

1 **ON THE SUBSTRATE SPECIFICITY AND SOME**
2 **PROPERTIES OF THE EXTRACELLULAR**
3 **OXIDASE FROM THE *NEONOTHOPANUS***
4 ***NAMBI* BASIDIOMYCETE**

8 **ABSTRACT**

9 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 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.

10 **Keywords:** aromatic compounds, basidiomycetes, biocatalysis, DTT, EDTA, extracellular oxidase

11 **1. INTRODUCTION**

12 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].

13 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. MATERIAL 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].

2.2.1. 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". [38]

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.

92 The native molecular weight of extracellular oxidase of the basidiomycete *N. nambi* IBSO 2391 was determined by
93 chromatography of protein markers – BSA (Sigma, U.S.A.) and cytochrome c (Fluka, Germany) with molecular weights of
94 66.5 kDa and 12.3 kDa, respectively – under the above conditions (column size, volume of the sample applied, eluent,
95 and flow rate). The native molecular weight of the study enzyme was calculated using the values of the maximum oxidase
96 activity in chromatographic samples and the maxima of elution of protein markers recorded as absorbance at 280 nm [31].
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98 **2.3 Analytical methods**

100 **2.3.1. Oxidase activity determination**

101 The presence of extracellular oxidase in the samples (aqueous extract from the *N. nambi* IBSO 2391 mycelium,
102 concentrated enzymes, and chromatographic fractions) was determined by oxidation reaction of veratryl alcohol (Sigma,
103 U.S.A.), which was used as substrate. An aqueous solution of veratryl alcohol was prepared in situ in DI water. In assay of
104 oxidase activity, the 600- μ l reaction mixture contained 10 mM veratryl alcohol and 50 μ l of the tested sample. After all
105 reaction ingredients were added, the samples were agitated for 3 s on a Vortex-Genie 2 g-560E mixer (Scientific
106 Industries, Inc., U.S.A.) and incubated for 30 min at a temperature of 25 °C. The level of oxidase activity in the samples
107 was determined by measuring the yield of veratryl alcohol oxidation reaction product using the spectral method
108 (spectrophotometer UV-1800, Shimadzu, Japan), as absorbance at 309 nm.
109

111 **2.3.2. Protein concentration measurement**

112 “Protein concentrations in the samples were determined by the well-known biuret method, using Benedict’s reagent and
113 BSA as the standard. The samples were incubated for 15 min at 25 °C with Benedict’s reagent, and then their absorbance
114 was measured at a wavelength of 330 nm using a UV-1800 spectrophotometer” [32].
115

117 **2.3.3. UV-visible spectral analysis of oxidase**

118 The absorption spectrum of the extracellular oxidase isolated from the basidiomycete *N. nambi* IBSO 2391 was
119 determined from 200 to 700 nm at 25 °C in DI water using the UV-1800 spectrophotometer.
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122 **2.3.4. Determination of Kinetic Parameters, Optimum pH and Temperature**

123 The temperature optimum of the catalytic activity of the isolated oxidase was determined within the temperature range of
124 the reaction medium between 20 and 50 °C. The yield of the product was estimated using the spectral method (the UV-
125 1800 spectrophotometer) after the reaction mixtures containing the enzyme and the substrate (veratryl alcohol) had been
126 incubated for 15 min at different temperatures (TB-85 Thermo Batch, Shimadzu, Japan). The effectiveness of the enzyme
127 at different pH values of the medium was studied using 50 mM Na-acetate buffers with pH between 3 and 8.

