



Estimating levels of light emission and extracellular peroxidase activity of mycelium of luminous fungus *Neonothopanus nambi* treated with β -glucosidase

Mogilnaya OA*, Ronzhin NO and Bondar VS

Institute of Biophysics, Siberian Branch of Russian Academy of Science, Federal Research Center “Krasnoyarsk Science Center SB RAS”, 660036 Krasnoyarsk, Russia

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Abstract

The present study estimates the level of extracellular peroxidase activity and light emission intensity of mycelium of luminescent basidiomycete *Neonothopanus nambi* treated with β -glucosidase. A hypothesis has been proposed that treatment with β -glucosidase may trigger biochemical mechanisms of activation of ROS (primarily hydrogen peroxide) generation in *N. nambi* mycelium. The results obtained indicate that the enzyme causes partial disintegration of the slimy sheath of fungal hyphae and intracellular matrix, which leads to release of the extracellular peroxidases to the incubation medium. Mycelial cells treated with the enzyme reach the peak of their luminescence sooner. It has been assumed that partial loss of extracellular peroxidases, as important enzymes of antioxidant defense, may be compensated for by an increase in the level of light emission by the fungus.

Key words – basidiomycetes – cell wall – luminescence – polysaccharide sheath

Introduction

Previously (more than 20 years ago), Shimomura and colleagues (Shimomura 1992, Shimomura et al. 1993) suggested that reactive oxygen species (ROS) and oxidase enzymes played a role in the mechanism of luminescence of higher fungi. Our results of studying luminous basidiomycete *Neonothopanus nambi* also provided evidence for involvement of ROS and oxidase enzymes in the mechanism of fungal luminescence. We found that addition of millimolar concentrations of H₂O₂ to mycelium samples (with steady-state or declining luminescence intensities) caused a 3–5-fold increase in light emission by the fungus (Bondar et al. 2011, 2013, 2014). Incubation of *N. nambi* mycelium in deionized water, usually for 1–5 days, mechanical injury, and exposure to ionizing radiation resulted in a multifold increase in light emission (Bondar et al. 2013, Kobzeva et al. 2014, Medvedeva et al. 2014, Mogilnaya et al. 2016). Experiments with extracts of brightly and dimly luminous *N. nambi* mycelium showed the same trends of change in light emission intensity and activity level of enzymes of antioxidant defense system – peroxidases and catalase (Mogilnaya et al. 2015, 2016). These findings suggested an assumption about the metabolic interrelationship between luminescence enzymes of basidiomycete *N. nambi* and

enzymes of antioxidant defense such as peroxidases (including extracellular peroxidases), which neutralize the damage done by ROS. Previously, in the *in vivo* staining of *N. namibi* pellets for peroxidase activity, we also showed that fungal pellets differing in the luminescence intensity contained different amounts of extracellular peroxidases (Mogilnaya et al. 2016).

Extracellular fungal peroxidases can be found at a certain distance from the hyphae in the hyphal cell wall and in the slime matrix around the hyphae (Ruel & Joseleau 1991, Takano et al. 2009).

The cell wall of higher fungi is an extremely complex structure consisting of an elastic framework of microfibrillar polysaccharides (chitin and β -D-glucan), whose mass constitutes between 30 and 80% of the dry weight of cell wall (Klis et al. 2007). The cell wall also contains glycoproteins, lipids, proteins, and other minor components. Chitin chains form a framework that has considerable tensile strength (Abu Ali et al. 1999, Bowman & Free 2006, Feofilova 2010, Free 2013). Outside, the cell wall of the hyphae is covered by polysaccharide slime, which has pores and also consists of β -D-glucan (Pielken et al. 1990, Ruel & Joseleau 1991). Ascomycetes and basidiomycetes show a high degree of variability in β -D-glucans that they synthesize. Although they consist of only one type of sugar – D-glucose, the structural diversity is caused by the presence of anomeric forms (α -/ β -), positions and sequences of glucoside linkages along the polymer chain, the degree of branching of the polymer, and its molecular weight (Synytsya & Novak 2013, Giavasis 2014, Osin'ska-Jaroszuk et al. 2015). In basidiomycetes, the major polymers of exopolysaccharides of the hyphal sheath are (1–3)/(1–6) β -D-glucans. Some basidiomycetes synthesize β -D-glucans with (1–4)/(1–6) or (1–3)/(1–4) linkages in the main polysaccharide chains (Liu & Wang 2007, Fraga et al. 2014, Silveira et al. 2015). 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 enzymes immobilized here such as fungal extracellular peroxidases (Ruel & Joseleau 1991, Barrasa et al. 1998, Latge' & Beauvais 2014).

