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Isolation and identification of bacteria from contamination by hydro-sites to produce AgNPs

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Abstract

Bacteria are widely used in the manufacture of nanoparticles as an alternative means for chemical and physical manufacturing. Studies have begun to use the biomass of bacterial cells for manufacturing. Bacteria are effective factories for the manufacture of various types of nanoparticles, such as silver, gold, platinum, and palladium. In the present study, three bacterial isolates were obtained from petroleum-contaminated soils and sewage water. The isolates were diagnosed using the Vitek 2 system and screened for AgNPs. The AgNPs were characterized by visible ultraviolet (UV) spectroscopy and scanning electron microscopy (SEM) analysis. The results showed that three isolates, B1, B4, and B5, were *Pseudomonas aeruginosa* with 79, 99 %, and 99% identity, respectively. The results showed that all isolates had the ability to synthesize AgNPs, and the color of the supernatant of all isolates changed from green to dark brown; B1 was the best for the production of AgNPs with the highest weight of 0.005 g /100 ml and absorption peak 420 nm compared with other isolates B4, and B5 0.0024 g and 0.003 g/100 ml at absorption peaks 410 nm and 365 nm, respectively.

Keywords: *Pseudomonas aeruginosa*, biochemical test, contaminated site, silver nanoparticles

Introduction

Bacteria spread in environments contaminated with crude oil and its derivatives can degrade petroleum hydrocarbon compounds (Tamis *et al.*, 2011). Pantidos and Horsfall (2014) explained the rapid and abundant growth of bacteria in the environment, and their ability to adapt to harsh environmental conditions. They are inexpensive to grow, easy to manipulate with their genetic system, and their growth conditions such as temperature, oxygen, and incubation time can be easily controlled. Bacteria are often exposed to diverse, and sometimes harsh, environmental conditions. Survival under these conditions ultimately depends on the ability to resist them. They use defense mechanisms to deal with the toxicity caused by high concentrations of metal ions in the environment, turning them into nanoparticles (Durán *et al*., 2007; Husseiny *et al*., 2007).

Shende *et al*. (2017) explained that chemical and physical methods for producing Ag nanomaterials are very costly and involve the use of toxic chemicals, which may lead to potential environmental and biological risks. The growing need to develop environmentally friendly and economically

feasible techniques for nanomaterial synthesis has led to the search for biological methods of production, and there are three main sources of AgNPs: bacteria, fungi, and plant extracts.

Bacteria are widely used in the manufacture of nanoparticles as an alternative means for chemical and physical manufacturing, and studies have begun to use the biomass of bacterial cells for manufacturing. Magnetite and cadmium sulfide Most of the metal ions are toxic to bacteria, so the reduction processes of metal ions or the formation of insoluble complexes from them is one of the defense mechanisms or means that bacteria follow to eliminate the toxicity of these metals (Klaus-Joerger et *al*., 2001 ; Lengke *et al* .,2007) . Several bacterial species, such as *Klebsiella pneumoniae*, *Escherichia coli, Acinetobacter sp.*, *Lactobacillus sp.*, *Bacillus cereus*, *Corynebacterium sp.*, *and Pseudomonas sp.*, could manufacture nanoparticles (Mohanpuria *et al*., 2008; Prasad *et al*., 2011; Iravani, 2014).

Kannan *et al*., (2010) showed that biosynthesis of Nanoparticles is conducted either inside or outside the cell (intra- or extracellularly). Depending on the organism's cellular metabolism, additional steps, such as ultrasound treatment or detergent reaction, are required to release the synthesized nanoparticles (Kalimuthu *et al*., 2008). AgNP accumulation within the cell nucleates and synthesis continues with the growth of microbes. After the optimum time for bacterial growth, nanoparticles with live cells are harvested, which requires special treatment compared with extracellular biosynthesis, which is cheap and requires simpler downstream processing. The reduction of Ag+ ions occur by combining biomolecules, polysaccharides, amino acids, proteins, enzymes, and vitamins present in the extracts; however, the most accepted mechanism is based on the presence of the nitrate reductase enzyme (Kumari et al. 2016). One of the nanomaterials with the highest level of marketing is AgNPs, which have the highest degree of commercialization in emerging nanotechnologies, as approximately 30% of all products currently registered in nano-product databases claim to contain nano-silver (Bogumiła *et al*., 2013). Therefore, the present study aimed to isolate and evaluate the efficiency of *Pseudomonas aeruginosa* isolates for producing AgNPs.

