ISSN 0026-2617, Microbiology, 2017, Vol. 86, No. 4, pp. 503–511. © Pleiades Publishing, Ltd., 2017.
Original Russian Text © N.M. Myasoedova, Zh.V. Renfeld, E.V. Podieiablonskaia, A.S. Samoilova, A.M. Chernykh, T. Classen, J. Pietruszka, M.P. Kolomytseva, L.A. Golovleva, 2017, published in Mikrobiologiya, 2017, Vol. 86, No. 4, pp. 494–503.

EXPERIMENTAL ARTICLES

Novel Laccase–Producing Ascomycetes

N. M. Myasoedova[a], Zh. V. Renfeld[b], E. V. Podieiablonskaia[a], A. S. Samoilova[a], A. M. Chernykh[a], T. Classen[c], J. Pietruszka[c, d], M. P. Kolomytseva[a, *], and L. A. Golovleva[a]

[a] *Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Russia*
[b] *Pushchino State University of Natural Sciences, Pushchino, Russia*
[c] *Institute of Bio- and Geosciences (IBG-1), Forschungszentrum Jülich GmbH, Germany*
[d] *Institute of Bioorganic Chemistry, Heinrich Heine University Düsseldorf, Germany*
**e-mail: mkolomytseva@rambler.ru*

Received November 3, 2016

Abstract—Screening of ascomycetes producing laccases during growth on agar medium or submerged cultivation in the presence of various natural sources of carbon and energy (grain crops and potato) was carried out. The conditions of submerged cultivation of the most active strains (*Myrothecium roridum* VKM F-3565, *Stachybotrys cylindrospora* VKM F-3049, and *Ulocladium atrum* VKM F-4302) were optimized for the purpose of increasing laccase activity. The pH-optima and substrate selectivity of laccases in the culture liquid of the strains in relation to ABTS and phenolic compounds (2,6-dimethoxyphenol, syringaldazine, ferulic acid, *p*-coumaryl alcohol, and coniferyl alcohol) were investigated. High laccase activity at neutral pH was shown for the culture liquids of *M. roridum* VKM F-3565 and *S. cylindrospora* VKM F-3049 strains that provides prospects for using laccases of these strains in various cell biotechnologies.

Keywords: laccase, laccase activity, fungi, ascomycetes, submerged cultivation, pH-optimum, substrate selectivity, phenolic compounds

DOI: 10.1134/S0026261717030110

Laccase (EC 1.10.3.2, *para*-diphenol: oxygen oxidoreductase) is a copper-containing enzyme, which catalyzes oxidation of a wide range of substrates with the involvement of molecular oxygen and reduces it to water. This enzyme has been found in bacteria, fungi, plants, and insects (Baldrian, 2006).

The fungal laccase, in contrast to laccases isolated from other organisms, has a high redox potential and, as a result, can oxidize a wide range of substrates with different structures and properties (Baldrian, 2006): mono-, di-, and polyphenols, amino- and methoxy-substituted phenols, aromatic amines, or inorganic ions ($[Mo(CN)_8]^{4-}$, $[Fe(CN)_6]^{4-}$, Mn^{2+}). These properties have made laccase one of the most widespread tools in modern biotechnology, where it is used for degradation of xenobiotics, delignification and bleaching of plant fiber, decolorization of dyes in textile wastewater, in production of high-density fiberboards and cardboard without using toxic binding agents, in cosmetics, food industry, organic synthesis of compounds having novel properties, and in biosensor development (Baldrian, 2006; Riva, 2006; Kunamneni et al., 2008; Mikolasch and Schauer, 2009; Kudanga et al., 2011; Polak and Jarosz-Wilkolazka, 2012; Margot et al., 2013).

Therefore, the search of new enzymes and their producers is still an urgent issue. Among all laccases produced by the known microorganisms, the laccases of basidiomycete fungi have been studied most thoroughly (Baldrian, 2006). The laccases of other higher fungi (ascomycetes) have been least studied (Banerjee and Vohra, 1991; Hao et al., 2007; Junghanns et al., 2008; Chakroun et al., 2010; Elshafei et al., 2012; Feng et al., 2013). In contrast to basidiomycete fungi, ascomycetes can grow more rapidly and produce laccases at the early stages of cultivation (Banerjee and Vohra, 1991; Hao et al., 2007; Elshafei et al., 2012; Feng et al., 2013).

The goal of the present work was to search for novel ascomycete strains producing laccases, as well as to optimize the cultivation conditions for the most productive strains in order to increase the yield of laccases and their characterization.

MATERIALS AND METHODS

Microorganisms. The ascomycetes (39 strains) used in the work were obtained from the All-Russian Collection of Microorganisms of the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (Table 1).