128
129 Michaelis–Menten kinetic parameters for *N. nambi* oxidase were performed by assaying various concentrations of veratryl
130 alcohol (0.1 to 10 mM), hydroquinone (0.05 to 10 mM) and guaiacol (0.5 to 15 mM). The oxidation of substrates was
131 measured by an increase in absorption at 309 nm for veratryl alcohol ($\epsilon_{309} = 9300 \text{ M}^{-1} \cdot \text{cm}^{-1}$), at 246 nm for hydroquinone
132 ($\epsilon_{246} = 19000 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and at 467 nm for guaiacol ($\epsilon_{467} = 3800 \text{ M}^{-1} \cdot \text{cm}^{-1}$) [24,33]. To determine the kinetic constants, the
133 obtained Michaelis–Menten dependences were transformed into a linear Lineweaver–Burk graphs.
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135 **2.3.5. Substratespecificity and factors**

136 Substrate specificity of the extracellular oxidase from the basidiomycete *N. nambi* IBSO 2391 was estimated using the
137 following aromatic compounds as model substrates: phenol derivatives, aromatic acids, and aromatic amines (Table 1).
138 Solutions of reagents were prepared in situ in DI water. Concentrations of the aromatic compounds in reaction mixtures
139 were varied to determine the dependencies of product yields and calculate kinetic characteristics of enzymatic reactions.

140 The catalytic efficiency of the enzyme with model substrates was estimated using the spectral method (UV-1800
141 spectrophotometer) by the maximal yields of reaction products recorded as the absorbance at the corresponding
142 wavelengths (Table 1) and expressed in absorbance units per 1 mg protein per 1 min. In the experiment with monophenol
143 as the model substrate, the level of activity of the *N. nambi* IBSO 2391 oxidase was determined in reaction of co-oxidation
144 of phenol with 4-aminoantipyrine (4-AAP) by measuring the yield of reaction product – quinoneimine, recorded as
145 absorbance at 506 nm. In this experiment, the 600- μ l reaction mixture contained 5.96 mM phenol, 0.49 mM 4-AAP, and
146 100 μ l of the enzyme sample. After all reaction ingredients were added, the samples were agitated for 3 s on a Vortex-
147 Genie 2 g-560E mixer and incubated for 30 min at a temperature of 25 °C. The experiments were performed with 4-AAP
148 (1-phenyl-2,3-dimethyl-4-aminopyrazolone) of analytical grade (Reakhim, Russia) and phenol (Fluka, Germany); the
149 solutions were prepared in situ in DI water [31].
150

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 $M \pm m$, $n = 3$ 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

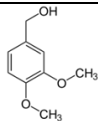



Micrographs of the pellets and hyphae were obtained by using the PowerShot S50 camera (Canon, Japan) and an Axiolmager M2 microscope (Carl Zeiss, Germany) in the fluorescent mode of viewing.

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.

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:

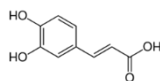
Guaiacol (2- methoxyphenol) (Sigma-Aldrich, U.S.A.)



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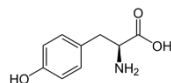
Aromatic acids:

Caffeic acid(3,4 - hydroxycinnamic acid) (Sigma-Aldrich, U.S.A.)



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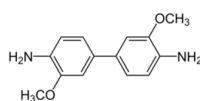
L-Tyrosine (L-2-Amino-3-(4-hydroxyphenyl) propanoic acid) (Fluka, Germany)



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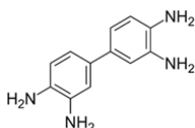
Aromatic amines:

o-Dianisidine (Fluka, Germany)



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Diaminobenzidine (DAB) (AppliChem, Germany)



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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

213 fraction containing an enzyme with oxidase activity (Fig. 2). That oxidase catalyzed oxidation reaction of veratryl alcohol
 214 with no exogenous hydrogen peroxide or other mediators added to the reaction mixture. Calculations based on the
 215 maximum oxidase activity and absorption maxima of protein markers recorded when protein components were eluted
 216 from

217 the column showed that extracellular oxidase of the fungus *N. nambi* IBSO 2391 catalyzing oxidation of veratryl alcohol
 218 was detected in the protein fraction with the native molecular

219 weight of the protein of 80 kDa. After chromatography, fractions with the highest oxidase activity (Fig. 2) were pooled, and
 220 the pooled sample was subjected to ultrafiltration through a 30 kDa membrane to concentrate the enzyme and remove
 221 residual eluent (NaCl). The concentrated enzyme (final oxidase preparation) was used in further studies. Parameters of

