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Characterization of laccase from *Coriolopsis byrsina* GRB13 and application of the enzyme for synthesis of redox mediators

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Eleven fungal isolates were screened for their ability to oxidize 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid). The highest laccase activity levels (about 6 200 U l^{-1}) were obtained in *Coriolopsis byrsina* GRB13 liquid cultures in the presence of 4-dimethylaminobenzoic acid and oak leaf extract. The enzyme from *Coriolopsis byrsina* GRB13 purified to homogeneity had a molecular mass of 57.7 kDa and an absorption maximum at 604 nm that is characteristic of blue copper proteins. It oxidized various substrates including 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), syringaldazine, promazine, phenoxazines, *p*-hydroxyphenylacetic acid and caffeic acid as well as Phenol Red and potassium hexacyanoferrate(II) (apparent bimolecular constants covered a range of 0.0002–25 $\mu M^{-1} s^{-1}$). The laccase catalyzed synthesis of various substituted quinones starting from the simple *o*- and hydroquinone. Enzymatic synthesis of 3*H*-phenothiazin-3-one and 10*H*-phenazin-2-one was achieved for the first time. Two synthesized quinones (2-(*N*-methylanilino)-1,4-benzoquinone and 2-(3-nitroanilino)-1,4-benzoquinone) were powerful redox mediators applicable for bioelectrocatalytic systems based on pyrroquinoline quinone-dependent glucose dehydrogenase.

Key words: laccase, redox mediators, pyrroquinoline quinone-dependent glucose dehydrogenase, *Coriolopsis byrsina*

Abbreviations: ABTS – 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), AMB – *N*,*N*'-dimethylamine-4-(4-morpholine)benzene, BQ – benzoquinone, CAA – caffeic acid, CbL – laccase from *Coriolopsis byrsina*, DCPIP – 2,6-dichlophenol indophenol, DB – Chicago Sky Blue (Direct Blue), HPAA – *p*-hydroxyphenylacetic acid, HRMS – high-resolution mass spectra, MS – methyl syringate, PET – 2-(10*H*-phenoxazin-10-yl)ethanol, PMS – phenazine methosulphate, PPA – 3-(10*H*-phenoxazin-10-yl)propanoic acid, PPAM – 3-(10*H*-phenoxazin-10-yl)propylamine, PPSA – 3-(10*H*-phenoxazin-10-yl)-1-propanesufonic acid sodium salt, PQQ – pyrroloquinoline quinone, PQQ-GDH – pyrroloquinoline quinone-dependent glucose dehydrogenase, PZ – promazine hydrochloride, rPpL – recombinant laccase from *Polyporus pinsitus*, SYR – syringaldazine.

INTRODUCTION

The enzyme-based electrocatalysis has showed great potential for many applications including biosensors, biofuel cells and bioelectrosynthesis [1, 2]. Various oxidoreductases are used for bioelectrocatalysis, however, due to insensitivity to oxygen and tightly bound cofactor PQQ-dependent enzymes have clear advantages when it comes to development of various electrocatalytic processes [3, 4]. Of primary importance in bioelectrocatalysis is the problem of electron transfer from the active center of the enzyme to the electrode. A direct or mediated electron transfer is used to com-

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bine the enzyme and electrode into a catalytically active system [3]. Various redox mediators including BQ, DCPIP, PMS, metal complexes, ferrocene derivatives, conducting polymers and various nanostructures can be used to shuttle the electrons from the substrate via PQQ-dependent enzymes to the electrode [3, 5–9], however, many of these mediators either suffer of low efficiency or are expensive for application in a bulk synthesis.

Laccases (E.C. 1.10.3.2) belong to the family of copper-containing polyphenol oxidases, enzymes widespread in nature that catalyse the four electron reduction of molecular oxygen to water [10]. The high stability of laccases in aqueous solutions, the mild reaction conditions used in enzyme-catalyzed reactions and oxidative selectivity for phenolic structures make laccases attractive for chemical synthesis. The use of laccases to create new fine chemicals, biologically active compounds and biomaterials is a fast developing field of biocatalysis [11, 12]. While the fungal laccases have been thoroughly studied, and many of them have been purified and characterized at the protein and gene level [10, 13], the discovery and application of novel laccases are important for both analytical and industrial applications.

We present here the purification and characterization of the laccase from *Coriolopsis byrsina* GRB13 (CbL) and evaluation of its potential for the synthesis of the new redox mediators applicable for PQQ-dependent glucose dehydrogenase.

EXPERIMENTAL

Materials

ABTS was obtained from Boehringer Mannheim GmbH (Germany). MS, a product of Lancaster Synthesis, was additionally recrystallized from ethanol. Methanol, CAA, 2-, 3- and 4-hydroxypyridine, *p*-, *o*- and *m*-coumaric acids, *N*,*N*-dimethylaniline, 2,3-, 2,5- and 3,4-dimethylaniline were obtained from Fluka, Switzerland. AMB was synthesized according to [14]. PPSA, PPA, PET and PPAM were synthesized as described in [15]. Phenol Red, benzoic acid, 6-nitrobenzimidazole and potassium hexacyanoferrate(II) were obtained from Reachim, Russia. PZ, SYR and HPAA, DB, *N*-ethyl-5-hydroxy-2-methylaniline, 4-dimethylaminobenzoic acid, 3-dimethylaminophenol, *N*,*N*-diethylaniline, 4-dimethylaminobenzaldehyde, 4,4'-diaminobiphenyl dihydrochloride, 1-naphthol, sodium acetate, potassium dihydrogen phosphate, citric, succinic and maleic acids, sodium hydroxide and other chemicals were purchased from Sigma-Aldrich, Switzerland. Malt agar medium was purchased from Oxoid, England. Sodium acetate, acetic acid and $CaCl_2$ were obtained from J. T. Baker (Holland, NL). D-glucose was purchased from Riedel-de Haen (DE). Graphite rods were purchased from Sigma-Aldrich (Saint Louis, USA).

The solutions of the investigated substrates were prepared by weight in triple distilled water (ABTS, HPAA, DB, Phenol Red, potassium hexacyanoferrate(II), PPSA, PZ and 1-naphthol) or methanol (AMB, MS, CAA, PPAM, PET, PPA, SYR, coumaric acid and 4-hydroxypyridine). The final methanol concentration in the reaction mixture did not exceed 1% (v/v).

PQQ-GDH (specific activity 1 290 U mg^{-1}) was purified from *Acinetobacter calcoaceticus* as desribed [9]. The PQQ-GDH solution of 2 460 U ml^{-1} was used for the experiments.

Media and culture conditions

The solid medium used for the isolation of the fungal strain and detection of ligninolytic activity contained (g l^{-1}) glucose 10, KH_2PO_4 2, $MgSO_4 \cdot 7H_2O$ 0.5, $CaCl_2$ 0.1, NH_4Cl 0.5, yeast extract 0.5, ABTS 0.2 and agar 22. The pH was adjusted to 5.0 before autoclaving. For the preparation of the extracts, 300 ml of distilled water was added to 2 or 20 g of dried maple (*Acer* sp.) or oak (*Quercus* sp.) leaves and the mixture was autoclaved at 121 °C for 30 min. Insoluble material was removed by centrifugation at 6 000 × g for 30 min.

