Asian Journal of Biochemistry, Genetics and Molecular Biology

Volume 16, Issue 4, Page 35-48, 2024; Article no.AJBGMB.114023
ISSN: 2582-3698

On the Substrate Specificity and Some Properties of the Extracellular Oxidase from the *Neonothopanus nambi* Basidiomycete

Olga Mogilnaya [a*], Nikita Ronzhin [a], Ekaterina Posokhina [a], Violeta Le [b], Yulia Zakharova [c], Andrey Sukhikh [b] and Vladimir Bondar [a]

[a] Institute of Biophysics FRC KSC SB RAS, Krasnoyarsk, Russia.
[b] Kemerovo State University, Kemerovo, Russia.
[c] Kemerovo State Medical University, Kemerovo, Russia.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJBGMB/2024/v16i4369

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/114023

Original Research Article

Received: 01/01/2024
Accepted: 06/03/2024
Published: 13/03/2024

ABSTRACT

The present study reports experimental data on substrate specificity and some properties of the extracellular enzyme with oxidase activity isolated from the mycelium of the higher fungus *Neonothopanus nambi* IBSO 2391 by treating the biomass with β-glucosidase. Gel-filtration chromatography showed that molecular weight of the isolated enzyme was 80 kDa. Spectral analysis did not reveal any chromophore components in the enzyme. The extracellular oxidase of the basidiomycete *N. nambi* IBSO 2391 was active with most of the aromatic compounds chosen

**Corresponding author: E-mail: ol_mog@mail.ru;*

as model substrates. An important fact is that the enzyme exhibited catalytic activity with no hydrogen peroxide or any other mediators added to the reaction mixture. The highest activity of the enzyme was observed in reactions with veratryl alcohol and hydroquinone. In reactions with guaiacol and aromatic amines (diaminobenzidine, o-dianisidine), the level of activity of the extracellular oxidase was considerably lower – by a factor of 2.5–3.5. In reactions with resorcinol, phenol, and caffeic acid, the catalytic efficiency of the enzyme was no greater than 6% of its activity with veratryl alcohol. Kinetic parameters of enzymatic reactions were determined for the most efficiently oxidized substrates. The addition of the chelating agent of divalent metal ions (EDTA) did not affect the activity of the extracellular oxidase from the fungus *N. nambi* IBSO 2391, indicating the absence of divalent metal ions in the molecule of the enzyme. At the same time, addition of the SH reagent (DTT) increased catalytic efficiency of the enzyme. The study showed that the extracellular oxidase of the fungus *N. nambi* IBSO 2391 functions in wide ranges of temperature and pH of the reaction medium, showing the highest catalytic activity at temperatures between 22 and 35 °C and pH 6.0. Results obtained in the current study provide the basis for studying potential uses of the isolated enzyme in biomedical analytics and bioremediation.

Keywords: Aromatic compounds; basidiomycetes; biocatalysis; DTT; EDTA; extracellular oxidase.

1. INTRODUCTION

Organic compounds are among the major pollutants of the environment, which they enter as wastes of the chemical, food, and pharmaceutical industries, agricultural chemicals used to protect crops, etc. Therefore, increasing research effort is currently devoted to developing biological methods of neutralizing toxic chemicals. Higher fungi (Ascomycetes and Basidiomycetes), which have unique enzyme systems capable of degrading plant polymers (lignin, cellulose), are considered as promising agents for management of toxic substances. Because of the considerable redox potential of fungal enzyme systems, researchers have focused on their potential uses in degradation of xenobiotics and bioremediation of contaminated natural environments. Over the last few decades, fungal oxidoreductases have aroused serious interest as biotechnological tools, as they catalyze reactions that often cannot take place in the presence of traditional chemical catalysts [1-6]. Enzymes secreted by basidiomycetes have properties that make them useful for practical applications: the products formed during the reactions catalyzed by them are of low toxicity; they retain their functional activity over wide ranges of pH, temperature, and concentrations of pollutants; fungal enzymes are resistant to peptidolysis; and, what is important, they function without the involvement of mediators. Thus, fungal enzymes (and enzyme systems) can be regarded as promising tools of “green” biotechnologies [7-9]. Extensive research is conducted to test the enzymes secreted by higher fungi – FAD-, heme-, and copper-containing oxidoreductases – as components of sensors and analytical systems for biomedical applications [10-12].

Because of insufficient stability of fungal oxidases, though, it may be difficult to use them for analytical purposes. On the other hand, this is an incentive to research aimed at finding new species of higher fungi for producing oxidoreductases [13,14], studying the ways to increase the production of enzymes with oxidative properties in fungal biomass during the cultivation stage, and discovering fungal oxidases with new properties [15,16]. Studies are conducted to develop and improve the methods of isolating these enzymes from the biomass and immobilizing them on various carriers in order to enhance their resistance to negative factors and enable them to retain their catalytic function when reused multiple times [17-20]. An important fact is that oxidoreductases secreted by higher fungi have a great number of isoforms and are capable of oxidizing a wide range of substrates. Therefore, various new enzymes with oxidative properties secreted by higher fungi are described in the literature: laccases [21-24], peroxidases [25,26], alcohol oxidases [15,27,28]. At the same time, this opens up opportunities for adding new enzymes with oxidative properties secreted by higher fungi to the pool of the already known oxidoreductases, which can be used both for purposes of scientific research and in analytical and biocatalytic processes.

The present study reports experimental data on certain properties and substrate specificity of the extracellular oxidase from the basidiomycete *N. nambi* IBSO 2391.

2. MATERIALS AND METHODS

2.1 Strain and Culture Medium

The mycelium of the higher fungus *N. nambi* IBSO 2391, which is maintained in the CCIBSO 836 collection of the Institute of Biophysics of the Federal Research Center "Krasnoyarsk Science Center SB RAS" (Krasnoyarsk, Russia), was used in the study. Experiments were performed with 2–7 mm diameter spherical pellets of mycelium produced by submerged cultivation of the fungus in PDB (potato extract – 4 g/L, glucose – 20 g/L) purchased from HiMedia Laboratory (India). The prepared liquid medium was autoclaved at 120 °C for 15 min immediately before use.

2.2 Procedures

2.2.1 Submerged cultivation

Submerged cultivation of mycelium was performed in 250-ml conical flasks containing 100 ml of the broth. Mycelium, which had been grown in Petri dishes in potato sucrose agar medium for 8–10 d, was crushed and used as inoculum for submerged cultivation of the fungus. The volume of the inoculum was 2–5% of the broth volume. Cultivation process was conducted for 8 d at a temperature of 27–28 °C under continuous stirring at 180–200 rpm (Environmental Shaker-Incubator ES-20, Biosan, Latvia) [29].

