Rapid and environment-friendly preparation of silver nanoparticles and their inhibition against phytopathogenic fungi

INTRODUCTION

Metal nanoparticles have been receiving considerable attention as a result of their unique physical and chemical properties. They have been used in various fields like medicine, environmental protection, textiles, cosmetics and catalysis [1]. Among different metal nanoparticles, silver nanoparticles (AgNPs) are the most extensively studied ones because of their antimicrobial functions against the increasing threat posed by antibiotic resistant microbes [2, 3]. They are also considered to be important nanomaterials due to extensive application in biotechnology and environment remediation.

AgNPs are usually synthesized by using chemical and physical methods. But most of these methods employed toxic chemicals as reducing and stabilizing agents, organic solvents or non-biodegradable agents [4, 5], which may pose environmental impact [5, 6]. Therefore, the alternatives methods, which are less hazardous and economically viable, are being explored to overcome these drawbacks [1]. Biological methods may be relatively simple, reliable, eco-friendly and promising for the growth of advanced nanomaterials. Recently, scientists have endeavoured to make metal nanoparticles including synthesis of AgNPs, using microorganism such as bacteria, fungi and yeasts [8, 9]. Among these microbes, fungi are the most popular choice for synthesis of AgNPs due to their advantages of secreting reducing proteins and easy treatment in downstream processing [10, 11]. In addition, it can produce metal nanoparticles in a large scale within a short time [12]. And various sized monodispersed nanoparticles synthesized from fungi show antimicrobial properties against various pathogens [13].

However, some filamentous fungi used for extracellular synthesis of AgNPs, such as Fusarium oxysporum and Aspergillus fumigatus, are pathogenic to either plants or humans [14]. Thus, the successful synthesis of nanosilver grains calls for the development of a novel approach using a non-pathogenic fungus. Trichoderma spp. have been used as potent biocontrol agents for a variety of soilborne phytopathogenic fungi including soilborne, foliar and postharvest pathogens, and they have the ability for promotion of plant growth [15]. Fungal pathogens may cause substantial losses to crop production, and then the efficacious control of phytopathogens is an important issue for all agricultural systems. The traditional method to control the fungal pathogens is chemical fungicide, which may led to human health hazards and environmental pollution. In addition, the resistance to fungicides by phytopathogenic fungi has emerged in the past few decades, and it is a major challenge for industry. The spores of Trichoderma can be produced by submerged or solid state fermentation. In order to get a clear supernatant containing biologically active molecules like enzymes, the separation of biomass is much easier, and the extracellular synthesis of nanoparticles by fermentation broth has its advantage [16]. If the supernatant of the fermentation broth can be fully utilized to enhance the antifungal efficiency, it will be of great importance in industry process to reduce the cost and increase productivity.

Trichoderma asperellum Q1 is a kind of biocontrol and plant growth promoting fungi that has been isolated and identified in our lab [17]. It has been reported that some species of Trichoderma show the ability to synthesize AgNPs. For example, previous studies have confirmed that the Trichoderma atroviride based synthesis of anisotropic structural AgNPs showed remarkable antibacterial, antioxidant and cytotoxicity activities [18]. As the synthesis of nanoparticles by fungi is an emerging field of biological research technology, it is necessary to clarify the exact mechanism of silver nanoparticles synthesis [19]. Therefore, our aim in this study is to biosynthesize AgNPs rapidly using T. asperellum Q1, and to clarify the synthesis mechanism. AgNPs have shown a strong biocidal action against many bacterial strains and microorganisms [20, 21]. The antibacterial function of AgNPs is attributed to their surface interaction with bacterial cells and suppress the virulence related genes, proteins and damage the cell membrane in bacterial pathogens [22]. And studies have shown that the silver nanoparticles synthesized by Trichoderma have an inhibitory effect on tomato and chill soilborne plant pathogens [23, 24]. In this study, we will illustrate whether the AgNPs synthesized by T. asperellum would be beneficial in enhancing the activity against plant pathogenic fungi or not. If this was the case, the fungi would be of great important for further improving the antifungal ability of T. asperellum Q1.
2 | MATERIALS AND METHODS

2.1 | Source of microorganisms

2.1.1 | Species and chemicals

Pure culture of fungus *T. asperellum* Q1 were stored on sterile potato dextrose agar (PDA) at 4°C and sub-cultured every month.

