Synthesis and characterization of silver nanoparticles by *Serratia marcescens* strains isolated from different sources in Egypt

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Abstract: Nine red pigmented bacterial isolate which different environmental sources and preliminary identified as *S. marcescens* in previous studies were subjected to 16S rDNA sequencing to confirm identification and to know the phylogenetic relationships between these sequences and those available for *S. marcescens* in gene databank. The obtained results revealed that these isolates are closely related to each other and to *S. marcescens* with more than 94% similarity. Silver nanoparticles synthesized by *S. marcescens* isolate WSE were found to have maximum absorbance at 412 nm, their size distribution was determined by dynamics light scattering (DLS) and the average particle size was found to be 11.5 nm. Transmission electron microscopy (TEM) showed a uniform distribution of silver nanoparticles with a mean particle diameter of 10.72 nm. X-ray diffraction (XRD) spectrum of the silver nanoparticles recorded 2θ value corresponding to silver nanocrystal. Characterization of the nanosilver was also carried out by Fourier transform infrared spectroscopy (FTIR).
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1. Introduction

The ability of some microorganisms such as bacteria and fungi to control the synthesis of metallic nanoparticles should be employed in the search for new materials (Mandal et al., 2006; El-Batal et al., 2013, 2014; El-Batal and Al Tamie, 2016). Biological synthesis of nanoparticles may have many advantages over their more traditional industrial synthesis (Thakkar et al., 2010). Besides, they are used as antimicrobial agents in surgically implanted catheters in order to reduce the infections caused during surgery and are recommend to own antibacterial, antifungal, antiinflammatory, antiangiogenic and antipermeability activities (Gurunathan et al., 2009a, 2009b; Kalishwaralal et al., 2009; Sheikpranbabu et al., 2009). Bacteria are among the most extensively exploited natural resources for the synthesis of metallic nanoparticles (Musarrat et al., 2011). The key reason for bacterial preference for nanoparticle synthesis is their relative ease of manipulation. The interactions between metals and microbes have been exploited for various biological applications in the fields of bioremediation, biomineralization, bioleaching, and biocorrosion (Klaus-Joerger et al., 2001). Biosynthesis of silver nanoparticles can be categorized into intracellular and extracellular synthesis according to the place where nanoparticles are formed (Simkiss and Wilbur, 1989; Vert, 1996; El - Baz et al., 2015). *S. marcescens* is a member of the family Enterobacteriaceae. It is red pigmented, Gram negative rod shaped motile bacteria. It has been isolated from soil (Mazzafera et al., 1996), water (Matsushita et al., 2009), plant (Rascoe et al., 2003; Selvakumar et al., 2008) and animal (Li et al., 2011). This investigation was carried out to characterize nine *S. marcescens* isolates isolated from different sources by 16S rDNA gene sequances and examine their ability to synthesize silver nanoparticles.

2. Material and methods

Bacterial isolates

S. marcescens HIM307-2, was kindely provided by professor W.Mannhein, Med. Zentrum Fur Hygiene, Universitat Marborg / Lahn. Germany. In addition, 8 red pigmented bacterial isolates were isolated by members of the Bacteriology Laboratory, Botany and Microbiology Department, Faculty of Science, Helwan University, Cairo, Egypt, and preliminary identified as *S. marcescens*. Isolates were maintaind on Nutrient agar (NA) slopes and passaged on fresh NA slopes monthely. All isolates were kept at 4°C.

Sequancing and phyllogentic analysis of 16S rDNA.

The genomic DNA of these isolates was extracted by Wizard® Genomic DNA Purification Kit. According to the manufacturer's recommended procedure. PCR was performed in a thermal cycler (Biometra® cycler personal). PCR amplification of the 16S rDNA gene was performed using two universal oligonucleotide bacterial primers, 16S rDNA forward

