Isolation, Purification, and Investigation of Some Properties of Glucose Oxidase of the Wood-Degrading Fungus *Lentinus (Panus) tigrinus* Strain VKM F-3616D

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The activity and some properties of glucose oxidase (EC 1.1.3.4) of the wood-degrading fungus *Lentinus (Panus) tigrinus* VKM F-3616D were studied. The results showed that 12 days of dynamic liquid-phase cultivation increased the activity and the biosynthetic levels of the enzyme with maximal activity at day 6. After 6 days, a decrease in the glucose oxidase activity was observed. The enzyme isolated *via* ion exchange chromatography had an optimal pH of 4.0 and a temperature optimum of 50 °C. Spectrophotometry, fluorescence analysis, and IR Fourier spectroscopy showed that the enzyme was a flavoprotein and that its prosthetic group contains flavin adenine dinucleotide. The relative kinetic parameters of the enzyme were determined: the Michaelis constant (K_m) was 4.1×10^{-3} M, and the maximal rate of the enzymatic reaction (V_{max}) was 0.36 IU. Results of electrophoresis in native and denaturing conditions were consistent with an enzyme structure having two equal subunits with a total molecular mass of approximately 160 kDa.

INTRODUCTION

Plant (lignocellulosic) biomass is a high-tonnage renewable resource that has a huge economic and environmental significance. The main world trends in the development of scientific and technological research utilizing plant biomass are focused on deep processing, which includes the manufacturing of second generation bioethanol. These methods are associated with the development of new principles and methods for the integrated use of all its components (starch, cellulose, hemicelluloses, lignins, and extractives) and the utilization of the waste generated during processing (Sun 2009; Han *et al.* 2011; Veettil *et al.* 2016).

The complex supramolecular structure of the lignocellulosic raw materials has led to the use of energy-consuming and environmentally dangerous methods to isolate sugars suitable for fermentation, *i.e.*, acid hydrolysis, steam explosion, alkaline delignification, and ultrasonic pretreatment (Fang *et al.* 2014; Pan *et al.* 2014; Carvalho *et al.* 2017). However, as a result of such treatments, lignin decomposition products are formed, including furfural and hydroxymethyl-furfural, which inhibits the subsequent hydrolysis and fermentation processes (Palmqvist and Hahn-Hägerdal 2000; Klinke *et al.* 2004; Kim 2018; Kim *et al.* 2018). Degradation of the plant biomass lignins would be used for the

production of bioethanol, cellulose, and other materials (Taherzadeh and Karimi 2007). Most lignin biodegradation studies have been conducted using the white-rot basidiomycete, *Phanerochaete chrysosporium* (Bugg *et al.* 2011; Wang *et al.* 2016). Intensive study of this organism has yielded results, and basic information on the physiology and enzymology of the lignin decomposition *via* fungal enzymes was obtained in experiments utilizing *Phanerochaete chrysosporium*. However, *P. chrysosporium* does not produce laccase under normal conditions. Thus, it differs from typical ligninolytic fungi. Despite the concentration of research interest on a single, although well-studied fungus in all aspects of the body, there has been limited scientific research on a variety of enzymatic and metabolic systems of other fungi.

During the last two decades, more attention has been given to lignin biodegradation enzymology of another species of fungi, *Lentinus (Panus) tigrinus* (VKM F-3616D), which possesses a high level of ligninolytic activity. The authors, and other researchers, have shown that the fungus *Lentinus (Panus) tigrinus* produces laccase, peroxidase, and Mn-peroxidase (Maltseva *et al.* 1989; Revin *et al.* 2000; Cadimaliev *et al.* 2005). This fungus has a powerful extracellular ligninolytic enzyme complex, which destroys aromatic compounds, including lignins (Kadimaliev *et al.* 2003; 2006; 2010). This enzyme complex can reduce the lignin derivative inhibitory effects on yeast in the subsequent fermentation of the lignocellulosic biomass. These fungi also produce cellulases, which are promising agents for the depolymerization of cellulose, converting it to monosaccharide (Atykyan *et al.* 2016).

Numerous studies have shown that the extracellular enzymes are from a class of oxidoreductases: lignin peroxidase, Mn-peroxidase, plant-type peroxidase, and laccase, which are defined as ligninolytic enzymes of the white-rot fungi. These enzymes work as part of an extracellular enzymatic complex similar to cellulases, but unlike the latter, they need an active form of oxygen to perform the radical oxidation of the lignin. According to some authors, extracellular oxidases perform this function and restore oxygen to hydrogen peroxide while simultaneously oxidizing a suitable substrate. Such peroxide-generating enzymes can include glyoxal oxidase, an extracellular enzyme from the ligninolytic culture *Ph. chrysosporium* (Kersten and Cullen 2007), glucose oxidase (GOx), pyranose oxidase (synonymous with glucose-2-oxidase (EC 1.1.3.4.)), methanol oxidase and aryl-alcohol oxidase (Ferreira *et al.* 2015), cellobiose oxidase and cellobiose:quinone dehydrogenase isolated only from fungi that caused white rot in wood (Henriksson *et al.* 1993), as well as cellobiose oxidoreductases (Mason *et al.* 2003). Another source of H_2O_2 is possible, in the form of acyl-CoA oxidase of fatty acids (Greene and Gould 1984). The activity of extracellular GOx was also detected in the ligninolytic culture of *Lentinus (Panus) tigrinus* (Maltseva *et al.* 1989). It can be assumed that GOx may be one of the possible producers of hydrogen peroxide and that this may be the case for the strain of the fungus *Lentinus (Panus) tigrinus* (VKM-F-3616D) studied.

