

Synthesis of Pullulan-Mediated Silver Nanoparticles (AgNPs) and their Antimicrobial Activities

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ABSTRACT: The synthesis of silver nanoparticles (AgNPs), using plant extracts, bacteria, fungi and yeasts, and their antimicrobial activities have been widely investigated and well documented. However, pullulan AgNPs and their antimicrobial activities have not received much attention. The objective of this study was to synthesize pullulan AgNPs, characterize them, and test their antibacterial and antifungal activities. Pullulan was extracted from *Aureobasidium mangrovei* isolated from Oman and, using UV-Vis spectroscopy and Fourier Transform Infrared (FT-IR), found to be identical to the commercial pullulan obtained from Sigma, USA. Transmission electron microscopy (TEM) showed that most of the synthesized particles were poly-dispersed, irregular in shape, and most were spherical with an average size of 9.76 nm. Pullulan-mediated AgNPs were found to have antibacterial activities, and the ANOVA test showed that there were no significant differences between AgNO₃, Pullulan and pullulan-mediated AgNPs for all the bacteria tested. Pullulan-mediated nanoparticles were found to have antifungal activity against *Curvularia lunata*, *Fusarium incarnatum*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus ochraceus* and *Penicillium* sp. The ANOVA test also revealed that there was a significant difference in antifungal activity between pullulan and pullulan-mediated AgNPs, pullulan-mediated nanoparticles having shown a higher inhibitory activity than pullulan. Pullulan and pullulan-mediated nanoparticles could be used in the food industry and are safer than silver nitrates.

Keywords: Pullulan; Pullulan-mediated silver nanoparticles; Antibacterial and antifungal activities; *Aureobasidium mangrovei*.

إنتاج الجسيمات النانوية الفضية من البولولون وأنشطتها المضادة للميكروبات

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المخلص: إنتاج الجسيمات النانوية الفضية (AgNPs) باستخدام المستخلصات النباتية والبكتيريا والفطريات والخميرة وأنشطتها المضادة للميكروبات تم بحثها على نطاق واسع وتوثيقها بشكل جيد. ومع ذلك، لا تلقى الجسيمات النانوية الفضية من بولولون وأنشطتها المضادة للميكروبات اهتماماً كبيراً. الهدف من هذه الدراسة هو تجميع جزيئات الفضية النانوية بولولون، وتمييزها، واختبار أنشطتها المضادة للبكتيريا والفطريات. تم استخراج البولولون من *Aureobasidium mangrovei* المعزولة من عمان وتبين أنها مطابقة للبولولون التجاري الذي تم الحصول عليه من سيغما، الولايات المتحدة الأمريكية، باستخدام التحليل الطيفي للأشعة فوق البنفسجية UV و Fourier Infrared (FT-IR). أظهر الفحص المجهر الإلكتروني (TEM) أن معظم الجسيمات المركبة منتظمة الشكل، ومعظمها كروية بمتوسط حجم 9.76 نانومتر. أثبتت الدراسة أن AgNPs لها أنشطة مضادة للجراثيم وأظهر اختبار ANOVA أنه لا توجد فروق ذات دلالة إحصائية بين AgNO₃، بولولون و AgNPs في جميع البكتيريا التي تم اختبارها. أثبتت الدراسة أن الجسيمات النانوية لها نشاط مضاد ضد الفطريات *Curvularia lunata*، *Fusarium incarnatum*، *Aspergillus niger*، *Aspergillus flavus*، *Aspergillus ochraceus* و *Penicillium* sp. وقد أظهر اختبار ANOVA أيضاً أن هناك اختلافاً كبيراً في النشاط المضاد للفطريات بين بولولون و AgNPs المتواسطة بالبولولون حيث أظهرت الجسيمات النانوية بوساطة البولولون نشاطاً مثبطاً أعلى من نشاط بولولون. يمكن استخدام بولولون والجزيئات النانوية بوساطة البولولون في صناعة الأغذية وهي أكثر أماناً من نترات الفضة.