Material and methods

1. Samples collection

Water and soil samples were collected from the Hamdan sewage treatment plant and Shuaiba area in Basrah and Iraq, respectively. Water samples (500 ml) were collected in sterile containers, while ten grams soil samples were collected from different points at a depth of 5 cm using a sterile shovel and placed in sterile containers. All the samples were immediately transferred to the laboratory for analysis.

2. Isolation of bacteria.

One milliliter of serially diluted water sample was 10-3 10^{-5}**,** 100 µl of 10^{-4} and 10^{-5} dilution was withdrawn and spread on a sterile plate of nutrient agar and MacConkey agar media. For soil samples, 1 g was taken and diluted to 10^{-6}, 100 µl from 10^{-6} and 10^{-4} dilution was spread onto the same plates mentioned above, and all plates were left to dry and incubated at 34°C for 24 h. The obtained colonies were taken and subcultured by streaking on nutrient agar to obtain pure isolates, which were then kept on nutrient agar slants (Mulamattathil *et al*., 2014).

3. Phenotypic and biochemical traits

3.1. Gram stain

The shapes of the bacterial isolates and their Gram staining properties were recorded for all isolates using the Gram staining protocol (Vincent, 1970).

3.2. Catalase test

Bacterial colonies were grown at 33 °C for 24 h. Subsequently, part of the colony was placed on a clean glass slide, and a few drops of hydrogen peroxide reagent were added to the bacteria on the glass slide. A positive result is the formation or appearance of gas bubbles from bacterial growth (Finegold and Baron, 1986).

3.3. Oxidase test

A portion of the bacterial colonies that were 24 h old aseptically, using a toothpick, was placed on a filter paper, to which the oxidase reagent was added. The color of the colonies changed to violet, indicating a positive result (Cowan, 1974).

Identification of Isolates by Using Vitek 2 System

The vitek 2 system is implemented using the Vitek 2 NG REF 21341 kit, as Following (Divd 2007):

1- The bacterial culture was grown for application in NA. The cells were then incubated at 37 °C for 24 h.

2. One pure bacterial colony was transferred to 3 ml of sterile saline in a sterile canister tube.

3- The suspension was shaken by a vortex and the Vitek 2 density was measured; the degree of turbidity should be between 5.0 - 6.0.

4- The tube containing the bacterial suspension was placed in Vitek 2 case, and the label depended on the type of bacteria.

5- The Vitek 2 strips were transferred to the Vitek 2 device for diagnosing bacteria through 64 biochemical tests.

6- The results of the bacterial diagnosis appeared after 24 h.

Screening of bacterial isolates to produce AgNPs.

The bacterial isolates were sieved to produce AgNPs as follows:

Preparation of bacterial inoculum

The bacterial inoculum was prepared for each isolate by inoculating 25 ml of nutrient broth medium with 24-hour-old bacterial growth discs and incubating in a shaking incubator at a temperature of 34°C and a rotation speed of 120 rpm for 24 h.

Production of silver nanoparticles from bacterial isolates

Approximately 250 ml containing 100 ml nutrient broth medium were prepared and inoculated with 5% (v/v) of activated bacterial inoculum and incubated at 34°C with a rotation speed of 120 rpm for 24 h. After incubation, the bacterial culture was centrifuged at 6000 rpm for 25 min to obtain cell-free supernatant. A final concentration of 1 mM $AgNO_3$ was added to 100 ml of the cell-free supernatant in a 250 ml beaker volume and incubated in a dark room for 24 h to avoid any photochemical reactions. The control was maintained in a cell-free supernatant without the addition of AgNO3. After the incubation period, the change in color indicated the formation of AgNPs observed in the culture for three days. The AgNP-forming solution was centrifuged at 6000 rpm for 25 min. The filtrate was discarded and replaced with deionized distilled water. The precipitate was washed three times, dried at 40 °C, and the dried powder is gently collected and weighed (Sarvamangala *et al.,*2013).