The aim of this study was to study the GOx activity of the fungus *Lentinus (Panus) tigrinus* strain VKM F-3616D. The isolation, purification, identification, and characterization of some properties of this enzyme are reported.

EXPERIMENTAL

Organism and Maintenance

The fungus *Lentinus (Panus) tigrinus* strain VKM F-3616 D was isolated in the

Department of Biotechnology, Bioengineering, and Biochemistry from the National Research center at the Ogarev Mordovia State University, from the dry fruit bodies of fungi growing on birch trees, and deposited in the All-Russian collection of microorganisms as strain identification VKM F-3616 D. The microorganism was maintained on wort agar at 4 °C to 6 °C and subcultured every 60 days.

Preculture of Lentinus tigrinus

Inoculum of the fungus *Lentinus tigrinus* was grown for 4 days on a modified liquid Czapek-Dox medium containing: 3.0 g/L of $NaNO_3$, 0.5 g/L of $MgSO_4$, 0.5 g/L of KCl, 1.0 g/L of K_2HPO_4, 0.01 g/L of $FeSO_4 \times 7H_2O$, 30.0 g/L of sucrose, and 50.0 g/L of corn extract, with a pH of 6.0. Cultivation of the inoculum was carried out in Erlenmeyer flasks filled with 20% medium, on orbital shakers at rotation speed of 235 rpm at 26 °C. A piece of agar, overgrown with a fungus culture ($\approx 1 \times 1$ cm), was introduced into the culture medium.

Cultures condition

Into 500 mL Erlenmeyer flasks, 100 mL of medium was added (Eggert *et al.* 1996) containing: 3.0 g/L of glucose, 1.0 g/L of KH_2PO_4, 0.26 g/L of NaH_2PO_4, 0.0005 g/L of $CuSO4 \times 7H_2O$, 0.074 g/L of $CaCl_2 \times 2H_2O$, 0.006 g/L of $ZnSO_4 \times 7H_2O$, 0.005 g/L of $FeSO_4 \times 7H_2O$, 0.005 g/L of $MnSO_4 \times 5H_2O$, and 0.001 g/L of $CoCl_2 \times 7H_2O$, with a pH of 4.5. Then, 0.5 mL of a vitamin solution was added into the medium, containing: 2.0 g/L of biotin, 2.0 g/L of folic acid, 5.0 g/L of riboflavin, 5.0 g/L of thiamine, 5.0 g/L of pyridoxine, 0.1 g/L of cyanocobalamin, 5.0 g/L of nicotinic acid, and 5.0 g/L of calcium-D-pantothenate (Kirk *et al.* 1978). The 4-day inoculum was introduced into flasks at an amount of 5% v/v.

Determination of GOx Activity and Protein

GOx activity was determined using the o-dianisidine-peroxidase system under standard conditions (Worthington and Worthington 1993). The reaction velocity is determined by an increase in absorbance at 460 nm resulting from the oxidation of o-dianisidine through a peroxidase coupled system. One unit causes the oxidation of one micromole of o-dianisidine per minute at 25 °C and pH 6.0 under the conditions specified. The spectrophotometer was set to 460 nm and 25 °C. Cuvette amounts were as follows:

Dianisidine-buffer mixture, pH 6.0 (oxygenated)	2.5 mL
18% Glucose	0.3 mL
Peroxidase	0.1 mL

The mixture was incubated in the spectrophotometer for 3 to 5 min to achieve temperature equilibration and establish blank rate if any. Then 0.1 mL of appropriately diluted enzyme was added, and the increase in A_{460} was recorded for 4 to 5 min. The ΔA_{460} value was calculated from the initial linear portion of the curve, according to the following expression:

$$\text{International units (IU)/mg} = \frac{\Delta A_{460}/\text{min}}{11.3 \times \text{mg enzyme/ml reaction mixture}}$$

The activity was expressed in International units (IU), μm / min × mL. The specific activity was expressed in mg of protein.

The concentration of protein was estimated by the Bradford method (Bradford 1976) using bovine serum albumin (BSA) as the standard.

Isolation, Purification, and Determination of the Molecular Weight of the Enzyme

The GOx was isolated and purified using a NGC medium-pressure liquid chromatography system (Bio-Rad, Hercules, CA, USA). The culture medium of the fungus (0.5 L) was harvested at maximal GOx activity; on day 6, it was filtered and precipitated with a 90% solution of ammonium sulfate with constant stirring. The precipitate obtained by centrifugation at 2,500 g for 30 min was re-dissolved in 5 mM potassium phosphate buffer, with a pH of 6.0 and desalted in dialysis bags (OrDial D-Clean, Orange Scientific, Braine-l'Alleud, Belgium) with a pore size of 10 kDa.