Six different forma specialis (f. sp.) of phytopathogenzistic fungi, including four that may cause severe plant wilt disease: *F. oxysporum* f. sp. *conglutinis*, *F. oxysporum* f. sp. *ecumerinum*, *F. oxysporum* f. sp. *nivum*, and *F. oxysporum* f. sp. *vasinfectum*, and two plant pathogenic fungi: *Fusarium graminearum*, which can cause wheat scab disease, and *Pythium ultimum*, which can cause the damping-off and root rot diseases of hundreds of diverse plant hosts.

2.1.2 | Chemicals

All chemicals (analytical grade), for example, silver nitrate, potassium nitrate, potassium dihydrogen phosphate (KH$_2$PO$_4$), dipotassium hydrogen phosphate (K$_2$HPO$_4$), magnesium sulphate heptahydrate (MgSO$_4$·7H$_2$O), ammonium sulphate (NH$_4$)$_2$SO$_4$ and glucose, are all purchased from Guoyao Company, China.

2.2 | The preparation of AgNPs

2.2.1 | Induction of nitrate reductase

1 ml spore suspension (1 × 10$^7$ spores ml$^{-1}$) of *T. asperellum* Q1 was inoculated into flasks containing sterile PDA medium including KNO$_3$ at various concentrations, which are 0.1, 0.2 and 0.3 mol/L, respectively. A KNO$_3$-free PDA medium was used as a control and all above were incubated at 28°C in a rotary shaker at 180 rpm. The cell-free culture broth was obtained by centrifugation at 10,000 rpm for 10 min at 24 h intervals for nitrate reductase analysis.

2.2.2 | Nitrate reductase activity assay

Nitrate reductase is an enzyme that can convert nitrate to nitrite. The activity of the nitrate reductase in culture filtrate of *T. asperellum* Q1 was assayed according to previous methods (Devi et al., 2013). Culture filtrate (1 ml) was sampled every 24 h, and mixed with 1 ml assay medium (30 mM KNO$_3$ and 5% propanol in 0.1 M phosphate buffer, pH 7.5), and then incubated in the dark for 60 min. After that, the nitrates formed in the assay mixture were estimated by adding 0.5 ml of sulphanalamide and α-naphthylamine solutions. After 20 min, the developed pink colour was measured in an UV–vis spectrophotometer at 540 nm. The activity was measured by putting in the substrate for the enzyme (nitrate), and then the amount of nitrite was measured 1 h later. The net increase in nitrite at 1 h is the amount of nitrate reductase activity.

2.2.3 | Biosynthesis of AgNPs

A weighted quantity of silver nitrate was then added into the 100 ml of cell filtrate with the highest nitrate reductase activity to yield overall Ag$^+$ ions concentration of 10–3 M. The reaction was allowed to proceed in the dark at 28°C under constant agitation at 180 rpm in an orbital shaker for 72 h. The control sample, which consisted of the culture filtrate without the silver nitrate, was maintained under same conditions.

2.3 | Synthesis mechanism of AgNPs

2.3.1 | Isolation of extracellular protein involved in AgNPs biosynthesis

The reaction mixture containing the AgNPs was filtered through a 0.22 μm Millipore filter to remove residual cellular debris. The samples were centrifuged at 14,000 rpm for 30 min to separate the AgNPs, washed with sterile water and ethyl alcohol, and then re-dissolved in distilled water. The AgNPs solution was first sonicated for 5 min, then heat treated at 60°C for 5 min, and finally centrifuged at 14,000 rpm for 15 min to collect the supernatant for SDS-PAGE analysis [25].

2.3.2 | Partial purification of enzyme preparations

Culture filtrate of *T. asperellum* Q1 was prepared from KNO$_3$-inducing medium for 48 h. Following filtration through a membrane (0.22 μm), proteins in the supernatant fluid were precipitated with (NH$_4$)$_2$SO$_4$ (∼80% saturation). The precipitate was recovered by centrifugation at 10,000 rpm for 30 min, dissolved in 20 mM phosphate buffer (pH 7.5), dialyzed three times against the same buffer at 4°C overnight, and then used as the crude enzyme solution [26]. The dialyzed solution was loaded onto a DEAE Sepharose Fast Flow column that had been previously equilibrated with phosphate buffer (20 mM, pH 7.5) and then washed with phosphate buffer until the absorbance of the effluent at 280 nm reached baseline levels. After that, the solution was then eluted with the gradient of NaCl (0→1 M) prepared in the same buffer at a flow rate of 3 ml/min [16]. The fractions with nitrate reductase activity were collected for SDS-PAGE.