Characteristics of nanoparticles produced from isolates.

1. Ultraviolet-Visible Spectroscopy

The cell-free supernatant containing AgNPs was subjected to absorption

analysis in the range of 200-700 nm using a spectrophotometer (Perkin Elmer Lambda 650) (Birla *et al*., 2013).

2. Scanning electron microscope (SEM)

Silver nanoparticles synthesized from cell-free supernatant were imaged using electron microscopy (SEM) to determine the size and shape of silver nanoparticles at the University of Tehran, Iran.

Results and discussion

Isolation and identification of bacteria

The results showed the isolation of 10 gram-negative bacterial isolates from both soil and water samples. Isolates B1, B2, B3, B4, and B5 were positive for oxidase and catalase. All the bacterial isolates were identified using the Vitek 2 system (Table 1).

Samples of soil and water contaminated with hydrocarbons were used to isolate Pseudomonas *aeruginosa.* This bacterium can thrive in different environments and utilize a wide range of aromatic hydrocarbon molecules as energy and carbon sources (Puskarova *et al*., 2013). These results agree with those of previous studies (Al-Razn, 2021; Alyousif *et al*., 2021; Bader *et al*., 2021) in which P. aeruginosa was isolated from hydrocarbon-contaminated soils. Wasi *et al*. (2013), Rado *et al*. (2017) and Gbashii (2020) isolated *P. aeruginosa* from water contaminated with industrial and petroleum wastes.

The results of biochemical tests Oxidase and Catalase showed that all isolates were positive for catalase by forming air bubbles, which is evidence of the production of the enzyme catalase that breaks down hydrogen peroxide O_2H_2 and raises oxygen (Mansour *et al*.,2015).

In the oxidase test, all samples yielded a positive result, except for isolate B2, which was negative for the oxidase test, as shown in Table (1). This result was consistent with the findings of Zeb *et al*. (2017).

Table 1. Biochemical tests for diagnosing bacteria

Isolates	Catalase test	Oxidase test	Gram stain
B1	+	+	_
B2	+	_	_
B3	+	+	_
B4	+	+	_
B5	+	+	_

The bacterial isolates were diagnosed using the VITEK 2 system. The results revealed that isolates B1, B4, and B5 are belonging to the species of *P. aeruginosa* with an identical ratio, 97, 99 and 99% respectively except for B2 and B3, it was *Acinetobacter baumannii* with identity of 93% and *Pseudomonas stutzeri* with identity of 99% (Table 2). The results compatible with Al-Razn (2021), who used a Vitek 2 system to identify *P. aeruginosa*. Other studies by Matiny *et al*. (2012); Sanaa, (2017) and Gbashii, (2020) diagnosed different species of *Pseudomonas* such as *P. fluorescens*, and *P. aeroginosa* using the Vitek 2 system.

Table 2. Identification of bacterial isolates using system Vitek 2

Isolates	symmetry ratio	solitude sex
B1	97%	*Pseudomonas aeruginosa*
B2	93%	*Acinetobacter baumannii*
B3	99%	*Pseudomonas stutzeri*
B4	99%	*Pseudomonas aeruginosa*
B5	99%	*Pseudomonas aeruginosa*

Production of silver nanoparticles from bacterial isolates

The bacterial isolates that were identified as *Pseudomonas aeruginosa*, such as B1, B4, and B5, were tested for the screening of AgNPs. The results showed that the color of the culture supernatant changed after 24 h of incubation from yellow to brown and dark brown, with the formation of a black precipitate with increasing incubation period compared with the control (free cell supernatant only). The B1 isolate was the best in terms of production, with a productivity of 0.005g/100 ml; the supernatant with AgNO3 changed from green to very dark brown, while the productivity of isolate B4 was 0.0024 g, where the color was pale green and turned to a very light brown. The productivity of B5 isolate was 0.003 g/100 ml with a dull brown color that turned reddish brown, as shown in (Table 3) and (Figure 1).

The change in color indicates the production of AgNPs formed because of the bioreduction process. These results agree with those reported by Wang *et al.* (2016). Bachii *et al*., (2021) used *P. stutzeri* to synthesize AgNO3, where the color of the supernatant changed after adding silver nitrate from yellow to very dark brown. Li *et al.* (2011) showed that microbial enzymes play a direct and real role in biosynthesis of nanoparticles, where nanoparticles are biosynthesized when microorganisms pick up target ions from their environment and then convert metal ions into a metal element through enzymes generated by cell activities.