After 24 h of dialysis against 5 mM potassium phosphate buffer, with a pH of 6.0, the GOx preparation was concentrated *via* osmotic dehydration. The enzyme solution in the dialysis bags (OrDial D-Clean, Orange Scientific, Braine-l'Alleud, Belgium) with a pore size of 10 kDa was immersed in dextran with a molecular weight of 670 kDa (Merck KGaA, Darmstadt, Germany) at 4 °C. The concentrated preparation was loaded into an anion-exchange TEAE-cellulose column (2.6×25 cm); after that they were eluted with 1 M NaCl. One major fraction (from 1 to 8) with GOx activity was eluted. The fractions with GOx activity (from 1 to 8 fraction) were combined, re-chromatographed on a 2 cm x 20 cm column of DEAE-Toyopearl 650M (Sigma), and equilibrated with 5 mM potassium phosphate buffer (pH of 7.2). The protein was eluted with a linear gradient of potassium phosphate buffer (2 mL × 250 mL) with molarity from 5 to 200 mM and a pH of 7.2. The elution rate was 3 mL/min.

The elution of the protein from the column was monitored spectrophotometrically at 280 nm. Fractions were collected, analyzed for the presence of enzymatic activity, and eight fractions with GOx activity were selected.

To determine the molecular weight and purity of the enzymes, gel electrophoresis was performed in 12% polyacrylamide gel (PAGE) in the presence of sodium dodecyl sulfate (SDS-Na) on a Mini-Protean device (Bio-Rad). The protein bands in the gels were stained with Coomassie R-250. The standard for electrophoresis was the standard mixture of proteins # 26634 - a prestained mixture of 10 recombinant proteins ranging from 10K to 260K (ThermoFisher Scientific, Waltham, MA, USA) and a commercial preparation of GOx from *Aspergillus niger* with a molecular weight of 160 kDa (Merck KGaA, Darmstadt, Germany).

Identification of the Enzyme and Prosthetic Group

Enzyme absorption spectra were obtained in the wavelength range of 200 nm to 500 nm using a UV-3600 spectrophotometer (Shimadzu, Kyoto, Japan).

The fluorescence of the enzyme prosthetic group was studied on a Shimadzu RF 5301-PC spectrophotometer under excitation with light at 470 nm.

The IR spectra of the samples were recorded using a Shimadzu IR-Fourier spectrometer IR Prestige 21 in the range of 400 cm^{-1} to 4000 cm^{-1}, with a sensitivity of 4 cm^{-1}.

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Investigation of pH and Temperature Effect

pH-dependence studies were carried out in 0.1 M sodium acetate buffer (pH = 3.5 to 6.0) and 0.1 M potassium phosphate buffer (5.5-7.0) by using the o-dianisidine-peroxidase system (Worthington and Worthington 1993) at a temperature of 25 °C. The temperature dependence studies were carried out in sodium acetate buffer (pH 4.0) in the range of 25 °C to 70 °C using the o-dianisidine-peroxidase system (Worthington and Worthington 1993).

Determination of Kinetic Parameters

To determine the kinetic constants of V_{max} and K_m, experiments were carried out at optimal pH (4.0) and temperature (25 °C) with D-glucose in the range of 2 mM to 40 mM concentrations, and the kinetic constant were determined by Lineweaver–Burk plot (Odebunmi and Owalude 2007).

All experiments were carried out in five replicates with statistical processing of the data calculated using Microsoft Excel (Redmond, WA, USA).

RESULTS AND DISCUSSION

The dynamics of the GOx activity in the fungus studied were determined during 12 days of cultivation under dynamic conditions. In the first stage, standard medium without additional additives was used (Eggert *et al.* 1996). During the first three days, there was a gradual accumulation of biomass and extracellular protein. Beginning with three days of culture in the liquid, GOx was detected in the medium, and on the 6th day, its quantity reached the maximal value – 0.28 IU/ml, after which the activity decreased to 0.05 IU/ml, as shown in Fig. 1. Specific activity had same character of synthesis.
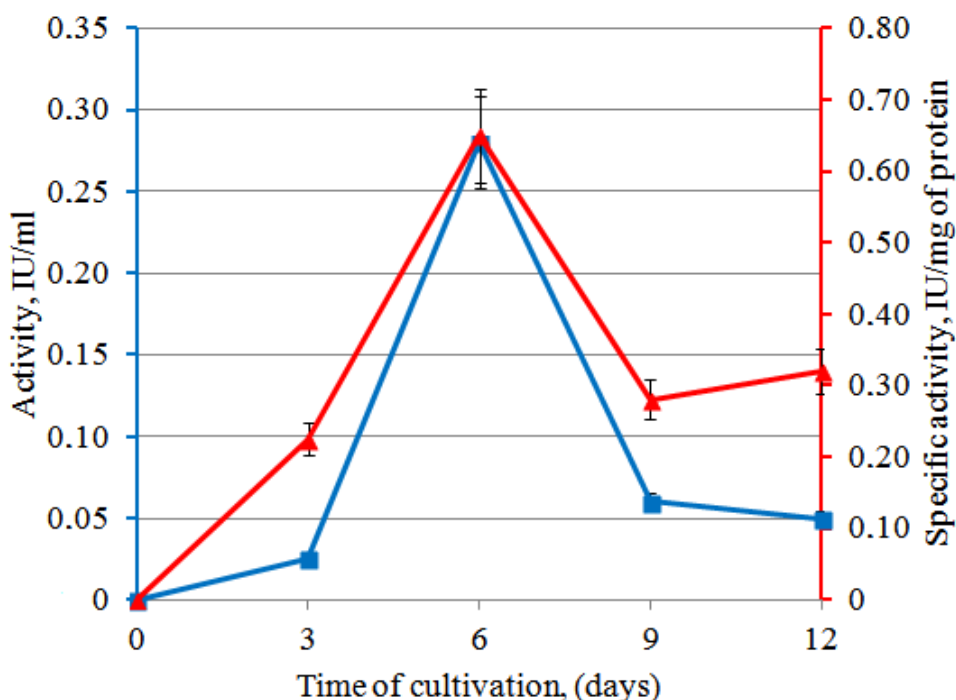