Taking into account the above fact, we assumed that the outflow of extracellular peroxidases from the glucan matrix could affect the light emission intensity of the pellets. This effect could be primarily achieved by partially destroying the polysaccharide slime and perhaps the glucan fibrils in the cell wall by enzymatic treatment of the fungal mycelium with, for example, β -glucosidase.

The enzyme of the hydrolase class – β -glucosidase – catalyzes hydrolysis of terminal residues in β -D-glucans, releasing glucose molecules. β -glucosidase is detected in lower and higher fungi and in plants (van den Brink & de Vries 2011). In this study, we evaluated extracellular peroxidase activity and light emission of *N. namibi* pellets treated with β -glucosidase.

Materials & Methods

Culture and procedures

The strains of luminous basidiomycete *N. namibi* IBSO 2307 used in this study are available in the Collection of Microorganisms (CCIBSO 836) of the IBP SB RAS.

Submerged cultivation of mycelium was performed in potato sucrose broth (potato broth 200 g/L, sucrose 20 g/L) 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% (w/v) of the broth volume. Cultivation was performed for 8 d at a temperature of 27–28°C under continuous stirring at 180–200 rpm (Environmental Shaker-Incubator ES-20, Biosan, Latvia).

At regular intervals (every day), 2-3 flasks with pellets were taken to determine growth parameters of the culture. Glucose concentration in the culture medium during cultivation was measured by the glucose oxidase method, using an enzyme kit for measuring glucose (Diakon DS, Russia). Before measurements, the culture medium was diluted 25-fold with deionized (DI) water. Deionized water was produced using a Milli-Q system (Millipore, U.S.).

Luminescence measurement

Luminescence of the pellets was measured using a Glomax 20/20 luminometer (Promega, U.S.) calibrated with the radioactive standard of Hastings and Weber (Hastings & Weber 1963) (one luminescent unit was 2.7×10^3 photons per second). After luminescence measurements, the pellets were dried in a rotational vacuum concentrator (Concentrator 5301, Eppendorf, Germany) at 45°C for 1.5 h, to determine their dry weight. Specific luminescence activity of mycelium was determined as the ratio of the light emission intensity of the sample to its dry weight.

Enzymatic treatments of pellets and measurement of peroxidase activity

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 hyphal cell envelope. At Day 5 of cultivation, pellets were taken out of the nutrient medium, placed into DI water, and rinsed at 25°C for 3 days, with the water changed twice a day. Every 24 h, some of the pellets were taken for experiments. Pellets were placed in DI water containing β -glucosidase (0.5 – 1.0 IU/ml) and incubated for three hours at a temperature of 25°C under slow, continuous stirring at 80 rpm on an OS-10 shaker (Biosan, Latvia). Control samples of mycelium were incubated in DI water under identical conditions but without an enzyme. After incubation, we measured light emission intensities and levels of extracellular peroxidase activity of the control and treatment pellets and peroxidase activity in incubation media. Peroxidase activity was measured by using the azo coupling reaction between 4-aminoantipyrine (4-AAP) and phenol, which is catalyzed by peroxidase in the presence of H_2O_2 and accompanied by formation of chromogen (quinoneimine). In our experiments only phenol and 4-AAP were introduced into the reaction mixture. For this, three pellets of the same size were taken out of the incubation medium and placed into test tubes with 1 ml of the reaction mixture containing phenol (0.56 mg/ml) and 4-AAP (0.1 mg/ml). The samples were maintained at 25°C for one hour. Then, the liquid was collected, and the amount of the reaction chromogen was estimated in a UV-1800 spectrophotometer (Shimadzu, Japan) by measuring optical density at a wavelength of 506 nm. To determine peroxidase activity of the incubation medium, 8 mM H_2O_2 was added to it, along with phenol and 4-AAP. Peroxidase activity was expressed in terms of optical density. Each measurement experiment was performed in triplicate.