primer: 5-GAG TAA TGT CTG GGA AAC TGC CT-3, 16S rDNA reverse primer: 5-CCA GTT TCG AAT GCA GTT CCC AG-3. PCR reactions mixures contained 1 µL of a10 µM working solution of each primer, 1 µL of genomic DNA, 12.5 µL of a DreamTaq Green DNA Polymerase (2x) (© 2012 Thermo Fisher Scientific Inc.) and 9.5 µL of Water, nuclease-free following the manufacturer's guidelines. PCR conditions used in the amplification of 16S rDNA gene were: 95°C for 5 min., followed by 35 cycles of 95°C for 1.5 min, 59°C for 1 min and 72°C for 1.5 min, with final 10 min extension at 72°C. Then DNA molecules were separated in 0.8% agarose-TBE according to **Sharp et al. (1973)** (90 mM Tris-borate, pH 8.0, 2 mM EDTA). The approximate size of PCR products was estimated by comparison to DNA marker, 100 bp Gene Ruler ™, which was electrophoresed alongside the samples. DNA bands were visualized by using a UV transilluminator (LMS-26, 302 runs, Wolf laboratories. UK), and photographed. The PCR products were purified by using Thermo Scientific Gene JET PCR purification kit (© 2013 Thermo Fisher Scientific, Inc). The purified PCR product was sequenced in one direction using the previously forward designed universal primer in an automated sequencer ABI prism 3730XL (applied Biosystems, Foster City, CA, USA) at Macrogen Inc., Korea. Sequences were analyzed by using Geneious Pro 8.1.1. The 16S rDNA gene sequences were compared to those of the GeneBank and EMBL databases by advanced BLAST (Megablast) searches from the National Center for Biotechnology Information (NCBI). The phylogenic relationship of the isolates was determined by comparing the sequencing data with the related 16S rDNA gene sequences in the GenBank database of the National Center for Biotechnology Information, via BLAST search. The phylogenetic tree was constructed by the Geneious Pro 8.1.1 program.

Synthesis of silver nanoparticles

This was carried out according to **Kalishwaralal et al. (2008) and Vaidyanathan et al. (2010)** with slight modification. In 250 ml Erlenmeyer flasks, *S. marcescens isolates* were grown in 50 ml nutrient broth and incubated at 28°C for 24 h with shaking at 150 rpm. The overnight culture was centrifuged at 6000 rpm and 4°C for 10 min. The supernatant was filter sterilized throught 0.45 µm membrane filter and 10 ml were transfered to a test tube and silver nitrate solution was added to a final concentration of 1mM then the solution was incubated at 28°C for 24 h. After incubation, the colour change was recorded if the brown colour appeared this indicates production of silver nanoparticles **(Shankar et al., 2004; Manivasagan et al., 2013).**

Characterization of Synthesized Silver Nanoparticles (AgNPs)

Silver nanoparticles produced by *S. marcescens isolates* were characterized according to **El-Batal et al. (2013) as follows:** UV-vis spectroscopy was carried out by using a JASCO V-630 UV-VIS spectrophotometer and the absorption maxima were analyzed at a wavelength of 200-800 nm. Average particle size and size distribution of very fine particles dispersed or dissolved in a liquid were analyzed by Dynamic Light Scattering (DLS) (PSS-NICOMP 380-ZLS particle sizing system St. Barbara, California, USA). Transmission electron microscopy (TEM) studies were performed using a JEOL electron microscopy JEM-100 CX (FEI, America) electron microscope operating at an accelerating voltage of 200 kv. For the TEM measurements, a drop of the resulting solution was placed on a copper grid covered with amorphous carbon. After allowing the film to stand for 2 min, the extra solution was removed by blotting with filter paper and the grid allowed to air dry before measurement. The resulting solutions were dried at 60 °C and then analyzed by the Fourier transform infrared spectroscopy (**FTIR**) spectra (4000-400 cm^{-1}) at JASCO FT-IR-3600 (Bruker, Germany). The crystal structures of the silver nanoparticles was analyzed by X-ray diffraction technique (6000 - shimadzu - Japan) using Cu Kα radiation.

3. Results

PCR amplification of 16S rDNA

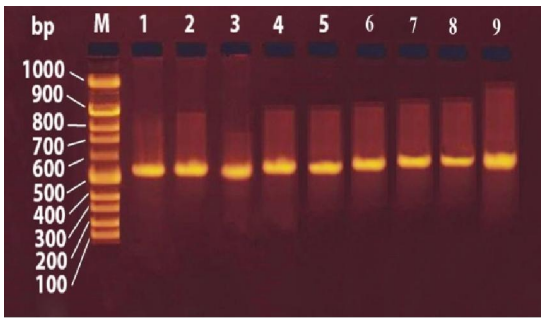


Figure 1. PCR amplification of 16S rDNA from 9 *S. marcescens* bacterial isolates using universal primers. Lane M: 100 bp molecular weight DNA marker; Lanes 1-9 *S. marcescens* isolates *(Lane 1:* isolate *RC, Lane 2:* isolate *On1D, Lane 3:* isolate *On1E, Lane 4:* isolate *Cab12A, Lane 5:* isolate *Or1, Lane 6:* isolate *Sm6, Lane 7:* isolate *HIM307-2, Lane 8:* isolate *WS2 and Lane 9:* isolate *WSE).*

DNAs of *S. marcescens* isolates were amplified with universal primers. They produced bands of 391-610 bp on agarose gel electrophoresis (Figure 1). The purified PCR products were sequenced in one direction using forward universal primers. Based on the alignment of 16S rDNA gene sequences from the