Reagents. The following reagents were used in the work: 2,2-azino-bis(3-ethylbenzothiazoline 6-sulfonic acid) (ABTS) (AppliChem, Germany); syringal-

Table 1. Screening of laccase—producing ascomycetes (25-day cultivation)

No.	Strain	Origin	Mineral medium*			
			mixture of 7 cereal crops (20 g/L), agar		mixture of 7 cereal crops (20 g/L), liquid	
			activity with ABTS*	day of emergence	specific activity with ABTS, U/mL	day of emergence
1	*Acrostalagmus luteoalbus* VKM F-3548	Volcanic soil	++	1	0	–
2	*Acrostalagmus luteoalbus* VKM F-3552	Potato fields	++	1	0.49 ± 0.04	17
3	*Biscogniauxia nummularia* VKM F-1247	Plant, *Fagus* sp.	+	3	0	–
4	*Botryosphaeria rhodina* VKM F-1175	Plant, *Citrus aurantium*	+	3	0	–
5	*Ceratocystis paradoxa* VKM F-413	Plant, *Elaeis guineensis*, on roots	+	2	0	–
6	*Ceratocystis pilifera* VKM F-1453	Plant, *Pinus* sp.	+	6	0	–
7	*Chaetomium globosum* VKM F-109	Plant, *Linum usitatissimum*	+	3	0	–
8	*Chrysosporium merdarium* VKM F-3547	Volcanic soil	–	–	0	–
9	*Cladosporium herbarium* VKM F-1692	Rhizosphere of pine, *Pinus* sp.	+	6	0	–
10	*Cladosporium herbarium* VKM F-2290	*Querqus acorn*, acorn	++	2	0.085 ± 0.03	14
11	*Cladosporium colocasiae* VKM F-767	Plant, *Colocasia esculenta,* on leaf	+	2	0	–
12	*Cladosporium halotolerans* VKM F-2804	Fungi, *Aureobasidium caulivorum*	+	6	0	–
13	*Cladosporium herbarium* VKM F-235	Plant, *Scirpus* sp.	+	1	0	–
14	*Cryphonectria parasitica* VKM F-3897	Plant, *Castanea saliva*	++	1	0	–
15	*Doratomyces nanus* VKM F-4326	Coniferous litter (3–5 cm), in subtaiga, on mountain slopes	+	2	0	–
16	*Humicola grisea* VKM F-3571	Potato, *Solanum tuberosum*	–	–	0	–
17	*Gonytrichum caesium* VKM F-1570	Plant, *Querqus* sp., deadwood	+	1	0.146 ± 0.04	14
18	*Gonytrichum macrocladum* VKM F-3847	Chernozem	+	2	0	–
19	*Lophodermium pinastri* VKM F-3221	Plant, *Pinus* sp.	–	–	0	–
20	*Myrothecium cinctum* VKM F-1489	Plant, *Avena sativa*, on roots	+	3	0	–

Table 1. (Contd.)

No.	Strain	Origin	Mineral medium*			
			mixture of 7 cereal crops (20 g/L), agar		mixture of 7 cereal crops (20 g/L), liquid	
			activity with ABTS*	day of emergence	specific activity with ABTS, U/mL	day of emergence
21	*Myrothecium roridum* VKM F-3564	Potato field	+	2	0	–
22	*Myrothecium roridum* VKM F-3565	Soil of thermal landscapes	+	1	1.7 ± 0.06	7
23	*Neonectria galligena* VKM F-1187	Plant, *Malus sylvestris*	+	1	0	–
24	*Ophiostoma piceae* VKM F-3181	Plant, *Querqus robur*	+	1	0	–
25	*Pseudogymnoascus roseus* VKM F-1158	Plant, woody detritus	–	–	0	–
26	*Sclerotinia ricini* VKM F-100	Plant, *Ricinus communis*	+	1	0	–
27	*Stachybotrys chartarum* VKM F-3839	Desert soil	+	1	0	–
28	*Stachybotrys cylindrospora* VKM F-3049	DuraSil silicone adhesive (Germany), wood	++	1	0.846 ± 0.05	8
29	*Talaromyces luteus* VKM F-308	Plant, *Citrus limon*	+	6	0	–
30	*Talaromyces stipitatus* VKM F-2090	Plant, decaying wood	–	–	0	–
31	*Thielavia appendiculata* VKM F-1733	Rhizosphere of maize, *Zea mays*	+	3	0	–
32	*Thielavia terricola* VKM F-1719	Plant, *Ficus* sp.	+	1	0	–
33	*Ulocladium atrum* VKM F-4301	Plant, *Solanum tuberosum*, on leaves	+	1	0	–
34	*Ulocladium atrum* VKM F-4302	Plant, *Triticum aestivum*	+	1	0.69 ± 0.08	8
35	*Ulocladium botrytis* VKM F-2131	Flax fiber, *Linum usitatissimum*	+	3	0	–
36	*Ulocladium botrytis* VKM F-543	Plant, *Scirpus* sp.	+	3	0	–
37	*Ulocladium chartarum* VKM F-1866	Plant, *Daucus carota*	+	2	0	–
38	*Ulocladium chartarum* VKM F-1875	Plant, *Citrus limon*	++	1	0	–
39	*Ulocladium chartarum* VKM F-3837	Plant, *Picea* sp.	+++	2	0	–

* The diameter of stained area around the fungal colony: "+"—less than 0.5 cm, "++"—0.5–1 cm, "+++"—more than 1 cm.

dazine (Sigma-Aldrich, United States); 2,6-dimethoxyphenol (Fluka, Germany). *p*-Coumaryl alcohol was synthesized from *p*-coumaric acid via preliminary esterification in methanol under acidic conditions with microwave irradiation, followed by reduction according to the method described previously (Pickel et al., 2010). Ferulic acid and coniferyl alcohol were produced by Serva (Germany). All other reagents used in the work were analytically pure and produced in Russia.

Cultivation techniques. The strains were maintained in test tubes with glucose-potato agar slants (g/L: glucose, 10; fresh finely grated potato, 200; agar, 20) and stored at 4°C.

