Biosynthesis of silver nanoparticles by cell-free extract from *Trichoderma reesei* - study on the influence of growth media

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**Abstract.** *Trichoderma reesei* is a non-pathogenic microorganism able to reduce the toxic for the microorganism silver ions (Ag\(^+\)) to non-toxic silver nanoparticles (AgNPs). The effect of components of the nutrient medium on which the biomass from *T. reesei* was obtained on the biosynthesis of AgNPs from Ag\(^+\) was monitored. Five media were studied. The main medium (1) contained glucose-2\%, NH\(_4\)Cl-0.1\%, Co(NH\(_2\))\(_2\)-0.3\%, (NH\(_4\))\(_2\)SO\(_4\)-0.1\%, KH\(_2\)PO\(_4\)-0.2\%, (NH\(_4\))\(_2\)SO\(_4\)-0.14\%, MgSO\(_4\).7H\(_2\)O-0.03\%, CaCl\(_2\).2H\(_2\)O-0.04\%. The other were medium 1+0.1% yeast extract (2), medium 1+0.1% corn steep liquor (3), medium 1+0.1% peptone (4), medium 1+0.1% casamino acids (5). After culturing the strain, the resulting biomass was subjected to aqueous extraction, and the cell-free extract - CFE (containing biologically active metabolites) was used for extracellular biotransformation (performed with 10\% extracted biomass and 10 mM AgNO\(_3\)) of Ag\(^+\) to AgNPs. The AgNPs formation was monitored by UV-Vis. The isolated nanoparticles were characterized by TEM, ICP-AES and FTIR-ATR. The slowest reduction rate of Ag\(^+\) was obtained with the CFE from biomass grown in medium 1 and the highest - with CFE from biomass grown in medium 3. Nearly mono disperse spherical AgNPs (2-6 nm) were synthesized with the aid of CFE of fungi cultivated in media 1 and 3. The AgNPs obtained with CFE from the other media were 4-11 nm.

1. Introduction

The use of silver nanoparticles (AgNPs) is considered a promising approach for solving different medical [1, 2] environmental [3] and agricultural [4, 5] problems.

AgNPs can be synthesized by chemical route, employing reagents to reduce the silver (Ag\(^+\)) ions and stabilize the nanoparticles formed. Production and use of these reagents can cause health and environmental risks. AgNPs can be produced by physical methods but usually this is linked with high energy consumption and related environmental problems. The mentioned issues have led to enhanced interest in biogenic synthesis (biosynthesis) methods. Biosynthesis of AgNPs can be carried out by using plants extracts, microorganisms (bacteria, fungi) or their metabolites, which operate as reducing agents and AgNPs stabilizing reagents. These methods are relatively simple and clean and can be sustainable and economical if optimal conditions are found for microorganisms’ growth. Fungi, with their high tolerance to metals, good biomass production, easy handling and suitability for large-scale syntheses represent very attractive agents for biosynthesis of AgNPs [6]. Moreover, according to some studies, by optimizing conditions for fungi cultivation it is possible to influence the metabolism of fungi thus to vary the properties of the fungi themselves or of their metabolites used as reducing and capping reagents – with the final result - production of AgNPs with desired characteristics [7]. Biosynthesis of AgNPs by fungi can be realized by intracellular or extracellular mechanism. In the former the metal precursor is
added to the mycelia culture and the nanoparticles are formed inside the fungal biomass. As a result, extraction of the nanoparticles is obligatory after the synthesis, applying centrifugation, filtration and chemical treatment to disrupt the biomass cells and release the nanoparticles. In the extracellular synthesis, the precursor Ag\(^+\) ions are added to the aqueous cell-free extract (CFE) containing only the bio-molecules released from the carefully washed fungal biomass. This method is widely used. Some authors apply the term extracellular synthesis for nanoparticles production by using only filtered media where the fungi were cultivated\([7, 8]\). However, in this case strict difference has to be made between the action of the bio-molecules released by the fungal biomass and the action of the cultivation medium components.