2.2.2 Extraction and separation of extracellular proteins

Sweet almond β-glucosidase (EC 3.2.1.21) (Serva, Germany) dissolved in 10 mM phosphate buffer (pH 6.0) was used for enzymatic treatment of the envelope of the hyphal cells of the mycelium. Extracellular enzymes (including oxidases) were isolated from the biomass of *N. nambi* IBSO 2391 mycelium as follows. "Mycelial pellets were taken out of the nutrient broth and rinsed many times in deionized (DI) water to remove residual nutrient medium and metabolites. DI water was produced using a Milli-Q system (Millipore, U.S.A.). Then, the pellets were placed in a new portion of DI water containing β-glucosidase (0.5 IU/ml) and incubated at 25 °C for 24 h under slow agitation at 80 rpm (Shaker OS-20, Biosan, Latvia). After incubation of pellets, the liquid (aqueous extract containing extracellular proteins and enzymes of fungus) was separated from the pellet biomass by filtration through filter paper. To concentrate the enzymes and remove low-molecular-weight compounds, the extract was subjected to ultrafiltration through a 30 kDa cutoff membrane (EMD Millipore Amicon, Darmstadt, Germany). To remove low-molecular-weight compounds more effectively during ultrafiltration, DI water in the extract was replaced three times. The retentate, which contained concentrated extracellular enzymes, was collected and used in experiments [30].

The extracellular enzymes extracted from the mycelium were separated by gel-filtration chromatography of the concentrated enzymes on a Sephacryl S-200 column (1.6 × 33 cm) (Pharmacia, Sweden) equilibrated with a 50 mM NaCl solution. NaCl was added to the concentrated sample to reach a concentration of 50 mM, and 2 ml of the sample was applied onto the column. Chromatography was performed at a flow rate of 0.2 ml/min (EP-1 Econo Pump, Bio-Rad, U.S.A.); a 50 mM NaCl aqueous solution was used as eluent; 2-ml fractions were collected. Protein elution from the chromatographic column was controlled using a 2138 Uvicord S photometer (LKB, Sweden) at a wavelength of 280 nm [31].

After screening of oxidase activity in chromatographic fractions, the fractions with the highest activity of the enzyme were pooled and concentrated by ultrafiltration of the pooled sample through a 30 kDa membrane (EMD Millipore Amicon). To remove the eluent (sodium chloride) more effectively during ultrafiltration, DI water in the sample was replaced twice. The retentate (concentrated oxidase) was collected and used in experiments.

The native molecular weight of extracellular oxidase of the basidiomycete *N. nambi* IBSO 2391 was determined by chromatography of protein markers – BSA (Sigma, U.S.A.) and cytochrome c (Fluka, Germany) with molecular weights of 66.5 kDa and 12.3 kDa, respectively – under the above conditions (column size, volume of the sample applied, eluent, and flow rate). The native molecular weight of the study enzyme was calculated using the values of the maximum oxidase activity in chromatographic samples and the maxima of elution of protein markers recorded as absorbance at 280 nm [31].

2.3 Analytical Methods

2.3.1 Oxidase activity determination

The presence of extracellular oxidase in the samples (aqueous extract from the *N. nambi* IBSO 2391 mycelium, concentrated enzymes, and chromatographic fractions) was determined by oxidation reaction of veratryl alcohol (Sigma, U.S.A.), which was used as substrate. An aqueous solution of veratryl alcohol was prepared in situ in DI water. In assay of oxidase activity, the 600-µl reaction mixture contained 10 mM veratryl alcohol and 50 µl of the tested sample. After all reaction ingredients were added, the samples were agitated for 3 s on a Vortex-Genie 2 g-560E mixer (Scientific Industries, Inc., U.S.A.) and incubated for 30 min at a temperature of 25 °C. The level of oxidase activity in the samples was determined by measuring the yield of veratryl alcohol oxidation reaction product using the spectral method (spectrophotometer UV-1800, Shimadzu, Japan), as absorbance at 309 nm.

2.3.2 Protein concentration measurement

Protein concentrations in the samples were determined by the well-known biuret method, using Benedict's reagent and BSA as the standard. The samples were incubated for 15 min at 25 °C with Benedict's reagent, and then their absorbance was measured at a wavelength of 330 nm using a UV-1800 spectrophotometer [32].

2.3.3 UV-visible spectral analysis of oxidase

The absorption spectrum of the extracellular oxidase isolated from the basidiomycete *N. nambi* IBSO 2391 was determined from 200 to 700 nm at 25 °C in DI water using the UV-1800 spectrophotometer.

2.3.4 Determination of Kinetic Parameters, Optimum pH and Temperature

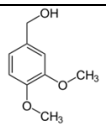
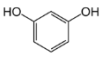
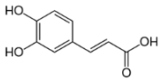
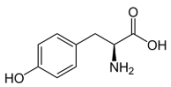
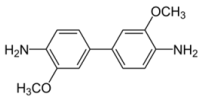
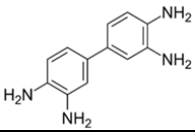
The temperature optimum of the catalytic activity of the isolated oxidase was determined within the temperature range of the reaction medium between 20 and 50 °C. The yield of the product was estimated using the spectral method (the UV-1800 spectrophotometer) after the reaction mixtures containing the enzyme and the substrate (veratryl alcohol) had been incubated for 15 min at different temperatures (TB-85 Thermo Batch, Shimadzu, Japan). The effectiveness of the enzyme at different pH values of the medium was studied using 50 mM Na-acetate buffers with pH between 3 and 8.

Michaelis–Menten kinetic parameters for *N. nambi* oxidase were performed by assaying various concentrations of veratryl alcohol (0.1 to 10 mM), hydroquinone (0.05 to 10 mM) and guaiacol (0.5 to 15 mM). The oxidation of substrates was measured by an increase in absorption at 309 nm for veratryl alcohol (ε_{309} = 9300 $M^{-1}\cdot cm^{-1}$), at 246 nm for hydroquinone (ε_{246} = 19000 $M^{-1}\cdot cm^{-1}$) and at 467 nm for guaiacol (ε_{467} = 3800 $M^{-1}\cdot cm^{-1}$) [24,33]. To determine the kinetic constants, the obtained Michaelis–Menten dependences were transformed into a linear Lineweaver–Burk graphs.

2.3.5 Substrate specificity and factors

Substrate specificity of the extracellular oxidase from the basidiomycete *N. nambi* IBSO 2391 was estimated using the following aromatic compounds as model substrates: phenol derivatives, aromatic acids, and aromatic amines (Table 1). Solutions of reagents were prepared in situ in DI water. Concentrations of the aromatic compounds in reaction mixtures were varied to determine the dependencies of product yields and calculate kinetic characteristics of enzymatic reactions. The catalytic efficiency of the enzyme with model substrates was estimated using the spectral method (UV-1800 spectrophotometer) by the maximal yields of reaction products recorded as the absorbance at the corresponding wavelengths (Table 1) and expressed in absorbance units per 1 mg protein per 1 min. In the experiment with monophenol as the model substrate, the level of activity of the *N. nambi* IBSO 2391 oxidase was determined in reaction of co-oxidation of phenol with 4-aminoantipyrine (4-AAP) by measuring the yield of reaction product – quinone imine, recorded as absorbance at 506 nm. In this experiment, the 600-µl reaction mixture contained 5.96 mM phenol, 0.49 mM 4-AAP, and 100 µl of the enzyme sample. After all reaction ingredients were added, the samples were agitated for 3 s on a Vortex-Genie 2 g-560E mixer and incubated for 30 min at a temperature of 25 °C. The experiments were performed with 4-AAP (1-phenyl-2,3-dimethyl-4-aminopyrazolone) of analytical grade (Reakhim, Russia) and phenol (Fluka, Germany); the solutions were prepared in situ in DI water [31].

