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Biosynthesis of silver nanoparticles by Enterobacter sp. and their antifungal activity against the phytopathogenic fungus Fusarium oxysporum f. sp. lycopersici

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Abstract

This study was concerned with the isolation of bacterial strains from different water sources. Among these strains; Enterobacter sp. exhibited extracellularly biosynthesized silver nanoparticles with good monodispersity. Enterobacter sp. has produced extremely stable nanoparticles within 72 hrs at 35°C in dark conditions. These nanoparticles are characterized by UV–vis spectrophotometer, transmission electron microscopy, Zeta potential analyzer, and size distribution by volume. Enterobacter sp. synthesized 9.45-17.15 nm sized spherical shaped silver nanoparticles. The presence and binding of stabilized proteins with nanoparticles were confirmed by the zeta size distribution by volume. Our study demonstrates that silver nanoparticles are potent inhibitors of the plant pathogenic fungus, Fusarium oxysporum f. sp. lycopersici.

Keywords: nanoparticle, silver, Enterobacter sp., antifungal.

Introduction

Nanobiotechnology is a branch of nanotechnology at which biological systems as microorganisms and plant extracts are used to generate nanoparticles (NPs) with specific functions that are featured by safety, non-toxicity, and eco-friendly processing (Ahmad *et al*., 2005; Boisselier and Astruc, 2009; Sharma *et al*., 2012; Kafshgari *et al*., 2015). Particles that are created with a size range of 1–100 nanometers, the materials' chemical, physical, mechanical, electronic, thermal, electrical, magnetic, dielectric, optical, and biological properties change from those at larger scales (Morais *et al*., 2014). Among biological systems, bacteria are more advantageous because of its rapid growth and high efficiency in NPs biosynthesis as bio-factors (Abbaszadegan *et al*., 2015). The supernatant broth culture of different bacterial strains is recorded as a fast bioreactor for NPs biosynthesis (Shahverdi *et al*., 2007).

The bacterial extracellular biosynthesis method has a great deal of interest due to its simplicity and

lesser time consumption in contrast to intracellular biosynthesis (Balakumarana *et al*., 2016). The shape and size of extracellular biosynthesis can also be controlled by different factors such as metal ions concentration, temperature, pH (Sathishkumar *et al*., 2010; Krishnaraj *et al*., 2012). Extracellular biosynthesis of NPs can be achieved by the reduction of metal ions in a solution by the soluble secreted enzymes then it is obvious to find NPs extracellular (He *et al*., 2007). Because of the previous advances, the bacterial extracellular biosynthesis method is often considered as the best resource for higher productivity of NPs (Shanmugaiah *et al*., 2015).

The extracellular biosynthesis of silver nanoparticles (AgNPs) has been reported using varieties of bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Lactobacillus* strains, *Morganella* sp. and others as bionanofactories (Lengke and Southam, 2006; Parikh *et al*., 2008). *Pediococcus pentosaceus* and *Enterococcus faecium* were found to synthesize AgNPs (Sintubin *et al*., 2009). The antifungal activity of AgNPs against *Fusarium oxysporum* f. sp. *lycopersici* was observed by Dar *et al*. (2013) and Devi and Joshi (2012). Silver NPs form complexes with bases contained in DNA and is a potent inhibitor of fungal DNases (Devi and Bhimba, 2014). It has been proposed that silver ions interact strongly with the thiol (-SH) group of membrane bound proteins or the lipid bilayer and that it destabilizes the membrane, causing ion leakage and cell rupture (Matsumura *et al*., 2003). As a part of our continuing study to isolate the bacterial strain with the ability to biosynthesize AgNPs with efficient biological properties, water microbes were selected. Water bacteria are relatively unexplored as a resource of bioreductants for the extracellular biosynthesis of AgNPs. This study was concerned with the isolation of bacterial strains from different localities in Damietta Governorate, Egypt with the better extracellular biosynthesis of new functional AgNPs and showing their antifungal activity *Fusarium oxysporum* f. sp. *lycopersici*.