GeneBank database, the 16S rDNA gene sequence of the isolates showed the highest similarity to that of *S. marcescens* (Figure 2) and 16S rDNA gene sequences of other Enterobacteriaceae. Isolate *RC* (392 bp), isolate On1D (391 bp), isolate On1E (431 bp), isolate HIM307-2 (584 bp) and isolate WS2 (507 bp), are closely related to each other and showed the highest similarity to that of *Serratia sp. MIE2 (KF647218) and S. marcescens strain TC-1 (KF700093).* Interestingly, isolate Cab12A and isolate Or1, are closely related to each other, isolate Cab12A with a partial nucleotide sequence of the 16S rDNA gene (430 bp) showed the highest identity (100%) in the BLAST search to *S. marcescens strain T237 (KC764987)* and *S. marcescens strain CM96 (KF782799)*. However, isolate Or1 with a partial nucleotide sequence of the 16S rDNA gene (507 bp) showed 98% identity to *S. marcescens strain T237 (KC764987)* and *S. marcescens strain CM96 (KF782799)*. Partial nucleotide sequence of the 16S rDNA gene (610 bp) of isolate Sm6 showed 94% identity to both of *S. marcescens strain PS-1 (KF258679)* and *S. marcescens* strain SA30 (KF686740). Also, partial nucleotide sequence of the 16S rDNA gene (508 bp) of isolate WSE showed 99% identity to both of *S. marcescens strain DUCC3751* (KP318498) and *S. marcescens strain IARI-THW-5 (KF054974)* (Figure 2).

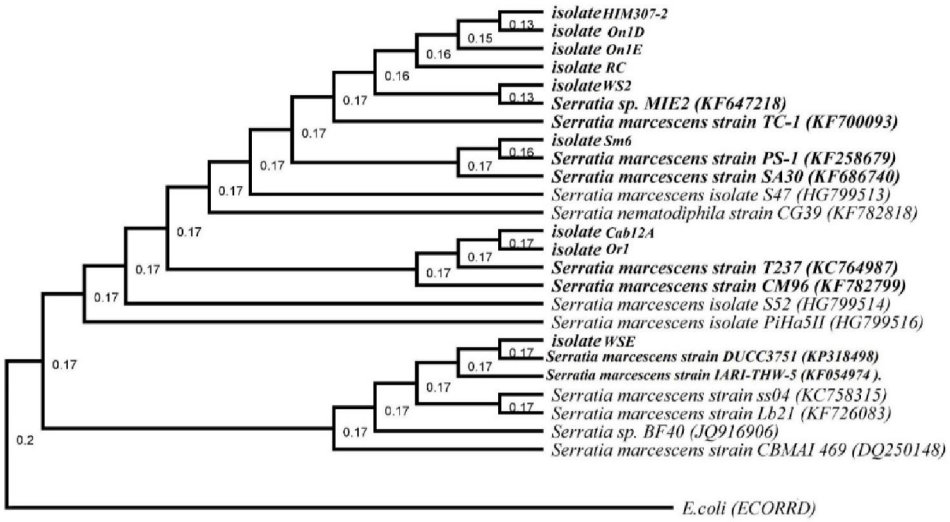


Figure 2. Rooted phylogenetic tree showing the relationship of randomly selected bacterial 16S rDNA isolates, the 16S rDNA gene sequence aligned in MUSCLE (Edgar, 2004).

Characterization of Synthesized Silver Nanoparticles (AgNPs)

The silver nanoparticles were synthesized by using sterilized supernatant of *S. marcescens* (**Figure 3).** The appearance of yellowish-brown color in aqueous silver solution in the reaction vessels is due to the surface plasmon resonance (SPR) exhibited by the silver nanoparticles and has suggested the formation of the silver nanoparticles **(Shankar et al., 2004; Manivasagan et al., 2013).**

UV-VIS spectral analysis

The nanoparticles were characterized by the UV-Vis spectroscopy. The absorption spectrum **(Figure 4 and Table 1)** of silver nanoparticles prepared by biological reduction showed a surface plasmon absorption spectrum band in the range of 420 nm to 480 nm indicates the formation of silver nanoparticles **(Hyllested et al., 2015).** The maximum absorption of the studied nanoparticles syntheised was found to be 412 nm for the isolate WSE and 413 nm for the isolate WS2 **(Figure 4)**, corresponding to the surface plasmon resonance of silver nanoparticles. However, the UV-VIS spectral analysis of *S. marcescens* isolates RC, On1D, On1E, Cab12A, Or1, Sm6 and HIM307-2 showed the absorbance peaks at 425nm, 432nm, 435nm, 439nm, 441nm, 443nm and 445nm, respectively.