Visualization of luminescence and images of pellets

Visual observation of luminescence of native pellets was conducted by using a ChemiDoc™ XRS System (Bio Rad, U.S.) in a dark cabinet, in the signal accumulation mode. Exposure time, 300 seconds, was chosen by trial and error. Images of pellets were made with a PowerShot S50 camera (Canon, Japan).

Micrographs of the pellets and hyphae were obtained by using an AxioImager M2 (Zeiss, Germany) microscope in the transmitted and reflected light mode. Before microscopy, the pellets were fixed for 15 minutes in a 3.7% solution of paraformaldehyde prepared in 10 mM PBS (pH 6.9), washed several times in buffer solution, and then rinsed in DI water. For fluorescent microscopy, the pellets were stained for 15 minutes in the aqueous solution of acridine orange dye (20 μ g/ml). Samples were examined with an AxioImager M2 with a Filter set 09 (450–490 nm excitation filter, a 510 nm chromatic beam splitter, 515 nm emission filter) and a Plan-Neofluar 100 \times /1.30 Oil M27 lens.

Results

Submerged cultivation of the fungus *N. nambi* under continuous orbital stirring produced white or off-white mycelial pellets 2–7 mm in diameter (Fig. 1). The pellets were of regular spherical shape, with hyphal bundles, some of them extending for a few millimeters, on their surface. Microscopic examination revealed that the inner portion of the pellet was rather dense, consisting of interwoven hyphae and intercellular substance.

In submerged culture, *N. nambi* rapidly grew in the nutrient medium containing glucose, consuming the greater part of glucose over the first four days of mycelial growth. After that,

glucose concentration in the medium changed insignificantly (Fig. 2). Our findings show that light emission of the pellets began to increase during the exponential growth of biomass, reaching its maximum by days 5–6. Then, the growth of mycelium slowed, biomass stopped increasing, and mycelium luminescence intensity decreased.

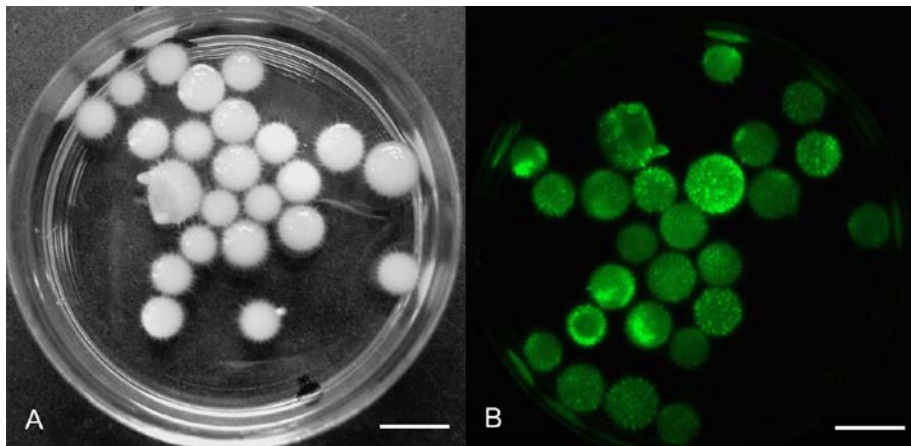


Fig. 1 – Images of *Neonothopanus nambi* IBSO 2307 pellets grown under submerged cultivation. Reflected-light images of fungal pellets (A); images of the pellets taken using their own light in the dark cabinet, in the signal accumulation mode (B). – Bars = 10 mm.

