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简 报

Biosynthesis of Silver Nanoparticles Using the Phototrophic Bacteria *Rhodopseudomonas palustris* and Its Antimicrobial Activity Against *Escherichia coli* and *Staphylococcus aureus*

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Abstract: The use of *Rhodopseudomonas palustris* in biosynthesis of silver nanoparticles (AgNPs) emerges as a reliable and eco-friendly approach in recent years. This report focuses on extracellular biosynthesis of AgNPs using cell filtrate of *Rhodopseudomonas palustris*. These nanoparticles were characterized by UV-vis spectrum, X-ray diffraction (XRD) spectrum and transmission electron microscopy (TEM). UV-vis spectrum of the aqueous medium containing silver ion showed a peak between 420 nm−460 nm corresponding to the plasmon absorbance of AgNPs. TEM micrograph showed formation of the AgNPs in the range of 5 nm−20 nm. XRD of the nanoparticles confirmed the formation of metallic silver. The AgNPs were evaluated for their antimicrobial activities against *Escherichia coli* and *Staphyloccocus aureus*.

Keywords: *Rhodopseudomonas palustris*, Biosynthesis, Characteration, Silver nanoparticles, Antimicrobial activities

沼泽红假单胞菌生物合成银纳米粒子及其抗菌作用

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摘 要: 近年来, 利用沼泽红假单胞菌合成银纳米粒子作为一种可靠和环境友好的方法出现。主要利用沼泽红假单胞菌的细胞滤液来还原银离子。制备的纳米粒子用紫外可见光谱(UV-vis)、X 射线衍射光谱(XRD)和透射电镜(TEM)进行表征。含有银粒子溶液的 UV-vis 光谱显示在 420 nm−460 nm 处出现银纳米粒子的吸收峰。TEM 图像表明所形成的银纳米粒子的粒径范围为 5 nm−20 nm。纳米粒子的 XRD 图谱证明产物为金属银。所制备的银纳米粒子对大肠杆菌和金黄色葡萄球菌作抑菌性试验。

关键词: 沼泽红假单胞菌, 生物合成, 表征, 银纳米粒子, 抗菌活性

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1 Introduction

Metal nanoparticles have received extensive attentions due to their unique performance in catalytic, optical, electronic, magnetic, biomedical and many other fields compared to their macro scaled counterparts[1,3].

Many methods have been applied to synthesize AgNPs, such as chemical reduction, photochemical or radiation-chemical reduction, metallic wire explosion, sonochemical method and plasma method. To achieve the objective of developing simple and eco-friendly technology, researchers in this field have turned to biological systems[4]. The biological method is recently developed as a promising method because of its special advantages such as sufficient material sources, mild reaction conditions, good dispersion of nanoparticles as well as few chemical addictives and poisonous byproducts. There are several reports in the literature on the cell-associated biosynthesis of AgNPs using various microorganisms, such as the fungus *Aspergillus flavus*[5], airborne bacteria *Bacillus* sp.[6], *Staphylococcus aureus*[7] and *Phaenerochaete chrysosporium*[8]. The current interest in the field of material miniaturization is to develop a protocol to synthesize metal nanoparticles with controlled size and shape, because the nanomaterials exhibits different properties which depend on their sizes and shapes. Fayaz *et al* have demonstrated that an effective approach to biosynthesize silver nanoparticles using fungus *Trichoderma viride* and the effect of temperature on controlling size of silver nanoparticles[9]. Lee *et al* have shown the effect of temperature and pH over the preparation of silver nanorods[10]. AgNPs have been used for their well-known antimicrobial properties. It is currently used to control bacterial growth in a variety of applications, including dental work, catheters, and burn wounds[11–12]. Nanoparticles generally exhibit inherent unique characteristics, such as large specific surface area, modified structure, controlled surface composition and reactivity[13]. The present research work mostly emphasized *Escherichia coli* and *Staphyloccocus aureus*, because they are found to be resistant to a wide range of broad-spectrum antibiotics[14]. Hence, an antibacterial activity test was conducted to observe the different inhibitory effect between *Escherichia coli* and *Staphyloccocus aureus.*

