Novel bio-fabrication of silver nanoparticles using the cell-free extract of *Lysinibacillus fusiformis* sp. and their potent activity against pathogenic fungi

Pei Liu$^{1,2,3,4}$, Rongying Zhou$^{1,4}$, Tingting Yin$^{1,2}$, Qiang Wang$^{1,2}$, Zhen Guo$^{1,2}$, Tian Qiwen$^{1,2}$, Muhammad Bilal$^{2}$, Shuai He$^{1,2,3}$, Xiaoyan Zhu$^{1,2,3}$, Hao Shi$^{1,2,3}$ and Xiangqian Li$^{1,2,3}$

$^1$ Jiangsu Provincial Key Construction Laboratory of Probiotics Preparation, Huaiyin Institute of Technology, Huaian, 223003, People’s Republic of China

$^2$ Faculty of Life Science and Food Engineering, HuaiYin Institute of Technology, Huaian, 223003, People’s Republic of China

$^3$ Jiangsu Provincial Engineering Laboratory for Biomass Conversion and Process Integration, Huaian, 223003, People’s Republic of China

$^4$ Authors of equal contribution.

E-mail: lixq2002@126.com

Keywords: green biosynthesis, cell-free extract, *Lysinibacillus fusiformis* sp., silver nanoparticles, characterization, antifungal activity

Abstract

Green synthesis of nanoparticles is considered an efficient method when compared with chemical and physical methods. Herein, an extracellular green method was adopted to synthesize silver nanoparticles (AgNPs) by using the cell-free extract of a new silver-resistant bacterium, *Lysinibacillus fusiformis* sp., which was isolated from the sewage treatment plant and identified by 16S rRNA gene sequencing. Without using any toxic organic solvents and reducing agents, the cell-free extract effectively reduced Ag$^+$ to AgNPs with a conversion rate of 72.21% under the optimal condition of pH 9.0, 50°C, and 5 mM of silver nitrate solution. The newly-developed AgNPs were characterized by the methods of ultraviolet-visible spectroscopy (UV-vis), Fourier transform infrared spectroscopy (FTIR), x-ray diffraction (XRD), transmission electron microscopy (TEM), zeta potential/size distribution analysis and energy-dispersive x-ray spectroscopy (EDS). Characterization results showed that the as-prepared AgNPs were spherical in shape and well-dispersed with an average size of ∼50 nm. The results of the Oxford cup plate assay used for measuring the antifungal activity indicated a pronounced antifungal activity of the biosynthesized nanoparticles against pathogenic fungi of tinea pedis, i.e., *Candida albicans*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*. In conclusion, the biosynthesized AgNPs might display a high potential as novel antifungal agents in medicine. However, it is indispensable to conduct *in vivo* studies to explore the biocompatibility of AgNPs for their future therapeutic applications.

1. Introduction

Fungi constitute a large group of organisms that are ubiquitous in natural environments. Some fungi are probiotics, such as *Saccharomyces cerevisiae*, while others may cause severe infections in human beings [1]. The dermatophytoses, skin infections caused by some fungi (dermatophytes) have the highest morbidity rate globally [2]. Infections which incurred by dermatophytes are generally linked with recurrence after successful antifungal therapy. Moreover, the overuse and misuse of antibiotics may lead to the emergence and spread of resistant strains [3]. Therefore, it pressingly needs to explore and develop a novel generation of antifungal agents with unique mechanisms [1].

Among inorganic antimicrobial agents, silver and silver nanoparticles (AgNPs) are widely employed to fight off bacterial and fungal infections [2]. The Ag$^+$ has an obvious inhibitory effect against an array of pathogenic microbes [4]. The use of AgNPs as antimicrobial agents has recently gained considerable attention, around the globe [5–7]. Generally, AgNPs are synthesized with physical/chemical methods [8, 9]. Although these two
methods can easily synthesize AgNPs with various sizes and shapes, they rely heavily on using harmful reducing or capping agents, which pose a serious environmental risk [10]. Furthermore, it is the most challenging issue in current nanotechnology to prepare AgNPs with good monodispersity and a range of sizes [11].

