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Bio-Production of silver nanoparticles by a *Brevibacillus* sp. and testing of its inhibitory efficacy on pathogenic fungi

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ABSTRACT

Seeking to find innovative technologies and treatments to eradicate pathogenic fungi that pose a hazard to human health has been a global problem given their antibiotic resistance. Currently, using silver nanoparticles that are antifungal in biocontrol methods is giving us a possible way to deal with this problem. Because metal nanoparticles have a high surface area to volume ratio, they have potent antimicrobial capabilities. This article describes a new, simple, and long-lasting way to use Brevibacillus sp. supernatants to make antifungal silver nanoparticles. We explored the microbiological composition of these nanoparticles. The AgNPs that were made had a peak absorption spectrum at 420 nm and a size range of 30.12 to 38.59 nm, as shown by spectroscopic and microscopic analysis. In vitro tests showed strong antifungal activity against *Aspergillus ochraceus* and *Alternaria alternata*, stopping the growth of colonies by 73.5% and 59%, respectively, compared to controls., reducing colony growth by 73.5% and 59%, respectively, compared to controls. This study shows that silver nanoparticles made by *Brevibacillus* sp. might be useful for fighting fungal diseases and environmental fungal contaminants. They could be a new way to deal with the worldwide problem of fungal infections.

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Introduction

Nanotechnology has emerged as a prominent topic with a wide range of applications, Across many different scientific fields, (Husen & Siddiqi 2014; Siddiqi & Husen 2016). Since they differ from bulk materials in terms of electrical, physical, optical, electromagnetic, and chemical properties, nanoparticles have been the focus of a lot of research in recent years (Mazur 2004).

The use of microorganisms like bacteria and fungi, which present a viable path for sustainable nanoparticle manufacturing, has been investigated by researchers as an eco-friendly and economical way to synthesize nanoparticles in recent years (Saifuddin et al. 2009; Shahverdi et al. 2007; Abdel-Azeem et al. 2020a; Srivastava et al. 2021). Because of their special physicochemical characteristics and possible antibacterial effects, silver nanoparticles (AgNPs) in particular have attracted a lot of research (Wei et al. 2015; Mishra et al. 2022).

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Brevibacillus species are gram positive and rod-like shaped bacteria (Carrascosa et al. 2021). *Brevibacillus* spp. can be found in a diversity of habitats, such as soil, animals the digestive tracts, the ocean, and a various kind of foods (Ruiu 2013). Based on a genetic reclassification of isolates formerly recognized as *Bacillus brevis*, the genus *Brevibacillus* was established in 1996 (Panda et al. 2014). Based on their 16S rRNA gene sequence and in-depth phylogenetic analyses, 20 species of the *Brevibacillus* genus have been identified as of the time of writing (Panda et al. 2014; Hatayama et al. 2014).

For a long time, *Brevibacillus* species were utilized as probiotics, and their metabolites were employed in biotechnology to make enzymes, amino acids, antibiotics, fermented foods, and insecticides, among other things (Sanders et al. 2003). Due to their shared probiotic characteristics, such as gut viability, certain strains were being added to a variety of food products (Tam et al. 2006). For instance, *B. laterosporus* is probiotic bacteria that is isolated from honeybees' digestive system which is shown to boost host growth (Khaled et al. 2018). While most *Brevibacillus* spp. have shown not to be involved in human health risks, few species have been linked to food spoiling, particularly milk products (Gopal et al. 2015; Yuan et al. 2012).

Brevibacillus sp. is also known for its versatile biochemistry and ability to transform metal ions into nanoparticles, making it an ideal candidate for this biogenic synthesis approach **(**Ghiuță et al. 2017). The silver nanoparticles produced through this method are expected to possess distinct characteristics that can be harnessed for various biomedical and environmental applications (Songnaka et al. 2021; Chalasani et al. 2015).