The fungal strains showing laccase activity were initially screened on an agar mineral medium (g/L: NH_4NO_3, 0.2; KH_2PO_4, 0.2; K_2HPO_4, 0.02; $MgSO_4 \cdot 7H_2O$, 0.12; agar, 20) containing 20 g/L of the mixture of seeds of 7 cereal plants (equal amounts of rye, wheat, oats, barley, buckwheat, rice, and maize) as a carbon and energy source and 0.5 mM ABTS as an indicator of laccase activity. Laccase activity of the fungal strains was assessed by ABTS staining in the agar.

Submerged cultures of the strains were screened in test tubes with 20 mL of a liquid mineral medium (g/L: NH_4NO_3, 0.2; KH_2PO_4, 0.2; K_2HPO_4, 0.02; $MgSO_4 \cdot 7H_2O$, 0.12) containing 20 g/L of the mixture of seeds of 7 cereal plants (the equal portions of rye, wheat, oats, barley, buckwheat, rice, and maize). The inocula were washouts (1 mL) of 5-day cultures grown in the test tubes with glucose-potato agar.

In the experiments on optimization of the cultivation conditions in order to increase the laccase activity, the inocula were grown in 750-mL flasks under stirring (200 rpm) for 5–7 days at 29°C in 100 mL of soy-glycerol medium (Myasoedova et al., 2015). The medium contained the following (g/L): NH_4NO_3, 0.2; KH_2PO_4, 0.2; K_2HPO_4, 0.02; $MgSO_4 \cdot 7H_2O$, 0.1; peptone, 0.5; soy flour, 0.5; and glycerol, 4.0; pH 5.0. The mycelium separated from the culture liquid was homogenized using porcelain beads under stirring (200 rpm) for 10 min and added as inocula into 750-mL flasks at the ratio of 10 mL of homogenized mycelium per 100 mL of the liquid mineral medium (g/L: NH_4NO_3, 0.2; KH_2PO_4, 0.2; K_2HPO_4, 0.02; $MgSO_4 \cdot 7H_2O$, 0.12) containing one of the carbon and energy sources at different concentrations (the ground seeds of rye, wheat, oats, barley, buckwheat, rice, maize, pea, bean, or fresh finely grated potato tubers).

Determination of the activity and substrate selectivity of fungal laccases in the culture liquid. During the screening of productive strains, laccase activity was detected using an UV-160 spectrophotometer (Shimadzu, Japan) in a quartz cuvette with an optical path length of 10 mm by the rate of ABTS oxidation, using the molar extinction coefficient: $\varepsilon_{436} = 29300\ M^{-1}\ cm^{-1}$ (Perez et al., 1990). The reaction mixture contained 1 mM of the substrate, the enzyme preparation (culture liquid), and 20 mM of Na-acetate buffer, pH 5.0. The average rate of transformation of 1 μM of substrate per 1 min in 1 mL of the buffer was accepted as a unit of laccase activity.

The substrate specificity of laccases in the fungal culture liquid was determined spectrophotometrically by the rate of product accumulation or oxidation of 0.02 mM of the initial substrate with the following molar extinction coefficients: $\varepsilon_{470} = 35645\ M^{-1}\ cm^{-1}$ for 2,6-dimethoxyphenol (Eggert et al., 1996); $\varepsilon_{525} = 65000\ M^{-1}\ cm^{-1}$ for syringaldazine (Leonowicz and Grzywnowicz, 1981); $\varepsilon_{259} = 13210\ M^{-1}\ cm^{-1}$ for *p*-coumaryl alcohol (Sterjiades et al., 1992); $\varepsilon_{263} = 13400\ M^{-1}\ cm^{-1}$ for coniferyl alcohol (Paszczyński et al., 1986); and $\varepsilon_{287} = 12483\ M^{-1}\ cm^{-1}$ for ferulic acid (Sterjiades et al., 1992). The measurements were made in 20 mM Na-acetate buffer, pH 5.0, or in 50 mM Tris-HCl buffer, pH 7.2, at 25°C.

Determination of the pH-optimum of laccases in the culture liquid of the fungal strains. The changes in laccase activity of the culture liquid depending on pH value of the medium were determined spectrophotometrically by the rate of formation of stained products during substrate oxidation: 0.1 mM ABTS (Perez et al., 1990); 0.02 mM syringaldazine (Leonowicz and Grzywnowicz, 1981); or 0.1 mM 2,6-dimethoxyphenol (Eggert et al., 1996) in the universal Britton-Robinson buffer (Xu, 1996) within the pH range from 2.5 to 8.0 at 25°C.

RESULTS

Screening of active laccase producers. Laccase producers were searched among 39 ascomycete strains isolated from diverse natural habitats (Table 1): woody and herbaceous plants, soil, and extreme biotops.

Assuming that the most of ascomycetes are the parasites of cereals, a mixture of seven cereal crops was used for initial screening as a growth substrate for the fungi. During cultivation of the fungi on a mineral agar containing 20 g/L of the mixture of ground seeds of seven cereal crops and the indicator of laccase activity, ABTS, 36 strains with different degrees of laccase activity were selected (Table 1). The tested fungi demonstrated laccase activity on day 1–6 of mycelium cultivation on the agar medium.