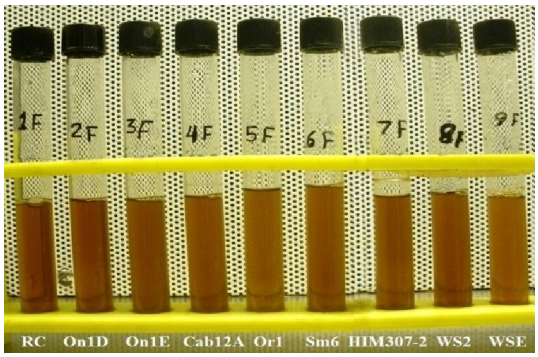


Figure 3. Formation of silver nanoparticles in the culture supernatant of *S. marcescens* isolates grown in NB for 24 h at 28°C

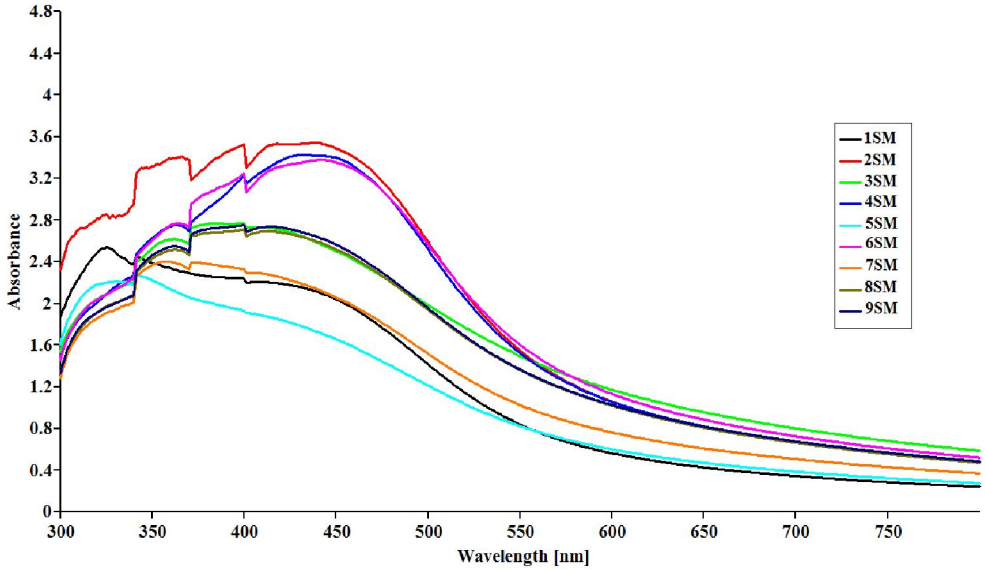
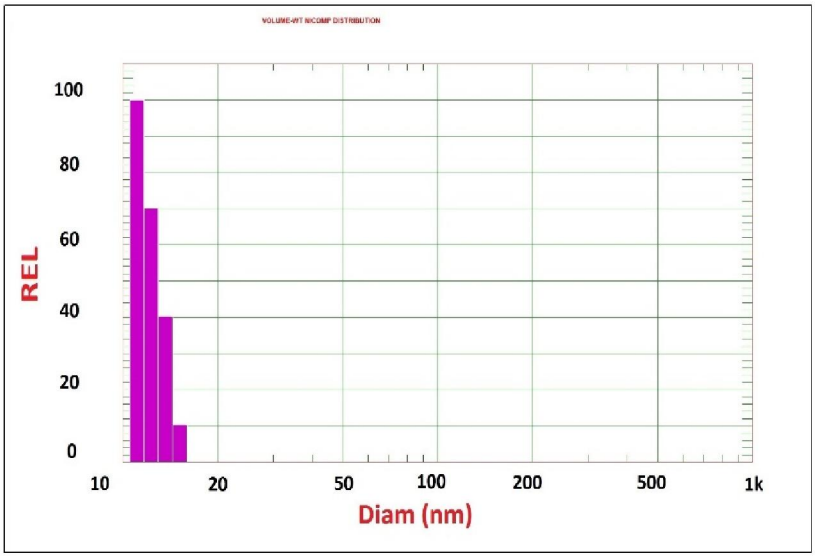


Figure 4. UV-Vis absorption of silver nanopartical synthesis for supernatant of *S. marcescens* isolates after incubation at 28°C for 24 h.

Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) of the nanoparticles obtained from nine *S. marcescens* isolates was performed. The average mean diameter of silver nanoparticles is summarized in Table 1. Dynamic Light Scattering (DLS) has provided further investigation for size details of studied nanoparticles.