Table 1. The aromatic compounds used as the model substrates for the extracellular oxidase from *Neonothopanus nambi* IBSO 2391

Substrate	Structure	Registration wavelength (nm)
Aromatic alcohol: Veratryl alcohol (3,4-dimethoxybenzyl alcohol) (Sigma-Aldrich, U.S.A.)		309
Mono- and Dihydric phenols: Phenol (Fluka, Germany)		506
Hydroquinone (Reakhim, Russia)		246
Resorcinol (Reakhim, Russia)		273
Metoxyphenols: Guaiacol (2- metoxyphenol) (Sigma-Aldrich, U.S.A.)		422, 467
Aromatic acids: Caffeic acid (3,4 - hydroxycinnamic acid) (Sigma-Aldrich, U.S.A.)		412 478
L-Tyrosine (L-2-Amino-3-(4-hydroxyphenyl) propanoic acid (Fluka, Germany)		
Aromatic amines: o-Dianisidine (Fluka, Germany)		442 462
Diaminobenzidine (DAB) (AppliChem, Germany)		

Laccase from the fungus *Agaricus bisporus* (EC 1.10.3.2.) and horseradish peroxidase (HRP) (EC 1.11.1.7) (Sigma, U.S.A.) were used in this study to perform comparative assessment of substrate specificity to the tested aromatic compounds. In supplementary experiments, we also compared catalytic efficiencies of *N. nambi* IBSO 2391 oxidase, *A. bisporus* laccase, and HRP treated with the chelating agent of divalent metal ions ethylenediamine tetraacetic acid (EDTA) (Serva, Germany) and SH reagent dithiothreitol (DTT) (AppliChem, Germany). EDTA and DTT solutions were prepared in DI water; reagent concentrations between 0.1 and 1 mM were used to treat the enzymes [24].

2.3.6 Statistical values

The data are normalized to the maximal values of the product yield in measurement series and represented as M ± m, n = 3 for each measurement. Error bars were generated as a standard deviation of the mean from 3 replicates.

2.3.7 Micrographs of the pellets and hyphae

Micrographs of the pellets and hyphae were obtained by using the PowerShot S50 camera (Canon, Japan) and an AxioImager M2 microscope (Carl Zeiss, Germany) in the fluorescent mode of viewing.

3. RESULTS AND DISCUSSION

3.1 Morphology of the Mycelium after β-Glucosidase Treatment

The current study showed that the technique of submerged cultivation that we used to grow the fungus *N. nambi* IBSO 2391 produced spherical

mycelial pellets with the rough surface created by numerous bundles of hyphae (Fig. 1a). The diameter of the pellets produced under the cultivation conditions used in this study (the composition and the volume of the nutrient solution, the rate of agitation, the temperature and the time of cultivation) usually ranged between 2 and 7 mm. Surface roughness of the pellets is caused by bundles of long hyphae extending for distances that may reach a few millimeters. Outside, the cell wall of the hyphae is covered by polysaccharide slime, which has pores and consists of β-D-glucan (Fig. 1b). Branched glucans form a gel-like network in the outer hyphal sheath and intercellular space. This network serves as a supporting structure and retains water, which is needed for the function of extracellular enzymes immobilized here. After incubation of the *N. nambi* pellets in DI water containing β-glucosidase, they showed noticeable destructive changes of the slimy layer on the surface of the hyphae as β-glucosidase catalyzed hydrolysis of β-D-glucans (Fig. 1c). Hence, β-glucosidase disintegrated the polysaccharide matrix (slimy layer) covering the outside surface of the hyphal cell wall, enabling release of the extracellular enzymes of the fungus localized there to the outer environment [34]. In our opinion, the treatment of mycelium with β-glucosidase may be a promising method for biotechnology, enabling, under relatively mild conditions (without complete destruction of biomass), production of extracts enriched with extracellular fungal enzymes and containing minor amounts of impurities.

3.2 Extraction and Purification of the Extracellular Oxidase

Gel-filtration chromatography of the concentrate of extracellular proteins extracted from N. nambi IBSO 2391 performed on the Sephacryl S-200 column showed the presence of the protein fraction containing an enzyme with oxidase activity (Fig. 2). That oxidase catalyzed oxidation reaction of veratryl alcohol with no exogenous hydrogen peroxide or other mediators added to the reaction mixture. Calculations based on the maximum oxidase activity and absorption maxima of protein markers recorded when protein components were eluted from the column showed that extracellular oxidase of the fungus *N. nambi* IBSO 2391 catalyzing oxidation of veratryl alcohol was detected in the protein fraction with the native molecular weight of the protein of 80 kDa. After chromatography, fractions with the highest oxidase activity (Fig. 2) were pooled, and the pooled sample was subjected to ultrafiltration through a 30 kDa membrane to concentrate the enzyme and remove residual eluent (NaCl). The concentrated enzyme (final oxidase preparation) was used in further studies. Parameters of purification of the extracellular oxidase of *N. nambi* IBSO 2391 using gel-filtration chromatography on the Sephacryl S-200 column (total and specific activities, yield, and purification level) are summarized in Table 2. They show that one stage of chromatography resulted in tenfold purification of the enzyme: the specific activity of the oxidase in the final enzyme preparation was higher by a factor of 10 compared to the specific activity of the oxidase in the initial concentrate of extracellular proteins from *N. nambi* IBSO 2391 mycelium.

3.3 Spectral Characteristic, Optimum pH and Temperature

Having examined the spectral characteristics of the final preparation of the extracellular oxidase isolated from the fungus *N. nambi* IBSO 2391, we found that the enzyme did not contain any chromophore components. Results of spectral analysis show (Fig. 3) that the absorption spectrum of the aqueous sample of the oxidase final preparation corresponded to the typical protein spectrum with a single absorption maximum at 280 nm.

Table 2. Parameters of purification of the extracellular oxidase from the fungus *N. nambi* IBSO 2391 using gel-filtration chromatography on the Sephacryl S-200 column

Sample	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification level (x-fold)
Concentrated extract	5.39	62.9	11.7	100	1.0
Final enzyme preparation	0.23	27.1	116.8	43.1	10.0

Total enzyme activity was calculated in micromoles of the reaction product formed per minute. The specific enzyme activity was calculated as total enzyme activity per one milligram of protein. The molar extinction coefficient for veratryl aldehyde used for calculation was $\varepsilon_{309} = 9300\ M^{-1} \cdot cm^{-1}$

Experiments demonstrated that the extracellular oxidase isolated from the basidiomycete *N. nambi* IBSO 2391 was able to function effectively in a wide range of pH of the medium, oxidizing veratryl alcohol most efficiently at pH 6.0 (Fig. 4a). Another finding was that the enzyme was functionally active in a wide range of temperatures of the reaction medium, showing the highest efficiency of oxidation of the substrate (veratryl alcohol) at temperatures between 22 and 35 °C (Fig. 4b).