الكلمات المفتاحية: الجسيمات النانوية الفضية، بولولون، إنتاج، أنشطة مضادة للميكروبات.



1. Introduction

The antibacterial and antifungal properties of AgNPs have been used for many purposes, such as in biomedical and scientific applications, water purification, and food preservation [1]. This is because of their low toxicity to animal cells and high toxicity to microorganisms. Nanoparticles have a broad spectrum of antibacterial and antifungal activities and have not been associated yet with microbial resistance [2]. They have the potential of being used against multi-drug resistant microbial strains.

The synthesis of AgNPs using plant extracts, bacteria, fungi, yeasts and their antimicrobial activities has been widely investigated [3, 4] and there is a growing interest in the synthesis of nanoparticles from polymers such as polyester, cellulose, starch, dextran, alginate, and agar [2, 6]. The combined efficacy of synthesized *Aspergillus flavus* nanoparticles and different antibiotics against multi-drug resistant bacteria has been reported [5]. However, there is still very little information available on pullulan-mediated nanoparticles and their antimicrobial activities. The synthesized and characterized pullulan-mediated nanoparticles and their antibacterial activities against *Escherichia coli*, *Pseudomonas aeruginosa*, *Listeria monocytogenes* and against fungal isolates *Aspergillus* spp. and *Penicillium* spp. have been reported [6] and in addition there has been some study [2] on the activity of transparent nanocomposite films of pullulan and AgNPs against *Aspergillus niger*, which has shown that the film can be used as an antifungal packaging material.

Aureobasidium (A.) mangrovei S. Nasr is a saprophytic black yeast-like fungus that was isolated from healthy leaves of *Avicinnia marina* in Iran [7]. The species has some resemblance to *Aurebasidium pullulans* which is also a yeast-like fungus capable of producing pullulan. Pullulan, an exopolysaccharide (EPS), is a linear homopolymer with a chemical structure composed of maltotriose subunits interconnected with α -1,6 glucosidic linkages [8]. It is non-toxic, biodegradable, water soluble, non-carcinogenic, non-immunogenic and edible [9-12]. Pullulan has been applied in various fields, ranging from food manufacturing to pharmaceutical applications, and even used as a source of edible monosaccharaides. Pullulan is an excellent film former, producing a biodegradable film, which is heat sealable with good oxygen barrier properties [13]. As a result, pullulan film is sometimes referred to as "edible packaging." Coloring, flavoring, and other functional ingredients can be entrapped in the film matrix and are very stable. It has also been reported that pullulan can inhibit fungal growth in food [14]. Pullulan polymer is exploited commercially in the food industry, cosmetics, and manufacturing industries [15]. With its high viscosity, pullulan is also a candidate for enhanced oil recovery [16].

The objective of this research was to extract pullulan from *Aureobasidium mangrovei*, synthesize pullulan-mediated AgNPs, characterize them and test their antibacterial activities against *Staphylococcus aureus*, *E.coli*, *P. aeruginosa*, and *Bacillus cereus*, and their antifungal activities against *A. niger*, *A. flavus*, *A.ochraceus*, *Curvularia lunata*, *Penicillium* spp., and *Fusarium incarnatum*.

2. Materials and Methods

The fungus was isolated from *Nerium oleander* leaf at Sultan Qaboos University botanic garden (Oman). It was identified using molecular techniques as *A. mangrovei* Nasr. It was deposited at CBS, the Netherlands, having an accession number CBS 142327 and a Sultan Qaboos University number, SQU 30. It was cultured in yeast malt extract broth, placed in a shaker at a speed of 200 rpm and incubated at 25 °C for 4 to 5 days [14]. This is the second report of the existence of this species.

