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Extracellular Oxidase from the *Neonothopanus nambi* Fungus as a Promising Enzyme for Analytical Applications

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Abstract
The extracellular enzyme with oxidase function was extracted from the *Neonothopanus nambi* luminescent fungus by using mild processing of mycelium with β-glucosidase and then isolated by gel-filtration chromatography. The extracted enzyme is found to be a FAD-containing protein, catalyzing phenol co-oxidation with 4-aminoantipyrine without addition of H_2O_2, which distinguishes it from peroxidases. This fact allowed us to assume that this enzyme may be a mixed-function oxidase. According to gel-filtration chromatography and SDS-PAGE, the oxidase has molecular weight of 60 kDa. The enzyme exhibits maximum activity at 55–70 °C and pH 5.0. Kinetic parameters K_m and V_{max} of the oxidase for phenol were 0.21 mM and 0.40 μM min^{-1}. We suggest that the extracted enzyme can be useful to develop a simplified biosensor for colorimetric detection of phenol in aqueous media, which does not require using hydrogen peroxide.

Keywords Extracellular oxidase · Flavoprotein · Fungi · Phenol

Abbreviations

4-AAP	4-Aminoantipyrine (1-phenyl-2,3-dimethyl-4-aminopyrazolone)
BSA	Bovine serum albumin
DI	Deionized water
FAD	Flavin adenine dinucleotide
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis

1 Introduction

Basidiomycetes have considerable biotechnological potential owing to their unique enzyme systems, which take part in degradation of plant-derived biopolymers–lignin and cellulose [1−3]. The cell wall and the outer slimy layer of fungal hyphae contain various enzymes, which are used by the cell to decompose nutrients. Among other enzymes, this slimy network includes heme-, copper- and FAD (flavin adenine dinucleotide)-containing oxidoreductases [4, 5]. In nature, laccases and peroxidases are the first to attack lignin. FAD-containing oxidoreductases cooperate, providing extracellular peroxidases with the necessary co-substrate—hydrogen peroxide—during lignin degradation [3, 6−8].

Considerable research efforts have been devoted to the use of extracellular enzymes of higher fungi, such as FAD-, heme- and copper-containing oxidoreductases, as analytical enzymes for immobilization and construction of sensory and analytical systems [9–12]. For example, FAD-containing oxidoreductases are commonly used to construct biosensors and develop processes of enzymatic production of various carbohydrate products [13–15]. Glucose oxidase and pyranose oxidase have long been successfully used in analytical and diagnostic systems in various branches of bio-industry [16–18]. Aryl-alcohol oxidases are extensively used in biotechnology, as this enzyme exhibits broad substrate specificity, and reaction catalyzed by it is simple and irreversible. Aryl-alcohol oxidases offer great potential for application in biocatalytic processes, as they only require molecular oxygen for substrate oxidation and generate hydrogen peroxide as byproduct, without the need for any added cofactors [19–21].

At the same time, analytical use of secreted fungal oxidases is hindered by the insufficient stability of these enzymes, which, however, can be enhanced by improving their properties [16, 22]. This, in turn, encourages researchers to discover new fungal species to be used as sources of oxidoreductases, to find ways to increase production of

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these enzymes in fungal biomass, and to reveal fungal oxidases with novel properties [2, 23–27]. Approaches to constructing fungal producers of oxidases are being developed using protein engineering technologies, resulting in enzyme mutants with higher stability and activity but also increased expression levels [12, 21, 28, 29]. To increase the resistance of fungal oxidases, they are immobilized on different types of carriers (matrices) including nanoparticles [9, 30–32].

The *Neonothopanus nambi* bioluminescent fungus is a model fungus used to study not only the fundamental aspects of bioluminescence in higher fungi but also their applicability in the biotechnological production of valuable target products, for example, for analytical purposes. Our previous studies demonstrated that treatment of mycelium of *N. nambi* incubated in water with β-glucosidase caused release of fungal extracellular enzymes to the incubation medium [33]. In our previous studies, we used the crude extracts of extracellular oxidases of *N. nambi* to create indicator systems applicable for glucose detection and environmental monitoring of phenolic pollution in the aqueous medium [34, 35]. In those works, we tested peroxidase activity using the well-known oxidative azo coupling reaction (phenol—4-AAP–H_2O_2), which is catalyzed by peroxidases to generate quinone imine. In our subsequent experiments with the extracts, however, we observed a very interesting effect: quinone imine was produced when we added only phenol and 4-AAP to the extracts. That was a complete surprise for us. Although the product yield was not as high as when the extract was supplemented with phenol, 4-AAP, and H_2O_2, we had reason to assume that the extracts contained, in addition to peroxidases, an enzyme that catalyzed reaction of co-oxidation of phenol with 4-AAP producing quinone imine, with no exogenous hydrogen peroxide added to the extract [36].

In the present study, the enzyme catalyzing co-oxidation of phenol with 4-AAP was extracted from the pool of extracellular proteins obtained by mild processing of *N. nambi* mycelium with β-glucosidase. The study describes conditions of extraction and purification of the enzyme and provides the data on its physicochemical and catalytic properties.

