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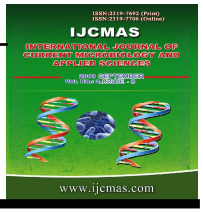

Original Research Article

Exploiting of *Streptomyces bottropensis* as creator for silver nanoparticles inhibiting growth of microbes

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Keywords

AgNPs; *Streptomyces bottropensis*, silver nanoparticles; *Staphylococcus aureus and Streptococcus pyogenes;* SEM.

A B S T R A C T

Synthesis of nanoparticles with interesting physico-chemical properties using efficient as well as eco-friendly technology is one of the main objectives of nanotechnology. In the current study, *Streptomyces bottropensis* was isolated from soil, Riyadh, Saudi Arabia, and identified by 16S rRNA. Our study showed that shape of silver nanoparticles (AgNPs) was predominantly monodispersed and spherical shapes in the size range of 5–35 nm upon addition of 1 mM silver nitrate. The AgNPs were characterized by determining Fourier transform infrared spectroscopy (FTIR), Energy Dispersive Spectroscopy (EDS) and transmission electron microscopy (TEM). Furthermore, the biosynthesized AgNPs at 5µl significantly inhibited the growth of medically important pathogenic gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus pyogenes*), gram-negative bacteria (*Klebsiella pneumoniae* and *Salmonella typhi*) and yeast (*Candida albicans*). Thus, bioconversion of silver nanoparticles by *Streptomyces diastaticuschromogenes* could be employed as a potential nanomedicine to eliminate pathogenic microorganisms.

Introduction

Nanotechnology can be defined as an investigation for the design, synthesis, and manipulation of structure of particles with dimension smaller than 100 nm. Nanotechnology has a variety of applications in fields such as optics, electronics, biomedicine (Jain *et al.,* 2011; Klaus-Jeorger *et al.,* 2001), magnetics, mechanics, catalysis, energy science (Dura´n *et al.,* 2005), etc. Thus, developing different branches of nanotechnology definitely results in developing the related sciences, and is a significantly goal of scientific research (Mohanpuria *et al.,* 2008; Bhattacharya and Gupta, 2005). Nanoparticles are at the important advantage of the quickly emergent field of nanotechnology and they are currently made by chemical and physical approaches. The development of environmentally friendly, kindly and green process technologies for the production of nanoparticles with a range of chemical and physical properties is one of the challenges

in the newly emerging field of nanobiotechnology. The expensive and extensive use of toxic solvents and hazardous reducing agents in chemical procedures to synthesize nanoparticles has augmented the necessity in view of eco-friendly and green chemistry approach. Hence, a well estab-lished non-toxic and eco-friendly potent methodology for the synthesis of nanoparticles has mounted to a level of supreme importance (Mukherjee *et al.,* 2008).

Over the last few years, the biosynthesis of nanoparticles by bacteria, fungi, and plant has gained much of interest of good conductivity, chemical, stability, catalytic and antimicrobial activity (Sivalingama *et al.,* 2012; Antony *et al.,* 2011). Sadhasivam *et al.,* (2010) used the extracellular components from a *Streptomyces hygroscopicus* culture medium to synthesize silver nanoparticles, and characterized their antimicrobial activity. *Streptomyces* sp. is members of gram positive, soil inhabiting filamentous actinomycetes characterized based on its complex life cycle. The genus is well known for its unique potential ability to produce a wide variety of secondary metabolites, such as antibiotics, immunosuppressors and many other biologically active compounds (Chater, 1993). Exploitation of *Streptomyces* in nanotechnology has recently received considerable attention (Sadhasivam *et al.,* (2010).

The present study aims to biosynthesize silver nanoparticles by *Streptomyces bottropensis*, and to determine their characterization and potential role as antimicrobial activity.

Materials and Methods

Collection of soil samples and isolation of *streptomyces*

Soil sample was collected in sterile airlock polythene bags and stored at 4 ◦C from Eldrieh, Riyadh, Saudi Arabia. Soil sample was inoculated on starch casein agar medium (Pisano *et al.,* 1986; Hayakawa and Ohara, 1987) supplemented with antibiotics such as cycloheximide (40 g/ l), nystatin (30 g/ l) and nalidixic acid (10 g/ l). The plates were incubated at 30 ◦C until the appearance of colonies with a tough leathery texture, dry or folded appearance, and branching filaments with aerial mycelia (Mince *et al.,* 2002). Pure colonies were isolated and subcultures were carried out by streaking the particular isolate directly on ISP2 agar medium.

