

## CULTURE CHARACTERISTICS AND ENZYMATIC ACTIVITY OF *SARCODONTIA CROCEA* (*BASIDIOMYCOTA*) STRAINS COLLECTED FROM THE CENTRAL RUSSIAN UPLAND

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The biological properties of the xylotrophic fungus *Sarcodontia crocea* (*Polyporales*, *Basidiomycota*), which develops basidiocarps on the trunks of fruit trees (mainly *Malus domestica*) and exhibits phytopathogenic activity, have been investigated under pure culture conditions. Morphological and molecular verification was performed for *Sarcodontia crocea* strains isolated in 2019 by seeding the basidiospores and directly from specimens of fresh basidiocarps growing on *Malus domestica* in Belgorod and Oryol Oblasts (the Central Russian Upland). New data on micromorphological (presence of chlamydospores and anastomoses on dikaryotic mycelium) and molecular (complete ITS1–5.8S–ITS2 nrDNA sequences) characteristics of this species have been obtained. The maximum growth rate of mycelium on the agarized MEA medium was determined (up to 5.5 mm/day) and the oxidative and cellulolytic activity of *Sarcodontia crocea* was assessed. It was revealed that all strains had the ability to ABTS oxidation as a result of activity of oxidative enzymes. Strains of *S. crocea* LE-BIN 4342, 4343, 4346, 4350, and 4365 showed the high activity of oxidoreductases.

**Keywords:** cellulases, fungal cultivation, growth rate of mycelium, ligninases, micromorphology, wood-inhabiting fungi, xylotrophic phytopathogens

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### INTRODUCTION

Basidial macromycetes represent one of the key evolutionary-established groups of living organisms that actively decompose wood. Among xylotrophic basidiomycetes, so-called xyloparasites can be distinguished that grow on the trunks, branches or roots of living trees, both wild and cultivated *Sarcodontia crocea* (Schwein.) Kotl. (*Polyporales*, *Basidiomycota*) is a xylotrophic basidiomycete with pronounced phytopathogenic activity that develops predominantly on fruit trees, mainly *Malus* (Eriksson et al., 1981) and rarely on *Pyrus*, *Sorbus*, and *Prunus*, causing white rot. The most obvious features of infestation of fruit trees with phytopathogenic xylotrophs, as *S. crocea*, in the absence of developed fungal fruit bodies are the individual branches drying up and crown asymmetry, while the tree retains its limited ability to fruit. The dead basidiomata of *S. crocea* have also been found on thick, dry branches and dry tree trunks, for which this species is the main cause of drying and death (Volobuev et al., 2019). For this reason, *S. crocea* is considered to be a saprotroph. *S. crocea* is relatively rare in Central and Northern Europe (Szczechowski et al., 2017), and the species is assessed as Vulnerable (VU) A2c+3c+4c according to the IUCN criteria because the habitat for *S. crocea* has declined and continues to be so due to the

intensification of orchard and garden management (Iršénaitė, 2019). At the same time, in the conditions of the Central Russian Upland (Eastern Europe), there are significant areas of neglected gardens or orchards that do not receive proper horticultural and phytosanitary care and thus are areas of mass distribution of this phytopathogen (Fig. 1). To date, the biology and ecology of *S. crocea* have not been sufficiently studied, although it is important for developing measures to control and prevent its spread in orchard agroecosystems.

The aim of this study is to verify new strains of *Sarcodontia crocea*, to obtain their cultural characteristics and to evaluate oxidative and cellulolytic enzymes that determine the xylotrophic activity of the species and its ecology. This study is also intended to supplement the current knowledge on the biology of the xylotrophic basidial fungus *S. crocea* by obtaining new DNA sequences and *ex situ* collection.