2 Materials and Methods

2.1 Strain and Culture Medium

The *Neonothopanus nambi* IBSO 3293 luminous fungus available in the CCIBSO 836 collection of the Institute of Biophysics, Siberian Branch of Russian Academy of Sciences, Federal Research Center "Krasnoyarsk Science Center SB RAS", was used in the experiments.

PDB medium (dextrose—20 g/L, potato extract—4 g/L) was used for cultivation of mycelium. The prepared liquid medium was autoclaved at 120 °C for 15 min before use. Sweet almond β-glucosidase (Serva, Germany) (EC 3.2.1.21) dissolved in 10 mM phosphate buffer (pH 6.0) was used for enzymatic treatment of mycelium.

2.2 Procedures

Submerged cultivation of *N. nambi* IBSO 3293 mycelium was performed in 300-ml flasks containing 100 ml of PDB medium. Mycelium, which had been grown in Petri dishes in PDB medium for 8–10 days, 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 was carried out at 27 °C and under continuous agitation at 160–180 rpm using an Environmental Shaker-Incubator ES-20 (BIOSAN, Latvia). The process was stopped after 7–8 days, during the transition of fungal culture to a stationary growth stage. This cultivation procedure produced spherical granules of *N. nambi* mycelium with diameter of 2 to 9 mm.

Extracellular proteins (including enzymes) were extracted from the mycelium using the following procedure. Mycelial pellets were taken out of the nutrient medium and rinsed many times in deionized (DI) water to remove residual nutrient broth and metabolites. DI water was produced using a Milli-Q system (Millipore, U.S.). After that, the pellets were placed in fresh DI water containing β-glucosidase (0.5 IU/ml) and incubated at 25 °C for 24 h under slow agitation at 80 rpm using an ES-20 shaker. After incubation of pellets, the aqueous extract containing extracellular proteins was separated from the pellet biomass by filtration through filter paper. To concentrate the proteins and remove low-molecular-weight ballast 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, DI water in the extract was replaced three times. The retentate, which contained concentrated extracellular proteins (concentrate), was collected and used in experiments.

The extracellular proteins were separated by gel-filtration chromatography of the concentrate on a Sephadex G-200 column (1.6 × 33 cm) (Pharmacia, Sweden) equilibrated with a 50 mM NaCl aqueous solution. NaCl was added to the concentrated sample to reach a concentration of 50 mM, and 2 ml of the sample was applied to the column. Chromatography was performed at a flow rate of 0.2 ml/min (EP-1 Econo Pump, Bio-Rad, U.S.); 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. Chromatography of protein

markers—bovine serum albumin, BSA (Sigma, U.S.) and cytochrome c (Fluka, Germany)—was performed under the above conditions to determine the native molecular weight of the enzyme studied. After gel-filtration chromatography, the fractions with the highest oxidase activity were collected and further concentrated by ultrafiltration through a 10 kDa cutoff membrane. For a more complete removal of the eluent residues (sodium chloride), DI water in the sample was replaced three times. The resulting retentate (final enzyme preparation) was placed in plastic tubes (Eppendorf, Germany) and stored at -20 °C until used in experiments.

Protein contents of the samples (extracellular protein concentrate, chromatographic fractions, and the final enzyme preparation) were determined by the well-known biuret method, using Benedict's reagent and BSA as the standard [37]. 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 (Shimadzu, Japan).

2.3 Assessment of Catalytic Activity of the Enzyme

The presence of the oxidase in the samples was determined using reaction of co-oxidation of phenol with 4-aminoantipyrine (4-AAP). High quality reagents, 4-AAP (1-phenyl-2,3-dimethyl-4-aminopyrazolone) (Reakhim, Russia) and phenol (Fluka, Germany), were used. Solutions of reagents were prepared in situ in DI water. The reaction mixture (total volume 600 µl) contained 6 µl (5.96 mM) of phenol and 6 µl (0.49 mM) of 4-AAP. In the assay of the oxidase activity, the reaction was started by adding an aliquot of a sample—100 µl of the concentrate, or 300 µl of the chromatographic fractions, or 50 µl of the final enzyme sample. After all reaction ingredients were added, the samples were stirred for 3 s on Vortex-Genie 2 g-560E (Scientific Industries, Inc., U.S.) and incubated for 30 min at 25 °C. The level of oxidase activity in the samples was evaluated by the rate of formation of the reaction product (quinone imine), which was determined spectrophotometrically (UV-1800) by the absorption value at 506 nm. The enzyme activity in the samples was calculated as a ratio between the increase in the absorbance of the reaction product per minute and the amount of protein in the reaction.

To check the formation of H_2O_2 during the reaction catalyzed by the oxidase, we used catalase from bovine liver (80,000 U/mg protein) (Serva, Germany). The starting enzyme solution was prepared in situ in a 10 mM phosphate buffer (pH 6.0). The reaction mixture (600 µl) contained 6 µl (5.96 mM) of phenol, 6 µl (0.49 mM) of 4-AAP, and 6 µl (2 µg) or 12 µl (4 µg) of catalase. The reaction was started by adding an aliquot (50 µl) of the final enzyme (oxidase) sample to the reaction mixture; after being stirred for 3 s (Vortex-Genie 2 g-560E), the reaction mixture was incubated for 30 min at 25 °C. The formation of the reaction product was registered spectrophotometrically as described above.

