

## XYLOTROPHIC FUNGUS *Trichoderma atroviride*: CULTIVATION, EXTRACELLULAR HYDROLYTIC AND ANTIMICROBIAL ACTIVITY

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Xylotrophic fungi are well known by their ability to excrete enzymes into environment. These fungi have important biotechnological potential and some of them produce industrial enzymes. Besides, xylotrophic fungal species have recently attracted a lot of attention among researchers as a source of antibacterial drugs.

**Aim.** The aim of this work was to analyze the effect of the carbon source in the culture medium, as well as the conditions of deep cultivation on the mycelium yield, proteolytic, cellulolytic and antimicrobial activity of the culture liquid of *Trichoderma atroviride*.

**Methods.** Deep culture methods were used, partial purification was carried out with salting and subsequent dialysis, the cellulolytic activity was determined spectrophotometrically, antimicrobial activity was determined using the disc diffusion technique. Statistical analysis was performed using STATISTICA 6.0 software.

**Results.** The highest cellulolytic activity ( $0.50 \pm 0.03$  units/ml), mycelium yield and the smallest colony diameter were detected when cellulose was used as a carbon source. However, the highest proteolytic activity of the culture liquid was observed with dextrose as a carbon source. The optimal temperature range for hydrolase activity was shown to be in the range of 25–30 °C. In comparison with *Pleurotus ostreatus*, the culture liquid of *T. atroviride* not only has more pronounced antimicrobial activity, but also inhibits the growth of *Candida albicans*.

**Conclusions.** The culture liquid of isolated strain *T. atroviride* is a promising source of hydrolytic enzymes that can be used in organic farming and industry. The purified preparation obtained from the culture liquid of *T. atroviride* showed significant antimicrobial activity and can be successfully used for drug development in the future.

**Key words:** *Trichoderma atroviride*; cultivation; hydrolytic and antimicrobial activity.

*Trichoderma* spp., due to their ability to actively suppress competitors, are widely distributed in nature in almost all geographical areas of the world. They are most often found in the soil, on tree trunks and on the fruit bodies of edible and cultivated fungi, on which they parasitize [1]. These fungi are known as producers of a wide range of secondary metabolites, among which hydrolytic enzymes and antibiotics occupy an important place [2]. *Trichoderma* spp. are able to hydrolyze cellulose, hemicellulose and lignin. In addition, these fungi can serve as a source of proteolytic enzymes, which are

mainly represented by serine and aspartic proteases [3]. In addition to participating in the assimilation of complex substrates, *Trichoderma* proteases play an important role in mycoparasitism [3]. Special attention is paid to the ability of these fungi to produce substances that inhibit the growth of bacteria and other fungi [4–5]. All of the above allows us to consider *Trichoderma* as an attractive object for biotechnological production and for use in agriculture. In this paper, we developed approaches for the preparation of *T. atroviride* culture liquid with high activity of hydrolytic enzymes, and also carried out a study of

the antimicrobial activity of the obtained preparation.

### Materials and Methods

**Fungus isolation.** Wild strain of *T. atroviride* was isolated from the soil of the vicinity of Pinsk (52°06'33.5" N 26°06'46.2"E), as described in [6]. Sterile strips of filter paper were used as a carbon source [7].

**Fungus determination.** The determination of the type of fungus was carried out on the basis of a set of cultural and morphological characteristics using a key-determinant [8].

**Chemicals.** All the used reagents were Pro Analyti (p.a). Fragments of filter paper No. 1 (Whatman, UK) were used both to determine cellulase activity and as a carbon source; sucrose (Sigma-Aldrich, USA), dextrose (Sigma-Aldrich, USA) and lactose (Sigma-Aldrich, USA) were used as a carbon source for deep cultivation. For protease activity determination gelatin (Merck, Germany), casein (Merck, Germany), and milk (Biotium USA) were used as substrates. Salting out was performed using NaCl (Biochem, France). For DNS-reagent preparation 3,5-dinitrosalicylic acid (Sigma-Aldrich, USA) and potassium sodium tartrate tetrahydrate (Sigma-Aldrich, USA) were used.