In order to attain mono-disperse and stable AgNPs the parameters of the synthesis process have to be optimized. The following parameters have been widely studied in the literature: (i) during the fungi cultivation - fungus type, temperature, agitation, and time, and (ii) during the nanoparticles synthesis process - temperature, agitation, time, light availability, biomass to liquid mass ratio, concentration of the metal precursor and pH\([2, 6, 9, 10]\). Influence of the cultivation media composition on the AgNPs production was less studied and mainly – in the case when in the extracellular synthesis the filtered media where the fungi were cultivated was used\([7 - 10]\). Such studies have been carried out for different microorganisms, such as\[ Fusarium oxysporum\][9],\[ Sclerotinia sclerotiorum\] MTCC 8785\[2\],\[ Klebsiella pneumoniae\],\[ E. coli\] top 10 strain\[8\],\[ Escherichia coli\] and\[ Pseudomonas jessini\][7, 10]. The findings of different authors imply that the media for microorganisms’ growth play an important role in the synthesis of metallic nanoparticles in terms of yield and nanoparticles size and shape. It is expected that the impact of microorganisms’ cultivation medium will be more pronounced in the case of extracellular synthesis when the precursor Ag\(^+\) ions are added to the aqueous CFE containing only the bio-molecules released by the carefully washed fungal biomass. It is also known that the biological synthesis of AgNPs is a reaction catalyzed by the biomolecules\[9\]. It is recognized that in different culture media compositions and under different circumstances microbial cells secrete diverse metabolites and specific kinds of proteins. That is why it can be anticipated that the chemical nature and concentration of those bio-molecules will depend on the cultivation medium composition.

Seventy percent of fungi which have been investigated for their use in AgNPs biosynthesis are pathogenic for plant, human or animals\[11\]. Where these fungi are used, measures and the corresponding costs are needed to deal with their eventual negative effects on the environment and humans. In order to produce nanoparticles on large scale, at preserving the environment, non-pathogenic fungi are needed. The fungus \[Trichoderma reesei\](T. reesei) is a non-pathogenic, environmentally friendly microorganism, able to produce a variety of biologically active substances, including extracellular metabolites at high scale\[12\]. \[T. reesei\] for its own survival secrets extracellular reductase enzymes which are able to reduce toxic Ag\(^+\) ions to nontoxic AgNPs\[11\]. The fungus is well-known for its high growth rate, easy management in large-scale production and inexpensive production procedures. Different authors used different media to cultivate \[Trichoderma\] species with the aim to use the bio-molecules released by the fungal biomass in water as reducing and capping reagents. Some examples are given in table 1. Studies on \[Trichoderma\]-based synthesis of AgNPs are still incomplete\[13\]. Especially this holds true for the studies on the impact of fungi growth medium on the biosynthesis of AgNPs. On the other hand, the feasibility of AgNPs biosynthesis depends on the successful production of the fungal metabolites in large scale\[5\].

As it can be seen in the table 1, glucose is most often used carbon source, yeast extract, peptone and corn steep liquor are often used as additives to the “classical” medium based on inorganic salts. In the pioneering work\[17\] on the application of \[T. reesei\] for synthesis of AgNPs by using the fungus mycelium the fungus was grown in medium composed of 0.5 % glucose and 0.4 % casein hydrolysate. However, no data are presented in that paper on the AgNPs synthesis by use of CFE.

In the study presented here, an extracellular biosynthesis of AgNPs from AgNO\(_3\) solution by using the CFE of \[T. reesei\] biomass has been performed. The generalized effect of components of the nutrient medium on the \[T. reesei\] metabolite profile, that influences the biosynthesis of AgNPs from Ag\(^+\), was monitored. According to our knowledge, until now such study is not available.
Table 1. Media used to cultivate *Trichoderma* species.

| Fungus       | Medium                                           | Ref   |
|--------------|--------------------------------------------------|-------|
| *T. inhamatum* | Liquid broth containing 0.3% w/v malt extract, 1.0% w/v glucose, 0.3% w/v, yeast extract and 0.5% w/v peptone | [14]  |
| *T. harzianum* | Liquid medium containing malt extract (0.3%), yeast extract (0.3%), glucose (1.5%) and peptone (0.5%). |       |
| *T. viride*    | Liquid broth containing (g/L) KH$_2$PO$_4$ – 7; K$_2$HPO$_4$ – 2; MgSO$_4$·7H$_2$O – 0.1; (NH$_4$)$_2$SO$_4$ – 1; yeast extract – 0.6; glucose – 10. | [15]  |
| *T. atroviride*| Liquid broth containing, (g/L) KH$_3$PO$_4$ (7), K$_2$HPO$_4$ (2), MgSO$_4$·7H$_2$O (0.1), (NH$_4$)$_2$SO$_4$ (1); yeast extract (0.6), glucose (10). | [13]  |
| *T. harzianum* | Liquid broth containing, in g/L: 9.0 Oat, 5.0 yeast extract, 1.0 NaNO$_3$, 1.0 KH$_2$PO$_4$, 1.0 peptone, 0.3 MgSO$_4$·7H$_2$O, pH 5.5, 4.2% (w/v) (NH$_4$)$_2$SO$_4$, 2% (w/v) glucose | [5]   |

*According to the authors this medium gave the best results (out of 6 studied media) both in terms of biomass growth and AgNPs yield*

2. Materials and methods

The fungal strain *T. reesei* PF, used in the present study, was received from the collection of the Biotechnology Department, Faculty of Biology, Sofia University "St. Kliment Ohridski". Studies were carried out with seven days old culture of *T. reesei* cultivated on potato-dextrose agar at 29±1°C.