2.4 SDS-PAGE Analysis of the Final Enzyme Sample and Determination of its Kinetic Parameters, Optimum pH, and Temperature

Protein composition of the final sample of the extracellular oxidase extracted from the *N. nambi* IBSO 3293 fungus was analyzed by electrophoresis in a 12.5% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate (SDS-PAGE), according to Laemmli [38]. Ten µl of the sample (the amount of protein—4 µg) was applied to the track. The protein electrophoresis kit (Bio-Rad, U.S.) that contained proteins of molecular weights 250, 150, 100, 75, 50, 37, and 25 kDa, respectively, was used as the standard. The gel was stained with Coomassie Brilliant Blue G-250 (Serva, Germany) and washed in 10% acetic acid prepared in ethanol.

Kinetic constants K_m and V_{max} were determined for phenol at varying concentrations at 25 °C in 50 mM Na-acetate buffer solutions with pH 5.0. The substrate concentrations in the reactions were 0.026, 0.053, 0.106, 0.212, 0.531, and 1.062 mM. Concentration of chromogen (4-AAP) was constant: 0.49 mM. To determine the kinetic constants, the obtained Michaelis–Menten dependence was transformed into a linear Lineweaver–Burk graph. The molar extinction coefficient for quinone imine used for calculation was $\varepsilon_{506} = 6580\ M^{-1} \cdot cm^{-1}$.

The optimum pH of the purified oxidase was assayed by measuring its activity at 25 °C in 50 mM Na-acetate buffer solutions of different pH values (pH 3.0—8.0). The temperature optimum of the oxidase was examined by measuring its activity in a 50 mM Na-acetate buffer solution (pH 5.0) in the temperature range of 25–80 °C using TB-85 Thermo Batch (Shimadzu, Japan). Test tubes with the reaction mixture containing reaction components (phenol and 4-AAP) were placed into a thermostatically controlled cabinet at the temperatures mentioned above. Then, reaction was started by adding the enzyme preparation. After 15 min, the test tubes were taken out of the cabinet, and the amount of the product formed in the reaction mixture was determined by spectrophotometry. The enzyme thermal stability was determined: (i) during storage of aqueous samples of the enzyme at a temperature of 4 °C, (ii) during incubation of samples of the enzyme at 30 °C and 50 °C for 60 min with the residual activity in them measured at 10 min intervals. In all the above treatments, the reaction mixture (600 µl) was composed of buffer solution, 6 µl of phenol (5.96 mM), 6 µl of 4-AAP (0.49 mM), and 50 µl of enzyme sample containing 20 µg of protein. After the reactions were completed, oxidase activity was determined according to the assay procedure described previously.

2.5 Spectral Analysis of FAD and Oxygen Requirement of the Enzyme

Spectral measurements of the enzyme extracted from the *N. nambi* mycelium were conducted at room temperature using a UV-1800 spectrophotometer. The final enzyme sample (600 µl) was placed into a quartz cell (optical pass length 1 cm), and the absorption spectrum in the 250–650 nm wavelength range was recorded.

The strength of the binding between the cofactor (FAD) molecule and the protein molecule was assessed as follows. An aqueous enzyme sample was incubated for 10 min at 100 °C in the TB-85 Thermo Batch, and then the sample was cooled at 4 °C and subjected to ultrafiltration through a 10 kDa cutoff membrane. The permeate was collected, and FAD presence was determined by spectrophotometry, based on the absorption spectrum in the 300–600 nm wavelength range.

The effect of reducing agents on spectral parameters of the cofactor contained in the enzyme was estimated using sodium dithionite (Serva, Germany). Two or three sodium dithionite crystals were added to the measurement cell containing 300 µl DI water and 300 µl aqueous enzyme preparation; the absorption spectrum of the sample in the 300–600 nm wavelength range was recorded immediately.

In a supplementary study, sodium dithionite was also used to determine whether oxygen was needed for the reaction catalyzed by the extracted oxidase. Immediately after 50 µl of the enzyme preparation was added to the aqueous reaction mixture (of total volume 600 µl), which contained phenol and 4-AAP at the concentrations mentioned above, 2–3 sodium dithionite crystals were added to it. After that, dynamics of reaction product formation was recorded by spectrophotometry at a wavelength of 506 nm.

Each measuring experiment was performed in triplicate. Error bars were generated as a standard deviation of the mean from 3 replicates.