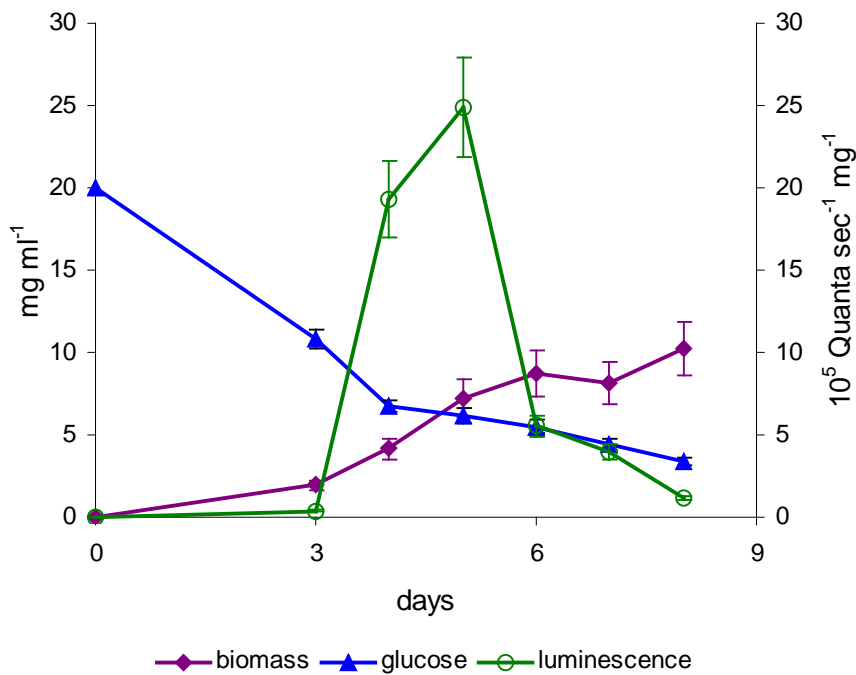


Fig. 2 – Time-course data of biomass production, glucose consumption, and changes in bioluminescence during submerged cultivation of *Neonothopanus nambi* IBSO 2307 in potato sucrose broth under continuous stirring at 180–200 rpm at a temperature of 27–28°C. Here and in Figures 4 and 6, the data are presented as $M \pm m$, $n=3$ for each measurement.