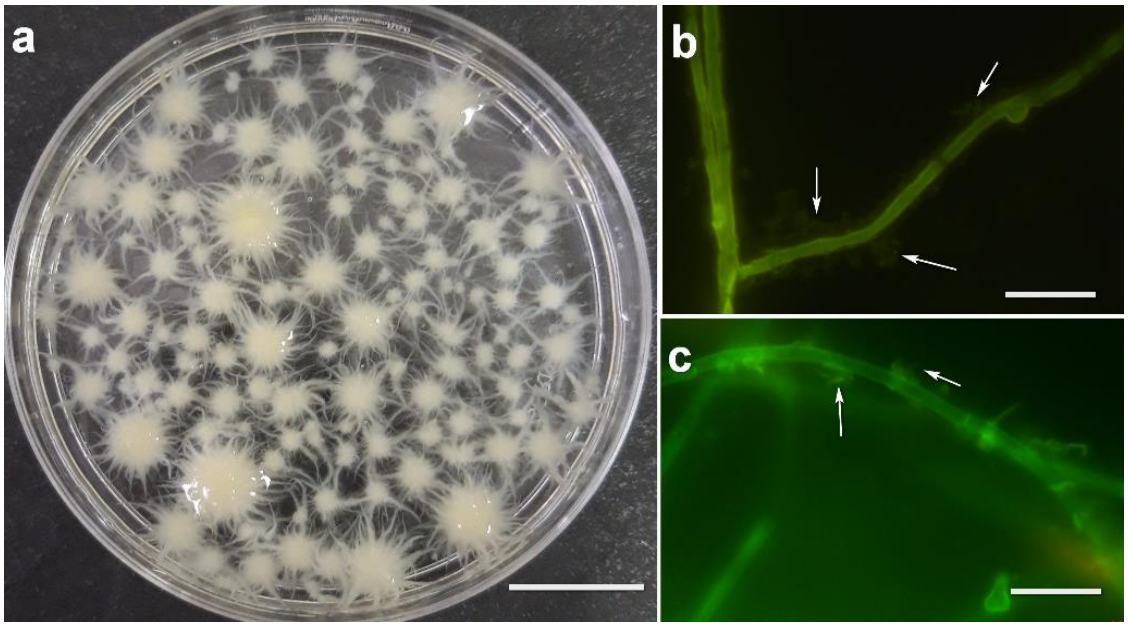


Fig. 1. Images of *N. nambi* IBSO 2391 mycelium grown under submerged cultivation
*(**a**) Mycelial pellets (PowerShot S50 camera, Canon, Japan); (b) fungal hypha with a clearly visible slimy layer (polysaccharide matrix) (arrows); (c) fragments of slimy layer on the surface of the hypha (arrows) after β-glucosidase treatment. Images (b) and (c) were obtained in the fluorescent mode of viewing with an AxioImager M2 (Carl Zeiss, Germany), filter set 09. Bars – 10 mm (a), 10 µm (b, c)*

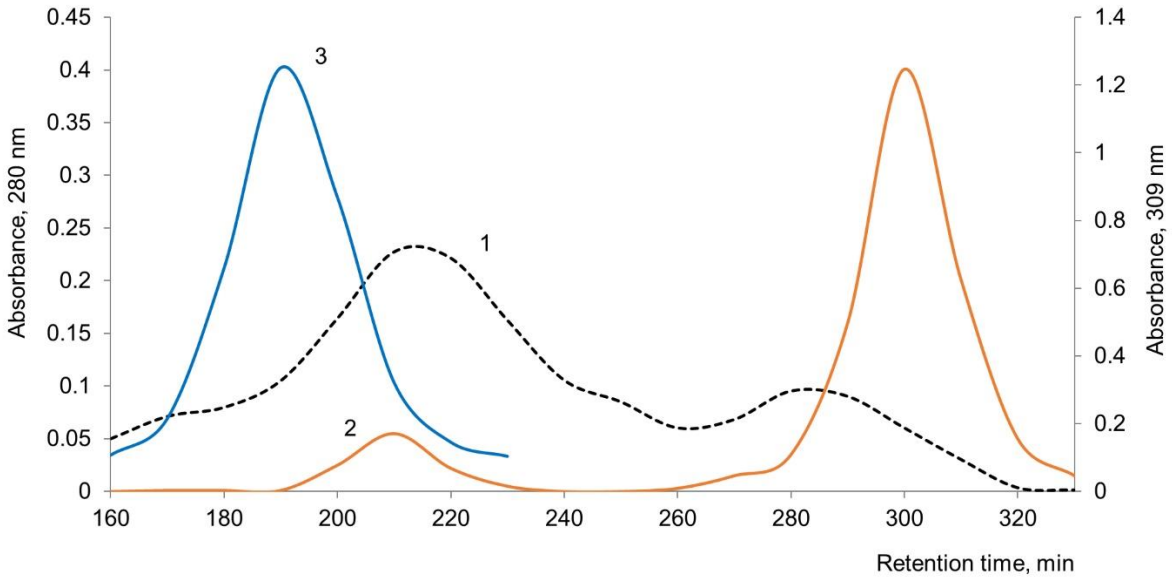


Fig. 2. Gel-filtration chromatography of protein preparations on the Sephacryl S-200 column
1 – profile of elution of concentrated extracellular proteins from N. nambi IBSO 2391 mycelium, 2 – profile of elution of protein markers (BSA – 66.5 kDa, and cytochrome C – 12.3 kDa), 3 – the distribution of activity of the extracellular oxidase catalyzing oxidation of veratryl alcohol in chromatographic fractions

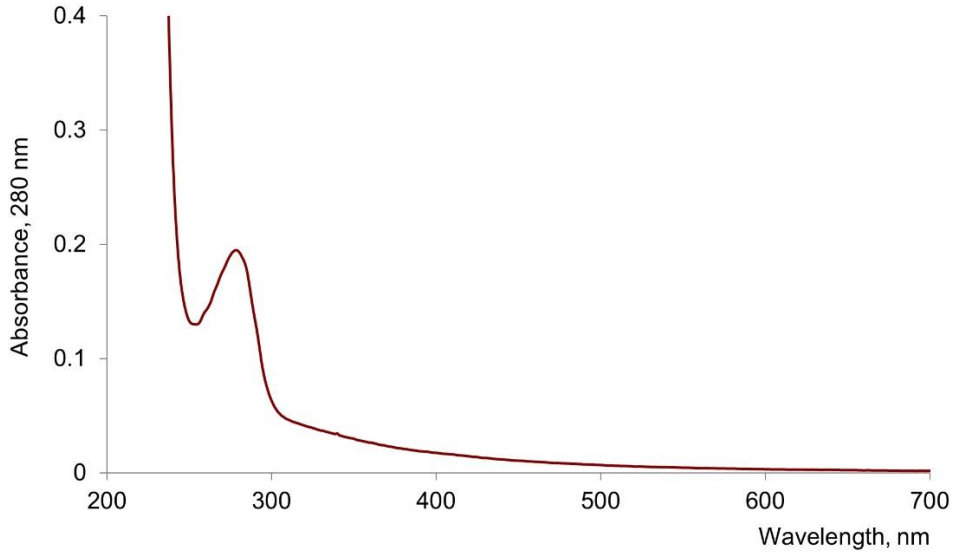


Fig. 3. Absorption spectrum of the final aqueous sample of the extracellular oxidase isolated from the basidiomycete *N. nambi* IBSO 2391