A basal medium consisting of (g l^{-1}) yeast extract 0.2, NH_4NO_3 2.0, KH_2PO_4 2.0, Na_2HPO_4 0.4, $MgSO_4 \cdot 7H_2O$ 0.5, $CuSO_4 \cdot 5H_2O$ 0.025 was used for testing the putative laccase inducers.

The inoculum for laccase production was prepared by cultivating *Coriolopsis byrsina* GRB13 on a rotary shaker (Innova 44, New Brunswick, USA) at 180 rpm in 250 ml flasks containing 50 ml of the medium (g l^{-1}: yeast extract 2.5, malt extract 3.5, KH_2PO_4 2.0; glucose 10) at 30 °C for 7 days. The flasks were inoculated with agar disks of active mycelia, 10 mm in diameter. For the production of laccase, 50 ml of inoculum was used to inoculate 500 ml Erlenmeyer flasks containing 200 ml of production medium consisting of (g l^{-1}) yeast extract 5, NH_4NO_3 2.0, KH_2PO_4 2.0, Na_2HPO_4 0.4, $MgSO_4 \cdot 7H_2O$ 0.5, $CuSO_4 \cdot 5H_2O$ 0.025, 4-dimethylaminobenzoic acid 0.165 and supplemented with the autoclaved and centrifuged extract from 2 g of dry ground oak leaves. The pH was adjusted to 6.0 before autoclaving. The fungus was cultivated on a rotary shaker (180 rpm) at 30 °C for 7 days.

Isolation of the fungal strains

The soil samples collected from forest sites in Lithuania were diluted in sterile distilled water, the aliquots were spread on solid mineral medium with ABTS, and the plates were incubated at 30 °C for five days. Microorganisms showing positive reaction were selected and purified by streaking repeatedly on the malt agar medium. The cultures were maintained on the malt agar medium at room temperature and transferred every month.

DNA manipulations and data analysis

Chromosomal DNA was extracted according to [16]. 18S rRNA encoding genes (1.6 kb fragments) were amplified using primers EukA (5'-tcctctaaatgaccaagtttg-3') and EukB (5'-ggaagggrtgtatttattag-3') [17]. Cycling conditions were as follows: initial denaturation step at 94 °C for 3 min, followed by 40 cycles each consisting of 94 °C for 1 min, annealing

temperature 48 °C for 1 min, and 72 °C for 3 min, with the final extension at 72 °C for 10 min. The PCR product was purified with a DNA purification kit (ThermoFisher, Lithuania) and sequenced in both orientations (Sequencing Centre, Institute of Biotechnology, Vilnius, Lithuania). The sequences were compared with rDNA sequence data from strains available at Genbank (http://www.ncbi.nem.nih.gov) by using the BLAST N sequence match routines. The sequences were aligned and phylogenetic analysis was conducted using MEGA version 4.0 [18]. The Kimura two-parameter model [19] was used to estimate the evolutionary distance. The phylogenetic reconstruction was performed using the neighbour-joining algorithm with bootstrap values calculated from 1 000 replicate runs, using software routines included in the MEGA software. The nucleotide sequences of the fungi determined in this study have been deposited in the EMBL-Bank under the accession numbers FN666089–FN66099.

Purification of CbL

All procedures were performed at 4 °C. Chromatographic separations were performed using an AKTA Purifier system (GE Healthcare, USA). The culture was harvested after 7 days of cultivation and mycelium was removed by centrifugation at 3 000 × g for 60 min. The culture liquid was clarified by adding in consecutive order K_2HPO_4 (final concentration 50 mM) and $CaCl_2$ (final concentration 30 mM). Then the Ca phosphate gel was removed by centrifugation at 3 000 × g for 20 min. The clear supernatant was concentrated by ultrafiltration through a polyether sulfone membrane (10 kDa cut off, Millipore, USA) and a buffer was replaced with 10 mM potassium phosphate, pH 7.0. The retentate was applied to DEAE-Toyopearl 650 M (Toyo-Soda, Japan) column (2.6 cm × 20 cm), equilibrated with the same buffer. The elution of laccase was carried out with a step gradient of 0.1 M NaCl in the same buffer. Fractions with laccase activity were concentrated by ultrafiltration through a polyether sulfone membrane (30 kDa cut off) in a stirred cell (Amicon Inc., USA), $(NH_4)_2SO_4$ was added to the final concentration of 1.5 M and the enzyme was applied to the Phenyl Sepharose 6 Fast Flow column (1.5 cm × 4 cm, GE Healthcare). Laccase was eluted with a linear gradient of 1.5–0 M $(NH_4)_2SO_4$ in 10 mM potassium phosphate buffer, pH 7.0. Fractions with activity were pooled, concentrated by ultrafiltration and dialyzed against 10 mM potassium phosphate buffer, pH 7.0. The enzyme solution was applied to Source 15Q column (1.5 cm × 3 cm, GE Healthcare) and eluted with a linear gradient of 0–100 mM potassium phosphate buffer, pH 7.0. Fractions with laccase activity were pooled, concentrated by ultrafiltration and stored at –20 °C.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried-out as described by Laemmli [20]. The gels were stained with Coomassie brilliant blue R-250. Protein concentrations were measured by the method of Lowry et al. [21].

Temperature optimum and stability of enzyme

The temperature optimum for the enzyme was measured at various temperature values in the range 20 to 90 °C using 0.1 M potassium succinate buffer, pH 3.0. The reaction mixture was incubated at the corresponding temperature for 3 min prior to the addition of the enzyme solution. To estimate the thermal stability of the enzyme, the remaining activity was measured at 30 °C, as described below, after preincubation at temperatures between 30 and 70 °C.

Laccase assay, kinetic measurements and calculations

Laccase activity was routinely assayed by examining the oxidation of ABTS to its cation radical $ABTS^+$ at 420 nm ($\varepsilon_{414} = 36\ mM^{-1}\ cm^{-1}$). The reaction mixture contained 0.5 mM ABTS in 0.1 M potassium succinate buffer, pH 3.0, and a suitable amount of enzyme. 1 U of activity was defined as the amount of enzyme forming 1 μmol of $ABTS^+$ per one min at 30 °C.

The oxidation of the selected substrates was monitored spectrophotometrically using a computer-controlled Nicolet Evolution 300 spectrophotometer (Thermo Electron Corporation, USA) and a MPF-4 spectrofluorimeter (Hitachi, Japan) in 50 mM acetate buffer solution, pH 5.5, at 25 °C. The kinetic curves were recorded at the wavelength corresponding to the maximum of absorbance. The concentration of the oxidized substrates was calculated using the molar absorption coefficients: $\varepsilon_{280} = 9.8\ mM^{-1}\ cm^{-1}$ for MS (determined experimentally), $\varepsilon_{514} = 8.9\ mM^{-1}\ cm^{-1}$ for the radical cations of phenothiazine and $\varepsilon_{530} = 16\ mM^{-1}\ cm^{-1}$ for phenoxazine derivatives, $\varepsilon_{420} = 1.02\ mM^{-1}\ cm^{-1}$ for hexacyanoferrate(II), $\varepsilon_{440} = 37.22\ mM^{-1}\ cm^{-1}$ for Phenol Red, $\varepsilon_{530} = 65\ mM^{-1}\ cm^{-1}$ for SYR. The value of the molar absorption coefficient of the oxidation product of caffeic acid ($\varepsilon_{410} = 1.42\ mM^{-1}\ cm^{-1}$) was determined experimentally. DB concentration was determined spectrophotometrically by using the molar absorption coefficient $9.1\ mM^{-1}\ cm^{-1}$ at 610 nm.

