Bioorganic Chemistry 93 (2019) 102803

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

The possible mechanism of the formation of silver nanoparticles by *Penicillium cyclopium*

Ewelina Wanarska, Irena Maliszewska*

Division of Medicinal Chemistry and Microbiology, Faculty of Chemistry, Wrocław University of Science and Technology, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland

ARTICLE INFO

Keywords:
Biosynthesis
Fungi
Penicillium
Silver nanoparticles
Mechanism

ABSTRACT

This contribution describes the biomineralization of silver nanoparticles by the microbial reduction of Ag (I) ions using the mycelium and the cell-free extract of *Penicillium cyclopium*. Different techniques, such as UV–Vis, SEM, TEM, FT-IR and GPC were used to characterize the obtained nanoparticles and understand the mechanism of their biosynthesis. The SEM and TEM images demonstrated the presence of silver nanoparticles on the mycelia surface suggesting that these particles are synthesized on the fungal cell wall. FT-IR analysis of the mycelium revealed two main types of compounds (saccharides and proteins) and these molecules might be involved in the formation of silver nanoparticles on the surface of mycelium. Ultraviolet–visible spectroscopy and TEM analysis confirmed the formation of silver nanoparticles with different shapes by the cell-free extract of *P. cyclopium*. Their size ranges from 12 to 25 nm and possess an average size of 16 ± 6 nm. GPC analysis of this filtrate revealed a few peaks responsible for polysaccharides and proteins presence. The only protein fraction with the mass approximately to 5000 Da indicated the formation of silver nanoparticles. Polypeptide(s) as the major molecules involved in biomineralization of silver by the cell-free extract of *P. cyclopium* are suggested. Enzymatic synthesis of silver nanoparticles by the mycelium and the cell-free extract of *P. cyclopium* is excluded.

1. Introduction

The term “biomimetics” is defined as the studies on the formation, structure or function of biologically produced materials and biological mechanisms and processes for the purpose of synthesizing similar products by artificial mechanisms that mimic natural ones [1]. Biomimetic synthesis of nanomaterials is important branch of biomimetics and this synthesis is the fastest growing and the most promising field [1]. Living-organism biomimetic synthesis via microorganisms mimics several higher life processes, including substance uptake, transport, stimulus and responses, and biomineralization offering an efficient way for the controlled synthesis of metallic nanoparticles. Among the numerous nanomaterials synthesized by living microbes, silver nanoparticles are the most attractive due to their high electrical and thermal conductivity, surface enhanced Raman scattering, non-linear optical behavior, chemical catalytic and stability [2,3]. These properties make them of potential value in microelectronics, inks and medical imaging [4]. Besides, silver nanoparticles show a broad spectrum of antibacterial and antifungal activity [5,6] that has made them very popular in a diverse range of consumer products, such as plastics, soaps, pastes and textiles [7].

It has been known for long time that fungi, due to their tolerance and bioaccumulation ability of metals, are taking the center-stage of studies on the biological formation of silver nanoparticles. Biosynthesis of silver nanoparticles by fungi is a complex process involving biomolecules as reducing and stabilizing agents. This process involves the reduction of the metal ion as the first step in which Ag(I) is reduced to Ag(0). Afterwards, the particles have to be stabilized so as to maintain their distance, thereby avoiding aggregation, which would result in fusion and finally the formation of bigger particles [8]. This is accomplished by electrostatic repulsion or steric hindrance. Although several factors acting together determine synthesis of nanoparticles, the exact reaction mechanism leading to the formation of silver nanoparticles by fungi is yet to be elucidated. Understanding these process is essential to combine microbial cells with artificial control for efficient biomimetic synthesis of this material.

Generally, in fungi the NADH/NADPH-dependent enzyme is known to be involved in reduction of silver ions. In one of the first studies related to the formation of silver nanoparticles by *Fusarium oxysporum*, Ahmad et al. [9] pointed out a possible participation of NADPH

* Corresponding author at: Division of Medicinal Chemistry and Microbiology (A-2), Faculty of Chemistry, Wrocław University of Science and Technology, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland.
E-mail address: irena.helena.maliszewska@pwr.edu.pl (I. Maliszewska).

https://doi.org/10.1016/j.bioorg.2019.02.028
Received 15 December 2018; Received in revised form 9 February 2019; Accepted 12 February 2019
Available online 22 February 2019
0045-2068/ © 2019 Elsevier Inc. All rights reserved.

dependent reductase in silver ion reduction. Moreover, it seems that this reaction is not specific to *F. oxysporum*, but it is also involved in the reduction of Ag(I) to Ag(0) in the case of the fungus *Fusarium semitectum* under the similar experimental conditions [10]. A band detected at 260 nm attributed to electronic excitations in tryptophan and tyrosine residues in the proteins suggests the release of proteins into solution by *F. semitectum*, indicating a possible mechanism for the reduction of the metal ions present in the colloidal solution mainly due to a coupling of the electron shuttle with the involvement of NADH-dependent reductase [10]. Durán et al. confirmed the presence of nitrate reductase in silver reduction by *F. oxysporum* [11]. The direct evidence for the role of nitrate reductase in the synthesis of silver nanoparticles by moulds was provided by Kumar and co-workers [12], who used the nitrate reductase purified from *F. oxysporum*, phytochelatin, 4-hydroxyquinoline and silver nitrate (as a precursor) for the synthesis of silver nanoparticles. Shaligram et al. [13] and Jain et al. [14] have demonstrated the extracellular synthesis of stable silver nanoparticles using *Penicillium brevicompactum* WA 2315 and *Aspergillus flavus* NJP08, respectively. The reduction may occur by means of the electrons from NADH, where the NADH-dependent reductase can act as a carrier.

The further example of bio-reduction of Ag(I) ions to Ag(0) nanoparticles has been demonstrated by Mukherjee et al. [15]. These authors described the "green synthesis" of highly stabilized silver particles by the biomass of *Verticillium*. It was shown that the exposure of the *Verticillium* biomass to aqueous solution of silver ions resulted in the intracellular reduction of these metal ions and formation of silver nanoparticles of dimensions 25 ± 12 nm. The authors speculated that the silver particles were formed on the cell wall surface, possibly due to reduction of the metal ions by some enzymes present in the cell wall membrane. It is also possible that some amount of silver ions is able to diffuse through the cell wall and are reduced by some enzymes present on the cytoplasmic membrane and in the cytoplasm. An interesting aspect of this study is the mechanism of formation of the silver nanoparticles by a non-pathogenic and agriculturally important fungus *Trichoderma asperellum* [16]. It is most likely that cysteine undergoes dehydrogenation upon the reaction with the mild oxidizing agent $AgNO_3$ to produce silver nanoparticles. The β-carbon in cysteine loses one hydrogen radical and one electron in a concerted or two consecutive steps and reduces Ag(I) ions to Ag(0) nanoparticles, possibly in the presence of an enzyme like NADPH-dependent reductase. Kathiresan et al. found the presence of a prominent protein band of 70 kDa in the culture filtrate of *Penicillium fellutanum* and it was believed that nitrate reductase might have been involved in the reduction of the silver ions to silver nanoparticles [17].

A number of recent publications also show the involvement of the NADH-dependent reductase produced by fungi in the synthesis of silver nanoparticles [18–21].

Quite recently, some authors have shown that the synthesis of silver nanoparticles by fungi does not have to be an enzymatic process. For example, Moteshafi et al. [22] described the silver nanoparticles biosynthesis by *F. oxysporum* and concluded that different mechanism rather than the reduction of metal ions by a NADH-dependent reductase takes place. Metabolic products secreted by the fungus, such as proteins, organic acids and polysaccharides are expected to be involved in silver nanoparticles formation by *Cladosporium cladosporioides* [23]. Similarly, Maliszewska et al. [24] showed that synthesis of silver nanoparticles by the cell-free filtrate of *Penicillium nalgiovense* AJ12I is non-enzymatic process and the proteins containing cysteine in their structure play a significant role in the reducing of silver ions and stabilization of silver nanoparticles. Thus, as shown above the actual biosynthetic pathway of silver nanoparticles by fungi remains a scientific area for further research.