Materials and methods

Chemicals

Silver nitrate was purchased from Panreac Quimica S.L.U, Barcelona, Spain.

Collection of water samples

Twenty water samples were collected from different localities; The River Nile, Ezbet EL-Borg canal, Ezbet EL-Borg sewage treatment station and Masraf Sittah in Damietta Governorate, Egypt. These water samples were taken from places along water source and different depths in sterile glass bottles. All samples were immediately transported to microbiology laboratory for isolation of bacteria.

Isolation of water bacteria

Samples of water were mixed well for each place separately under aseptic conditions. The bacteria were isolated by plating dilutions. Petri dishes containing nutrient agar (NA) medium were mixed well with 0.1 mL of the water sample, and incubated at 37˚C for 24 hrs. After the incubation period, the developed single colonies of bacteria which vary in shape and color were picked up and purified. The purified bacterial isolates were regularly subcultured and stored on NA slants at 4˚C for further use.

Identification of the isolated water bacteria

The purified bacterial isolates were stained with Gram and endospore stains and identified on the basis of colony characters; size, form, pigment production, elevation, surface, edge, color, opacity and physiological properties according to Bergey's Manual of Systematic Bacteriology (Garrity *et al*., 2006).

Screening of water bacteria for extracellular biosynthesis of silver nanoparticles

All the bacterial isolates were screened for the biosynthesis of AgNPs extracellularly. To prepare the bacterial supernatant, the isolates were grown aerobically in nitrate broth medium containing (g/L): peptone, 5.0; meat extract, 3; KNO_3, 1, pH 7.0±0.2 and incubated at 37˚C for 24 hrs at 150 rpm. After incubation, bacterial isolates were centrifuged at 4000 rpm for 20 min under aseptic conditions to collect the culture supernatants. A volume of 5 mL of an autoclaved aqueous solution of 1mM silver nitrate was mixed with 50 µL of culture supernatant (1% v/v) in a test tube, in addition to a test tube containing 5 mL of silver

nitrate solution and 50 µL of the nitrate broth medium as a control. All experiments were done in triplicates. The whole samples were kept in the shaking incubator at 150 rpm and maintained in dark conditions for 5 days at 37°C. After the incubation period, the reduction of silver nitrate was monitored by visible color change of the solution into brown color (Shahverdi *et al*., 2007).

16S rDNA sequencing and phylogenetic analysis

Bacterial genomic DNA was extracted by phenol/chloroform technique according to Ausubel *et al*. (2003). The 16S rRNA gene was amplified by using the prokaryotic universal pairs of primers (27F: $^{5'}$AGAGTTTGATCMTGGCTCAG$^{3'}$) and (1492R: $^{5'}$TACGGYTACCTTGTTACGACTT$^{3'}$) (Jiang *et al*., 2006). The amplified PCR product was sequenced by Macrogen, Korea using the same previous primers. BLAST (Altschul *et al*., 1990 and 1997) was performed for the resulting 16S rDNA sequence to match the best similarities with other related sequences in database. The best DNA sequence similarities with our 16S rDNA region were obtained from NCBI GenBank and aligned using CLUSTAL Omega (Sievers *et al*., 2011). Unalienable regions were excluded manually and the sequences from the same species and unidentified organisms were discarded. Finally, phylogenetic tree analyses were viewed and analyzed using MEGA version 4 (Tamura *et al*., 2007). The neighbor-joining was performed using the maximum composite likelihood methods (Tamura and Nei, 1993). The values 20 or above were only considered and represented next to the phylogenetic tree branches with confidence levels estimated by 1000 bootstrap replicates.