Human health is seriously threatened by fungal infections, and conventional antifungal medications frequently face problems like toxicity and drug resistance **(**Vitiello et al. 2023). *Aspergillus ochraceus* is known to produce the mycotoxins Ochratoxin A (OTA). Due to the natural occurrence of these mycotoxins in the environment, food is frequently contaminated **(**Nerva et al. 2019**)**.

OTA commonly co-occurs with citrinin. Citrinin and OTA have been linked to the development of Endemic Balkan Nephropathy in humans as well as the nephropathy of animals and birds. In comparison to ochratoxin B and ochratoxin C or citrinin, Ochratoxin is ten times more toxic. In addition to nephrotoxicity, Ochratoxin has been linked to numerous processes that can lead to immunotoxicity, hepatotoxicity, teratogenicity, neurotoxicity, and carcinogenicity.

OTA and citrinin are both harmful to development and reproduction. mitochondrial respiration, Protein synthesis, and ATP synthesis are all inhibited by OTA. Additionally, it stimulates the production of free radicals and lipid peroxidation (Nerva et al. 2019). *Alternaria alternata*, the most common mold in warm, dry areas, it has aerobic spores that usually spread in warm, dry air and temperate environments, the summer is typical when the most *A. alternata* spores are present **(**Ozdemir 2015). The outdoor fungus *A. alternata* often develops on vegetation. The species, however, may also be found indoors, where it grows in moist environments like bathrooms and frequently releases huge spores that are known to aggravate asthma and allergies diseases. **(**Fukutomi &Taniguchi 2015).

Because silver nanoparticles have a different method of action from traditional medications and are less likely to cause resistance, their use as possible antifungal drugs has promise **(**Huang et al. 2023). In contrast to physical or chemical synthesis, biosynthetic pathway mediated by bacterial supernatant culture supernatant provides the benefits of being rapid, friendly to the environment (minimal production of waste), as well as being energy efficient, as evidenced by related study (Sani et al. 2018). Although silver (Ag) ion or salts have antibacterial capabilities, the effect of silver nanoparticles (AgNPs) on bacteria and fungi and the antifungal mechanisms have yet to be extensively investigated (Kim et al. 2007).

The main objective of this research is twofold: a) Investigate the efficiency of the silver nanoparticle production by *Brevibacillus* sp. b) evaluate the ability of the silver nanoparticles produced to suppress environmental hazardous (pathogenic) fungi.

Materials and Methods

Isolation and maintenance of *Brevibacillus* isolates

Brevibacillus were isolated in the laboratory of the Environmental Research and Pollution Prevention Unit at the College of Science, Al-Qadisiyah University in September 2022, from yogurt samples using *Lactobacillus* MRS agar medium and incubated under anaerobic conditions at 37 °C for 48 hours. The diagnosis of the bacteria was confirmed based on biochemical tests and the isolates were stored in the refrigerator at 4 °C until use (Qiyi et al. 2024). Isolates were cultivated on Lactobacillus (MRS) broth medium and incubated for 48 h. at (37°C), 10 ml of broth was centrifuged for 15 minutes at 6000 rpm and supernatant (cfs) was collected (Singh et al. 2013).

Fungal isolates

Aspergillus ochraceus and *Alternaria alternata* used in this study were isolated from air. Both fungi were

isolated using Sabouraud dextrose agar (SDA) in petri dish by deposition plate technique (Abdel-Azeem & Rashad 2013). The SDA petri dishes were left open inside the laboratory, on the bench, for a period of 3 minutes, after which the dishes were closed and incubated for (5-7) days at (28°C). The phenotypic identification of both taxa were confirmed by the relevant identification keys (Simmon 2007; Abdel-Azeem et al. 2020b).