Figures 5 show the average mean diameter of the silver nanoparticles prepared by WSE isolate. The DLS analysis showed that the smallest particle size recorded mean diameter 11.5 nm for isolate WSE and mean diameter recorded 14.9 nm for isolate WS2.



Mean Diameter = 11.5 nm Fit Error = 76.434 Residual = 91.132

Figure 5. Mean diameter of silver nanopartical produced by *S. marcescens* isolate WSE using dynamic light scattering

Table 1. UV-VIS spectral analysis of synthesized silver nanoparticles and DLS mean diameter obtained by *S. marcescens* isolates, Peaks of synthesized silver nanoparticles at 28°C after 18 h

Isolates code	UV-VIS spectral analysis Synthesized silver nanoparticles		DLS (Mean Diameter)
	Wavelength of silver nanoparticles [nm]	Absorbance	
RC	425	2.2440	27.7 nm
On1D	439	3.5420	37.6 nm
On1E	435	2.7690	34.5 nm
Cab12A	432	3.4260	32.5 nm
Or1	441	2.2680	45.3 nm
Sm6	443	3.3760	53.2 nm
HIM307-2	445	2.3950	54.1 nm
WS2	413	2.6920	14.9 nm
WSE	412	2.7360	11.5 nm

Transmission electron microscopy (TEM) analysis

Transmission electron microscopy has provided further insight into the morphology and size details of silver nanoparticles. The TEM micrographs of nanoparticle obtained in culture supernatant showed that silver nanoparticles are spherical shaped, with an average mean diameter of about 10.72nm for WSE isolate Figure 6.

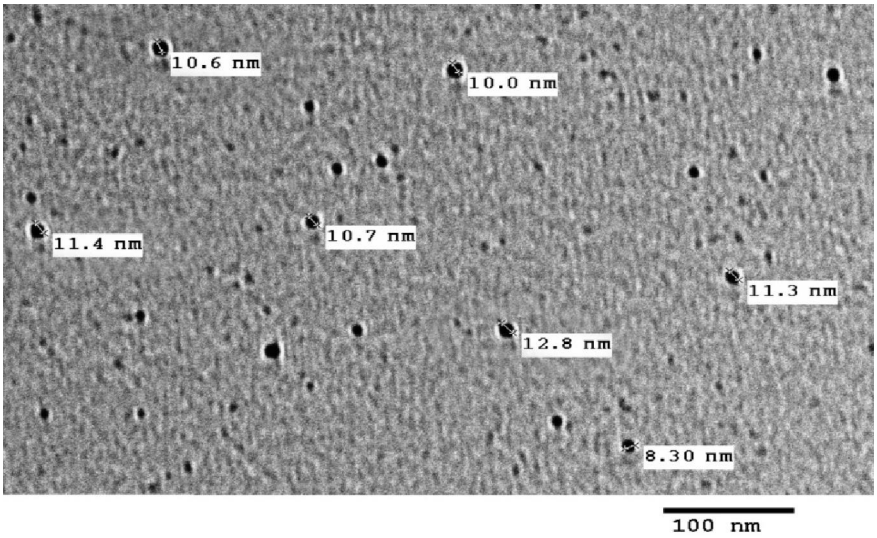


Figure 6. Transmission electron microscopy (TEM) image of silver nanoparticles produced by *S. marcescens* isolate WSE

Fourier transform infrared spectroscopy (FTIR) spectral analysis

After 24 hours of incubation of the culture supernatant and silver nitrate solution, the silver nanoparticles are subjected to FTIR analysis in the range of 400-4000 cm^{-1}. Spectrum is shown in the Figure 7. The FTIR spectrum resulted in a peak value at 3332.39 cm^{-1} corresponding to N-H stretching vibrations primary and secondary amines or amide linkages in the protein. The peak seen at 1384.64 cm-1 are identified as the aromatic amines and nitro groups due to the N=O bending vibrations. FTIR spectra are summarized in Table 2.

X-ray diffraction pattern (XRD) of silver nanoparticles

XRD measurements were carried out in order to characterize the crystal structure of the silver nanoparticles as shown in Figure 8. The major peaks at 38.2°, 44.1°, 64.72° and 77.4° (2θ values) correspond to the reflections from the (111), (200), (220), and (311) planes, respectively, and confirm the crystalline phase of the AgNPs., respectively, and confirm the crystalline phase of the AgNPs.

Table 2: FTIR with Wavenumber of characteristic bonds and corresponding assignments for culture supernatant and silver nanoparticles as indicated by FTIR (Krithika et al., 2014; Sumathi et al., 2014).