To remove the fungal growth, the culture was centrifuged at 15000 g for 20 min. For the extraction of pullulan, six ml of cold ethanol was transferred to three ml of cell-free culture medium in a test tube and kept at 4 °C for 12 h. The precipitate was separated by centrifugation at 5000 rpm at 4 °C for 10 min. After removal of the residual ethanol, the precipitate was dissolved in distilled water and distributed in test tubes. The tubes were kept in the freezer at -30 °C for 3 days and then lyophilized to get dry pullulan powder.

2.1 Characterization of pullulan

The extracted pullulan was scanned using Fourier Transform Infrared (FT-IR) spectroscopy [6]. It was compared with pullulan obtained from Sigma, USA (Number p4516). FT-IR spectra were recorded over a range of 4000-400 cm⁻¹. Ultraviolet-visible spectroscopy was used to identify the different concentrations of the pullulan.

2.2 Synthesis of pullulan-mediated AgNPs

For the synthesis of pullulan-mediated AgNPs, three different concentrations of pullulan (1, 3 and 5%) were mixed with 1mM freshly prepared silver nitrate solution using a magnetic stirrer. The formation of AgNPs was recognized when the color turned brown [17]. The solutions were stored at room temperature (25 °C).

Primary characterization of synthesized silver nanoparticles was carried out by UV-Vis spectroscopy. The stability of AgNPs was tested at different periods of time, 1.5 h, 3 days, 7 days and 14 days. The UV-Vis absorbance spectrum of the solution was in the range of 300-700 nm; AgNO₃ solution was used as a blank.

To examine the possible functional groups of pullulan which are responsible for the reduction and stabilization of AgNPs, FT-IR spectrum measurement was used. Dry AgNPs were prepared by lyophilization. Then, a sufficient amount of potassium bromide powder was added and placed in a mortar with a small amount of AgNPs of about the tip

of the micro spatula. The sample was crushed until it was uniformly distributed throughout the KBr. The mixture was placed in a stainless steel container and a hydraulic press was applied to produce a disk. The disk was put in the sample holder which was located in the sample compartment for scanning. FT-IR spectra were recorded at 4000-400 cm^{-1} .

Transmission electron microscopy (TEM) was used to determine the size, shape, morphology and distribution of AgNPs. For the analysis of TEM, a drop of AgNP-containing aqueous solution was placed on a carbon-coated copper grid, and then the drop was allowed to air dry completely prior to the TEM observations. At 300 kV of accelerating voltage, TEM images of AgNPs were obtained using a transmission electron microscope [6].

3. Results and Discussion

3.1 Production of pullulan

A. mangrovei had grown well in yeast malt extract broth after five days of incubation. The fungus produced pullulan together with melanin. This is the first report for this species to produce pullulan. Many strains of *A. pullulans* reported in the literature produced melanin. Pigment accumulation typically occurs late in culture growth, possibly associated with the formation of chlamyospore [9]. For commercial production, companies prefer to use strains which are non-melanin producers (mutant strains) or other non-pigment producers. However, pullulan can be purified of melanin by the addition of activated charcoal or hydrogen peroxide, sodium permanganate or other chemicals, but this makes pullulan more expensive.

3.2 Extraction of pullulan

Pullulan is synthesized intracellularly and secreted into media by *A. pullulans* strains. Most pullulan producing *A. pullulans* strains were reported from tropical countries. Different strains have different abilities in pullulan production depending on their genetics and cultural conditions and pullulan production depends both on species and strain performance and the nature of the medium. The pullulan produced by different strains have ranged from 1.3 g/L to 29 g/L when harvested from a batch culture using stirred tank fermenter [18]. About 1.6 g/L of pullulan was extracted from *A. mangrovei* after five days of incubation.

3.3 Characterization of pullulan by FT-IR analysis

The FT-IR analysis of pullulan produced by *A. mangrovei* was compared to pullulan obtained from Sigma. The strong absorption at 3435.75 cm^{-1} indicated that both the pullulans had some repeating units of $-\text{OH}$ as in sugars (Fig. 1). The other strong absorption at 2920.23 cm^{-1} indicated a $-\text{C-H}$ bond, and the absorptions at 1633 cm^{-1} a $-\text{COOH}$ bond, at 1383 cm^{-1} a C-OH bond, and at 1066 cm^{-1} the $-\text{C-O}$ bonds in the ketone compounds. The extracted pullulan peaks and the standard Sigma pullulan peaks were almost identical, which proves that our extracted compound from *A. mangrovei* was pullulan. Our results were similar to those of previous studies [19].