Biogenic production of metal nanoparticles

Extracellular silver nanoparticles were generated by combining a 50 ml cell free supernatant (Cfs) of 48h *Brevibacillus sp* culture supernatant (filtered through a filter membrane with a 0.2mm pore sizes) with a 50ml of aqueous silver nitrate $AgNO_3$ solution at 1mM. An orbital shaker was used to swirl the mixture, in the dark, in Erlenmeyer flasks at 200rpm for 10 minutes at 70°C .In addition to the flask used in the studies, a control flask containing cell free supernatant with no $AgNO_3$ was prepared as well (Al- Ziadi et al. 2015).

Characterization of AgNPs

Visual and spectrophotometric analysis of silver nanoparticles

After adding $AgNO_3$ and after the reaction was completed, the color of the generated nanoparticle in liquid was visually checked for change to brown color, a phenomenon because silver nanoparticle surface plasmon resonance is activated (Singh & Raja 2011). The Optical Density (O.D.) of AgNPs solution incubated in the darkness for 24 h and measured at wavelengths of 420 nm using a Carl-Zeiss Jena Spectrophotometer.

Scanning Electron Microscopy (SEM)

For size and morphological characterization of the biogenic silver nanoparticles, scanning electron microscopy (SEM) was used. AgNPs were ground or sonicated to create a colloidal solution, and then a droplet of this suspension was added to the fixing matrix to create the specimen. The sample was analyzed using SEM after drying, imaging was performed under different magnification powers considering analysis conditions including spot size (5), working distances (5-10) mm, accelerating voltage (12.5-15) kV. (Caroling et al. 2013).

Energy Dispersive Spectroscopy (EDS)

Utilizing energy dispersive spectroscopy (EDS), the composition of silver nanoparticles was examined. At compositional analysis of points and maps were examined, low vacuum mode, spot size of 5, distances of 5-10 mm and acceleration voltage (12.5-15) kV. (Caroling et al. 2013).

Evaluating the antifungal properties of AgNPs

We used the food poisoning method (Dixit et al. 1976) to test the effectiveness of 50 mg/ml how well bacteriogenic silver nanoparticles kill *A. alternata* and *A. ochraceus* isolates. 3ml of biosynthesized AgNPs were put on agar dishes with test fungus and Sabouraud dextrose agar (SDA). The dishes were then kept at 25 °C for 7 days. Control groups were also prepared and incubated at the same temperature and for the same period. We measured and recorded the inhibitory zone dimensions after incubation.

Results and Discussion

Production of bacteriogenic AgNPs

Brevibacillus was used in this study to biosynthesize AgNPs by reducing Ag ions into AgNPs. *Brevibacillus* sp. cell-free supernatants (Cfs) were combined with 1 mM $AgNO_3$ and incubated for 10 minutes at 70°C in a water bath. $AgNO_3$ reacted visually with the cell-free supernatant of *Brevibacillus* sp. and gradually changed color from colorless to brown after 10 minutes of incubation (Fig. 1). This refers to the OH groups of metabolites that are bioactive in the *Brevibacillus* sp. Cfs were electronically drawn to the positively charged (Ag) ions; these subsequently decreased to Ag0, which produced AgNPs. The emergence of a dark-brown color was a definite sign that AgNPs had been synthesized in the reaction solution. Attributed to stimulation of the surface plasmon resonance (SPR) in the metal nanoparticles; the emergence of the brown hue may be explained by the SPR characteristics of AgNPs (Raut et al. 2010).

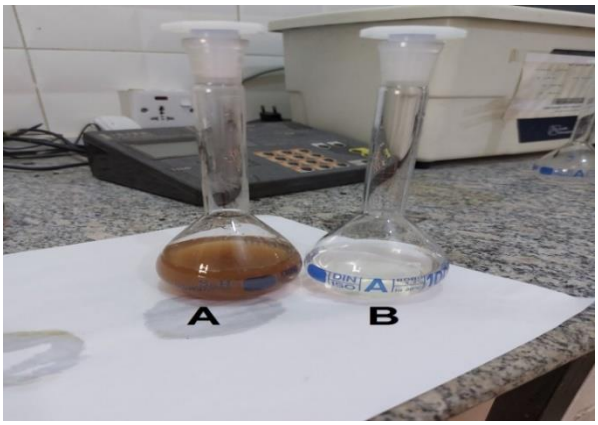

Fig 1. Color change in reaction solution comprising (A) *Brevibacillus* sp supernatants with 1mM silver nitrate in a 1:1 ratio compared with the control (B).

