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The Synthesis of Silver Nanoparticals from Streptomyces sp. with Antimicrobial Activity

Dina E. El-Ghwas[1] and Amr A. El-Waseif[2]*

[1]Chemistry of Natural and Microbial products Dept., National Research Center, Dokki, Egypt.
[2]Botany and Microbiology Dept., Faculty of Science (Boys), Al-Azhar University, Cairo, Egypt.

Abstract: In the present work, Ten Actinomycetes strains isolated from Egyptian soil were screened for their antimicrobial activity against the selected pathogen microorganisms. Five Actinomycetes strains showed mild to moderate antimicrobial activity and One isolate (No. 6) showed broad spectrum of activity against all tested microbial pathogens. The culture filtrate and the mycelium of the Ten Actinomycetes isolated strains were also screening for the biosynthesis of silver nano-particles. Three isolate strains are helpful in the biosynthesis of silver nano-particles. The most potent isolate No. 6 AgNPs producer strain and that achieved the highest antimicrobial activity was identified by molecular identification and it was Streptomyces coerulescens. The filtrate and the mycelium containing silver nano-particals were characterized by using the UV-Vis spectrum and showed a sharp narrow absorption spectrum located between 420-440 nm for both respectively. Also, the Fourier Transform Infrared Spectroscopy (FTIR) analysis was studied.
Keywords : Actinomycetes, Antimicrobial activity, AgNPs, UV-visible, FTIR analysis.

Introduction

Nanotechnology is mainly concerned with the synthesis of nano-materials using different systems and their applications. Nowadays, nano-particles are used in the areas of agriculture[1], health care, environment and consumer goods[2]. Nano-particals which synthesized by chemical and physical pathways are low yielding, energy intensive, difficult to scale up, producing high levels of hazardous wastes, requires the use of costly organ metallic precursors and yield extremely expensive materials. Also, the produced nano-particles exhibit undesirable aggregation with time. Chemical syntheses of nano-particals resulting in some toxic chemical species adsorbed on the surface that could have effects in medical applications. Thus, the need for clean, cost-effective, eco-friendly and biocompatible synthesis of metal nanoparticles encouraged the investigatore to exploit the biological sources as nanofactories [3, 4]. Various microorganisms (bacteria, yeast and fungi) are known to synthesize silver nanoparticles. The produced nanoparticles have different size and shape. Among the microorganisms, actinomycetes producing nano-particals that has not much work done. Actinomycetes are gram positive free living saprophytic bacteria, and it is a major source for production of natural metabolites with different biological activity such as antibiotics. Also, it play important role in control of infectious diseases, in enzymes, vitamins, antitumor agents, enzyme inhibitors and production of novel pharmaceuticals compounds[5]. Silver nanoparticles are high important due to their used as antimicrobial activity against the multidrug resistant microorganisms due to their small size. Therefore, AgNPs are extensively used in dental

materials[6], coating stainless steel in medical devices[7], cosmetics[8] and water treatment[9]. Antibacterial activity of AgNPs was studied by various groups [10, 11]. Due to vast emerging applications of AgNPs in distinct fields, there is increase in searching for synthesis of AgNPs and there is a pressing need to increase their yield, for which optimization of the process is very important step. The present work involves screening of Actinomycetes sp. isolated from Egyptian soil for both the extracellular biosynthesis of potent AgNPs and antimicrobial activity of the isolated strains. Also, the characterization of the resulted AgNPs was studies.

Materials and methods

Collection of samples:-

Soil samples were collected for the isolation of actinomycetes from heavy metal-polluted and non-polluted areas. The heavy metal-polluted sample was collected from tanneries area at Cairo, Egypt and non-polluted sample was collected from garden of the Al-Azher University, faculty of Science, Cairo Egypt. The samples were collected in sterilized containers and immediately transported to Chemistry of Natural and Microbial products laboratory and stored at refrigerator at 4ºC until further processing.

Isolation of Actinomycetes strains:-

The isolation was carried out by serial dilution method on starch casein agar medium[12] containing the following compositions (g/l):- soluble starch, 10.0; casein, 1.0; K_2HPO_4, 0.5 and agar, 20.0. The media are supplemented with cyclohexamide to avoid fungal contamination. The inoculated plates were incubated for 6-7 days at 37ºC. After incubation period the Actinomycetes colonies were separate and purified.