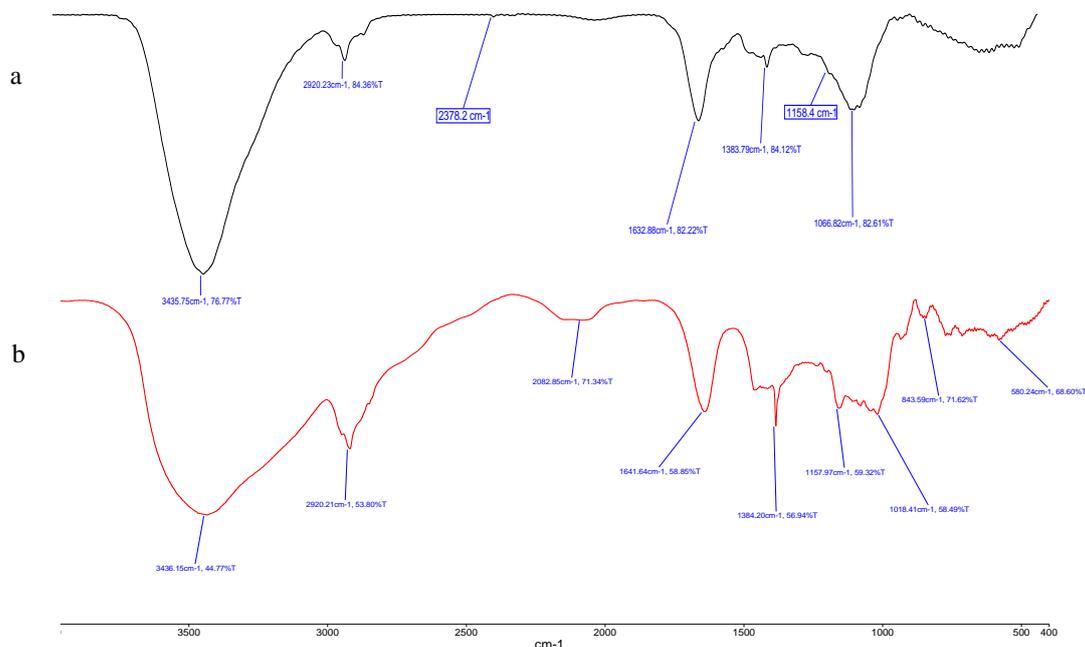


Figure 1. FT-IR of a) Standard Sigma pullulan and b) Extracted pullulan.

SYNTHESIS OF PULLULAN MEDIATED-SILVER NANOPARTICLES (AGNPS)

3.4 Characterization of AgNPs

3.4.1 UV –Vis spectroscopy

By the addition of pullulan into silver nitrate, AgNPs were synthesized by the reduction of Ag^+ into Ag^0 . The colorless solution turned to brown, which indicated the formation of AgNPs [17, 20]. The synthesis of AgNPs were tested by UV-Vis spectroscopy. Pullulan concentration (0.01%) formed AgNPs when mixed with AgNO_3 . A clear peak between 400-450 nm is an indication of AgNPs. The pullulan mediated AgNPs were tested for stability at 1.5 h, 3 days, 7 days and 14 days. The results showed that they were stable at room temperature (Figure 2).

