Fungus-mediated synthesis of silver nanoparticles (AgNP) and inhibitory effect on *Aspergillus* spp. in combination with antifungal agent

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Abstract

In this study, *A. niger* synthesised silver nanoparticles (AgNP) were characterised by using UV-Vis Spectrophotometry, Atomic Force Microscopy (AFM) and Transmission Electron Microscope (TEM) Analysis. The antifungal effect of synthesised AgNP and antifungal agent Amphotericin B (Amp-B) combination were investigated against *Aspergillus* spp. Antimicrobial efficiency were evaluated by Kirby Bauer Agar Disk Diffusion Test. In the end of this study, the particle size of AgNP which biosynthesised on *A. niger* were measured between 13.2-646.8 nm by AFM. The TEM analysis of AgNPs synthesised on *A. niger* were determined as a spherical in shape with different sizes 25.5-543.3 nm in the examined regions. The development of antifungal inhibition zone on *A. niger* and *A. flavus* was respectively carried out to evaluate on application of *A. niger*-AgNP; between 0-0.67 mm, 0-0.42 mm, Amp-B; 0.70-1.50 mm, 0-0.65 mm, *A. niger*-AgNP+Amp-B; 1.14-2.00 mm, 0-1 mm. According to this study data, antifungal effect of were respectively determined %0.4, %1.4, %2.4; %0.1, %0.45, %0.65 on *A. niger* and *A. flavus*. The antifungal inhibition zone occurrence indicated depent of both fungi results, the Amp-B were increase %43.91 of *A. niger*-AgNP, %40.84 of Amp-B, %84.75 of *A. niger*-AgNP. The statistical evaluation of this study showed that multiple comparison of three application on *A. niger* and *A. flavus* were significant (p<0.005).

1. Introduction

Mycotoxins are a group of toxic chemical secondary metabolites produced by some fungal species such as *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp. when they grow under favourable conditions on commonly foods and feeds [1,2,3].

Contamination of agricultural products is thought to be caused by infections in which toxin-producing fungi play a role. Mycotoxins generally cause acute and chronic effects on humans and animals. Therefore, they significantly affect human health, food safety and trade. In recent years, pathogenic bacteria and fungi have begun to develop resistance to commercially available antimicrobial agents. This has risen to the point of concern and has become a serious problem today [4,5].

Aflatoxins are mycotoxins mainly produced by *Aspergillus parasiticus* and *Aspergillus flavus*. These toxins are naturally present in feeds and foods and may show teratogenic, mutagenic and carcinogenic effects. Major naturally occurring AFs produced by aflatoxigenic fungi are known to be G1 (AFG1), G2 (AFG2), B1 (AFB1) and B2 (AFB2) [6-8]. The International Agency for Research on Cancer (IARC) announced that AFB1 is highly carcinogenic [9,10].

The biosynthesis of silver nanoparticles by different biomass is rapidly gaining importance due to ease of formation of nanoparticles and eco-friendly applications [11]. (Reference) The reactivity of silver ions is very high. In addition to inhibiting respiratory and metabolic activity by microorganisms, these ions are also highly capable of preventing physical damage caused by these organisms [12]. In addition, it is claimed that silver ions interact with the bacterial DNA after the bacterium enters the cell, thereby preventing it from proliferating within the cell [13].

Nowadays, both bacteria and fungi have been found to produce silver nanoparticles. Fungi are known to exhibit a very high tolerance to metals and accumulate them quite effectively [14]. Therefore, researchers have begun to focus on these microorganisms in the biological production of metallic nanoparticles [15]. The fungi are extremely efficient producer of extracellular enzymes, due to possible to easily obtain
production of enzymes. Producing metallic nanoparticles through fungi is a highly eco-friendly approach. This type of production also provides ease of use of biomass and economic viability. Numerous fungal species have been reported to date for the synthesis of AgNPs. These include *Rhizopus stolonifer, Penicillium citrinum, Aspergillus fawus* and Neurospora crassa [16-18].