### MATERIALS AND METHODS

**Isolation and verification of fungal cultures.** We studied nine strains of *Sarcodontia crocea* obtained in 2019 from basidiocarps growing on different parts of *Malus domestica* as a host tree on the territories of Belgorod and Oryol Oblasts (Table 1). *Ex situ* isolation was car-



Fig. 1. Basidiocarp of *Sarcodontia crocea* on old apple tree.

ried out by traditional methods of solid-phase culturing: by seeding of basidiospores or by placing small fragments of basidiocarps on an agarized medium (4% ale-wort “Severnaya Pivovarnya”, pH 5.8 and 2% w/v agar “Difco”), with addition of kanamycin water solution (final concentration in the medium – 0.5 mg/ml) in order to prevent bacterial contamination. Dikaryotic strains were deposited in the Komarov Botanical Institute Basidiomycetes Culture Collection (LE-BIN,

St. Petersburg, Russia) and are stored using the subculture method, the disk method (in distilled water at 4°C), and the cryopreservation method at –80°C (Psurtseva et al., 2012; Shakhova, Volobuev, 2020). The morphological description and photo-documentation were carried out for 14 days old colonies using the AxioScope A1 microscope (Carl Zeiss) with ×40 and ×60 magnifications. Strains of *S. crocea* were characterized by cultural and morphological parameters using the method and terminology of J.A. Stalpers (1978).

**Molecular study of pure cultures.** Genomic DNA was extracted using the FitoSORB DNA extraction kit (Syntol, Russia) according to the manufacturer’s instructions from 14 days old cultures, which were grown on standard liquid medium Malt Extract (ME, Conda) in the dark at 25°C. Amplification of the ITS1–5.8S–ITS2 region of the nrDNA, and sequencing were reformed as described in Zmitrovich et al. (2019). Newly generated sequences were deposited in GenBank.

**Growth measurement.** To characterize the linear growth rate, pure cultures of *S. crocea* were grown in Petri dishes 90 mm diam. on standard Malt Extract Agar (MEA, Conda) in the dark at 25°C. Cultivation of strains was carried out with mycelial blocks (7 mm diam.) cut from the edge zone of the actively growing colony, by placing them on the nutrient medium in the center of Petri dishes with the mycelial layer downwards. The growth of strains was characterized by the

Table 1. Characteristics of fungal strains studied

Strain numbers in LE-BIN	Origin of strains		The growth in the standard MEA medium			GenBank accessions
	Locality	Substrate	10 days diam., (mm)	Petri dish (days)	AGR (mm/days)	
4342	Russia, Belgorod Oblast, Korochansky District, vicinity of the Popovka village	alive tree of <i>M. domestica</i>	50.7 ± 1.8	21	4.2	MW042103
4343	Russia, Belgorod Oblast, Korochansky District, vicinity of the Popovka village	dry branches of alive tree of <i>M. domestica</i>	52.5 ± 2.2	17	5.5	MW042104
4346	Russia, Belgorod Oblast, Korochansky District, vicinity of the Popovka village	trunk of alive tree of <i>M. domestica</i>	36.7 ± 3.1	27	3.3	–
4350	Russia, Oryol Oblast, Glazunovsky District, vicinity of the Lovchikovo village	trunk of a dying tree of <i>M. domestica</i>	39.5 ± 2.5	19	4.9	MW042105
4355	Russia, Oryol Oblast, Glazunovsky District, vicinity of the Lovchikovo village	branch of alive tree of <i>M. domestica</i>	58.7 ± 2.3	19	4.8	MW042106
4365	Russia, Oryol Oblast, Mtsensky District, vicinity of the Volya village	trunk of alive tree of <i>M. domestica</i>	32.3 ± 2.1	25	3.5	MW042107
4367	Russia, Oryol Oblast, Mtsensky District, vicinity of the Volya village	trunk of alive tree of <i>M. domestica</i>	29.3 ± 2.2	23	4.0	MW042108
4378	Russia, Oryol Oblast, Orlovsky District, vicinity of the Zhilina village	trunk of a dying tree of <i>M. domestica</i>	17.2 ± 1.5	30	3.0	MW042109
4382	Russia, Oryol Oblast, Orlovsky District, vicinity of the Zhilina village	dead tree of <i>M. domestica</i>	56.3 ± 4.2	19	4.9	–

diameter of the colony (mm), measuring them every two days starting from the third day until the Petri dish was completely overgrown. The average growth rate of the vegetative mycelium of *S. crocea* strains (AGR, mm/day) was calculated according to the formula (Badalyan et al., 2015):  $AGR = (D_1 - D_0)/(t_1 - t_0)$ , where  $D_1$  is the diameter of the colony at the end of the growth, mm;  $D_0$  – colony diameter at the beginning phase of linear growth, mm;  $t_1 - t_0$  – the duration of the linear phase of the colony growth, days.

