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PHENOLOXIDASE ACTIVITY OF DARK PIGMENTED YEAST-LIKE FUNGI OF *AUREOBASIDIUM* AND *HORMONEMA* GENERA

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The phenoloxidase activity as a possible taxonomic feature of *Aureobasidium* and *Hormonema* strains was studied by qualitative method on agar media (potato-dextrose agar, Czapek's medium and water agar), where 0.05 % of α-naphthol, guaiacol, and D-tyrosine were added, respectively. Tyrosinase and laccase activities of 13 tested strains of dark pigmented yeast-like fungi *Aureobasidium pullulans*, *Hormonema dematioides*, *Hormonema* sp. and *Rhinocladiella* sp. were intracellular, which cannot be detected outside of the cells. These activities didn't depend on the radioactivity level of habitats of isolated strains. The laccase presence for the studied *A. pullulans* strains was not detected on Czapek's medium and water agar, the tyrosinase reaction was absent or very low. *Hormonema* sp., *H. dematioides* and *Rhinocladiella* sp. strains detected significant level of laccase activity, the tyrosinase activity was medium or high. The quantitative determination of laccase and tyrosinase activities was carried out by using 11 substrates (catechol, L-DOPA, pyrogallol, p-cresol, D-tyrosine, p-phenylenediamine, guaiacol, 2,6-DMOP, phloroglucinol, resorcinol and hydroquinone) at pH 4.0 and 7.0. The tyrosinase and laccase activities were absent in culture filtrates of studied strains and were observed only in cell extracts. The tyrosinase and laccase activities of *Hormonema* sp., *H. dematioides* and *Rhinocladiella* sp. strains were absent or significantly lower at pH 7.0 than at pH 4.0. The tyrosinase activity prevails for *A. pullulans* and laccase activity – for *Hormonema* strains, that can be used as an important additional criterion for identification of fungi of *Aureobasidium* and *Hormonema* genera.

Keywords: fungi identification, *Aureobasidium*, *Hormonema*, laccase, tyrosinase.

INTRODUCTION

Studies of many scientists were devoted to the presence of single laccase and tyrosinase genes and peculiarities of their expression, characterization of phenoloxidase inducers, physical and chemical characteristics of these enzymes [6, 13, 14]. At the same time, identification of dark pigmented fungus *Aureobasidium pullulans* is difficult because other morphologically similar to species of this group from genus *Hormonema*

Lagerb. et Melin are isolated from different substrates. The genera *Aureobasidium* and *Hormonema* differ from each other by the formation of conidia on multi-locus hyphal cells (2–14 conidiogenous loci per cell for *Aureobasidium*, and 1–2 for *Hormonema*). These genera and their species characterize by significant pleomorphism and variability of morphological structures, hence it is difficult to distinguish them [15, 17]. Thus, for identification of this fungal group is necessary to use not only morphological and cultural features, but also supplemental criteria: physiological, ecological or genetic peculiarities. The taxonomic status of *Aureobasidium* and *Hormonema* remains controversial, however, as these two genera are not well-differentiated using molecular techniques and physiological characteristics [4, 17].

Despite the wide distribution of these fungi in nature, this group is not enough studied. So, the study of the biological characteristics of these fungi and their physiological features is important. For isolation of dark pigmented yeast-like species and to study their morphological and cultural features, monitoring of mycobiota of soil and forest litter of the Chornobyl nuclear power plant (ChNPP) zone was carried out [18]. The aim of this work was to study the phenoloxidase activity of *Aureobasidium* and *Hormonema* strains as a possible taxonomic feature.

MATERIALS AND METHODS

As the objects of this study 13 strains of dark pigmented yeast-like fungi were used: *Aureobasidium pullulans* (de Bary) Arnaud (5), *Hormonema dematioides* Lagerberg et Melin (3), *Hormonema* sp. (3), and *Rhinocladiella* sp. (2) strains (Table 1).

Qualitative laccase and tyrosinase activity determination of 13 strains of dark pigmented yeast-like fungi was carried out on potato-dextrose agar (PDA), Czapek's medium (CM) and water agar (WA) with addition of 0.05% of α-naphthol, guaiacol, and D-tyrosine, respectively [1, 9, 10]. Change of color of nutrient medium with α-naphthol in blue and purple, and medium with guaiacol in red-brown indicates the presence of laccase. Change of color of the nutrient medium with D-tyrosine to brown or red-brown indicates the presence of tyrosinase. The nutrient media without α-naphthol, guaiacol and D-tyrosine were used as the controls.