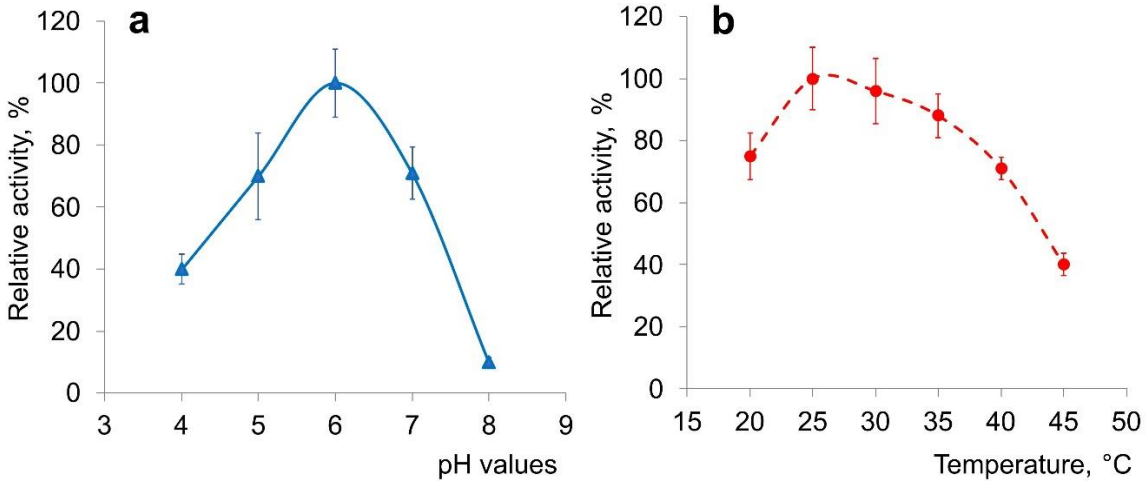


Fig. 4. The yield of the product of veratryl alcohol oxidation reaction catalyzed by extracellular oxidase from the basidiomycete *N. nambi* IBSO 2391 as dependent on pH (a) and temperature (b) of the reaction medium

The data are normalized to the maximal values of the product yield in measurement series.

3.4 Substrate Specificity Study

To study substrate specificity of the oxidase secreted by *N. nambi* IBSO 2391, we used a number of aromatic compounds (Table 1) as model substrates. The compounds were chosen based on the following considerations. It is well known that oxidoreductases secreted by higher fungi oxidize a wide range of cyclic compounds such as polyphenols, methoxy-substituted phenols, aromatic amines [4]. On the other hand, some of the aromatic compounds chosen as substrates in this study are commonly used in scientific research and in practical analytics to detect and identify enzymes with oxidase function (veratryl alcohol [28,33], hydroquinone, guaiacol [23,24], resorcinol [24], phenol [35,36], DAB [37]). Aromatic acids (caffeic acid and tyrosine) are metabolites of plants and fungi, taking part in lignification processes; in addition, tyrosine is a precursor in melanin biosynthesis [37].

The current study showed that the oxidase isolated from the basidiomycete *N. nambi* IBSO 2391 catalyzed oxidation of most of the aromatic

compounds chosen as model substrates (Fig. 5), which suggested broad substrate specificity of the enzyme. Moreover, the enzyme exhibited catalytic activity with no hydrogen peroxide or other mediators added to the reaction mixture. The greatest catalytic activity of the enzyme was observed in reactions with veratryl alcohol and hydroquinone. In reactions with guaiacol and aromatic amines (diaminobenzidine, o-dianisidine), the level of activity of the oxidase was considerably lower – by a factor of 2.5–3.5. In reactions with resorcinol, phenol, and caffeic acid, the catalytic activity of the enzyme was no greater than 6% of its activity with veratryl alcohol. No catalytic activity of the oxidase was observed in the reaction with tyrosine, suggesting that the extracellular oxidase from the basidiomycete *N. nambi* IBSO 2391 has no tyrosinase activity.

For the aromatic compounds that were oxidized by the *N. nambi* IBSO 2391 oxidase most effectively (veratryl alcohol, hydroquinone, and guaiacol), we obtained relationships between enzymatic reaction rates and concentrations of those substrates (Michaelis–Menten kinetics). Calculations of the kinetic parameters of enzymatic reactions based on the obtained relationships indicated (Table 3) that the *N. nambi* IBSO 2391 oxidase has the highest affinity for veratryl alcohol and catalyzes its oxidation with the greatest efficiency. At the same time, the enzyme has lower affinity for hydroquinone and guaiacol and oxidizes these compounds less effectively.

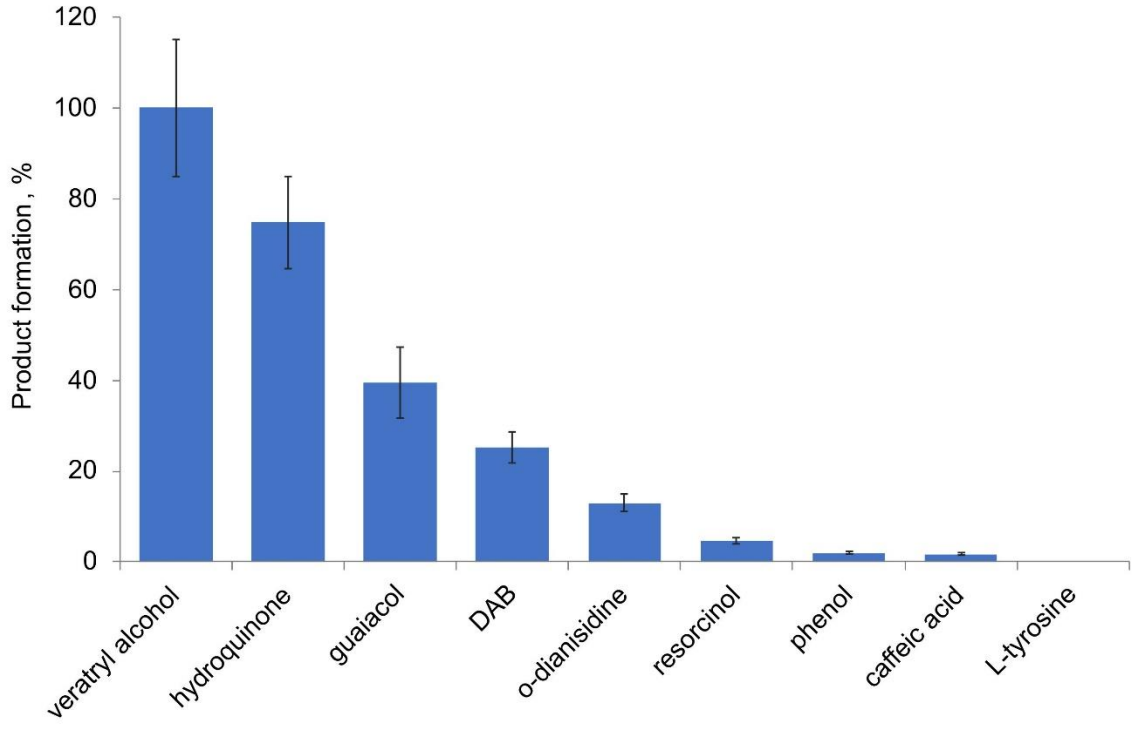


Fig. 5. The yields of reaction products showing the efficiency of oxidation of model aromatic substrates by the extracellular oxidase from the basidiomycete *N. nambi* IBSO 2391