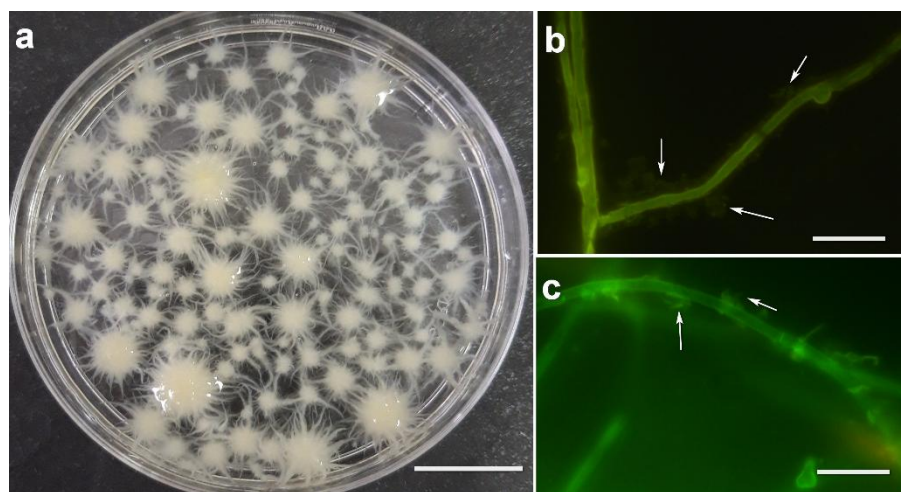
222 purification of the extracellular oxidase of *N. nambi* IBSO 2391 using gel-filtration chromatography on the Sephacryl S-200
 223 column (total and specific activities, yield, and purification level) are summarized in Table 2. They show that one stage of

224 chromatography resulted in tenfold purification of the enzyme: the specific activity of the oxidase in the final enzyme
 225 preparation was higher by a factor of 10 compared to the specific activity of the oxidase in the initial concentrate of

226 extracellular proteins from *N. nambi* IBSO 2391 mycelium.

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

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

240 **Table 2. Parameters of purification of the extracellular oxidase from the fungus *N. nambi* IBSO 2391 using gel-**
 241 **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

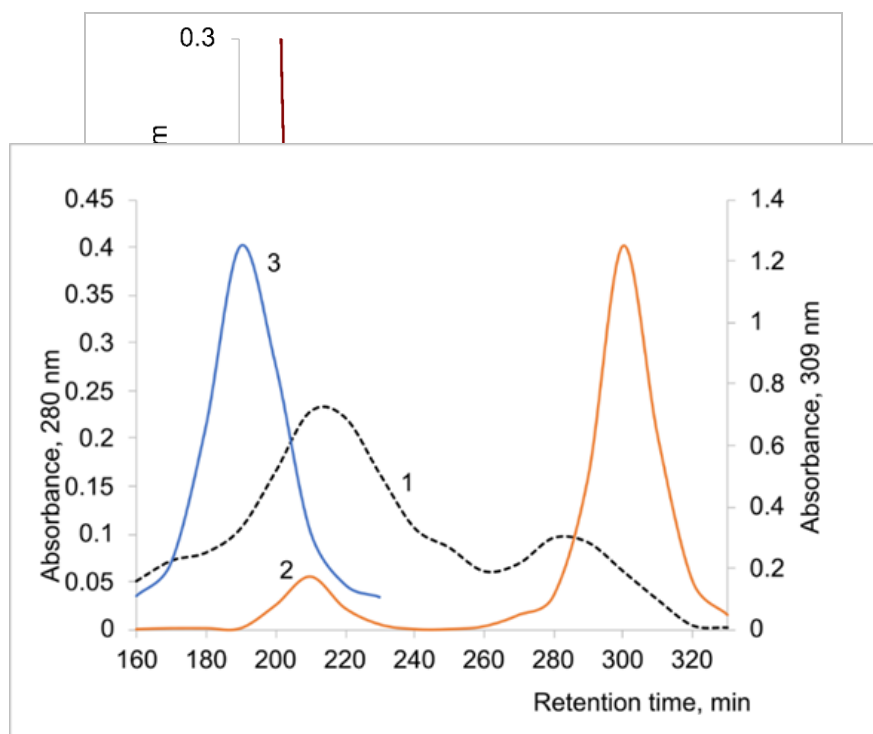
243 Total enzyme activity was
 244 calculated in micromoles of
 245 the reaction product formed
 246 per minute. The specific
 247 enzyme activity was
 248 calculated as total enzyme
 249 activity per one milligram of
 250 protein. The molar extinction
 251 coefficient for veratryl
 252 aldehyde used for calculation