For quantitative determination of laccase and tyrosinase activities, the cultures of micromycetes on stubble potato-dextrose agar in tubes during 14 days at 26±2 °C were grown. Then, the spore suspensions of studied strains (1×10^6 conidia/ml), which were transferred into Erlenmeyer flasks (750 ml volume capacity) with 100 ml of liquid Czapek's medium (with 3 % glucose as a single carbon source) were prepared. The strains were cultivated during 96–100 h on a shaker at 220 rpm rotation and 25 °C [1].

Biomass of strains was separated by centrifugation at 3,000 g for 30 min at 4 °C [16], washed with 0.7 M phosphate buffer (pH 7.0), centrifuged and were suspended in a small volume (5 ml) of the same buffer. Cells were destroyed in liquid nitrogen (–196 °C). Homogenates were centrifuged at 4,500 g for 30 min at 4 °C. The obtained cell extracts and culture filtrates were used for assay of the laccase and tyrosinase activities.

Assay of laccase and tyrosinase activities on spectrophotometer SPECORD UV VIS (Germany) at the temperature 25 °C was carried out. As a unit of activity was the amount of enzyme that catalyzed the conversion of 1 nmol substrate per 1 min. Specific activity was calculated as the number of units of enzyme activity per 1 mg of protein. The protein content in culture filtrates and cell extracts was determined by the method of Lowry et al. [8], and bovine serum albumin was used as a standard (control).

Table 1. **The studied dark pigmented yeast-like fungi strains**

Таблиця 1. **Досліджені штами темнопігментованих дріжджоподібних грибів**

Species, strains	Habitat, year of isolation	Radioactivity of substrate
Aureobasidium pullulans 2274	Forest soil, Kyiv region, 1970	Natural background
A. pullulans 7331	Soil from salt marshes, Kherson region, 1967	Natural background
A. pullulans 28M	Ancient sculpture, Crimea, 1996	Natural background
A. pullulans 2318	Soil, the Chornobyl nuclear power plant (ChNPP) zone, 1989	1.9×10^4 Bk/kg
A. pullulans 2388	Soil, village Novo-Shepelychi, 1991	1.4×10^3 Bk/kg
Hormonema dematioides 185	Internal wall of 4th block ChNPP,1997	2×10^4–1×10^5 Bk/ sm^2
H. dematioides 184	Internal wall of 4th block ChNPP,1997	2×10^4–1×10^5 Bk/ sm^2
H. dematioides 23a	Internal wall of 4th block ChNPP,1997	2×10^4–6×10^4 Bk/ sm^2
Hormonema sp. 1161	Forest litter, village Leliov, 1999	1.5×10^4 Bk/kg
Hormonema sp. 1162	Sphagnum moss, Zhytomyr region, 1999	8×10^3–1×10^4 Bk/kg
Hormonema sp. 1125	Sphagnum moss, Zhytomyr region, 1999	8×10^3–1×10^4 Bk/kg
Rhinocladiella sp. 359M	Forest litter, Poltava region 1998	Natural background
Rhinocladiella sp. 863	Soil, ChNPP zone, 1999	3.7×10^5 Bk/kg

The volume of reaction mixtures was 2.7 ml. The reaction mixture for assay of laccase and tyrosinase activities included: 0.7 M phosphate buffer (pH 7.0) or 0.1 M NaOH-citrate buffer (pH 4.0) – 0.3 ml, substrate solution (5 mM) – 2.0 ml, and culture filtrate or cell extract of fungus – 0.4 ml [19].

For assay of tyrosinase activity, 0.7 M phosphate buffer (pH 7.0) and 0.1 M NaOH-citrate buffer (pH 4.0) were used, in which respective reaction substrates were dissolved. The tyrosinase activity with catechol as substrate was measured at 410 nm, L-DOPA – 475 nm, pyrogallol – 450 nm, p-cresol – 410 nm, and with D-tyrosine – 475 nm [19].

Assay of laccase activity was carried out in 0.7 M phosphate buffer (pH 7.0) and 0.1 M NaOH-citrate buffer (pH 4.0). The oxidation of substrates in a concentration of 5 mM was studied: p-phenylenediamine at 410 nm, guaiacol at 470 nm, 2,6-DMOP at 469 nm, phloroglucinol at 410 nm, resorcinol at 410 nm, hydroquinone at 410 nm [10, 11, 19].