The pH optimum of laccase was determined using ABTS as a substrate in 50 mM buffers: citric acid-NaOH (pH 2.2–6.5), citric acid-phosphate (pH 2.7–7.0), succinic acid-NaOH (pH 2.6–6.5) and sodium acetate (pH 3.0–7.0).

Fluorescence intensity changes during *p*-hydroxyphenylacetic acid oxidation were studied at excitation λ = 320 nm and emission – 410 nm. The concentration of oxidation products was calculated using a fluorescence intensity coefficient, which was determined from the calibration curve.

The initial rate (V) of substrate oxidation was calculated by curve fitting. In the case of exponential function $V = k \times C_0$, k is the first order reaction constant and C_0 is the initial concentration of a substrate. For the linear dependence the initial rate was calculated as a slope. To analyze the dependence of V on the substrate concentration and determine the apparent kinetic parameters V_{max} and K_m of the reactions, the Michaelis-Menten equation was used. Catalytic constant (k_{cat}) was calculated as ratio V_{max} and the total enzyme concentration. Bimolecular enzyme and substrate

reactivity constant (k_{ox}) was calculated as a ratio of catalytic constant (k_{cat}) and K_m.

Electrochemical measurements

Electrochemical measurements were performed using an electrochemical system PARSTAT 2273 (Princeton Applied Research, USA) with a conventional three-electrode system comprised of a platinum plate electrode as auxiliary, a saturated Ag/AgCl electrode as reference and graphite (5.9 mm diameter rods molten into Teflon tube) as working electrode. Graphite electrodes were cleaned prior to use by a procedure of polishing with alumina powder and washing with bidistilled water followed by sonication for 5 min. The enzyme electrode was designed by the adsorption on the cleaned surface of 3 µl (10 mg/ml) of mediator compound and further drying at room temperature. After that, the electrode was coated with 3 µl of the enzyme solution and incubated for 1 hour at 4 °C. The response of the prepared enzyme electrodes to the addition of substrate and redox mediator was investigated under potentiostatic conditions at 400 mV (vs. Ag/AgCl) in a stirred 0.05 M acetate buffer solution, pH 6.0, containing 10 mM Ca^{2+} at 20 °C.

General procedure for the synthesis of aminated quinones

Laccase-catalyzed reaction was performed at room temperature in 20 mM sodium acetate buffer pH 5.0. A 4 mM solution of catechols or hydroquinone in 100 mL buffer was oxidized with 50 µL of laccase (activity 1.2 U/µL) for 2–3 hours. When oxidation was completed, according to TLC and UV/VIS spectrum, an equimolar amount of the appropriate amine was added. Thin-layer chromatography was carried out on 25 TLC aluminum sheets coated with silica gel 60 F_{254} (Merck). Products were isolated approximately 20 h after addition of amine. Products with low solubility in buffer were collected by centrifugation (4 000 g, 20 min), soluble products were purified by column chromatography (silica gel 60 (0.04–0.0630 nm) (Merck), eluent hexane / ethyl acetate). The structure and the purity of the synthesized compounds were evaluated by spectroscopic (NMR, IR, UV/VIS and HRMS) methods.

Spectrometric identification of isolated compounds

HRMS were recorded on a Dual-ESI Q-TOF 6520 mass spectrometer (Agilent Technologies). The IR spectra were recorded on KBr surface on a Perkin-Elmer FT-IR System Spectrum GX spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75.4 MHz, respectively, on a Varian Unity Inova 300 spectrometer in $CDCl_3$ or deuterated DMSO. The UV/VIS spectra were recorded on a Perkin-Elmer spectrometer Lambda-35 UV/VIS.

2-Anilino-1,4-benzoquinone (3). UV/VIS (EtOH), λ_{max} (nm) 258, 455. IR, ν_{max} (cm^{-1}) 3 231, 2 920, 2 853, 1 740, 1 674. ^{1}H NMR ($CDCl_3$): δ = 6.23 (d, *J* = 1.8 Hz, 1H), 6.76 (d, *J* = 2.4 Hz, 2H), 7.24 (m, 2H), 7.27 (m, 2H), 7.42–7.44 (s, 1H). ^{13}C NMR ($CDCl_3$): 70.8, 101.2, 122.6, 125.9, 130.0, 132.7, 136.8, 140.0, 183.6, 186.8. HRMS calcd. for $C_{12}H_9NO_2$ ($[M+H]^+$): 200.0706, found: 200.0706.

2-(3-Nitroanilino)-1,4-benzoquinone (4). UV/VIS (EtOH), λ_{max} (nm) 258, 464. IR, ν_{max} (cm^{-1}) 3 088, 2 917, 2 849, 1 678, 1 625, 1 572, 1 521. ^{1}H NMR (DMSO): δ = 6.1 (s, 1H), 6.77 (d, *J* = 10.2 Hz, 1H), 6.89 (d, *J* = 10.2, 1H), 7.69 (t, *J* = 8.1 Hz, 1H), 7.83 (d, *J* = 9.6 Hz, 1H), 8.0 (d, *J* = 9.6 Hz,1H), 8.2 (s, 1H), 9.33 (s, 1H). HRMS calcd. for $C_{12}H_8N_2O_4$ ($[M+H]^+$): 245.0557, found: 245.0554.

2-(4-Methoxyanilino)-1,4-benzoquinone (5). UV/VIS (EtOH), ν_{max} (nm) 258, 510. IR, ν_{max} (cm^{-1}) 3 312, 2 920, 2 850, 1 675, 1 577, 1 509, 1 245. ^{1}H NMR (DMSO): δ = 3.86 (s, 3H), 5.85 (d, 1H, *J* = 3.6 Hz), 6.71 (m, 2H), 7.05 (m, 2H), 7.34 (m, 2H), 8.09 (s, 1H). HRMS calcd. for $C_{13}H_{11}NO_3$ ($[M+H]^+$): 230.0812, found: 230.0811.

2-(N-Methylanilino)-1,4-benzoquinone (6). UV/VIS (EtOH), λ_{max} (nm) 249, 499. IR, ν_{max} (cm^{-1}) 3 338, 3 043, 2 916, 2 849, 1 674, 1 636. ^{1}H NMR (DMSO): δ = 3.27 (s, 3H), 5,92 (s, 1H), 6.66 (m, 2H), 7.27 (m, 5H). HRMS calcd. for $C_{13}H_{11}NO_2$ ($[M+H]^+$): 214.0863, found: 214.0860.