Phototrophic bacteria are metabolically the most versatile among all procaryotes: anaerobically photoautotrophic and photoheterotrophic in the light and aerobically chemoheterotrophic in the dark, so they can use a broad range of organic compounds as carbon and energy sources[15–16]. Various microorganisms have been reported to reduce silver ions to silver nanoparticles. However, some of them are pathogenic bacteria. As one kind of probiotics, photosynthetic bacteria make the production more productive and safe. Furthermore, the biomasses have many applications in wastewater treatment, animal feed, etc. Thus, no second pollution is caused. There are few reports on biosynthesis of silver nanoparticles using photosynthetic bacteria. In this study, phototrophic bacteria *Rhodopseudomonas palustris*, a typical purple non-sulfur bacterium, has been chosen to synthesize AgNPs at room temperature with a single step process.

2 Experimental

2.1 Source of microorganisms

The strains used in this study were obtained from College of Life Science and Technology, Shanxi University, Taiyuan, China. The *Rhodopseudomonas palustris* was used to synthesize AgNPs. The *Staphylococcus aureus* (*S. aureus*, ATCC 6358) and *Escherichia coli* (*E. coli*, ATCC 8099) were used for vitro antimicrobial test of AgNPs.

2.2 Preparation of cell filtrate

Photosynthetic bacteria *Rp. Palustris* were cultured in the medium containing 1.0 g NH_4Cl, 0.5 g Na_2HPO_4, 0.2 g $MgCl_2$, 2 g NaCl, 2 g yeast extract, 6 mL of 80% sodium lactate, dissolved in 1000 mL of distilled water; the pH was adjusted to 7.2 with NaOH before autoclaving. The bacteria were cultured anaerobically at 35°C under continuous illumination with incandescent lamps at a light intensity of about 2000 lux[16]. All chemicals used were of analytical grade. After 5 days of incubation, the biomass was separated by filtration and washed thrice with deion-

ized water to remove all interfering components existed in the medium from the biomass[17]. The washed biomass (10 g fresh weight) was kept in contact with deionized water for 72 h at 35°C. After the incubation, the cell filtrate was obtained by centrifugation and filtration.

2.3 Synthesis of AgNPs

The cell filtrate was challenged with 1 mmol/L silver nitrate solution, and then incubated in light condition at 35°C. Simultaneously, the control containing only silver nitrate solution were maintained under same conditions[18].

2.4 Characteration of AgNPs

Our investigation used ultravilolet-visible (UV-vis) spectroscopy (UNICO UV-2000 spectrophotometer) to ascertain the formation and stability of silver nanoparticles in aqueous solution[19]. The purified Ag nanoparticles were used for powder X-ray diffraction (XRD) analysis. The spectra were recorded on a Rigaku Dmax-γA automatic instrument. The diffracted intensities were recorded from 30° to 80° angles[20]. The morphology and size of as-formed AgNPs were further determined by transmission electron microscopy (TEM) images. TEM was performed on a Hitachi H-600 instrument operated at an accelerating voltage of 120 kV[21].

2.5 Disk diffusion assay to evaluate combined effects

The antimicrobial activity of AgNPs was tested against *Staphylococcus aureus* as the model gram-positive bacterial and *Escherichia coli* as the model gram-negative bacterial, which are the main bacteria being responsible for wound infection[22]. The silver samples were diluted and added to nutrient agar plate. After shaking uniformly, dip the pieces of round filterpaper of 6 mm diameter in the two kinds of tested bacterials suspensions and put these filter paper on the surface of agar plates. Positive control plates contained medium with tested bacterial concentrations of 10^6−10^7 CFU/mL. Negative control plates contained only medium. The AgNPs samples were used as prepared and tested at final concentrations of 20, 50, 60, 90, 100 mg/L. Each concentration has thrice parallel tests to reduce errors. All plates were incubated for 24 h at 37°C[23].

The minimum inhibitory concentration (MIC) was read by the visual change of inhibitory circle on the plates noted both before and after incubation. After being properly diluted, it appeared to have little or no cell growth on agar plates. The minimum bactericidal concentration (MBC) was determined as the lowest concentration that inhibited the visible growth of the used bacterium[24].

