Journal of Microbiology (2013) Vol. 51, No. 6, pp. 829–835
DOI 10.1007/s12275-013-2689-5

Reduction of Organic and Inorganic Selenium Compounds by the Edible Medicinal Basidiomycete *Lentinula edodes* and the Accumulation of Elemental Selenium Nanoparticles in Its Mycelium

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(Received December 13, 2012 / Accepted June 9, 2013)

We report for the first time that the medicinal basidiomycete *Lentinula edodes* can reduce selenium from inorganic sodium selenite (Se^{IV}) and the organoselenium compound 1,5-diphenyl-3-selenopentanedione-1,5 (DAPS-25) to the elemental state, forming spherical nanoparticles. Submerged cultivation of the fungus with sodium selenite or with DAPS-25 produced an intense red coloration of *L. edodes* mycelial hyphae, indicating accumulation of elemental selenium (Se^0) in a red modification. Several methods, including transmission electron microscopy (TEM), electron energy loss spectroscopy (EELS), and X-ray fluorescence, were used to show that red Se^0 accumulated intracellularly in the fungal hyphae as electron-dense nanoparticles with a diameter of 180.51±16.82 nm. Under designated cultivation conditions, shiitake did not reduce selenium from sodium selenate (Se^{VI}).

***Keywords*:** shiitake, elemental selenium, nanospheres, intracellular synthesis

Introduction

Nanoparticles are finding ever-widening application in medicine (synthesis, delivery, and utilization of drugs; cancer therapy), biology (immunological research and the use of nanoparticles as biomarkers to study intracellular processes *in vivo*), and technology (electronic engineering, information technology, and preparation of new materials with improved properties) (Krumov *et al*., 2009). Biological synthesis of nanoparticles by using microorganisms offers advantages over chemical and physical methods, which are costly and require the use of hazardous substances (Narayanan and Sakthivel, 2010). An active search is in progress to find effective biological agents for the preparation of nanoparticles of various chemical natures (Musarrat *et al*., 2011; Rai and Duran, 2011).

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Selenium is a trace element with unique biological functions that forms part of several proteins of living organisms. Previously, elemental selenium (Se^0) had been thought to be biologically inaccessible to living organisms, but recent research has demonstrated that selenium nanoparticles have biological activity. Artificial nanoparticles of Se^0 display antitumor activity and antioxidant properties *in vivo* and *in vitro* (Gao *et al*., 2002; Wang *et al*., 2007; Zhang *et al*., 2008; Wang, 2009). Size plays an important role in the biological activity of selenium nanoparticles; for example, Peng *et al.* (2007) found that particles of 5 to 200 nm can neutralize free radicals *in vitro*. Current research has produced evidence for the possibility of using bacteria to obtain selenium nanoparticles (Oremland *et al*., 2004; Pearce *et al*., 2008; Prakash *et al*., 2009; Fesharaki *et al*., 2010; Prokisch and Zommara, 2010; Eszenyi *et al*., 2011). Along with bacteria, fungi are promising biological agents for preparing nanoparticles of various elements (Sastry *et al*., 2003; Durán *et al*., 2007; Philip, 2009; Popescu *et al*., 2010). The use of higher edible fungi to obtain selenium nanoparticles may, therefore, be of great theoretical and practical importance, as these fungi have the important advantage of being nontoxic to humans.

Several lower fungi degrade inorganic selenium compounds with the formation of red Se^0, and their mycelia acquire a red or pink coloration in the process of degradation (Ramadan *et al*., 1988; Gharieb *et al*., 1995; Brady *et al*., 1996). It has been suggested that the mechanism of detoxification is the breakdown of selenium-containing salts to produce free selenium. We have not found any published studies pertaining to the use of fungi in the preparation of selenium nanoparticles.

The past few years have seen the beginning of extensive research into the peculiarities of the chemical composition of fungi grown on substrates supplemented with selenium salts. Selenium has been found to be present in the fruiting bodies of macromycetes, mostly as protein-bound or free selenium-containing amino acids and also as inorganic compounds (Ogra *et al*., 2004; Zhao *et al*., 2004; Díaz Huerta *et al*., 2006; Munoz *et al*., 2006). Basidial fungi can accumulate large amounts of selenium; therefore, selenium enrichment of fungal fruiting bodies and mycelia may be of great practical value in correcting shortages of this trace element in humans and animals. Turło *et al.* (2010) reported that the antioxidant activity of *Lentinula edodes* mycelial extracts increased by 100 to 400% when the fungus was grown with sodium selenite. They suggested that the activity increase may have been due to the accumulation of both selenium-containing

amino acids and Se^0 in the mycelium. Still, fungal transformation of inorganic selenium compounds to yield Se^0 remains poorly studied. Almost no attention has been paid to the effect on fungi of organoselenium compounds, some of which [e.g., diacetophenonyl selenide (DAPS-25)] are less toxic than selenates and selenites and so can serve as a better source of selenium for fungi.