During the last 5 years, many efforts have been devoted to developing new, cheaper and greener approaches to synthesize nanoparticles [12–15]. The biosynthesis of nanoparticles is suggested as an environmentally friendly and cost-efficient alternative method [14]. A variety of microorganisms such as bacteria, fungi, yeasts, and plants were found to be capable of synthesizing diverse nanoparticles [1, 9, 11, 16, 17]. Bacteria, in particular, their cell-free extracts, were better when compared with other biological methods because bacteria are ubiquitously present and easy to be cultured [12]. Furthermore, bacteria generally secrete large amounts of biomolecules, such as proteins, enzymes, peptides, and numerous other bioactive substances [18, 19], which are involved in reducing metal ions, thus controlling nanoparticle sizes [20]. Above all, using bacteria to biosynthesize AgNPs is highly interesting and may have a significant impact on further advances in nanoscience [12]. However, the biosynthesis of AgNPs by bacteria is usually associated with a low Ag concentration (i.e., 1 mM), which is the major hindrance to rapid and large-scale synthesis of AgNPs [21]. Qian et al. reported the reduction of 1 mM of AgNO3 solution into AgNPs with the cell extract of an endophytic fungus—Epicoccum nigrum [22]. Similarly, Magdalena and co-authors also carried out the biosynthesis of AgNPs from Streptomyces xinghaiensis OF1 strain using 1 mM of AgNO3 [23]. Consequently, the low yield and inefficient AgNP biosynthesis must be addressed for the purpose of commercial exploitation.

In this study, we screened a strain with silver tolerance, Lysinibacillus fusiformis sp. (L. fusiformis sp.) and investigated a novel approach to biosynthesize AgNPs rapidly with the cell-free extract of L. fusiformis sp. This extract reduced Ag⁺, of which the concentration was as high as 5 mM, into AgNPs. The biosynthesized AgNPs were characterized by methods of ultraviolet-visible absorption spectroscopy (UV–vis), x-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), Transmission electron microscopy (TEM), Zeta potential and size distribution analysis, together with energy dispersive x-ray spectroscopy (EDX). Finally, the possible mechanism of AgNPs formation during the synthesis was also discussed.

2. Materials and methods

2.1. Reagents and fungal strains
Silver nitrate (AgNO₃, 99,98%) was purchased from Merck (Germany), whereas fluconazole and ketoconazole were from Hangzhou microbial reagent co., Ltd China. Fungal strains of tinea pedis, namely Candida albicans, Trichophyton rubrum, and Trichophyton mentagrophytes were preserved by our laboratory. Other chemicals used in this research were used without further purification, and all strains were maintained in Sabouraud Dextrose Agar (SDA).

2.2. Screening and isolation of strain with silver tolerance
Water samples were collected from a wastewater treatment plant in Huaiian for the isolation of silver tolerating microbes. Water samples were diluted gradient-wise and inoculated on sterile enrichment nutritional agar plates (1% of peptone, 0.3% of beef extract, 0.5% of NaNO₃, 1.5% of agar and pH 7.2) at 37 °C. The colonies that grew on the plates were further purified by transferring into selective media (same with enrichment nutrient agar plates) containing different concentrations of silver. Molecular identification of the bacteria was conducted, and the 16S rRNA gene was partially sequenced by Sangon Biotech Co., Ltd (Shanghai) and compared with sequences deposited in databases; the molecular phylogenetic tree was constructed.

2.3. Biosynthesis of AgNPs
After the cultivation of L. fusiformis sp. at 37 °C, 200 rpm for 24 h, the cell-free extract was centrifugated at 10 000 rpm for 10 min under room temperature. Then, a 5 ml AgNO₃ solution (0.1 M) was added into 95 ml cell-free extract, and the reaction was carried out in dark at 37 °C on a rotary shaker (200 rpm). The selective medium with 5 ml 0.1 M of AgNO₃ solution and the cell-free extract without AgNO₃ was applied under the same conditions as the control.