Characterization of biosynthesized silver nanoparticles

Characterization of AgNPs was done through visual observation of change in color and observed using UV-vis spectrophotometer (Beckman DU-40). The biosynthesized AgNPs was confirmed by sampling the reaction mixture at regular intervals and the absorption spectra was scanned at the wavelength of 370-750 nm using Unicam UV-vis Spectrometer UV2, USA (Chou *et al*., 2005). Silver nanoparticles were analyzed using transmission electron microscopy at an accelerating voltage of 200 kv using TEM JEOL JEM-2100, Japan in Electron Microscope Unit, Mansoura University, Egypt according to the method of Wang (2000). In addition, zeta potential studies and size distribution by volume, the nanocolloidal solution stability and surface charge of nanoparticles were measured by Zeta Potential Analyzer (Model Malvern Zeta-size Nano-zs90, USA) according to the method of Hanaor *et al*. (2012).

Antifungal activity of biosynthesized silver nanoparticles

The antifungal activity of the biosynthesized AgNPs against *Fusarium oxysporum* f. sp. *lycopersici*, was performed. 100 µL of each; AgNPs solution, the antifungal miconazole nitrate (conc.) (positive control), distilled water (negative control), 1 mM silver nitrate (bulk salt solution) and a mixture of AgNPs solution and miconazole nitrate (1:1 v/v) were added into separate flasks containing sterile cold melted Dox agar medium. Media were poured into sterile petri dishes in triplicates. Each plate was inoculated by a 5 mm disc of testing fungi grown on Dox agar medium after incubation at 30°C for 5 days (check???). Plates were incubated at 30°C for 5 to 7 days and radial growth of fungal mycelium was recorded in mm.

Results

Screening of water bacteria for biosynthesis of silver nanoparticles

Twenty water samples were collected from Damietta Governorate. Totally, 75 bacterial strains were isolated using NA medium. These isolates were screened for their ability to biosynthesize AgNPs using visible color change of the solution into brown color. Among them, 20 bacterial strains had the ability to produce AgNPs within 72 hrs (Table 1).

Table 1: Characteristics and screening of some isolated water bacteria for the biosynthesis of silver nanoparticles. IN=Isolate number, GM= Gram stain, EF= Endospore formation, SOA= Synthesis of AgNPs

NO.	IN	GS	SHAPE OF CELL	EF	SOA	LOCATION
1	A1	G +ve	Rod	-	-	Water of The River Nile
2	A2	G +ve	Rod	+	-	
3	A3	G +ve	Rod	-	-	
4	A4	G +ve	Rod	-	-	
5	A5	G +ve	Rod	+	-	
6	A6	G +ve	Cocci cluster	-	-	
7	A7	G +ve	Rod	-	-	
8	A8	G +ve	Cocci cluster	-	-	
9	A9	G +ve	Rod	-	+	