Table 3. The productivity of bacterial isolates of silver nanoparticles

Isolates	Weight 100ml (/g)
B1	0.005
B4	0.0024
B5	0.003

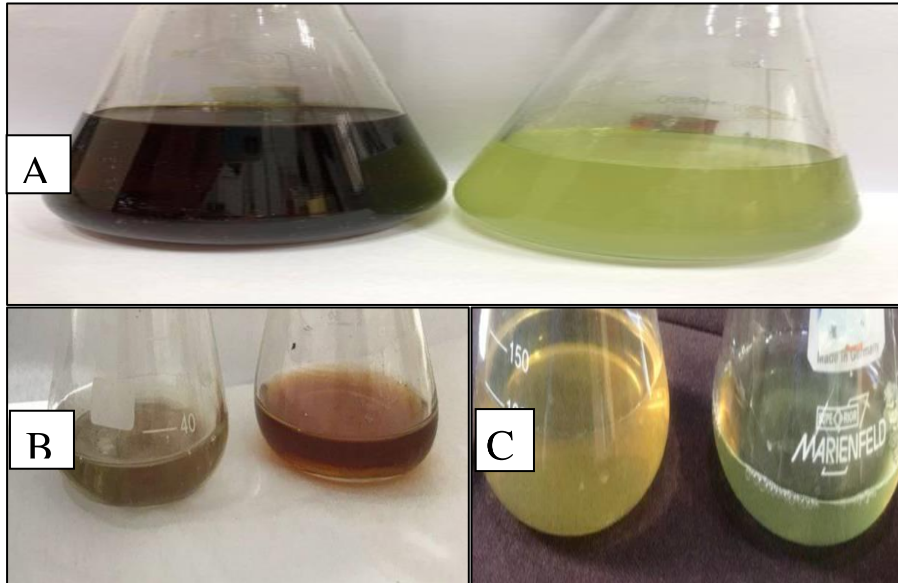


Fig.1: Production of AgNPs by *P. aeruginosa* bacteria: (A): B1, (B): B3, (C): B5

Characterization of Biosynthesis Silver Nanoparticles

1. UV-Visible Spectroscopy

The results of UV-Visible Spectroscopy showed that the highest absorption peak for B1 isolate was 420 nm, while the absorption peaks of isolates B4 and B5 were 410 nm and 365 nm, respectively, as shown in figures (2, 3, and 4). Thyagarajan *et al.*, (2017) used different types of bacteria including *Bacillus megaterium* (SIV01) and *Bacillus subtilis* (SIV02) in the production of AgNPs with absorption peaks of 450 nm. This result is consistent with that of El-Saadony *et al.* (2019), who showed the biosynthesis of silver nanoparticles using free cell supernatant of *Bacillus pseudomycoides* with an absorption peak at 420 nm. The current results agree with those of Huq (2020). Bachii *et al.*, (2021) showed AgNPs synthesis by culture supernatant of *pseudomonas stutzeri* which observed because of the color change with light wave 417 nm.

According to the bioreduction process carried out by microorganisms, the absorbance values differ because UV spectroscopy is a very important technique for detecting the formation of AgNPs because of the optical properties resulting from SPR, which are shown by nanoparticles (Othman *et al.*, 2019). The absorbance curve also shows a difference in the wavelength at which the silver particles had the highest absorbance of the B1 isolate at 420 nm. This is due to the shape and increased diameter of the silver nanoparticles suspended in a solution that increases the specific wavelength, which is the highest peak of the curve absorbance.