3 Results and Discussion

Our study showed that the technique of submerged cultivation that we used to grow the fungus *N. nambi* IBSO 3293 produced spherical mycelial pellets with the rough surface created by numerous bundles of hyphae (Fig. 1). The diameter of the pellets produced under the cultivation conditions used in this study (the composition and the volume of the nutrient medium, the rate of agitation, the temperature and the time of cultivation) usually ranged between 2 and 9 mm. Surface roughness was caused by bundles of long hyphae extending for distances that may reach a few millimeters. After incubation of the *N. nambi* IBSO 3293 pellets in DI water containing β-glucosidase, extracellular proteins of the fungus, including enzymes with oxidase activity, were detected in the incubation medium. The assay for oxidase activity in the concentrate of the extracted enzymes after phenol and 4-AAP were added to them showed formation of reaction product—quinone imine. That suggested that β-glucosidase disintegrated the polysaccharide matrix covering the outside surface of the hyphal cell wall, enabling release of the extracellular proteins (including enzymes) of the fungus localized there to the outer environment (Fig. 2).

Fig. 1 Image of the *N. nambi* IBSO 3293 pellets grown under submerged cultivation of the fungal mycelium in the PDB medium. Image of the pellets was obtained by using a PowerShot S50 camera (Canon, Japan). Bar—10 mm

Gel-filtration chromatography of the concentrate of extracellular proteins from *N. nambi* IBSO 3293 revealed protein fractions with oxidase activity (Fig. 3). The enzyme that catalyzed reaction of co-oxidation of phenol with 4-AAP was detected in the protein fraction with a native molecular weight of the protein of about 60 kDa (Fig. 3). It is important that the detected enzyme catalyzed co-oxidation reaction of phenol with 4-AAP without addition of exogenous hydrogen peroxide, and that distinguished this enzyme from other peroxidases. This indicates the prospects of using this oxidase in analytics, as assays for phenol and its analogs will require a smaller number of reagents (in particular, there will be no need to add exogenous hydrogen peroxide). After chromatography, the fractions with the highest enzyme activity were pooled, concentrated, and dialyzed to remove the residue of the eluent (NaCl). As a result, the specific activity of the final enzyme preparation was higher by a factor of 5. The total activity, specific activity, percentage of yield, and purification factor are summarized in Table 1.

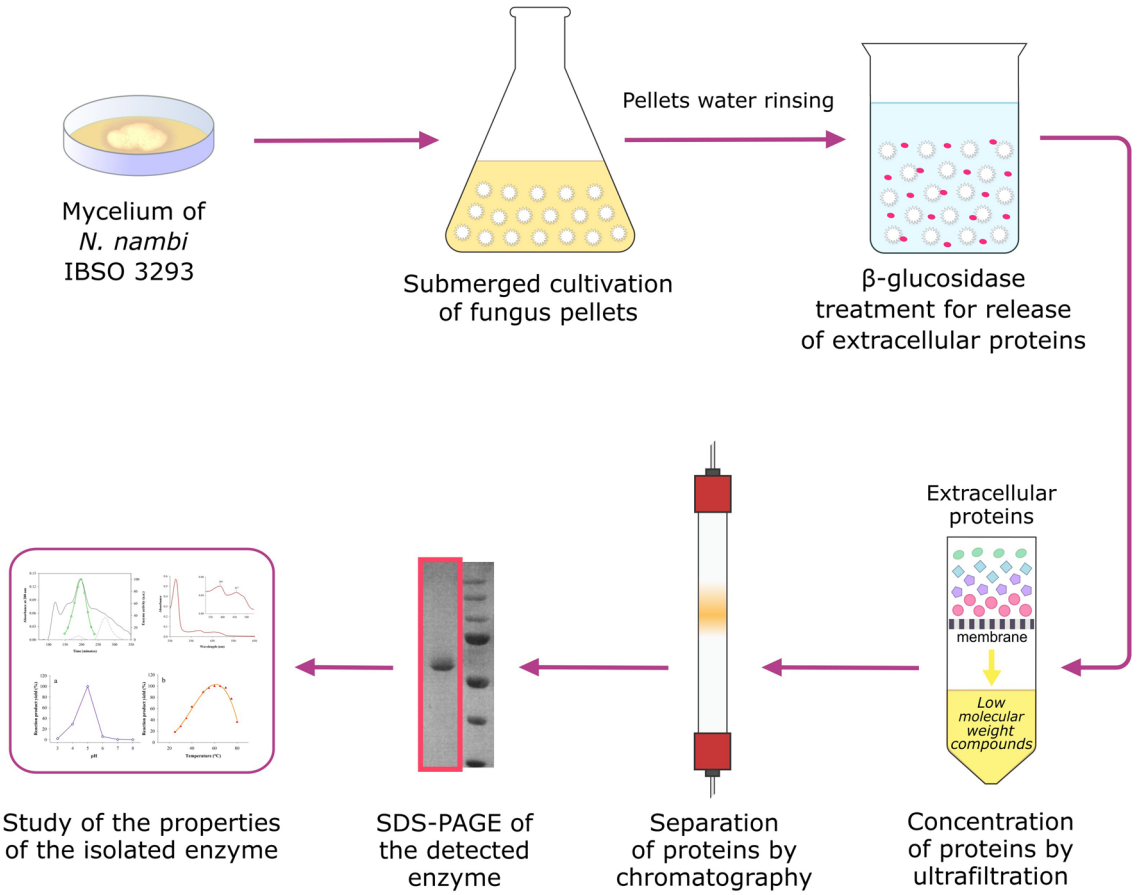

Fig. 2 A schematic representation of extraction of extracellular proteins from the *N. nambi* IBSO 3293 mycelium under β-glucosidase treatment and isolation of the detected oxidase