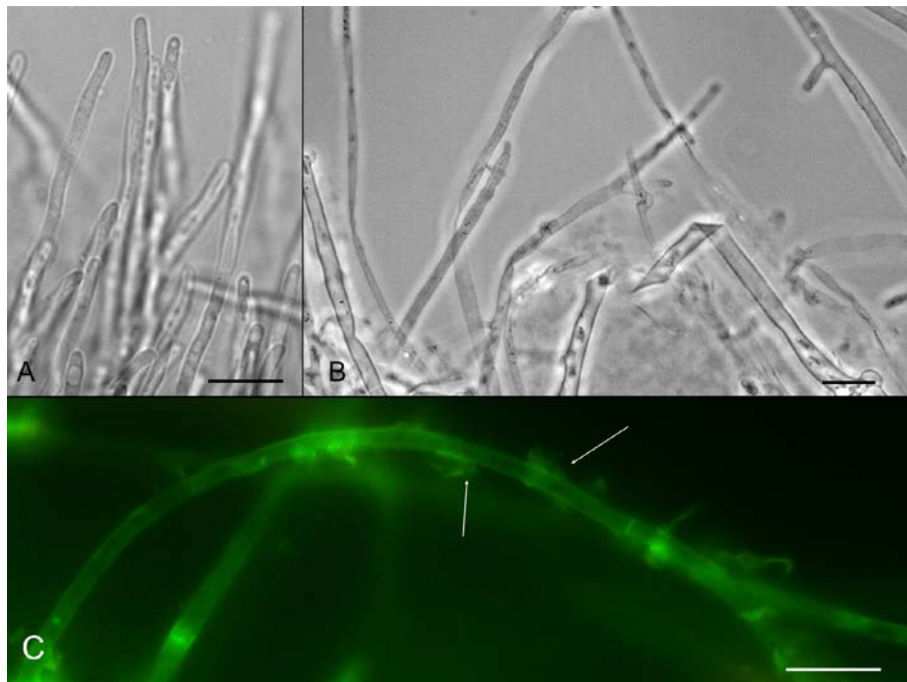


Fig. 3 – Light-optical images of destructive changes in *Neothopanus nambi* IBSO 2307 pellet treated with β -glucosidase. Bright-field modes of viewing the peripheral hyphae of the pellet in DI water (control) (A); flattened hyphae with impaired elasticity after β -glucosidase treatment (B); fragments of slimy layer on the surface of the hypha (arrows) in the fluorescent mode of viewing with an AxioImager M2, Filter set 09 (C). – Bars = 10 μ m.

For experiments, *N. nambi* pellets were taken out of the nutrient medium at Day 5 of the culture. As shown above (Fig. 2), at that time, mycelial growth was entering the steady-state phase, and the highest level of luminescence was observed for the pellets in submerged culture.

We did not observe any visible destructive changes in the mycelial pellets incubated in DI water for three days. Microscopic examination did not reveal any noticeable changes in the appearance and shape of the hyphae, including peripheral hyphae, which were in contact with the external medium (DI water) (Fig. 3a).

After *N. nambi* pellets had been incubated in β -glucosidase-containing DI water for three hours, they showed noticeable morphological destructive changes: the slimy layer on the surface of the hyphae was breaking into fragments, the hyphae lost their shape, becoming flattened and bent (Fig. 3b, c). In addition, the integrity of intercellular matrix was destroyed: pellets lost their rigidity and were easily flattened when pressed by cover glass for viewing with a light microscope. We assume that this is primarily due to the fragmentation of the network of glucan fibrils, which are known to be responsible for the elasticity of the hyphae.

Results of microscopic examination were consistent with results of testing extracellular peroxidase activity in control and treatment samples of mycelium and incubation media (Figs 4, 5). Control pellets taken out of the nutrient medium and incubated in DI water showed a gradual rise in extracellular peroxidase activity – an increase in chromogen release (Fig. 4a). At the same time, pellets exposed to β -glucosidase showed a considerably lower increase in extracellular peroxidase activity (Figs 4a, 5a), suggesting that exposure to β -glucosidase caused substantial destructive changes, in the polysaccharide matrix, which can be aggravated by the long-term osmotic stress. Thus, the fungus may have lost part of extracellular peroxidases due to their release from the destroyed hyphal polysaccharide sheath to the incubation medium. This assumption was confirmed by results of measurements of peroxidase activity in the media where control and treatment pellets were incubated (Figs 4b, 5b). We detected the product of azo coupling reaction in control and treatment incubation media, which indicated the presence of enzymes with peroxidase activity in them. However, in the treatments, where mycelium was exposed to β -glucosidase, we detected

considerably larger amounts of chromogen, which may be indicative of the higher content of peroxidases.