Fig. 1. Dynamics of accumulation of GOx activity by the fungus *Lentinus tigrinus* VKM F-3616 D

The decrease in the activity of the enzyme can be explained by the fact that over time, the glucose is gradually depleted. Laccase, a plant-type secretory peroxidase, and Mn-peroxidase primarily mediate the biodegradation of lignocellulosic substrates by *L. tigrinus* (Kadimaliev *et al.* 2003; 2006). The enzyme activity at the initial stage of cultivation increases and reaches its maximum on day 9 and then decreases (Kadimaliev *et al.* 2006). A comparison of this data showed that, despite the decrease in the activity of GOx from day 6 to day 9 of cultivation, a source of hydrogen peroxide for the ligninolytic enzymes of the fungus and the activity of the peroxide-dependent enzymes of the ligninolytic complex increased.

This discrepancy can be explained as follows. For ligninolytic enzymes, glucose is a source of carbon, but GOx generates the hydrogen peroxide necessary for the functioning of these enzymes.

During the initial stages of cultivation, with an enough amount of glucose, GOx provides the main role in the formation of hydrogen peroxide. When glucose is deficient in the medium, the source of hydrogen peroxide can include fatty acids. Compared with other organisms, the phospholipids of basidiomycetes, including the fungus *L. tigrinus*, contain a large amount of unsaturated fatty acids, especially linoleic acid (60% of all fatty acids) (Kadimaliev *et al.* 2006). Under the action of nonspecific esterases (phospholipase A2) and lipoxygenases, peroxide radicals of lipids are formed, which are strong oxidants for many toxic compounds. From previous research (Kadimaliev *et al.* 2006, 2010), studies of the dynamics of the phospholipase activity of the fungus *L. tigrinus* provided evidence of a similar mechanism. In the initial stages of cultivation, the phospholipase activity was not very pronounced. However, from 6 to 9 days of cultivation, there was a significant increase in the activity of phospholipase A2. This mechanism has been suggested by other researchers (Kapich *et al.* 1999; Kapich *et al.* 2005; Korneichik and Kapich 2011).

Because the maximal activity of GOx was observed on the 6th day, a 6 day culture liquid with an initial GOx activity of 0.65 IU/mg protein was used to further isolate, purify, and study its enzymatic properties. The elution profile of the enzyme at the last stage is presented in Fig. 2, and the purification results are shown in Table 1.
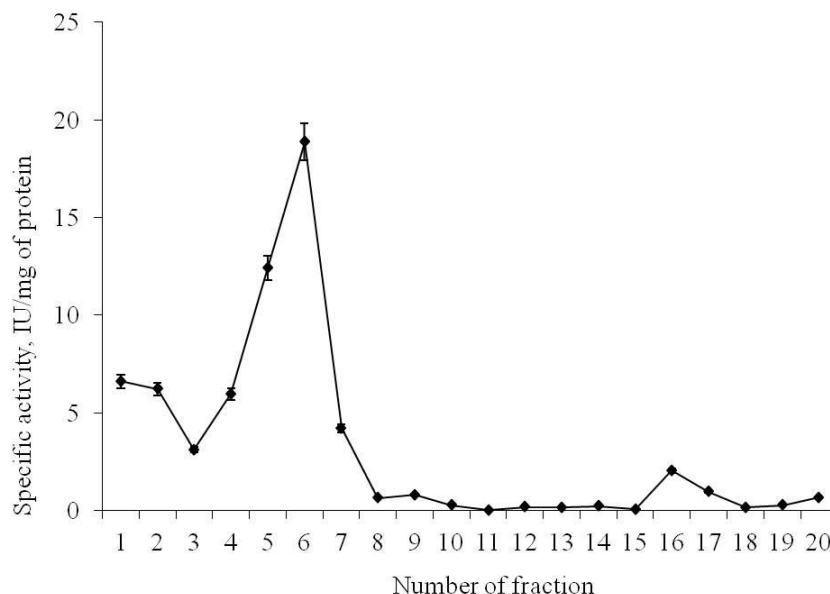