Characterization and culture conditions

Total genomic DNA of actinomycete isolate was extracted by a modification of a method of Smoker and Barnum (Smoker and Barnum, 1988). Actinomycete isolate was grown in 10 ml International Streptomyces Project Medium 2 (ISP 2) (Shirling and Gottlieb, 1966) with agitation at 30 °C for 96 h. Cells (4 ml) were harvested by centrifugation (12000 rpm for 2 min), washed once with 500 ml of 50 mM Tris-HCl/1 mM EDTA (TE) buffer (pH 8.0), the pellet was resuspended in 500 ml of 50 mM Tris-HCl (pH 8.0) –5 mM EDTA (pH 8.0) –50 mM NaCl, add 20 µl lysozyme (1 mg/ml), and the solution was incubated at 55°C for 30 min. After the addition of 10 µl of proteinase K (10 mg/ml) and 20 µl of 10% sodium dodecyl sulfate, the mixture is incubated at 55°C for 10 min or until the solution cleared (complete cell lysis).

The solution was chilled on ice and extracted with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1). The organic extraction was repeated. The supernatant is added to an equal volume of 4 M ammonium acetate. Total genomic DNA is precipitated by the addition of 2 volumes of isopropanol followed by centrifugation (13000 rpm) for 10 min at room temperature**.** Supernatant was removed after centrifugation (13000 rpm) and pellet was washed with 70% ethanol. The pellet was dissolved in 100 µl of TE buffer or double distilled water The 16S ribosomal DNA gene was amplified by PCR using the universal primer pair 27F $^{5-}$AGAGTTTGATCMTGGCTCAG^{-3} and 1492R $^{5-}$ACCTTGTTACGACTT^{-3}. The amplified products were analyzed by Macrogen Inc., South Korea. DNA sequence analysis was then performed by BLAST network services at the NCBI. The 16S rRNA gene sequence of the isolate was aligned with reference sequences obtained from GenBank using ClustalW (Thompson *et al.,* 1997). Phylogenetic tree was generated using the neighbor-joining method with MEGA 5 package (Saitou and Nei, 1987; Kumar and Yadav, 2009). The evolutionary distance matrix was derived with Jukes and Cantor model (Jukes and Cantor 1969). Topology of phylogenetic tree was evaluated by bootstrap analysis based on 1000 replicates (Felsenstein, 1985).

Biosynthesis and characterization of AgNPs

The isolate was further cultured in ISP2 medium (pH 7.2) and grown for 72 h at 30 ◦C in an orbital shaker at 220 rpm. Cell filtrate (CF) was obtained by centrifugation at 4 ◦C, 10,000 rpm for 10 min. Bioreduction process to occur, $AgNO_3$ (Qualigens 99.8%) was added to 100 ml CF at molarity 1 mM and incubated at 30 ◦C in dark for 48 h. Followed by initial observation of color change in the bioreduction process, UV– visible spectrometric measurements were performed on Hitachi double beam equipment (Model Lambda 35) at range 210 to 800 nm. The bio-transformed products present in cell-free filtrate after 72 h of incubation were freeze-dried and diluted with potassium bromide in the ratio of 1: 100. FTIR spectrum of samples was recorded on FTIR instrument mode Nicolet 6700 spectrometer at a resolution of 4 cm^{-1}in the range of 400– 4000 cm^{-1}. For energy dispersive spectroscopy (EDS), sample was prepared on a copper substrate by drop coating of silver nanoparticles, and was carried out using JEOL (JSM-6380 LA) equipped with scanning electron microscopy. Transmission electron microscopy was performed on JEOL (JEM-1010) instrument, with an accelerating voltage of 80 kV after drying of a drop of aqueous AgNPs on the carbon-coated copper TEM grids Samples were dried and kept under vacuum in desiccators before loading them onto a specimen holder. The particle size distribution of silver nanoparticles was evaluated using ImageJ 1.45s software.