10	A10	G +ve	Cocci cluster	-	-	
11	A11	G +ve	Rod	-	-	
12	A12	G +ve	Rod	+	-	
13	A13	G +ve	Rod	+	-	
14	A14	G +ve	Diplococci	-	-	
15	A15	G +ve	Cocci	-	-	
16	A16	G +ve	Rod	-	-	
17	A17	G +ve	Rod	-	-	
18	A18	G +ve	Cocci	-	-	
19	A19	G +ve	Cocci	-	-	
20	A20	G +ve	Diplococci	-	-	
21	A21	G +ve	Rod	+	-	
22	A22	G +ve	Cocci cluster	-	-	
23	A23	G +ve	Rod	-	-	
24	B1	G +ve	Rod	-	-	Water of Ezbet EL-Borg canal
25	B2	G -ve	Rod	-	-	
26	B3	G +ve	Rod	+	-	
27	B4	G +ve	Rod	+	-	
28	B5	G +ve	Rod	+	-	
29	B6	G +ve	Rod	+	-	
30	B7	G -ve	Rod	-	-	
31	B8	G +ve	Rod	-	-	
32	B9	G +ve	Rod	+	-	
33	B10	G +ve	Rod	-	-	
34	B11	G +ve	Rod	-	-	
35	B12	G +ve	Rod	-	-	
36	C1	G +ve	Rod	-	++	Water of Ezbet EL-Borg sewage treatment station
37	C2	G +ve	Rod	+	-	
38	C3	G -ve	Rod	-	++	
39	C4	G +ve	Rod	-	-	
40	C5	G +ve	Rod	+	+	
41	C6	G +ve	Rod	+	-	
42	C7	G +ve	Rod	-	+	
43	C8	G +ve	Rod	+	-	
44	C9	G +ve	Rod	-	-	
45	C10	G +ve	Rod	-	+	
46	C11	G +ve	Rod	+	-	
47	C12	G +ve	Rod	+	-	
48	C13	G +ve	Rod	+	-	
49	C14	G +ve	Rod	-	++	
50	C15	G +ve	Rod	-	+	
51	C16	G +ve	Cocci	-	+++	
52	C17	G -ve	Rod	-	+++	
53	C18	G -ve	Rod	-	-	
54	C19	G +ve	Rod	+	-	
55	C20	G +ve	Rod	+	+	
56	C21	G +ve	Rod	+	-	
57	C22	G +ve	Rod	-	-	
58	C23	G +ve	Rod	-	-	
59	C24	G +ve	Rod	-	-	
60	C25	G +ve	Rod	-	-	
61	C26	G +ve	Cocci	-	+	
62	C27	G +ve	Rod	-	+	
63	D1	G -ve	Rod	-	-	Water of Masraf Sittah
64	D2	G -ve	Rod	-	-	
65	D3	G -ve	Rod	-	++	
66	D4	G -ve	Rod	-	++	
67	D5	G -ve	Rod	-	-	
68	D6	G -ve	Rod	-	++++	
69	D7	G -ve	Rod	-	-	
70	D8	G -ve	Rod	-	++++	
71	D9	G -ve	Rod	-	+++	
72	D10	G -ve	Rod	-	-	
73	D11	G -ve	Rod	-	++++	
74	D12	G -ve	Rod	-	+	
75	D13	G -ve	Rod	-	-	

Symbols: −, negative; +, very weak; ++, weak; +++, medium; ++++, high AgNPs biosynthesis.

Further, identification of the best AgNPs producer (D11 isolate) was performed using morphological characteristics as shown in Table 2 and molecular characterization. Interestingly, the isolate D11 showed clustering with different species of *Enterobacter* with 99.4-99.8 % bootstrap value in phylogram (Figure 1).

Characterization of silver nanoparticles

The absorption peaks recorded in the range 370 - 750 nm in the UV-vis spectra with the maximum peak at 4٦7 nm (check??) further confirmed the formation of AgNPs as shown in Figure 2. TEM image confirmed spherical shaped, well-dispersed AgNPs with average size of 9.45–17.15 nm diameters (Figure 3a). Zeta potential measurement showed the negative charge of the nanoparticles (Figure 3b) and size distribution by volume showed in Figure 3c.

Table 2: Morphological characteristics for identification of bacterial isolate D11.

MICROSCOPIC AND CULTURAL CHARACTERS	**REACTIVITY**
Optimum temperature	35˚C
Air condition	Facultative anaerobic
Gram stain	Negative
Shape	Rod
Spore formation	Absence
Pigmentation	Yellow pigment
Growth on isolation medium	Grow on nutrient agar medium, circular white moist smooth opaque colonies with entire edge accompanying with yellow pigment