2.4. Optimization of the conditions to biosynthesize AgNPs
The effects of different concentrations of AgNO₃ (1 ~ 10 mM), pH (4 ~ 10) and temperature (20 ~ 60 °C) in the biosynthesis of AgNPs were studied separately [24]. For example, 5 ml AgNO₃ solutions (1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM and 10 mM) were added into 95 ml cell-free extracts individually, and allowed to react in dark at 200 rpm, 37 °C. After the reaction completion, 1 ml of the as-prepared AgNPs were characterized by UV–vis (UV-2401PC, Shimadzu, Japan).
2.5. Calculation of silver conversion

After the recovery of silver nanoparticles, the residual Ag\(^+\) concentration was analyzed with an atomic absorption spectrophotometer (Pgeneral, China) \=[6]. The Ag\(^+\) conversion was calculated with the relation given in equation (1).

\[
Q = \frac{C_i - C_f}{C_i} \times 100\%
\]

Where Q is the conversion of Ag\(^+\); \(C_i\) denotes the initial concentration of AgNO\(_3\), and \(C_f\) denotes the final concentration of Ag\(^+\).

2.6. Characterization of AgNPs

The as-prepared AgNPs were purified by centrifugation at 10 000 rpm for 10 min and washed three times. The AgNPs were firstly characterized by UV–vis in the wavelength range of 300–600 nm \=[25]. The zeta potential and hydrodynamic diameter of AgNPs were carried out by DLS with Malvern Zeta Sizer Nano ZS 90 (Worcestershire, UK) \=[24]. The TEM images were captured by a Tecnai 12, which was carried out at an accelerating voltage of 200 kV \=[2]. The FTIR spectra \([24]\) were recorded with Nicolet 5700 (Thermo Electron Co., USA) in the range of 500–4000 cm\(^{-1}\) at the resolution of 4 cm\(^{-1}\). XRD \([24]\) was performed with a Bruker D8 Advance instrument (Germany) with Cu K\(_\alpha\) radiation in the range of 20–90° 2\(\theta\). EDX was carried out by Quanta 250 FESEM (USA). All the data were processed by OriginPro 9.0.

2.7. Antifungal activity of the AgNPs

Antifungal activity was performed against C. albicans, T. Rubrum, and T. mentagrophytes. Sabouraud Dextrose (SD) medium was used to cultivate the test fungi using an Oxford cup plate assay. When cultured with a bacterial suspension of \(10^2 \sim 10^6\) CFU/mL, these three fungi were diluted and coated on solid SDA plates. Then placed the sterilized Oxford cups with 200 \(\mu\)l of different concentrations of biosynthesized AgNPs (i.e., 10, 30, 50, 60, 80, 100, 200 and 1000 \(\mu\)g ml\(^{-1}\)). AgNPs with the standard antifungal antibiotics (fluconazole or ketoconazole) were also tested in parallel. Double distilled water was performed as blank control. Each SDA plate was placed with 3 Oxford cups in a parallel experiment to eliminate errors. The antifungal activity of AgNPs was estimated by surveying the inhibition zone in millimeters \([23]\).

3. Results and discussion

3.1. Isolation and identification of the bacterium

The bacterium was isolated from wastewater. The screening of the microbe for silver tolerance was performed using isolated colonies that were plated on selective agar plates with varying concentrations of silver. Strains that grew on selective media were shortlisted for further study. At last, we acquired a strain with pronounced silver tolerance. The strain was Gram-positive and pure pale-white rod-shaped bacterium, as shown in figures 1(A) and (B). The isolate was further characterized as Lysinibacillus fusiformis sp. based on the 16S rRNA sequencing and phylogenetic studies (figure 2).