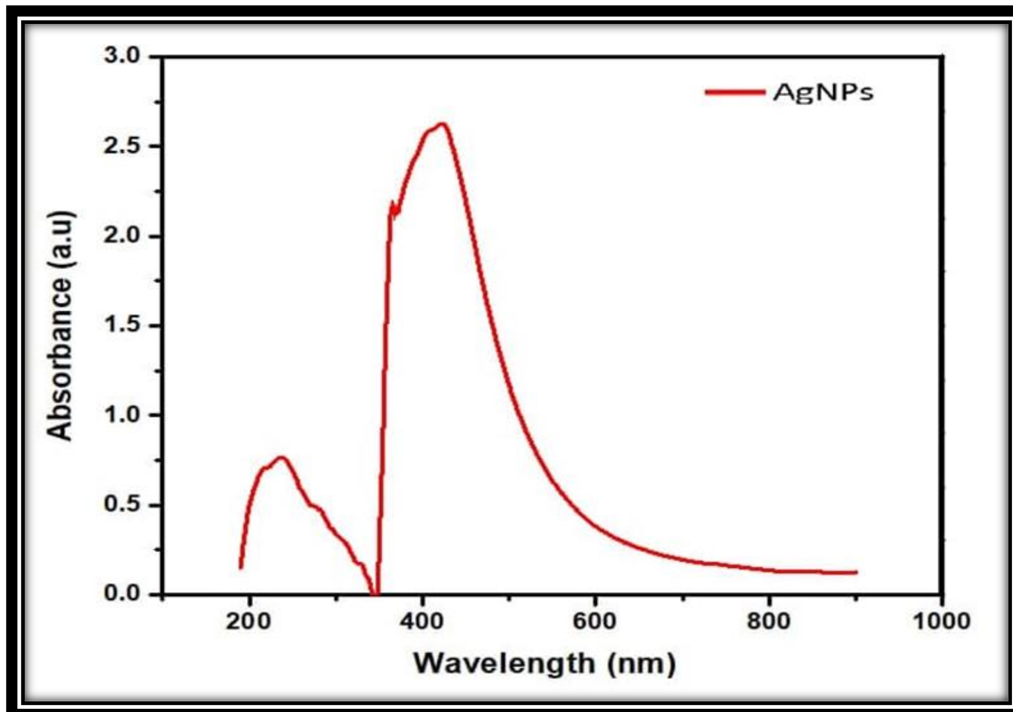


Fig.2: Absorption spectrum of AgNPs synthesized by *P. aeruginosa* (B1)