The data are normalized to the yield of the product of veratryl alcohol oxidation reaction. Absolute value of yield of product of veratryl alcohol oxidation was 2.92 ± 0.58 a.u./mg/min

Table 3. Michaelis–Menten kinetic parameters calculated for the oxidase from *N. nambi* IBSO 2391

Substrate	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (sec^{-1} mM^{-1})
Veratryl alcohol	0.52	97	187
Hydroquinone	0.67	88	131
Guaiacol	0.72	47	65

3.5 Comparative Studies with HRP and Laccase

In experiments with the aromatic compounds used in the present study (Table 1), we compared substrate specificity of the extracellular oxidase from the fungus *N. nambi* IBSO 2391 with the substrate specificity of the enzymes laccase and peroxidase, which are commonly used in analytical and biotechnological applications. High-purity enzymes produced by Sigma (U.S.A.) – laccase from the fungus *Agaricus bisporus* and horseradish peroxidase (HRP) – were used in experiments. The enzymes tested in this study noticeably differed in their substrate specificity to the aromatic compounds used as substrates (Fig. 6). The only substrate oxidized by laccase from the fungus *A. bisporus* with high efficiency was dihydric phenol resorcinol; in the phenol and 4-AAP co-oxidation reaction, laccase catalyzed phenol oxidation with considerably lower efficiency. *A. bisporus* laccase showed very low efficiency of oxidation of aromatic amines (DAB and o-dianisidine), methoxy-substituted phenol guaiacol, and caffeic acid; it did not catalyze oxidation of veratryl alcohol and dihydric phenol hydroquinone; however, it oxidized tyrosine. At the same time, the substrate specificity of HRP was considerably broader and comparable to that of the extracellular oxidase from the fungus *N. nambi* IBSO 2391. Yet, these two enzymes differed substantially in the efficiency of oxidation of some aromatic compounds used as substrates. The results obtained demonstrate that in contrast to the oxidase from the fungus *N. nambi* IBSO 2391, HRP did not catalyze oxidation of veratryl alcohol although the catalytic activity of peroxidase in oxidation of hydroquinone and guaiacol was comparable with the catalytic activity of the *N. nambi* IBSO 2391 oxidase. On the other hand, HRP catalyzed oxidation of resorcinol, phenol, and caffeic acid much more effectively than the extracellular oxidase from *N. nambi* IBSO 2391. An important difference was that HRP catalyzed oxidation of the tested aromatic compounds only after the addition of hydrogen peroxide to the reaction mixture while the extracellular oxidase from N. nambi IBSO 2391 exhibited high catalytic activity without the addition of that mediator. On the one hand, results of the present study (Fig. 6) are indicative of substantial differences between catalytic properties of the extracellular oxidase from the basidiomycete *N. nambi* IBSO 2391 and the catalytic properties of laccase from the fungus *A. bisporus* and HRP. On the other hand, these results suggest that the *N. nambi* IBSO 2391 oxidase can be a useful addition to the pool of enzymes used in enzymatic analytics.

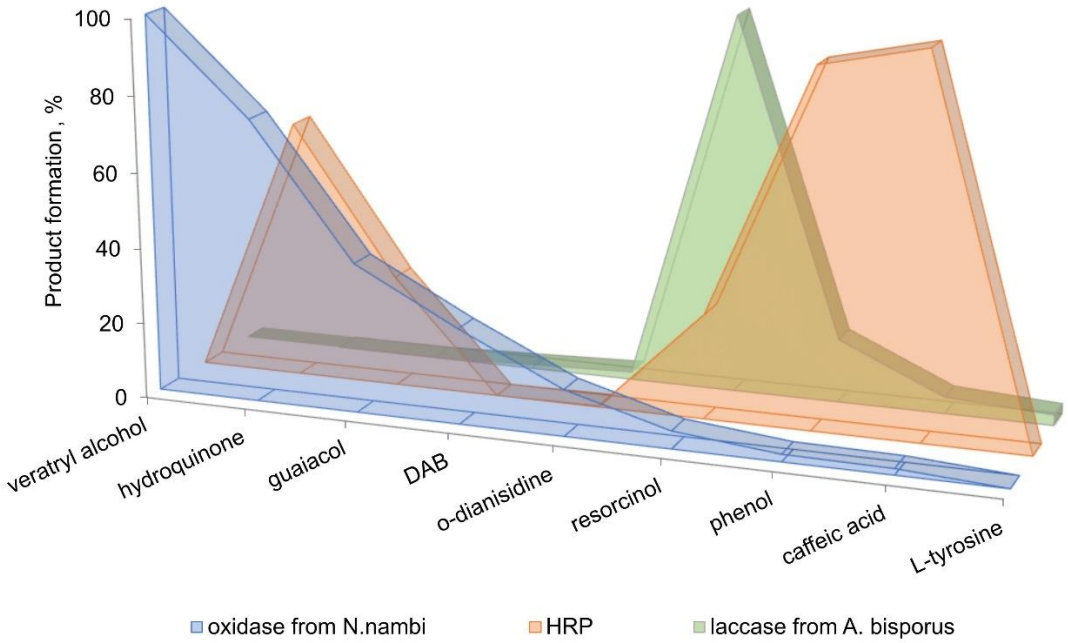


Fig. 6. The yields of reaction products showing the efficiency of oxidation of aromatic compounds by the extracellular oxidase from the basidiomycete *N. nambi* IBSO 2391, HRP, and laccase from the fungus *A. bisporus*

Values of the maximal product yields in measurement series are taken for 100%

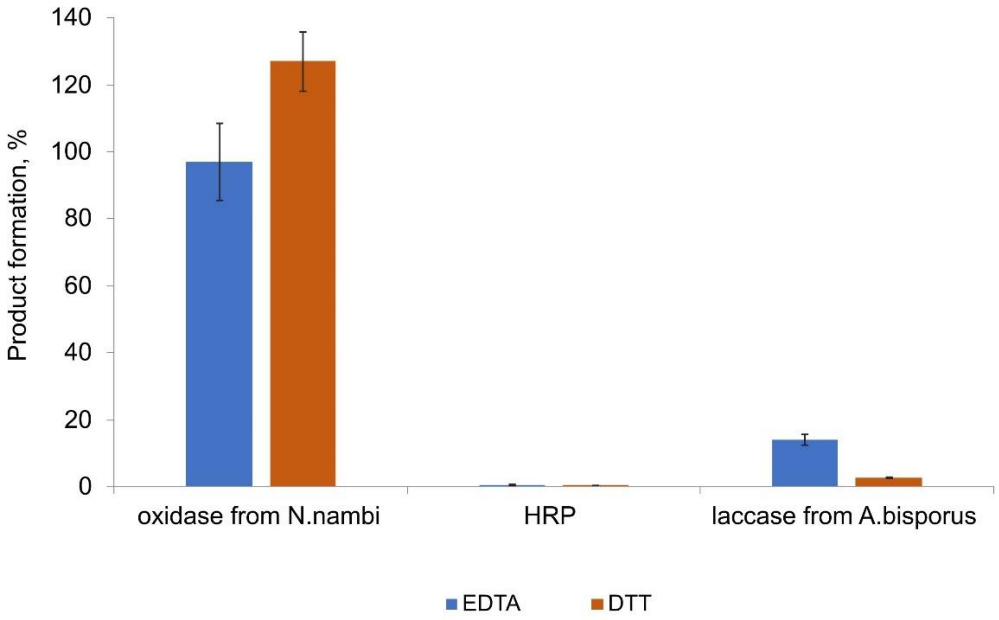


Fig. 7. The yields of products of reactions catalyzed by the extracellular oxidase from the basidiomycete *N. nambi* IBSO 2391, HRP, and *A. bisporus* laccase treated with EDTA or DTT at a concentration of 1 mM