2.3.3 | SDS-PAGE analysis of proteins associated with AgNPs

30 μl of the crude enzyme solution, the fractions containing nitrate reductase, the supernatant possessing proteins associated with AgNPs, and a standard molecular weight marker (10–200 kDa) were loaded on 10% SDS-PAGE gel, respectively.
low molecular weight marker was used to calculate the apparent molecular weight of the enzyme. Silver staining method was employed to visualize the protein bands in the gel.

2.4 Characteristics of AgNPs

2.4.1 UV-vis spectroscopy

A UV–vis spectrophotometer was used for preliminary identification of AgNPs. The analyses were done in quartz cuvettes, using distilled water as a reference. The reaction mixture was monitored spectrophotometrically at every 30 min interval from 0 to 150 min. The reduction of Ag$^+$ ions was monitored by measuring the UV–vis spectroscopy spectrum operated at a resolution of 1 nm from 300 to 800 nm.

2.4.2 Scanning electron microscopy

Scanning electron microscopy (SEM) was used to study the size and shape of the obtained AgNPs. Samples were mounted on specimen stubs with double-sided adhesive tape, coated with palladium in a sputter coater, and examined under SEM at a voltage of 8.0 kV [16].

2.4.3 X-ray diffraction

X-ray diffraction (XRD) was performed using freeze-dried reaction mixture embedded with the AgNPs [21], and the XRD patterns were recorded on X’Pert Pro (PANalytical, USA) operating at 40 kV and a current of 30 mA with Cu Ka radiation ($\lambda = 1.54\text{Å}$).

2.5 Antifungal activity of AgNPs

The antifungal assay was performed by the agar well diffusion method [27]. All plant pathogenic fungi were maintained on PDA slants and were subcultured before use. They were grown in PDA at 28°C on a rotary shaker at 180 rpm for 24 h and then inoculated on the PDA plates by spread plating. After that, 5 mm diameter wells were made in the plates using a sterile borer. The 50 μl of synthesized AgNPs solution, cell-free supernatant, AgNO$_3$ aqueous solution (1 mM) and sterile water were loaded into the wells, respectively. After incubation at 30°C for 48 h, the diameter of the zone around each well was measured, and the results were calculated as the mean diameter of zone of inhibition (mean ± standard deviation).

3 RESULTS AND DISCUSSION

3.1 Preparation of AgNPs

3.1.1 Induction of nitrate reductase

For synthesis of AgNPs, freshly prepared AgNO$_3$ was incubated with culture supernatant of fungus T. asperellum Q1 containing nitrate reductase at room temperature. Formation of nitrate reductase activity could be detected in the supernatant broth of T. asperellum Q1 [28]. Previous studies by Bacillus licheniformis have shown that the presence of nitrate reductase is related to the synthesis of silver nanoparticles [29] and nitrate reductase is an induced enzyme [4, 29, 30]. In this study, the activity of nitrate reductase in culture supernatant of inducing and non-inducing medium was assayed. The result of the induction test showed that the activity of the enzyme was increased at different concentration of KNO$_3$, and it presented different change rule with the induction time in Figure 1. Among the three treatments, 0.1 mol/L KNO$_3$ exhibited the highest activity of the enzyme after 48 h, followed by 0.2 mol/L and 0.3 mol/L KNO$_3$. Therefore, 0.1 M KNO$_3$ within 48 h was chosen as the optimal condition for the formation of AgNPs synthesis. It is the first report that the AgNPs synthesis efficiency could be increased by using potassium nitrate as the inducible reagent in T. asperellum Q1 media.

3.1.2 Biosynthesis of AgNPs

A. (I) T. asperellum Q1 cell-free supernatant obtained from KNO$_3$-free medium after 24 h exposure to AgNO$_3$ solution, (II) T. asperellum Q1 cell-free supernatant obtained from 0.1 M KNO$_3$ medium after 24 h exposure to AgNO$_3$, (III) T. asperellum Q1 cell-free supernatant.