253 was $\epsilon_{309} = 9300 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

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255 3.3. Spectral characteristic, optimum pH and temperature

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



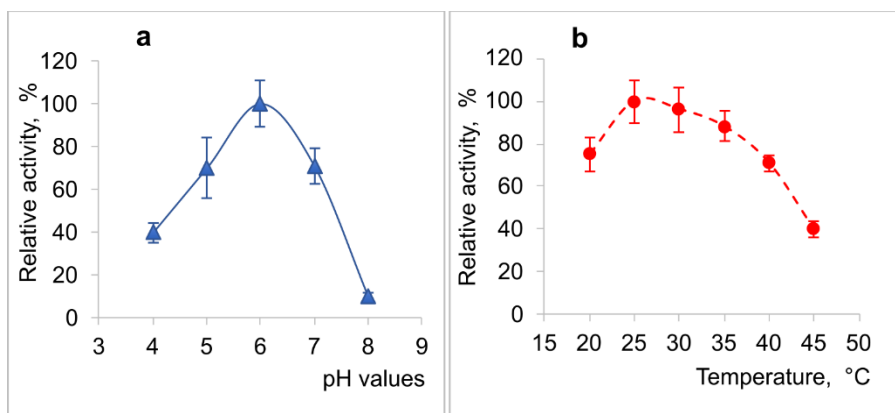
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Fig. 3. Absorption spectrum of the final aqueous sample of the extracellular oxidase isolated from the basidiomycete *N. nambi* IBSO 2391.

263 Experiments demonstrated that the extracellular oxidase isolated from the basidiomycete *N. nambi* IBSO 2391 was able
264 to function effectively in a wide range of pH of the medium, oxidizing veratryl alcohol most efficiently at pH 6.0 (Fig. 4a).
265 Another finding was that the enzyme was functionally active in a wide range of temperatures of the reaction medium,
266 showing the highest efficiency of oxidation of the substrate (veratryl alcohol) at temperatures between 22 and 35 °C (Fig.
267 4b).
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271 **Fig. 4. The yield of the product of veratryl alcohol oxidation reaction catalyzed by extracellular oxidase from the**
272 **basidiomycete *N. nambi* IBSO 2391 as dependent on pH (a) and temperature (b) of the reaction medium.**

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

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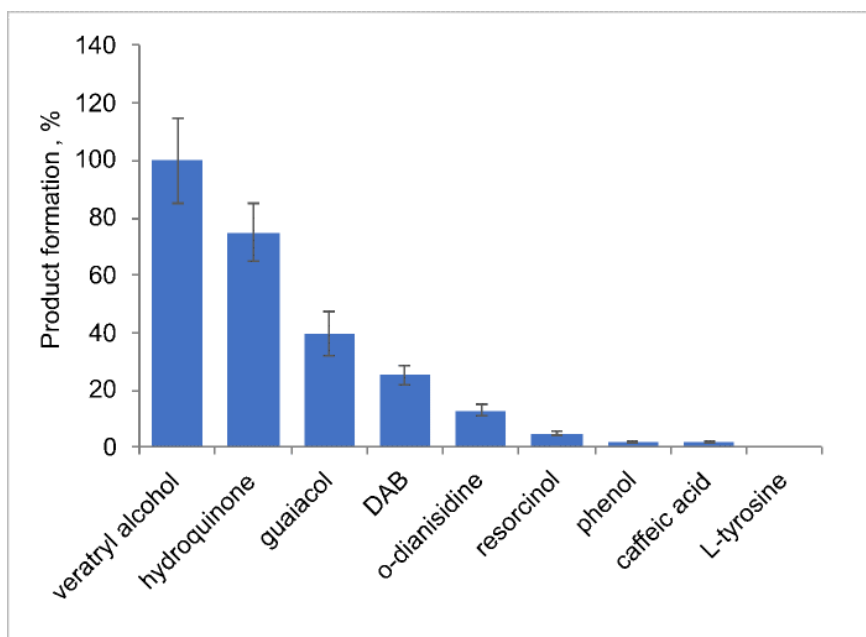
275 **3.4. Substrate specificity study**