Values of the product yields in reactions catalyzed by initial enzymes (untreated with EDTA and DTT) are taken for 100%

In supplementary experiments, commercial preparations of laccase from the fungus *A. bisporus* and HRP were used to answer the question whether the extracellular oxidase from the basidiomycete *N. nambi* IBSO 2391 is a metal-containing enzyme. As laccase and HRP contain ions of copper and iron, respectively, their catalytic activity was tested after the treatment with the chelating agent of divalent metal ions EDTA and SH reagent DTT. It is commonly known that these reagents bind divalent metal ions by forming coordinate bonds. The effects of EDTA and DTT on the activity of the *N. nambi* IBSO 2391 oxidase, *A. bisporus* laccase, and HRP were studied using veratryl alcohol, resorcinol, and phenol as substrates. Incubation of *A. bisporus* laccase and HRP in the presence of EDTA (or DTT) at concentrations between 0.1 and 1 mM resulted in a considerable decline in the functional activities of both enzymes and a decrease in the yields of reaction products (Fig. 7). A similar EDTA treatment of the extracellular oxidase from the fungus *N. nambi* IBSO 2391 did not affect the functional activity of the enzyme. This may suggest the absence of divalent metal ions in the molecule of this enzyme. At the same time, the treatment of the study oxidase with DTT resulted in a considerable (25% or greater) increase in its catalytic efficiency compared to the control. The mechanism of this effect is not yet clear and needs to be studied.

4. CONCLUSION

Thus, the treatment of the mycelium of the basidiomycete *N. nambi* IBSO 2391 with β-glucosidase followed by gel-filtration chromatography of the extract on Sephacryl S-200 resulted in isolation of an extracellular enzyme of the fungus exhibiting oxidase function, with native molecular weight 80 kDa. The findings of the present study suggest that the extracellular oxidase isolated from *N. nambi* IBSO 2391 does not contain chromophore compounds and divalent metal ions; functions in wide ranges of temperature and pH of the reaction medium, showing the highest catalytic activity at temperatures between 22 and 35 °C and pH 6.0; catalyzes oxidation of diverse aromatic compounds with no mediators (such as hydrogen peroxide) added to the reaction mixture; has substrate specificity that differs from the substrate specificity of metal-containing oxidases – laccase from the fungus *A. bisporus* and HRP. The current study provides the basis and opens up opportunities for producing homogeneous extracellular oxidase of the fungus *N. nambi* IBSO 2391, in order to determine its structure and mechanism of catalytic function

and to study its potential uses in biotechnological and analytical applications.

ACKNOWLEDGEMENTS

The study was funded by State Assignment of the Ministry of Science and Higher Education of the Russian Federation (project No. FWES-2024-0018).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Wang X, Yao B, Su X. Linking enzymatic oxidative degradation of lignin to organics detoxification. Int J Mol Sci. 2018;19(11):3373.
DOI: 10.3390/ijms19113373
2. Torres-Farradá G, Manzano-León AM, Rineau F, Thijs S, Jambon I, Put J, et al. Biodegradation of polycyclic aromatic hydrocarbons by native Ganoderma sp. strains: identification of metabolites and proposed degradation pathways. Appl Microbiol Biotechnol. 2019;103:7203–7215.
DOI:10.1007/s00253-019-09968-9
3. Morsi R, Bilal M, Iqbal HMN, Ashraf SS. Laccases and peroxidases: The smart, greener and futuristic biocatalytic tools to mitigate recalcitrant emerging pollutants. Sci Total Environ. 2020;20:714:136572.
DOI: 10.1016/j.scitotenv.2020.136572
4. Savino S, Fraaije MW. The vast repertoire of carbohydrate oxidases: An overview. Biotechnol Adv. 2021;51:107634.
DOI: 10.1016/j.biotechadv.202.107634
5. Urlacher VB, Koschorreck K. Pecularities and applications of aryl-alcohol oxidases from fungi. Appl Microbiol Biotechnol. 2021;105:4111–4126.
DOI: 10.1007/s00253-021-11337-4
6. Berger RG; Ersoy F. Improved foods using enzymes from basidiomycetes. Processes. 2022;10:726.
DOI: 10.3390/pr10040726
7. Hu Y, Zhu M, Tian G, Zhao L, Wang H, Ng TB. Isolation of a protease-resistant and pH-stable a-galactosidase displaying hydrolytic efficacy toward raffinose family oligosaccharides from the button mushroom *Agaricus bisporus*. Int J Biol Macromol. 2017;104:576–583.
8. Yin L, Ye J, Kuang S, Guan Y, You R. Induction, purification, and characterization of a thermo and pH stable laccase from *Abortiporus biennis* J2 and its application on the clarification of litchi juice. Biosci Biotechnol Biochem. 2017;81:1033–1040.
9. Sousa AC, Martins LO, Robalo MP. Laccases: Versatile biocatalysts for the synthesis of heterocyclic cores. Molecules. 2021;26:3719.
DOI: 10.3390/molecules26123719
10. Li F, Ma W, Wu X, Wang Y, He J. Luminol, horseradish peroxidase, and glucose oxidase ternary functionalized graphene oxide for ultrasensitive glucose sensing. Analyt Bioanalyt Chem. 2018;410:543–552.
DOI: 10.1007/s00216-017-0752-5
11. Naghdi M, Taheran M, Brar SK, Kermanshahi-Pour A, Verma M, Surampalli RY. Removal of pharmaceutical compounds in water and wastewater using fungal oxidoreductase enzymes. Environ Pollut. 2018;234:190–213.
DOI: 10.1016/j.envpol.2017.11.060
12. Nikolaivits E, Dimarogona M, Karagiannaki I, Chalima A, Fishman A, Topakas E. Versatile fungal polyphenol oxidase with chlorophenol bioremediation potential: characterization and protein engineering. Appl Environ Microbiol. 2018;84:e01628-18.
DOI: 10.1128/AEM.01628-18
13. Shebanova AD, Chernykh AM, Baskunov BP, Gaidina AS, Myasoedova NM, Renfeld ZV, et al. Novel biocatalyst from *Microthielavia ovispora* VKM F-1735 for industrial dye decolorization in the absence of mediators. Process Biochem. 2021; 109:186–197.
DOI:10.1016/j.procbio.2021.07.009
14. Kobayashi N, Wada N, Yokoyama H, Suzuki T, Habu N et al. Extracellular enzymes secreted in the mycelial block of Lentinula edodes during hyphal growth. AMB Expr. 2023;13:36.
DOI: 10.1186/s13568-023-01547-6
15. Jankowski N, Koschorreck K, Urlacher VB. High-level expression of aryl-alcohol oxidase 2 from *Pleurotus eryngii* in *Pichia pastoris* for production of fragrances and bioactive precursors. Appl Microbiol Biotechnol. 2020;104:9205–9218.
DOI: 10.1007/s00253-020-10878-4