Fig. 2. Profile of elution of GOx (fraction from 1 to 8) on a column with TEAE-cellulose

Table 1. Results of GOx Purification from the Culture Liquid

| Purification Step	Total Protein, mg	Total Activity, IU	Specific Activity, IU/mg of Proteins	Yield, %	Purification Degree
Culture Fluid	215.0	140.0	0.65	100.0	1.0
DEAE- Toyopearl 650M (elution by potassium phosphate buffer with molarity from 5 to 200 mM and a pH of 7.2)	3.6	18.0	5.00	12.8	7.7

Compared with previous reports, the yield of the enzyme was small, but the degree of purification was quite high (Kelley and Reddy 1986; Abbas and Mahmood 2007; Zia *et al.* 2007; Bhatti and Saleem 2009; Singh and Verma 2010; Hwang *et al.* 2011; Singh and Verma 2013).

To determine the optimal pH, studies were performed in 0.1 M sodium acetate buffer and 0.1 M potassium phosphate buffer in the pH range 3.5 to 7.0 (temperature 25 °C) and the optimal temperature studies were performed in 0.1 M sodium acetate buffer (pH 4.0) in range 25-70 °C The results are shown in Fig. 3 to 4. The optimal pH was 4.0, and the temperature optimum was 55 °C.
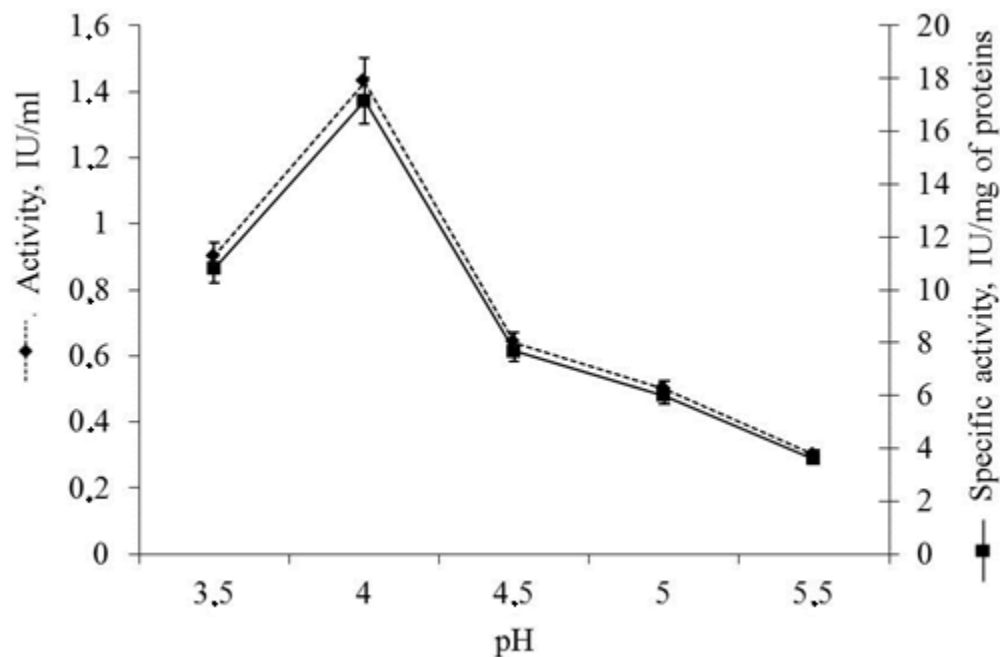
Fig. 3. Effect of pH on the activity of isolated GOx

The results confirmed that an acidic medium was optimal for most GOxs of fungi, and the optimal temperature was 55 °C to 60 °C. In the most studied fungus, *Phanerochaete chrysosporium*, as well as in this research, the optimum pH was shifted into the acidic region, and the optimum temperature was 50 °C (Kelley and Reddy 1986).