**Deep cultivation.** Deep cultivation of *T. atroviride* was carried out in 250 ml of Erlenmeyer flasks (100 ml of medium) on a shaker (70 rpm) at a temperature of 25 °C and 30 °C for 7 days. A standard Czapek-Dox culture medium (pH 5.0±0.2) was used as a control. The medium was modified by adding various carbon sources (2% by weight). The biomass of the fungus was separated from the medium and weighed in a dry state. Drying of the mycelium was carried out at 37 °C until complete loss of moisture. The culture liquid was used without further dilutions.

**Protein determination.** The protein concentration was determined spectrophotometrically [9].

**Cellulase activity determination.** The cellulolytic activity was determined by colorimetric method using DNS-reagent [10]. The amount of the enzyme that catalyzes the hydrolysis of cellulose to form 1 micromole of reducing sugars (in terms of dextrose) in 1 h at a temperature of 50 °C and a pH of 4.7±0.02 was taken as a unit of cellulolytic activity.

**Protease activity determination.** The total proteolytic activity (PA) was determined by the lysis of substrates in a thin layer of

agar gel [11]. For 1 unit of activity (E) of the enzyme was taken such an activity that leads to hydrolysis of the substrate in the area of the gel size 1 cm<sup>2</sup>.

**Preparation of preparations and their purification** The mycelium extract of *P. ostreatus* 451 was obtained as described in [12]. For further purification of the enzymes, the methods of salting out (NaCl) and dialysis were used.

**Determination of antimicrobial activity.** The antibacterial activity was determined using the disk diffusion technique [13].

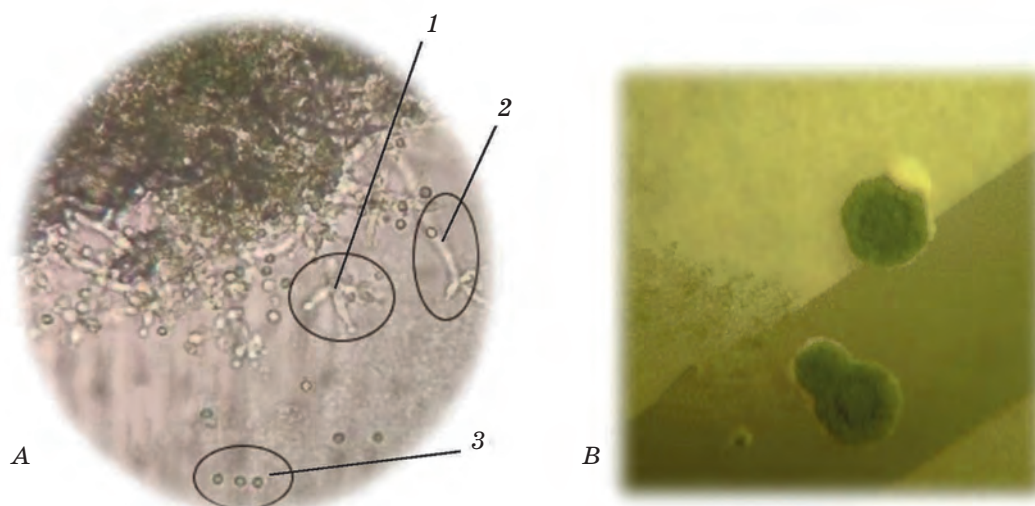
**Strains.** To evaluate antimicrobial activity *Staphylococcus aureus* ATCC 25923, *Streptococcus pneumoniae* ATCC 49619, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Klebsiella pneumoniae* ATCC 13883 and saccharomycete *Candida albicans* ATCC 885-653 were used. All used strains are control avirulent strains, their use is recommended by the ATCC. Strains were obtained from the collection of microorganisms of the Ivan Franko Lviv National University.