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277 To study substrate specificity of the oxidase secreted by *N. nambi* IBSO 2391, we used a number of aromatic compounds
278 (Table 1) as model substrates. The compounds were chosen based on the following considerations. It is well known that
279 oxidoreductases secreted by higher fungi oxidize a wide range of cyclic compounds such as polyphenols, methoxy-
280 substituted phenols, aromatic amines [4]. On the other hand, some of the aromatic compounds chosen as substrates in
281 this study are commonly used in scientific research and in practical analytics to detect and identify enzymes with oxidase
282 function (veratryl alcohol [28,33], hydroquinone, guaiacol [23,24], resorcinol [24], phenol [35,36], DAB [37]). Aromatic acids
283 (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].

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



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295 **Fig. 5. The yields of reaction products showing the efficiency of oxidation of model aromatic substrates by the**
 296 **extracellular oxidase from the basidiomycete *N. nambi* IBSO 2391.** *The data are normalized to the yield of the product of*
 297 *veratryl alcohol oxidation reaction. Absolute value of yield of product of veratryl alcohol oxidation was 2.92 ± 0.58 a.u./mg/min.*

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

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

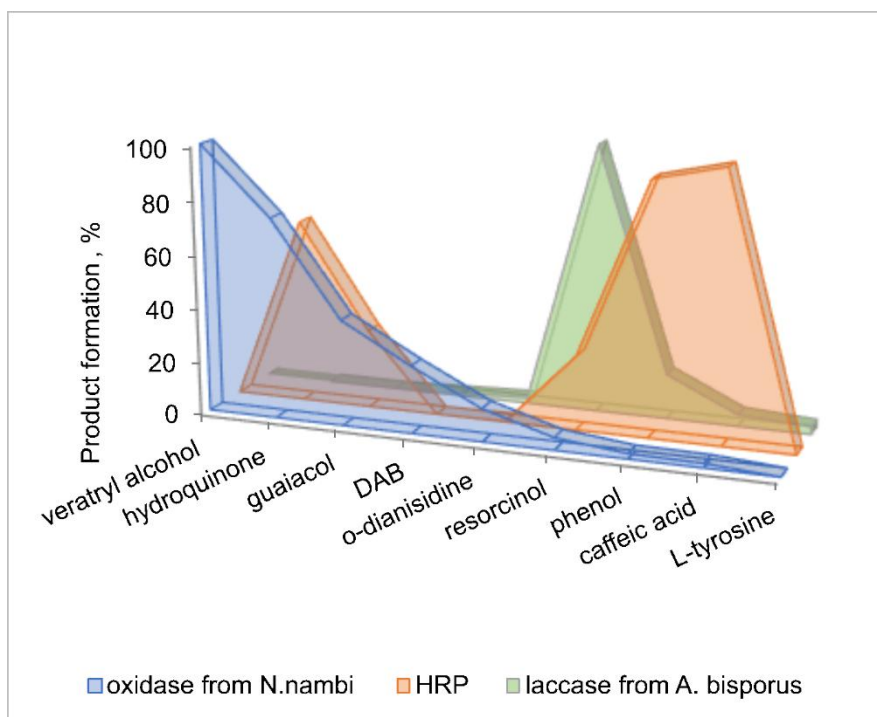
Substrate	K_m (mM)	k_{cat} (sec ⁻¹)	k_{cat}/K_m (sec ⁻¹ mM ⁻¹)
Veratryl alcohol	0.52	97	187
Hydroquinone	0.67	88	131
Guaiacol	0.72	47	65

