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Efficient continuous biosynthesis of silver nanoparticles by activated sludge micromycetes with enhanced tolerance to metal ion toxicity

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ABSTRACT

The method for producing AgNPs by granules of activated sludge micromycetes with enhanced tolerance to metal ion toxicity – *Penicillium glabrum*, *Fusarium nivale* and *Fusarium oxysporum* has been developed; the optimum conditions for AgNP biosynthesis being found: the Ag^+ ion concentration, duration of the contact of microbial cells with silver ions, a growth phase of microorganisms, medium composition, a pH value, mixing conditions, and also lighting intensity. The effect of Cl^-, SO_4^{2-} and HPO_4^{2-} ions binding Ag^+ ions was eliminated, that brought to significant increase of the yield of NPs. Under batch conditions, silver particles of 60–110 nanometers in size were formed with a 65% yield. It was established that the nanoparticles were covered with microbial cell membrane proteins composed up to 70% by weight of the NPs that prevented their aggregation. In addition, it was the first time stable AgNPs had been formed by continuous AgNP biosynthesis by living cells of *F. oxysporum* with an 80% yield for a long time.

1. Introduction

There is a set of techniques for obtaining nanoparticles (NPs) which can be subdivided into physical and chemical methods, and green (biotechnological) ones. Chemical synthesis of NPs is widespread; however, biosynthesis proves to be more promising. Biological synthesis of NPs takes place in the presence of oxygen whereas chemical synthesis is often carried out under vacuum or the atmosphere of inert gas; that increases the cost of NP production [1–7]. The particles formed by biological methods are mainly stable for a long time, and, on the contrary, NPs produced by chemical way are unstable and they tend to aggregate. Natural reducing agents and stabilizers are used at NP biosynthesis that ensures safety of production as for people and the environment [8–14].

The mechanism of NP biosynthesis is rather complex: metal NP formation is thought to be a way of microbial self-protection: cells convert toxic metals from their ionic form into atomic one, thereby decreasing their solubility and preventing their penetration into the cell [15]. First, microorganisms capture metal ions by electrostatic interaction of cellular surface charged negatively with silver ions charged positively, and then binding of ions by sticky polysaccharide compounds secreted by the cell takes place [16,17]. Further, the ions are reduced into their atomic metal form with cellular enzymes. Metal clusters are formed, adsorbing new ions, and then these metal clusters grow, forming NPs. The proteins form a protective cover on the surface of the particles. This cover prevents from repeated solution of metal and NP aggregation. As a result, NPs that are stable within several months are formed [17–19].

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It is very important to pay special attention to the development of eco-friendly methods for synthesis of silver NPs with unique chemical and physical properties as silver NPs are widely applicable in catalysis, electronics and instrument making industry (production of optical devices, sensors of superficial plasma resonance, and sensors of Raman spectroscopy) [20]. Silver NPs have a broad range of antimicrobial activity, e.g. against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. AgNPs have a potent bactericidal effect on bacteria that are resistant to antibiotics, for example, *E. coli* that are resistant to ampicillin or *Streptococcus pyogenes* that are tolerant to erythromycin [21,22]. They have also antifungal activity against *Aspergillus*, *Mucor*, *Saccharomyces*, and *Candida* [23]; they affect HIV, interfering with process of its replication more effectively than gold NPs applied to this purpose earlier. AgNPs cause death of viruses of herpes and hepatitis B [24]. The bactericidal effect of AgNPs is applied in production of medical equipment, fabrics, cosmetics, washing and disinfectants, water and air filters, and also furniture and household appliances. The global annual production of AgNPs is estimated to be more than 500 tons and their production volumes continue increasing [24,25].

A great number of methods for green synthesis of AgNPs with use of plant, fungi or bacterial cells, products of their metabolism, biopolymers, and viral particles have already been developed [1–8]. For example, sunlight exposing to the reaction mixture of silver nitrate solution and grape or geranium leaf extracts allowed producing stable AgNPs covered with a protective protein layer [1,3]. Mono-dispersed NPs of 10–15 nanometers in size were produced by bacterial synthesis by *Corynebacterium* sp. [6]. The fungus *Fusarium oxysporum* was capable of active extracellular synthesis of stable AgNPs [5]. However, to introduce the methods mentioned into practice for a large scale it should be taken into account the efficiency of conversion of raw materials into NPs, stability and mono-dispersion of NPs. Further search of the most effective producers of nanosilver and optimization of biosynthesis could lead to increasing the yield of silver NPs, their stability and reducing the process duration.