Test microorganisms:-

The antimicrobial activity was done using various pathogenic microorganisms such as *Escherichia coli NCTC 10416*, *Klebsiella pneumonia ATCC 13883* and *Pseudomonas aeruginosa ATCC 10145* as models for Gram-negative bacteria; *Staphylococcus aureus ATCC 29213* and *Bacillus subtilis NRRL B-543* as models for Gram-positive bacteria, *Aspergillus niger NRRL-363* as models for filamentous fungi and *Candida albicans ATCC 10231* as models for unicellular fungi.

Antimicrobial activity media:-

The media used for the antimicrobial activity of the strains under study have the following compositions (g/l):- Nutrient agar medium[13]:- D-glucose, 5.0; peptone, 5.0; meat extract, 5.0; NaCl, 5.0 and agar, 20.0; the pH was adjusted to 7. Used for growth of bacterial strains, Sabouroud agar medium[14]:- dextrose, 40.0; peptone, 10.0 and agar, 20.0; the pH was adjusted to 7. Used for growth of unicellular fungi and Czapek-Dox agar medium[15]:- sucrose, 20.0; $NaNO_3$, 2.0; K_2HPO_4, 1.0; KCl, 0.5; $MgSO_4.7H_2O$, 0.001 and agar, 20.0; the pH was adjusted to 7. Used for growth of Filamentous Fungi.

Screening for Antimicrobial activity:-

At the end of incubation period the secondary metabolite of Ten Actinomycetes strains were investigated for antimicrobial activity by using agar well diffusion method as follow: 40.0 ml of the media (Nutrient agar medium for bacterial strain, Sabouroud agar medium for unicellular fungi and Czapek-Dox agar medium for filamentous fungi) at (55-60◦C) was inoculated with 200.0 µl of the prepared test microorganisms suspensions and poured in 150.0 mm diameter plates and mixed well and allow to solidify. After solidification, holes of 9.0 mm diameter were made in the agar plate by the aid of a sterile Cork-borer. For each sample, duplicate holes were made and then 100.0 µl of the culture filtrate was poured in the prepared holes using an automatic micropipette. The Petri-dishes were kept in a refrigerator for one hour to permit homogenous diffusion of the antimicrobial agent before growth of the test microorganisms, and then the plates were incubated at 37 ◦C for 24 hours for Gram positive and Gram negative bacteria and at 28 ◦C for 72 hours for unicellular and filamentous fungi. The antimicrobial activities of the isolates under study were determined by measuring the diameter of inhibition zone[16].

Molecular identification of the most active producer strains:-

The most active Actinomycetes isolates strains were subjected to the molecular identification. The Chromosomal DNAs were isolated by a versatile quic-prip method for of genomic DNA from G-positive bacteria (*Streptomyces*) according to [17, 18] with some modification. Then, the polymerase chain reaction (PCR) was performed using primers designed to amplify about 1000 pb fragment of the DNA region of *Streptomyces*. The forward primer was StrepB, 5'- ACAAGCCCTGGAAACGGG T-3' and the reverse primer was SterF 5'-ACGTGTGCAGCCCAAGACA-3' [19, 20]. Also, the Blast program (www.ncbi.nlm.gov/blast) was used to assess the DNA similarities. Multiple sequence alignment and molecular phylogeny were performed using BioEdit software [21]. The phylogenetic tree was displayed using the TREEVIEW program [22].

Screening for extracellular synthesis of AgNPs:-

Ten isolated Actinomycetes strains were screened for the potent silver nano-particle production, and further biosynthesis of silver nano-particles was carried out by using starch casein broth medium at 37ºC under shaking cultivation conditions. After 6-7 days of incubation the mycelia (biomass) was separated from the culture broth by filtration, then the mycelia were washed twice with distilled water under sterile conditions. The biomass was taken again in the Erlenmeyer flask containing 50 ml sterile distilled water. Thereafter, the supernatant and the mycelium were used for the synthesis of AgNPs. A solution of silver nitrate (0.1mM) was prepared by dissolving 0.017 g of the compound in 100 ml of distilled water. Then, 50 ml of silver nitrate solution was added to 50 ml of both mycelium and supernatant respectively and incubated again for 24 hours at room temperature under dark conditions and observed for color changed.