In addition to the antimicrobial activities of AgNP, the researchers reported that they have antifungal activity against different fungal species [19-22]. According to the results of some studies, *Aspergillus terreus* is a highly capable organism for the production of silver nanoparticles [23-26]. Researchers claim that AgNPs used at low concentrations inhibit fungal growth but do not have any toxic effect on human cells [27].

Amphotericin B (AmpB), produced by Streptomyces nodosus, is one of the most potent antifungal compounds currently used, although it has serious side effects [28,29]. AgNPs have been shown to have strong antifungal activity against *Candida albicans, Trichosporon beigelli* and *Trichophyton mentagrophytes* when compared with commonly used antifungal agents such as fluconazole and AmpB [30].

In this study, *A. niger* was selected for the biosynthesis of AgNPs due to its easy isolation, growth on simple medium and has stable biochemical characteristics. AgNPs produced by using cell-free filtrate from *A. niger*, antifungal agent Amp-B alone and in AgNP-Amp-B combination against toxigenic *Aspergillus* species. Additive effects were respectively observed *A. niger* and *A. flavus*.

### 2. Materials and Methods

#### 2.1. Fungi

*A. niger* and *A. flavus* were obtained from Sivas Cumhuriyet University (SCU) Engineering Faculty, Food Engineering Department, Microbiology and Nanofood Technology Lab., Sivas. Cultures were grown in potato dextrose agar (PDA) were incubated for 7 days at 25 °C.

Identification of *Aspergillus* species is based on microscopic investigations and morphological characteristics of the colonies they produce. *Aspergillus* strains were purified through single spore isolation. The single conidial isolates were maintained on Potato Dextrose Agar (PDA) medium. Morphological features of *Aspergillus* cultures were evaluated. The major and remarkable macroscopic features used in the identification of species were colony texture, conidia and reverse colour and colony diameter according to the reports published elsewhere [31, 32].

#### 2.2. Chemicals

AgNO₃ and Amphotericin B (Amp-B) powder has been provided by Sigma &Aldrich Company, PDA, Potato Dextrose Broth Agar (PDB) Broth agar from Oxoid Company. All chemical used were of analytical grade and solutions were prepared with deionised water in experiments.

#### 2.3. Biosynthesis of AgNPs

Biosynthesis of AgNP on *A. niger* filtrate was performed according to Basavaraja et al [33]. For these experiments 250 μl of a suspension containing *A. niger* 1×10⁶ spores/ml were added to 250 ml erlenmeyer flasks containing 100 ml of Potato Dextrose Broth (PDB). Following the addition of each solvent, the cultures were shake-agitated at 120 rpm for approximately 5 days. At the end of the incubation period, mycelial biomass was separated by using filtration method. Then, in order to remove the media components completely, it was washed with sterile distilled water. After resuspending in 100 mL distilled water, it was incubated at 25°C. The suspension was filtered by using a Whatman filter paper No. 1 after 24 hours incubation period. The cell filtrate collected and used further AgNP synthesis. For the synthesis 50 ml of 1 mM AgNO₃ aqueous solution was mixed with 50 ml of *A. niger* cell filtrate in 250 ml erlenmeyer flask and agitated at 120 rpm 25°C for 72 hours in dark. A control that does not contain silver ion (contains only biomass) was also run in parallel to the experiments. All solutions were kept in dark to avoid any photochemical reactions during the experiment.

#### 2.4. Characterization of AgNPs

##### 2.4.1. UV-VIS analysis

Preliminary detection of *A. niger*-AgNP was carried out by visual observation of colour change in the cell filtrate after incubation process. The reduction of silver ion was confirmed 1 ml of sample was withdrawn after 24 h by UV-Vis spectrophotometer.