In this study, we examine the effect of selenium-containing compounds on the medicinal basidiomycete *L. edodes* and the ability of this xylotroph to reduce these compounds to the elemental state and accumulate selenium particles in its mycelium.

Materials and Methods

Fungus and cultivation conditions

Lentinula edodes (Berk.) Sing (shiitake), strain F-249, was obtained from the collection of higher fungi held by the Department of Mycology and Algology at Moscow State University, Russia. The fungal culture was maintained on beer-wort agar plates (4 degrees on the Balling scale for sugar content) (Ball, 2006) at 4°C.

The fungus was grown submerged in beer wort (4 degrees on the Balling scale for sugar content) and in a synthetic medium of the following composition (g/L): d-glucose, 5; l-asparagine, 1; KH_2PO_4, 2; K_2HPO_4, 3; $MgSO_4 \times 7H_2O$, 2.5; $FeSO_4 \times 7H_2O$, 0.03 (pH 5.8). Growth was conducted in 100-ml flasks containing 50 ml of medium at the mycelial growth temperature optimal for this species (26°C). For inoculation, we used a 14-day-old *L. edodes* culture grown on agar-supplemented synthetic medium at 26°C.

The effect of organic and inorganic selenium compounds on *L. edodes* growth and the xylotroph's reduction ability were examined by adding the following compounds to both beer wort and liquid synthetic medium:

i. Selenate (Se^{VI}, as Na_2SeO_4) and selenite (Se^{IV}, as Na_2SeO_3) (both from Sigma), added as aqueous solutions (concentration range, 10^{-6} to 10^{-2} mol).
ii. The selenium-containing formulation DAPS-25 (1,5-diphenyl-3-selenopentanedione-1,5), added as a solution in 50% ethanol (concentration range, 10^{-7} to 10^{-3} mol).

The solutions of the compounds were sterilized by UV irradiation and were added under sterile conditions to each of the growth flasks individually just before seeding. The indicated sterilization method was chosen to prevent heating of the compounds and their possible chemical breakdown during autoclaving.

Determination of *L. edodes* growth characteristics

L. edodes growth was characterized by the accumulation of dry biomass. The mycelium was passed through filters that had been preweighed on an analytical balance, and then it was dried to a constant weight and weighed again. The increment in biomass was compared between control and experimental trials. Experiments to measure fungal growth characteristics and biomass accumulation were done in 10 replicates.

X-ray fluorescence analysis

To detect Se^0 and/or selenium compounds in the fungal mycelium, an X-ray fluorescence study of mycelial samples was performed. To this end, *L. edodes* was grown in the synthetic medium (control) and in the same medium supplemented with 0.3 mmol of Na_2SeO_4, 0.3 mmol of Na_2SeO_3, or 0.3 mmol of DAPS-25. Growth was conducted at 26°C for 7 days. The grown mycelial samples were collected by centrifugation and were dried at room temperature. The content of selenium in the dry biomass and in the reference samples of DAPS-25, Na_2SeO_3, and Na_2SeO_4 were analyzed with an ED 2000 energy-dispersive spectrometer (Oxford Instruments, UK). The measuring conditions were as follows: element detection range, Na–U; X-ray tube, silver anode; tube voltage, 35 keV (medium elements); primary X-ray beam filter, thin Ag; exposure time, 600 sec; spectral path, air. The content of the elements being detected was evaluated by the basic parameters method included in the instrument's software.

TEM microscopy and EELS analysis

For TEM studies, the mycelium was washed twice with PBS (g/L: NaCl, 8.5; $Na_2HPO_4 \times 2H_2O$, 1.34; $NaH_2PO_4 \times 2H_2O$, 0.39; pH 7.4) and then with distilled water that had been filtered through a 0.22-μm-pore-size membrane filter (Millipore Corp., USA). Next, the mycelial hyphae were resuspended in a minimal volume of distilled water and were placed onto nickel grids coated with formvar (1% formvar in dichloroethane). After the sample had dried, the cells were contrasted with 1% aqueous uranyl acetate (Tandler, 1990).