![Figure 1](image-url)
3.2. Biosynthesis of AgNPs

The reduction of Ag\(^+\) into colloidal silver by microbial strains in aqueous solutions is a sequential process. In this process, a variety of Ag\(^+\) complexes are reduced to metallic silver atoms (Ag\(^0\)) following by Ag\(^0\) agglomerating into oligomer clusters. These clusters eventually result in the formation of colloidal AgNPs [11]. The stepwise reduction process of Ag\(^+\) to colloidal silver yields color and UV–vis spectrum of the aqueous solutions changed apparently [26]. In this paper, preliminary biosynthesis of AgNPs was verified with the visual identification method. The supernatant of the *L. fusiformis* sp. culture was collected into two separate 250 mL Erlenmeyer flasks to fabricate AgNPs. One flask contained an AgNO\(_3\) solution, while the other one was maintained as the standard/control. The Erlenmeyer flask containing the supernatant of the *L. fusiformis* sp. with Ag\(^+\) ions changed to be yellowish-brown after the addition of AgNO\(_3\) (figure 3(a)). The color change indicated the formation of AgNPs, where the reduction of Ag\(^+\) took place extracellularly [27]. Furthermore, the as-prepared AgNPs were analyzed with UV–vis spectroscopy. Some previous studies reported that AgNPs gave obvious absorption peak at 420–450 nm because of its surface plasmon resonance characteristics [6, 25, 28]. The maximum absorption at 420 nm further corroborated the formation of silver nanoparticles. Insert: active plots of maximum absorbance at 420 nm as a function of time.

3.3. Optimization of the conditions to biosynthesize AgNPs

Environmental conditions affect the apparent biosynthesis of silver nanoparticles [29]. The synthesis conditions, such as the concentration of AgNO\(_3\), pH, and temperature, directly determine the biosynthesis of AgNPs, which can be expressed on the maximum absorbance by UV–visible spectra. Firstly, the effects of different AgNO\(_3\) concentrations (1–10 mM) on the fabrication of AgNPs were evaluated. As shown in figure 4(a), OD\(_{420nm}\) (the maximum absorbance) increased by increasing AgNO\(_3\) concentration from 1 mM to 5 mM, whereas it...
indicated that moderately raised temperatures expedited the process of Ag biosynthesis was as high as 72.21%, which was comparable with the physicochemical methods et al. much higher than the general biological method to produce AgNPs by some plants.

420 nm appeared with 5 mM of Ag decreased when AgNO3 concentration was increased from 5 mM to 10 mM. The maximum absorbance peak of

we performed a detailed analysis of pH changes for AgNPs biosynthesis. It was found that OD420nm increased by increasing pH from 6.0 to 9.0, as shown in figure 4(b), whereas it decreased from 9 to 10 and no peak was noted at pH below 5.0. Therefore, pH 9.0 was chosen as the optimal pH for AgNPs biosynthesis. At last, the influence of temperature on AgNPs biosynthesis by L. fusiformis sp. was evaluated. The OD420nm of the reaction mixture was found increased from 20 °C to 50 °C, then decreased between 50 °C and 60 °C (figure 4(c)). These results indicated that moderately raised temperatures expedited the process of Ag reduction. Besides, L. fusiformis, which was originally known as Bacillus fusiformis before 2007 was reclassified to the genus Lysinibacillus, along with its close relative B. sphaericus [30]. Similar to Bacillus with spores, L. fusiformissp. exhibits the heat-resistant property [31], which has been verified by our above-mentioned results. Generally, the relatively higher reaction temperature is conducive for L. fusiformis to produce some bioactive substances, such as enzymes, polypeptides, etc [32].