Test pathogens

The antibacterial activity of biosynthesized AgNPs was assessed against four bacterial species: Gram positive (*Streptococcus pyogenes* ATCC 19615, *Staphylococcus aureus* ATCC 25923) and Gram negative (*Salmonella typhi* ATCC 6539 and *Klebsiella pneumoniae* ATCC 700603), and *Candida albicans* strain ATCC 90028 for antifungal activity which was growing sabouraud agar. The test pathogen samples were procured from Khalid hospital, Riyadh. All the test pathogen samples were maintained in Brain Heart Infusion

medium (BHI) at – 20 °C. 300 µL of each stock-culture were added to 3 mL of BHI broth. Overnight cultures were kept for 24 h at 37°C ± 1°C and the purity of cultures was checked after 8 h of incubation.

Antimicrobial activity of AgNPs

The antimicrobial effects of the microbiologically synthesized AgNPs were evaluated using method of disk inhibition zone. In disk inhibition zone method, the nutrient agar medium was inoculated with freshly prepared cells of each bacteria, and *Candida albicans* on sabouraud agar. After solidification of the agar, a number of sterilized disks were dipped into CF as positive control and biosynthesized AgNPs, and placed on the plates. After incubation at 37˚C for 24 h, the antimicrobial activity was measured as diameter of the inhibition zone formed around the disk.

Scanning Electron Microscopy

The bacteria that were inhibited by biosynthesized silver nanoparticles were prepared for scanning electron microscope (SEM). The paper disc containing bacteria were fixed in 3 % (v/v) glutaraldehyde buffered with 0.1 M sodium phosphate buffer (pH 7.2) for an hour at room temperature and then washed four times in sodium phosphate buffer. It was then post-fixed in 1 % (w/v) osmium tetroxide (OsO_4) for an hour and then washed four times in the buffer. They were dehydrated in a graded alcohol series. The last stages of dehydration were performed with propylene oxide. The specimens were dried and were mounted onto stubs using double-sided carbon tape, and then were coated with a thin layer of gold by a Polaron SC 502 sputter coater, and were examined in a Jeol JSM 6060 LV scanning electron microscope (Hayat, 1981).

Results and Discussion

The total length of the 16 S rRNA gene sequenced in the present study possess 1446 base pair, and showed 100% similarity index with *S. bottropensis* strain xsd08097 (GeneBank, Accession No. J481054.1). The result of cladistic analysis of generated sequence together with the related sequences accession retained from geneBank was consistent with the result of Blast search (Fig. 2); and here, the isolate was named as *S. bottropensis strain* xsd08097 isolate Essam (Fig. 1).

Fig.3 shows the change in color of a mixture of the cell filtrate after mixing with silver nitrate solution occurred, and this indicates the synthesis of silver nanoparticles by *S. bottropensis*. Mulvaney (1996) also reported that the dark brown color is the result of excitation of surface plasmon vibration in the metal nanoparticles and is typical of the silver nanoparticles. It was observed that the color development was result as different reductase enzymes in filtrate of *streptomyces* sp.. The role of NADH dependent nitrate reductase from fungi in the biosynthesis of silver nanoparticles was recently reported (Kumar *et al.,* 2007; Bai *et al.,* 2011); however, different NADH-dependent reductases may be produced also by *S. bottropensis*. There was previously study used a *streptomyces* filtrate to produce Ag nanoparticles (Sadhasivam *et al.,* 2010).

Results of energy dispersive spectroscopy (EDS) of the silver nanoparticles was confirmed the presence of elemental silver signal shown in Fig. 4. The presence of an optical absorption band at ~3ev reveals the presence of pure metallic silver nanoparticles. Magudapathy *et al.,* (2001) also reported that the spectrum of