In the present study *Penicillium cyclopium* was used to develop a biomimetic protocol for the formation of silver nanoparticles and a plausible mechanism behind the synthesis of these particles and their subsequent stabilization is discussed.

Fig. 1. The color of *P. cyclopium* mycelium before incubation with the Ag(I) ions (*left*); after incubation with the Ag(I) ions (*right*).

2. Results and discussion

As previously mentioned, the biomineralization of silver nanoparticles by fungi has been observed by many researchers, but the mechanism of this process has remained not fully clarified. This contribution describes the deposition of silver nanoparticles by microbial reduction of Ag(I) ions using the mycelium and cell-free extract of *P. cyclopium*.

2.1. Synthesis of silver nanoparticles by the mycelium of P. cyclopium

In our experiments, the mycelium of *P. cyclopium* was incubated with Ag(I) ions and color change of the biomass from pale white to dark brown suggesting the formation of silver nanoparticles was observed (Fig. 1). The aqueous solution remained colorless, strongly indicating that extracellular reduction of Ag(I) ions had not occurred. Besides, the color of solution without mycelium retained unchanged, pointing that the synthesis of silver nanoparticles was mediated by the mycelium. It is known that silver nanoparticles exhibit a characteristic color which can vary between yellow, pale orange, via reddish brown to brown [25]. These colors arise as a result from interaction of conduction band electrons in the metallic nanoparticles with the electric field vector of the incident light. It is well known that metallic nanoparticles, such as silver, gold and cooper, can absorb visible and infrared light in particular regions owing to localized surface plasmon resonance (LSPR) [26]. In simple terms, LSPR is made up of collective oscillations of free electrons in metal nanoparticles driven by the electromagnetic field of incident light. Moreover, the color of plasmonic nanoparticles can be systematically tuned, owing to changes in the LSPR absorption wavelength, by varying the size and morphology of the particles [27].

To corroborate the arrangement of silver nanoparticles in the mycelium we analyzed the dry biomass by SEM microscopy. The SEM images (Fig. 2) clearly show the formation of silver nanoparticles on the mycelia surface, suggesting that mycelium acts as a template in the formation of metallic particles. The synthesized silver nanoparticles are seen as bright dots due to their electron dense metallic character. It was considered that the white spots are silver nanoparticles, which is consistent with the color of biomass shown in Fig. 1. Moreover, it is clearly visible that an organic matrix is present among the silver nanoparticles. It should be noted that the morphology of the mycelium of *P. cyclopium* has changed after incubation with $AgNO_3$. The surface became more compact compared to the original due to adsorption of metal ions. Probably toxic silver ions present in the environment of the fungus affect the structure of the outer layers of the cell wall. The location of the silver nanoparticles in relation to the *P. cyclopium* cells was examined by transmission electron microscopy (TEM). Fig. 3 shows a single cell of *P. cyclopium* with silver nanoparticles localized on the cell wall. The particles were also seen outside the cell may be due to weakly

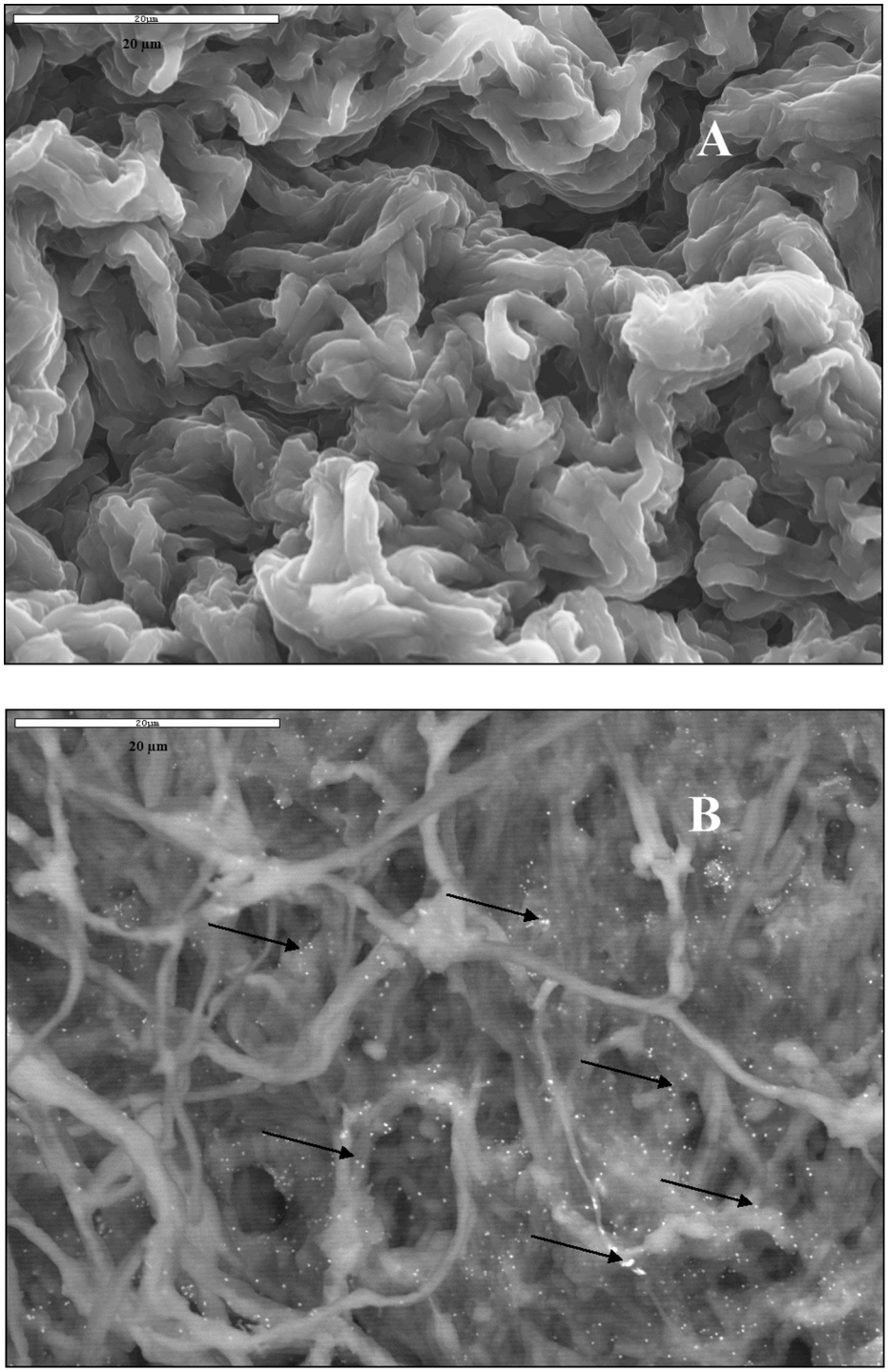

Fig. 2. SEM images of the surface of *P. cyclopium* mycelium before (**A**) and after incubation with Ag(I) ions (**B**); numerous white dots depict silver nanoparticles; some of them are marked with arrows; scale bar 20 µm.

bound silver nanoparticles dislodged from the mycelium during preparation of the sections for TEM studies. Our results clearly indicate that the reduction of the silver ions occurs on the surface of the mycelia leading to the formation of silver nanoparticles. Most of the previous studies relating to binding of metallic ions by fungi indicated that toxic metal ions are primary restricted to cell surface or cell membrane [28,29].