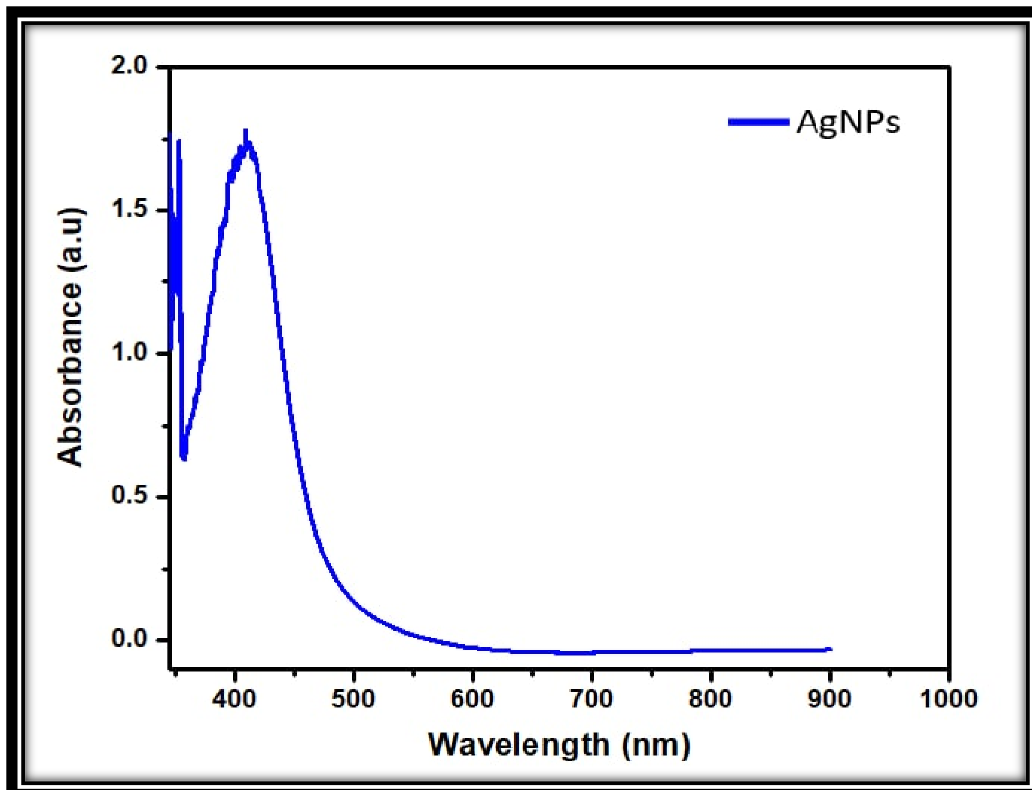
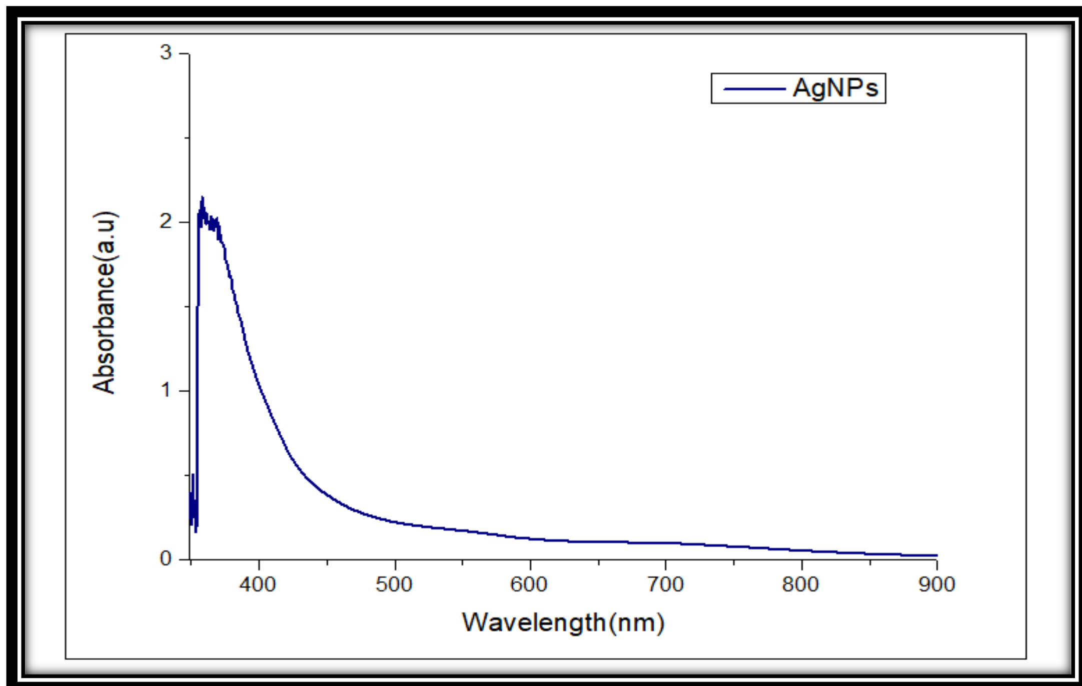


Fig.3: Absorption spectrum of AgNPs synthesized by *P. aeruginosa*(B4)



Fig, 4: Absorption spectrum of AgNPs synthesized by *P. aeruginosa*(B5)

2. Scanning electron microscope (SEM)

The shape and size of the biosynthesized AgNPs are demonstrated by the SEM micrographs shown in (Figure 5), which appear spherical in shape with sizes ranging from (46 to-48) nm. Furthermore, it was well distributed without aggregation, and the current results are consistent with those of Nithya and Ragunathan. (2011) and Bachii *et al*. (2021). Electron microscopy is an essential tool for describing the shape and basic physical properties of an adsorbed surface. It can be used to determine the particle shape, porosity, and particle-size distribution of an adsorbent (Diab *et al*., 2019).