Laccase and tyrosinase activity was calculated using the formula:

$$A = \frac{\Delta E \cdot V}{\varepsilon \cdot t \cdot a \cdot V_1},$$

where ΔE is change of extinction; V is volume of reaction mixture (ml); ε is molar extinction coefficient ($M^{-1}\times cm^{-1}$); t is time of incubation (min); a is protein concentration in studied solution (mcg); V_1 is volume of studied solution (ml).

Molar extinction coefficients were determined for each of studied substrates in phosphate and citrate buffers at the specified above wavelengths.

Obtained experimental data were statistically processed by using the software of Microsoft Excel and Statistica 6.0. The differences between values were calculated by the methods of variation statistics using Student *t*-test (for n = 9 $t_{0.95}$ = 2.262).

RESULTS AND DISCUSSION

For majority of the macromycetes (*Pleurotus ostreatus*, *Lentinula edodes*, *Agaricus bisporus*, *Amanita muscaria*, *Tuber magnatum*, *T. excavatum*), the maximum of their tyrosinase and laccase activities were at pH from 6.0 to 7.0. The high tyrosinase activity of the genus *Portabella* species was observed at pH 7.0, laccase activity at pH 3.5 [19], and the optimum of laccase activity of *Aureobasidium* fungi at pH 4.0 [5]. So, the above mentioned activities were studied at pH 4.0 and 7.0.

The activity of some enzymes of redox complex (laccase and tyrosinase) was determined by qualitative method for 13 strains of dark pigmented yeast-like fungi: *Aureobasidium pullulans* (5 strains), *Hormonema dematioides* (3 strains), *Hormonema* sp. (3 strains), and *Rhinocladiella* sp. (2 strains).

The presence of extracellular tyrosinase was detected for studied strains of micromycetes only on the PDA medium. On the other hand, reactions with guaiacol and α-naphthol (laccase tests) were absent (Table 2), except strain *Hormonema* sp. 1162, which was demonstrated the tyrosinase and laccase presence in the two tests with α-naphthol and guaiacol respectively. Tyrosinase and laccase activities of two *Rhinocladiella* sp. (359M, 863) and two *H. dematioides* strains (1125, 1161) were not detected.

Laccase presence in *A. pullulans* strains was not detected on CM and WA, the tyrosinase reaction was absent or very low. *Hormonema* sp. (strains 1125, 1161, 1162), *H. dematioides* (strains 184, 185) and *Rhinocladiella* sp. (strain 863) differed substantially from the others. These cultures had very strong laccase reactions, especially on WA. The tyrosinase reaction was medium or strong on these nutrient media. Notably, the color change of the nutrient medium on CM and WA was not observed, but the mycelium of the studied cultures was colored (Fig. 1). These data give the reason to conclude that tyrosinase and laccase of 13 tested strains of dark pigmented yeast-like fungi are intracellular, which are almost impossible to detect outside of the cell.

These activities did not depend on the radioactivity level of habitats of isolated strains (Tables 1, 2).

For quantitative determination of tyrosinase and laccase activities, 3 strains of different species were selected, which were characterized by the highest stability of morphological structure (*A. pullulans*, *Hormonema* sp. and *H. dematioides*) [18].

Laccase and tyrosinase activities of the 3 strains were investigated in culture filtrates and cell extracts. For determination of laccase and tyrosinase activities 11 substrates (phenolic compounds from plants and animals) were used. It was shown that tyrosinase and laccase activities were absent in culture filtrates of studied strains. These enzyme activities were observed only in cell extracts, except the reaction of *Hormonema* sp. 1125 with 2,6–dimethoxyphenol, laccase activity was three times higher in the culture filtrate almost than in supernatant (1.052 nmol/min×mg^{-1} protein) (Table 3). Thus, the obtained data of the qualitative determination of laccase and tyrosinase activities in strains grown on agar nutrient media confirms that these enzymes are produced by the studied fungi mainly intracellularly.