Submerged cultivation of the fungi in a liquid mineral medium with 20 g/L of the mixture of seeds of seven cereal crops made it possible to select a much lesser number of ascomycete strains with laccase activity (Table 1): *Acrostalagmus luteoalbus* VKM F-3552, *Myrothecium roridum* VKM F-3565, *Cladosporium herbarium* VKM F-2290, *Gonytrichum caesium*VKM F-1570, *Stachybotrys cylindrospora* VKM F-3049, and

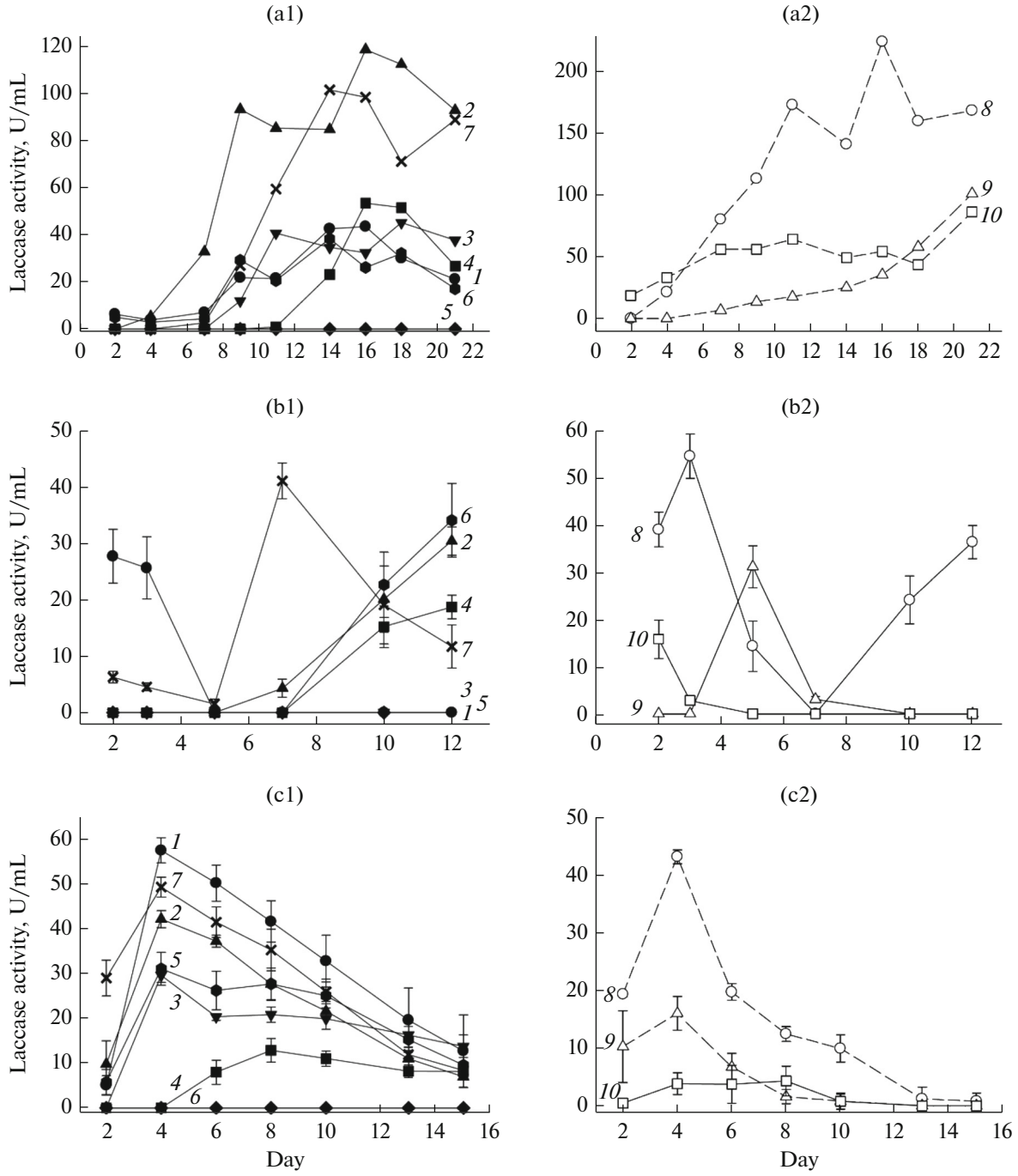

Fig. 1. The dynamics of laccase activity of selected fungi during their submerged cultivation in the presence of 20 g/L of one of the carbon and energy sources: buckwheat (*1*), wheat (*2*), barley (*3*), maize (*4*), rice (*5*), oats (*6*), rye (*7*), pea (*8*), bean (*9*), and potato (*10*) for *M. roridum* VKM F-3565 (a1, a2); *S. cylindrospora* VKM F-3049 (b1, b2); and *U. atrum* VKM F-4302 (c1, c2).

Ulocladium atrum VKM F-4302. Laccase activity was observed in these strains on day 7–17 of submerged cultivation, i.e., much later compared to the cultivation of the same fungi on agar (Table 1).

Optimization of cultivation conditions of the fungi in order to increase the laccase activity. Initially, the dynamics of laccase activities in the selected fungi was investigated during submerged cultivation in a liquid mineral medium with one of the carbon and energy sources of plant origin (20 g/L): the ground seeds of rye, wheat, oats, barley, buckwheat, rice, maize, pea, or kidney bean, as well as fresh finely grated potato tubers (Fig. 1).