**Detection of enzymatic activity.** The activity of oxidative and cellulolytic enzymatic complexes was studied using the rapid screening method. The strains were grown on the MEA medium in a thermostat at 25°C for 2 weeks. Cultivation of strains was carried out with mycelial blocks 7 mm diam. cut from the edge zone of the actively growing colony, by placing them in the center of the Petri dish with the mycelial layer upwards. The qualitative activity of oxidative enzymes in the studied strains was determined by the method of application with modifications. The activity of oxidoreductases was registered 48 hours after placing the mycelial blocks on Petri dishes with plain agar (1% w/v; Difco) containing ABTS (in 0.1% concentration) by the presence of emerald-greenish staining around inoculum (d, mm). The criteria to determine the intensity of the substrate oxidation reaction were described in Shakhova and Volobuev (2020). The qualitative activity of cellulolytic enzymes in cultures was determined using the medium containing carboxymethyl cellulose (1% w/v, CMC; Chemapol) and agar (1% w/v; Difco) by the application method and estimated by the presence of a clear zone around inoculum (d, mm). The clear zone was detected 48 hours after inoculation using solution I in KI (0.5% w/v I in 2% KI) (Shakhova, Volobuev, 2020). The same parameters were used to estimate the intensity of the reaction as in determining the activity of oxidoreductases.

## RESULTS AND DISCUSSION

The growth characteristics of the studied strains on the standard MEA medium are presented in Table 1 and Fig. 2. When comparing the data on the linear growth rate of the vegetative mycelium of *S. crocea* strains with the results of a study on the growth rate of other cultivated xylotrophic fungi obtained previously (Shakhova, Volobuev, 2020) and the literature data (Petre, Tănase, 2013), it should be noted that *S. crocea* belongs to slow-growing species. The most rapidly growing strains were LE-BIN 4343, LE-BIN 4350, LE-BIN 4355, and LE-BIN 4382. By the 10th day of cultivation, the diameter of the colonies of these strains exceeded 50 mm, and the Petri dishes ( $d \geq 75$  mm) were completely overgrown in 17–19 days. The average mycelium growth rate (AGR) of these strains was 4.8–5.5 mm/day. The strain LE-BIN 4378 had the lowest growth rate, with a colony diameter of 17.2 mm after 10 days, AGR was 3.0 mm/day, and the colonies of the

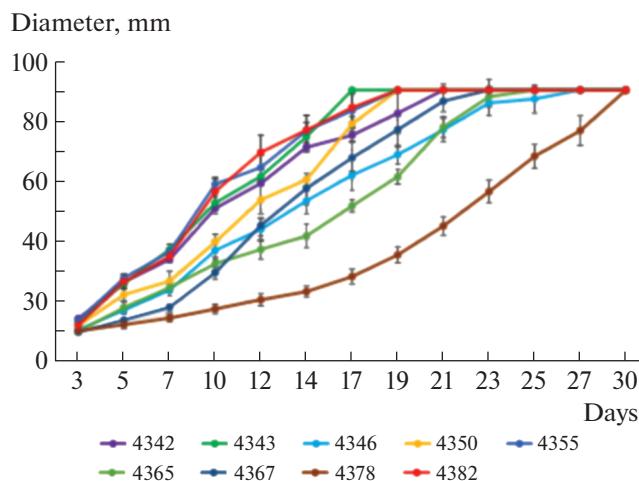


Fig. 2. Linear growth rate of the studied LE-BIN strains on Malt Extract Agar (MEA).

strain covered the whole Petri dish after 30 days (Table 1, Fig. 2).

