firth; *6* — sixth; *7* — seventh ($P \leq 0.05$)

and *Fennellia* sp. 2806. Thus, there is certain relation between endoglucanase and xylanase activities. Xylanase activity, as cellulase one, was inhibited by lactose (Fig. 4).

Thus, the best substrate for induction of cellulolytic enzymes in *Fennellia* sp. 2806 and *Fusarium* sp. 5 was wheat straw, and in *Trichoderma* sp. 17/1 were FP and wheat straw. Nonspecific inducers stimulated the synthesis of cellulases in *Trichoderma reesei* [17], had no such effect in studied strains of microscopic fungi. Exoglucanase and xylanase activities were the highest in *Fennellia* sp. 2806 and *Trichoderma* sp. 17/1, and endoglucanase activity in *Fusarium* sp. 5. The studied fungal strains were characterized by long-term (2–3 days) phase of adaptation to cellulosic substrates.

Partially purified enzyme preparations from culture filtrates of *Fusarium* sp. 5 and *Fennellia* sp. 2806 were obtained. These preparations did not lose their activity for three months at +4 °C (Table).

Enzyme activity of partially purified preparations of studied microscopic fungi

Enzyme activity	Total activity, U/ml		Specific activity, U/mg protein	
	Fusarium sp. 5	*Fennellia* sp. 2806	*Fusarium* sp. 5	*Fennellia* sp. 2806
Endoglucanase	12.6 ± 0.95	11.3 ± 2.38	2.55 ± 0.19	3.12 ± 0.66
Exoglucanase	2.32 ± 0.42*	1.41 ± 0.10*	0.47 ± 0.06	0.39 ±0.06
Xylanase	1982 ± 53*	638 ± 197*	400 ± 93*	176 ± 47*
β-Glucosidase	22.10 ± 0.95*	27.50 ± 1.40*	4.60 ± 0.44*	2.79 ± 0.42*

Note: $P \leq 0.05$; * — significant difference between the two strains.

Notably, *Fusarium* sp. 5 was characterized by sufficiently high level of xylanase activity and high thermostability of endoglucanase. Wild strain of *Fennellia* sp. 2806, unlike modified strains of *Trichoderma reesei* [18], was characterized by relatively high level of β-glucosidase activity. Total and specific xylanase and exoglucanase activities were higher in the preparation from *Fusarium* sp. 5 than in the one from *Fennellia* sp. 2806. However, in the culture filtrates levels of these activities were higher in *Fennellia* sp. 2806.

Total endoglucanase activity was higher in the preparation from *Fusarium* sp. 5. Preparation from *Fennellia* sp. 2806 had higher specific endoglucanase and total β-glucosidase activities.

Notably, the comparative evaluation of the experimental data on levels of enzymatic activity of cellulolytic complex *Fusarium* sp. 5 and *Fennellia* sp. 2806 (Table) to known producers were complicated at this stage of research because of using different methods of cellulase activity determination. It was confirmed by information about the twelve most widely represented commercial preparations of cellulase [19], as well as differences in the efficiency of hydrolysis of standard and natural cellulosic substrates by cellulases [7, 20].

An interesting fact is that the lactose was not an inducer of cellulase synthesis in studied micromycetes, unlike *T. reesei*, for that the mechanisms of induction of cellulases and xylanase synthesis were well studied. To date there are 7 separate regulons in Ascomycota genome responsible for the induction of synthesis of cellulase and hemicellulase complexes, where the most important factors are transcription regulators Xyr1 and Cre1. Despite the presence of identified in the genomes of *Aspergillus*, *Penicillium*, *Fusarium* and *Neurospora* genera orthogonal sequences, the regulation of synthesis of these enzymes has different mechanisms [21].

The fact, that the metabolism of lactose was not related to signaling mechanisms of induction, and was mainly observed in studied strains of micromycetes on natural hemicellulosic substrates, makes it possible to conclude that studied strains have other mechanism type of induction than *T. reesei*. The obtained data indicate the functional diversity of the regulator ways of the hemicellulase synthesis in micromycetes at species level that may be explained by different mechanisms of adaptation in natural habitats.

Thus, the research results indicate the perspectives of using wild strains of *Fusarium* sp. 5 and *Fennellia* sp. 2806 for obtaining of microbial enzyme preparations, that can accelerate decomposition of plant residues after harvest of crop, further optimization of the synthesis, and production of enzyme preparations with the full range of cellulase and xylanase activities.

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УТВОРЕННЯ ПОЗАКЛІТИННИХ ЦЕЛЮЛОЗОЛІТИЧНИХ КОМПЛЕКСІВ МІКРОСКОПІЧНИМИ ГРИБАМИ

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Метою роботи було провести скринінг серед мікроскопічних грибів на здатність утворення комплексу целюлозолітичних ензимів та визначити вплив на цей процес деяких індукторів. Целюлозолітичну та ксиланазну активність визначали на редукуючих цукрах з використанням реактиву на основі динітросаліцилової кислоти, β-глюкозидазну активність — за гідролізом пара-нітрофеніл-β-D-глюкопіранозиду. Ензимні препарати одержували осадженням сульфатом амонію. Встановлено, що з 32 досліджених штамів мікроскопічних грибів 14 синтезують комплекс целюлозо- та ксиланолітичних ензимів. Максимальну активність виявляють штами *Fusarium* sp. 5 та *Fennellia* sp. 2806. Із цих штамів отримано ензимні препарати з високою ендо-, екзоглюканазною, ксиланазною та β-глюкозидазною активністю. Штами *Fusarium* sp. 5 та *Fennellia* sp. 2806 є активними продуцентами ензимів целюлазного комплексу на природних субстратах. Встановлено, що індуктори целюлозолітичних ензимів у *Fusarium* sp. 5 та *Fennellia* sp. 2806 відрізняються від індукторів у *Trichoderma reesei*.

Ключові слова: мікроскопічні гриби, ендоглюканаза, екзоглюканаза, ксиланаза, β-глюкозидаза.

ОБРАЗОВАНИЕ ВНЕКЛЕТОЧНЫХ ЦЕЛЛЮЛОЗОЛИТИЧЕСКИХ КОМПЛЕКСОВ МИКРОСКОПИЧЕСКИМИ ГРИБАМИ

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Цель работы — проведение скрининга среди микроскопических грибов на способность образования внеклеточных комплексов целлюлозолитических энзимов и определение влияния на этот процесс некоторых индукторов. Целлюлозолитическую и ксиланазную активность определяли на редуцирующих сахарах с использованием реактива на основе динитросалициловой кислоты, β-глюкозидазную активность — по гидролизу пара-нитрофенил-β-D-глюкопиранозида. Энзимные препараты получали осаждением сульфатом аммония. Установлено, что из 32 исследованных штаммов микроскопических грибов 14 синтезируют комплекс целлюлозо- и ксиланолитических энзимов. Максимальную активность проявляют штаммы *Fusarium* sp. 5 и *Fennellia* sp. 2806. Из этих штаммов были получены энзимные препараты с высокой эндо-, экзоглюканазной, ксиланазной и β-глюкозидазной активностью. Штаммы *Fusarium* sp. 5 и *Fennellia* sp. 2806 являются активными продуцентами энзимов целлюлазного комплекса на природных субстратах. Установлено, что индукторы целлюлозолитических энзимов у *Fusarium* sp. 5 и *Fennellia* sp. 2806 отличаются от индукторов у *Trichoderma reesei*.

Ключевые слова: микроскопические грибы, эндоглюканаза, экзоглюканаза, ксиланаза, β-глюкозидаза.