Statistical analysis was performed using STATISTICA 6.0 software.

### Results and Discussion

**Characteristic of isolated strain *T. atroviride*.** Colonies on the Czapek-Dox medium grew rapidly at a temperature of 26 °C, visible growth was observed on the 1st day of incubation. The final stage of fungus development was observed on the 5th day of incubation. The profile was convex. The texture was velvety. The mycelium was colorless, creeping, cotton-like. Sporulation occurred on the 4th day of growth. The colonies reached the edges of the cup, the color of the colonies was green. The reverse side of the colonies had a lemon-yellow color. The pigment was not released into the medium. No exudate was observed. The smell was sharp, musty, earthy. Hyphae were colorless. Conidiophores were unpainted, smooth, and sinuous. Branching often, regularly, twigs were arranged in two, rarely one, straight, sometimes bent. Phialides were ampoule-shaped, arranged on twigs in whorls of 2–5, rarely singly. The neck was elongated and narrow. Conidia were green, collected in slimy heads, rounded, smooth (Fig. 1). The described morphological and cultural features allow us to identify the fungus as *T. atroviride* [8].

**Deep cultivation.** Depending on the selected nutrient medium, the cultural and morphological properties of the *T. atroviride*



**Fig. 1. Microscopy of isolated strain *T. atroviride*:**

A — increase  $\times 400$ ; B — increase  $\times 56$

1 — phialides on conidiophores; 2 — conidiophores; 3 — conidia

strain, as well as the growth dynamics, were changed (Fig. 2–3).

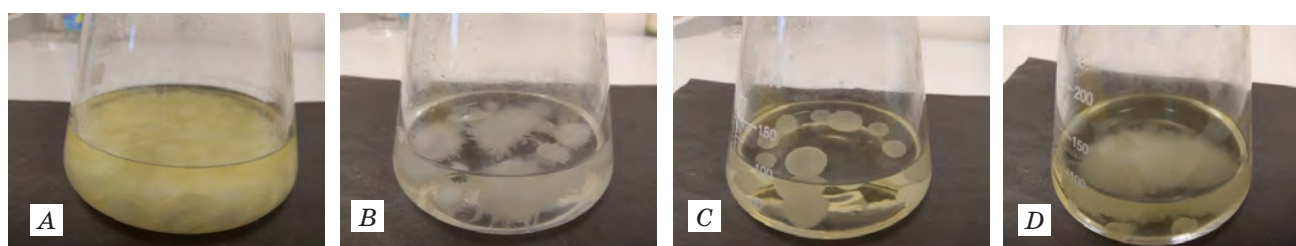
At the same time, the structure of the colonies in most cases had the form of rounded glomeruli, with the exception of colonies grown on a medium with lactose, which had the form of glomeruli (at 30 °C) or a jellyfish-like formation (at 25 °C). The presence of outgrowths was observed in fungi when using all carbon sources except dextrose. On the medium with dextrose, the colonies had a smooth texture, without outgrowths.

The color of the culture liquid also varied depending on the carbon source in the medium. Thus, the color of the culture liquid varied from colorless on the medium with sucrose (at 25 °C) to intensely yellow on the medium with cellulose (at 30 °C). In most cases, on the 7th day of incubation, the color of the culture liquid has a yellowish tint, on the medium with sucrose, the culture liquid remained colorless at 25 °C.

To evaluate the effect of temperature on the growth dynamics, the strain was grown on Czapek-Dox medium (pH  $5.0 \pm 0.2$ ) and the growth parameters were evaluated by dry weight indicators at temperatures of 25 °C and 30 °C, as recommended in the literature (Table 1).