Therefore, the optimal conditions thus achieved to synthesize AgNPs by L. fusiformissp. were 5 mM of AgNO3, pH 9.0, and 50 °C. Under these optimal conditions, the conversion rate of silver nitrate to AgNPs during biosynthesis was as high as 72.21%, which was comparable with the physicochemical methods (70%–85%) and much higher than the general biological method to produce AgNPs by some plants [2, 24]. For example, Maria et al. [2] achieved a 60% conversion rate by using the stem bark extract of Z. xylopyrus to produce AgNPs at the optimal condition of pH 9.0, 50 °C, and 5 mM of AgNO3. Although biogenic silver nanoparticles were found to be interesting alternatives to physically and chemically synthesized AgNPs, the large-scale biosynthesis of AgNPs by the green microbial method still remains a challenge to be overcome. The cell-free extract of L. fusiformis sp., which can be easily obtained through fermentation, was used as a reducing agent to biosynthesize AgNPs. Besides, this microorganism is renewable and easily cultivable. Therefore, the proposed extracellular biosynthesis is suitable for large-scale commercial production of AgNPs.

Furthermore, proteases and esterases are the most frequently discovered hydrolytic enzymes from L. fusiformis that catalyze the hydrolysis of peptide bonds and acylglycerols [32, 33]. Prabha et al [32] isolated a bacterial strain, L. fusiformis AU01 that simultaneously produced an intracellular esterase and extracellular protease under the same conditions. Gong et al [34] screened a protease-producing strain-L. fusiformis from a high protein silicone waste brewery. The protein degradation characteristics of L. fusiformis were also studied using rice protein as the sole nitrogen source. The maximum protein degradation activity was achieved under the optimal conditions, and the content of free amino acids, such as Pro, Lys, Ala, Lys, Phe, Tyr, His and Thr was found to accelerate the protein hydrolysis process. Therefore, we speculated that proteases and esterases secreted by L. fusiformis, or the bioactive substances, such as polypeptides, acids, and alcohols produced by these two enzymes might play a reducing role in the development of AgNPs.

3.4. Characterization of AgNPs
Nanoparticles characterization is vital to manage nanoparticle synthesis and their applications [35]. After optimization of AgNPs synthesis conditions by L. fusiformis sp., the crystalline nature of the biosynthesized AgNPs was characterized by XRD, as shown in figure 5(a). Five diffraction peaks found at 20 values of 38.11°, 44.29°, 64.41°, 77.38°, and 81.53° were respectively corresponded to (111), (200), (220), (311), and (222)

Figure 4. Effects of (a) different AgNO3 concentration (1–10 mM), (b) pH(4–10) and (c) temperature (20–60 °C) on silver nanoparticle synthesis.
crystalline planes of the face-centered cubic crystalline structure of metallic silver (JCPDS card number 04-0783) [36].

The functional groups responsible for the bioreduction of AgNO₃ into Ag were characterized by FTIR spectroscopy. Notably, FTIR spectra ascertained the involvement of various functional groups in the synthesis and stabilization of AgNPs [25]. It was clear from the FTIR spectra, which indicated the emergence of six obvious peaks at 3685, 1602, 1363, 1153, 1049, and 678 cm⁻¹ (figure 5(b)). The emergence of a strong absorption peak at 3685 cm⁻¹ indicated the existence of polyphenols because of binding Ag⁺ with a hydroxyl group, referring to the stretching of the O–H group or the free hydroxyl group. Besides, these two characteristic absorption bands at 1363 and 1049 cm⁻¹ corresponded respectively to the C–N and –O– stretching vibrations of aromatic and aliphatic amines [24]. The presence of C=O stretching vibration around 1602 cm⁻¹ substantiated the existence of a broad range of alkene groups in biosynthesis of AgNPs. The alkene groups were speculated to play an effective role in the green biosynthesis of AgNPs [24]. The presence of characteristic functional groups such as protein, polypeptides, phenols, and acids, which presented in the L. fusiformis sp. cell-free extract would have participated in the bioreduction process of AgNPs biosynthesis. Specifically, it was found that proteins and polypeptides can bind onto nanoparticles via free amine groups or the residues of cysteine, or through the electrostatic combination of the negatively charged carboxylate groups in proteins and polypeptides [37, 38]. Therefore, we deduced that proteins or polypeptides in the cell-free extract were the dominant bioactive molecules participated in the bioreduction and stabilization of the AgNPs. The chemical reaction equation may be as follows:

\[ \text{Proteins/Polypeptides} + \bigcirc \text{Ag}^{+1} \rightarrow \text{Peptides/Amino acid} + \bullet \text{AgNPs} \]