##### 2.4.2. Atomic force microscopic (AFM) analysis

AFM reveals the three dimensional picture to characterised nanoparticles. The silver nanoparticles, which are extracted according to the protocol given above, were visualized by using an AFM. A thin layer film of the sample was prepared on a glass slide by
dropping 100 μL of the sample on the slide and was allowed to dry for 5 min. The slides were then scanned with the AFM.

2.4.3. Transmission electron microscope (TEM) analysis

Transmission Electron Microscopy (TEM) was used in microscopic evaluation of particle size and shape properties of A. niger-AgNP. AgNP to be prepared in aqueous suspension, 400 mesh carbon film coated copper grids after being removed with aqueous part evaporator, was studied at 120 kV.

2.5. Antifungal activity

Kirby Bauer Disk Diffusion method was used according to Guatam et al., [34]. The in vitro antifungal activity of AgNPs, Amp-B and AgNP+Amp-B against A. niger and A. flavus were evaluated on PDA medium. The standard Amp-B disks (Amphotericin B 20; 20 μg/disk) were purchased from Oxoid. To determine the combined effect, each standard paper disk was further impregnated with 20 μL of the freshly prepared A. niger-AgNPs. Potato dextrose agar plates were inoculated with a fungal suspension (20 μL) of the test fungi. As positive control, standard antifungal Amp-B disks were used, and Amp-B disks impregnated with A. niger-AgNP were placed onto the PDA medium inoculated with test fungi. Fungal cell filtrate, which is used for the synthesis of silver nanoparticles, was used as negative control. The plates were then incubated at 25°C for 48 hours. In the case of A. niger-AgNPs, a similar experimental protocol was applied. The diameters of the zone of inhibition were measured after incubation. The assays were performed in duplicate.

2.6. Statistical analysis

The data were subjected to statistical analysis by using SPSS (Ver. 14.00). The antifungal activity were performed the double comparison (A. niger and A. flavus) by Mann Whitney U and comparison in working groups (A. niger-AgNP, Amp-B and A. niger-AgNP+Amp-B) Friedman test.

3. Results and Discussion

The strains belonging to the genus Aspergillus Section characteristically present dark-brown to black conidia, with uniseriate or biseriate conidiophores, spherical vesicles and hyaline or lightly pigmented hyphae. A. niger and A. flavus were identified through the examination of their morphological features as per the key descriptions recommended by Raper and Fennel [31] and Klich [32] presented in figure 1.

The yellow colour of the A. niger fungal cell can clearly be observed before immersion in AgNO₃. The colour changed from its natural colour to yellowish brown after 24h of incubation as well as of agitation with increasing intensity during the incubation period (Figure 2). The appearance of the brown colour was an indication of the formation of silver nano particles in the medium. Similar colour observations were noted in the several studies [24,25]. Gade et al., [35], reported that the form of silver ions using A. niger fungus filtrate AgNP thanks to the nitrate reductase and anthraquinone of the fungi.

The UV-visible spectroscopy studies could be considered as the most useful technique for structural characterization of silver nanoparticles. The technique outlined above has proved to be very useful for the analysis of nanoparticles [27].
It has been found a strong surface plasmon resonance biosynthesized AgNPs at 420 nm. This value confirms the formation of silver nanoparticles. Absorption in the 435-445 nm range is thought to result from electronic excitation generated by the amino acids tryptophan and tyrosine in the protein [36,38]. This absorption value appears to be very close to that exhibited by silver nanoparticles produced by different experimental methods [39-41]. Colloidal silver nanoparticles show strong absorbance between 390 and 420 nm. This is due to mie scattering [40].

**Figure 3.** UV-Vis spectrum of *A. niger* biosynthesized AgNPs.

The mechanism of AgNP synthesis has not yet been fully elucidated. However, it was later proposed that silver ions were necessary for the catalytic activity of NADH-dependent nitrate reductase. This enzyme is secreted into the extracellular environment by the fungus. In line with this hypothesis, the presence of NADH-dependent nitrate reductase enzyme in the extracellular filtrate of fungi tested for the synthesis of nanoparticles was determined. This data is believed to be an important step in validating the hypothesis put forward [42,43].