Fig. 1: a) *S. bottropensis* on SPI-2 medium, b) Under light microscope

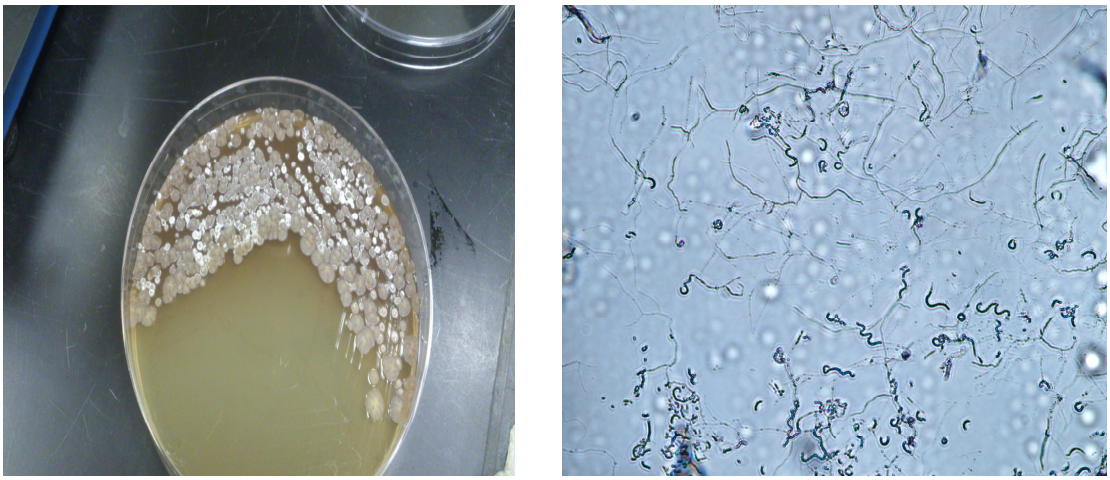


Fig.2 The phylogenetic tree of *S. bottropensis* was constructed using the neighbor-joining method with aid of MEGA 5.0 program. The Bootstrap values above 50%, presented as percentages of 500 replications, are shown at the branch points. Bar 0.5 substitutions per nucleotide position.