B. UV–visible absorption spectra of produced AgNPs. (I) Non-induced culture supernatant of T. asperellum Q1, (II) Induced culture supernatant of T. asperellum Q1.

AgNPs is easily discernible due to the colour change of the solution (Figure 2A). The supernatant of T. asperellum Q1 was slight yellow before the reaction with silver ions (Figure 2AIII). When the obtained supernatant from the broth, which used KNO$_3$ as the inducer, was subjected to AgNO$_3$, the colour of the solution turned to yellowish brown within 24 h(Figure 2AII). In contrast, the colour of the control obtained from the broth, which has no inducer, remained practically unchanged throughout this time period (Figure 2A).
3.1.3 | UV–visible spectroscopy analysis

The synthesis of AgNPs in the presence of nitrate reductase can be spectrophotometrically analyzed as the results presented in Figure 2B, which showed that the AgNPs synthesized by inducing supernatant had a stronger and broader peak. The presence of AgNPs was determined by UV–visible spectroscopy absorption spectra ranging from 300 to 800 nm wavelengths with the maximum absorbance at 430 nm corresponding to the surface plasmon resonance of silver [31]. The results showed that AgNPs produced in both inducible and non-inducible fermentation liquid had an absorption peak at 430–440 nm of the full wavelength.

It is reported that the size and shape of AgNPs can influence their optical properties [32], and the results of UV–visible spectroscopy indicated that the obtained AgNPs were spherical and nano-sized. The intensity of the plasmon peak is proportional to the concentration of AgNPs produced. In addition, higher peak intensity was observed in AgNPs produced in inducible than non-inducible fermentation liquid. These demonstrate that the intensity of the plasmon peak initially increases due to increasing concentration of the AgNPs. The activity of nitrate reductase was relatively high in inducible fermentation liquid, suggesting a positive relationship between nitrate reductase and AgNPs production.

3.2 | Synthesis mechanism of AgNPs

3.2.1 | Partial purification of nitrate reductase and SDS-PAGE analysis

Ion exchange chromatography was used to purify nitrate reductase. The column was packed with DEAE Sepharose Fast Flow pre-equilibrated with phosphate buffer (20 mM, pH 7.5). The dialyzed solution was loaded onto the column and then washed with phosphate buffer until the absorbance of the effluent at 280 nm reached baseline level. After that, it was eluted with the gradient of NaCl (0→1 M) prepared in the same buffer at a flow rate of 3 ml/min [16]. The fractions with nitrate reductase activity were collected and concentrated for SDS-PAGE.

Lane 1: extracellular proteins in cell-free culture filtrate of T. asperellum Q1; Lane 2: proteins associated with AgNPs; Lane 3: fraction from DEAE Sepharose Fast Flow

The crude enzyme solution (Lane 1), the isolated nanosilver supernatant with nitrate reductase activity (Lane 2), and the fractions obtained from the elution (Lane 3) were all loaded to a SDS-PAGE gel for electrophoresis. Upon analyzing the gel with silver staining (Figure 3), it was observed that each tested liquid showed two identical bands at 70 kDa and 55 kDa, and the two proteins in Lane 3 was at the same position as Lane 2, which implies that nitrate reductases were involved in the synthesis of AgNPs. It is the first direct evidence that the nitrate reductase produced by T. asperellum Q1 participates in the synthesis of AgNPs.

3.3 | Characteristics of AgNPs

3.3.1 | SEM analysis

(A) Induced culture supernatant of T. asperellum Q1, and (B) non-induced culture supernatant of T. asperellum Q1

The SEM micrograph provided further information about the morphological characteristics of the synthesized particles. SEM analysis showed that the majority of AgNPs produced by either the inducible or non-inducible method were relatively spherical. However, the particle size of AgNPs generated by the
inducible method was small, which was approximately 20 nm, while AgNPs synthesized using the culture supernatant of *T. asperellum* Q1 were found to be aggregates with an average size of 50 nm (Figure 4). Better control of particle size and monodispersity of nanoparticles synthesis by microbes is still being sought. It showed that the particle size of AgNPs is associated with reagents reducing or stabilizing Ag⁺ in fermentation liquid [32, 33], but the mechanism remains unclear. Moreover, the small size of AgNPs facilitates the penetration of the particle through cell membranes to affect and inhibit the normal budding process. This will result in a higher percentage of interaction than encountered with larger particles, which enhances antimicrobial activity [34, 35]. Our study indicates that the induction of nitrate reductase can either increase the productivity of AgNPs or reduce the size compared with no inductor treatments. So it is of great importance for industrial synthesis of AgNPs.