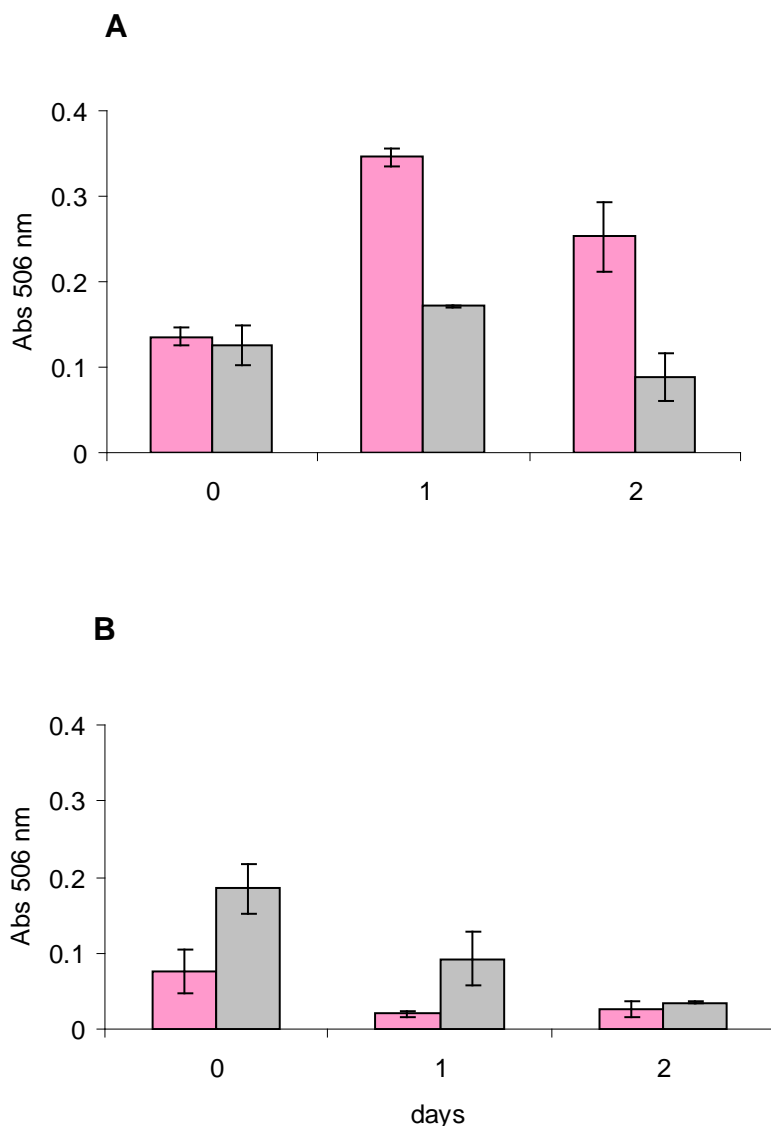


Fig. 4 – Comparative evaluation of formation of chromogen in azo coupling reaction by *Neonothopanus nambi* IBSO 2307 pellets (A) and in the incubation medium (B): without β -glucosidase treatment (pink columns) and after β -glucosidase treatment (gray columns). The data on the X-axis show the total time of incubation of mycelium in DI water; the zero point corresponds to the day when the pellets were taken out of the nutrient medium.

N. nambi pellets incubated in DI water with β -glucosidase, and the control mycelium demonstrated similar patterns of change in light emission (Fig. 6). However, our results showed that in the presence of β -glucosidase, luminescence of mycelium reached its maximum sooner than luminescence of the control mycelium. The greatest differences between light emission by the control and treatment samples were revealed in the initial luminescence, measured after the pellets had been taken out of the nutrient medium and immediately incubated in the DI water with and without the enzyme. Differences in the initial levels of light emission between the control and treatment samples of mycelium reached several orders of magnitude.