DLS revealed the surface charge and hydrodynamic size of nanoparticles in aqueous colloidal dispersion [25]. As shown in figure 6, the charge and average size of the AgNPs were found to be −32.2 mV and 71.39 nm, respectively. The zeta potential degree corresponded with an initial fluctuation of AgNPs in the colloidal dispersion, but the value of −32.2 mV indicated a high static charge on the surface of AgNPs that was enough to prevent further aggregation. Notably, the Zeta potential and average size of AgNPs give an obvious character that can be applied in the biomedical field as biosensors and/or drug carriers [39].

Analysis of L. fusiformis sp. cell-free extract mediated AgNPs synthesis by TEM evidenced that the AgNPs were approximately spherical in shape with an average diameter of 50 nm (figure 7). The AgNPs were also observed to be in physically close contact but dispersed by a sufficiently uniform distance between AgNPs. The EDX analysis further confirmed the synthesis and presence of AgNPs by a single sharp signal at 3 keV (figure 8), which represents a typical optical absorption band of metallic nanocrystallites [40].

3.5. Antifungal activity of AgNPs

In order to assess the antifungal activity of the synthesized AgNPs at different concentrations, the oxford cup plate assay was performed in SDA plates by detecting the inhibition zones around AgNPs. The appearance of clear inhibition zones around AgNPs clearly indicated the potential antifungal activity of AgNPs (figure 9; table 1). As shown in table 1, the growth inhibition of cells was increased by increasing the amount of AgNPs, and the diameter of inhibition zones was different for various tinea pedis fungi. Specifically, the minimum inhibitory concentration (MIC) of AgNPs on C. albicans, T. rubrum, and T. mentagrophytes were 30, 50,
Figure 6. (a) DLS spectra on the hydrodynamic size distribution and (b) Zeta potential (mV) of synthesized AgNPs.

Figure 7. TEM image of biogenically synthesized silver nanoparticles, which were about 50 nm in size, spherical, and well dispersed.

Figure 8. EDX spectrum of silver nanoparticles, which shows a strong peak at 3 keV confirmed the presence of silver.
60 $\mu g \text{ml}^{-1}$, respectively. Therefore, we concluded that the antimicrobial activity of AgNPs on tinea pedis fungi was dependent on the concentration of the AgNPs used, and was closely associated with the formation of pits in the cell wall of fungi [41]. Following this, AgNPs accumulate in the fungi membrane and cause permeability, resulting in cell death.

The rapid spread and emergence of antibiotic-resistant strains have made the use of conventional treatment of many communicable diseases challenging, and, in rare cases, impossible to cure. Besides, the widespread of this problem has led the researchers and scientific community to predict a ‘post-antibiotic era’ [42]. It is worthy to mention that the occurrence of antibiotic resistance is an inevitable consequence of humans’ overuse and misuse of antibiotics. Several studies have reported the combined action of nanomaterials and antibiotics to overcome the problem of antibiotic abuse. Ma et al investigated the selective improvement of antibacterial effects of Bi$_2$S$_3$ nanospheres with 3 ineffective antibiotics, beta-lactam, quinolone and aminoglycoside against methicillin-resistant Staphylococcus aureus (MRSA) [43]. Naheed et al synthesized AgNPs from Desmodium triflorum and found that combining AgNPs with antibiotics showed an approximately 2-fold increase in MIC values [44]. In this work, we combined AgNPs with several antifungal antibiotics (fluconazole and ketoconazole) and found that combining nanoparticles with antibiotics showed a synergistic effect on the inhibition of fungal growth (table 2). The silver nanoparticles can be freeze-dried into powders and combined with antifungal antibiotics, such as fluconazole and ketoconazole, and will be a promising remedy for the improvement of antifungal drugs.