To obtain ultrathin sections, the fungal hyphae were fixed in epoxy resin, as follows. The mycelial hyphae were collected by centrifugation (13,000×*g* for 20 min) at room temperature, and the material was then transferred to 2-ml polypropylene test tubes and fixed in 0.1% glutaraldehyde. Subsequent fixation was done for 12 h in a 2.5% glutaraldehyde solution made with phosphate buffer (0.1 mol, pH 7.2). The material was then held in a 0.1% OsO_4 solution made with the same buffer but supplemented with 34 mg/ml sucrose.

Samples were dehydrated in increasing concentrations of acetone. After dehydration, the samples were held in propylene oxide for 45 min, and then they were embedded in Epon 812 and propylene oxide (each time for 24 h) at ratios of 1:2, 1:1, and 2:1. Next, the material was embedded in pure epoxy resin. Polymerization was done at 37, 45, and 57°C for 24 h each. Ultrathin sections were cut on an LKB-III microtome (Sweden), placed on nickel grids, and stained with aqueous uranyl acetate (Tandler, 1990) and lead citrate (Reynolds, 1963).

For nanoparticle studies, the fungal mycelium was grown as above for 14 days in the presence of 0.3 mmol of Na_2SeO_3 or 0.3 mmol of DAPS-25. After that, it was washed free of the cultivation medium with distilled water, collected by centrifugation, and lyophilized. The fungal hyphae were then mechanically disrupted and were separated from nanoparticles through a membrane filter (0.4 μm pore size) (Millipore).

A Libra 120 electron microscope (Carl Zeiss, Germany)

operating at 120 keV was used to take photomicrographs and electron energy loss spectra for selenium and to prepare a selenium map.

Statistical analysis

All experiments had no less than 10 replications. Data are presented as means±95% confidence intervals.

Results

Effect of Na_2SeO_4, Na_2SeO_3, and DAPS-25 on the growth characteristics of *L. edodes* F-249

We first tested the sensitivity of *L. edodes* F-249 to Na_2SeO_4, Na_2SeO_3, and DAPS-25 and the effect of these compounds on its growth characteristics and on biomass accumulation as fungal mycelium under submerged cultivation. The inhibition of culture growth in liquid medium was determined by the change in the biomass increment. We found that 0.1 to 1 mmol of DAPS-25 suppressed fungal growth but that 0.1 to 1 μmol had a positive effect. The culture growth rate was much higher on beer wort than it was on synthetic medium, and this relatively fast-growing mycelium was more responsive to the selenium addition, which was evident as a greater enhancement of growth (up to 23%).

When *L. edodes* was grown with 0.1 to 1 mmol of Na_2SeO_3 and DAPS-25, the mycelium acquired a red or reddish-orange coloration. The color intensity depended on the concentration of the compound used. The change in the color of microbial colonies growing in the presence of selenium-containing compounds to various hues of pink and red is the first sign of reduction of these compounds to the red modification of Se^0 (Roux *et al*., 2001; Hunter and Manter, 2009). Such coloration is characteristic of bacteria and fungi growing on selenium-containing media, indicating that the culture has accumulated Se^0 (Gharieb *et al*., 1995; Turner *et al*., 1998; Hunter and Kuykendall, 2007; Fernández-Martínez and Charlet, 2009; Turło *et al*., 2010).

In experiments with 0.3 mmol of Na_2SeO_3 or 0.3 mmol of DAPS-25, the mycelium had a bright red coloration. The growth medium did not change its color, indicating that (i) the red coloration was associated with the mycelial hyphae, (ii) the enzymes reducing selenium from Na_2SeO_3 and DAPS-25 were probably localized inside the cells, and (iii) selenium had accumulated inside the cells. For this reason, the 0.3 mmol concentration was used in all subsequent experiments.