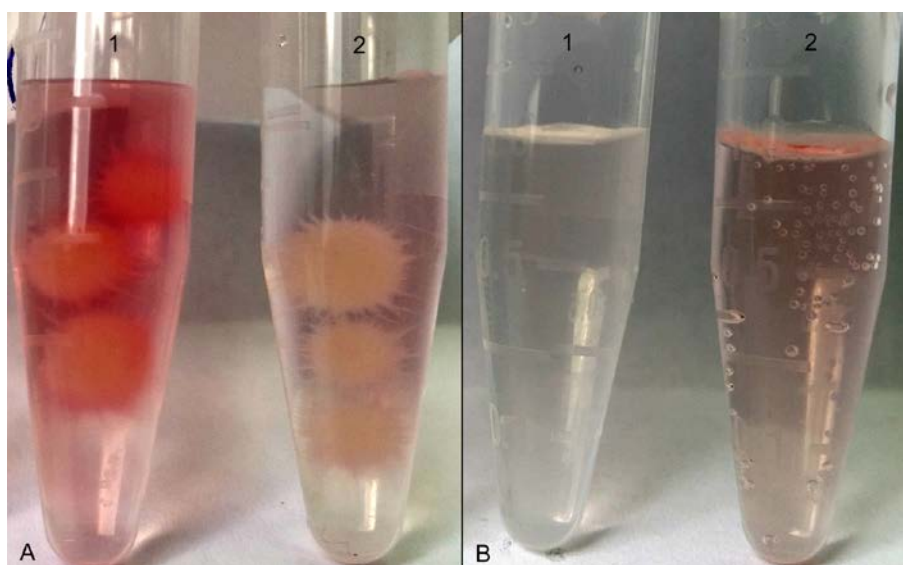


Fig. 5 – Images of *Neonothopanus nambi* IBSO 2307 pellets (A) and incubation medium (B) after β -glucosidase treatment and azo coupling reaction: without β -glucosidase treatment (1) and after β -glucosidase treatment (2).

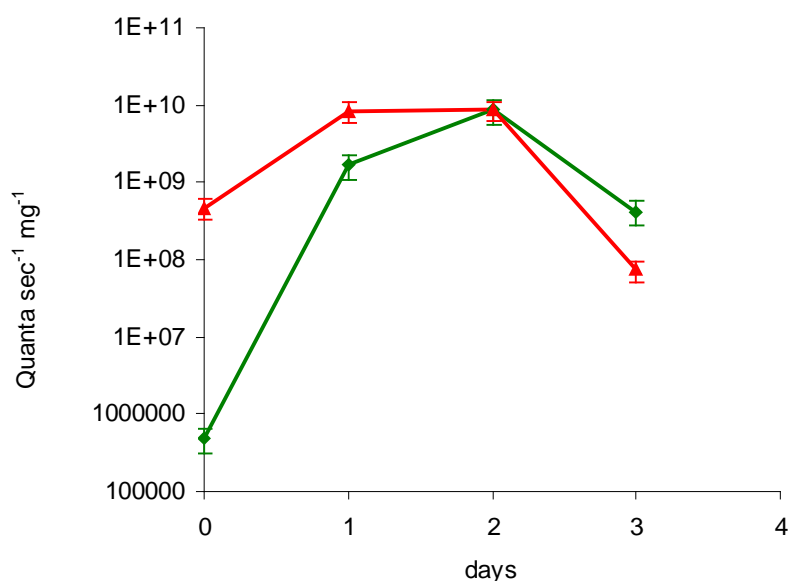


Fig. 6 – Changes in light emission by *Neonothopanus nambi* IBSO 2307 pellets during long-term incubation in DI water: without β -glucosidase treatment (green line) and after β -glucosidase treatment (red line). The data on the X-axis show the total time of incubation of mycelium in DI water; the zero point corresponds to the day when the pellets were taken out of the nutrient medium. The data on the Y-axis show the specific luminescence activity, determined as the ratio of the light emission intensity of the sample to its dry weight.

Discussion

Previously we showed that luminescence intensity of *N. nambi* pellets incubated in DI water for long periods of time (1–5 days) was increasing, and the mycelium luminescence peak was generally reached in 2–3 d after the fungus was placed in the DI water (Bondar et al. 2013, Mogilnaya et al. 2016). Other authors also used a similar approach to enhance luminescence of fungal mycelium (Mori et al. 2011). Morphological integrity of the hyphae was preserved during

long-term incubation in hypo-osmotic medium because of resilience of their cell walls and, hence, due to osmotic pressure remaining balanced. Another study demonstrated elasticity and dynamism of the cell wall of yeast under osmotic stress, when the chitin-glucan layer was compressed and relaxed several times within a short period of time (Ene et al. 2015). Destructive morphological changes of *N. namibi* hyphae and pellets after enzyme treatment may be primarily explained by the fragmentation of the network of glucan fibrils in the sheath and intercellular matrix, which are known to be responsible for the elasticity of the hyphae.