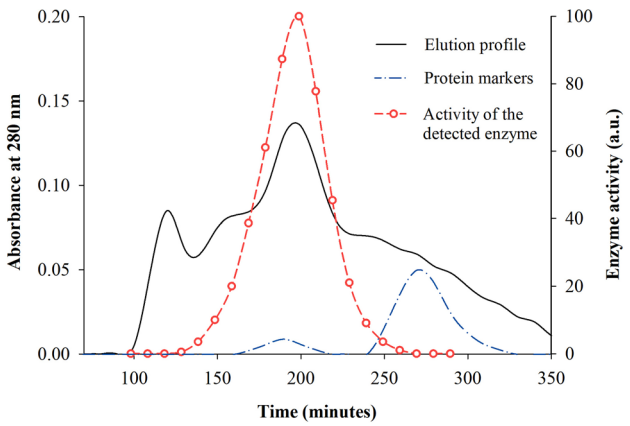

Fig. 3 Gel-filtration chromatography of protein preparations on a Sephadex G-200 column and distribution of the *N. nambi* IBSO 3293 oxidase activity in the chromatographic fractions catalyzing co-oxidation of phenol with 4-AAP without added hydrogen peroxide

SDS-PAGE analysis of the sample with a major peak of oxidase activity after chromatography revealed one dominant protein band, and its molecular weight was estimated to be about 60 kDa (Fig. 4). The electrophoresis data showed that the enzyme obtained was not homogenous but of high purity. As molecular weight of this protein was the same under denaturing and native (chromatographic) conditions, we assumed that the extracellular enzyme extracted from the *N. nambi* IBSO 3293 was a monomeric protein.

Spectral analysis of the final sample (Fig. 5) demonstrated the presence of a chromophore component in the oxidase molecule. In the absorption spectrum of the aqueous sample of that enzyme, in addition to the protein peak (280 nm), there were absorbance peaks at 390 and 457 nm and a shoulder around 490 nm, which corresponded to the spectral characteristics of FAD molecule. This indicates that the oxidase isolated from the *N. nambi* IBSO 3293 fungus is a flavoprotein and contains FAD as a cofactor.

After the enzyme sample was incubated at 100 °C for 10 min and then dialyzed through a 10 kDa cutoff membrane, FAD was detected by spectrophotometry in the permeate. In the absorption spectrum of FAD found in the permeate, instead of peaks at 390 and 457 nm, peaks at 372 and 446 nm were detected, and that corresponded more closely to the absorption peaks of free flavin (Fig. 5 inset). These

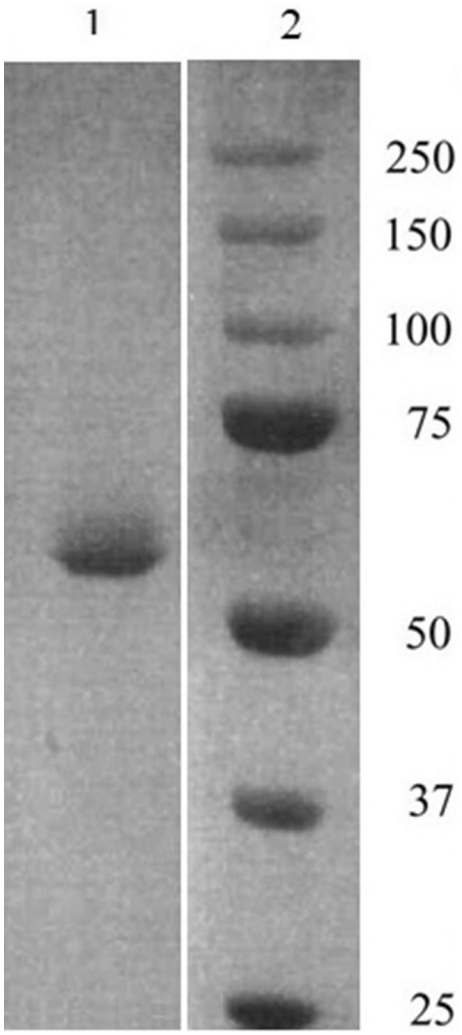

Fig. 4 SDS-PAGE of the final sample of the extracellular oxidase extracted from the *N. nambi* IBSO 3293 mycelium. Electrophoresis was performed using 12.5% polyacrylamide gel supplemented with 0.1% SDS. Tracks: 1—sample of isolated enzyme (the amount of protein in the well was 4 µg); 2—protein standards with molecular weights of 250, 150, 100, 75, 50, 37, and 25 kDa

data can suggest the absence of a covalent bond between FAD and the protein molecule of the enzyme.

The addition of sodium dithionite to the sample of extracted enzyme caused changes in its absorption spectrum, which were observed in the 300–600 nm wavelength range, suggesting reduction of the oxidized cofactor (FAD) in the enzyme molecule to $FADH_2$. There were no characteristic peaks of the cofactor at 390 and 457 nm in the spectral range mentioned above (Fig. 5 inset).