The synthesized AgNPs were characterized by AFM for its detail size, morphology and agglomeration of silver. It was observed that the silver nanoparticles agglomerated and formed distinct nanoparticles. The particle size of the silver nanoparticles ranges in size from 13.2-646.8 nm (Figure 4). Formation of silver nanoparticles and its agglomeration was clearly observed in figure.

**Figure 4.** Atomic Force Microscopy result of *A. niger* synthesized AgNPs.

As a result of TEM analysis of AgNPs synthesized on *A. niger*, spherical AgNP formations of different sizes between 25.5-543.3 nm were determined in the examined regions (Figure 5).

**Figure 5.** TEM images of *A. niger* synthesized AgNPs in examined regions.

In the in vitro antifungal activity, Amp-B an antifungal agent that is widely used against many fungal infections, was used as a combination with AgNP and positive (Amp-B) –negative (fungal cell filtrate) control for comparison with AgNP and Amp-B alone. The diameter of inhibition zones and increase in fold area for all the test fungi (*A. niger* and *A. flavus*) was measured. However no inhibition zone was obtained in case of the negative control (fungal cell filtrate) (Fig 6).

**Figure 6.** Antifungal activity of *A.niger*-AgNP, AMP-B against *A. niger* and *A. flavus*.

The antifungal activity of Amp-B increased significantly in presence of AgNP. The statistical evaluation of this study showed that multiple comparison of *A. niger*-AgNP, Amp-B and *A.niger* and *A. flavus* were significant (p<0.005) (Table 1; Figure 7).
Table 1. Results of statistical analysis of inhibition zones.

| Fungi     | Application      | x ± S  | Result       |
|-----------|------------------|--------|--------------|
| A. niger  | A. niger-AgNP    | 0,33±1,18 | p = 0.0001  |
|           | Amp-B            | 0,99±0,31 |               |
| A. niger  | A. niger-AgNP+B  | 1,67±0,27 | χ² = 24,00   |
| A. flavus | A. niger-AgNP    | 0,09±1,13 | p = 0.0001   |
|           | Amp-B            | 0,31±0,29 |               |
| A. flavus | A. niger-AgNP+B  | 0,45±0,36 | χ² = 18,00   |

The maximum antifungal activity was observed against 68% A. niger followed by 31% A. flavus. This was also shown through the assessment of increases in fold area of activity. It was observed that A. niger had the highest increase in the fold area from three applications (AgNP, Amp-B and AgNP+Amp-B) as respectively 0.67 mm; 1.50 mm; 2 mm while a much smaller increase in fold area 0.42 mm; 0.70 mm; 1 mm was observed for A. flavus. These findings collaborate the results obtained by several researchers [44-48]. Noorbakhsh [49], reported that combination of AgNP + Flucanazole and Griseofulvin showed a significant increase as 50% of AgNPs effect on Trichophyton rubrum. Shahverdi et al [50], investigated that antibacterial effect of AgNP and AgNP+Antibiotics combination on Staphylococcus aureus and Escherichia coli. They reported thatAgNP+Antibiotics combination increased antimicrobial effect of AgNP.

4. Conclusions

As a result, A. niger are among some fungi being used biosynthesis of AgNPs and their antifungal activities. AgNPs can be synthesized with cheapest and eco-friendly methods using A. niger. Moreover, the combination effect of a standard antifungal agent (AMP-B) with A. niger biosynthesised AgNP was observed antifungal effect against A. niger whereas much smaller effect on A. flavus. This investigation can be used in the treatment of foods and feeds to reduce the hazards of mycotoxigenic fungi, so more research work especially on experimental needs to be done. In the light of findings is promising the usage of biosynthesized AgNPs has potential as substitutes for food preservatives, pharmaceutical and agrochemical applications.

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Conflicts of interest

The author state that did not have conflict of interests.

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