During submerged cultivation of the ascomycete *M. roridum* VKM F-3565, the peak of laccase activity was observed in the cultures of different age depending

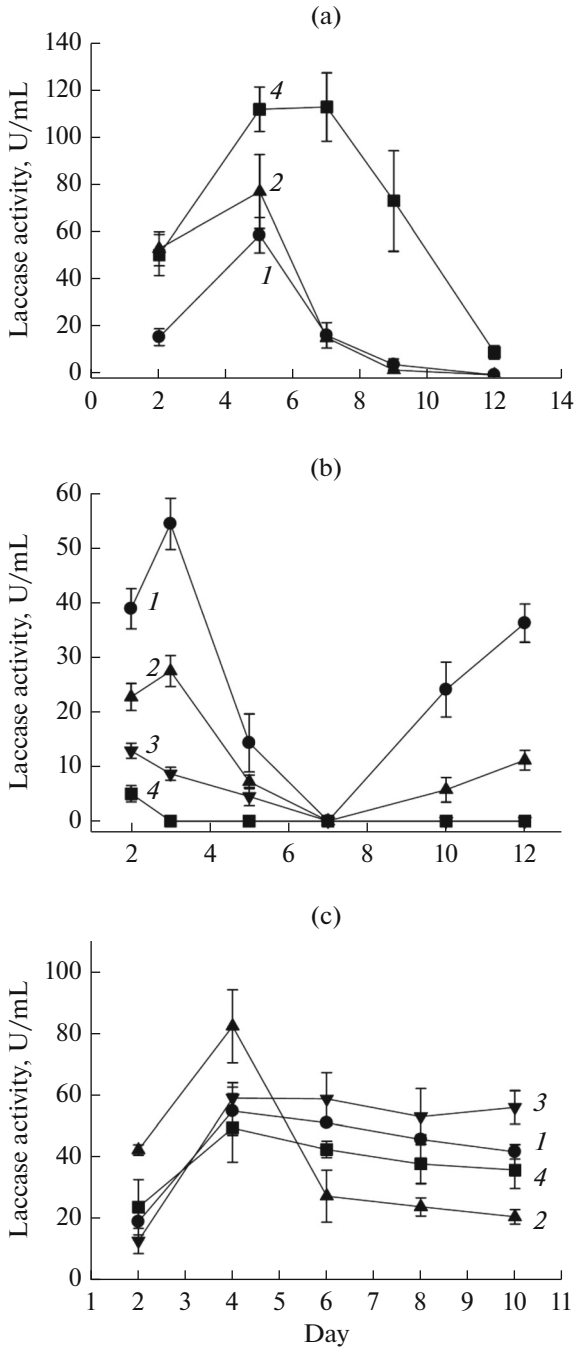

Fig. 2. The dynamics of laccase activity of selected fungi during their submerged cultivation in the presence of different concentrations of optimal substrates: *M. roridum* VKM F-3565 in the presence of 20 (*1*), 40 (*2*) and 80 (*4*) g/L pea (a); *S. cylindrospora* VKM F-3049 in the presence of 20 (*1*), 40 (*2*), 60 (*3*) and 80 (*4*) g/L pea (b); *U. atrum* VKM F-4302 in the presence of 20 (*1*), 40 (*2*), 60 (*3*) and 80 (*4*) g/L buckwheat (c).

on the substrate (Fig. 1a). In the medium with 20 g/L of pea or potato, laccase activity peaked after 7–11 days of cultivation; in the media with other substrates, the peak of laccase activity appeared later—on days 9–18 of growth. Pea was the optimal growth substrate yielding the maximum laccase activity (66.5 U/mL of activity on day 7 of cultivation).

The maximum laccase activity of the ascomycete *S. cylindrospora* VKM F-3049 during submerged cultivation in the presence of pea, kidney bean, or potato was observed at the beginning of cultivation (day 1–5); in the media with other carbon sources (cereal crops), the peak of laccase activity appeared after 7–12 days of cultivation (Fig. 1b). Pea was the optimal carbon source yielding the maximum laccase activity (54.4 U/mL on day 3 of cultivation).

The study of dynamics of laccase activity of the ascomycete *U. atrum* VKM F-4302 during submerged cultivation with different natural carbon sources revealed one peak of laccase activity on day 4 of cultivation on most substrates except for maize; with the latter, the fungus showed the maximum activity on day 8 of cultivation (Fig. 1c). The best substrate yielding the maximum laccase activity was buckwheat (57.3 U/mL of activity on day 4 of cultivation).

Thereafter, the optimal substrate concentrations yielding maximum laccase activities in the selected fungal strains during their submerged cultivation were determined (Fig. 2).

The optimal concentration of the best substrate (pea) during submerged cultivation of the fungus *M. roridum* VKM F-3565, with the maximum laccase activity of 113.0 U/mL on day 7 of cultivation, was 80 g/L (Fig. 2a). The maximum laccase activity (54.4 U/mL) of the fungus *S. cylindrospora* VKM F-3049 was reached during submerged cultivation in the medium with 20 g/L of pea (Fig. 2b). Different concentrations of the best carbon source (buckwheat) were tested during submerged cultivation of the fungus *U. atrum* VKM F-4302, and the optimal concentration (40 g/L) yielding the maximum laccase activity of this strain (83.0 U/mL) was observed on day 4 of cultivation (Fig. 2c).

The pH-optimum of laccases in the culture liquid of fungal strains. The culture liquids of all strains under study were most active with ABTS at low pH, which is in agreement with the literature data for most of the known laccases and is associated with the nature of ABTS (Baldrian, 2006).