When the colored cultures were sedimented by centrifugation, the supernatant liquid remained uncolored and the mycelial sediment was bright red. This also suggests that selenium reduction was not associated with a chemical reduction occurring under the influence of culture-medium or culture-liquid components (various low- and high-molecular-weight compounds from the fungus's biological activity, including extracellular enzymes). Instead, it was associated directly with the fungal mycelium, with the reduction and the subsequent accumulation of Se^0 occurring inside the mycelial hyphae. In no-fungus control flasks containing synthetic medium of the same composition plus

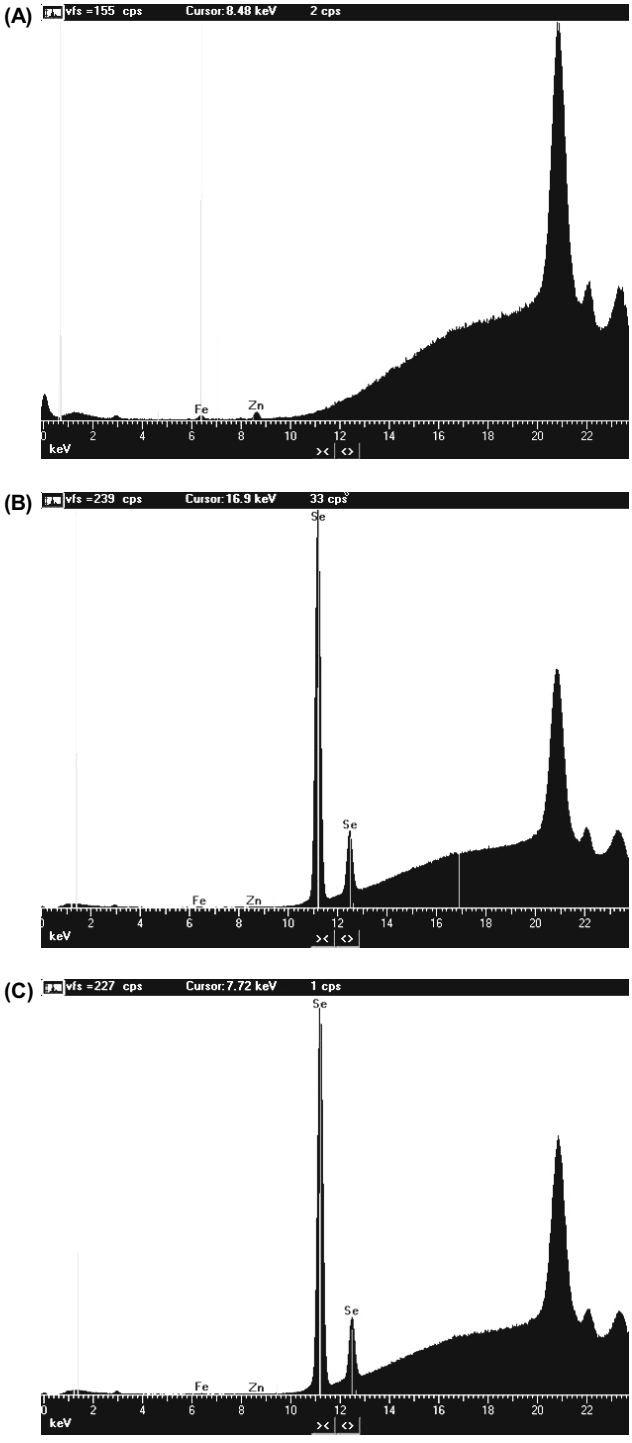

Fig. 1. X-ray fluorescence analysis of dry biomass of *L. edodes* F-249 mycelium grown in liquid synthetic medium in the absence (A, control) and presence of 0.3 mmol of Na_2SeO_3 (B) and 0.3 mmol of DAPS-25 (C) at 26°C for 14 days. The emission lines at 11.22 and 12.49 keV (Kα and Kβ lines, respectively) correspond to selenium.

Na_2SeO_4, Na_2SeO_3, or DAPS-25 at 0.001 to 10 mmol, no color change or sedimentation occurred for 90 days of selenium reduction at 26°C.