The cell wall is the first cellular site of interaction with metal species. The fungal cell wall is an extremely complex structure mainly compose of polysaccharides, which constitute typically about 80% of the dry weight [30]. Fungal cell walls are structurally unique consisting of glycoproteins and polysaccharides, mainly chitin and glucan [30]. The glycoproteins are extensively modified with both N- and O-linked carbohydrates and many examples show a presence of glycosylphosphatidylinositol (GPI) anchor as well [30]. The glucan component is predominately beta-1,3-glucan, long linear chains of beta-1,3-linked glucose. Glucans having alternate linkages, such as β-1,6-glucan are also found. Chitin containing beta-1,4-linked N-acetylglucosamine residues is typically less abundant [31]. The composition of the cell wall is not constant and may be change depending of a fungal isolate, culture conditions and growth stage. It should be noticed that the cell surface of fungi is negatively charged owing to the presence of various anionic

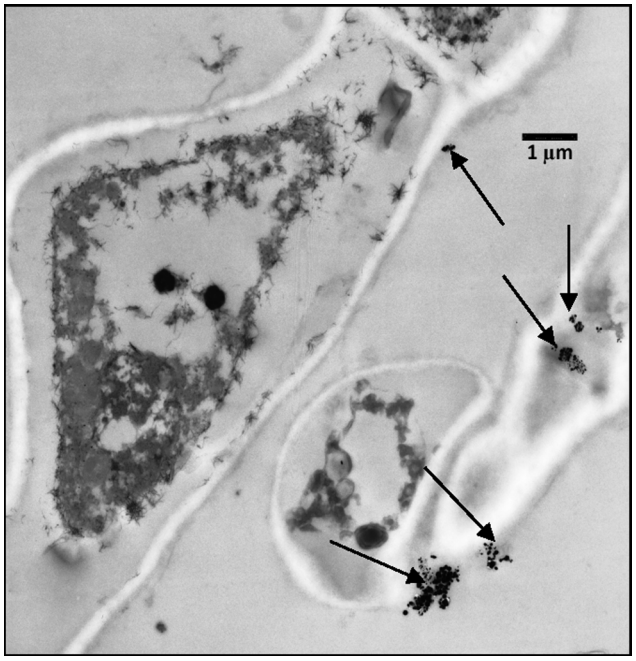

Fig. 3. TEM image of the *P. cyclopium* mycelium after incubation with the Ag(I) ions (the synthesized silver nanoparticles are marked with arrows).

structures, such as glucan and chitin [32]. This gives microorganisms the ability to bind metal cations. Taking into account the complexity of the structure of the fungal cell wall a variety of potential binding sites may be involved in silver ions sequestration, including carboxyl, amine, hydroxyl, phosphate and sulphydryl groups. It should be emphasized that physicochemical factors such as pH and temperature have an influence on the process [33].

To understand the mechanism of interaction of Ag(I) ions with the mycelium of *P. cyclopium*, the formation of silver nanoparticles was repeated at pH between 3.0 and 8.0. Studies on the effect of pH on the formation of silver nanoparticles by the mycelium demonstrated that the process occurs at pH range of 5.0–8.0 (Fig. 1S; Supplementary Information). When the mycelium was incubated with Ag(I) ions at pH 3.0–4.0, the formation of silver nanoparticles was very diminished. Our results are in agreement with some authors. For example, Akthar et al. [34] showed that intensity of silver ion sorption by *A. niger* was not influenced by pH between 5 and 7. This phenomenon is explained by the formation of covalent complexes with N and S containing ligands. Almeida et al. [18] believed that under alkaline conditions, the proteins involved in the synthesis may bind to silver in the thiol regions (−SH) forming an S−Ag bond, assisting the conversion of Ag(I) to Ag(0). In addition, the alkaline ion (−OH) is very much required for the reduction of metal ions.

On the basis of our current research, we believed that the biosorption and bioreduction of Ag(I) ions could occur on the cell wall and it is justified to hypothesize that Ag(I) ions was first trapped on the cell wall through biosorption. Thereafter, some groups on the cell wall reduced Ag(I) to Ag(0) forming Ag(0) nuclei onto the cell wall, which further grew into Ag(0) nanoparticles.

The functional groups on the surface of *P. cyclopium* cell wall, before (A) and after adsorption of Ag(I) ions (B) by FT-IR spectroscopy in order to verify the binding mechanism of silver were determined (Fig. 4). Both the spectra exhibit a broad intense band at ~3400 cm^{-1} assigned to the N−H stretching frequency arising from the peptide linkages. The bands noticed at 2847 cm^{-1} and 2943 cm^{-1} respectively assigned to the symmetric and asymmetric stretching vibration of sp^3 hybridized CH_2 groups. It was found that intensity of bands significantly diminishes in B, indicating a decrease in the concentration of the peptide linkages on the surface of *P. cyclopium* wall. The spectrum of the mycelium before incubation with silver ions (A) exhibit an intense bands at 1638 cm^{-1} and 1540 cm^{-1} corresponding to the amide I and II bands of proteins, respectively. Following reaction with $AgNO_3$ (B) the amide I band at 1638 cm^{-1} shifted to 1619 cm^{-1}, while the band at 1541 cm^{-1} disappeared. The two peaks observed at ~1361 cm^{-1} and ~1024 cm^{-1} can be assigned to the C−N stretching vibrations of aromatic and aliphatic amines respectively [35,36]. The peaks at 1083 cm^{-1} and 1024 cm^{-1} (bands also diminishes in B) correspond to P−OH and P−O−C stretching respectively, indicate the presence of protein phosphate groups. The position of these bands are close to those reported for native proteins [37]. It should be emphasized that detailed interpretation in the fingerprint region (~1300–900 cm^{-1}) is difficult due to extensive overlapping of the amide-III band with in-plane deformational modes of O−H bond (1450–1250 cm^{-1}) as well as C−O vibrations of esters (1330–1050 cm^{-1}) and of aromatic anhydrides (1282–1220 cm^{-1}). It seems that FT-IR analysis of the mycelium confirmed two main types of compounds (saccharides and proteins) present in the analyzed samples. At this stage of research the mechanism of reduction of Ag(I) to Ag(0) by mycelium could not be explained. Considerably more study involving separation of the different compounds present in the cell wall of *P. cyclopium* followed by assaying each component is required before an unequivocal statement on their roles in the nanoparticle synthesis can be made. These efforts are currently in progress. An important guidelines provide scientific data that polysaccharides can be reducing agents as well as capping agents, i.e. neutral polysaccharide like (1 → 6)-β-D-glucan [38], carboxymethylated-curdlan-the polysaccharide composed of (1 → 6)-β-linked carboxymethyl-D-glucose units [39], but also naturally occurring polysaccharides with some charge, like chitosan [40,41] or ulvan [42]. Large amount of hydroxylic groups in the structure of polysaccharides are responsible for capping and stabilization of silver nanoparticles.

Noteworthy is the synthesis of silver nanoparticles using the heat-denatured mycelium of *P. cyclopium*. It was found that the incubation of denatured mycelium with Ag(I) ions caused the change of biomass color from pale yellow to brown after 120 h (Fig. 2S; Supplementary Information). This observation indicates that the non-living cells of *P. cyclopium* are able to synthesize of silver nanoparticles which is consistent with our previous studies [24], but the process demands more time. Prolonged time of silver nanoparticles synthesis by the heat-denatured mycelium of *P. cyclopium* is probably due to the destroy of some binding sites by the high temperature required for inactivation of the mycelium. Several previous results showed that sorption of metal ions by nonviable cells is very efficient [43]. These authors believed that the damaged cells would offer a larger available surface area and expose the intracellular components and more surface binding sites because of the destruction of the cell membranes. On the other hand, the capability of the heat-denatured mycelium to silver nanoparticles formation indicated that native three-dimensional structure of proteins was not essential in this process. So, it was concluded that the formation of silver nanoparticles by mycelium of *P. cyclopium* is non-enzymatic process as the activity of enzymes depends on their structure that is changing during thermal destruction of cells. Our results are not in agreement with the previous assumptions that Ag(I) ions are trapped and reduced by enzymes present on/in the cells of fungi [9–11,12–17].