To study the influence of growth medium on the AgNPs synthesis, the fungal biomass was harvested after inoculating the strain in 100 mL of five different growth media. The main medium contained inorganic salts and glucose as carbon source. It is referred to as medium 1, with the following composition: glucose – 2 %, NH$_4$Cl 0.1 %, CO(NH$_2$)$_2$ - 0.3 %, (NH$_4$)$_2$SO$_4$ – 0.1 %, KH$_2$PO$_4$ - 0.2 %, (NH$_4$)$_2$SO$_4$ - 0.14 %, MgSO$_4$·7H$_2$O - 0.03 %, CaCl$_2$ 2H$_2$O - 0.04 %. The other media were medium 1 to which different additives were added. Medium 2 was 1 + 0.1% yeast extract, medium 3 was medium 1 + 0.1% corn steep liquor, medium 4 was medium 1 + 0.1% peptone, and medium 5 was medium 1 + 0.1% casamino acids. All reagents used were p.a., purchased from Sigma Aldrich. Sterile distilled water was used as diluent, extractant and for fungi washing. The fungal culture was incubated in 500 cm$^3$ flasks for 72 h at 29±1 °C and under continuous shaking at 220 rpm. Then the fungus biomass was separated from the culture broth by using sterile Whatman No. 1 filter paper and washed more than twenty times with distilled water. Further, 20 g of the wet biomass was suspended in 200 mL sterile distilled H$_2$O and it was extracted for 24 h, under continuous shaking at 150 rpm. Then the utilized fungus biomass was separated by filtration and the obtained CFE (containing a mixture of fungal metabolites) was used in the further experiments on extracellular biotransformation of Ag$^+$ to AgNPs. For this purpose AgNO$_3$ (p.a.) was dissolved in the produced different CFE to obtain a concentration of 10 mM AgNO$_3$. The extracellular fungi cell-free biosynthesis of AgNPs was carried out in 500 cm$^3$ flasks, at 29±1°C, under continuous shaking at 150 rpm, in dark condition.

The process of AgNPs formation was monitored from 0 h till 144 h by medium sampling for conducting UV-Vis absorption spectroscopy by using BOECO S-220 UV/VIS spectrophotometer at the wavelength from 200 to 600 nm. Samples of CFE, containing the produced AgNPs, were taken at every 24 hours and their absorbance was recorded. Further, the synthesized AgNPs were isolated from the CFE by centrifugation (30 min, 13300 rpm), washed several times with distilled water, re-suspended and their spectra were also recorded. The amount of bio-transformed Ag$^+$ ions was measured by Inductively-Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) after dissolving the carefully washed AgNPs that were separated from known volume of CFE, containing the prepared AgNPs.

The synthesized and isolated nanoparticles were characterized by Transmission Electron Microscopy (TEM) and infrared spectroscopy (FTIR-ATR). For the TEM analysis the washed AgNPs were re-suspended in distilled water. Further a drop of this suspension was casted on carbon-coated copper grids and dried under ambient conditions prior the measurement. JEOL model JEM 2100, 200 kV analytical electron microscope was used. For the IR analysis, biosynthesized AgNPs were washed with distilled
water several times. FTIR spectra were recorded on Bruker Tensor 27 spectrometer by direct deposition of the samples on a diamond attenuated total reflectance (ATR) accessory (INFRAMAT Equipment - DO1-284/17.12.2019). The spectra were collected in the frequency region 4000 – 600 cm\(^{-1}\) with 64 scanning at resolution of 2 cm\(^{-1}\). Air spectrum was used as a background.

3. Results and discussion
Under the same cultivation conditions different media gave different yield of \(T.\ reesei\) biomass, that generally could be presented by the order: medium 2 \(\cong\) medium 3 \(\cong\) medium 4 > medium 1 >>> medium 5. For the period of the \(Ag^+\) ions reduction experiments the pale yellow color of the all CFEs changed to dark yellow and then through yellowish-brown to brown color. The color appearance is an obvious sign of the formation of silver nanoparticles in the reaction mixture. The excitation of surface plasmon vibrations in the AgNPs is the cause for the color change of the solution [9, 17]. The reaction was followed by recording the UV–Vis absorption spectra of the five studied media with biosynthesized AgNPs. Figure 1 depicts the recorded spectra.