In the presence of Na_2SeO_4, the color of the fungal culture

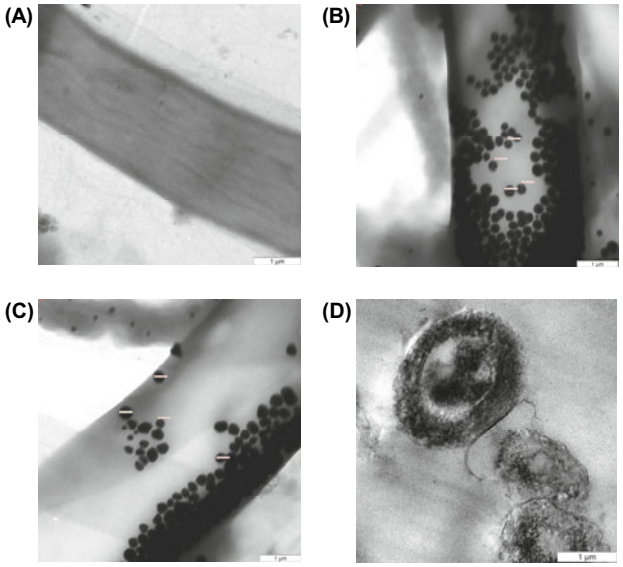

Fig. 2. TEM images of the hyphae of *L. edodes* F-249 mycelium grown in liquid synthetic medium in the absence (A, control) and presence of 0.3 mmol of Na_2SeO_3 (B) and 0.3 mmol of DAPS-25 (C) at 26°C for 14 days. A cross section of fungal hyphae embedded in epoxy resin after selenium reduction (D). Bar marker = 1 μm.

and of the growth medium remained at the control level, suggesting that under submerged cultivation conditions, *L. edodes* did not reduce selenium from this compound to Se^0.

Selenium determination in the mycelium by X-ray fluorescence

L. edodes was grown submerged in synthetic medium at 26°C for 14 days in the presence of 0.3 mmol of Na_2SeO_3, 0.3 mmol of Na_2SeO_4, or 0.3 mmol of DAPS-25. The mycelium was then air dried at 20°C. X-ray fluorescence analysis of the fungal cells showed considerable accumulation of selenium in the mycelium growing with Na_2SeO_3 and DAPS-25, which was reflected in the spectra as intense emission lines corresponding to selenium (Figs. 1A and 1B). The presence of selenium was determined by two lines, at 11.22 and 12.49 keV. The spectra taken for the control cultures showed absence of selenium (Fig. 1C). Na_2SeO_4 at 0.3 mmol had no pigmenting effect on the mycelium, and selenium was present in the mycelium only in trace amounts.

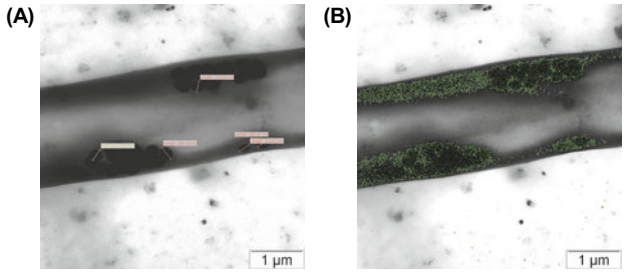

Fig. 3. TEM image of selenium particles (A, control) and a selenium distribution map created by EELS (B). Bar marker = 1 μm.

TEM and EELS

We performed TEM of *L. edodes* hyphae grown under the same conditions in the presence of 0.3 mmol of Na_2SeO_3 or 0.3 mmol of DAPS-25. As seen in Fig. 2, the cells contained spherical electron-dense formations, which were absent from the control. Most of these formations had an average diameter of 180.51±16.82 nm and were localized inside the mycelial hyphae. Isolated spherical particles could be observed outside the cells, most probably resulting from the rupturing of a small number of hyphae and release of elemental selenium particles.

The map of selenium distribution obtained by electron spectroscopic imaging showed that the inclusions were composed of selenium (Fig. 3). The spectra obtained by EELS confirmed this finding (Fig. 4).