Extracellular cell-free filtrates wave number (cm^{-1})	Silver nanparticles wave number (cm^{-1})	Comment
3598.52	3332.39	This beak attributed to the –N–H stretch in vibrations primary and secondary amines or amide linkages in the protein.
	1635.34	The peaks at 1635.34 cm^{-1} which found only in silver nanoparticles combination due to the conjugation of silver nanoparticles in the –NH function groups.
1469.49		The visible peak at 1469.49 cm−1 is due to the presence of the CH stretch, CH_2 and CH_3 bending modes.
----------	1384.64	The peak seen at 1384.64 cm-1 are identified as the aromatic amines and nitro groups due to the N=O bending vibrations.
1099.23	1114.65	This peak is attributed to carbon-carbon double bond
821.527	----------	Also, this peak is attributed to carbon-carbon double bond.

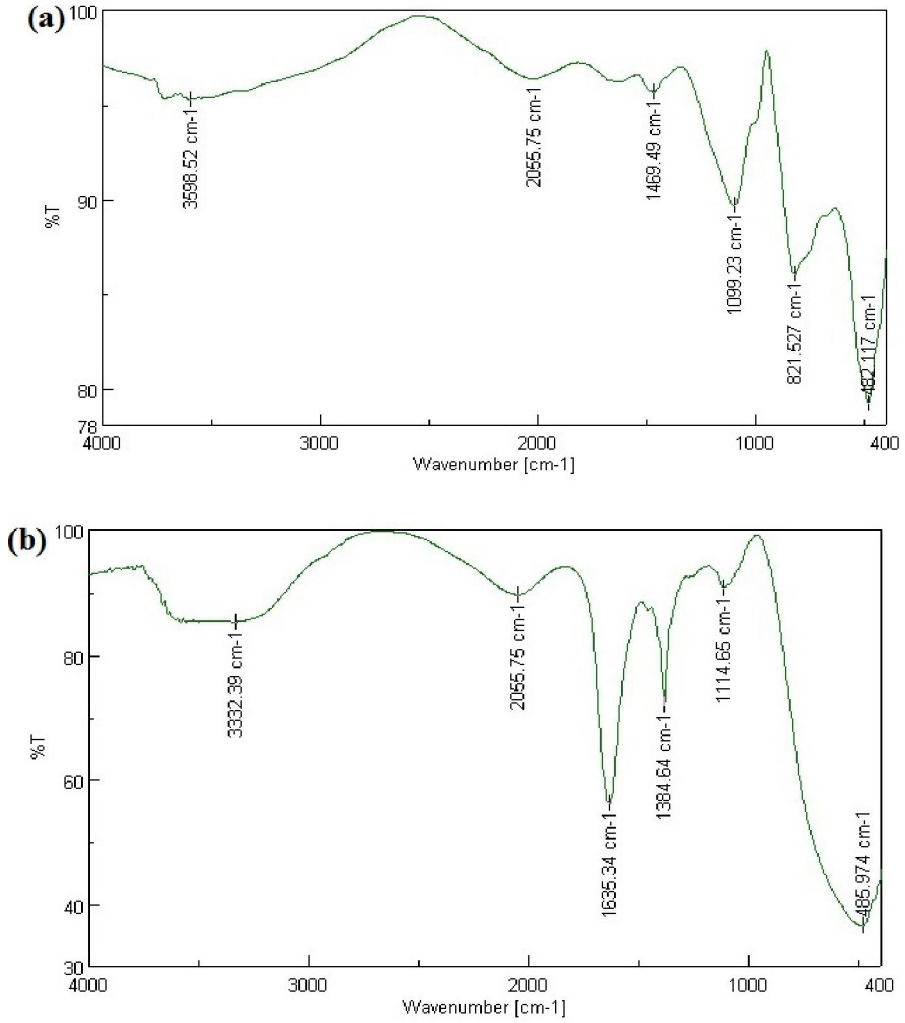


Figure 7. FTIR analysis of (a) culture supernatant of *S. marcescens* isolate WSE (b) silver nanoparticles