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3.5. Comparative studies with HRP and Laccase

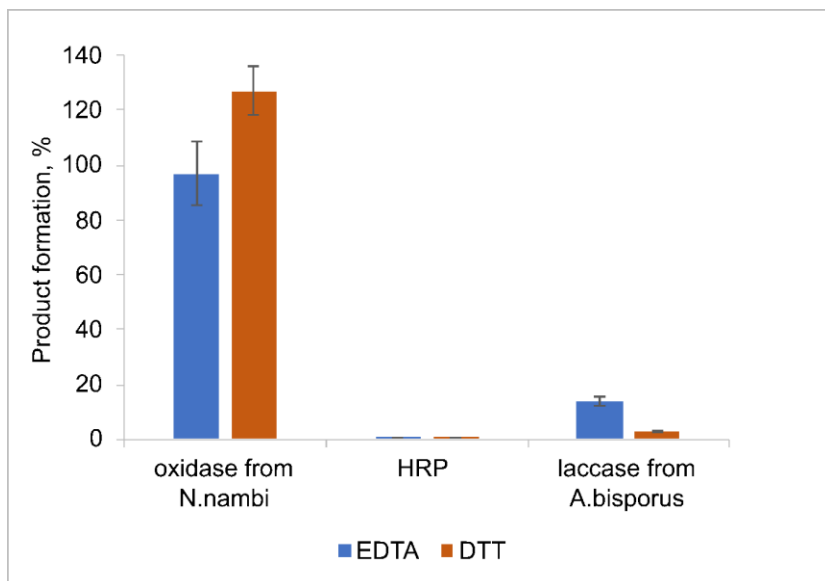
310 In experiments with the aromatic compounds used in the present study (Table 1), we compared substrate specificity of the
 311 extracellular oxidase from the fungus *N. nambi* IBSO 2391 with the substrate specificity of the enzymes laccase and
 312 peroxidase, which are commonly used in analytical and biotechnological applications. High-purity enzymes produced by
 313 Sigma (U.S.A.) – laccase from the fungus *Agaricus bisporus* and horseradish peroxidase (HRP) – were used in
 314 experiments. The enzymes tested in this study noticeably differed in their substrate specificity to the aromatic compounds
 315 used as substrates (Fig. 6). The only substrate oxidized by laccase from the fungus *A. bisporus* with high efficiency was
 316 dihydric phenol resorcinol; in the phenol and 4-AAP co-oxidation reaction, laccase catalyzed phenol oxidation with
 317 considerably lower efficiency. *A. bisporus* laccase showed very low efficiency of oxidation of aromatic amines (DAB and o-
 318 dianisidine), methoxy-substituted phenol guaiacol, and caffeic acid; it did not catalyze oxidation of veratryl alcohol and
 319 dihydric phenol hydroquinone; however, it oxidized tyrosine. At the same time, the substrate specificity of HRP was
 320 considerably broader and comparable to that of the extracellular oxidase from the fungus *N. nambi* IBSO 2391. Yet, these
 321 two enzymes differed substantially in the efficiency of oxidation of some aromatic compounds used as substrates. The
 322 results obtained demonstrate that in contrast to the oxidase from the fungus *N. nambi* IBSO 2391, HRP did not catalyze
 323 oxidation of veratryl alcohol although the catalytic activity of peroxidase in oxidation of hydroquinone and guaiacol was
 324 comparable with the catalytic activity of the *N. nambi* IBSO 2391 oxidase. On the other hand, HRP catalyzed oxidation of
 325 resorcinol, phenol, and caffeic acid much more effectively than the extracellular oxidase from *N. nambi* IBSO 2391. An