Table 2. **Presence of the phenoloxidase activity in dark pigmented yeast-like fungi**

Таблиця 2. **Наявність фенолоксидазної активності темнопігментованих дріжджоподібних грибів**

Strains	Nutrient medium								
	Potato-dextrose agar			Water agar			Capek agar		
	1	2	3	1	2	3	1	2	3
Aureobasidium pullulans									
2274	–	–	++	–	–	–	–	–	–
2318	–	–	+	–	–	–	–	–	–
2388	–	–	++	–	–	–	–	–	+
7331	–	–	+	–	–	–	–	+	+
28M	–	–	+	–	–	±	–	–	±
Hormonema dematioides									
184	–	–	++	++	+++	+	+++	±	+
185	–	–	+	++	++	+	–	+	+
23a	–	–	++	–	–	+	–	–	+
Hormonema sp.									
1125	–	–	–	+++	+++	+	+++	+	+
1161	–	–	–	+	+++	+	+++	–	±
1162	+++	+	+	+++	+++	+	+++	+++	+
Rhinocladiella sp.									
359M	–	–	–	–	–	±	–	–	±
863	–	–	–	++	+	–	–	–	–

Comments: 1. – no reaction, ± low, **+** medium, **++** strong, **+++** very strong reaction; **2**. 1 – α-naphthol, 2 – guaiacol, 3 – D-tyrosine.

Примітки: 1. – реакції немає, ± слабка, **+** чітка, **++** сильна, **+++** дуже сильна реакція; **2**. 1 – α-нафтол, 2 – гваякол, 3 – D-тирозин.

The tyrosinase and laccase activities in the studied strains were absent at pH 7.0 or significantly lower than at pH 4.0. Tyrosinase is able to use mono-, di-, and trihydroxyphenols as substrates; among these, dihydroxyphenols (catechols) provide the maximum enzymatic activity, indicating the enzyme, which is most active with catechol as a substrate [12, 20]. In our case, *A. pullulans* 2274 was characterized by the maximum of tyrosinase activity in tests with catechol, and laccase activity with 2,6-dimethoxyphenol.

The ability to produce tyrosinase or laccase with all 11 substrates, and laccase activity of *Hormonema* sp. strain 1125 was the highest in the presence of resorcinol.

The lowest tyrosinase and laccase activities had *H. dematioides* 185. Reactions were observed only with 4 plant substrates: resorcinol, catechol, pyrogallol, and phloroglucinol. Maximum activity was also achieved with resorcinol.