3*H*-Phenothiazin-3-one (7). UV/VIS (EtOH), λ_{max} (nm) 236, 272, 370, 516. IR, ν_{max} (cm^{-1}) 2 955, 2 914, 2 849, 1 732, 1 620, 1 597. ^{1}H NMR (DMSO): δ = 6.79 (s, 1H), 6.98 (dd, 1H, *J* = 10.2 Hz, 1.8 Hz), 7.5 (s, 3H), 7.66 (d, 1H, *J* = 9.9 Hz), 7.94 (t, 1H, *J* = 4.2 Hz). ^{13}C NMR (DMSO): 120.3, 124.0, 125.3, 128.3, 131.3, 134.3, 135.5, 135.6, 139.5, 140.1, 146.7, 182.8. HRMS calcd. for $C_{12}H_7NOS$ ($[M+H]^+$): 214.0321, found: 214.0321.

2-[(2-Aminophenyl)disulfanyl]aniline (8). UV/VIS (EtOH), λ_{max} (nm) 342. IR, ν_{max} (cm^{-1}) 3 459, 3 362, 2 956, 2 914, 2 849, 1 602. ^{1}H NMR (DMSO): δ = 4.2 (s, 4H), 6.62 (dt, 2H, *J* = 9 Hz, 1.5 Hz), 6.75 (dd, 2H, *J* = 8.4 Hz, 1.2 Hz), 7.18 (dd, 4H, *J* = 7.8 Hz, 1.2 Hz). ^{13}C NMR (DMSO): 115.5, 118.5, 119.0, 131.9, 137.1, 148.9. HRMS calcd. for $C_{12}H_{12}N_2S_2$ ($[M+H]^+$): 249.3807, found: 249.3803.

10*H*-Phenazin-2-one (9). UV/VIS (EtOH), λ_{max} (nm) 257, 359, 408. IR, ν_{max} (cm^{-1}) 3 272, 2 971, 2 920, 2 851, 1 705, 1 632, 1 602. ^{1}H NMR (DMSO): δ = 5.8 (dd, 1H, *J* = 15.3 Hz, 1.5 Hz), 6.8 (m, 1H), 7.35 (d, 1H, *J* = 2.7 Hz), 7.8–7.9 (m, 2H), 8.1–8.2 (m, 2H). ^{13}C NMR (DMSO): 107.7, 127.2, 129.3, 129.7, 130.1, 131.4, 131.5, 140.2, 141.6, 143.5, 145.5, 160.3. HRMS calcd. for $C_{12}H_8N_2O$ ($[M+H]^+$): 197.0709, found: 197.0706.

Phenazine-2,3-diamine (10). UV/VIS (EtOH), λ_{max} (nm) 260, 430. IR, ν_{max} (cm^{-1}) 3 314, 3 193, 2 918, 2 849, 1 656, 1 495. ^{1}H NMR (DMSO): δ = 6.27 (s, 4H), 6.99 (s, 2H), 7.78–8.02 (q, 4H, *J* = 3.3 Hz). ^{13}C NMR (DMSO): 102.8, 127.1, 128.6, 141.0, 142.8, 144.8. HRMS calcd. for $C_{12}H_{10}N_4$ ($[M+H]^+$): 211.2469, found: 211.2464.

(4E)-5-Anilino-2-hydroxy-4-phenylimino-cyclohexa-2,5-dien-1-one (11). UV/VIS (EtOH), λ_{max} (nm) 264, 305. IR, ν_{max} (cm^{-1}) 3 265, 2 920, 2 852, 1 735, 1 614, 1 579, 1 516, 1 201. ^1H NMR ($CDCl_3$): δ = 6.15 (s, 2H), 7.1–7.2 (m, 4H), 7.26 (t, *J* = 7.5 Hz, 2H), 7.46 (t, *J* = 7.5 Hz, 4H), 8.63 (s, 1H). HRMS calcd. for $C_{18}H_{14}N_2O_2$ ($[M+H]^+$): 291.1128, found: 291.1128.

(4E)-5-Anilino-2-hydroxy-3-methyl-4-phenylimino-cyclohexa-2,5-dien-1-one (12). UV/VIS (EtOH), λ_{max} (nm) 260, 300, 411. IR, ν_{max} (cm^{-1}) 3 276, 2 923, 2 853, 1 719, 1 661, 1 580, 1 500, 1 247, 1 197. ^1H NMR ($CDCl_3$): δ = 1.65 (s, 3H), 6.1 (s, 1H), 7.01–7.04 (m, 4H), 7.25 (m, 2H), 7.4 (m, 4H), 8.5 (s, 1H). HRMS calcd. for $C_{19}H_{16}N_2O_2$ ($[M+H]^+$): 305.1285, found: 305.1280.

RESULTS AND DISCUSSION

Isolation of laccase producing microorganisms

The screening resulted in the isolation of 11 ABTS oxidizing fungal strains, which were characterized taxonomically by using molecular methods. The data derived from BLAST and phylogenetic analyses (Fig. 1) identified these fungi as *Fusarium oxysporum* strains T50, PUS and KAL1, *Trichoderma viride* strain LAV3, *Pseudallescheria* sp. strain T55, *Chaetomium elatum* strain T53, *Phaeosphaeria* sp. strain UZK, *Penicillium* sp. strain LAC1, *Galerina* sp. strain KAL2, *Coriolopsis byrsina* strain GRB13 and *Mucor hiemalis* strain PLS. Although all laccase-positive strains exhibited fast oxidation of ABTS on agar plates, cultivations in liquid media of the same constitution showed that *Coriolopsis byrsina* strain GRB13 produced laccase at the highest level (up to 200–300 U l^{-1}). The laccase activity of other isolates was lower and varied from 4 to 20 U l^{-1}. Consequently, strain GRB13 was used for further studies.

Optimization of cultivation medium for laccase production

Laccase production by fungi had been previously shown to depend markedly on the composition of the cultivation medium, for example, carbon source, nitrogen content and phenolic inducer compounds were reported to have significant effects on laccase production [22]. In order to define the best conditions for the production of laccase by *Coriolopsis byrsina* GRB13, several possible inducers were tested using the basal medium (Table 1). The production of laccase started on the third day and reached its maximum activity on the seventh day. The maximum level of laccase activity (6 200 U l^{-1}) was observed in the concomitant presence of the oak leaf extract and 4-dimethylaminobenzoic acid (Table 1).