2.2. Synthesis of silver nanoparticles by the cell-free extract of P. cyclopium

In order to avoid the difficult step of isolation of silver nanoparticles from the mycelium, it is reasonable to use the cell-free extract obtained from mycelium of *P. cyclopium* for formation of these nanostructures. Moreover, the kinetic of silver nanoparticles formation by the cell-free extract is more amenable to analysis, allowing the roles of various biomolecules to be better elucidated. The fungal biomass of *P. cyclopium*, as described above, after incubation with deionized water was separated by filtration and to the obtained cell-free extract aqueous

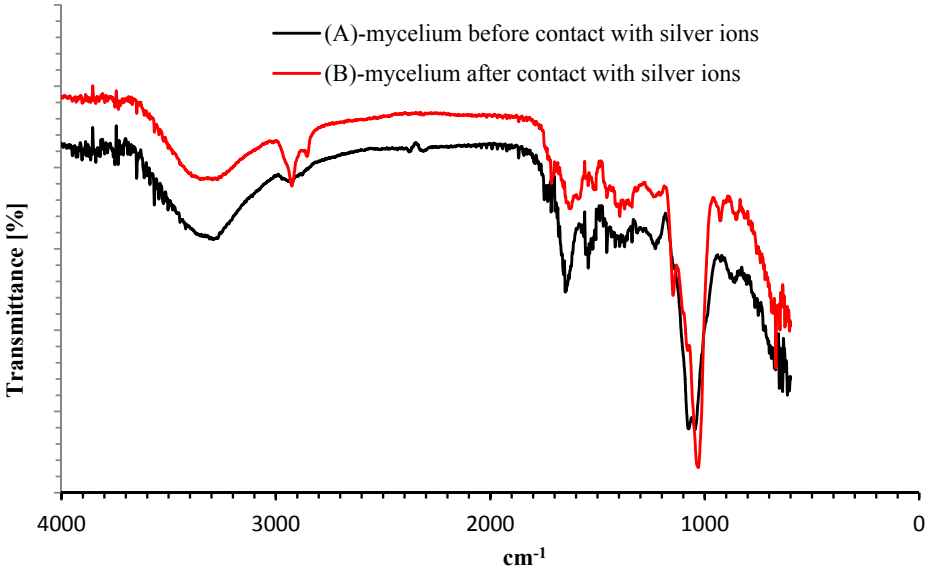

Fig. 4. FT-IR spectra of the surface of *P. cyclopium* mycelium before (**A**) and after incubation with silver ions (**B**).

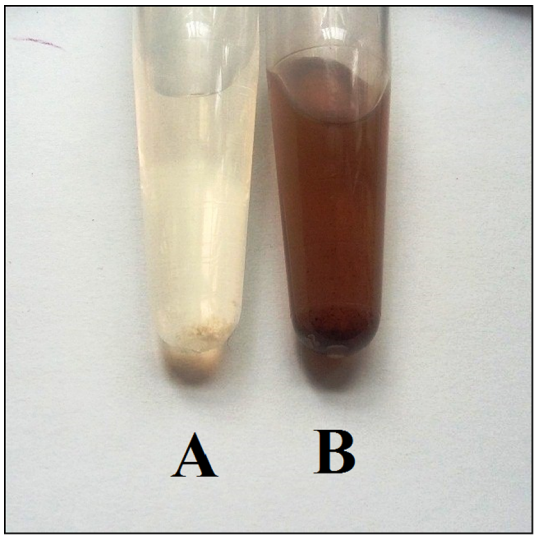

Fig. 5. The cell-free extract of *P. cyclopium* before incubation (**A**) and after incubation (**B**) with the Ag(I) ions.

$AgNO_3$ was added. The incubation of Ag(I) ions with the cell-free extract induced gradual color change of the solution from light yellow to brown, indicating the formation of silver nanoparticles. In contrast, the control without the cell-free extract remained yellow, pointing that the synthesis of silver nanoparticles was mediated by the extract (Fig. 5).

Formation of the colloidal silver nanoparticles was also monitored from their absorption spectra as the small noble metal particles reveal absorption band in the UV–vis spectral region due to localized surface plasmon resonance (LSPR) [27].

Fig. 6A shows the evolution of the optical density at 418–420 nm obtained at different time intervals after mixing the cell-free extract of *P. cyclopium* with the silver ions. This indicates the presence of silver nanoparticles which is due to the excitation of surface plasmon, typical of silver nanoparticles. Fig. 6B represents the plot of absorbance at 420 nm at different time intervals of reaction. As can be seen from this plot, after 72 h of incubation, no further increase in intensity was recorded indicating complete reduction of the silver ions. The kinetics of silver nanoparticles showed that more than 70% of the nanoparticles were formed within the 48 h of the reaction which suggest that the formation of silver nanoparticles is exponential in nature. Relatively

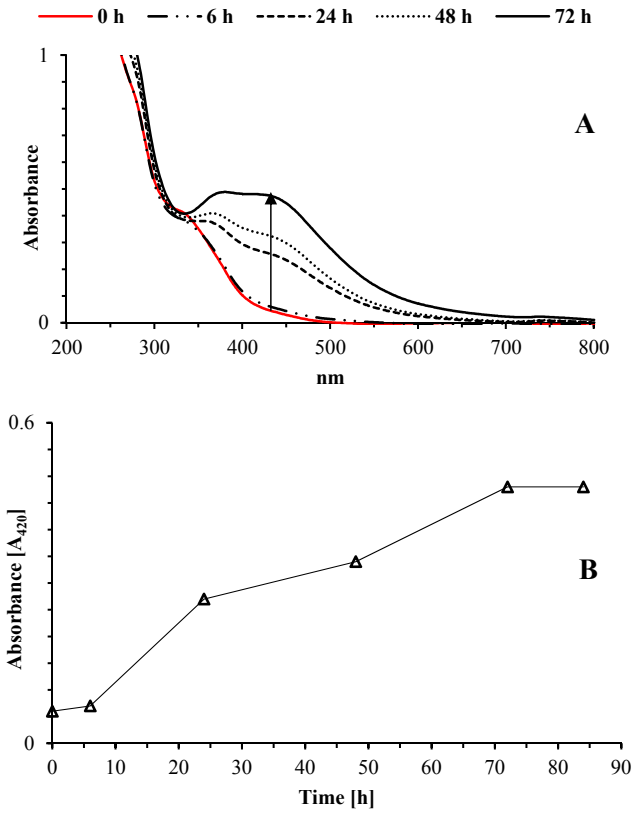

Fig. 6. UV–vis spectra of silver nanoparticles synthesized by the cell-free extract obtained from 4 days old culture of *P. cyclopium* (**A**); Absorbance of silver nanoparticles at 420 nm in different reaction time intervals.

broad SP band of the silver nanoparticles synthesized by the cell-free extract of *P. cyclopium* indicates the broad size distribution which is mainly due to the mixture of molecules present in the extract.