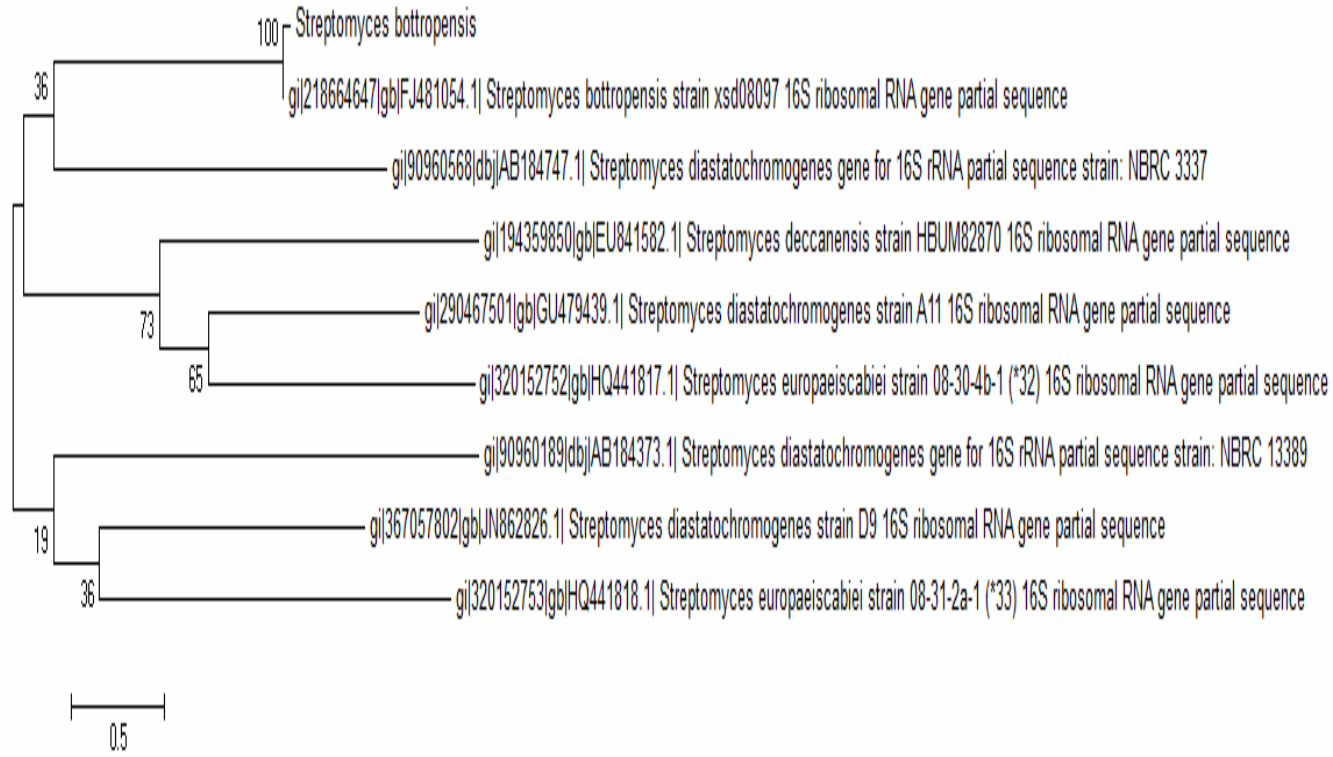


Fig.3 Test tubes containing the filtrate of the *S. bottropensis* at before addition 1 mM AgNO3 (right) and after 72 h incubation with 1 mM AgNO3 (left).

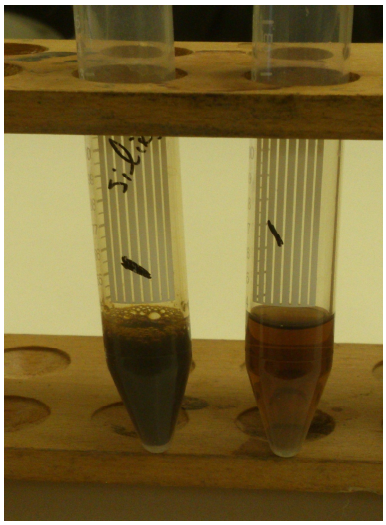


Fig.4 EDS spectra of silver nanoparticles

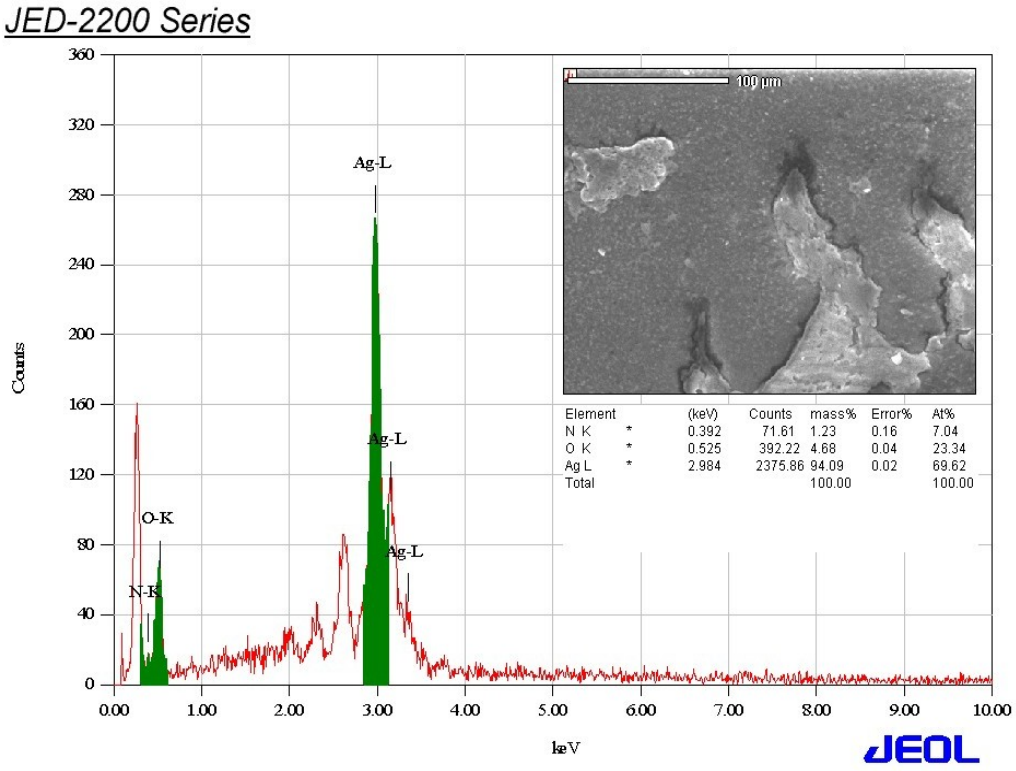


Fig.5 FTIR spectrum of AgNPs synthesized by using cell free supernatant of *S. bottropensis*.