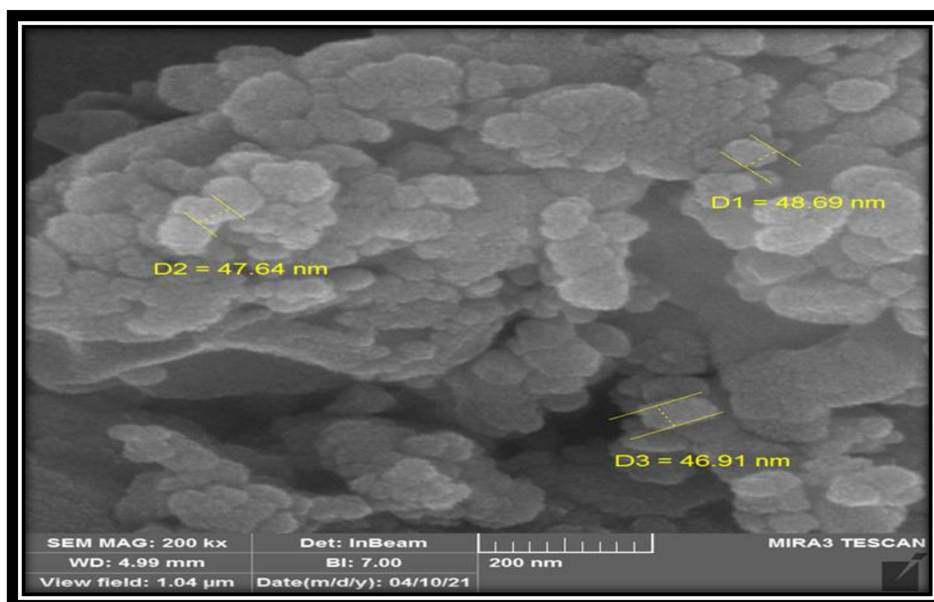


Fig. 5: Scanning electron microscopy image of AgNPs synthesized from B1 isolates B1.

Conclusions

Three bacterial isolates were obtained from petroleum-contaminated soils and sewage water. Studies have begun to use the biomass of bacterial cells for manufacture of various types of nanoparticles. The isolates were diagnosed and screened for AgNPs. The AgNPs were characterized by visible ultraviolet (UV) spectroscopy and scanning electron microscopy (SEM) analysis. The results showed that three isolates, B1, B4, and B5, were, respectively. The results showed that all isolates had the ability to synthesize AgNPs, and the color of the supernatant of all isolates changed from green to dark brown; B1 was the best for the production of AgNPs

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عزل وتشخيص البكتريا من المواقع المائية الملوثة المنتجة للنانو فضة AgNPs

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المستخلص:

تستخدم البكتيريا على نطاق واسع في تصنيع الجسيمات النانوية كوسيلة بديلة للتصنيع الكيميائي والفيزيائي. بدأت الدراسات في استخدام الكتلة الحيوية للخلايا البكتيرية في التصنيع. البكتيريا هي مصانع فعالة لتصنيع أنواع مختلفة من الجسيمات النانوية، مثل الفضة والذهب والبلاتين والبلاديوم. في هذه الدراسة، تم الحصول على ثلاث عزلات بكتيرية من التربة الملوثة .تم تشخيص العزلات باستخدام نظام Vitek 2 وفحصها بحثًا عن .AgNPs تميزت AgNPs بالتحليل الطيفي المرئي للأشعة فوق البنفسجية (UV) وتحليل الفحص المجهري الإلكتروني (SEM) أظهرت النتائج أن ثلاث عزلات، B1و B4 وB5 ، كانت *Pseudomonas aeruginosa* بنسبة 79 و 99٪ و 99٪ على التوالي. أظهرت النتائج أن ثلاث عزلات، B1و B4 وB5 ، كانت *Pseudomonas aeruginosa* بنسبة 79 و 99٪ و 99٪ على التوالي. أظهرت النتائج أن جميع العزلات لديها القدرة على تصنيعAgNPs ، وتغير لون العامل الخارق لجميع العزلات من الأخضر إلى البني الداكن ؛ كان B1هو الأفضل لإنتاج AgNPs بأعلى وزن 0.005 غم/100 مل وذروة الامتصاص 420 نانومتر مقارنة بالعزلات الأخرى B4 و B5 0.0024 غم و 0.003 غم/100 مل عند قمم الامتصاص 410 نانومتر و 365 نانومتر على التوالي.

الكلمات المفتاحية*Pseudomonas aeruginosa* ، اختبار الكيمياء الحيوية، الموقع الملوث، جسيمات الفضة النانوية