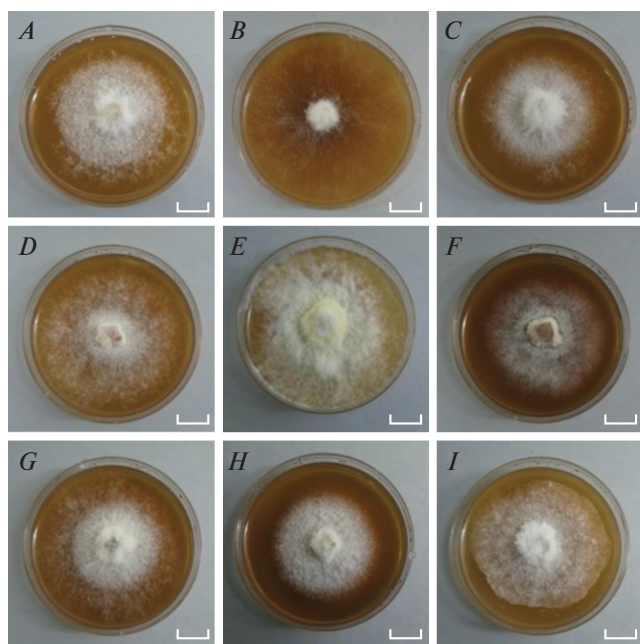
Morphological characteristics of cultures of studied strains are listed in Table 2, photos of the colonies are shown in Fig. 3. The main features of *S. crocea* in the cultivation on the MEA medium are floccose (occasionally pinnate as in LE-BIN 4343 and 4346) colonies with a strong characteristic sweet-fruity odour (Table 2, Fig. 3). The fruity odour, which indicates the presence of volatile compounds in strains (Kokubun et al., 2007), can play an important physiological role in the development of *S. crocea* basidiocarps and the spread of its substrate mycelium (in particular, in the distant inhibition of *S. crocea* fungal opponents during wood colonization). The vast majority of strains had unclear colony zones (except for LE-BIN 4343 and 4346). More than half of the strains had a bleached reverse. The colour of the colonies ranged from white to creamy (LE-BIN 4343, 4346, 4367, 4378) and lemon creamy (LE-BIN 4355) (Fig. 3). It should be noted that pigmentation manifested itself well with the age of the colonies. The outline of the colonies also varied significantly from fringe to wavy (LE-BIN 4382) and flat (LE-BIN 4365).

Among the characteristic features of the micromorphology of the vegetative mycelium of *S. crocea* is the presence of asexual reproduction structures on the dikaryotic mycelium, such as chlamydospores, along with the presence of clamps and anastomoses (LE-BIN 4346 and 4367). Terminal chlamydospores were found in strains LE-BIN 4350 and 4365. The beginning of growth of apical chlamydospores occurred after their separation from the parent hyphae by a transverse septum. No thickening of the protoplasm was observed. The presence of chlamydospores on the dikaryotic mycelium of *S. crocea* strains which we have described is in accordance with the micromorphological study of this basidiomycete by J.A. Stalpers (1978).