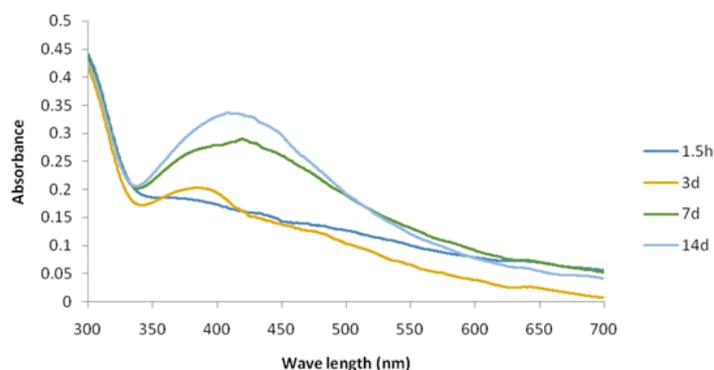


Figure 2. UV–Vis spectra of AgNPs at different periods.

3.4.2 Transmission Electron Microscopy (TEM)

TEM was used to visualize the size and shape of the pullulan mediated AgNPs. It is evident from Figure 3 that most of the synthesized particles were poly-dispersed, irregular in shape, and that most were spherical. The average diameter of the AgNPs was between 2-24 nm. The average size of the pullulan-mediated AgNPs was 9.76 nm; this figure was obtained from measuring 45 particles. It was found that the pullulan produced spherical AgNPs had a size of between 50-55 nm. [17], while in another study, the average size of the spherical AgNPs was reported to be between 2-40 nm [6].

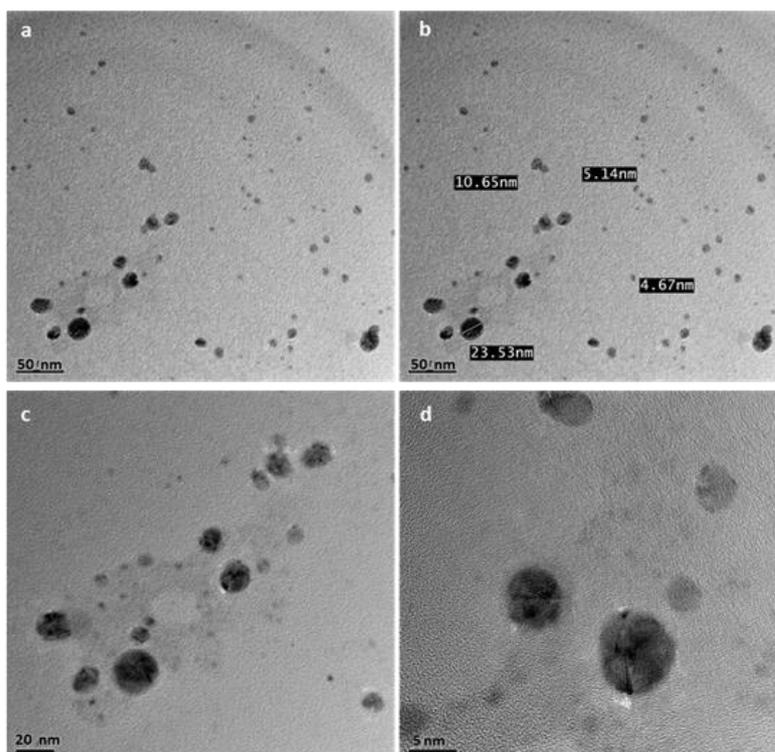


Figure 3. TEM micrograph showing the size of synthesized AgNPs. a,b) 50 nm scale, c) 20 nm scale and d) 5 nm scale.

3.4.3 Scanning Electron Microscope (SEM)

The surface morphology of the AgNPs was investigated using a SEM. The micrographs in Figure 4 show that the synthesized AgNPs had rough surfaces, and were spherical with uneven, irregular beads of different sizes. The results indicate that the reduction process takes place at the surface. The rough surface may be an advantage for the immobilization of enzymes [6]. The micrograph shows that the nanoparticles did not aggregate, but were dispersed as individuals. This means that they were stable [6].