Further characteristic of the silver nanoparticles synthesized by the cell-free extract obtained from *P. cyclopium* was provided by TEM analysis. Fig. 7 shows representative TEM picture recorded from drop-coated films of the silver nanoparticles synthesized by treating silver nitrate solution with the cell-free extract. The silver nanoparticles synthesized by the cell-free extract various shapes, mostly irregular

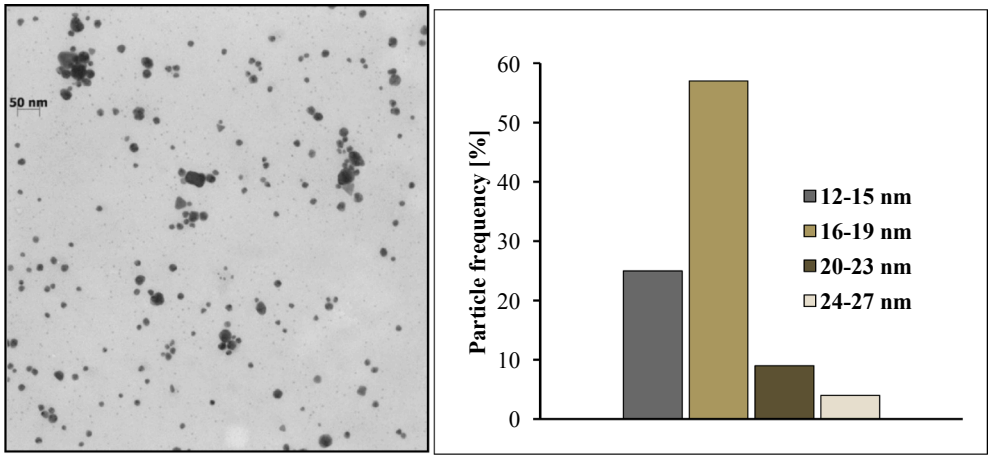

Fig. 7. TEM image of the silver nanoparticles synthesized by the cell-free extract of *P. cyclopium* obtained from 4 days old culture (*left*); the particle size histogram of the silver nanoparticles (*right*).

(Fig. 7). Some large aggregates of particles are created. Their size ranges from 12 to 25 nm and possess an average size of 16 ± 6 nm. The size distribution given by the histogram indicates that almost 60% of the silver particles are in the 16–19 nm sized range (Fig. 7).

2.3. Identification of components in the fungal cell-free filtrate active for silver nanoparticles formation

The reduction of Ag(I) ions and the resulting growth of Ag(0) into nanoparticles must have been driven by some active biomolecules in the fungal cell- free extract. Most of the previous work have identified enzymes as the molecules involved in formation of silver nanoparticles [9–21]. GPC analysis of the cell-free extract of *P. cyclopium* **(PE)** from carbohydrate point of view revealed a few peaks, detected using phenol-sulfuric method [44], as well as few narrow peaks responsible for proteins presence, detected in Lowry assay [45] (Fig. 8). The results suggest the presence of polysaccharides – some homogenous one with molecular mass much bigger than 2 000 000 Da (95 mL volume of the eluate; **PE 1**) and the second much smaller with mass of 10 000 Da (232.5 mL volume) (**PE 6**), and a group of polysaccharides with molecular mass in the range of 1 000 000 – 70 000 Da (120–175 mL volume) (**PE 2**). The presence of proteins was also detected, but as some narrow peaks, at 217.5 mL volume of the eluate – with the molecular mass of 20 000 Da (**PE 5**), and at 245 mL of the eluate – with the mass approximately to 5 000 Da (**PE 7**). Moreover, some peaks of saccharides and proteins with the same elution volume were detected. At 195 mL of the eluate the higher peak of proteins than of carbohydrates was detected. It might suggest the presence of a glycoprotein with molecular mass of 20 000 Da (**PE 3**). In the range of 197.5–215 mL of the eluate the high wide peak of some polysaccharide was detected, probably connected with two smaller peaks of proteins (**PE 4**). This group of peaks might be responsible for some proteoglycan presence. Their average molecular mass was 30 000 Da. Each fraction was then individually tested for reaction with Ag(I) in the aqueous solution. The formation of colloidal silver nanoparticles was evaluated based on visual observation of the solutions turning brown. After 24 h of incubation the color change occurred only in the protein fraction with the mass approximately to 5 000 Da (**PE 7**) indicated the formation of silver nanoparticles (data not shown). The presence of silver nanoparticles was confirmed by UV–vis spectroscopy. The nanoparticles formed by

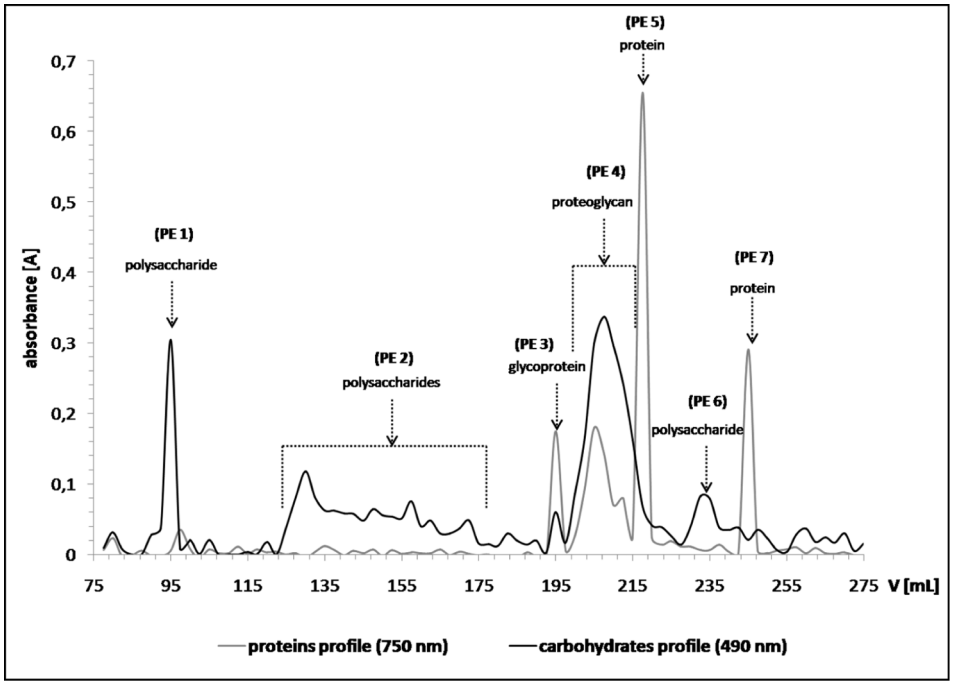

Fig. 8. Gel permeation chromatogram (GPC) of the cell-free extract (**PE**) of *P.cyclopium*. The fractions received in the separation procedure: fraction I (**PE 1**), fraction II (**PE 2**), fraction III (**PE 3**), fraction IV (**PE 4**) and fraction V(**PE 5**). The collected fractions were detected from proteins point of view [45], and using method based on their carbohydrate character, measured by phenol-sulfuric method [44]

the protein fraction (**PE 7**) exhibit an intense peaks at 418–422 nm (data not shown) corresponding to the surface plasmon resonance frequency of nanocrystalline silver particles implying that bioreduction of the silver ions has taken place following incubation of the Ag(I) ions in the presence of the examined fraction PE 7.

Taking into account the results described above and the fact that the molecular weight of NADPH-dependent enzymes (for example nitrate reductase) is higher than 5 000 Da it is believed that formation of silver nanoparticles by the cell-free extract of *P. cyclopium* is non-enzymatic process. These findings lead up to the conclusion that polypeptides might be involved in the formation of silver nanoparticles by the cell-free extract of *P. cyclopium*. An interesting observations have been made by Kumar et al. [46]. These authors found that 2 kDa peptide produced by *Streptomyces clavuligerus* acts as a reducing and stabilizing agent in the formation of gold nanoparticles by this microorganism. Further, this peptide was characterized based on SDA-PAGE and MALDI-TOF-MS analysis in the mass/charge ratio ranging from *m/z* 400–5000. This peptide is comprised of 18 amino acids and the sequence was identified as GCSAAQGQGLCALKLSRL (Gly-Cys-Ser-Ala-Ala-Gln-Gly-Gln-Gly-Leu-Cys-Ala-Leu-Lys-Leu-Ser-Arg-Leu). The homology search with the peptide databases showed that this peptide sequence was not present in the database.