**Table 2.** Culture characteristics of the *Sarcodontia crocea* strains

LE-BIN strains	Macromorphology	Micromorphological features
4342	The aerial mycelium is white. Reverse bleached. Colony growing edge pressed. Outlines of colonies fringed. Mycelial mat has an approximately zonal development. The colony has a floccose texture with small hyphal tufts, standing out from the agar. Odor is very intense, strong, sweetish-fruity	Hyphal system monomitic. The mycelium has two types of hyphae: leading hyphae d 3.5–4.8 µm and thin-walled and branched exploiting hyphae d 2.3–3.0 µm. Branched generative hyphae, differentiated with septate and rare clamp connections, d 4.3–5.8 µm
4343	The aerial mycelium is white (becomes dark cream color with age). Reverse bleached. Colony growing edge pressed. Outlines of colonies fringed, broken. Mycelial mat immersed (sometimes downy in more differentiated hyphal). The colony has a plumose texture, mycelial tufts with long groups of hyphae radiating from the central axis, fan-like arrangement. Odor is very intense, strong, sweetish-fruity	Hyphal system monomitic. The mycelium consists of straight, smooth, rarely branching hyphae d 2.5–5.0 µm, often arranged with partitions, with thickened walls. Generative hyphae, differentiated with rare clamp connections, d 5.6–8.1 µm
4346	The aerial mycelium is white (becomes cream color with age). Reverse unchanged. Colony growing edge pressed. Outlines of colonies fringed, broken. Mycelial mat has an approximately zonal development. The colony has a floccose texture with small hyphal tufts, standing out from the agar. Odor is very intense, strong, sweetish-fruity	Hyphal system monomitic. The mycelium has thin-walled and branched hyphae (d 1.7–3.0 µm) forming anastomosis. Branched generative hyphae, differentiated with septate and frequent clamp connections, d 4.3–5.8 µm
4350	The aerial mycelium is white (becomes cream color with age). Reverse unchanged. Colony growing edge pressed. Outlines of colonies fringed. Mycelial mat has an approximately zonal development. The colony has a floccose texture with small hyphal tufts, standing out from the agar. Odor is very intense, strong, sweetish-fruity	Hyphal system monomitic. The mycelium consists of straight, smooth, rarely branching hyphae d 3.3–4.7 µm, often arranged with partitions, with thickened walls. Generative hyphae, differentiated with rare clamp connections, 6.0–8.2 µm in diameter. Chlamydo spores broadly ellipsoid: d 5.0–6.6 µm, length –15.0–24.0 µm
4355	The aerial mycelium is white (becomes lemon-cream color with age). Reverse bleached. Colony growing edge pressed. Outlines of colonies fringed. Mycelial mat has an approximately zonal development. The colony has a floccose texture with small hyphal tufts, standing out from the agar. Odor is very intense, strong, sweetish-fruity	Hyphal system monomitic. The mycelium has thin-walled and branched hyphae (d 1.7–2.7 µm) forming frequent curling. Branched generative hyphae, differentiated with septate and rare clamp connections, d 5.2–6.3 µm
4365	The aerial mycelium is white. Reverse bleached. Colony growing edge pressed. Outlines of colonies smooth. Mycelial mat has an approximately zonal development. The colony has a floccose texture with small hyphal tufts, standing out from the agar. Odor is very intense, strong, sweetish-fruity	Hyphal system monomitic. The mycelium consists of straight, smooth, rarely branching hyphae d 3.4–4.9 µm, often arranged with partitions, with thickened walls. Generative hyphae, differentiated with rare clamp connections, 5.3–8.5 µm in diameter. Chlamydo spores globose (d 9.0–12.6 µm) or broadly ellipsoid (d 5.0–6.6 µm, length –15.0–24.0 µm)
4367	The aerial mycelium is white (becomes cream color with age). Reverse unchanged. Colony growing edge pressed. Outlines of colonies fringed. Mycelial mat has an approximately zonal development. The colony has a floccose texture with small hyphal tufts, standing out from the agar. Odor is very intense, strong, sweetish-fruity	Hyphal system monomitic. The mycelium has thin-walled and branched hyphae (d 3.2–4.3 µm) forming anastomosis. Sometimes hyphae forming curling. Sometimes there are empty swollen hyphae with thickened walls. Branched generative hyphae, differentiated with septate and frequent clamp connections, 4.9–6.3 µm in diameter
4378	The aerial mycelium is white (becomes cream color with age). Reverse unchanged. Colony growing edge pressed. Outlines of colonies fringed. Mycelial mat has an approximately zonal development. The colony has floccose-cottony texture, rather long, single mycelial hyphae spreading in all directions. Odor is very intense, strong, sweetish-fruity	Hyphal system monomitic. The mycelium has thin-walled and branched hyphae (d 1.8–2.8 µm) forming frequent curling. Branched generative hyphae, differentiated with septate and rare clamp connections, d 4.5–5.6 µm
4382	The aerial mycelium is white. Reverse bleached. Colony growing edge pressed. Outlines of colonies wavy. Mycelial mat has an approximately zonal development. The colony has a floccose texture with small hyphal tufts, standing out from the agar. Odor is very intense, strong, sweetish-fruity	Hyphal system monomitic. The mycelium has thin-walled and branched hyphae (d 2.3–3.7 µm) forming frequent curling. Branched generative hyphae, differentiated with septate and rare clamp connections, 4.5–5.6 µm in diameter

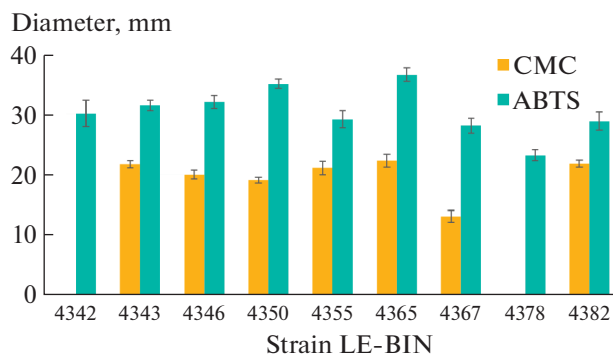




**Fig. 3.** Morphology of *Sarcodontia crocea* cultures which were incubated for 14 days on Malt Extract Agar (MEA). Scale bars = 1 cm. *A* – LE-BIN 4342, *B* – LE-BIN 4343, *C* – LE-BIN 4346, *D* – LE-BIN 4350, *E* – LE-BIN 4355, *F* – LE-BIN 4365, *G* – LE-BIN 4367, *H* – LE-BIN 4378, *I* – LE-BIN 4382.