16. Tu T, Wang Y, Huang H, Wang Y, Jiang X, Wang Z, et al. Improving the thermostability and catalytic efficiency of glucose oxidase from *Aspergillus niger* by molecular evolution. Food Chem. 2019;281:163–170.
DOI: 10.1016/j.foodchem.2018.12.099
17. Asgher M, Noreena S, Bilal M. Enhancing catalytic functionality of *Trametes versicolor* IBL-04 laccase by immobilization on chitosan microspheres. Chem Eng Res Des. 2017;119:1–11.
DOI: 10.1016/j.cherd.2016.12.011
18. Zdarta J, Meyer AS, Jesionowski T, Pinelo M. Developments in support materials for immobilization of oxidoreductases: a comprehensive review. Adv Colloid Interface Sci. 2018;258:1-20.
DOI: 10.1016/j.cis.2018.07.004
19. Giannakopoulou A., Patila M., Spyrou K., Chalmpes N., Zarafeta D., Skretas G., et al. Development of a Four-Enzyme Magnetic Nanobiocatalyst for Multi-Step Cascade Reactions. Catalysts. 2019;9:995.
DOI: 10.3390/catal9120995
20. Shakerian F, Zhao J, Li S-P. Recent development in the application of immobilized oxidative enzymes for bioremediation of hazardous micropollutants – A review. Chemosphere. 2020;239:124716
DOI: 10.1016/j.chemosphere.2019.124716
21. Olajuyigbe FM, Fatokun CO. Biochemical characterization of an extremely stable pH-versatile laccase from Sporothrix carnis CPF-05 Enzyme. Int J Biol Macromol. 2017;94:535–543.
DOI: 10.1016/j.ijbiomac.2016.10.037
22. Othman AM, Elsayed MA, Elshafei AM, Hassan MM. Purification and biochemical characterization of two isolated laccase isoforms from *Agaricus bisporus* CU13 and their potency in dye decolorization. Int J Biol Macromol. 2018;113:1142–1148.
DOI: 10.1016/j.ijbiomac.2018.03.043
23. Liu X, Deng W, Yang Y. Characterization of a Novel Laccase LAC-Yang1 from White-Rot Fungus *Pleurotus ostreatus* Strain Yang1 with a Strong Ability to Degrade and Detoxify Chlorophenols. Molecules. 2021;26:473.
DOI: 10.3390/molecules26020473
24. Zerva A, Pentari C, Termentzi A, America AHP, Zouraris D, Bhattacharya SK, et al. Discovery of two novel laccase-like multicopper oxidases from *Pleurotus citrinopileatus* and their application in phenolic oligomer synthesis. Biotechnol Biofuels. 2021;14:83.
DOI: 10.1186/s13068-021-01937-7
25. Kimani V, Ullrich R, Büttner E, Herzog R, Kellner H, Jehmlich N, et al. First Dye-decolorizing peroxidase from an ascomycetous fungus secreted by *Xylaria grammica*. Biomolecules. 2021;11:1391.
DOI: 10.3390/biom11091391
26. Athamneh K, Alneyadi A, Alsadik A, Wong TS, Ashraf SS. Efficient degradation of various emerging pollutants by wild type and evolved fungal DyP4 peroxidases. PLoS ONE. 2022;17(1):e0262492.
DOI: 10.1371/journal.pone.0262492
27. Kadowaki MAS, Higasi PMR, de Godoy MO, de Araújo EA, Godoy AS, Prade RA, Polikarpov I. Enzymatic versatility and thermostability of a new aryl-alcohol oxidase from *Thermothelomyces thermophilus* M77. Biochim Biophys Acta Gen Subj. 2020;1864(10):129681.
DOI: 10.1016/j.bbagen.2020.129681
28. Ferreira P, Carro J, Balcells B, Martínez AT, Serrano A. Expanding the physiological role of aryl-alcohol flavooxidases as quinone reductases. Appl Environ Microbiol. 2023;89(5):e0184422.
DOI: 10.1128/aem.01844-22.27
29. Medvedeva SE, Artemenko KS, Krivosheenko AA, Rusinova AG, Rodicheva EK, Puzyr AP et al. Growth and light emission of luminous basidiomycetes cultivated on solid media and in submerged culture. Mycosphere. 2014;5: 565–577.
30. Mogilnaya OA, Ronzhin NO, Artemenko KS, Bondar VS. Morphological properties and levels of extracellular peroxidase activity and light emission of the basidiomycete *Armillaria borealis* treated with β-glucosidase and chitinase. Mycosphere 2017;8(4):649–659.
DOI: 10.5943/mycosphere/8/4/11
31. Mogilnaya O, Ronzhin·N, Posokhina E, Bondar V. Extracellular oxidase from the *Neonothopanus nambi* fungus as a promising enzyme for analytical applications. The Protein J. 2021;40:731–740.
DOI: 10.1007/s10930-021-10010-z
32. Kochetov GA. Practical guide to enzymology. Moscow: Higher School, (in Russian); 1980.
33. Ferreira P, Medina M, Guillén F, Martínez M, van Berkel W, Martínez A. Spectral and

catalytic properties of aryl-alcohol oxidase, a fungal flavoenzyme acting on polyunsaturated alcohols. Biochem J. 2005;389:731–738.

34. Mogilnaya OA, Ronzhin NO, Bondar VS. Estimating levels of light emission and extracellular peroxidase activity of mycelium of luminous fungus *Neonothopanus nambi* treated with β-glucosidase. Curr Res Environ Appl Mycol. 2018;8:75–85.
DOI: 10.5943/cream/8/1/6

35. Gilabert MA, Hiner AN, García-Ruiz PA, Tudela J et al. Differential substrate behaviour of phenol and aniline derivatives during oxidation by horseradish peroxidase: kinetic evidence for a two-step mechanism. Biochim Biophys Acta. 2004;1699(1-2): 235-243.
DOI: 10.1016/j.bbapap.2004.03.003

36. Maryskova M, Linhartova L, Novotny V, Rysova M, Cajthaml T, Sevcu A. Laccase and horseradish peroxidase for green treatment of phenolic micropollutants in real drinking water and wastewater. Environ Sci Pollut R. 2021;28:31566–31574.
DOI: 10.1007/s11356-021-12910-0

37. Marchiosi R, dos Santos WD, Constantin RP, de Lima RB, Soares AR, Finger-Teixeira A, et al. Biosynthesis and metabolic actions of simple phenolic acids in plants. Phytochem Rev. 2020;19:865–906.
DOI: 10.1007/s11101-020-09689-2

Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/114023