The main purpose of the present study was to develop a microbiological method for obtaining stable silver nanoparticles with a high yield in a continuous mode. Such a mode of synthesis is preferable because the constancy of the process parameters is the key to stability of the product properties, increasing the monodispersity of nanoparticles. Its implementation would make it possible to bring a microbiological method for silver NP preparation from a laboratory to a large-scale level.

2. Experimental

2.1. The cultures of microorganisms

Three fungal cultures isolated from the aerobic activated sludge of wastewater treatment facilities, the system, in some cases with enhanced tolerance to metal ion toxicity, especially to silver both in ionic and colloidal form were used in producing silver NPs [26,27]. Some of the activated sludge microorganisms were revealed earlier to become dominating, showing a rapid growth and more intensive biomass accumulation in comparison with other organisms of the activated sludge subjected to changes in a number of operating parameters of wastewater treatment facilities (non-stationary loading or starvation), or under oxidative stress.

Three cultures, which were the most stress-resistant ones, were selected at prolonged passivating of activated sludge under conditions of fractional introducing hydrogen peroxide [28], these cultures being identified as follows: *Penicillium glabrum*, *Fusarium nivale*, and *Fusarium oxysporum*. These microorganisms formed strong granules that could explain to some extent their survival under stress. These cultures had enhanced tolerance to silver ion toxicity [29] and they were able to reduce Ag^+ ions, forming metal NPs with high yield, and besides, the fungus *Fusarium oxysporum* is known to be an active producer of silver NPs [5].

2.2. Cultivation of microorganisms

The fungal cultures were grown in Gause's No1 medium. Moreover, its modified versions differed in lowered content of some salts (K_2HPO_4–0.05 g/l, $MgSO_4{\cdot}7H_2O$ – 0.05 g/l and NaCl – 0.05 g/l) in case of the poor medium, and in absents of not only the salts above mentioned, but also $FeSO_4{\cdot}7H_2O$ in case of the minimum medium. In some experiments, the content of starch in the medium was varied from 1.0 to 10.0 g/l, or starch was replaced with sucrose.

Growth mediums were autoclaved at 121°C for 30 min. Cultivation was carried out under batch conditions in flasks of 100 ml volume with a 30 ml volume of the medium, and inoculating material of 10% of a medium volume being introduced. The microorganisms were cultivated on the orbital platform shaker (Heidolph Unimax 2010, Germany) at 27°C and 150 rpm, and with background scattered visible lighting. The incubation time was three days, and it varied in a number of experiments from one to seven days. The culture growth and amount of microbial biomass accumulated were estimated by oven-dry weight measurements. For this purpose 25 ml of the cultural suspension containing granules was sampled; the granules were washed out twice with distilled water using velocity sedimentation (7000 rpm, 10 min). The supernatant was decanted, and the biomass was transferred into a weighing bottle and dried up to constant weight at 105 °C.

2.3. Obtaining silver NPs

In one case, the sample aliquots of microbial biomass after cultivation were separated from growth medium by centrifugation at 7000 rpm within 10 min and then added into the water solutions of silver nitrate ($AgNO_3$); the concentrations of $AgNO_3$ varied from 5.0 to 250 mg/l. In the other case, the sterile silver salt solutions were added directly into the cultural liquid with biomass. The mixtures were incubated on the orbital platform shaker at the rate of shaking in the range from 50 to 250 rpm. The incubation was carried out at 27°C in the darkness or at directed lighting with use of lamps of Philips TL-D 18W/33-640; lighting at the level of flasks changed from 400 to 1000 lx and it was measured with the luxmeter-UF-radiometer TKA-01/3 (TKA Scientific Instruments, Russia). The experiments with background scattered lighting or in the absence of light were conducted in parallel. The pH adjustment was fulfilled by adding either 2N HNO_3 or 2N NaOH into the suspensions in experiments with varying the pH values of the medium. The incubation time varied from 5 min to three days. The resulting NPs were separated from biomass and large metal particles by centrifugation at 7000 rpm within 10 min, the NPs being remained in the supernatant. If necessary, further additional purification of NPs from the low organic impurities and micro-particles was carried out by zonal sucrose gradient centrifugation at 12,000 rpm.

2.4. Detection of silver NPs

Silver particle formation was indicated visually by changing the color of solutions and microbial biomass into yellow, orange or brown that was typical for colloidal silver and by large silver particles precipitated. The biomass and large crystals of silver were separated at 7000 rpm for 10 min. It was silver NP presence or absence in the supernatant that was estimated not only on the solution color, but also by spectroscopic analysis of solutions with UV–vis Mini 1240 spectrometer (Shimadzu, Japan). The silver NP concentration and the efficiency of conversion of metal ions into the