This result was like those that took place for AgNPs produced by various cell-free supernatants of lactic acid bacteria (LAB) (Thiruneelakandan et al. 2014; Ranganath 2012) or by employing plant leaf extracts (Khoshnamvand et al. 2018). Furthermore, a study conducted by Liu et al. (2022) demonstrated that AgNPs were biosynthesized using *Lysinibacillus sphaericus* after 24 hours, while Nallal et al. (2021) achieved success in the biosynthesis of AgNPs by combining an *A. ampeloprasum* bulb extract with 1 mM of silver nitrate in 20 minutes.

Characterization of AgNPs (UV-vis spectrum) analysis

In our study, we examined the bacteriogenic nanoparticles under the UV to visible range and how they look and behave electrically. The silver nanoparticles themselves have a wavelength of 420 nm, which is where the absorbance peak was seen in a 1 mM solution of AgNPs (Fig. 2). According to these results, *Brevibacillus* sp. supernatant did help to form silver nanoparticles extracellularly. In the same way, Dawoud et al. (2021) found a link between *Nigrospora oryzae* producing AgNPs and an absorbance peak at 420 nm. A UV-Vis test on the AgNPs showed a plasmon peak at 420 nm as well (Yassin et al., 2021). Thiruneelakandan et al. (2014) say that the color changed from clear to brown when the number of metallic ions in AgNPs made by other lactic acid bacteria (LAB) decreased.

These particles also had absorption peaks at 430 nm and 437 nm. Moreover, our findings on changing colors and the UV-Vis spectral region agreed with those of Ahmed et al. (2020). The special optical features of AgNPs have a big effect on how they react with certain wavelengths of light (Zhang et al. 2016). Additionally, UV-vis spectroscopy can identify particles in colloidal suspensions without the need for calibration because it is quick, easy, sensitive, selective for different types of NPs, and only takes a short time to measure. Many studies have shown that the best way to characterize particles between 2 and 100 nm in size is in the absorbance band between 200 and 800 nm (Tomaszewska et al. 2013).

Analysis of SEM and EDS

A scanning electron microscope (SEM) was used to measure and identify the shape and size of biogenic AgNPs. The results showed that *Brevibacillus* sp. produced spherical AgNPs that were evenly distributed. The size, shape, and dispersion are variables that affect the biotechnological and biological activities of nanoparticles (Hashem et al. 2022). According to SEM images, these AgNPs ranged in size from 30.12 to 38.59 nm (Fig. 3). Previous research on AgNPs biosynthesized using supernatant from lactic acid bacteria (LAB) strains also found that they had a spherical shape that changed with strain and ranged in size from 2 to 20 nm to 40 nm (Ranganath 2012, Panda et al. 2014).

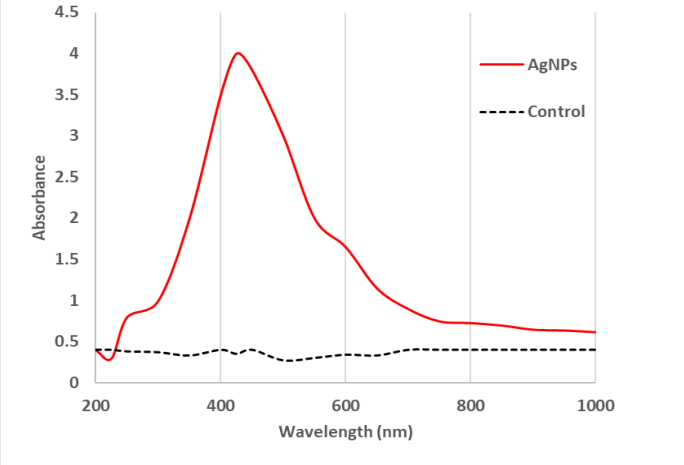

Fig 2. UV-vis spectrum analysis of biosynthesized silver nanoparticles. *Brevibacillus sp* supernatants with 1mM silver nitrate in a 1:1 ratio.