### 4. Conclusions

Biogenic synthesis of nanoparticles is a simple, and cost-efficient approach that has drawn much attention for its wide range of applications. In the current investigation, an extracellular green and novel approach was applied to synthesize AgNPs using a cell-free extract of L. fusiformis sp., which was isolated from a wastewater treatment plant. Without any hazardous reducing or capping agents, the cell-free extract effectively reduced Ag$^+ +$ to AgNPs
with a conversion rate as high as 72.21% with the optimal condition of pH 9.0, 50 °C, and 5 mM of AgNO₃. This conversion rate was comparable with the physical and chemical methods (70%–85%). The biosynthesis AgNPs were characterized by UV-vis, XRD, Zeta potential, FTIR, TEM, and EDX methods. Characterization results indicated that the prepared AgNPs were spherical, stable and well dispersed with a diameter of ~50 nm. The biosynthesized AgNPs were also found coated with some biological molecules, and proteins or polypeptides in the cell-free extract were deduced to play a vital role in reducing and protecting of AgNPs. The synthesized AgNPs also showed a strong antifungal activity towards pathogenic fungi of tinea pedis, i.e., *C. albicans*, *T. rubrum*, and *T. mentagrophytes*. The biomolecules binding on the surface of AgNPs provided ample room for modifications, indicating the significant potential of synthesized AgNPs for biomedicine application. The method adopted here for AgNPs biosynthesis was easy for scale-up and can be applied to many other metal nanoparticles because of the obvious oxidized nature of *L. fusiformis* sp. cell-free extract. In conclusion, the present work revealed that the biosynthesized AgNPs could be utilized as novel antimicrobial agents in medical care because of their obvious antifungal activity. Further in-vitro experiments should be carried out to investigate the biocompatibility and undesired effects of AgNPs for future therapeutic applications.

### Acknowledgments

This work was supported by the Chinese National Science Foundation (Grant No. 21576110, 21706089 and 31870952), the Natural Science Foundation of Jiangsu Province of China (Grant No. BK20171266 and BK20181480), Jiangsu Provincial Key Construction Laboratory of Probiotics Preparation Open Project (Grant No. JSYSZJ2017007 and JSYSZJ2018004), Opening Fund of the Jiangsu Provincial Engineering Laboratory for Biomass Conversion and Process Integration (Huaiyin Institute of Technology, Grant No. JPELBCP12015003), Funding of Natural Science Research Projects in Colleges and Universities of Jiangsu Province (Grant No. 19KJB550003 and 19KJB480008), and Jiangsu province Graduate student scientific research innovation projects (Grant No. SJCX18_0902). The authors would like to thank Professor Pan Changjiang for his assistance in the characterization of AgNPs.

### ORCID iDs

Pei Liu [https://orcid.org/0000-0003-1440-8851](https://orcid.org/0000-0003-1440-8851)
Muhammad Bilal [https://orcid.org/0000-0001-5388-3183](https://orcid.org/0000-0001-5388-3183)

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Table 2. Zone of inhibition of AgNPs with or without standard antifungal drugs against tested fungi.

|               | *C. albicans* | *T. rubrum* | *T. mentagrophytes* |
|---------------|---------------|-------------|---------------------|
| AgNPs (200 μg/mL) | 14.0          | 13.3        | 12.3                |
| AgNPs & fluconazole | 21.0          | 21.7        | 15.7                |
| AgNPs & ketoconazole | 23.7          | 20.0        | 17.7                |

*Table 2. Zone of inhibition of AgNPs with or without standard antifungal drugs against tested fungi.*
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