It was previously shown that proteins containing cysteine in their structure might play a significant role in the reducing of silver ions and stabilization of silver nanoparticles [24]. To obtain proof of the participation of free thiol groups in the formation of silver nanoparticles by the cell-free extract, the following set of experiment was carried out. The free thiol groups of polypeptide(s) (PE 7) were modified by reaction with DTNP. The UV–Vis spectrum of the initial mixture consisted of peak at 318 nm corresponding to DTNP is shown in Fig. 9A. After overnight incubation the reaction mixture became pale yellow and the UV–Vis spectrum showed a peak at 387 nm, which is characteristic for

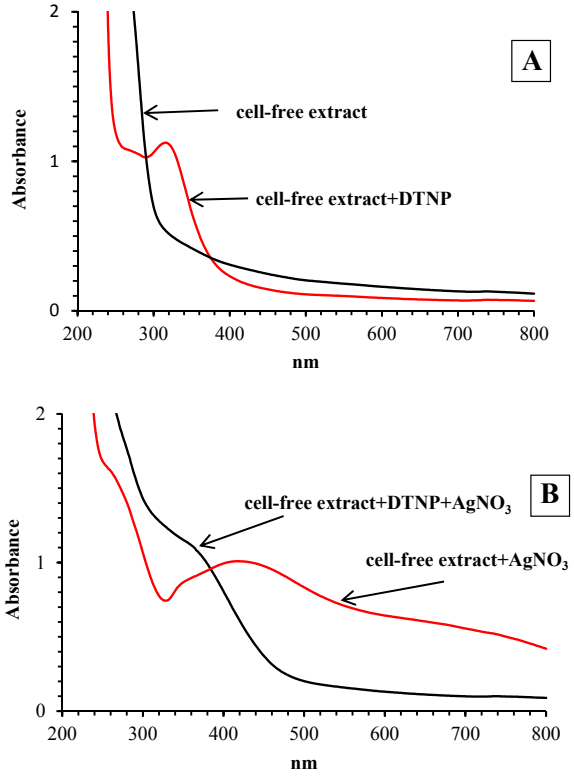

Fig. 9. UV–vis spectra of the cell-free extract of *P. cyclopium* and the cell-extract treated with DTNP after 24 h of incubation (**A**); the cell-free extract after 24 h of incubation with silver ions; the cell-extract treated with DTNP after 24 h of incubation with silver ions.

the product of the reaction of DTNP with thiols (5-nitropyridine-2-thione) and confirmed that the free thiol groups of polypeptides were modified [47,48]. The inhibition of silver nanoparticles synthesis by the modified polypeptides was further evidenced in the corresponding UV–Vis spectra (Fig. 9B). These results may be used as a indirect evidence that free thiol groups of polypeptides are involved in synthesis of silver nanoparticles by the cell-free extract of *P. cyclopium*.

3. Conclusions

This article for the first time shows the ability of *P. cyclopium* towards synthesizing metallic silver nanoparticles. The combination of five techniques: UV–vis, SEM, TEM, GPC and FT-IR allowed to characterize the basic mechanism of silver biomineralization and formulate the following conclusions. Silver nanoparticles can be synthesized by living and death fungal cells, and the formation of these structures can be easily monitored by visual observation of the browning mycelium. FT-IR mycelium analysis confirmed two main types of compounds (saccharides and proteins) present in the cell wall structure. These findings suggest that saccharides and/or proteins are the molecules involved in silver biomineralization by the mycelium of *P. cyclopium*. It is well known that saccharides dominate on the surface of the mycelium, therefore at this stage of research it is believed that these biomolecules are involved in the reduction of silver ions. Considering the complexity of the structure of the cell wall, we are convinced that further studies are needed to clearly state the role of saccharides in the biomineralization of silver nanoparticles.

Moreover, it was shown that silver nanoparticles can also be obtained with cell-free extract of *P. cyclopium*. Formation of colloidal nanoparticles can be monitored by changing the color of the extract and absorption spectra at 418–420 nm. GPC analysis confirmed the presence of saccharides and proteins in the cell-free extract of *P. cyclopium*. It was found that the nanoparticles are formed by a protein fraction of approximately 5000 Da, implying that bioreduction occurred after the incubation of Ag (I) ions in the presence of the polypeptide(s) containing free thiol groups.

These findings allow us to conclude that the different molecules are involved in the biomineralization of silver nanoparticles by the mycelium and cell-free *P. cyclopium* extract, but the enzymatic mechanism is excluded in both cases.

4. Experimental

All chemical agents including silver nitrate ($AgNO_3$) were obtained from (POCH) Poland. A wild strain of *Penicillium cyclopium* was used for the synthesis of silver nanoparticles.

4.1. Synthesis of silver nanoparticles by the mycelium of P. Cyclopium

The basal medium used in our study consisted of (%): KH_2PO_4 0.7; K_2HPO_4 0.2; $MgSO_4{\cdot}7H_2O$ 0.01; NH_4NO_3 1.0; yeast extract 0.06; glucose 1.0. The Erlenmeyer flasks were inoculated with spores (10^5/mL) of *P. cyclopium* and incubated at room temperature with shaking (200 rpm) for 4 days. Then the biomass was filtered (Whatman filter paper No. 1) and extensively washed with distilled water to remove any medium component. The clean mycelium (10 g) was taken into the Erlenmeyer flask, containing 100 mL of Milli-Q deionised water (UV Ultrapure Water System, Burnstead, USA). The silver nitrate (1 mM of final concentration) was added to the Erlenmeyer flask and agitated at 25 °C for 24 h (in dark). The control (without the mycelium) was also run along with the experimental flasks. To monitor the formation of silver nanoparticles the colour of biomass was observed. The silver nanoparticles synthesis was also performed with the denatured mycelium of *P. cyclopium*. Denaturation was achieved by heating the mycelium at 95 °C for 30 min. The mycelium without heat treatment was used as a control.

4.1.1. SEM analysis

For SEM study, the 4-days mycelium before and after incubation with Ag(I) ions was fixed in 2.5% glutaraldehyde for 24 h at 4 °C. The samples were centrifuged, the fixative was removed and the samples were rinsed three times with 0.1 M sodium phosphate at pH 7.0. The material was then dehydrated in graded ethanol series (from 50% to 100%). After dehydration, the excess fluid was removed, the samples were air-dried and coated with carbon. The surface topography of the samples was evaluated by scanning electron microscopy (Quanta 250, FEI).

4.1.2. TEM analysis

For TEM studies the mycelium was fixed with 2.5% (w/v) glutaraldehyde solution and post fixed in aqueous osmium tetroxide. The sample was then dehydrated in a graded series of ethanol, block stained in uranyl acetate, and embedded in Epon. Ultrathin sections (100 nm) were contrasted with lead citrate and imaged by TEM (Zeiss EM 900).

4.1.3. FT-IR analysis

The mycelium of *P. cyclopium* (before and after incubation with Ag (I) ions) was harvested by centrifugation for 5 min at 410 g at ambient temperature. The pellets were washed twice with distilled water and the centrifugation was repeated. The supernatant was discarded and the mycelia pellets were re-suspended in approximately 200 µL of 0.9% NaCl and were placed on ZnSe-crystals and dried for 30 min at 40 ± 0.5 °C in a cabinet dryer. Bruker Vertex 80v Spectrometer was used in this study.