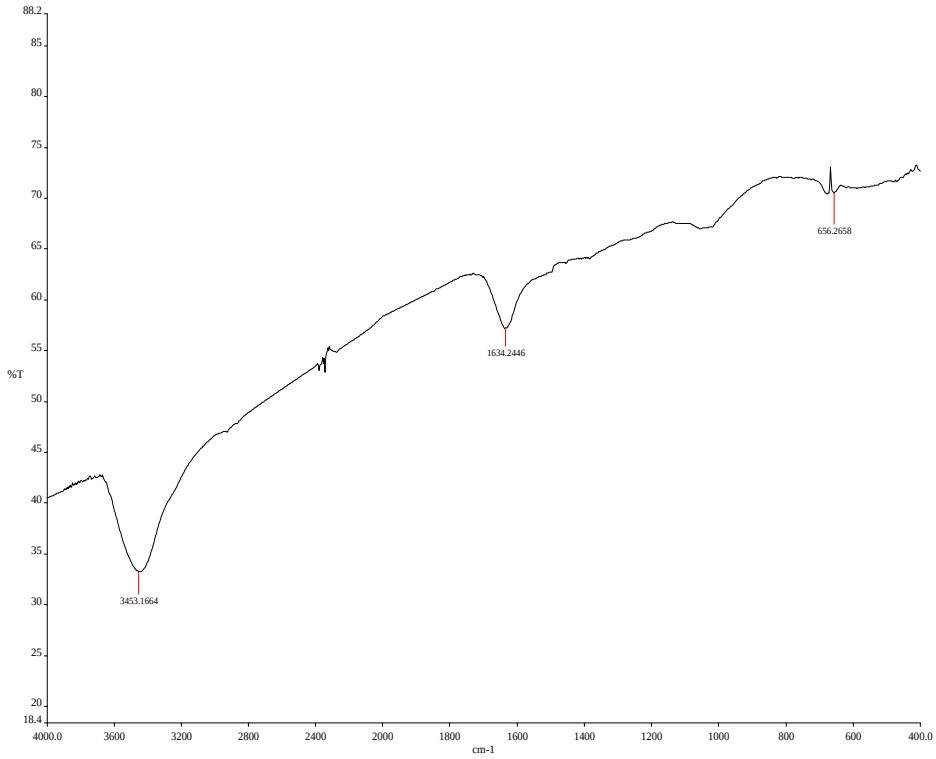


Fig.6 Images of biosynthesized silver nanoparticles *by* Transmission Electron Microscopy (TEM)

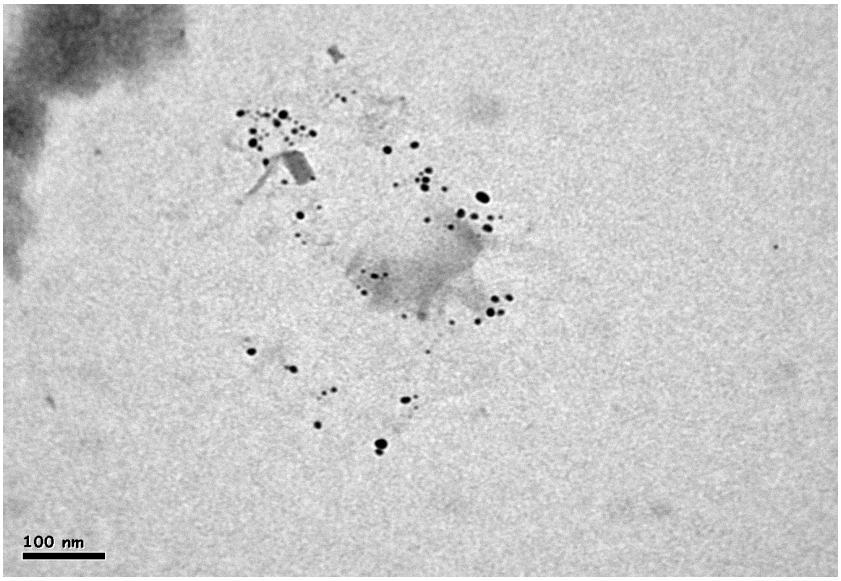


Fig.7 Histogram for different sizes of biosynthesized silver nanoparticles from TEM images