Thus, the macromorphological features of different strains of *S. crocea* on MEA fluctuated significantly, while their micromorphology remained more stable. The results obtained can be used to verify strains and control the purity of vegetative mycelium in a culture.

*S. crocea* is among the very few organisms that colonize fruit trees, mainly *Malus* spp., less often *Pyrus* spp. and members of the genus *Prunus* (Eriksson et al., 1981, Volobuev et al., 2015), and thanks to the complex enzymatic system can degrade lignin, one of the most abundant and resistant biopolymers. However, the enzymatic activity of this species has not been sufficiently studied. Therefore, we performed an assessment of lignocellulose-converting enzyme activity among *S. crocea* cultures. The results of a rapid test for the presence of cellulolytic enzymes showed that most of the strains studied have a medium activity of these enzymes. The cellulolytic enzyme activity was not revealed for the strains LE-BIN 4343 and LE-BIN 4378. It should be noted that in *S. crocea* strains the activity level of cellulolytic enzymes was significantly lower than that of oxidative enzymes (by 25–45%). This is probably due to the fact that during selective delignification with white rot fungi at an early stage of wood decomposition, more lignin breaks down than hemicellulose or cellulose (Żółciak, 2019). As can be seen in Fig. 4, all strains had the ability to oxidize ABTS as a result of the oxidative enzymes. Strains LE-BIN 4342, 4343, 4346, 4350, and 4365 showed high oxidoreductase activity in qualitative spot-test (Fig. 4).



**Fig. 4.** Results of the express assays of oxidative (ABTS) and cellulolytic (CMC) enzymes in pure cultures of *Sarcodontia crocea*.

As a result of the research, a collection of pure cultures of *S. crocea* with the expressed potential of ligno- and cellulolytic enzymes was formed and conditions were selected for reliable maintenance of strains in the LE-BIN collection with preservation of their growth and biosynthetic activity were selected. This study also allowed us to ensure a sufficient set of strains to further solve the problems of *S. crocea* ecology, for instance, connected with interspecies relations of different xylo-trophic pathogens.

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## Культуральные характеристики и ферментативная активность штаммов *Sarcodontia crocea* (*Basidiomycota*) с территории Среднерусской возвышенности

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В условиях чистой культуры исследованы биологические свойства ксилотрофного гриба *Sarcodontia crocea* (*Polyporales*, *Basidiomycota*), развивающего плодовые тела преимущественно на стволах семечковых плодовых культур (*Malus domestica*) и проявляющего фитопатогенную активность. Проведена морфологическая и молекулярная верификация штаммов *Sarcodontia crocea*, выделенных в чистую культуру в 2019 г. путем высева базидиоспор и непосредственно из образцов свежих базидиом, растущих на *Malus domestica* в Белгородской и Орловской областях (Среднерусская возвышенность). Получены новые сведения о микроморфологических (наличие хламидоспор и анастомозов на дикариотическом мицелии) и молекулярно-биологических (полные нуклеотидные последовательности ITS1–5.8S–ITS2 области ярдНК) характеристиках этого вида. Определены максимальная скорость роста мицелия на агаризованной среде МЭА (до 5.5 мм/сут) и проведена оценка окислительной и целлюлитической активностей *Sarcodontia crocea*. Установлено, что у всех штаммов наблюдалась способность к окислению АВТС в результате работы окислительных ферментов. Штаммы *S. crocea* LE-BIN 4342, 4343, 4346, 4350 и 4365 показали высокую активность оксидоредуктаз.

**Ключевые слова:** деревообитающие грибы, ксилотрофные фитопатогены, культивирование грибов, лигниназы, микроморфология, скорость роста мицелия, целлюлазы