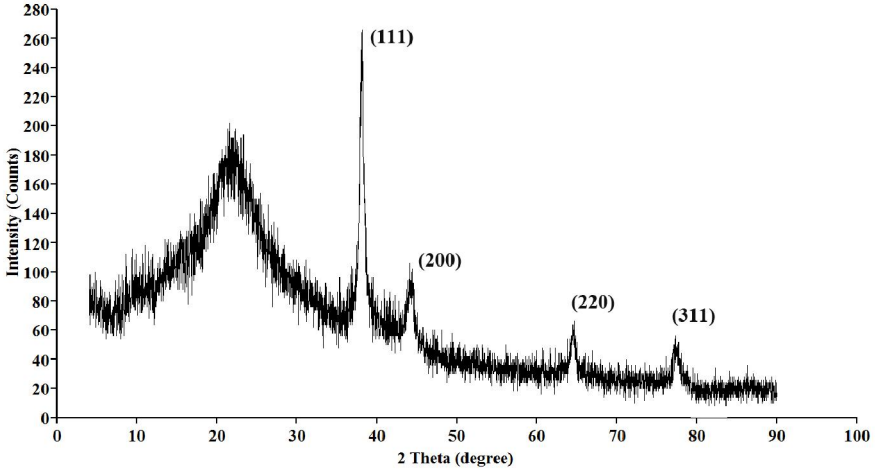


Figure 8. XRD pattern of synthesized silver nanoparticle obtained by of *S. marcescens* isolate WSE.

4. Discussion

The use of 16S rDNA gene typing has previously been reported to be useful for molecular subtyping of bacterial species and strains with the traditional methods that depend on morphology and biochemical studies that had defect in identifying those uncultivable organisms or those with biochemical characteristics which did not resemble any certain genus or species **(Gee et al., 2003; Acinas et al., 2004; Clarridge, 2004; Sacchi et al., 2002, 2005; Hellberg et al., 2012)**. The use of 16S rDNA gene sequencing in the clinical laboratory play a very important role in identifying biochemically unidentified bacteria or for providing reference identifications for unusual strains (Lane et al., 1985; Boudewijns et al., 2006; Janda and Abbott, 2007; Woo et al., 2000, 2008)Six endophytic strains of *S. marcescens* isolated from surface-sterilized rice roots and stems, of different rice varieties grown in the Philippines were identified as a taxonomically distinct subgroup by the phylogenetic analysis of 16S rDNA sequences (Tan et al., 2001). Deorukhkar et al. (2007) reported the isolation and identification of a red pigmented producing *Serratia marcescens ost3*. The complete genome sequence of *S. marcescens WW4*, which consists of one circular chromosome and one plasmid, was determined by Chung et al. (2013). **Iguchi et al. (2014)** reported the complete genome sequences of two carefully selected *S. marcescens* strains, a multidrug-resistant clinical isolate (strain SM39) and an insect isolate (strain Db11). **Nicholson et al. (2013)** reported the complete genome sequence of *S. liquefaciens* strain ATCC 27592, which was previously identified as capable of growth under low-pressure conditions. **(Liu et al., 2013)** report a high-quality draft genome sequence of *S. marcescens* strain VGH107, which was isolated from a patient with an infection from a snakebite wound.

In this study *S. marcescens* was subjected to analysis of 16S rDNA gene sequences to confirm their identification. The 16S rDNA genes of *S. marcescens* were highly conserved. Alignment of the 16S rDNA gene of *S. marcescens* the nine isolates showed the presence of some similarity or difference among nucleotides sequences that were aligned. Based on the concept of similarity or nucleotides difference between the query nucleotides and those compared, it is recommended when the sequences similarity is more than 90% or the nucleotides different between the query and those compared 1–1.5% (14–22 bp), the query should be categorized as the same species identified by the 16S rDNA gene (Bosshard et al., 2003; Suardana, 2014), however most taxonomists accept a percent identity score of 97% and 99% to classify a microorganism to genus and species, respectively **(Reller et al., 2007)**. A phylogenetic tree of the 16S rDNA gene was performed using geneious Tree Builder option with genetic distance model: Tamura Nei, tree builder methods: Neighbor joining (Saitou and Nei, 1987), and Outgroup: ECORRD. The tree was rooted with *E. coli* a member of the family Enterobacteriaceae (Drummond et al., 2014). The Phylogenetic tree showed that the *S. marcescens* isolate RC (392 bp), isolate On1D (391 bp), isolate On1E (431 bp), isolate HIM307-2 (584 bp) and isolate WS2 performed close clade with some strains of Serratia sp. MIE2 (KF647218) and *S. marcescens* strain TC-1 (KF700093). In the contrast, both isolates Cab12A and isolate Or1 also showed distinct clade toward *S. marcescens strain T237 (KC764987)* and *Serratia marcescens strain CM96 (KF782799)* strains that are available in gene databank. *Isolate WSE* (508 bp) show very closest clade with *Serratia marcescens strain DUCC3751* (KP318498) and *Serratia marcescens strain IARI-THW-5 (KF054974).*