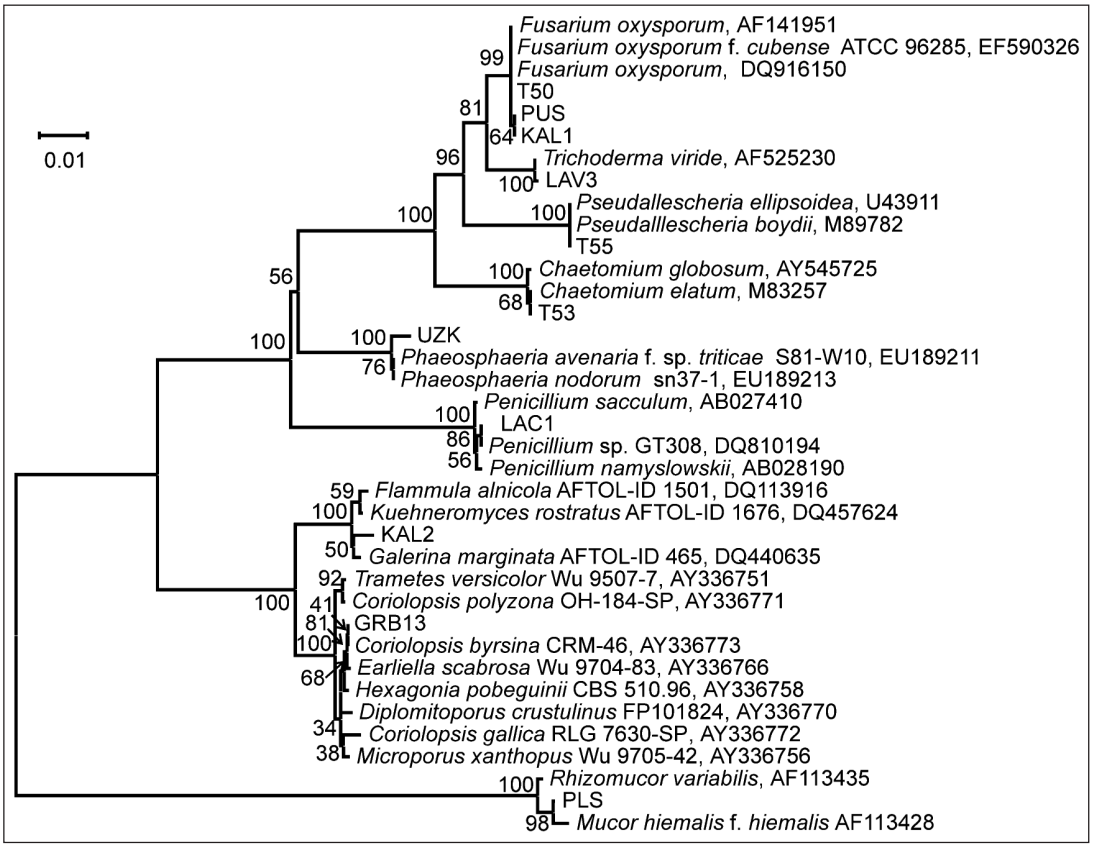

Fig. 1. A phylogenetic tree constructed from the alignment of the 18S rRNA encoding sequences showing the closest relatives of laccase-producing fungi isolates (Kimura two-parameter model; neighbour-joining algorithm and 1 000 replicate bootstrap). The bootstrap values are marked at nodes. The taxonomic names of fungi, strains and the GenBank accession numbers are shown near the branches. Scale bar: branch length proportional to genetic distance

Table 1. **Effect of inducers on *Coriolopsis byrsina* laccase production**

Inducer	Laccase activity in culture medium, U l^{-1}
Control	15
Benzoic acid (1 mM)	0
6-nitrobenzimidazole (1 mM)	3
4-dimethylaminobenzaldehyde (1 mM)	218
4,4′-diaminobiphenyl dihydrochloride (1 mM)	11
N,*N*-dimethylaniline (1 mM)	0
N,*N*-diethylaniline (1 mM)	87
4-dimethylaminobenzoic acid (1 mM)	2146
3-dimethylaminophenol (1 mM)	363
N-ethyl-5-hydroxy-2-methylaniline (1 mM)	507
2,3-dimethylaniline (1 mM)	40
2,5-dimethylaniline (1 mM)	186
3,4-dimethylaniline (1 mM)	386
Extract from 20 g of maple leaves (l^{-1})	1700
Extract from 20 g of oak leaves (l^{-1})	2357
Extract from 2 g of oak leaves (l^{-1})	3911
4-dimethylaminobenzoic acid (1 mM) + + extract from 2 g of oak leaves (l^{-1})	6200

Table 2. **Purification of CbL from culture broth of *Coriolopsis byrsina* GRB13**

Purification step	Total protein, mg	Total activity, U	Specific activity, U mg^{-1} protein	Yield, %	Purification fold
Culture broth	4400	17000	3.86	100	1
Ultrafiltration	1668	16440	9.8	96.7	2.54
DEAE-Toyopearl chromatography	92.9	13013	140	77	36.3
Phenyl-sepharose chromatography	33.7	11600	344	68.2	89.1
Source Q15 chromatography	21.8	10922	501	64.3	130

Purification and characterization of CbL

CbL was purified as described in the experimental part and the results were summarized in Table 2. The procedure resulted in a 130-fold purification of CbL with a yield of 64.3%. Purified CbL preparation showed typical laccase characteristics and the ability to oxidize phenolic and non-phenolic compounds. CbL exhibited high specific activity of 500 U/mg of protein with ABTS as a substrate. The activity was almost five times higher as compared to the enzyme from *Coriolopsis rigida* [23], but twice lower than that of the laccase from *Coriolopsis gallica* [24]. The molecular mass of CbL was estimated to be 57.7 kDa by SDS-PAGE analysis (Fig. 2) and it was slightly lower than that of other laccases [10]. The purified CbL had a colour typical of copper-containing proteins. The UV/VIS absorption spectrum of laccase showed two peaks at 280 and 604 nm. Peak at 604 nm indicated the presence of type I copper (Cu^{2+}) responsible for the blue colour of laccases (Fig. 3). There was a small shoulder around 330 nm, which is known as the ligand to copper charge transfer from type 3 site. The blue band of this laccase showed an absorption maximum at 604 nm (A_{280}/A_{604} ratio was 11.3) with the molar absorption coefficient of 3.81 $mM^{-1}cm^{-1}$, which was in the range of known copper oxidoreductases [25].

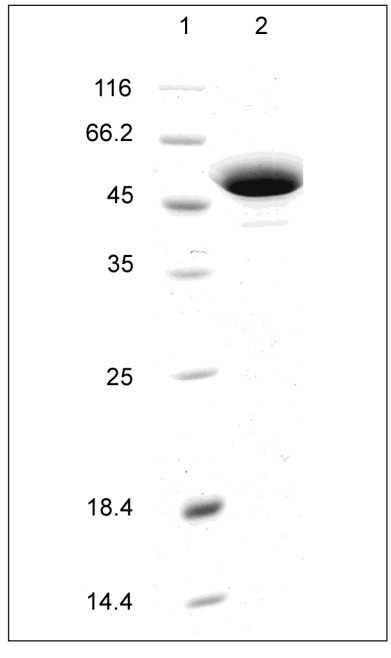

Fig. 2. SDS-PAGE of the purified laccase from *Coriolopsis byrsina* GRB13. Lane 1: molecular mass standards (kDa, Fermentas, Vilnius, Lithuania); lane 2: purified CbL (20 µg)