Interestingly, the maximal activity of the main ligninolytic enzymes of the fungus under study occurred in a neutral pH (Revin *et al.* 2000; Cadimaliev *et al.* 2005). This discrepancy is probably due to one of the products in the oxidation of glucose *via* enzyme GOx is gluconic acid. As a result, local acidification of the microenvironment of the enzyme occurs. Under such conditions, it is logical that the enzyme should be stable and active in an acidic environment for the effective hydrolysis of glucose under these conditions and to ensure normal cell metabolism.
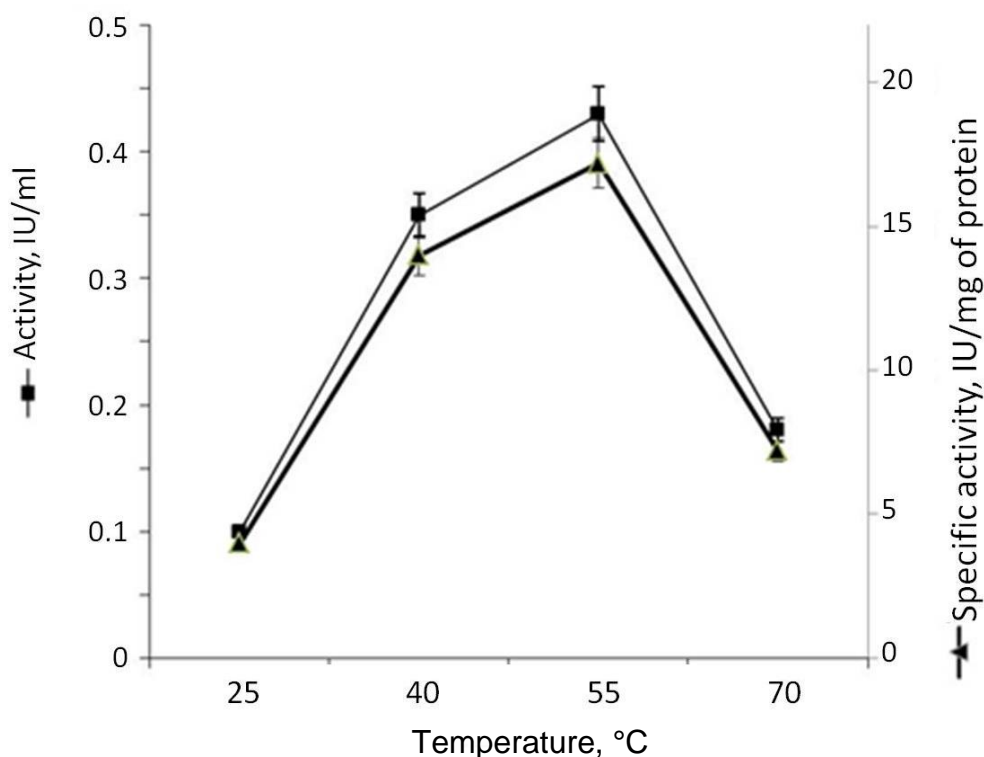
Fig. 4. Effect of temperature on GOx activity

The kinetic parameters of the enzyme K_m and V_{max} displayed the values of 4.1 mM and 0.36 IU, respectively. The K_m value determines the affinity of the enzyme for the substrate, with a smaller value indicating greater affinity. For enzymes isolated from highly active strains, this value ranges from 2 to 30 mM (Kersten and Cullen 2007). The obtained K_m values indicated a relatively high affinity of the isolated enzyme for D-glucose.

The absorption spectra and fluorescence IR spectra were recorded to identify the isolated enzyme and prosthetic group. The absorption spectrum of GOx, shown in Fig. 5, was typical for flavoproteins with an absorption maxima at 455 nm to 460 nm and 360 nm to 370 nm (Leitner *et al.* 2001). The ratio of the absorption maxima in the visible region of the spectrum (at wavelengths 370/455 nm) characterizes the degree of purification of the enzyme. For highly purified GOx preparations, this ratio is 1:32 (Yoshimura and Isemura 1971). For the enzyme studied herein, the ratio was 1:29, which indicates a sufficiently high degree of purification.

When excited by light with a maximal wavelength of 470 nm, GOx displayed a typical fluorescence spectrum for a flavoprotein with a maximum at 530 nm, as shown in

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Fig. 6, which is typical for GOx. The enzyme from *Aspergillus niger* indicated a maximum of 520 nm to 540 nm (Esposito *et al.* 2011).
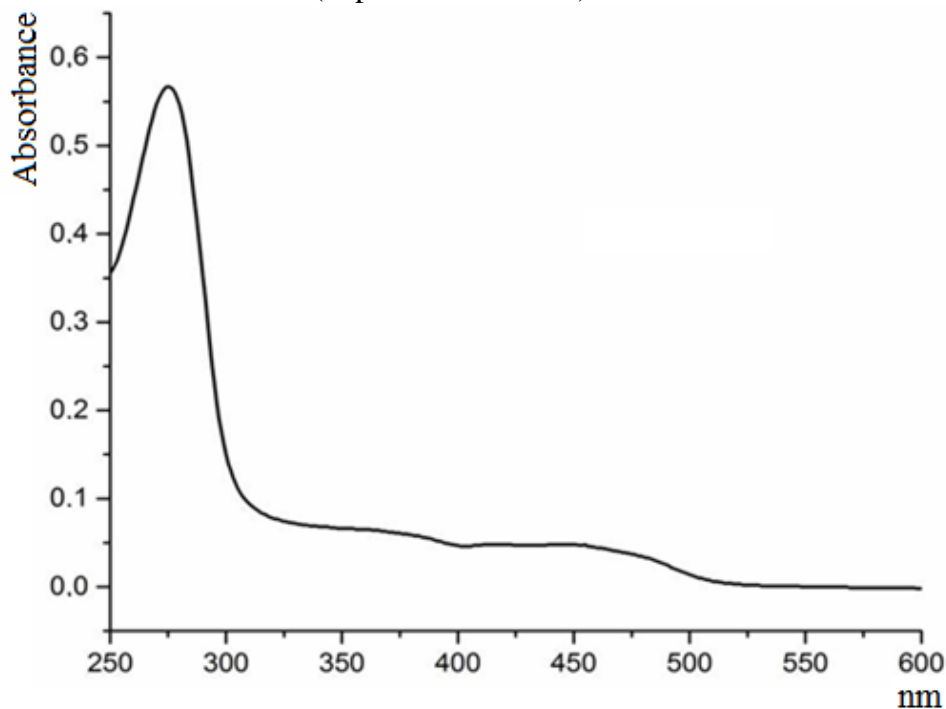