The study of dependence of laccase activity in the fungal culture liquid on pH of the reaction medium revealed one ascomycete strain, *M. roridum* VKM F-3565, producing laccases active with syringaldazine and 2,6-dimethoxyphenol under neutral pH conditions (Table 2). Most of the other tested strains exhibited laccase activities at low pH values (Table 2), similar to the typical laccases of basidiomycete fungi (Baldrian, 2006), except for the strain *A. luteoalbus* VKM F-3552: its culture liquid was more active with syringaldazine at pH 7.5 (Table 2).

Substrate selectivity of laccases in the fungal culture liquid. For verification of pH selectivity of laccases

Table 2. The pH-optima of laccases in the culture liquids of selected fungal strains

Substrate	pH-optimum		
	ABTS	2,6-dimethoxyphenol	syringaldazine
Acrostalagmus luteoalbus VKM F-3552	3.5	3.5	7.5
Cladosporium herbarium VKM F-2290	3.5	6.0	6.0
Gonytrichum caesium VKM F-1570	3.5	3.5	6.0
Myrothecium roridum VKM F-3565	4.3	6.5	7.0
Stachybotrys cylindrospora VKM F-3049	4.0	5.0	5.5
Ulocladium atrum VKM F-4302	3.0	4.0	6.5

Table 3. The substrate specificity of laccases in the culture liquid of the most active fungi

Substrate	Specific activity of the culture liquid of the strains, U/mL (V_{rel}, %)					
	Myrothecium roridum VKM F-3565		*Stachybotrys cylindrospora* VKM F-3049		*Ulocladium atrum* VKM F-4302	
	pH 5.0	pH 7.2	pH 5.0	pH 7.2	pH 5.0	pH 7.2
ABTS	37.2 (100.0)	4.6 (100.0)	28.4 (100.0)	0.0 (0.0)	4.2 (100.0)	0.0 (0.0)
Syringaldazine	0.0 (0.0)	4.2 (91.0/11.3*)	70.6 (249.0)	41.6 (146.9*)	3.7 (88.1)	2.3 (54.8*)
2,6-Dimethoxyphenol	4.5 (12.1)	13.2 (287.0/35.5*)	22.1 (78.0)	18.1 (63.7*)	2.2 (52.4)	0.6 (14.3*)
Ferulic acid	5.3 (14.3)	9.2 (200.0/24.7*)	31.4 (110.6)	17.5 (61.7*)	2.4 (57.1)	1.4 (33.3*)
p-Coumaryl alcohol	4.9 (13.2)	5.0 (108.7/13.4*)	2.7 (9.5)	3.3 (11.6*)	0.1 (2.4)	0.4 (9.5*)
Coniferyl alcohol	2.2 (5.9)	3.7 (80.4/10.0*)	27.1 (95.6)	27.7 (97.9*)	2.8 (66.7)	1.3 (31.0*)

* The value V_{rel} was calculated relative to the specific activity of laccases in the culture liquid of the strain with ABTS at pH 5.0.

produced by the fungal strains, the substrate specificity of laccases from the culture liquid of the most active fungi was also investigated in the acidic (pH 5.0) and neutral (pH 7.2) pH ranges (Table 3).

Our data showed that the culture liquid of the fungus *M. roridum* VKM F-3565 with the pH-optimum close to the neutral range (Table 2) had a high specific activity with the most of the phenol compounds under study in the neutral pH range, except for the activity with ABTS (Table 3). It should also be noted that only the culture liquid of the strain *M. roridum* VKM F-3565, among all of the tested fungi, was active with ABTS at pH 7.2, most likely due to the neutral shift of the pH-optimum of reaction of ABTS oxidation by laccases of this strain (Tables 2, 3).

The culture liquid of the fungus *S. cylindrospora* VKM F-3049 showed higher activity in the acidic pH range with the most of the tested substrates except for *p*-coumaryl and coniferyl alcohols (Table 3) but, in spite of this fact, laccases from the culture liquid of this strain had relatively high activity with the most of the substrates in the neutral pH range compared to other fungal strains under study (Table 3).

The culture liquid of the fungus *U. atrum* VKM F-4302 showed higher activity with all of the tested substrates in the acidic pH range (Table 3), remaining active with the most of them at pH 7.2, which suggested that the produced laccase was active within a broad pH range or that the culture liquid of the strain contained several laccase isoforms with different pH selectivity.

Laccases produced by the strains under study had also different substrate specificity to the tested compounds (Table 3). The culture liquid of the fungus *M. roridum* VKM F-3565 was most active with 2,6-dimethoxyphenol and ferulic acid at pH 7.2 but showed the maximum activity with ABTS at pH 5.0. The culture liquid of the fungus *S. cylindrospora* VKM F-3049 was most active with syringaldazine both at pH 5.0 and at pH 7.2, as well as with ferulic acid at

pH 5.0 (Table 3). The fungus *U. atrum* VKM F-4302 produced laccases with the highest activity in relation to ABTS and syringaldazine at pH 5.0 and 7.2, respectively (Table 3).

DISCUSSION

Investigation of the substrate selectivity of laccases in the culture liquid of selected fungi at acidic and neutral pH confirmed the data on the pH-optimum of laccase reactions with the tested substrates and made it possible to select two ascomycete strains: *M. roridum* VKM F-3565 and *S. cylindrospora* VKM F-3049, which produced laccases highly active with phenol compounds in the neutral pH range, thereby opening up new prospects for using the laccases of these strains in various cell biotechnologies, since the pH value of the cytosol is in the neutral region.