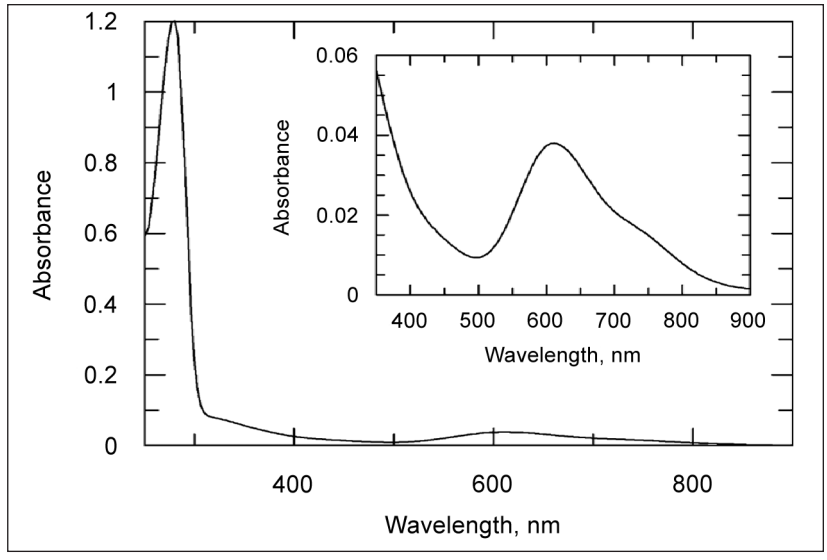

Fig. 3. UV-VIS spectrum of purified *Coriolopsis birsyna* GRB13 laccase (9.4 mg/ml) in 50 mM phosphate buffer, pH 7.0

CbL stability, temperature and pH optimum

CbL showed the maximum enzyme activity in potassium-succinate buffer, pH 3.0 at 50 °C with ABTS as the substrate. The activity of laccase started to decrease significantly after 5 min of incubation at 70 °C. CbL was stable for a long time at 50 °C retaining 33% of its initial activity even after 5 h of incubation at this temperature. Temperature stability of laccases can vary considerably and depend on the organism source [10]. The typical half-life of purified fungal laccases at 50 °C varies from minutes to hours [10, 26].

The effect of pH on laccase activity (initial ABTS oxidation rate) (Fig. 4) showed distinct activity maxima at pH 5.5 in acetate buffer solution. With pH decreasing (from 5.5 to 3.0), the laccase activity decreased constantly until the enzyme became non active at pH 3.5–3.0. With pH increasing (from 5.5 to 7.0), the laccase activity also decreased constantly, until it became almost undetectable at pH 7.0. In contrast, laccase activity in citric acid-NaOH, citric acid-phosphate and succinic acid-KOH buffer solutions increased with pH decreasing to pH 2.5. The maximum reaction rate in these buffer solutions was observed at pH 2.5–3.0. A similar effect of pH on enzyme activity was observed with rPpL [27]. The initial ABTS oxidation rate depended on the ionic strength of buffers (Fig. 5). The increase of concentration from 1 to 10 mM (citrate, maleate) and from 1 to 30 mM (succinate) increased the initial reaction rate about 1.5–2.3 fold, yet at higher ionic strength the reaction rate decreased.

Substrate oxidation catalysed by CbL

CbL oxidized a number of substrates such as complex phenol (e. g. SYR), the non-phenolic heterocyclic compound ABTS as well as inorganic compound hexacyanoferrate(II) (Table 3). The dependence of the initial rate on the substrate concentration largely fitted the Michaelis-Menten equation. K_m and

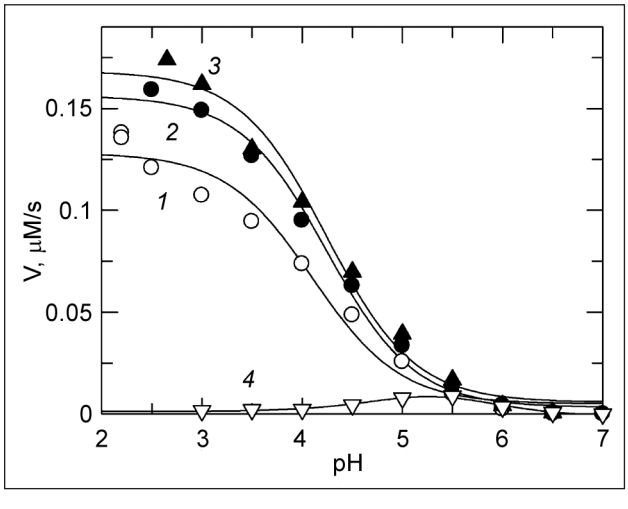

Fig. 4. Dependence of the initial ABTS oxidation reaction rate on the nature of the buffer and pH. Citric acid-NaOH (*1*), citric acid-potassium phosphate (*2*), succinic acid-KOH (*3*) and sodium acetate-HCl (*4*) buffer. ABTS – 50 μM; CbL – 0.5 nM

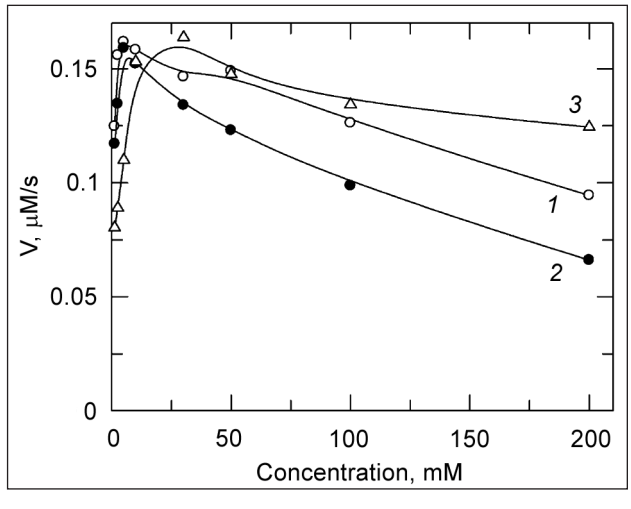

Fig. 5. Initial ABTS oxidation reaction rate dependence on the concentration of the buffer. Citrate (open circles), maleate (closed circles) and succinate (triangles); pH 3.0; ABTS – 50 μM; CbL – 0.5 nM

Table 3. **Kinetic constants of substrates oxidation catalysed by *Corioliopsis byrsina* GRB13 laccase**

Substrate	pH	K_m, μM	k_{cat}, s^{-1}	k_{ox}, $\mu M^{-1} \cdot s^{-1}$
ABTS	5.5	31.6 ± 2.0	37.4 ± 1.1	1.2 ± 0.05
PPA	5.5	2.2 ± 0.2	50.5 ± 0.8	23.4 ± 1.5
PZ	5.5	113 ± 9	27.4 ± 1.05	0.24 ± 0.02
SYR	5.5	1.5 ± 0.1	33.6 ± 0.6	22.4 ± 0.8
MS	5.5	74 ± 23	10.8 ± 1.6	0.15 ± 0.03
Potassium hexacyanoferrate(II)	5.5	283 ± 30	41.5 ± 1.0	0.15 ± 0.005
CAA	5.5	9.6 ± 1.6	27.0 ± 1.2	2.8 ± 0.4
DB	5.5	287 ± 73	0.6 ± 0.2	0.002 ± 0.001
	3.0	32.0 ± 7.0	23.7 ± 2.9	0.75 ± 0.12
Phenol Red	5.5	180 ± 40	0.07 ± 0.01	0.0004 ± 0.00004
	3.0	7.0 ± 1.3	0.016 ± 0.002	0.0022 ± 0.0004
HPAA	5.5	770 ± 120	75 ± 11	0.1 ± 0.003