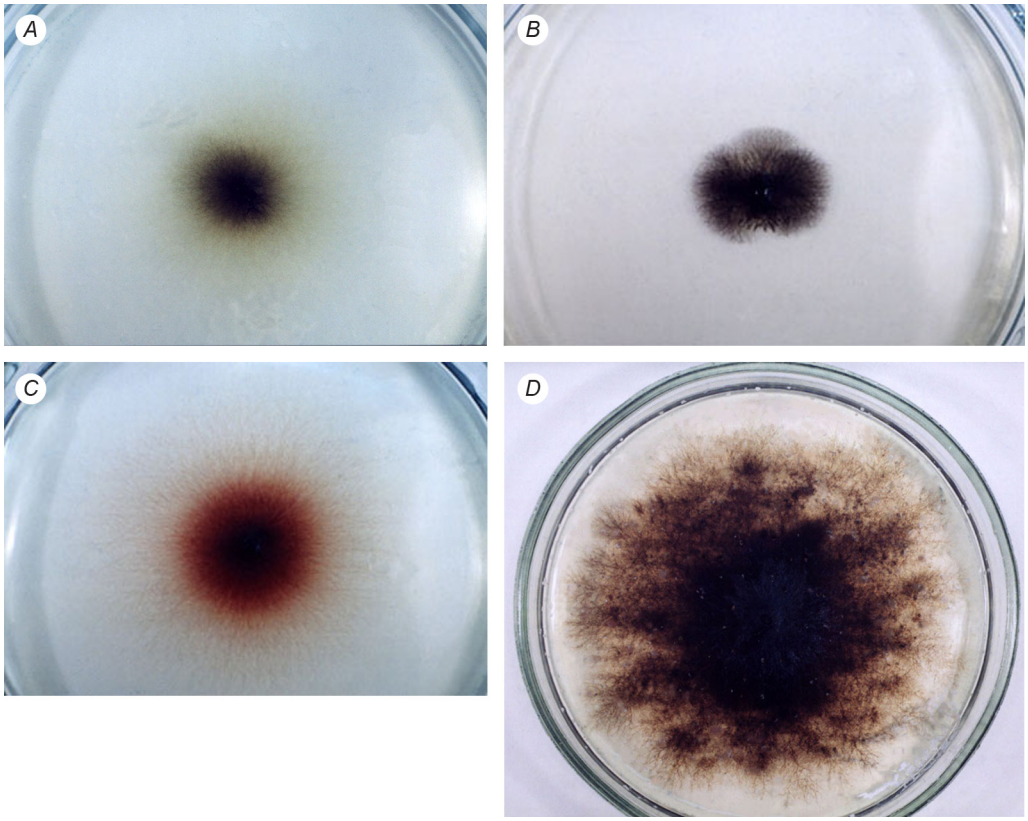

Fig. 1. Growth of *Hormonema* sp. 1161 on 21 days of cultivation at 25 °C:
water agar (control) (*A*); water agar with α-naphthol (purple coloration of mycelium indicates laccase presence) (*B*); water agar with guaiacol (red-brown coloration of mycelium indicates laccase presence) (*C*); and water agar with D-tyrosine (brown coloration of the mycelium indicates the tyrosinase presence) (*D*)

Рис. 1. Ріст *Hormonema* sp. 1161 на 21-шу добу культивування при 25 °C:
A – на водному агарі (контроль); *B* – на водному агарі з α–нафтолом (забарвлення міцелію у фіолетовий колір свідчить про наявність лакази); *C* – на водному агарі з гваяколом (забарвлення міцелію у червоно-коричневий колір свідчить про наявність лакази); *D* – на водному агарі з D–тирозином (забарвлення міцелію у коричневий колір свідчить про наявність тирозинази)

Laccase (EC 1.10.3.2), catecholoxidase (EC 1.10.3.1) and tyrosinase (EC 1.14.18.1) belong to phenoloxidases and have similar substrate specificity [3]. Some authors consider that catecholoxidase and tyrosinase is the same enzyme [2]. In the presence of molecular oxygen, enzymes of phenoloxidase complex catalyze not only the oxidation of different polyphenols and their derivatives, but also of monophenols to the quinones which in large quantities synthesize and produce plants in response to the presence inside other organisms like mycorrhizal, plant pathogenic and endophytic fungi. The distinction between polyphenoloxidases and tyrosinases is not always clear because under the specific conditions many polyphenol oxidases can hydroxylate monophenols [7].

Table 3. **Specific laccase and tyrosinase activities in cell extracts of the studied strains (nmol/min×mg^{-1} protein) ($P \geq 0.95$)**

Таблиця 3. **Питома лаказна і тирозиназна активності в клітинних екстрактах досліджених штамів (нмоль/хв×мг$^{-1}$ білка) ($P \geq 0,95$)**

Substrates	*Aureobasidium pullulans* 2274		*Hormonema* sp. 1125		*Hormonema dematioides* 185	
	1	2	1	2	1	2
Catechol	0.481± 0.053	–	0.392± 0.024	–	0.162± 0.021	–
Resorcinol	0.315± 0.039	–	1.143± 0.041	–	0.261± 0.008	–
p-Phenylenediamine	0.162± 0.032	–	0.639± 0.030	–	–	–
p-Cresol	–	–	0.660± 0.036	–	–	–
Hydroquinone	–	–	0.601± 0.029	0.550± 0.016	–	–
Pyrogallol	0.324± 0.017	–	0.415± 0.019	–	0.182± 0.033	0.149± 0.037
Phloroglucinol	–	–	0.519± 0.044	–	0.199± 0.016	–
2,6-DMOP	0.411± 0.026	–	0.345± 0.027	–	–	–
Guaiacol	0.279± 0.022	–	0.202± 0.037	–	–	–
L-DOPA	0.286± 0.016	–	0.569± 0.042	–	–	–
D-tyrosine	–	–	0.569± 0.024	–	–	–

Comments: **1**. «–» – activity was not shown; **2**. 1 – pH 4.0, 2 – pH 7.0.

Примітки: **1**. « » – активності не виявлено; **2**. 1 – pH 4.0, 2 – pH 7.0.

CONCLUSIONS

Tyrosinase and laccase of 13 tested strains of dark pigmented yeast-like fungi are intracellular and cannot be synthesized outside of the cells. Tyrosinase activity prevails for *A. pullulans*, and laccase activity for *Hormonema* strains, that can be used as an important additional criterion, – for the identification fungi of *Aureobasidium* and *Hormonema* genera.