As nanoparticle sizes shrank, their activity and biocompatibility increased (Lashin et al. 2021). Therefore, it's crucial to investigate the nanoparticles morphological traits.

EDS analysis has been used to search for silver-induced optical absorption peaks in AgNPs to measure their presence. The presence of elemental silver in the EDS spectrum indicated that the reaction solution's silver ions had changed into metallic silver. We detected several signals, with oxygen and silver being the strongest (Fig. 4). The optical absorption peak was compatible with the absorption of elemental silver. The presence of oxygen peaks alongside the Ag peak suggests that oxygen atoms coat the AgNPs as biomolecules. Other minor peaks were seen that were attributed to the biomolecules being synthesized in the cell-free media (Bonnia et al. 2016).

Evaluating the antifungal properties of AgNPs

The goal of this research is primarily to find new natural antimicrobial compounds that are effective against human infections, particularly drug-resistant fungal strains. The researchers put *A. ochraceus* or *A. alternata* on Petri dishes to show that biosynthesized AgNPs could kill these fungi over the course of seven days. The fungi being studied had their growth slowed down by silver nanoparticles made from crude extracts from the bacteria *Brevibacillus* sp (Fig. 5). For *A. ochraceus*, the growth diameters were 1.5 mm, and for *A. alternata*, they were 2.33 mm, which were smaller than the controls' diameters of 5.66 mm and 7.83 mm, respectively (Figs. 6 and 7).