The diameter of *T. atroviride* colonies on various nutrient media on the 7<sup>th</sup> day of deep cultivation varied from  $0.25 \pm 0.03$  cm on the medium with cellulose at 30 °C, to  $2.97 \pm 2.29$  cm on a medium with lactose under the same conditions. The colonies had the smallest diameter on a medium using cellulose as a carbon source. This morphology of the mycelium provides more favorable conditions for the growth of the fungus compared to large glomeruli, since a full supply of oxygen and nutrients occurs only on the outer surface of the glomeruli.

The pH of the culture medium, depending on the carbon used, varied from  $6.18 \pm 0.1$



**Fig. 2. Deep cultivation of *T. atroviride* at 7<sup>th</sup> day 25 °C:**

A — cellulose; B — sucrose; C — dextrose; D — lactose

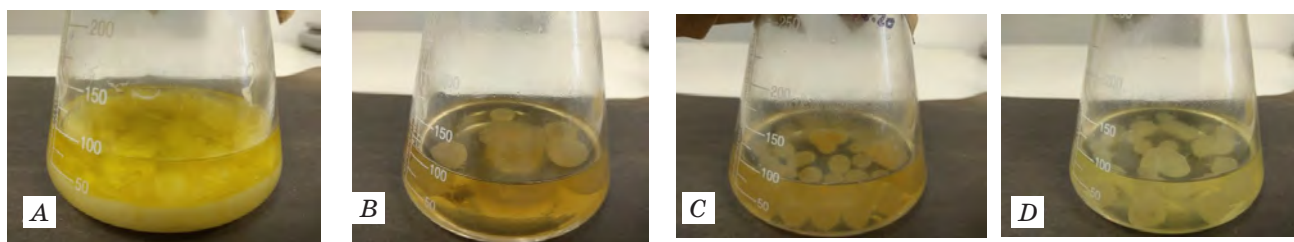


Fig. 3. Deep cultivation of *T. atroviride* at 7<sup>th</sup> day 30 °C: A — cellulose; B — sucrose; C — dextrose; D — lactose

units on the medium with sucrose at 25 °C to  $9.12 \pm 0.1$  (medium with sucrose at 30 °C).

The highest yield of mycelium by dry weight was ( $0.51 \pm 0.03$  g) on a medium with cellulose at 30 °C. The smallest one ( $0.05 \pm 0.03$  g) on a medium with dextrose, at 25 °C.

The conducted studies of the hydrolase activity of the *T. atroviride* culture liquid allowed us to establish the following. Significant values of cellulolytic activity were observed only when the fungus was grown on a medium using cellulose as a carbon source. Protease activity data of the *T. atroviride* culture liquid are presented in Table 2.

As can be seen from the above data, when using casein and gelatin as substrates, the highest protease activity was observed for preparations of *T. atroviride* grown on a

medium with dextrose. If the substrate was milk, the highest protease activity was shown for the culture liquid of the fungus grown on a medium with sucrose. It is noteworthy that the culture liquid of *T. atroviride* had higher values of proteolytic activity than the culture liquid of *P. ostreatus*.

Antibacterial activity data of *T. atroviride* and *P. ostreatus* 451 are presented in Tables 3 and 4 correspondently.

In our experiments, the preparation of *T. atroviride* culture liquid showed antimicrobial activity towards all tested strains. The purified preparation had a much more pronounced antimicrobial activity, in some cases more than 2 times. It should be noted that the culture liquid of *T. atroviride* suppressed not only the growth of the studied Gram-positive and Gram-negative bacteria,

Table 1. The effect of incubation temperature on *T. atroviride* growth parametres using different carbon sources in the medium ( $n = 5$ )