Characterization of silver nano-particals (AgNPs):-

Ultraviolet (UV) spectrum:

The synthesis of silver nano-particals was confirmed by ultraviolet (UV) spectrum analysis using: T80+UV/VIS Spectrometer, PG Instrument Ltd. Range: 190-1000 nm.

FTIR spectrum measurement:

FTIR spectrum was obtained by mixing with potassium bromide at 1 : 100 ratio which was compressed to a 2 mm semi-transparant disk for 2 min. spectra over the wavelenght (4000-400 cm^{-1}) were recorded using Nexus 670 FTIR spectrophotometer (Iclet Co., USA)

Results and discussion:-

Screening for antimicrobial activity:

Preliminary screening for antimicrobial metabolites production was conducted by agar well diffusion method as mentioned in material and method section. Ten Actinomycete isolates were grown in submerged culture to select the most potent isolates that have the ability to produce antimicrobial metabolites against the above mentioned test organisms. The results obtained in Table 1 indicated that, the best isolates producing antimicrobial metabolites against all test organisms under shaking conditions was isolates number 1, 2 and 6. However, isolates number 3 and 10 showed pronounced antimicrobial activities against Gram positive and negative bacteria but appeared to be inactive against the unicellular and filamentous fungi.

Table (1):-The antimicrobial activity of isolated Actinomycete strains

Actinomycete Isolate No.	Diameter of inhibition zone in (mm) Test organisms						
	E. Coli NCTC 10416	***K. pneumonia ATCC 13883***	***P. Aeruginosa ATCC 10145***	***B. Subtilis NRRL B-543***	***S. Aureus ATCC 29213***	***A. Niger NRRL-363***	***C. Albicans ATCC 10231***
1	20.0	19.0	17.0	20.0	15.0	20.0	10.0
2	18.0	20.0	15.0	19.0	15.0	20.0	21.0
3	35.0	19.0	14.0	18.0	20.0	-	-
4	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-
6	30.0	30.0	25.0	30.0	25.0	25.0	30.0
7	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-
10	20.0	18.0	16.0	20.0	15.0	-	-

(-) mean negative results

Screening for extracellular synthesis of AgNPs:-

Ten isolated Actinomycetes strains are inoculated into production media (starch casein medium) for screening of biosynthesis of silver nanoparticles (AgNPs) as mentioned in material and method section. After the end of the incubation period both the cultural filtrate and the mycelium were used for the synthesis of (AgNPs) under sterile conditions. After 24 hours of adding the silver nitrate the results showed that, among ten tested strains only three strains showed ability to synthesis AgNPs on neither the cultural filtrate nor the mycelium. As shown in Table 2, isolate No 2 and 3 were color changed from colorless before addition of silver nitrate to pale orange and green yellow after synthesis of AgNPs for both mycelium and cultural filtrate respectively. Also, isolate No. 6 changed from Dark yellow to pale orange after synthesis of AgNPs for both mycelium and cultural filtrate. On the other hand, isolate No. 1 changed from colorless to orange after synthesis of AgNPs by cultural filtrate only. However, no color change was observed in the cultural filtrate, mycelium and silver nitrate as controls. *Streptomyces sp.* No. 6 showed good production of AgNPs compared to other isolates. So, *Streptomyces sp.* No.6 is used for further characterization and application studies.

Table (2):- Screening for biosynthesis of AgNPs by Actionomycetes isolates

Actinomycete Isolate No.	Color before inoculation with silver nitrate	Color of mycelium after inoculation	Color of supernatant after inoculation
1	Colorless	-	Orange
2	Colorless	Pale orange	Pale orange
3	Colorless	Green yellow	Green yellow
4	Colorless	-	-
5	Colorless	-	-
6	Dark Yellow	Pale orange	Pale orange
7	Colorless	-	-
8	Colorless	-	-
9	Colorless	-	-
10	Colorless	-	-

(-) mean negative results

Molecular identification of the most active producer strains:-

The resulted sequence from PCR amplification process was aligned with the corresponding sequences of representive *Streptomyces* species. The sequence data of the isolate was then analyzed by Blast program (www.ncbi.nlm.gov/blast) to assess the DNA similarities. Multiple sequence alignment and molecular phylogeny were performed using BioEdit software and the phylogenetic tree (Figure 1). The obtained results revealed that the sequence of the isolate No. 6 showed highest similarity (98%) with *Streptomyces coerulescens*.