V_{max} values of the CbL were determined by measuring the initial rate with various concentrations of SYR (0.05–5 μM), ABTS (5–150 μM), PZ (20–200 μM), PPA (0.3–100 μM), MS (5–100 μM), hexacyanoferrate(II) (10–500 μM), CAA (5–100 μM), DB (5–100 μM), Phenol Red (5–50 μM) and HPAA (10–150 μM) as substrates. K_m and V_{max} were determined, and k_{cat} and k_{ox} were calculated. The calculated apparent bimolecular constants covered a range of 0.0002–25 $\mu M^{-1}\ s^{-1}$ (Table 3).

ABTS, SYR and PPA were shown to be good substrates for CbL, but hexacyanoferrate(II), HPAA, Phenol Red and DB dyes were not oxidized so rapidly. The apparent K_m for ABTS oxidation was relatively high compared to K_m values of other fungal laccases [10, 28]. The enzyme showed the highest affinity for SYR, which is considered to be a specific substrate for laccase. The determined k_{cat} value for SYR was very similar to the k_{cat} value obtained with *Trametes* sp. *C30* laccase [29]. Among the investigated substrates the CbL showed the highest k_{cat} and high affinity for phenoxazine PPA. In comparison, the rPpL exhibited a lower affinity (K_m = 37.8 ± 2.6 μM), but higher k_{cat} (102 ± 3 s^{-1}) [27]. CbL affinity for phenothiazine PZ was one of the lowest among the investigated compounds. A lower CbL affinity was determined for HPAA (770 ± 120 μM) and hexacyanoferrate(II) (283 ± 30 μM) only. Most of previously studied laccases showed both higher affinity and k_{cat} [10]. Only small laccase from *Streptomyces coelicolor* [30] showed a similar affinity (~400 μM), and rPpL exibited a lower affinity (750 ± 220 μM) [27] to that determined for CbL. As seen from Table 3, the lowest apparent k_{ox} was determined for Phenol Red (at pH 5.5), but k_{ox} value increased at pH 3 as it was in the case of DB, were pH optimum for ABTS was obtained. The pH also influenced the initial oxidation rate of other investigated substrates. Hence, the initial rate of oxidation of the investigated phenoxazines (PPAM, PET, PPSA and PPA) increased 4–18 fold at pH 3.0 (data not shown). The oxidation rate of vanillin and MS increased about 3-fold and 20-fold (data not shown), respectively, in comparison with the initial rate at pH 5.5. Whereas when CbL oxidized caffeic acid, AMB or PZ at pH 3.0, the initial rate decreased or changed insignificantly.

Reaction of hydroquinones with aromatic amines in the presence of CbL

The purified CbL was tested for the ability to synthesize various substituted quinones starting from the simple pyrocatechol and hydroquinone. Laccase-catalyzed amination of hydroquinone (**1**) with primary aromatic amines (**2a–f**) resulted in the formation of the corresponding monoaminated quinones (Fig. 6), while no diaminated products were obtained. The reaction giving of exclusively diaminated benzoquinones, catalyzed by immobilized commercial laccase Denilite IIBase (from *Aspergillus* sp.), had been reported previously by Wellington et al. [31]. A laccase-catalyzed intermediate formation of the corresponding benzoquinone and subsequent nonenzymatic amination with aromatic amines by Michael addition was proposed as a reaction mechanism [32]. A difference regarding the reactivity of hydroquinones and formation of the products could depend on the employed laccase [31, 32].

CbL catalyzed reaction of hydroquinone with bifunctional aromatic amines (2-aminothiophenol or *o*-phenylenediamine) proceeded in a different manner compared to reactions with substituted anilines. Not only Michael addition to BQ ring was observed, but also the reaction of amine with carbonyl group took place. Moreover, apart from the suspected products, 2-aminothiophenol and *o*-phenylenediamine dimerization byproducts 2-[(2-aminophenyl)disulfanyl]aniline (**8**) and phenazine-2,3-diamine (**10**) were also formed (Fig. 6). Reaction of hydroquinone with 2-aminophenol resulted in the formation of the dimerization product (data not shown) only. Enzymatic synthesis of 3*H*-phenothiazin-3-one (**7**) and 10*H*-phenazin-2-one (**9**) was achieved for the first time. Similar cyclization was observed during laccase catalyzed synthesis of 3-substituted 1,2,4-triazolo(4,3-b)(4,1,2) benzo-thiadiazine-8-ones from various 5-substituted-4-amino-3-mercapto-1,2,4-triazoles and-hydroquinone [33].

Laccase-catalyzed amination of 1,2-dihydroxybenzenes with primary aromatic amines resulted in the formation of the corresponding diaminated quinones and no monoaminated products were obtained. The structures of the diaminated products from the reaction in Table 4 are shown

in (Fig. 6). Amination with primary aliphatic amines (methylamine, *tert*-butylamine, 2-amino-1,3-propandiol) did not give satisfactory results.

Electrochemical analysis of aminated hydroquinones

All synthesized compounds were evaluated as potential redox mediators in PQQ-GDH catalyzed oxidation of glucose. The current response of the manufactured bioelectrocatalytic systems to glucose was evaluated measuring the difference between the steady state current and the background current. Efficiency as a factor of the successful electron transfer between the active site of the enzyme and electrode surface via redox conversions of mediator was calculated for each bioelectrocatalytic system under saturating concentration of glucose (Table 4). The efficiency of biocatalytic systems using newly synthesized compounds varied from $0.08 \pm 0.01 \times 10^5$ A M^{-1}