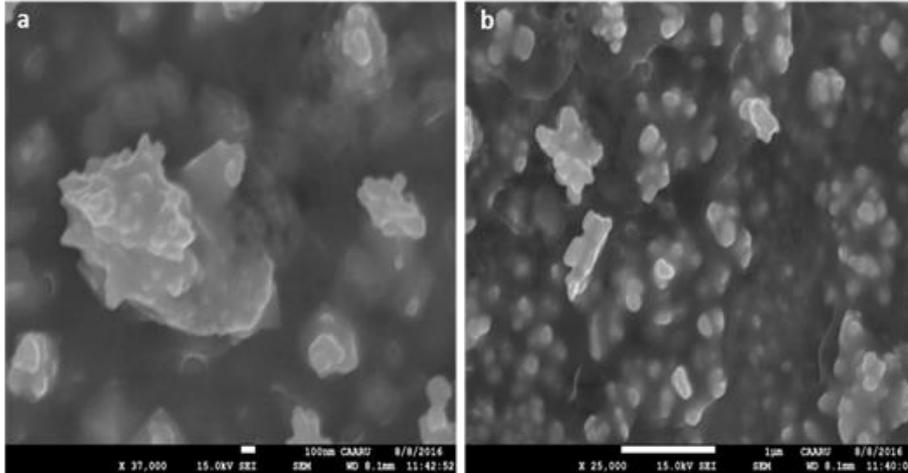


Figure 4. SEM imaging of AgNPs a) 100 nm scale; b) 1 μm scale.

3.4.4 Antibacterial activity of pullulan, AgNO₃ and AgNPs

Pullulan has been reported to have some antimicrobial properties and has been used as a coating agent. In this study, pullulan, AgNO₃ and AgNPs were tested against *E. coli*, *S. aureus*, *B. cereus* and *P. aeruginosa* obtained from The American Type Culture Collection (ATCC). Figure 5 shows that pullulan, AgNO₃, and AgNPs have antimicrobial activity against *E. coli*, *S. aureus*, *B. cereus* and *P. aeruginosa* but that the degree of inhibition, as measured by the average of inhibition zones, differs. AgNO₃ showed better antimicrobial activities against *E. coli*, *B. cereus* and *P. aeruginosa* compared to pullulan, and AgNPs, although not against *S. aureus*. The ANOVA test showed that there were no significant differences between AgNO₃, pullulan and AgNPs against any of the bacteria tested. It has previously been reported that AgNPs can inhibit the growth of Gram negative bacteria such as *E. coli* and *P. aeruginosa* [6], while other researchers [17] have reported that Gram positive bacteria such as *S. aureus* can also be inhibited by the AgNPs. It has also been reported that [20] there was no antibacterial activity or inhibition when pullulan was tested on a film of *S. aureus*, *L. monocytogenes*, *E. coli*, and *S. typhimurium*, while other researchers [6] have found that only about 5% of *E. coli* and *P. aeruginosa* is inhibited by pullulan. Our pullulan, which was extracted for the first time from *A. mangrovei*, showed better antibacterial activity against both Gram negative and Gram positive bacteria.

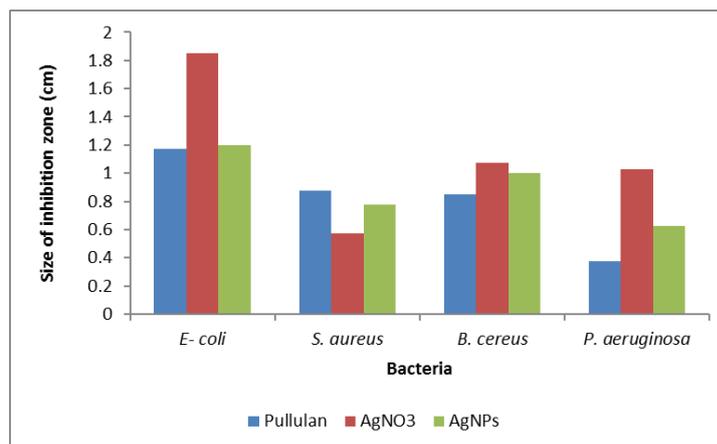


Figure 5. Antibacterial activity of pullulan, AgNO₃ and AgNPs (Average diameter of the inhibition zones).