We found that the reaction catalyzed by the enzyme studied was oxygen dependent. The addition of sodium dithionite to the reaction mixture containing all reaction components prevented formation of quinone imine for a few minutes. At least under the experimental conditions of this study

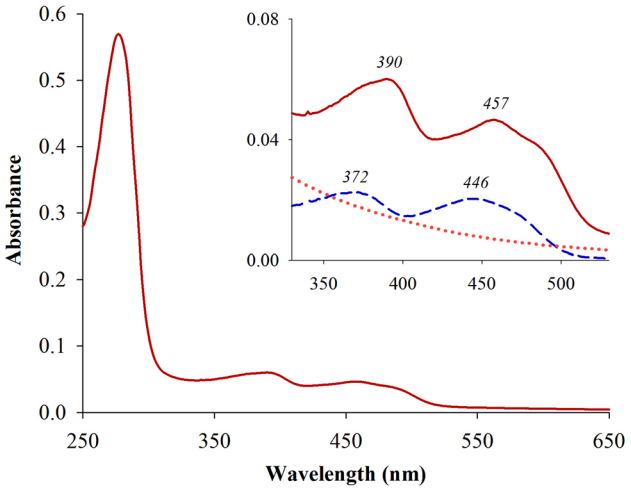

Fig. 5 UV–Vis absorption spectrum of the aqueous sample of the FAD-containing oxidase from the *N. nambi* IBSO 3293. The inset shows the fragments of spectra in the range of 330–550 nm, with characteristic absorption peaks of the cofactor before (solid line) and after (dashed line) thermal treatment of the enzyme followed by dialysis through a 10 kDa cutoff membrane. Dotted line shows the fragment of the spectrum of reduced cofactor form after the addition of sodium dithionite

(volume of the reaction mixture, concentrations of the necessary reaction ingredients, the amounts of the enzyme and sodium dithionite, and reaction temperature), we did not observe quinone imine formation for the first 5 min after the addition of the reducing agent (Fig. 6). This suggests that complete or incomplete removal of oxygen from the

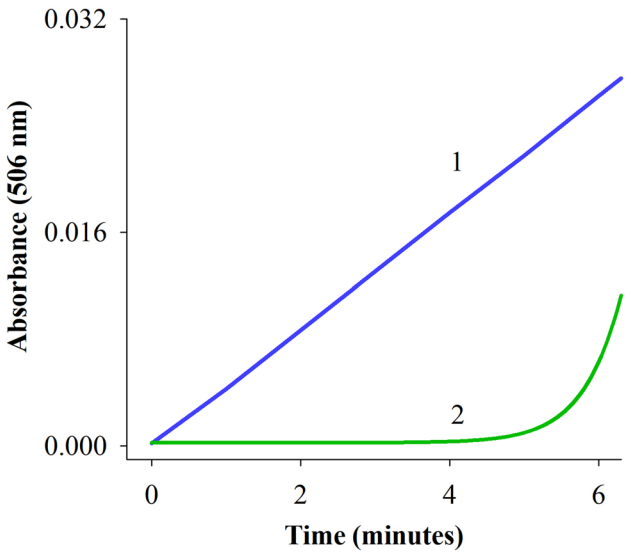

Fig. 6 Kinetics of formation of quinone imine in the reaction of co-oxidation of phenol with 4-AAP catalyzed by the oxidase from the *N. nambi* IBSO 3293: 1—reaction without sodium dithionite, 2—with added sodium dithionite

reaction mixture by sodium dithionite prevents the enzyme to generate hydrogen peroxide necessary for the reaction of co-oxidation of phenol with 4-AAP. After that latent period, we observed production of quinone imine (Fig. 6), as oxygen of the external air diffused into the reaction mixture. These cumulative data suggest that the enzyme extracted from the *N. nambi* IBSO 3293 fungus is an oxidase, and this enzyme requires oxygen to perform catalytic function in the reaction of co-oxidation of phenol and 4-AAP.

Based on the study of the oxidase described above, we assume that this enzyme may be a mixed-function oxidase. In this instance, in the presence of phenol in water, this oxidase first generates hydrogen peroxide, which is then used in the reaction of co-oxidation of phenol with 4-AAP. The assumption that this enzyme generates hydrogen peroxide is supported by experimental results, which show that the addition of exogenous catalase to the reaction mixture decreases quinone imine yield by 30–40% relative to the control (Fig. 7). This version seems quite valid to us, as it is consistent with the results of other researchers who studied FAD-containing oxidases from other fungal species. The literature data demonstrate that there are extracellular FAD-containing fungal oxidases that enable H_2O_2 formation by oxidizing cyclic OH-containing compounds followed by reduction of O_2 of the aqueous medium [3, 7, 19].