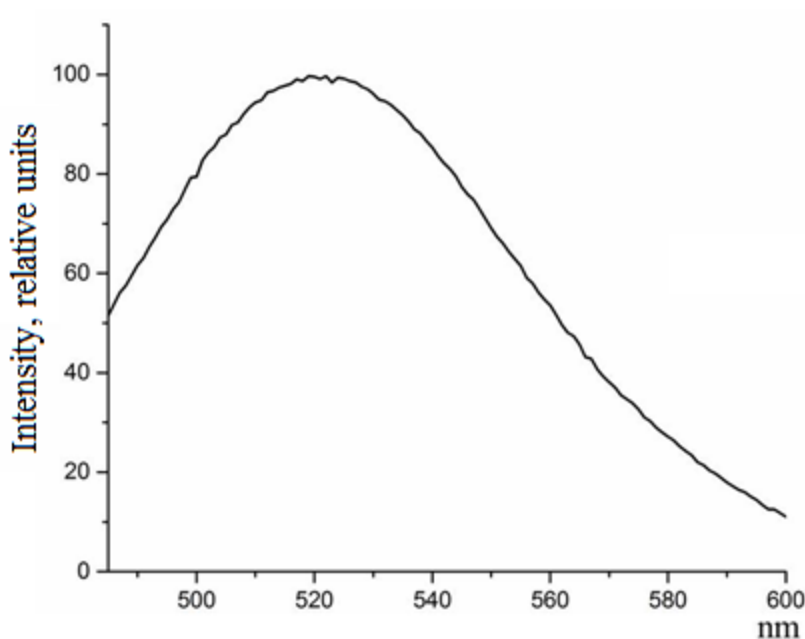**Fig. 5.** Absorption spectrum of purified GOx

Fig. 6. Fluorescence spectrum of purified GOx

To further confirm the presence in the molecule of the isolated GOx coenzyme FAD, an IR spectrum was recorded and compared with the IR spectrum of serum albumin, as shown in Fig. 7. The absorption spectra of all samples were represented by high-intensity bands characteristic for proteins in the frequency range of 3500 cm^{-1} to 3300 cm^{-1}, 1630 cm^{-1} to 1660 cm^{-1}, 1560 cm^{-1} to 1530 cm^{-1}, and 1400 cm^{-1} to 1380 cm^{-1},

which are commonly referred to as amide A (NH), amide I (Co + NH), amide II (CH$_2$ + NH), and amide III (CN + NH) (Arrondo *et al.* 1993; Yampolskaya *et al.* 2005).
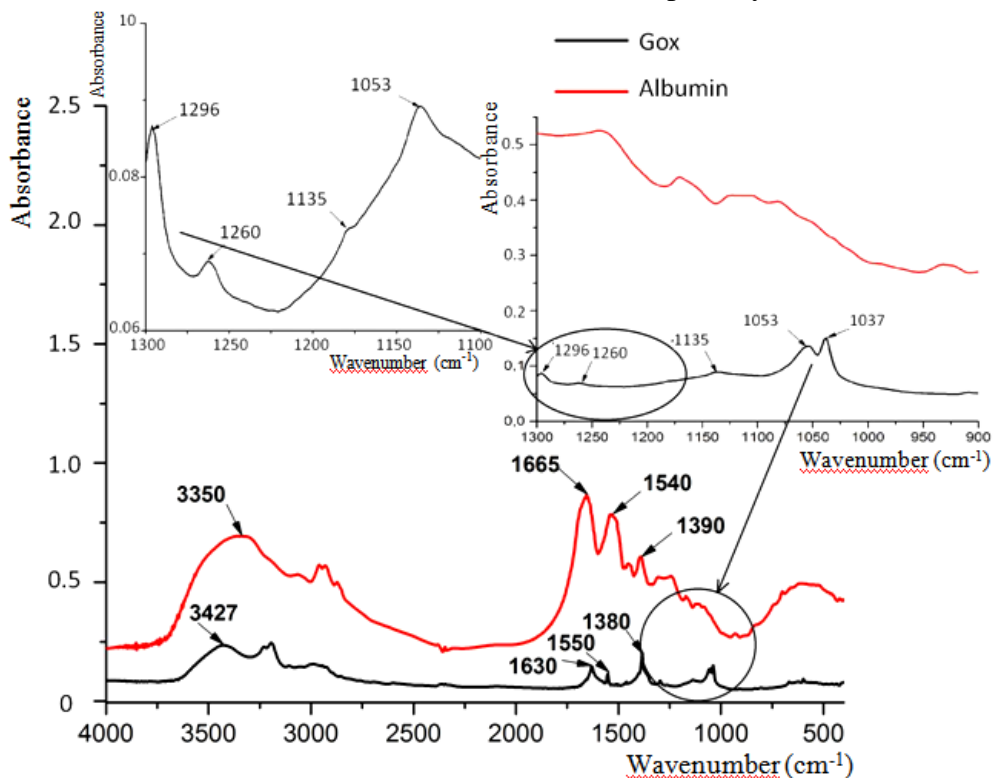