The spectra of all the samples (figure 1a – 1e) exhibited a pronounced peak around 280 nm in the beginning of the experiments. This peak is assigned to the superposition of the absorption bands of proteins and \(AgNO_3\). As it can be seen out of media containing different additives, the highest peak is observed for the CFE obtained from fungi grown on medium 3 (with addition of corn steep liquor). This could be related to the extraction and availability in the CFE of higher amount of proteins. Interestingly, this peak is the highest for the CFE from fungi cultivated on medium 1, however this has not resulted in higher production of AgNPs (see figure 1f). The intensity of this peak drops off with time implying conversion of the components of the reaction medium. This effect can be clearly seen at 24\(^{th}\) h for CFE obtained from fungi grown on media 5, 4, 3 and 2 (the most pronounced for medium 5 and the least – for medium 2) and after 72 h – for the CFE obtained from fungi grown on medium 1. At the same time a broad peak appears, starting from around 350 nm. This indicates the presence of AgNPs. For the mixtures of the CFE and synthesized AgNPs the peak appearance was observed at 24\(^{th}\) hour for CFE from fungi grown on media 4 and 5, at 48\(^{th}\) h for CFE from fungi grown on media 3, at 72\(^{th}\) h - for CFE from fungi grown on media 2, and at 96\(^{th}\) h - for CFE from fungi grown on media 1. Since it is recognized that metal nanoparticles synthesis by fungi is a bio-reduction process on the basis of the above mentioned observations it could be assumed, that either the CFE from fungi grown on media 4 and 5 contain bio-active molecules in higher concentration or contain some molecules with more pronounced catalytic activity compared to the CFE from fungi grown on the other media. The intensity of the peak that is characteristic for the AgNPs increases with the time for all media which is indicative for the continued reduction of the \(Ag^+\) ions and an increase in concentration of AgNPs. However, at 144h the highest relative increase in the absorption was observed for the mixture of AgNPs and CFE from fungi grown on medium 3, implying the higher total reduction ability of this CFE. The peak height of isolated AgNPs (figure 1f) hints to the same idea.

The peak indicative for the AgNPs is broad for the mixtures CFE + AgNPs, as well as for the isolated AgNPs from all media, except of medium 3, where it is relatively sharp – figure 1f. The latter finding is a sign for the formation of mono-disperse particles. The peaks recorded in the mixtures AgNPs + CFE from fungi grown on media 3, 4, and 5 are symmetric, which points at the stability of the synthesized nanoparticles [9]. The absorbance peak of AgNPs synthesized with CFE from fungi grown on media 4 and 5 are observed at a longer wavelength which shows the presence of larger nanoparticles [18], compared to those formed in CFE from fungi grown on media 3. The small blue and red shifts in the wavelength of the absorbance peak observed for AgNPs synthesized with CFE from fungi grown on media 4 and 5 (more pronounced for medium 5) could be related to obtaining AgNPs that are different in shape and size [9].

Based on the data presented in figure 1 it could be stated that the highest yield of AgNPs is achieved when the medium for \(T.\ reesei\) cultivation contained corn steep liquor (medium 3). This is confirmed by the data from ICP analysis – table 2. Our yield of AgNPs is in the range of the obtained by \(Ag^+\) ions extracellular reduction by the metabolites of the \(Fusarium oxysporum\) [9].
Figure 1 UV–Vis absorption spectra of CFEs containing AgNPs synthesized by T. reesei metabolites at various time and initial concentration 10 mM AgNO₃: synthesized AgNPs + CFE of fungi cultivated in: medium 1 - a), medium 2 - b) medium 3 - c) medium 4 - d), medium 5- e), Spectra of the isolated, washed and re-suspended AgNPs at 144 h - f). Arrows point the peak development in time.

Table 2. Degree of biotransformation of Ag⁺ to AgNPs by CFE from T. reesei.

| Medium for T. reesei cultivation | No 1 | No 2 | No 3 | No 4 | No 5 |
|--------------------------------|------|------|------|------|------|
| Biotransformation degree, %    | 2.4  | 4.4  | 6.1  | 5.2  | 5.4  |

Figure 2 depicts TEM micrographs of biosynthesized AgNPs obtained with CFE from fungi grown on media giving the higher degree of biotransformation, namely media 3, 4 and 5.

As it can be seen in the TEM micrographs the AgNPs formed were mostly spherical (till spheroid shape), with diameters in the range 2 - 6 nm – for AgNPs produced from CFE from fungi grown on media 3; and 4 - 11 nm – for AgNPs produced from CFE from fungi grown on the other media.
Figure. 2 TEM micrograph of biosynthesized AgNPs by reduction with a cell-free extract of T. reesei biomass for 144 hours: a) medium 3, b) medium 4, c) medium 5.