3 Results and discussion

Fig. 1 shows that upon addition of the silver ion (1 mmol/L) into the tube containing the cell filtrate, the color of the medium turned to brown. The appearance of the brown color was an indication of formation of colloidal silver particles in the medium. This colour is primarily due to the surface plasmon resonance of deposited AgNPs[25]. The controlling test was carried out by adding silver nitrate solution to deionized water, no change in color was observed.

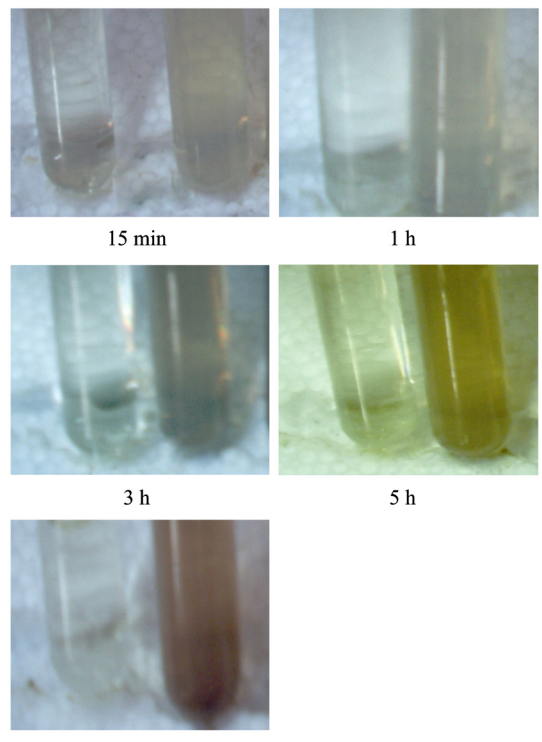

Fig. 1 Photographs of silver ions treated with deionized water (left) and silver ions subjected to cell filtrate (right) at different exposure time (15 min, 1 h, 3 h, 5 h, 16 h)

Formation and stability of AgNPs in aqueous colloidal solution are confirmed using UV–vis spectral analysis. Sample of 1 mL was withdrawn at different time intervals and the absorbance was measured in the range of 200 nm−800 nm using a UV-visible spectrophotometer. As illustrated in Fig. 2, a strong and broad peak located between 420 nm and 460 nm was observed. The increase in intensity could be due to increasing number of nanoparticles formed as a result of reduction of silver ions present in aqueous solution.

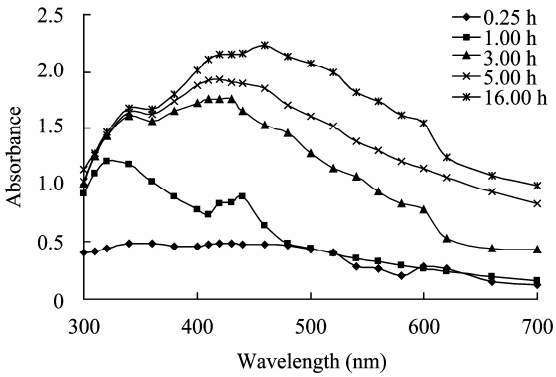

Fig. 2 UV-visible spectrum of aqueous medium containing cell filtrate and silver ion (1 mmol/L) after different reaction time

The XRD pattern of the silver nitrate-treated sample corresponds to that of AgNPs. Fig. 3 shows four intense peaks in the whole spectrum of 2θ values ranging from 30° to 80°. It is important to know the exact nature of the silver particles formed and this can be deduced from the XRD spectrum of the sample. XRD spectra of pure crystalline silver structure have been published by the Joint Committee on Powder Diffraction Standards (file nos. 04−0783). A comparison of our XRD spectrum with the standard confirmed that the silver particles formed in our experiments were in the form of nanocrystals, as evidenced by the peaks at 2θ values of 38.45°, 44.48°, 64.69° and 77.62°, corresponding to 111, 200, 220 and 311 planes for silver, respectively[26]. The full width at half maximum (FWHM) values measured for 111, 200, 220 and 311 planes of reflection were used with the Debye-Scherrer equation to calculate the size of the nanoparticles. The four intense peaks observed in the spectrum agree to the Braggs's reflection of silver nanocrystals reported in literature. In the XRD analysis, the four intense characteristic peak confirmed that the synthesized nanoparticles have Ag structure. Less mixed peaks presented in the XRD spectrum showed that silver nanocrystals of high purity was prepared.