4.2. Synthesis of silver nanoparticles in the cell-free extract

The procedure of silver nanoparticles synthesis by the cell-free extract from *P. cyclopium* was similar to that described in [24] with minor modifications. In detail, the 4-day biomass of *P. cyclopium* were filtered (Whatman filter paper No. 1) and extensively washed with distilled water to remove any medium component. The clean mycelium (10 g) was taken into the Erlenmeyer flask, containing 100 mL of Milli-Q deionised water (UV Ultrapure Water System, Burnstead, USA). The flasks was agitated at room temperature with shaking (120 rpm) for 72 h. The cell-free extract was collected by pre-filtration in Whatman No. 1 filter papers and filtered using Millex-GP filter (PES membrane, 0.22 µm). Then the silver nitrate (1 mM of final concentration) was mixed with the cell-free filtrate in an Erlenmeyer flask and agitated at room temperature in dark. The control (without the cell-free filtrate) was also run along with the experimental flasks.

4.2.1. UV–vis spectroscopy

To verify reduction of silver ions the solutions were scanned in the range of 200–800 nm in a spectrophotometer (Shimadzu, UV 3600, Japan).

4.2.2. TEM analysis

The size and morphology of the silver nanoparticles were analyzed with the transmission electron microscope TEM (Zeiss EM 900). The sample was prepared by placing a drop of the gold nanoparticles on a carbon-coated copper grid and subsequently drying in air before transferring it to the microscope. From electron micrographs the particle size was found for no less than 150 particles.

4.2.3. GPC analysis

The molecular mass estimation and the degree of heterogeneity of the cell-free extract (PE) from *P. cyclopium* were performed with gel permeation chromatography (GPC), using a chromatographic column (15 × 1000 mm) packed with Sepacryl 400HR, eluted with deionized water, with elution rate of 0.3 mL/min. A set of 5 dextran standards with molecular mass of 10 000, 40 000, 70 000, 500 000 and 2 000 000 Da was used for the column calibration. The solution of (PE) (50 mg) diluted in 0.5 mL of deionized water was applied on the top of the column, and was eluted through the resin with deionized water, with flow rate of 0.3 mL/min. The fractions collected using fraction collector Gilson C 203B were analyzed from carbohydrates point of view in phenol–sulfuric method described by Dubois et al. [44] at the wavelength of 490 nm. The spectrophotometer was auto-zeroed on the color of each fraction, to reduce the interference of yellowish color of the fractions on the measurements. In every fraction proteins presence using Lowry assay [45] was detected, where the reaction products were measured at the wavelength of 750 nm. All colorimetric assays were performed using Cecil CE 2021 spectrophotometer. The collected data were used to prepare the saccharide profile as well as the protein profile as the chromatograms.

4.2.4. Modifications of free cysteine thiol groups

In order to modify the free cysteine thiol groups of the cell-free extract of P. cyclopium, 400 µL of 6.0 mM 2,2′-dithiobis(5-nitropyridine) (DTNP) in dimethyl sulfoxide (DMSO) was added to 5 mL of the cell-free filtrate and the reaction mixture was incubated overnight. Then, the UV–Vis spectrum of the sample was measured to establish modification of the free cysteine thiol groups. The solution was then treated with silver ions and kept for 24 h at room temperature. A flask containing the cell-free extract and silver ions only in the presence of DMSO was used as control.

Conflict of interest

The authors declares no competing financial interest.

Acknowledgments

We would like to thank dr Izabela Pawlaczyk-Graja (Department of Organic and Pharmaceutical Technology, Wrocław University of Science and Technology) for GPC analysis and helpful consultation on manuscript.

This work was financed by a statutory activity subsidy from the Polish Ministry of Science and Higher Education (PMSHE) for the Faculty of Chemistry of Wrocław University of Science and Technology.

References

[1] G. Zan, Q. Wu, Biomimetic and bioinspired synthesis of nanomaterials/nanostructures, Adv. Mater. (2016), https://doi.org/10.1002/adma.201503215.
[2] Y. Ju-Nam, J.R. Lead, Manufactured nanoparticles: an overview of their chemistry, interactions and potential environmental implications, Sci. Total Environ. 400 (2008) 396–414.
[3] C.M. Niemeyer, Nanoparticles, proteins, and nucleic acids: biotechnology meets materials science, Angew. Chem. Int. Ed. 22 (2001) 4128–4158.
[4] Y.A. Krutyakov, A.A. Kudrynskiy, A.Y. Olenin, G.V. Lisichkin, Synthesis and properties of silver nanoparticles: advances and prospects, Russ. Chem. Rev. 77 (2008) 233–257.
[5] Q.L. Feng, J. Wu, G.Q. Chen, F.Z. Cui, T.N. Kim, J.O. Kim, A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*, J. Biomed. Mat. Res. Part A 4 (2000) 662–668.
[6] C. Baker, A. Pradhan, L. Pakstis, D.J. Pochan, S.I. Shah, Synthesis and antibacterial properties of silver nanoparticles, J. Nanosci. Nanotechnol. 5 (2005) 244–249.
[7] P. Dallas, V.K. Sharma, R. Zboril, Silver polymeric nanocomposites as advanced antimicrobial agents: classification, synthetic paths, applications, and perspectives, Adv. Coll. Inter. Sci. 1–2 (2011) 119–135.
[8] D.M. Ledwith, A.M. Whelan, J.M. Kelly, A rapid, straight-forward method for controlling the morphology of stable silver nanoparticles, J. Mater. Chem. 17 (2007) 2459–2464.
[9] A. Ahmad, P. Mukherjee, S. Senapati, D. Mandal, M.I. Khan, R. Kumar, M. Sastry, Extracellular biosynthesis of silver nanoparticles using the fungus *Fusarium oxysporum*, Coll. Surf. B: Biointerf. 28 (2003) 313–318.
[10] S. Basavaraja, S.D. Balaji, A. Lagashetty, A.H. Rajasab, A. Venkataraman, Extracellular biosynthesis of silver nanoparticles using the fungus *Fusarium semitectum*, Mat. Res. Bull. 43 (2008) 1164–1170.
[11] N. Durán, P.D. Marcato, O.L. Alves, G.I.H. De Souza, E. Esposito, Mechanistic aspects of biosynthesis of silver nanoparticles by several *Fusarium oxysporum* strains, J. Nanobiotechnol. 3 (2005) 8–14.
[12] S.A. Kumar, M.K. Abyaneh, S.W. Gosavi, S.K. Kulkarni, R. Pasricha, A. Ahmad, M.I. Khan, Nitrate reductase-mediated synthesis of silver nanoparticles from