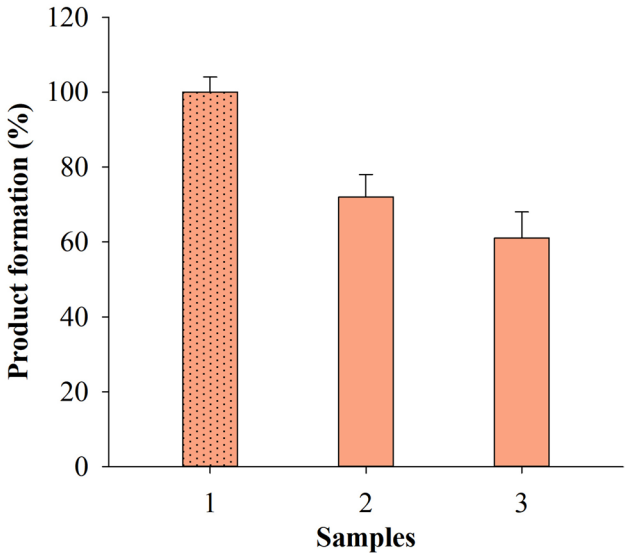

Fig. 7 Product formation in the reaction catalyzed by the oxidase from the *N. nambi* IBSO 3293 without (1) and with added catalase: 2 μg (2) and 4 μg (3). The data are calculated per 1 mg of protein and normalized to product formation in the reaction without catalase. Absolute value of product formation in the concentrate was 0.181 ± 0.008 a.u./mg

In addition, we conducted control experiments, in which we assessed the possibility of non-enzymatic quinone imine formation in the reaction mixture (of total volume 600 μl) that contained phenol (5.96 mM), 4-AAP (0.49 mM), and H_2O_2 (8 mM). At concentrations of the components used in those experiments, the rate of formation of quinone imine was very low—no more than 1% of the rate of formation of quinone imine in the reaction catalyzed by the enzyme (data not shown).

The effectiveness of *N. nambi* IBSO 3293 oxidase in reaction media with different pH levels was tested using 50 mM Na-acetate buffer solutions with pH between 3 and 8 (Fig. 8a). The oxidase was found to be the most effective within a very narrow range of weakly acidic pH levels, with the highest catalytic activity at pH 5.0. Apparently, the highest activity under weakly acidic conditions is a characteristic property of many (if not all) fungal FAD-contained oxidases. It is well known that most reported fungal FAD-containing oxidases from white-rot fungi have pH optima in the weakly acidic range from 4.0 to 6.0 [12, 19, 39].

Purified oxidase from *N. nambi* IBSO 3293 was active in a wide range of temperatures (25–80 °C) with the maximum of catalytic activity at 55–70 °C (Fig. 8b). It is interesting to note that the authors of an earlier study observed the maximum activity of FAD-dependent oxidase from the *Phanerochaete chrysosporium* fungus at 60 °C [39], which practically coincides with the temperature optimum for the activity of the oxidase from *N. nambi* IBSO 3293. At the same time, the authors of the work [23] showed that the maximum activity of FAD-dependent oxidase from the

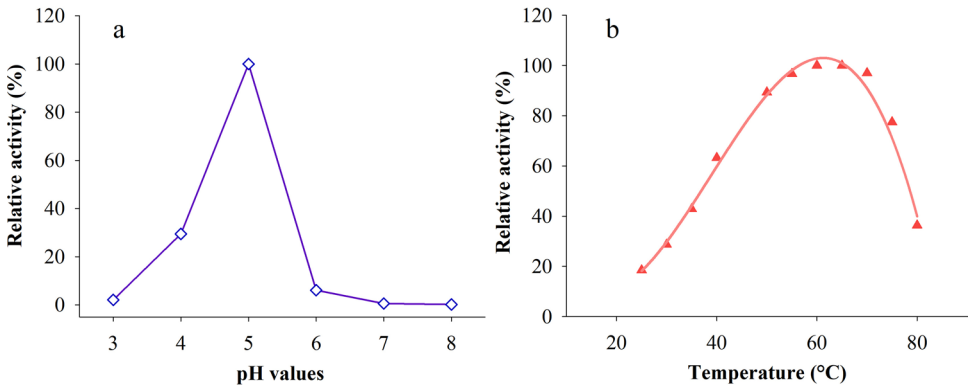

Fig. 8 Influence of pH (**a**) and temperature (**b**) on the activity of the oxidase from the *N. nambi* IBSO 3293 in the reaction of co-oxidation of phenol with 4-AAP. The activity was determined in a 50 mM acetate buffer. The data are normalized to the maximal oxidase activity in the series of measurements. Absolute activity values were 0.92 a.u./mg (**a**) and 5.0 a.u./mg (**b**)

Bjerkandera adusta fungus was observed at a much lower temperature – 45 °C. This indicates that the temperature optima of the activity of FAD-dependent oxidases from different species of basidiomycetes can differ considerably.