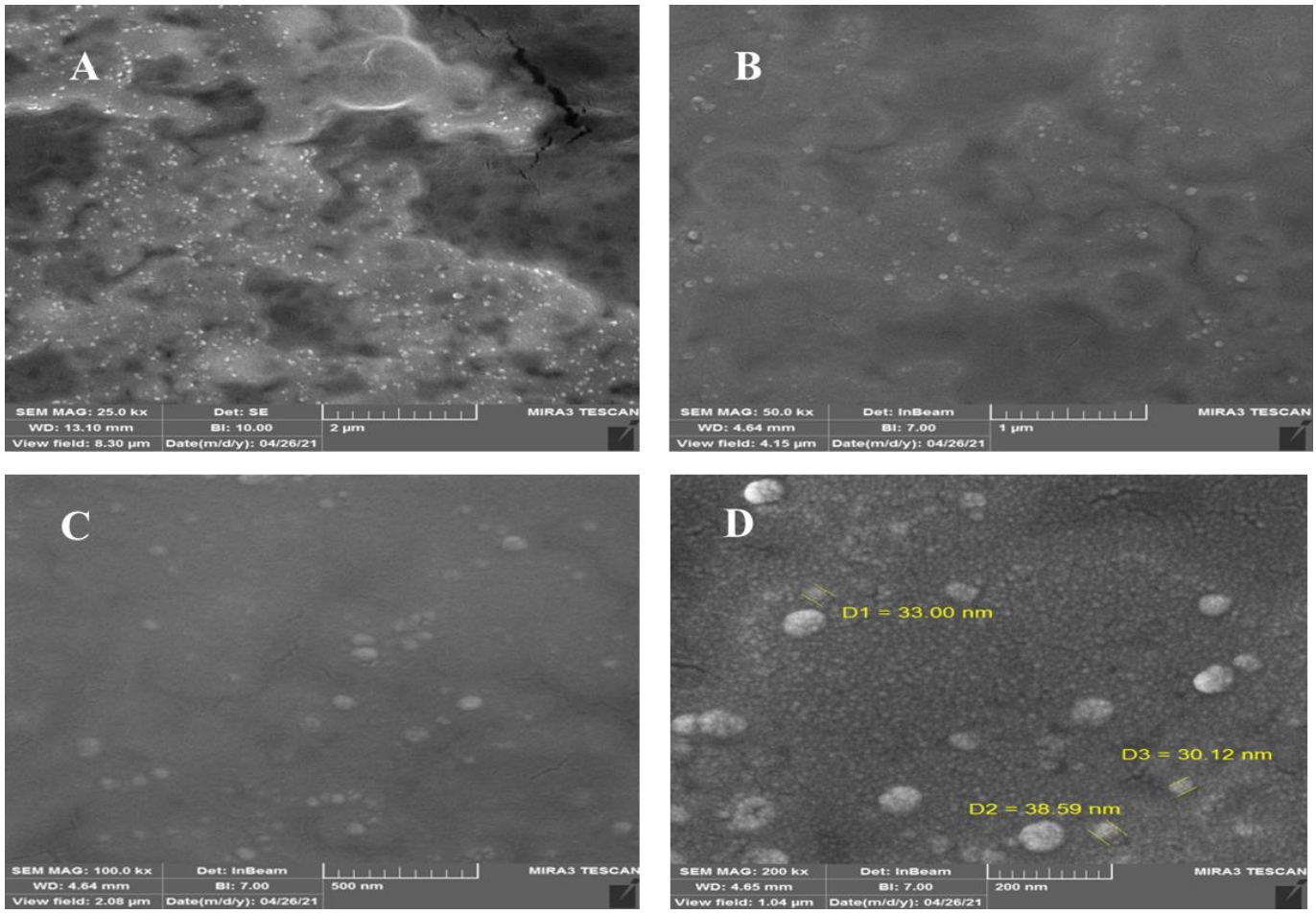

Fig 3. (SEM) micrographs of silver nanoparticles created by *Brevibacillus* sp supernatant at various magnifications, 25x (A), 50x (B), 100x (C) and 200x (D).

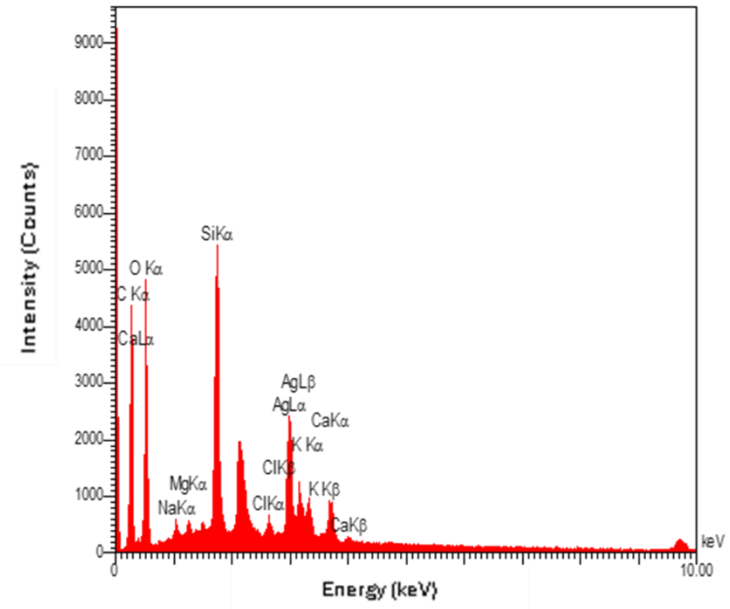

Fig 4. EDS point analysis spectrum of *Brevibacillus* sp supernatant film showing availability and abundance of AgNPs.