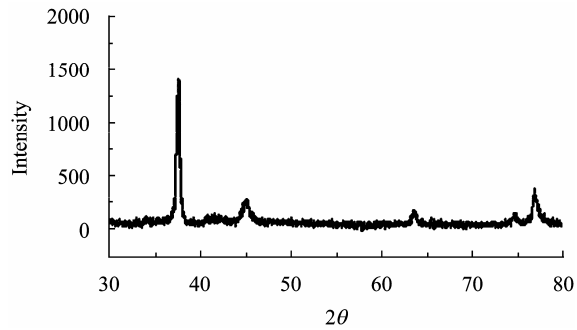

Fig. 3 XRD pattern of nanoparticles formed after addition of cell filtrate to silver nitrate

Transmission electron microscopy has provided further insight into the morphology and size details of the AgNPs. Fig. 4 shows that many particles were captured. The morphology of nanoparticles is highly variable, with spherical nanoparticles observed on micrographs. The TEM micrograph suggests that there is variation in the particle size. Almost 75% of the particles are in the 10 nm to 15 nm range. The size of 10% of the observed nanoparticles is 15 nm−20 nm, and about 15% of the nanoparticles are in the range of 2 nm−10 nm. The prepared AgNPs presented good uniformity. All the nanoparticles with occasional aggregation are well separated and narrow size distribution was noticed.

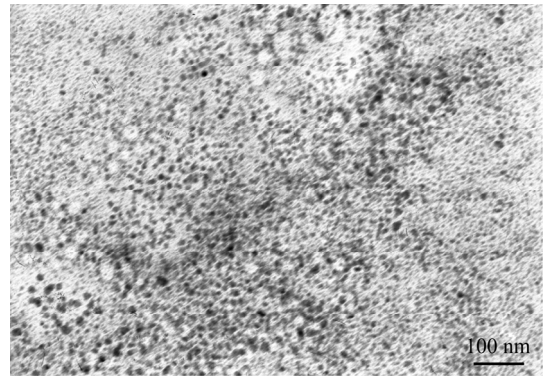

Fig. 4 TEM micrograph of silver particles synthesized by *Rp. palustris*

Our investigation has confirmed that silver nanoparticles were formed in aqueous solution. Accurate controls over pH and process conditions were required to increase output of AgNPs. pH could have an important effect on the size and shape of AgNPs through single factor experiment. At the same pH 7, more silver nanoparticles were acquired as the ratio of Ag^+ concentration was increased. However, the nanoparticles agglomerated significantly. Our findings on optimum biosynthetic conditions of silver nanoparticles will be reported later.

The enzyme involved in the synthesis of nanoparticles may be nitrate reductase present in *R. Palustris*. This nitrate reductase is induced by nitrate ions and reduces silver ions to metallic silver. NADH widely exists in the cells of microorganisms, which is important components of the electron-transport chains of redox reactions.

$$Ag^+ + NADH \xrightarrow{\text{Nitrate Reductase}} Ag + NAD^+$$

Previous studies have indicated that NADH and NADH-dependent nitrate reductase enzyme are important factors in the biosynthesis of metal nanoparticles. The nitrate reductase might be responsible for the bioreduction of Ag^+ to Ag^0 and the subsequent formation of silver nanoparticles. The reduction seems to be initiated by electron transfer from the NADH by NADH-dependent nitrate reductase as electron carrier. However, further experiments should be performed to elucidate the mechanism involved in the biosynthesis of silver nanoparticles[28].