The addition of silver ions into the culture supernatant of the nine isolates of *S. marcescens* changes its color from almost colorless to brown with increased intensity along the period of incubation **(Sadowski et al., 2008).** The wavelength of the plasmon absorption maximum in a given solvent can be used to indicate particle size. The broadness of the peak is the best indicator of the size of nanoparticles. As the particle size increases, the peak of plasmon resonance shifts to longer wavelengths. When the particle size increased, the absorption peak shifted towards the red wavelength, which indicated the formation of larger sized nanoparticles **(Peng et al., 2010).** UV–Vis spectral analysis after 18 h was carried out to confirm synthesis of silver nanoparticles. It showed that the typical absorption spectrum of AgNPs is in the band of 350 nm to 450 nm **(Aslan et al., 2005).** The spectral analysis for the nine isolates ranged from 412 nm for isolate WSE to 445 nm for isolate HIM307-2. It has been reported that the absorption spectrum of spherical silver nanoparticles exhibits a maximum between 420 nm and 450 nm with a blue or red shift when particle size decreased or increased, respectively **(Suber et al., 2005)**. For this reason, the silver nanoparticles produced by *S. marcescens* isolate WSE that present a plasmon, which is blue shifted is the most potent strain to produced silver nanoparticles. It is well known that a surface plasmon band of spherical silver nanoparticles appears around 420 nm region **(Suber et al., 2005).** The Dynamic Light Scattering (DLS) has provided further insight into the size details of the synthesized nanoparticle by isolate WSE. It confirm that the data investigated from UV–Vis spectroscopy that revealed the formation of silver nanoparticles with an average size of about 11.5 nm for isolate WSE. *S. marcescens* isolate WSE is the most potent strain for synthesizing silver nanoparticles. The morphology and size of silver nanoparticles had investigated by transmission electron microscopy (TEM), the majority of silver nanoparticles are spherical in shape. The mean diameter of the silver nanoparticle was found to be about 10.72 nm. Mainly protein is involved in the bioreduction process as observed from Nitrate reductase reported by**.** The synthesis mechanism of silver nanoparticles can be assumed due to the enzyme NADH dependent nitrate reductase that involved in the process **(Krithika et al., 2014)**.

The FTIR analysis was carried out for silver nanoparticle formed after 24h incubation with *S. marcescens* supernatant. The peak observed at 1384.65 cm^{-1} (400-4000), is similar to that obtained by **Krithika et al., (2014)**. The peak at 1635.34 cm^{-1} which obtained by silver nanoparticles combination due to the conjugation of silver nanoparticles in the –NH function groups **(Sumathi et al., 2014).** The mechanism involved in the transformation of silver ion could be the involvement of an enzyme probably nitrate reductase. The catalytic activity of NADH dependent reductase and the role of NADH as an electron carrier could be the silver ion reduction process. The free amine groups of cysteine residues in the protein bind to silver nanoparticles offering stability. However, the carbonyl group and peptides behave as a capping agent over the nanoparticles thereby avoiding aggregation of particles **(Krithika et al., 2014)**. X-ray diffraction (XRD) spectrum of the synthesized silver nanoparticles showed 2θ value of 38.2°, 44.1°, 64.72° and 77.4°, corresponding to [111], [200], [220] and [311], respectively, for silver nanocrystal. Most of the particles were spherical in shape, had a size of 10.72 nm, highly stable, and showed no visible aggregation and precipitation at room temperature for several months.

4. Conclusion

Use of 16S rDNA partial gene sequencing could greatly reduce the time and labor needed for confirmation and subtyping of *Serratia marcescens* isolates isolated from different vegetable and soil samples in order to know the phylogenetic relationships between these sequences and those between the sequences of bacteria available in gene databanks. The results of this analysis showed that the *isolates WS2*, *HIM307-2, Sm6, On1D, On1E, RC, WSE, Cab12A and Or1*, respectively, are closely related among them and with respect *S. marcescens* with more than 94% similarity values. The present study demonstrates the biological mechanism for synthesis of silver nanoparticles by *S. marcescens* isolates isolated from different sources in Egypt. The formation of nanosilver was characterized by using the UV-Vis spectra, Dynamic Light Scattering (DLS), Transmission electron microscopy (TEM), X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR). The synthesized silver nanoparticles yielded the maximum absorbance peak at 412 nm, the size distribution of nanoparticles was determined using DLS and the average particle size was found to be 11.5 nm. TEM characterization showed a uniform distribution of silver nanoparticles, with an average size of 10.72 nm. X-ray diffraction (XRD) spectrum of nanosilver showed 2θ value corresponding to the silver nanocrystal. Most of the nanoparticles produced by *S. marcescens* isolate WSE were spherical in shape, had a size of 10.72 nm, highly stable, and showed no visible aggregation and precipitation at room temperature for several months.

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