### 3.3.2  |  X-ray diffraction analysis

XRD was used to make structural analysis of the synthesized AgNPs. The XRD pattern showed four intense peaks in the whole spectrum of 2θ values ranging from 30 to 80 (Figure 5).
Scattering XRD spectra of the sample has been published by phase (JCPDS file no.04-0783). A comparison of our XRD spectrum with the standard confirmed that the silver particles synthesized in our experiments had an isotropic crystalline structure, which is demonstrated by the peaks at the $2\theta$ values of 38.12°, 46.08°, 64.42°, and 77.47°, corresponding to the 111, 200, 220, and 311 planes for silver, respectively.

3.4 Antifungal activity of *T. asperellum* Q1 synthesized AgNPs

(A) *F. oxysporum* f. sp. *cucumerinum*; (B) *F. graminearum*; (C) *F. oxysporum* f. sp. *vasinfectum*; (D) *P. ultimum*; (E) *F. oxysporum* f. sp. *conglutinans*; (F) *F. oxysporum* f. sp. *niveum*. The blank disks were impregnated with: (1) cell-free supernatant (control 1); (2) 1 mM AgNO$_3$ solution (50 μl/disk) (sample 3); (3) sterile water (control 2); and (4) synthesized AgNPs solution (50 μl/disk) (sample 4)

Antifungal activity of the synthesized silver colloid solution was determined on plant pathogenic fungi, and this test was performed on nutrient agar plates spread with microbial culture. AgNPs synthesized by *T. asperellum* Q1 showed strong antifungal activity against the plant pathogenic strains, which was confirmed by the formation of inhibition zones with varying diameters (Figure 6). In the agar well diffusion method of the antifungal activity test, the zone of inhibition was expressed as the mean ± SD ($n = 3$). The diameter of inhibition zones around each well containing AgNPs, AgNO$_3$, cell-free supernatant or sterile water, were recorded, and the data are shown in Table 1. The highest antimicrobial activity was observed against *F. oxysporum* f. sp. *niveum*, and the lowest was observed against *P. ultimum*. The result demonstrated that the inhibition rate was different among species. The control sample containing cell-free supernatant and sterile water did not show antifungal activity against any of the tested fungi. In comparison with the synthesized AgNPs, the silver nitrate solution showed lower antimicrobial activity against the tested fungi.

The use of microbial and plant extracts for the synthesis of AgNPs has been regarded as reliable and chemically friendly for the environment [36]. *Trichoderma* is a genus of fungi that is harmless to human and animals. Many species in this genus can be characterized as opportunistic avirulent plant symbionts. Conidia are the main active ingredients of *Trichoderma* preparations, and they have been registered in many countries. The liquid culture method can also be used to produce chlamydospores. The comprehensive utilization of the fermentation liquid is an effective way to improve the production efficiency.
The present study utilized agar diffusion as a way to investigate the inhibition effect of AgNPs obtained by the inducible method on several plant pathogens that cause serious harm to agricultural production. The results indicated that AgNPs inhibited pathogenic fungi. *Trichoderma* is effective bacteria for biocontrol, and AgNPs produced with its fermentation liquid hold strong potential for the development of new fungicides on plant pathogenic fungi.

4 | CONCLUSION

Nitrate reductase produced by *T. asperellum* Q1 exhibited the highest activity in inducer medium (0.1 mol/L KNO₃) after 48 h. The AgNPs were synthesized by the inducing supernatant, which lead to the development of a fast bioprocess. In addition, the result from UV–vis, SEM and XRD analysis showed that most of the particles synthesized by the inducing supernatant were spherical in shape with an average size of approximately 20 nm. The biosynthetic mechanism of AgNPs was confirmed by SDS-PAGE which showed that nitrate reductases were involved in. Antifungal activity of AgNPs was determined on plant pathogenic fungi by agar well diffusion method. The result showed that AgNPs had a stronger antifungal activity than both cell-free supernatant and silver nitrate solution. This provides a potential method for the development of new fungicides on plant pathogenic fungi.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

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