When *Brevibacillus* sp. metabolic products are turned into nanoparticles, they help kill the fungal cell by breaking down its cell wall. This is possible because the nanoparticles are very small and can easily get inside the cell wall. There are several ways that silver nanoparticles work. One way is by attaching to phosphate groups in DNA and the plasma membrane. This lets protons move around and kills cells in the end. (Neves et al. 2021; Salleh et al. 2020). Ag^{+} is also found to have an inhibiting action that largely impacts membrane-associated enzymes, like the respiratory chain enzymes. The gene expression of various microbial proteins and enzymes may likewise be impacted by Ag+. Through a process known as competitive inhibition, AgNPs can also engage with materials, deactivating the enzymes and inhibiting the synthesis of necessary products for normal functioning of cells (Hashem et al. 2022).

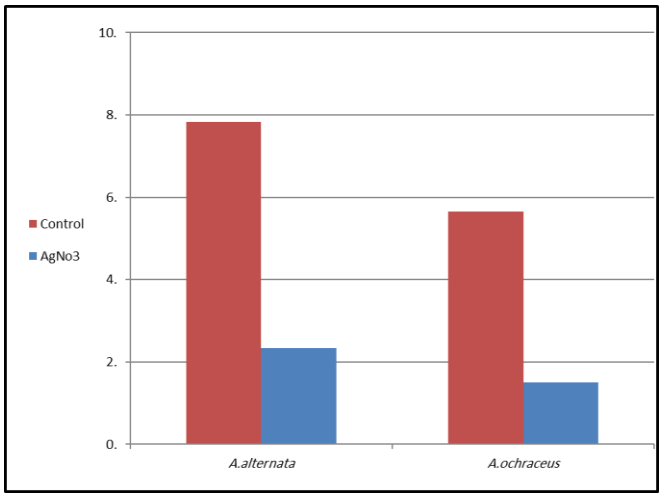

Fig 5. Bacteriogenic **A**gNPs supernatant against *A. ochraceus* and *A. alternata*.

Additionally, researchers discovered that AgNPs derived from *Bacillus subtilis* and *E. coli* culture filtrates exhibited antifungal properties against *Aspergillus fumigatus*, *Candida albicans*, and *Trichophyton* sp. These AgNPs killed the fungi by getting inside their cells (Matei et al. 2020). A study from 2019 also found that silver nanoparticles (AgNPs) made from *Allium ampeloprasum* extract could kill five types of mycotoxigenic *Aspergillus niger* with inhibition zones ranging from 8 to 14 mm (Al-Zahrani & Al-Garni 2019).

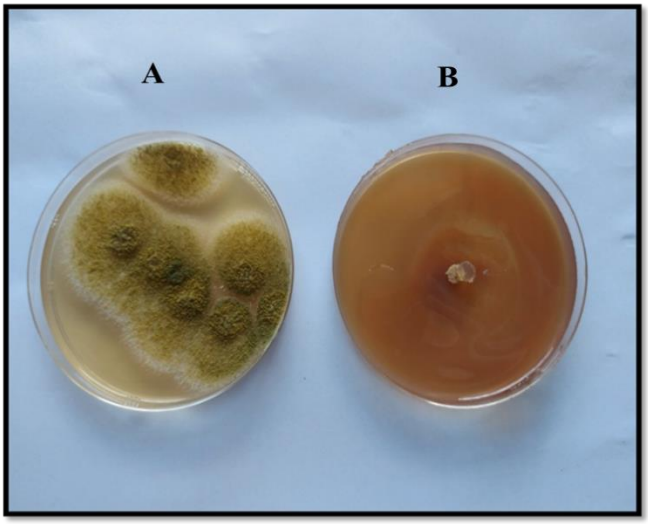

Fig 6. Antifungal activity of AgNPs- treated with *Brevibacillus sp* supernatant at ratio of 1:2 against *A. ochraceus* using agar well diffusion method. (A) represents the control (untreated) and (B) represents AgNPs treatment.