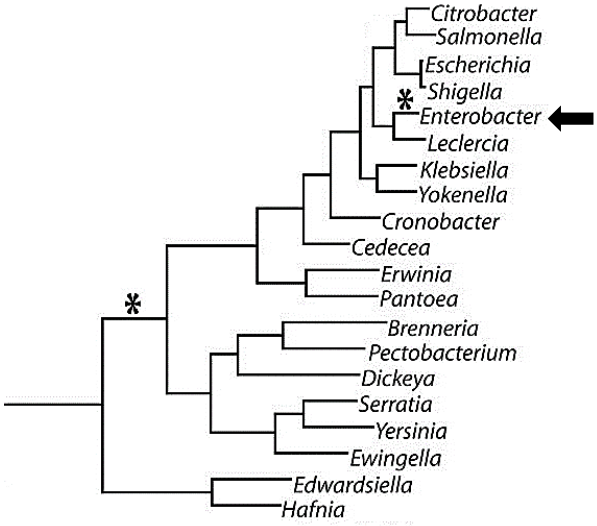


Figure 1. Phylogenetic tree analysis based on 16S rDNA sequence alignment for *Enterobacter* sp. with some other related species, which possessed the highest similarity. The neighbour-joining was performed using the maximum composite likelihood methods (Tamura and Nei, 1993).

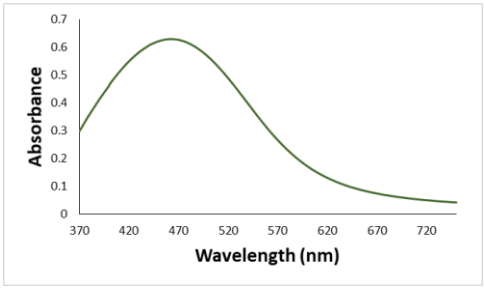
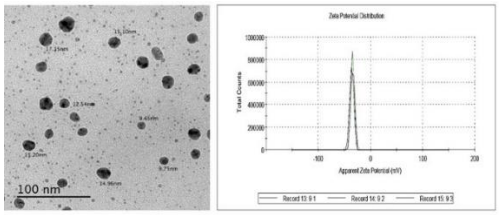


Figure 2. Silver nanoparticles synthesized using *Enterobacter* sp. bacterial supernatant. The tested production supported the nanoparticle synthesis as it can be seen from UV-vis spectra.

(a) (b)



(c)

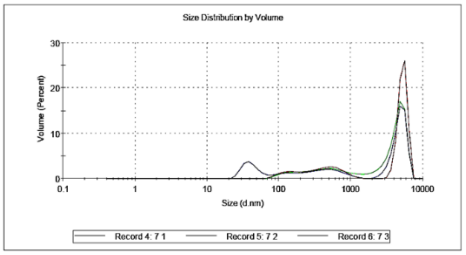


Figure 3. Characterization of silver nanoparticles synthesized using *Enterobacter* sp. supernatant. (a) Transmission electron microscopic image of spherical shaped silver nanoparticles. The size of the nanoparticles was 9.45-17.15 nm (scale bar = 100 nm). (b) Zeta potential measurement analysis of silver nanoparticles. (c) Size distribution by volume.

Antifungal activity of AgNPs against Fusarium oxysporum f. sp. lycopersici

AgNPs synthesized from *Enterobacter* sp. showed inhibitory activity against *Fusarium oxysporum* f. sp. *lycopersici* and the data are presented Table 3 and Figures 4 and 5.

Table 3: Radial growth of *Fusarium oxysporum* f. sp. *lycopersici* mycelium (mm)

Fungus	**Radial Growth of Fungal Mycelium (mm)**				
	control	AgNPs	miconazole nitrate	AgNPs + miconazole nitrate	$AgNO_3$ solution
Fusarium oxysporum f. Sp. *Lycopersici*	100	51.88	44.25	61.03	79.17

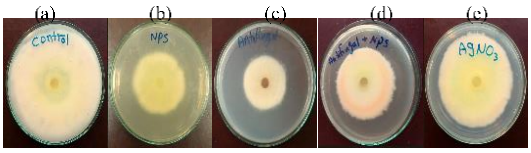


Figure 4: Radial growth of fungal mycelium that was recorded for tested fungus *Fusarium oxysporum* f. sp. *lycopersici*. (a) Control, (b) AgNPs, (c) miconazole nitrate, (d) AgNPs + miconazole nitrate, (e) silver nitrate solution.