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ФЕНОЛОКСИДАЗНА АКТИВНІСТЬ ТЕМНОПІГМЕНТОВАНИХ ДРІЖДЖОПОДІБНИХ ГРИБІВ РОДІВ *AUREOBASIDIUM* І *HORMONEMA*

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Досліджено фенолоксидазну активність як можливу таксономічну ознаку штамів *Aureobasidium* і *Hormonema* якісним методом на агаризованих середовищах (картопляно-глюкозному агарі, середовищі Чапека й водному агарі), до яких було додано 0,05% α-нафтолу, гваяколу та D-тирозину відповідно. Тирозиназа і лаказа 13 досліджених штамів темнопігментованих дріжджоподібних грибів *Aureobasidium pullulans*, *Hormonema dematioides*, *Hormonema* sp. та *Rhinocladiella* sp. є внутрішньоклітинними, які не визначаються за межами клітин. Ці активності не залежали від рівня радіоактивності місцеіснувань ізольованих штамів. Для досліджених штамів *A. pullulans* не було встановлено наявності лакази на середовищі Чапека та водному агарі, тирозиназної реакції не було або була дуже низькою. Штами *Hormonema* sp., *H. dematioides* і *Rhinocladiella* sp. виявили значний рівень лаказної активності, тирозиназна активність була помірною або високою. Кількісне визначення лаказної і тирозиназної активностей здійснено з використанням 11 субстратів (катехолу, L-DOPA, пірогалолу, р-крезолу, D-тирозину, р-фенілендіаміну, гваяколу, 2,6-DMOP, флороглюцину, резорцину і гідрохінону) при pH 4.0 і 7.0. Тирозиназної та лаказної активності не було в культуральних фільтратах штамів. Вони спостерігались тільки в безклітинних екстрактах. Тирозиназної і лаказної активності штамів *Hormonema* sp., *H. dematioides* і *Rhinocladiella* sp. не було при pH 7,0 або вони були суттєво нижчими, ніж при pH 4,0. Тирозиназна активність переважала у штамів *A. pullulans*, лаказна активність – у штамів *Hormonema*, що є важливим додатковим критерієм для ідентифікації грибів родів *Aureobasidium* і *Hormonema*.

Ключові слова: ідентифікація, *Aureobasidium*, *Hormonema*, лаказа, тирозиназа.

ФЕНОЛОКСИДАЗНАЯ АКТИВНОСТЬ ТЕМНОПИГМЕНТИРОВАННЫХ ДРОЖЖЕПОДОБНЫХ ГРИБОВ РОДОВ *AUREOBASIDIUM* И *HORMONEMA*

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Изучена фенолоксидазная активность как возможный таксономический признак штаммов *Aureobasidium* и *Hormonema* качественным методом на агаризованных средах (картофельно-глюкозном агаре, среде Чапека и водном агаре), к которым добавляли 0,05 % α-нафтола, гваякола и D-тирозина соответственно. Тирозиназа и лакказа 13 исследованных штаммов темнопигментированных дрожжеподобных грибов *Aureobasidium pullulans*, *Hormonema dematioides*, *Hormonema* sp. и *Rhinocladiella* sp. являются внутриклеточными, которые не определяются за пре-

делами клеток. Эти активности не зависели от уровня радиоактивности местообитаний изолированных штаммов. Для изученных штаммов *A. pullulans* не установлено наличие лакказы на среде Чапека и водном агаре, тирозиназная реакция отсутствовала или была очень низкой. Штаммы *Hormonema* sp., *H. dematioides* и *Rhinocladiella* sp. проявили значительный уровень лакказной активности, тирозиназная активность была умеренной или высокой. Количественное определение лакказной и тирозиназной активностей осуществлено с использованием 11 субстратов (катехола, L-DOPA, пирогаллола, р-крезола, D-тирозина, р-фенилендиамина, гваякола, 2,6-DMOP, флороглюцина, резорцина и гидрохинона) при pH 4.0 и 7.0. Тирозиназная и лакказная активности отсутствовали в культуральных фильтратах штаммов и наблюдались только в бесклеточных экстрактах. Тирозиназная и лакказная активности штаммов *Hormonema* sp., *H. dematioides* и *Rhinocladiella* sp. отсутствовали при pH 7,0 или были существенно ниже, чем при pH 4,0. Тирозиназная активность преобладала у штаммов *A. pullulans*, лакказная активность – у штаммов *Hormonema*, что является важным дополнительным критерием для идентификации грибов родов *Aureobasidium* и *Hormonema*.

Ключевые слова: идентификация, *Aureobasidium*, *Hormonema*, лакказа, тирозиназа.

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