Besides that are smaller compared to the AgNPs formed in CFE from fungi grown on the other media with comparable yield, i.e. media 4 and 5, the AgNPs obtained in CFE from fungi grown on media 3 are nearly mono-disperse - figure 2. This suggests that the composition of culture media is important both for the yield of AgNPs synthesis and for the particles size. However, even bigger synthesized nanoparticles are with relatively low-size. This finding implies that the enzymes extracted from the biomass act not only as reducing but also as capping reagents. As it can be seen in figure 2, the synthesized particles were not in direct contact, indicating good stability. The latter may be assigned to the functional groups of the protein molecules wrapping AgNPs and acting as capping reagents. They were identified using FTIR-ATR analysis - figure 3.

Figure. 3 FTIR-ATR spectra of AgNPs synthesized by using CFE obtained from T. reesei cultivated on medium 3 (solid line), medium 4 (dotted line) and medium 5 (dots).

The IR bands of isolated and carefully washed biocoated AgNPs practically appeared at the same cm\(^{-1}\) for the three studied media. The ATR-FTIR analysis showed the presence of IR bands at the typical positions for protein vibrations such as N-H stretching vN-H at app. 3270 cm\(^{-1}\), amide I bands (carbonyl stretching) at app. 1638 cm\(^{-1}\) and N-H deformation vibration at app. 1535 cm\(^{-1}\). Besides, weaker bands appeared at app. 1730 cm\(^{-1}\) for medium 5 which correspond to the carbonyl stretching vibration of ester groups. There is an evidence that some lipids were also deposited on the AgNPs along with the protein molecules. The C-H stretching vibrations of both protein and lipid molecules were found within the IR interval 3000-2800 cm\(^{-1}\). The IR spectra of the three samples showed very strong absorptions at app. 1300 cm\(^{-1}\) for P-O vibrations and 1030 cm\(^{-1}\) for C-O vibrations, pointing out to the presence of phospholipids. The IR spectra of the three samples have minor differences – mainly in the low-frequency region below 900 cm\(^{-1}\), where medium 3, shows an additional band at 801-809 cm\(^{-1}\). The characteristic infrared bands for biocoated AgNPs in the three samples and the corresponding vibrational mode assignments are shown in table 3.

The CFE is the only source of proteins and phospholipids available on the AgNPs. The biomolecules and/or their parts that present in filtrate are responsible for synthesis and stabilization of AgNPs by their capping. In addition to ensuring stability of the produced AgNPs the proteins can act in the anchoring of drugs to transport them into cells [6].
Table 3. Characteristic infrared bands and vibrational mode assignments for bio-coated AgNPs.

| Characteristic IR band positions | Assignments                  |
|----------------------------------|------------------------------|
| Medium 3            | Medium 4            | Medium 5            |                          |
| 3267                | 3277                | 3272                | N-H stretching          |
| 2956                | 2955                |                     | assym. C-H stretching of -CH₃ groups |
| 2918                | 2918                | 2917                | assym. C-H stretching of –CH₂- groups |
| 2850                | 2850                | 2850                | sym. C-H stretching of –CH₂- groups C=O stretching of ester groups |
| 1730                |                     |                     |                            |
| 1638                | 1639                | 1638                | C=O stretching of amide groups |
| 1541                | 1536                | 1536                | N-H deformation of amide groups |
| 1291                | 1307                | 1297                | P=O asymmetric stretching vibration |
| 1032                | 1032                | 1032                | C-O stretching vibrations |

4. Conclusions
As a result of the studies carried out the following conclusions can be drawn:
AgNPs can be synthesized by using CFE from the non-toxic and easy to cultivate in high amounts fungus *Trichoderma reesei*, grown on medium containing inorganic salts and glucose as carbon source, in presence of different nutrient additives (yeast extract, corn steep liquor, peptone or casamino acids).

Having in mind (i) the yield of *T. reesei* cultivated on the different media, (ii) the biotransformation degree of Ag⁺ ion by the CFE from *T. reesei* cultivated on the different media, and (iii) the size and size dispersion of the synthesized AgNPs, it can be concluded that the media containing as additive corn steep liquor is the most suitable (among the studied media) for the cultivation of *T. reesei* whose aqueous CFE can be used for the biosynthesis of AgNPs.

The metabolites released in sterile distilled water by the cultivated *T. reesei* act both as reducing and capping reagents for obtaining nearly monodispersed AgNPs with size in the range of 2-11 nm.

5. References
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