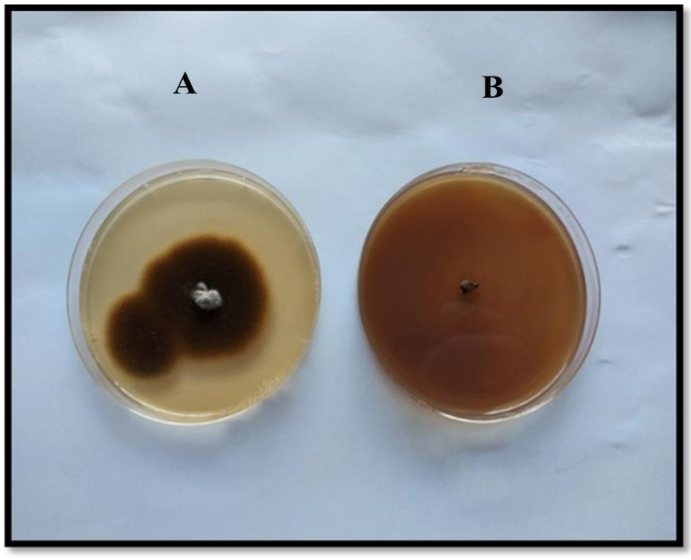

Fig 7. Antifungal activity of AgNPs- treated with *Brevibacillus sp* supernatant at ratio of 1:2 against *A. alternata* using agar well diffusion method. (A) represents the control (untreated) and (B) represents AgNPs treatment**.**

Indeed, *Brevibacillus* has yielded several bioactive chemicals including novel antimicrobial, antifungal, and antiviral agents (Ruiu 2013; Panda et al. 2014). A strain of *B. laterosporus* was shown to eliminate the development of a variety of fungi, such as *Fusarium* sp, *Aspergillus* sp and *Alternaria* sp (Saikia et al. 2011); as

well as *B. laterosporus* was reported effective against gram-negative and gram-positive bacteria (Zhao et al. 2012). An antibacterial lipopeptide known as tauramamide was recently identified in a *B. laterosporus* strain culture (Desjardine et al. 2007). This lipopeptide is a peptide chain with a specific lipophilic moiety linked to it. Indeed, these antimicrobials lipopeptide possess antibacterial, antifungal, or antiviral drugs (Evans et al. 1999; Huang et al. 2006). (Makovitzki et al. 2006) discovered that a series of lipo-peptides interactions with target pathogens, causing penetration of cell membrane and its disintegration. In a study evaluating antifungal effect of bacteriocin producing lactic acid bacteria (LAB), a zone of inhibition was evident throughout seven days (Adebayo & Aderiye 2010).

According to other investigations, *Brevibacillus* sp. facilitated AgNP synthesis, and their antibiotic efficacy against gram-positive and gram-negative humans, harmful microorganisms, which is demonstrated by varied growth zones of inhibition of 16 to 24 mm in diameter (Saravanan et al. 2018). Similar to our results, biosynthesized AgNPs by *Bacillus* MB353 shown remarkable anti-fungal activity against *Aspergillus fumigatus, A. niger,* and *Fusarium soleni* at nanoparticle concentrations of 50 µg/mL (Khan et al. 2020). Both strains of *Aspergillus flavus* and *Aspergillus parasiticus* exhibit antifungal activity in response to biogenic AgNPs derived from *Syzygium cumini* leaf extract (Asghar et al. 2020). According to Yassin et.al. (2021), AgNPs produced by *Penicillium verrucosum* prevented *Fusarium chlamydosporum* and *A. flavus* from growing radially.

Conclusions

The current study gives an insight that AgNPs can be successfully biosynthesized from *Brevibacillus* sp. is a non-toxic, inexpensive, and environmentally acceptable method. According to the attributions SEM and EDS results, the produced nanoparticles ranged in size from 30.12 to 38.59 nm and had a spherical shape. AgNPs revealed excellent performance in antifungal effectiveness against *A. ochraceus* and *A. alternata*, which is promising.

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Declaration of Competing Interest

The authors of this paper affirm that they conducted this work without any known potential conflicts of interest at the time.

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