Czapek-Dox medium*	Dry weight of the fungus after 7 days of cultivation, g	Diameter of colonies, cm	pH, Units
medium 1a	$0.32 \pm 0.02$	$0.34 \pm 0.04$	$7.07 \pm 0.1$
medium 1b	$0.51 \pm 0.03$	$0.23 \pm 0.03$	$7.07 \pm 0.1$
medium 2a	$0.07 \pm 0.02$	$2.53 \pm 1.45$	$6.18 \pm 0.1$
medium 2b	$0.10 \pm 0.04$	$1.48 \pm 0.61$	$9.12 \pm 0.1$
medium 3a	$0.05 \pm 0.03$	$1.47 \pm 0.63$	$6.20 \pm 0.1$
medium 3b	$0.06 \pm 0.01$	$0.67 \pm 0.33$	$6.26 \pm 0.1$
medium 4a	$0.13 \pm 0.03$	$2.43 \pm 1.93$	$6.55 \pm 0.1$
medium 4b	$0.15 \pm 0.05$	$2.97 \pm 2.29$	$6.74 \pm 0.1$

\* medium 1 — cellulose as carbon source in the medium; medium 2 — sucrose as carbon source in the medium; medium 3 — dextrose as carbon source in the medium; medium 4 — lactose as carbon source in the medium; a — temperature 25 °C; b — temperature 30 °C.

Table 2. Protease activity of *T. atroviride* and *P. ostreatus* culture liquid ( $n = 9$ )

Medium*	Protease activity (U/mg protein)		
	milk	casein	gelatin
<i>T. atroviride</i> (medium 1a)	12.0 ± 0.77	10.6 ± 0.68	5.9 ± 0.73
<i>T. atroviride</i> (medium 1b)	42.240 ± 0.75	58.2 ± 0.83	129.5 ± 0.87
<i>T. atroviride</i> (medium 2a)	47.735 ± 0.96	189.1 ± 0.77	72.2 ± 0.66
<i>T. atroviride</i> (medium 2b)	110.290 ± 0.86	142.5 ± 0.86	15.6 ± 0.79
<i>T. atroviride</i> (medium 3b)	83.3 ± 0.74	246.7 ± 0.69	180.0 ± 0.81
<i>T. atroviride</i> (medium 3b)	1.8 ± 0.65	167.14 ± 0.73	148.57 ± 0.62
<i>T. atroviride</i> (medium 4b)	4.5 ± 0.68	64.29 ± 0.89	160.0 ± 0.88
<i>T. atroviride</i> (medium 4b)	20.3 ± 0.73	69.0 ± 0.67	99.29 ± 0.70
<i>P. ostreatus</i>	164.1 ± 0.14	23.9 ± 0.2	22.7 ± 0.2

\* medium 1 — cellulose as carbon source in the medium; medium 2 — sucrose as carbon source in the medium; medium 3 — dextrose as carbon source in the medium; medium 4 — lactose as carbon source in the medium; a — temperature 25 °C; b — temperature 30 °C.

but also inhibited the growth of *Candida albicans*. Thus, preparations of xylophilic fungi (*P. ostreatus* and *T. atroviride*) possess a pronounced antimicrobial activity, and this activity is more pronounced for *T. atroviride*.

The obtained results are consistent with the data of previous authors.

The carbon source is one of the main elements influencing the growth and development of fungi [14]. For the closely related species *T. harzianum*, it was shown that the replacement of one sugar in the medium with another led to changes in both the colour of the culture liquid and the appearance of the

Table 3. Antibacterial activity of *T. atroviride* culture liquid ( $n = 9$ )

Test strains	Zone of inhibition by the culture liquid, mm	Zone of inhibition by the purified preparation, mm	Gram staining
<i>Staphylococcus aureus</i>	13.2 ± 0.12*	17.5 ± 0.14*	(+)
<i>Streptococcus pneumonia</i>	11.3 ± 0.2	23.4 ± 0.11*	(+)
<i>Echerichia coli</i>	18.8 ± 0.09*	24.8 ± 0.1	(-)
<i>Pseudomonas aeruginosa</i>	8.5 ± 0.13*	24.6 ± 0.17*	(-)
<i>Klebsiella pneumonia</i>	17.3 ± 0.21	18.7 ± 0.2	(-)
<i>Candida albicans</i>	19.2 ± 0.14*	21.4 ± 0.11*	

\* significantly at  $P \leq 0.005$ .