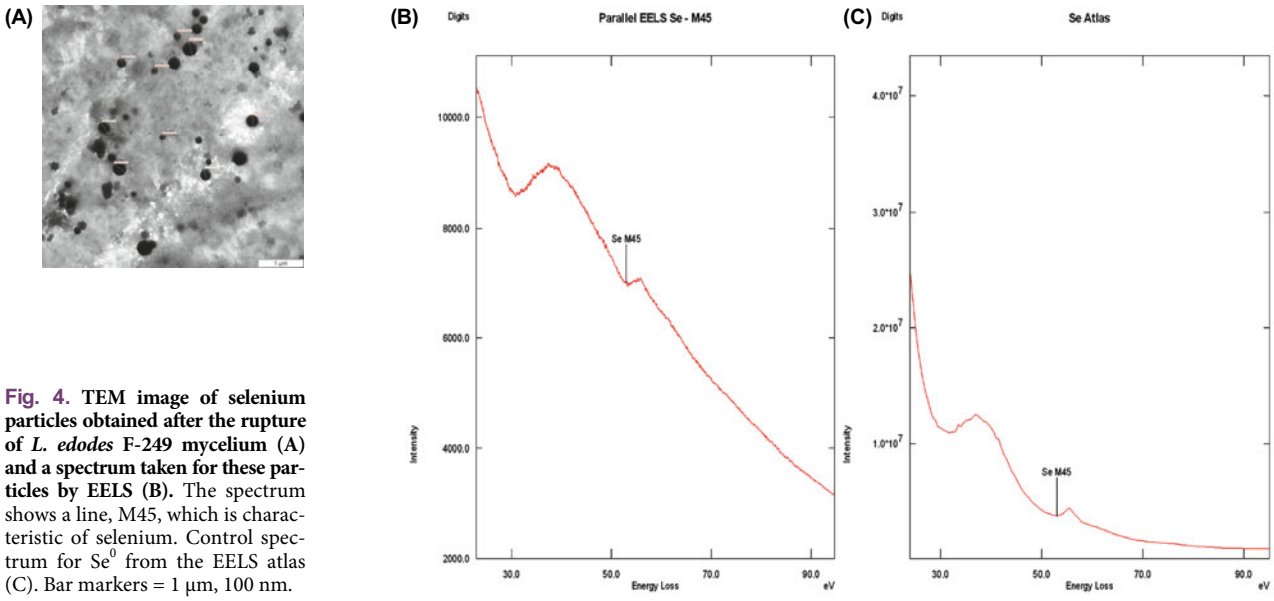

Fig. 4. TEM image of selenium particles obtained after the rupture of *L. edodes* F-249 mycelium (A) and a spectrum taken for these particles by EELS (B). The spectrum shows a line, M45, which is characteristic of selenium. Control spectrum for Se^0 from the EELS atlas (C). Bar markers = 1 μm, 100 nm.

Discussion

Determination of the chemical species of selenium in organisms belonging to various taxonomic groups is a hot area in current research. In the past two decades, much attention has been given to the study of selenium in agronomic crops and in other foodstuffs (Reilly, 2006; Rayman *et al.*, 2008). In several countries, selenium deficiency is relieved by generating agricultural plants and industrial microorganisms with increased contents of this trace element. In this respect, edible fungi hold much promise because they can accumulate high concentrations of selenium. In fungi, selenium is found mostly as selenium-containing amino acids (both free and forming part of the fungal proteins), which are highly bioavailable organic compounds with low toxicity (Falandysz, 2008). However, there are nearly no data on the biotransformation of selenium compounds by basidial fungi with release of Se^0. The literature available to us contains only assumptions as to the ability of (mostly lower) fungi to reduce selenium to Se^0, which are based on the observations that fungal mycelia have acquired a red coloration, indirectly pointing to the presence of reduced selenium. For basidiomycetes, we were able to find only two articles, from a Polish research group, mentioning the red modification of Se^0 on the basis of a mycelial color change (Turło *et al.*, 2007, 2010). However, those articles were addressed to the accumulation of selenium-containing amino acids in the fungal mycelium. The only report that we know to be devoted to the effect of the organoselenium compound DAPS-25 on the growth characteristics of fungal cultures (the basidiomycete *Pleurotus ostreatus* and several mold fungi) and to the biodegradation of DAPS-25 by *Aspergillus niger* to form Se^0 and acetophenone is that of Poluboyarinov *et al.* (2009). No information is currently available on Se^0 nanoparticles obtained from biological reduction of organic or inorganic selenium compounds by the medicinal basidiomycete *L. edodes*. We have not found any photos of these particles, description of their sizes, or evidence that they can accumulate in the interior of fungal mycelia.

It is well known that various microorganisms can synthesize nanoparticles. Up-to-date information on microbial nanoparticle synthesis is available from a recent review by Narayanan and Sakthivel (2010), in which a diversity of microorganisms have been described to produce nanoparticles of gold, silver, selenium, and other elements. However, it has been reported in articles on selenium reduction that not all microorganisms can reduce selenium to nanoparticle form and that most bacteria and fungi synthesize nanoparticles extracellularly.