Fig. 7. IR spectra of glucose oxidase and serum albumin
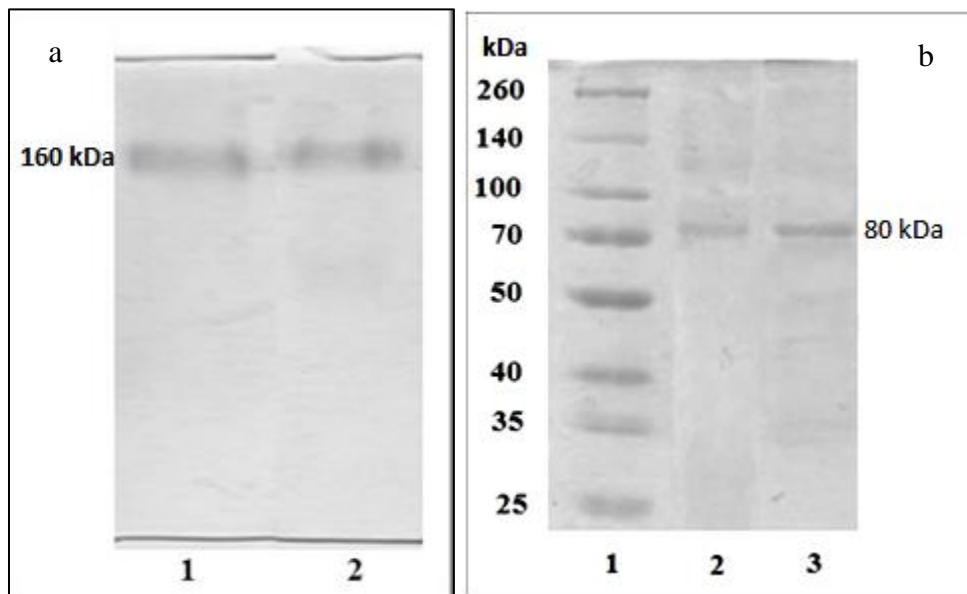

Fig. 8. Electrophoresis of glucose oxidases; a - in non-denaturing conditions (1 - standard enzyme from Aspergillus niger type VII, lyophilized powder, Mr 160 kDa (Sigma-Aldrich), 2 - enzyme isolated from *L. tigrinus*); b - in denaturing conditions (1 - standard mixture of proteins # 26634 - a prestained mixture of 10 recombinant proteins ranging from 25 K to 260 K (25ThermoFisher Scientific, Waltham, MA, USA), 2 - glucose oxidase standard, 3 - purified

glucose oxidase *L. tigrinus*)

However, the IR spectra of GOx in contrast to serum albumin, had quite distinct absorption bands in the 1135 cm^{-1} to 1000 cm^{-1} region and weak ones in the 1270 cm^{-1} to 1200 cm^{-1} region, which are due to symmetrical and asymmetrical vibrations of the bound phosphate groups entering in FAD (Wang *et al.* 2010).

The GOx of fungi have a molecular weight in the range of 140 kDa to 186 kDa and typically consist of two identical subunits of the polypeptide chain covalently bound by disulfide bonds (Kelley and Reddy 1986; Abbas and Mahmood 2007 Zia *et al.* 2007; Bhatti and Saleem 2009; Singh and Verma 2010; Hwang *et al.* 2011; Singh and Verma 2013). The molecular weight of GOx from *L. tigrinus* was calculated using electrophoresis, in non-denaturing conditions with 4% (stacking gel) and 12% (separating gel) of PAGE, and was determined to be 160 ± 10 kDa, as shown in Fig. 8a.

After processing the enzyme with mercaptoethanol, the preparation was run on a polyacrylamide gel with 0.1% DDS-Na, as shown in Fig. 8b. There was a clear band corresponding to a molecular mass of approximately 80 kDa. Based on published data (Kelley and Reddy 1986; Abbas and Mahmood 2007; Zia *et al.* 2007; Bhatti and Saleem 2009; Singh and Verma 2010; Hwang *et al.* 2011; Singh and Verma 2013), the enzyme probably consists of two subunits that differ insignificantly in molecular masses.

CONCLUSIONS

1. *Lentinus (Panus) tigrinus* VKM F-3616D produces a typical fungal GOx that serves as the peroxide-generating enzyme necessary for the ligninolytic enzyme complex to function.

2. The enzyme produced has an optimum pH of 4.0 and an optimum temperature of 50 °C. It is a flavoprotein, and the present analysis suggests that it consists of two equal subunits with a total molecular mass of approximately 160 kDa.

3. The kinetic characteristics of the enzyme indicate a high affinity for the substrate, glucose.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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