Table 4. Antibacterial activity of *P. ostreatus* 451 mycelium extract ( $n = 9$ )

Test strains	Zone of inhibition by the mycelium extract, mm	Zone of inhibition by the purified preparation, mm	Gram staining
<i>Staphylococcus aureus</i>	6.2±0.01*	7.5±0.11*	(+)
<i>Streptococcus pneumonia</i>	11.7±0.12*	19.6±0.21	(+)
<i>Echerichia coli</i>	6.8±0.21*	8.7±0.16*	(-)
<i>Pseudomonas aeruginosa</i>	8.5±0.13	21.4±0.13	(-)
<i>Klebsiella pneumonia</i>	7.1±0.09*	8.9±0.09*	(-)
<i>Candida albicans</i>	–	–	

\* significantly at  $P \leq 0.05$ .

mycelium [15]. In our case, the colour of the culture liquid had a yellowish tint, and on the medium with sucrose, the culture liquid remained colorless. There is no consensus in the literature on the effect of temperature on the growth of *Trichoderma*. According to some authors, *Trichoderma* are mesophilic and can multiply at 25–35 °C [16]. At the same time, other authors believe that the temperature optimum for the growth of these fungi is 25–30 °C [17]. In our case, the optimal growth was observed at 30 °C. According to some authors, pH of the culture liquid of *T. atroviride* may vary depending on the period of cultivation According to [18] when *T. atroviride* I-6-IIB was cultivated on Czapek-Dox medium with various carbon sources for 4 days the pH decreased during the first 16 hours of growth from the initial 6.7 to 6.4 units; after that, it increased, reaching 7.8 units at the end of cultivation. In our case, the pH of the culture liquid of *T. atroviride* on the 7th day of incubation was 6.18–7.07 units. The exception was the culture liquid on a medium with sucrose, its pH was 9.12±0.1 units. There is evidence that the protease activity of *Trichoderma* depends on the carbon source in the nutrient medium [15]. It is shown that the modification of the media activates the output of various endo- and exoproteases. The active production of cellulases is noticed when fungi are cultivated on a medium containing cellulose or a mixture of plant polymers [19] which is confirmed in our study. A number of authors note that cellulase is not formed until dextrose is removed from the medium [20].

## Conclusions

The results of the present study concluded that carbon source has a significant effect on the color of the culture medium and the colony structure of *T. atroviride*. The optimal growth parameters of the deep cultivation of *T. atroviride* were the following: Czapek-Dox medium with the addition of 2% cellulose, at 30 °C; the growth of the fungus was observed on all the studied carbon sources.

The highest protease activity of *T. atroviride* culture liquid (gelatin and casein as substrates) was shown for the fungus grown on a medium with dextrose, while significant value of cellulolytic activity (0.50±0.03 units/ml) was obtained only on a medium with cellulose.

The purified preparation obtained from the culture liquid of *T. atroviride* showed significant antimicrobial activity and can be successfully used for drug development in the future.

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**КСИЛОТРОФНИЙ ГРИБ  
*Trichoderma atroviride*:  
КУЛЬТИВУВАННЯ,  
ЕКСТРАЦЕЛЮЛЯРНА ГІДРОЛІТИЧНА  
ТА АНТИМІКРОБНА АКТИВНІСТЬ**

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Ксилотрофні гриби добре відомі своєю здатністю виділяти ензими в навколишнє середовище. Ці гриби мають важливий біотехнологічний потенціал, деякі з них використовують для промислового виробництва ензимів. Крім того, останнім часом ксилотрофні гриби привертають велику увагу дослідників як джерело антибактеріальних препаратів.