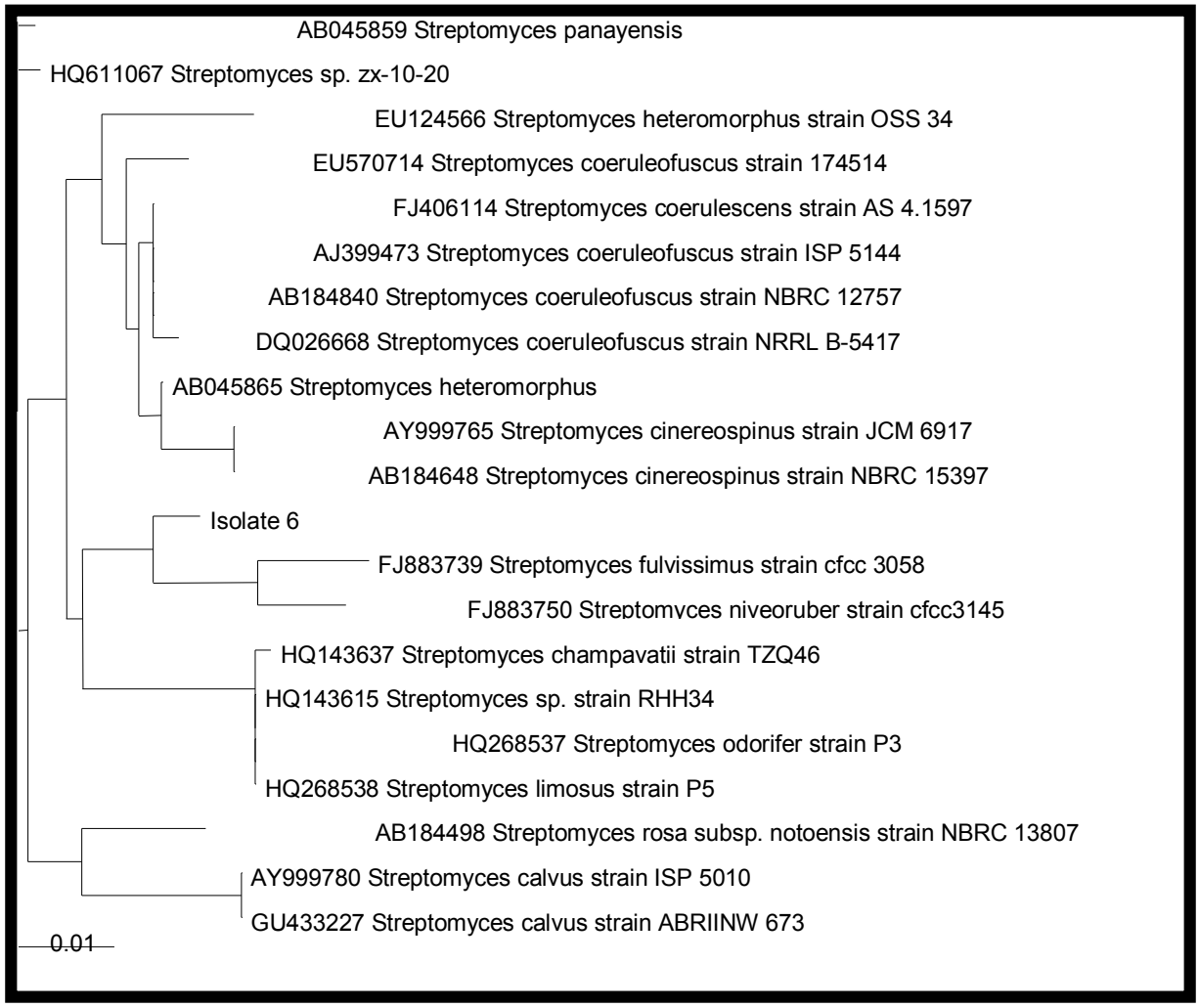

Figure (1):-The phylogenetic tree of isolate No. 6 compared to other *Streptyomyces sp*. based on 16S rDNA sequences.