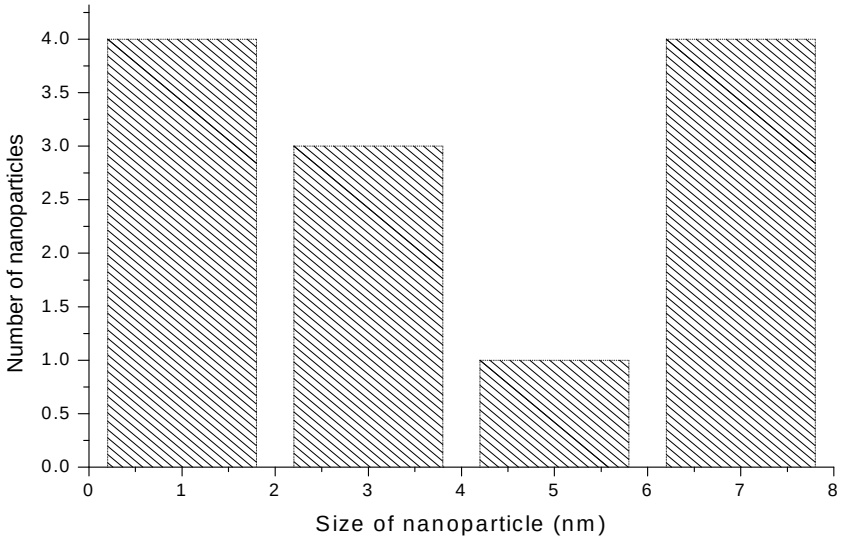


Fig.8 Antimicrobial activity of AgNPs (5 µl) against pathogens

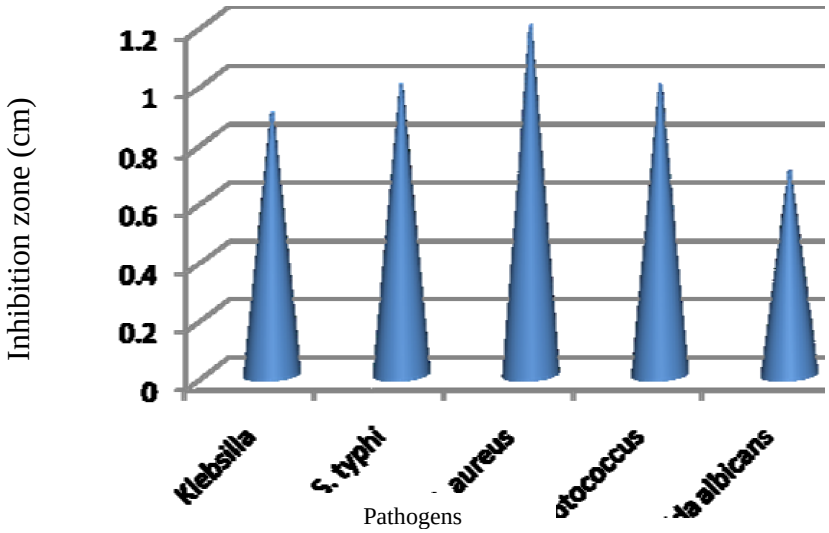
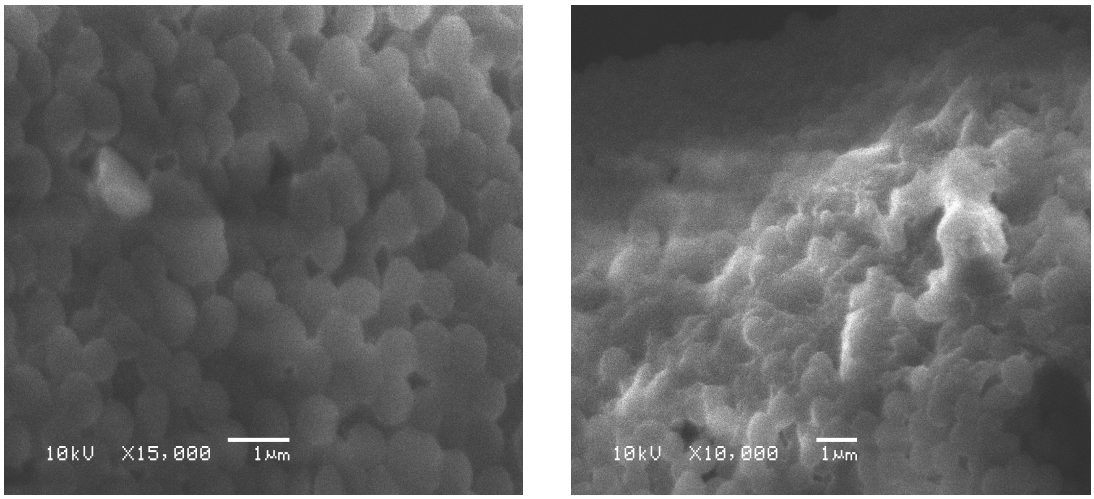