**Мета.** Проаналізувати вплив джерела вуглецю в живильному середовищі, а також умов глибинного культивування на вихід міцелю, протеолітичної, целюлолітичної та антимікробної активності культуральної рідини *Trichoderma atroviride*.

**Методи.** Використовували метод глибинного культивування, часткове очищення препарату проводили методом висолування з подальшим діалізом, целюлолітичну активність визначали спектрофотометрично, антимікробну активність — методом дискової дифузії. Статистичну обробку даних здійснювали з використанням пакета програм STATISTICA 6.0.

**Результати.** Найбільшу целюлолітичну активність ( $0,50 \pm 0,03$  Од/мл), вихід міцелю і найменший діаметр колоній було виявлено з використанням целюлози як джерела вуглецю. Однак найбільшу протеолітичну активність культуральної рідини спостерігали, застосовуючи як джерело вуглецю глюкозу. Показано, що оптимальний діапазон температур для активності гідролази 25–30 °С. Порівняно з *P. ostreatus* культуральна рідина *T. atroviride* не тільки має більш виражену антимікробну активність, але й пригнічує ріст *Candida albicans*.

**Висновки.** Культуральна рідина виділеного штаму *T. atroviride* є перспективним джерелом гідролітичних ензимів, які можуть бути використані в органічному сільському господарстві та промисловості. Очищений препарат, отриманий з культуральної рідини *T. atroviride* мав значну антимікробну активність і його можна успішно застосовувати в майбутньому для розроблення лікарських засобів.

**Ключові слова:** *Trichoderma atroviride*; культивування; гідролітична та антимікробна активність.

**КСИЛОТРОФНИЙ ГРИБ *Trichoderma atroviride*: КУЛЬТИВІРОВАНИЕ, ЭКСТРАЦЕЛЛЮЛЯРНАЯ ГИДРОЛИТИЧЕСКАЯ И АНТИМИКРОБНАЯ АКТИВНОСТЬ**

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Ксилотрофные грибы хорошо известны своей способностью выделять энзимы в окружающую среду. Эти грибы обладают важным биотехнологическим потенциалом, и некоторые из них используются для промышленного производства энзимов. Кроме того, в последнее время ксилотрофные грибы привлекают большое внимание исследователей в качестве сырья для антибактериальных препаратов.

**Цель.** Целью работы был анализ влияния источника углерода в питательной среде, а также условий глубинного культивирования на выход мицелия, протеолитической, целлюлитической и антимикробной активности культуральной жидкости *Trichoderma atroviride*.

**Методы.** Использовали метод глубинного культивирования, проводили частичную очистку препарата методом высаливания и последующего диализа, целлюлитическую активность определяли спектрофотометрически, антимикробную активность — с помощью метода дисковой диффузии. Статистическую обработку данных осуществляли с использованием пакета программ STATISTICA 6.0.

**Результаты.** Наибольшая целлюлолитическая активность ( $0,50 \pm 0,03$  ед/мл), выход мицелия и наименьший диаметр колоний были при использовании целлюлозы в качестве источника углерода. Однако наибольшую протеолитическую активность культуральной жидкости наблюдали, используя в качестве источника углерода глюкозу. Показано, что оптимальный диапазон температур для активности гидролаз находится в диапазоне 25–30 °С. По сравнению с *P. ostreatus* культуральная жидкость *T. atroviride* не только обладает более выраженной антимикробной активностью, но и подавляет рост *Candida albicans*.

**Выводы.** Культуральная жидкость выделенного штамма *T. atroviride* является перспективным источником гидролитических энзимов, которые могут применяться в органическом сельском хозяйстве и промышленности. Очищенный препарат, полученный из культуральной жидкости *T. atroviride*, показал значительную антимикробную активность и может успешно использоваться в будущем для разработки лекарственных средств.

**Ключевые слова:** *Trichoderma atroviride*; культивирование; гидролитическая и антимикробная активность.