The differences in the levels of peroxidase activity observed in incubation media of control and treatment mycelial samples do not contradict the data previously obtained in studies of fungi. Extracellular peroxidases of fungal mycelium are localized in the cell wall and slimy sheath enclosing hyphae (Daniel et al. 1989, Ruel & Joseleau 1991, Takano et al. 2009). Therefore, in our experiments, extracellular peroxidases of the control mycelium samples could diffuse from the hyphae to the aqueous medium because of disintegration of the water-soluble part of their polysaccharide sheath. In the treatments, this process could be intensified by the presence of β -glucosidase, which catalyzed hydrolysis of β -D-glucans in the outer slimy layer. Hence, larger amounts of extracellular peroxidases could have diffused to the aqueous medium. The fact that we detected larger amounts of chromogen in the water where treatment pellets had been incubated is the evidence in favor of this statement.

The reason for the differences in the initial levels of light emission between the control and treatment samples is that mycelium removed from the nutrient medium and placed into DI water containing β -glucosidase was subjected to much higher stress, caused by the simultaneous effect of two factors – hypo-osmotic shock and the enzyme with hydrolytic properties. This may imply a considerably higher level of ROS generated in the mycelium. At the initial instant of time (the zero point of the diagram in Fig. 4), extracellular peroxidases of the control and treatment pellets showed the same levels of activity (Fig. 4a), suggesting that neutralization of the ROS, whose increase had been initiated by β -glucosidase and hypo-osmotic shock, could occur during light emission reaction. A more considerable loss of extracellular peroxidases observed in the treatment pellets compared to the control ones (Fig. 4b) may be interpreted as evidence supporting this assumption. This partial loss of the enzymes of the defense system against ROS may be compensated for by the increase in the level of light emission. Later, the differences in the levels of light emission between the control and treatment pellets become less substantial (Fig. 6).

The increase in the level of ROS (H_2O_2 , in particular) in mycelium exposed to β -glucosidase can occur via the following biochemical processes. β -glucosidase catalyzes hydrolysis of β -D-glucans of the hyphal cell wall and, thus, supplies glucose molecules to mycelium. As we mentioned above, we collected samples of mycelium when the fungal culture was entering the steady-state growth phase, with the lowest glucose concentration in the nutrient medium. Carbon deficiency in the steady-state phase of growth of fungi causes their own endo- and exoglucanases and β -glucosidases to break up the slimy polysaccharide sheath, producing glucose molecules (Eriksson et al. 1986, Daniel et al. 1989, van den Brink & de Vries 2011). Meanwhile, glucose-oxidizing enzymes (glucose oxidase and pyranose oxidase) begin to diffuse from cytoplasm to both periplasm and extracellular slimy sheath. In this way, fungal extracellular peroxidases continuously receive the necessary substrate – hydrogen peroxide. On the other hand, disintegration of the polysaccharide framework caused by exposure to β -glucosidase and development of osmotic shock can be regarded as the damaging effect of the stressor. It is commonly known that this type of effect triggers the mechanism of ROS generation. In what order mechanisms of activation of ROS generation in mycelium of basidiomycete *N. namibi* start operating and what contribution each of them makes should be investigated in a separate study.

Thus, the data obtained in this study suggest several general propositions and conclusions. Experiments with luminous basidiomycete *N. namibi* showed that treatment of mycelium with β -glucosidase caused disruption of the slimy sheath and, perhaps partially, cell wall of the hyphae. Morphological changes were revealed by microscopy techniques. The disruption of the hyphal cell envelope was confirmed by results of comparative evaluation of extracellular peroxidase activity in

pellets and the presence of peroxidase activity in the incubation medium. Investigation of luminescence of the control and treatment mycelial samples showed similar patterns of change in light emission in both groups. However, treatment of mycelium with the enzyme considerably decreased the time over which light emission reached its maximum. A possible explanation for this is activation of generation of ROS (hydrogen peroxide, in particular) and an increase in the level of luminescence as the compensatory mechanism neutralizing the effect of ROS when the fungus loses part of its extracellular peroxidases. We obtained similar results earlier in the study of another species of luminous fungus *Armillaria borealis* (Mogilnaya et al. 2017).

The results of this study provide support for our hypothesis (Mogilnaya et al. 2015, 2016) suggesting a common metabolic basis of the function of the fungal luminescent system and antioxidant defense enzymes (including extracellular peroxidases), which neutralize the damaging effect of ROS on the fungus.

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