Among the fungi of the genus *Myrothecium*, laccase activity has been shown previously for *M. verrucaria* strains 24G-4 and NF-05 (Sulistyaningdyah et al., 2004; Zhao et al., 2012, 2014). The laccase of *M. verrucaria* 24G-4 had a pH-optimum of the reaction of dimerization of 4-aminoantipyrine with phenol around pH 9.0. Unfortunately, the substrate specificity of laccase with phenylpropanoids (ferulic acid, *p*-coumaryl and coniferyl alcohols) and typical laccase substrates (2,6-dimethoxyphenol and syringaldazine) was not investigated in this work, and the conditions of submerged cultivation were not optimized for increasing the laccase activity of the strain (Sulistyaningdyah et al., 2004). The laccase of *M. verrucaria* NF-05 (Zhao et al., 2012) was obtained during submerged cultivation in a glucose-potato medium with copper sulfate added at a final concentration of 1 mM on day 4 of growth: the yield of laccase was 40.2 U/mL on day 13 of cultivation, which was almost 3 times less than the optimal yield of laccases in the strain *M. roridum* VKM F-3565 (115 U/mL) obtained in the present work. The pH-optimum and substrate specificity to monolignols and typical laccase substrates (2,6-dimethoxyphenol and syringaldazine) were not studied for that laccase either. The pH-optimum of the reaction with ABTS was about 4.0. A recent publication (Zhao et al., 2014) describes the possibility of enhancing the laccase activity in the culture liquid of the fungus *M. verrucaria* NF-05 (up to 258.1 U/mL) by adding an inducer (10 μM 3,3'-dimethylbenzidine) to the cultivation medium. For other species of the genus *Myrothecium*, the information about laccase production ability is entirely absent.

There is even less information about laccase production by the fungi of the genus *Stachybotrys*. The presence of the laccase gene was shown only for the genome of the *S. chartarum*, and its heterologous expression was successfully performed (Janssen et al., 2004; Mander et al., 2006). However, the properties of this laccase and its production by the fungus during submerged cultivation have not been studied at all.

There are only single reports on the laccase production ability of representatives of the genus *Ulocladium* (*Ulocladium* sp. (Fillat et al., 2016) and *U. chartarum* (Bridžiuvienė and Raudonienė, 2013; Abd El Aty et al., 2015)); however, the dynamics of laccase activity, optimization of the yield of laccases and their properties have not been investigated.

Thus, the novel fungal strains of laccase-producing ascomycetes were found in this work by screening, the conditions of their submerged cultivation were optimized to increase the yield of laccases, and the substrate selectivity and pH-optima of laccases in the culture liquid of selected strains were studied in detail for the first time. Moreover, the ability to synthesize laccases active in the neutral pH range has not been previously reported for representatives of the genera *Myrothecium* and *Stachybotrys*, which opens up new prospects of using the enzymes synthesized by the fungi *M. roridum* VKM F-3565 and *S. cylindrospora* VKM F-3049 in different cell biotechnologies.

ACKNOWLEDGMENTS

The work was supported by the Ministry of Education and Science of the Russian Federation (Agreement no. 14.616.21.0001, RFMEFI61614X0001) and by a grant from the Federal Ministry of Education and Research of Germany (BioLiSy, FKZ 031A554).

REFERENCES

Abd El Aty, A.A., El-Shamy, A.R., Atalla, S.M.M., El-Diwany, A.I., and Hamed, E.R., Screening of fungal isolates for laccase enzyme production from marine sources, *Res. J. Pharm. Biol. Chem. Sci.*, 2015, vol. 6, pp. 221–228.

Baldrian, P., Fungal laccases—occurrence and properties, *FEMS Microbiol. Rev.*, 2006, vol. 30, pp. 215–242.

Banerjee, U.C. and Vohra, R.M., Production of laccase by *Curvularia* sp., *Folia Microbiol.*, 1991, vol. 36, pp. 343–346.

Bridžiuvienė, D. and Raudonienė, V., Fungi surviving on treated wood and some of their physiological properties, *Mater. Sci.* (*MEDŽIAGOTYRA*), 2013, vol. 19, no. 1, pp. 43–50.

Chakroun, H., Mechichi, T., Martinez, M.J., Dhouib, A., and Sayadi, S., Purification and characterization of a novel laccase from the ascomycete *Trichoderma atroviride*: application on bioremediation of phenolic compounds, *Process. Biochem.*, 2010, vol. 45, pp. 507–513.

Eggert, C., Temp, U., and Eriksson, K.E., The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase, *Appl. Environ. Microbiol.*, 1996, vol. 62, pp. 1151–1158.

Elshafei, A.M., Hassan, M.M., Haroun, B.M., Elsayed, M.A., and Othman A.M., Optimization of laccase production from *Penicillium martensii* NRC 345, *Advans. Life Sci.*, 2012, vol. 2, pp. 31–37.

Feng, X., Chen, H., Xue, D., and Yao, S., Enhancement of laccase activity by marine-derived deuteromycete *Pestalotiopsis* sp. J63 with agricultural residues and inducers, *Chinese J. Chem. Engin.*, 2013, vol. 21, pp. 1182–1189.

Fillat, Ú., Martín-Sampedro, R., Macaya-Sanz, D., Martín, J.A., Ibarra, D., Martínez, M.J., and Eugenio, M.E., Screening of eucalyptus wood endophytes for laccase activity, *Process Biochem.*, 2016, vol. 51, pp. 589–598.