Fig.9 Effect of AgNPs on growth of bacterial cell under scanning electronic microscope. untreated cells (left) and treated cells (right)



nanoparticles sample showed mainly Ag (94.1%) and only minor amounts of other elements (5.9 %), the signals for N and O indicate the presence of proteins as a capping material on the surface of silver nanoparticles.

The FTIR spectroscopy is very important to characterize the protein binding with the were seen at 3453.17 cm^{-1}. Similarity, Gole *et al.,* (2001) and Jain et al (2011) also reported the presence and binding of protein whith silver nanoparticles which can lead to their possible stabilization.

Result in Fig. 6 showed different sizes of nanoparicles, mostly spherical in shape but aggregated to a lesser extent. In Fig. 7, there were four distinct sizes of Ag nanoparticles produced by *S. bottropensis*. The size of Ag nanoparticles was approximately 4.2 nm with standard deviation 2.3nm, 33.3% was at 1 nm, 25.1% at 3 nm, 8.3% at 5 nm and 33.3 % at 7 nm. Sadhasivam *et al.,* (2010) reported nanoparticle sizes of 20–30 nm when produced by the extracellular from a *Streptomyces* culture. This result is similar to previous study (Saifuddin *et al.,* 2009) silver nanoparticles and it is possible to quantify secondary structure in metal nanoparticle-protein interaction. Fig. 5 shows FTIR spectrum of the present study which revealed two bands at 1634.92 cm^{-1} that corresponds to the bending vibrations of the amide I and amide II bands of the proteins while their corresponding stretching vibrations of primary amines for biosynthesis of silver nanoparticles from *Bacillus subtilis* using microwave radiation. The different size distribution of nanoparticles may be due to differences in reductases produced by *S. bottropensis*, or the effects of other proteins coating the nanoparticles. The size distribution of nanoparticles can be very important for application purposes. For example, smaller nanoparticles were more effective as an antimicrobial against pathogens (Elechiguerra *et al.,* 2005).

Figs. 8 and 9 clearly, showed that the biosynthesized nanoparticles induced the formation of inhibition zone of test pathogen. Shrinking of the cell and degradation of cell was observed under SEM (Fig. 10). Stoimenov *et al.,* (2002) also confirmed that reactive metal oxide

nanoparticles posses excellent bactericidal effects. The mechanism of inhibitory action of silver ions on microorganisms revealed that upon Ag^+ treatment, DNA loses its replication ability (Feng *et al.*, 2000) and expression of ribosomal subunit proteins as well as some other cellular proteins and enzymes essential to ATP production becomes inactivated (Yamanaka *et al.*, 2005). It had also been hypothesized that Ag^+ primarily affects the function of membrane-bound enzymes, such as those in the respiratory chain (Bragg *et al.*, 1974; McDonnell *et al.*, 1999). However, the mechanism of bactericidal actions of silver nanoparticles is still not well understood. In a previous report (Sondi and Salopek-Sondi, 2004) on the bactericidal activity of silver nanoparticles, it was shown that the interaction between silver nanoparticles and constituents of the bacterial membrane caused structural changes in or damage to membranes, finally leading to cell death.

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