326 important difference was that HRP catalyzed oxidation of the tested aromatic compounds only after the addition of
327 hydrogen peroxide to the reaction mixture while the extracellular oxidase from *N. nambi* IBSO 2391 exhibited high
328 catalytic activity without the addition of that mediator. On the one hand, results of the present study (Fig. 6) are indicative
329 of substantial differences between catalytic properties of the extracellular oxidase from the basidiomycete *N. nambi* IBSO
330 2391 and the catalytic properties of laccase from the fungus *A. bisporus* and HRP. On the other hand, these results
331 suggest that the *N. nambi* IBSO 2391 oxidase can be a useful addition to the pool of enzymes used in enzymatic
332 analytics.
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337 **Fig. 6. The yields of reaction products showing the efficiency of oxidation of aromatic compounds by the**
338 **extracellular oxidase from the basidiomycete *N. nambi* IBSO 2391, HRP, and laccase from the fungus *A.***
339 ***bisporus*. Values of the maximal product yields in measurement series are taken for 100%.**

340
341 In supplementary experiments, commercial preparations of laccase from the fungus *A. bisporus* and HRP were used to
342 answer the question whether the extracellular oxidase from the basidiomycete *N. nambi* IBSO 2391 is a metal-containing
343 enzyme. As laccase and HRP contain ions of copper and iron, respectively, their catalytic activity was tested after the
344 treatment with the chelating agent of divalent metal ions EDTA and SH reagent DTT. It is commonly known that these
345 reagents bind divalent metal ions by forming coordinate bonds. The effects of EDTA and DTT on the activity of the *N.*
346 *nambi* IBSO 2391 oxidase, *A. bisporus* laccase, and HRP were studied using veratryl alcohol, resorcinol, and phenol as
347 substrates. Incubation of *A. bisporus* laccase and HRP in the presence of EDTA (or DTT) at concentrations between 0.1
348 and 1 mM resulted in a considerable decline in the functional activities of both enzymes and a decrease in the yields of
349 reaction products (Fig. 7). A similar EDTA treatment of the extracellular oxidase from the fungus *N. nambi* IBSO 2391 did
350 not affect the functional activity of the enzyme. This may suggest the absence of divalent metal ions in the molecule of this
351 enzyme. At the same time, the treatment of the study oxidase with DTT resulted in a considerable (25% or greater)
352 increase in its catalytic efficiency compared to the control. The mechanism of this effect is not yet clear and needs to be
353 studied.
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357 **Fig. 7. The yields of products of reactions catalyzed by the extracellular oxidase from the basidiomycete *N. nambi***
358 **IBSO 2391, HRP, and *A. bisporus* laccase treated with EDTA or DTT at a concentration of 1 mM. Values of the product**
359 **yields in reactions catalyzed by initial enzymes (untreated with EDTA and DTT) are taken for 100%.**
360

361 4. CONCLUSION

362 Thus, the treatment of the mycelium of the basidiomycete *N. nambi* IBSO 2391 with β -glucosidase followed by gel-
363 filtration chromatography of the extract on Sephacryl S-200 resulted in isolation of an extracellular enzyme of the fungus
364 exhibiting oxidase function, with native molecular weight 80 kDa. The findings of the present study suggest that the
365 extracellular oxidase isolated from *N. nambi* IBSO 2391 does not contain chromophore compounds and divalent metal
366 ions; functions in wide ranges of temperature and pH of the reaction medium, showing the highest catalytic activity at
367 temperatures between 22 and 35 °C and pH 6.0; catalyzes oxidation of diverse aromatic compounds with no mediators
368 (such as hydrogen peroxide) added to the reaction mixture; has substrate specificity that differs from the substrate
369 specificity of metal-containing oxidases – laccase from the fungus *A. bisporus* and HRP. The current study provides the
370 basis and opens up opportunities for producing homogeneous extracellular oxidase of the fungus *N. nambi* IBSO 2391, in
371 order to determine its structure and mechanism of catalytic function and to study its potential uses in biotechnological and
372 analytical applications.
373

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380 AUTHORS' CONTRIBUTIONS

381 All authors made substantial contributions in conceptualizing, drafting, developing and reviewing the manuscript. The
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383

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