3.4.5 Antifungal activity of pullulan, AgNO₃, and AgNPs

The antifungal activity of pullulan, AgNO₃, and AgNPs was tested against *C. lunata*, *F. incarnatum*, *A. niger*, *A. flavus*, *A. ochraceus* and *Penicillium sp.* obtained from Sultan Qaboos University Biology Department Culture Collection. Figure 6 shows that pullulan, AgNO₃, and AgNPs have antifungal activities against *C. lunata*, *F. incarnatum*, *A. niger*, *A. flavus*, *A. ochraceus* and *Penicillium*. ANOVA testing showed that there were significant differences between the anti-fungal effects of AgNO₃, pullulan and AgNPs, where AgNO₃ had a higher inhibitory effect compared to both the AgNPs and pullulan, and that AgNPs were more strongly inhibitory than pullulan. Our results are similar to those of previous researchers [6] who showed that AgNPs inhibited *Penicillium sp.* and *Aspergillus sp.* Others [21] have also found that AgNPs inhibit some fungal pathogens such as *A. niger*, *A. flavus*, *Penicillium sp.* and *Curvularia sp.*, but their reducing agent was the leaf extract of *Cassia roxburghii* and not pullulan.

The pullulan-mediated AgNPs could be used as an antifungal agent in controlling various mycotoxigenic fungi such as *A. flavus*, which produces aflatoxin that causes liver cancer, *A. ochraceus*, which produces ochratoxin that causes kidney damage, and plant pathogens such as *F. incarnatum* and food spoilage fungi such as *Penicillium* and *Rhizopus*.

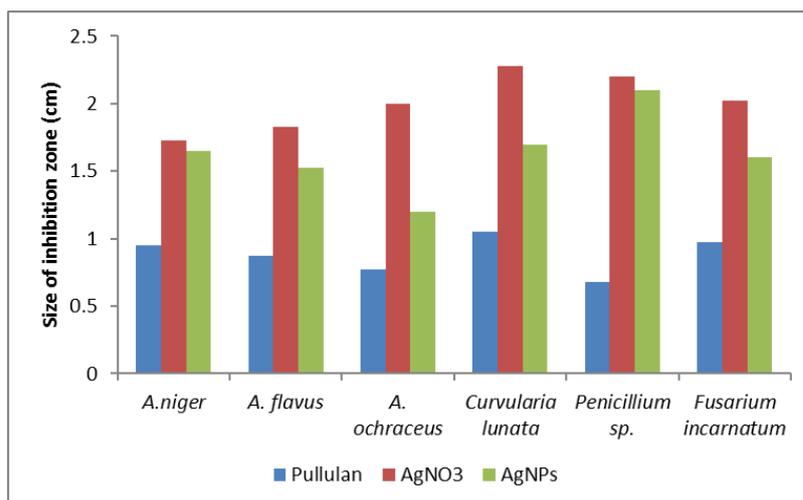


Figure 6. Antifungal activity of pullulan, AgNO₃, and AgNPs.

Conclusion

Pullulan-mediated AgNPs are of particular interest because of their ease of production, high antimicrobial activity, and ability to be incorporated into a diverse range of products. With the increasing number of antibiotic-resistant strains of bacteria and silver's high toxicity to humans, the use of AgNPs as an antimicrobial agent is an exciting topic with a great deal of relevance to many fields of study and to industry. Since only two papers have been published in using pullulan as reducing agent for the synthesis of nanoparticles, this study makes a contribution to this field. Pullulan was extracted for the first time from *A. mangrovei*, which was isolated from the SQU botanic garden. This species has shown better antibacterial and antifungal activities than other published strains of *A. pullulans*.

Conflict of interest

The authors declare no conflict of interest.

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