In this work, we have for the first time obtained information concerning selenium nanoparticles reduced from the organoselenium compound DAPS-25 (diacetophenonyl selenide) and the inorganic selenium compound Na_2SeO_3 by the medicinal basidiomycete *L. edodes* F-249 and concerning the ability of this fungus to synthesize and accumulate nanoparticles intracellularly. The reduction of selenium by the fungus during growth with Na_2SeO_3 and DAPS-25 was studied by X-ray fluorescence, TEM, and EELS. Because X-ray fluorescence is inadequate to identify selenium in its elemental state, we used TEM to take micrographs of the fungal mycelium (including cross sections) showing the accumulation of electron-dense nanoparticles inside the hyphae, and we also used EELS to confirm that these particles were indeed selenium. In our samples, Se^0 was detected by obtaining a characteristic spectrum that proved to be identical to the Se^0 spectrum included in the software database of the Libra 120 microscope. In addition, the selenium distribution map obtained by electron spectroscopic imaging showed that these inclusions were formed by selenium and that reduced selenium was localized inside the cells.

It has been reported in the literature that various microorganisms can reduce selenium both intra- and extracellularly. The reduction of selenites (Se^{IV}), which have a low oxidation–reduction potential, is a fairly easy process; accordingly, possible mechanisms of this phenomenon may include both simple chemical reduction, induced by specific components of the culture medium, and enzymatic microbiological reduction. To rule out the possibility that selenite was reduced by the components of the growth medium, we ran an additional control consisting of no-fungus flasks with synthetic medium of the above composition that had been supplemented with Na_2SeO_3 or DAPS-25 at 0.3 mmol. The flasks were incubated at 26°C for 90 days. During this period, the medium did not change its color, indicating that it did not reduce selenium by itself. To rule out any involvement of fungus-derived excretions into the culture liquid, we ran the following control: The culture liquid used to grow *L. edodes* for 14 days, which contained various products of the fungus's vital functions, was filtered thoroughly to remove the remaining mycelium. After 0.3 mmol of Na_2SeO_3 had been added, the culture liquid was incubated at 26°C for 14 days. Throughout the incubation time, the solution did not change its color, attesting that neither the culture liquid itself nor its components, including extracellular enzymes, were involved in selenium reduction. We conclude, therefore, that it is the fungal hyphae that possess reduction ability. This conclusion has also been confirmed by the results of TEM, showing accumulation of reduced selenium inside the fungal cells in the form of nanospherical particles. This work establishes that biochemical reduction is directly related to the metabolism of the higher basidiomycete and is not dependent on either extracellular synthetic products or chemical reduction induced by components of the culture medium.

It can be said with assurance that the degradation of Na_2SeO_3 and DAPS-25 by *L. edodes* F-249 yields Se^0 as its product. Because the accumulation of Se^0 in the mycelium correlated with increased levels of the selenium-containing compounds in the growth medium, it can be assumed that in *L. edodes*, as in many microorganisms (Garbisu *et al.*, 1996), the degradation of such compounds serves to relieve the toxic effect of high Se concentrations on the fungus.

The discovery of antitumor and antioxidant properties of selenium nanoparticles (Gao *et al.*, 2002; Fernández-Martínez and Charlet, 2009) makes them a promising object of study. Biosynthesis of selenium nanoparticles through the use of the medicinal higher fungus *L. edodes* holds particular promise because of the simplicity, ready accessibility, and environmental safety of this method.

Conclusions

When *L. edodes* is grown in synthetic medium in the presence of Na_2SeO_3 and the organoselenium compound DAPS-25 at initial concentrations above 0.01 mmol, the mycelium acquires a red coloration, with the color intensity and time to visual color detection being dependent on the concentration of the added substance. By X-ray fluorescence, TEM, and EELS, we have recorded the ability of a submerged *L. edodes* culture to degrade the selenium-containing compounds with the formation of Se^0. Red Se^0 is formed in the degradation of DAPS-25 and Na_2SeO_3 (but not Na_2SeO_4) in a submerged culture of *L. edodes*. The electron microscopy data show the presence of 180.51±16.82 nm Se^0 particles inside the fungal hyphae. This is the first time that accumulation of spherical Se^0 nanoparticles inside *L. edodes* (shiitake) cells as a result of transformation of selenium-containing organic and inorganic compounds has been found.

Acknowledgements

This work was supported by a Siberian Health Corporation grant (2012–2013).

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