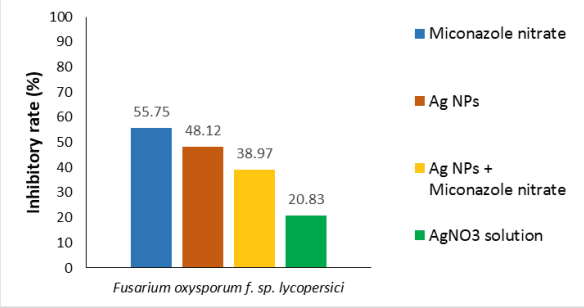


Figure 5: Inhibitory rate (%) against *Fusarium oxysporum* f. sp. *lycopersici*.

Discussion

Water environment is considered as a reservoir of biological as well as chemical diversity. The present study was aimed to isolate water bacteria from different water sources in Damietta Governorate, Egypt with an ultimate objective to produce antifungal nanoparticles. The results showed that there were a synthesis of AgNPs differed from bacterial isolates. There are about three bacterial isolates had the best production rates among others. Only three isolates have synthesized extremely stable nanoparticles. The isolate designated as D11 produced the metal nanoparticles within 72 hrs in dark conditions which was similar to the production using *Bacillus flexus* (Payal and Nikhilesh, 2017). Interestingly, the isolate D11 was identified as *Enterobacter* sp. The complete molecular studies together with classical taxonomy results strongly supported the identification of isolated strain as *Enterobacter* sp. In this study, based on the particle stability and faster rate of synthesis, *Enterobacter* sp. was recognized as a potential strain for the extracellular synthesis of silver nanoparticles.

The first indication for AgNPs biosynthesis is the color change. Brown color formed in the medium indicated the biosynthesis of AgNPs. The color change was due to the excitation of surface Plasmon vibrations, which is a characteristic feature of synthesized nanoparticles (Kalimuthu *et al*., 2008; Das *et al*., 2014). *Enterobacter* sp.

produced spherical shaped, well-dispersed AgNPs, which similar to *Bacillus* sp. AgNPs extracellular biosynthesis by Vithiya *et al*. (2014) and Gopinath and Velusamy (2013) in case of *Bacillus* sp. GP-23 which produced spherical shaped AgNPs in addition to *Acinetobacter baumannii* (Shaker and Shaaban, 2017) and *Leuconostoc lactis* (Saravanan *et al*., 2017). The particle size distribution analysis revealed an average size of 9.45-17.15 nm diameter which matched with Balakumarana *et al*. (2016) who obtained AgNPs which have size ranged from 8-20 nm. Zeta potential measurement confirmed the charge of the nanoparticles and it was found to be a negative for the metal nanoparticles. Much similar to our present study, Elbeshehy *et al*. (2015) have shown a negative zeta potential for *Bacillus* spp. that synthesized AgNPs. Wrótniak–Drzewiecka *et al*. (2014) found a surface zeta potential graph showing the negative zeta potential value for AgNPs synthesized by cell free extract of *Myxococcus virescens* and Boopathi *et al*. (2012) also showed a negative zeta potential for *Pseudomonas aeruginosa* M6 synthesized AgNPs. A common problem observed with nanoparticles production is aggregation, which greatly decreases the surface area of the nanoparticles and, in turn, affects their chemical, physical, and biological properties (Sintubin *et al*., 2012). To assess the stability of AgNPs formed in the medium, UV-vis spectrophotometric study was carried out. In case of silver nanoparticles, there was no alteration in the peak at 467 nm even after 2 months of incubation period. There was no sign of aggregation of nanoparticles observed (Balakumaran, 2016). The synthesized AgNPs from *Bacillus* spp. were found to be stable for up to 48 h (Elbeshehy *et al*., 2015). Thus, it is obvious from these observations that, the biosynthesized AgNPs are stabilized by the capping agent that is likely to be proteins present in the *Enterobacter* sp. supernatant.