Hao, J., Song, F., Huang, F., Yang, C., Zhang, Z., Zheng, Y., and Tian, X., Production of laccase by a newly isolated deuteromycete fungus *Pestalotiopsis* sp. and its decolorization of azo dye, *J. Ind. Microbiol. Biotechnol.*, 2007, vol. 34, pp. 233–240.

Janssen, G.G., Baldwin, T.M., Winetzky, D.S., Tierney, L.M., Wang, H., and Murray, C.J., Selective targeting of a laccase from *Stachybotrys chartarum* covalently linked to a carotenoid-binding peptide, *J. Peptide Res.*, 2004, vol. 64, pp. 10–24.

Junghanns, C., Parra, R., Keshavarz, T., and Schlosser, D., Degradation of the xenoestrogen nonylphenol by aquatic fungi and their laccases, *Eng. Life Sci.*, 2008, vol. 8, pp. 277–285.

Kudanga, T.G., Nyanhongo, S., Guebitz, G.M., and Burton, S., Potential applications of laccase-mediated coupling and grafting reactions: a review, *Enz. Microbiol. Technol.*, 2011, vol. 48, pp. 195–208.

Kunamneni, A., Plou, F.J., Ballesteros, A., and Alcalde, M., Laccases and their applications: a patent review, *Rec. Pat. Biotech.*, 2008, vol. 2, pp. 10–24.

Leonowicz, A. and Grzywnowicz, K., Quantitative estimation of laccase forms in some white-rot fungi using syringaldazine as a substrate, *Enz. Microb. Technol.*, 1981, vol. 3, pp. 55–58.

Mander, G.J., Wang, H., Bodie, E., Wagner, J., Vienken, K., Vinuesa, C., Foster, C., Leeder, A.C., Allen, G., Hamill, V., Janssen, G.G., Dunn-Coleman, N., Karos, M., Lemaire, H.G., Subkowski, T., et al., , Use of laccase as a novel, versatile reporter system in filamentous fungi, *Appl. Environ Microbiol.*, 2006, vol. 72, pp. 5020–5026.

Margot, J., Bennati-Granier, C., Maillard, J., Blánquez, P., Barry, D.A., and Holliger, C., Bacterial versus fungal laccase: potential for micropollutant degradation, *AMB Express*, 2013, vol. 3, pp. 63–77.

Mikolasch, A. and Schauer, F., Fungal laccases as tools for the synthesis of new hybrid molecules and biomaterials, *Appl. Microbiol. Biotechnol.*, 2009, vol. 82, pp. 605–624.

Myasoedova, N.M., Gasanov, N.B., Chernykh, A.M., Kolomytseva, M.P., and Golovleva, L.A., Selective regulation of laccase isoform production by the *Lentinus strigosus* 1566 fungus, *Appl. Biochem. Microbiol.* (Moscow), 2015, vol. 51, no. 2, pp. 222–229.

Paszczyński, A., Huynh, V.-B., and Crawford, R., Comparison of ligninase-I and peroxidase-M2 from the white-rot fungus *Phanerochaete chrysosporium, Arch. Biochem. Biophys.*, 1986, vol. 244, pp. 750–765.

Pickel, B., Constantin, M.A., Pfannstiel, J., Conrad, J., Beifuss, U., and Schaller, A., An enantiocomplementary dirigent protein for the enantioselective laccase-catalyzed oxidative coupling of phenols, *Angew. Chem. Int. Ed. Engl.*, 2010, vol. 49, pp. 202–204.

Perez, J. and Jeffries, T.W., Mineralization of C-ring-labeled synthetic lignin correlates with the production of lignin peroxidase, not of manganese peroxidase or laccase, *Appl. Environ. Microbiol.*, 1990, vol. 56, pp. 1806–1812.

Polak, J. and Jarosz-Wilkolazka, A., Fungal laccases as green catalysts for dye synthesis, *Process Biochem.*, 2012, vol. 47, pp. 1295–1307.

Riva, S., Laccases: blue enzymes for green chemistry, *Trends Biotechnol.*, 2006, vol. 24, pp. 219–226.

Sterjiades, R., Dean, J.F.D., and Eriksson, K.-E.L., Laccase from *Sycamore maple* (*Acer pseudoplatanus*) polymerizes monolignols, *Plant Physiol.*, 1992, vol. 99, pp. 1162–1168.

Sulistyaningdyah, W.T., Ogawa, J., Tanaka, H., Maeda, C., and Shimizu, S., Characterization of alkaliphilic laccase activity in the culture supernatant of *Myrothecium verrucaria* 24G-4 in comparison with bilirubin oxidase, *FEMS Microbiol. Lett.*, 2004, vol. 230, pp. 209–214.

Xu, F., Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition, *Biochemistry*, 1996, vol. 35, pp. 7608–7614.

Zhao, D., Cui, D.Z., Mu, J.S., Zhang, X., Wang, Y., and Zhao, M., Induction of a white laccase from the deuteromycete *Myrothecium verrucaria* NF-05 and its potential in decolorization of dyes, *Biocatal., Biotransform.*, 2014, vol. 32, pp. 214–221.

Zhao, D., Zhang, X., Cui, D., and Zhao, M., Characterization of a novel white laccase from the deuteromycete fungus *Myrothecium verrucaria* NF-05 and its decolorization of dyes, *PLos One,* 2012, vol. 7. e38817.

Translated by E. Makeeva