The oxidase from *N. nambi* IBSO 3293 has high stability at 4 °C. At least, when the aqueous samples of the enzyme were stored at this temperature for one week, we did not observe any decrease in the oxidase activity. Oxidase has sufficient thermal stability at 30 °C (pH 5.0) and retains about 70–75% of its initial activity after incubation for 60 min (Fig. 9). Moreover, even after incubation of purified enzyme samples at 50 °C for 1 h, they retain about 30% of the initial enzyme activity, which also indicates its sufficient thermal stability. We suppose that the stability of the oxidase from *N. nambi* IBSO 3293 can be additionally increased by immobilizing the enzyme on a suitable matrix (carrier). This approach has been successfully used in a number of studies to increase the stability of fungal oxidases [30–32].

Having studied the formation of the product of reaction catalyzed by oxidase as dependent on concentrations of the substrate, we determined Michaelis–Menten constant for this substance. The values of the kinetic parameters K_m and V_{max} of the oxidase from *N. nambi* IBSO 3293 were 0.21 mM and 0.40 μM min^{-1}, respectively, with phenol used as the substrate (Fig. 10).

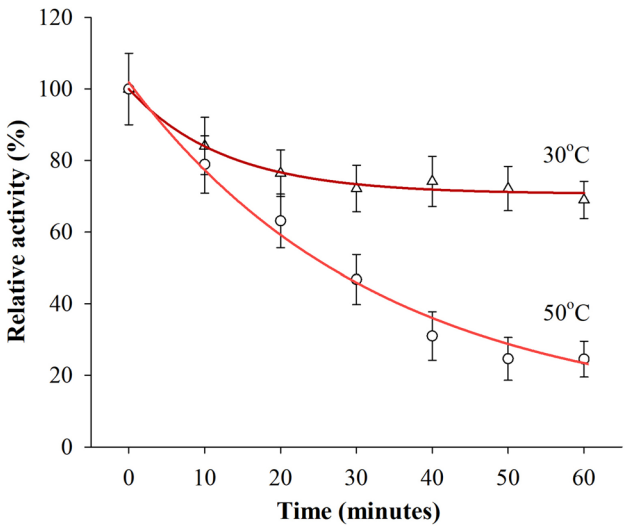

Fig. 9 Thermal stability of the oxidase isolated from the *N. nambi* IBSO 3293 during incubation of the enzyme samples at 30 °C and 50 °C. Residual activity of the enzyme was detected at 10 min intervals in a 50 mM acetate buffer (pH 5.0). The data are normalized to the maximal oxidase activity in the series of measurements. Absolute activity value was 1.30 ± 0.09 a.u./mg

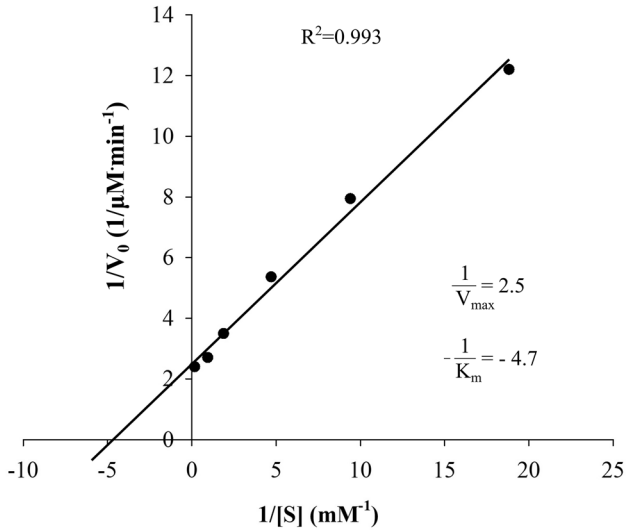

Fig. 10 Lineweaver–Burk graph obtained from kinetics of co-oxidation of phenol with 4-AAP by the *N. nambi* IBSO 3293 oxidase using different concentrations of phenol (0.026, 0.053, 0.106, 0.212, 0.531, and 1.062 mM)

4 Conclusion

The technique of mild enzymatic treatment of the *N. nambi* IBSO 3293 mycelium made it possible for us to receive a pool of extracellular enzymes containing small amounts of ballast admixtures, and the subsequent gel-filtration chromatography allowed us to isolate the desired oxidase with good apparent homogeneity. The present study showed that the isolated oxidase is a monomeric FAD-containing enzyme with a molecular weight of 60 kDa, which catalyzes phenol co-oxidation with 4-AAP without addition of H_2O_2,

Table 1 Purification of the extracellular oxidase from *N. nambi* IBSO 3293

Sample	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification level (x-fold)
Concentrate of extracellular proteins	19.23	109.8	5.7	100	1.0
Final enzyme preparation	0.79	22.8	28.9	21	5.1

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 quinone imine used for calculation was $\varepsilon_{506} = 6580\ M^{-1} \cdot cm^{-1}$

in contrast to the known peroxidases. This allowed us to hypothesize that this enzyme is an oxidase with a mixed function. Moreover, we suggest that the catalytic property of this enzyme is an advantage and can be useful in analytical applications such as development of a simplified biosensor for colorimetric detection of phenol in aqueous media, which does not require using exogenous hydrogen peroxide. Other findings were that the enzyme has the K_m value of 0.21 mM for phenol, exhibits maximum activity at 55–70 °C and pH 5.0, and shows sufficient thermal stability.

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Availability of data and materials The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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