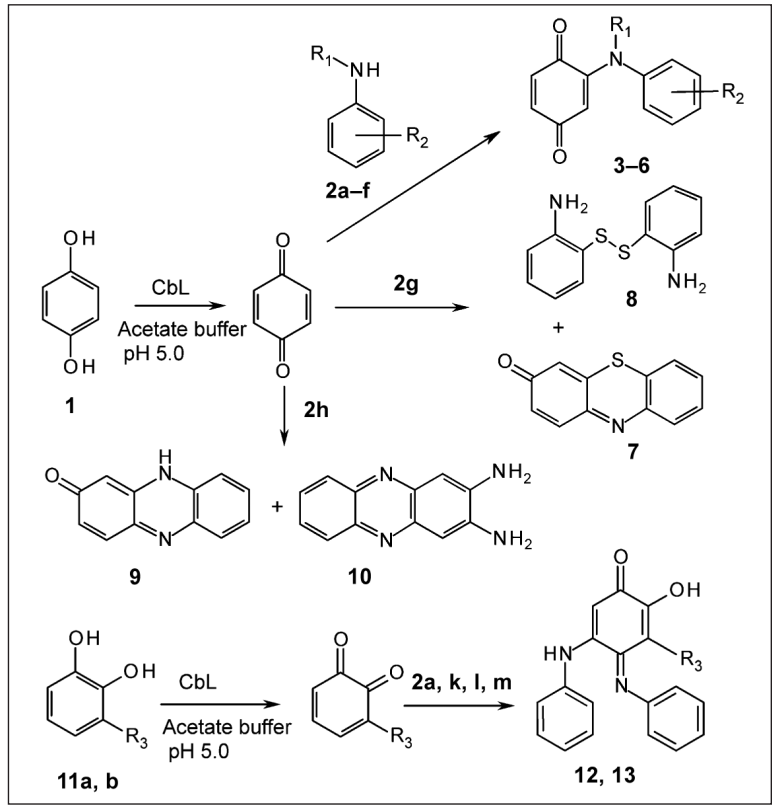

Fig. 6. Scheme of synthesis of aminated quinones

Table 4. **CbL-catalyzed amination of hydroquinones and efficiency of the electrocatalytic system in the presence of PQQ-GDH and the product**

Hydroquinone	Amine	Product (yield)	Maximal current per mol of the active centres of PQQ-GDH, A M^{-1}
1	**2a**: R_1 = H, R_2 = H	**3** (69%)	$0.82 \pm 0.05 \times 10^5$
1	**2b**: R_1 = H, R_2 = *m*-NO_2	**4** (74%)	$2.12 \pm 0.24 \times 10^5$
1	**2c**: R_1 = H, R_2 = *p*-NO_2	no products isolated	
1	**2d**: R_1 = H, R_2 = *o*-NO_2	no products formed	
1	**2e**: R_1 = H, R_2 = *p*-OCH_3	**5** (35%)	$1.50 \pm 0.06 \times 10^5$
1	**2f**: R_1 = CH_3, R_2 = H	**6** (39%)	$2.29 \pm 0.17 \times 10^5$
1	**2g**: 2-aminothiophenol	**7** (21%)	$1.50 \pm 0.07 \times 10^5$
		8 (18%)	$0.08 \pm 0.01 \times 10^5$
1	**2h**: *o*-phenylenediamine	**9** (21%)	$0.30 \pm 0.03 \times 10^5$
		10 (19%)	$0.12 \pm 0.02 \times 10^5$
11a: R_3 = H	**2a**: R_1 = H, R_2 = H	**12** (69%)	$0.12 \pm 0.01 \times 10^5$
11a: R_3 = H	**2k**: methylamine	no products isolated	
11a: R_3 = H	**2l**: *tert*-butylamine	no products formed	
11a: R_3 = H	**2m**: 2-amino-1,3-propandiol	no products isolated	
11b: R_3 = CH_3	**2a**: R_1 = H, R_2 = H	**13** (66%)	$0.36 \pm 0.05 \times 10^5$
11b: R_3 = CH_3	**2k**: methylamine	no products isolated	
11b: R_3 = CH_3	**2l**: *tert*-butylamine	no products isolated	
11b: R_3 = CH_3	**2m**: 2-amino-1,3-propandiol	no products isolated	

of the enzyme active centres for 2-[(2-aminophenyl)disulfanyl]aniline (**8**) to 2.29 ± 0.17 × 10^5 A M^{-1} for 2-(*N*-methylanilino)-1,4-benzoquinone (**6**), that was higher than the efficiency of biocatalysis based on PQQ-GDH and various mediators DCPIP, PMS, metal complexes and ferrocene derivatives [5, 6, 8, 9]. Recently, multi-walled carbon nanotubes modified with PQQ were applied for development of the powerful PQQ-GDH-based biofuel cell [34], however, an efficiency achieved under substrate saturation was 10-fold lower (approx. 0.2 × 10^5 A M^{-1} of the active centres of PQQ-GDH). The highest efficiency (approx. 19.9 × 10^5 A M^{-1} at 23 °C) was recorded in the case of the reagentless biosensor based on the entrapment of PQQ-GDH into an anodic electrodeposition paint modified with Os-complexes as redox relays between the enzyme and the electrode [35]. The enzyme efficiency was approximately 9-fold higher from that observed in the presence of 2-(*N*-methylanilino)-1,4-benzoquinone (**6**) or 2-(3-nitroanilino)-1,4-benzoquinone (**4**). Hence, the application of the redox mediators described during this work could be much more cheaper, especially for the bulk bioelectrocatalytic conversions.

CONCLUSIONS

Eleven fungal isolates were screened for their ability to produce laccases. The laccase from *Coriolopsis byrsina* was analyzed in detail. By employing CbL catalyzed reactions, monoaminated quinones starting from hydroquinone and aryl amines could be synthesized in good yields. Moreover, CbL was applicable for synthesis of 3*H*-phenothiazin-3-one and 10*H*-phenazin-2-one. New synthesized quinones (2-(*N*-methylanilino)-1,4-benzoquinone and 2-(3-nitroanilino)-1,4-benzoquinone) were powerful redox mediators applicable for bioelectrocatalytic systems based on PQQ-GDH.

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CORIOLOPSIS BYRSINA GRB13 LAKAZĖS CHARAKTERIZAVIMAS IR PANAUDOJIMAS REDOKSO MEDIATORIŲ SINTEZEI

S a n t r a u k a

Vienuolika grybų izoliatų buvo atrinkta pagal jų gebėjimą oksiduoti 2,2'-azino-bis(3-etilbenztiazolino-6-sulfonatą). Didžiausias lakazinis aktyvumas (apie 6200 U l^{-1}) buvo aptiktas *Coriolopsis byrsina* GRB13 kultūriniame skystyje auginant su 4-dimetilaminobenzoine rūgštimi ir ąžuolo lapų ekstraktu. Išgryninus homogenišką *Coriolopsis byrsina* GRB13 lakazę, nustatyta jos molekulinė masė (57,7 kDa), o absorbcijos maksimumas 604 nm srityje rodė, kad fermentas priklauso žydrųjų vario baltymų grupei. Lakazė oksidavo 2,2'-azino-bis(3-etilbenztiazolino-6-sulfonatą), siringaldaziną, promaziną, fenoksazinus, *p*-hidroksifenilacto ir kavos rūgštis, Fenolio raudonąjį dažą bei kalio heksacianoferatą(II) (menamosios bimolekulinės konstantos nuo 0,0002 iki 25 $\mu M^{-1}\ s^{-1}$). Lakazė katalizavo įvairių pakeistų chinonų sintezę, substratais naudojant *o*-, *p*- ir kitus chinonus. Pirmą kartą buvo atlikta fermentinė 3*H*-fenotiazin-3-ono ir 10*H*-fenazin-2-ono sintezė. Du susintetinti chinonai (2-(*N*-metilanilino)-1,4-benzochinonas ir 2-(3-nitroanilino)-1,4-benzochinonas) pasižymėjo kaip efektyvūs redokso mediatoriai, tinkami bioelektrokatalizei, panaudojant nuo pirolochinolino chinono priklausomą gliukozės dehidrogenazę.