AgNPs exhibit very strong bactericidal activity against both gram-positive and gram-negative bacteria including multiresistant strains. As shown in Fig. 5, the antimicrobial activity of the synthesized AgNPs was studied by disc diffusion qualitative assay. The evident inhibitory effect could be seen in the above figure. These results indicate that the higher AgNPs concentration, the smaller bacterial area around filter paper[29]. When AgNPs concentration is 20 mg/L, the minimum inhibitory concentration (MIC) could be read. Whereas, no difference can be seen from the positive control plate when lower than 20 mg/L. Visible bacterial growth could be seen in the plate of 100 mg/L AgNPs, so the minimum bactericidal concentration (MBC) is 100 mg/L. Hence, the synthesized nanoparticles could be of immense use as antimicrobial agent. AgNPs attach to phosphate and sulfur groups that are part of the phospholipid cell membrane or to membranal proteins and severely damage the cell and its major functions. AgNPs can penetrate the bacterial cell and accumulate to toxic levels that may cause death of the organism. In addition, AgNPs can bind to the DNA inside the bacterial cells, preventing its replication, or interact with the bacterial ribosome[13].

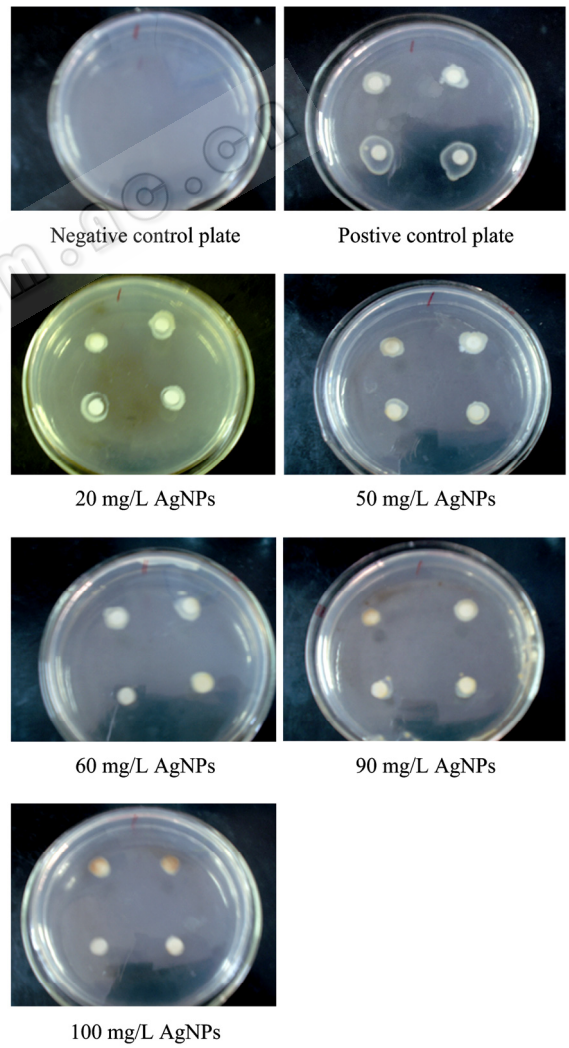

Fig. 5 Photographic images of bacterial zones against *S. aureus* (the two filter paper at the top of plate) and *E. coli* (the other two), produced by different final AgNPs concentrations (20, 50, 60, 90, 100 mg/L)

4 Conclusions

In this study, the AgNPs are synthesized by cell fitrate of phototrophic bacteria *Rhodopseudomonas palustris*. The UV-visible spectrum of AgNPs in aqueous colloidal solution are found to have a strong, broad peak located between 420 nm and 460 nm. The XRD pattern shows four peaks at 2θ values of 38.45°, 44.48°, 64.69° and 77.62°, corresponding to 111, 200, 220 and 311 planes for silver, respectively. The size of the nanoparticles ranged from 5 nm to 20 nm. The result shows that silver nanoparticles obtained in this experiment have the quality with low impurity content and high purity, the particles have the characters of centralized distribution and uniform size, basically presenting the spherical. This process of nanoparticle production is eco-friendly as it is free from any solvent or toxic chemicals, also easily amenable for large-scale production. The AgNPs exhibit obvious antimicrobial effect. The minimum inhibitory concentration (MIC) is 20 mg/L. The minimum bactericidal concentration (MBC) is 100 mg/L.

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