AgNO3, Biotechnol. Lett. 29 (2007) 439–445.
[13] N.S. Shaligram, M. Bule, R. Bhambure, R.S. Singhal, S.K. Singh, G. Szakac, A. Pandey, Biosynthesis of silver nanoparticles using aqueous extract from the compactin producing fungal strain, Process Biochem. 44 (2009) 939–943.
[14] N. Jain, A. Bhargava, S. Majumdar, J.C. Tarafdar, J. Panwar, Extracellular biosynthesis and characterization of silver nanoparticles using *Aspergillus flavus* NJP08: A mechanism perspective, Nanoscale 3 (2011) 635–641.
[15] P. Mukherjee, A. Ahmad, D. Mandal, S. Senapati, S.R. Sainkar, M.I. Khan, R. Ramani, R. Parischa, P.V. Ajayakumar, M. Alam, M. Sastry, R. Kumar, Bioreduction of AuCl4-ions by the fungus, Verticillium sp. and surface trapping of the gold nanoparticles formed, Angew. Chem. Int. Ed. Engl. 40 (2001) 3585–3588.
[16] P. Mukherjee, M. Roy, B.P. Mandal, G.K. Dey, P.K. Mukherjee, J. Ghatak, S.P.T. Kale, Green synthesis of highly stabilized nanocrystalline silver particles by a non-pathogenic and agriculturally important fungus *T. asperellum*, Nanotechnology 19 (2008) 075103.
[17] K. Kathiresan, S. Manivannan, M.A. Nabeel, B. Dhivya, Studies on silver nanoparticles synthesized by a marine fungus, *Penicillium fellutanum* isolated from coastal mangrove sedyment, Coll. Surf. B: Biointerf. 71 (2009) 133–137.
[18] E.S. Almeida, D. Oliveira, D. Hotza, Characterization of silver nanoparticles produced by biosynthesis mediated by *Fusarium oxysporum* under different processing conditions, Bioprocess Biosyst. Eng. 40 (2017) 1291–1303.
[19] S. Neethu, S.J. Midhun, E.K. Radhakrishnan, M. Jyothis, Green synthesized silver nanoparticles by marine endophytic fungus *Penicillium polonicum* and its antibacterial efficacy against biofilm forming, multidrug-resistant *Acinetobacter baumanii*, Microb. Pathog. 116 (2018) 263–272.
[20] B.K. Nayak, A. Nanda, V. Prabhakar, Biogenic synthesis of silver nanoparticle from wasp nest soil fungus, *Penicillium italicum* and its analysis against multi drug resistance pathogens, Biocatal. Agric. Biotechnol. 16 (2018) 412–418.
[21] M. Saravanan, S. Arokiyaraj, T. Lakshmi, A. Pugazhendhi, Synthesis of silver nanoparticles from *Phenerochaete chrysosporium* (MTCC-787) and their antibacterial activity against human pathogenic bacteria, Microb. Pathog. 117 (2018) 68–72.
[22] H. Moteshafi, S.M. Mousavi, S.A. Shojaosadati, The possible mechanisms involved in nanoparticles biosynthesis, J. Ind. Eng. Chem. 18 (2012) 2046–2050.
[23] D.S. Balaji, S. Basavaraja, R. Deshpande, D.B. Mahesh, B.K. Prabhakar, A. Venkataramanb, Extracellular biosynthesis of functionalized silver nanoparticles by strains of *Cladosporium cladosporioides* fungus, Coll. Surf. B: Biointerf. 68 (2009) 88–92.
[24] I. Maliszewska, A. Juraszek, K. Bielska, Green synthesis and characterization of silver nanoparticles using *Ascomycota* fungi *Penicillium nalgiovense* AJ12, J. Clust. Sci. 25 (2014) 989–1004.
[25] T. Huang, X.H.N. Xu, Synthesis and characterization of tunable rainbow colored colloidal silver nanoparticles using single-nanoparticle plasmonic microscopy and spectroscopy, J. Mat. Chem. 20 (2010) 9867–9876.
[26] J.P. Kottmann, O.J.F. Martin, D.R. Smith, S. Schultz, Plasmon resonances of silver nanowires with a nonregular cross section, Phys. Rev. B 64 (2001) 235402-1–235402-10.
[27] M.Á. Gracia-Pinilla, E. Pérez-Tijerina, J.A. García, C. Fernández-Navarro, A. Tlahuice-Flores, S. Mejía-Rosales, J.M. Montejano-Carrizales, M. José-Yacamán, On the structure and properties of silver nanoparticles, J. Phys. Chem. C 112 (2008) 13492–13498.
[28] S. Siddiquee, K. Rovina, S. Al Azad, L. Naher, S. Suryani, P. Chaikaew, Heavy metal contaminants removal from wastewater using the potential filamentous fungi biomass: a review, J. Microb. Biochem. Technol. 7 (2015) 6, https://doi.org/10.4172/1948-5948.1000243.
[29] P. Khandel, S.K. Shahi, Mycogenic nanoparticles and their bio–prospective applications: current status and future challenges, J. Nanostruct. Chem. 8 (2018) 369–391.
[30] P.H. Fesel, A. Zuccaro, Beta-glucan: crucial component of the fungal cell wall and elusive MAMP in plants, Fungal Genet. Biol. 90 (2016) 53–60.
[31] S.M. Bowman, S.J. Free, The structure and synthesis of the fungal cell wall, Bioessays 28 (2006) 799–808.
[32] J.P. Latge, The cell wall: a carbohydrate armour for the fungal cell, Mol. Microbiol. 66 (2007) 279–290.
[33] C. Mack, B. Wilhelmi, J.R. Duncan, J.E. Burgess, Biosorption of precious metals, Biotechnol. Adv. 25 (2007) 264–271.
[34] N. Akthar, S.K. Sastry, N.P. Mohan, Biosorption of silver ions by processed *Aspergillus niger* biomass, Biot. Lett. 17 (1995) 551–556.
[35] R. Sanghi, P. Verma, S. Puri, Enzymatic Formation of gold nanoparticles using *Phanerochaete chrysosporium*, Adv. Chem. Eng. Sci. 1 (2011) 154–162.
[36] B.A. Omran, H.N. Nassar, N.A. Fatthallah, A. Hamdy, E.H. El-Shatoury, N.S. El-Gendy, Characterization and antimicrobial activity of silver nanoparticles mycosynthesized by *Aspergillus brasiliensis*, J. App. Microb. 125 (2018) 370–382.
[37] A. Gole, C.V. Dash, V. Ramachandran, A.B. Mandale, R. Sainkar, M. Rao, M. Sastry, Pepsin-gold colloid conjugates: preparation, characterization and enzymatic activity, Langmuir 17 (2001) 1674–1677.
[38] H. Huang, X. Yang, Synthesis of polysaccharide-stabilized gold and silver nanoparticles: a green method, Carbohydr. Res. 339 (2004) 2627–2631.
[39] T.C.-Y. Leung, C.K. Wong, Y. Xie, Green synthesis of silver nanoparticles using biopolymers, carboxymethylated-curdlan and fucoidan, Mat. Chem. Phys. 121 (2010) 402–405.
[40] H. Huang, Q. Yuan, X. Yang, Preparation and characterization of metal-chitosan nanocomposites, Coll. Surf. B 39 (2004) 31–37.
[41] R. Kalaivani, M. Maruthupandy, T. Muneeswaran, A. Hameedha Beevi, M. Anand, C.M. Ramakritinan, A.K. Kumaraguru, Synthesis of chitosan mediated silver nanoparticles (AgNPs) for potential antimicrobial applications, Front. Labor. Med. 2 (2018) 30–35.
[42] A. Massironi, L. Morelli, D. Grassi, S. Puppi, G.A. Braccini, S. Maisetta, G. Esin, C. Batoni, P.F. Della, Chiellini, Ulvan as novel reducing and stabilizing agent from renewable algal biomass: application to green synthesis of silver nanoparticles, Carbohydr. Polym. 203 (2019) 310–321.
[43] S. Rangabhashiyam, E. Suganya, N. Selvaraju, L.A. Varghese, Significance of exploiting non-living biomaterials for the biosorption of wastewater pollutants, World J. Microbiol. Biotechnol. 30 (2014) 1669–1689.
[44] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Biochem. 28 (1956) 350–355.
[45] O. Lowry, N. Rosebrough, L. Farr, R. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[46] C.G. Kumar, Y. Poornachandra, Ch. Chandrasekhara, Green synthesis of bacterial mediated anti-proliferative gold nanoparticles: inducing mitotic arrest (G2/M phase) and apoptosis (intrinsic pathway), Nanoscale 7 (2015) 18738.
[47] A. Rangnekar, T.K. Sarma, A.K. Singh, J. Dek, A. Ramesh, A. Chattopadhyay, Retention of enzymatic activity of α-amylase in the reductive synthesis of gold nanoparticles, Langmuir 23 (2007) 5700–5706.
[48] P. Sharma, S. Sathyanarayana, P. Kumar, K.C. Gupta, A spectrophotometric method for the estimation of polymer-supported sulfhydryl groups, Anal. Biochem. 189 (1990) 173–177.