AgNPs synthesized from *Enterobacter* sp. showed a potent antimicrobial activity against *Fusarium oxysporum* f. sp. *lycopersici*. AgNPs synthesized from *Enterobacter* sp. exhibited potent inhibitory activity with a clear inhibition rate (48.12%). The synthesized AgNPs matched with significant antifungal activity against *Fusarium oxysporum* f. sp. *lycopersici* by Dar *et al*. (2013) and Devi and Joshi (2012).

Conclusions

To our knowledge, this is the first study in which, AgNPs were synthesized using the bacterial supernatant of *Enterobacter* sp., isolated from water samples in Damietta Governorate. The biosynthesis protocol employed in the present study was found to be simple, rapid, cost-effective, and ecofriendly. Thus, this extracellular synthesis method has huge potential to develop into the simple bioprocess system for sustainable production of nanoparticles at larger amount in addition to their high stability and a negative zeta potential. Moreover, the remarkable antimicrobial activity of AgNPs produced from *Enterobacter* sp. was detected against *Fusarium oxysporum* f. sp. *lycopersici*.

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الملخص العربي

عنوان البحث: تخليق جسيمات الفضة النانوية بواسطة بكتريا انتيروباكتر و دراسة قدرتها التضادية لفطر فيوزاريم اوكسيسبورم ليكوبيرسيسي

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تم أخذ عينات مياه من مناطق مختلفة بمحافظة دمياط (مثل مجرى نهر النيل برأس البر- محطة الصرف الصحي بمدينة عزبة البرج- ترعة طريق دمياط عزبة البرج- مصرف ٦ بمدينة دمياط الجديدة) وعزل بعض السلالات البكتيرية المختلفة على بيئة الآجار المغذي و التعرف عليها عن طريق الصفات المزرعية و المورفولوجية وبعض التجارب الحيوية اللازمة لتعريف البكتريا التي تم استخدامها في التخليق الخارجي لجسيمات النانو لعنصر الفضة .تم معالجة محلول معقم من نترات الفضة (١ مل مولر) بالسلالات المنتقاة بنسبة ١% من المستخلصات البكتيرية بدرجة حامضية تساوي ٧ و تم التحضين عند درجة حرارة ٣٧°م في حضانة هزاز بسرعة ١٥٠ لفة/دقيقة في غياب الضوء لمدة ٥ ايام مع الملاحظة اليومية للتجربة. تم التأكد من انتاج جسميات النانو من خلال تغير لون المحلول إلى اللون البني ومن خلال منحني الامتصاص للجسيمات عند طول موجي يساوى تقريبا ٤٦٧ نانومتر باستخدام الطيف المرئي والأشعة الفوق بنفسجية. بعد التأكد من عملية التخليق الخارجي للجسيمات تم دراسة بعض الخصائص المختلفة للجسيمات مثل شكل و حجم الجسيمات باستخدام الميكروسكوب الالكتروني النافذ و شحنة الجسيمات باستخدام جهاز زيتا. من خلال التجربة بواسطة بكتريا انتيروباكتر تم انتاج جسيمات نانو كروية يتراوح حجمها ما بين ٩,٤٥- ١٧,١٥ نانومتر و تحمل شحنة سالبة بعد ٧٢ ساعة من بداية التحضين. تم اختبار تأثير هذه الجسيمات المخلقة ضد فطر فيوزاريم اوكسيسبورم ليكوبيرسيسي المسبب لمرض الذبول في الطماطم و قد أظهرت التجربة القدرة التضادية القوية لجسيمات النانو ضد هذا الفطر. كما أكدت النتائج أن بكتريا انتيروباكتر تعتبر سلالة واعدة لإنتاج جسيمات النانو لعنصر الفضة ذات قدرة مضادة للفطريات مما يؤهل استخدامها للحد من الفطريات المسببة لأمراض النبات خاصة فطر فيوزاريم اوكسيسبورم ليكوبيرسيسي.