Characterization of AgNPs produced by using *Streptomyces coerulescens*: - Ultraviolet (UV) spectrum:-

The biosynthesis of Ag^+ in the culture filtrate and mycelium produced by Streptomyces coerulescens was detected by the reaction mixture at regular intervals by using UV spectroscopy. The UV-Vis spectrum of AgNPs showed sharp narrow absorption spectrum located between 420-440 nm for both culture filtrate and mycelium respectively (Figure 2 and 3). Several reports sated that, the biosynthesis of Ag^+ happened due to the electron shuttle quinines and reducing agents such as enzyme [23].

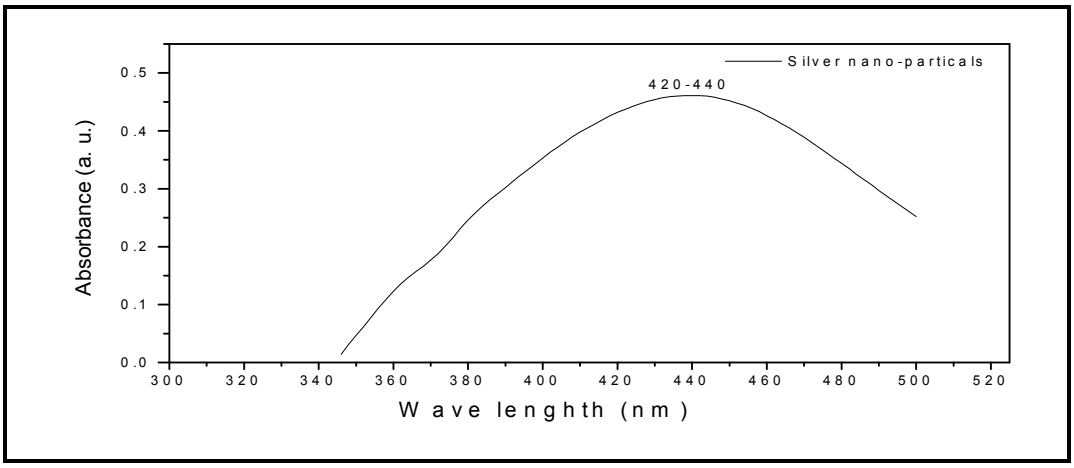

Figure (2): UV-Vis Spectra absorbance value of synthesis AgNPs by supernatent of *Streptomyces coerulescens*

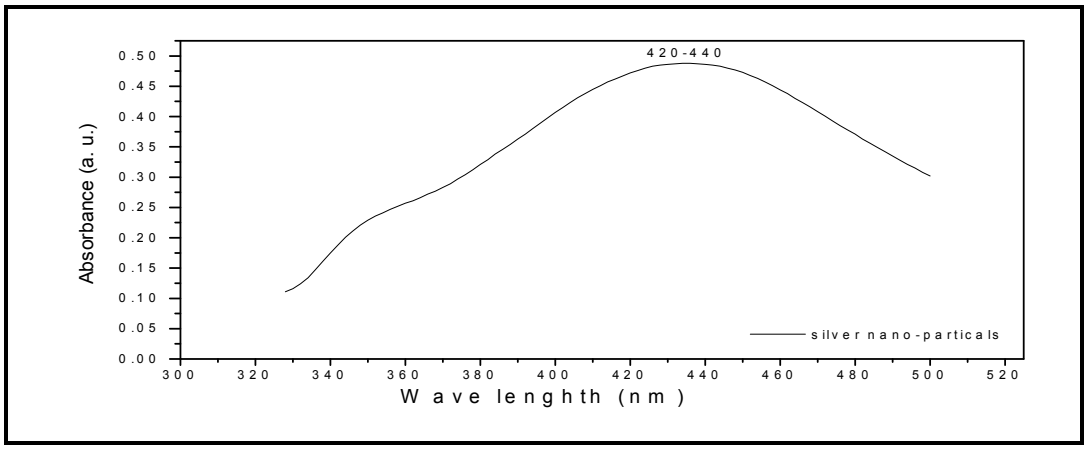

Figure (3): UV-Vis Spectra absorbance value of synthesis AgNPs by mycelium of *Streptomyces coerulescens*

FTIR spectrum measurement:

FTIR measurements were carried out to identify possible interaction between silver and protein molecules, which may be responsible for synthesis, stabilization and well dispersed silver nanoparticles in the reaction mixture[23]. FTIR spectral analysis showed array of absorbance bands in 400cm^{-1} - 4000 cm^{-1}. As shown in Figure 4 and 5 for both supernatant and mycelium respectively. There is a peak at 3445.21 and 3438.46 cm^{-1} for both supernatant and mycelium respectively, which is assigned to the primary amines (N-H stretch group) and its intensity showed large amounts of AgNPs were formed. This due to the presences of amide group in proteins which has strong ability to bind metal indicating that the proteins as capping agent to prevent agglomeration and there by stabilizing the nanoparticles[24]. Also there is a peak at 1638.23 and 1644.98 cm^{-1} for both supernatant and mycelium respectively which is assigned to primary amines (N-H bond).

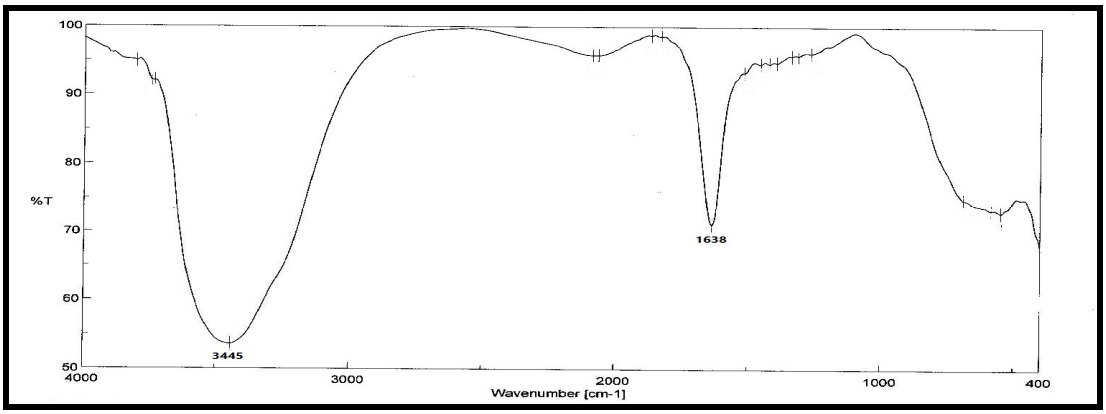

Figure (4):- FTIR analysis of silver nano-particles synthesized by supernatant of *Streptomyces coerulescens*

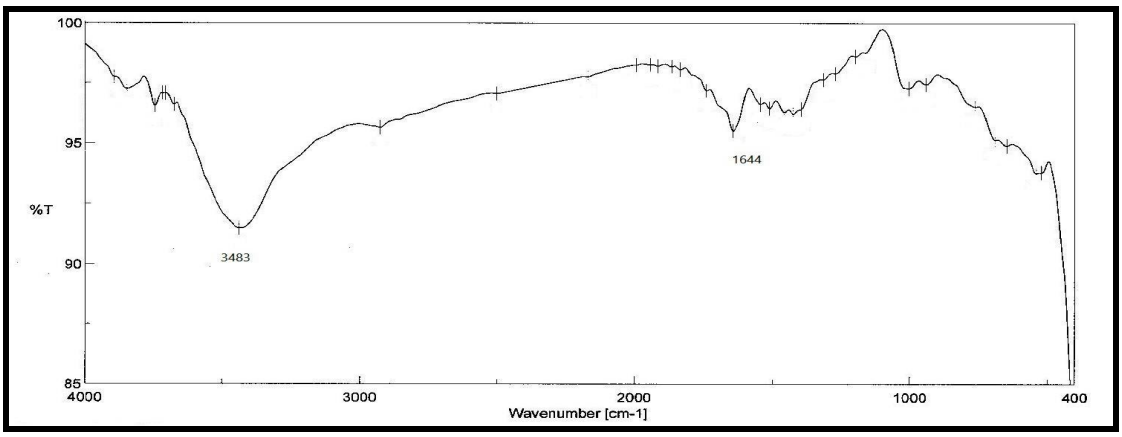

Figure (5):- FTIR analysis of silver nano-particles synthesized by mycelium of *Streptomyces coerulescens*

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