

# Biosynthesis, Characterization, Optimization of Silver Nanoparticles from Marine *S. griseoflavus* and Their Role in Improving Antibiotic Susceptibility of Multidrug Resistance Pathogenic Fungi

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## Abstract

Green synthesis of silver nanoparticles using marine microorganisms has gained profound interest because of their potential to synthesize nanoparticles of various size, shape and morphology. In the present investigate, 25 silver resistant bacterial isolates were isolated from different sites of red sea coast at Jeddah city, , bacterial strain BM3 was selected on the basis of its ability to produce stable extracellular silver nanoparticles ( AgNPs). Biochemical and Molecular characterization of this isolate indicated that BM3 strain is belonged to *Streptomyces* and identified as *S. griseoflavus*. The biosynthesis of AgNPs was monitored by UV–Visible spectrum that showed surface plasmon resonance (SPR) peak at 420 nm. Further characterization of synthesized AgNPs was carried out using the XRD, TEM and FTIR spectroscopy. TEM and XRD analysis revealed that the AgNPs synthesized by MB was spherical in shape with a size range of 5-45. nm FTIR confirmed the presence of proteins as the stabilizing agent surrounding the nanoparticles. Optimization of silver nanoparticles synthesis has been studied and the results demonstrated that the biosynthesis of AgNPs by bacterial supernatant increased with the increasing of the temperature, pH and Ag<sup>+</sup> ions concentration. The synthesized AgNPs and their combination with different commercial antibiotic were tested against *C. albicans* and *C.krusei*. All tested antibiotics showed synergistic inhibition against growth of the pathogenic bacteria. The biocide actions of AgNPs studied using SEM. The results showed damage in membrane and some pits that have been created cause inter cellular components leakage and finally cell death .

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UV-Vis absorption spectra of AgNPs alone, each antibiotic alone, and AgNPs-antibiotic combinations were studied. The results clearly point out that the synergistic effect of AgNPs-antibiotic combination against pathogenic is mediated by complexes that are produced from the interaction between AgNPs and the antibiotics, instead of individual or additive action of AgNPs or antibiotic. In other words, the pathogenic cells are killed more effectively by “AgNPs-antibiotic complexes”. However, the nature of the interaction between the AgNPs and the antibiotics needs further investigation.

**Keywords:** Marine bacteria; Antifungal activity; Optimization of silver nanoparticles; Biochemical and Molecular characterization.

## **1. Introduction**

The synthesis of metal nanoparticles using biological methods is an expanding research area due to the potential applications in nanomedicines [8]. Production of metallic nanoparticles for applications in catalysis, electronics, optics, environmental science, and biotechnology is an area of steady interest [13]. In general, various chemical and physical procedures have been used for synthesis of metallic nanoparticles [1,18]. However, these methods are fretful with many problems including use of toxic solvents, generation of hazardous by-products [13] and high energy consumption. Therefore, there is a growing need to develop environmentally benign methods for nanoparticles synthesis without using any toxic chemicals. Synthesis of uniform nano sized drug particles with specific requirements in terms of size, shape, and physical chemical properties is of great interest in the formulation of new pharmaceutical products [8]. Marine environments are considered a good source of metal tolerant microorganisms due to continuously release of the metals into marine environments by volcanoes, natural weathering of rocks and also by numerous anthropogenic activities, such as mining, combustion of fuels, industrial and urban sewage and agricultural practices. Recently, they are being explored as potential sources of metal tolerant microorganisms with the ability to synthesize metallic nanoparticles [3]. Silver nanoparticles (AgNPs), are the noble metal nanoparticles that has being studied extensively due to its various biological properties [15]. Antibiotic resistance among pathogenic bacteria is one of the most important human health problems worldwide, seriously threatens human life every passing day. Therefore, there is an immediate need to develop new approaches to overcome this problem. New drugs or at least new formulations of known drugs that provide better efficacy are urgently needed for a faster, more efficient, and less impairing treatment [4]. One of the promising approaches for overcoming bacterial resistance is the use of metallic nanoparticles [4,15,7]. Due to their small sizes and higher surface-to-volume ratio, metallic nanoparticles have an enough contact area with a bacteria [5]. Therefore, the present study is aimed to (1) Biosynthesis of silver nanoparticles using marine bacteria. (2) investigate the synergistic effect of produced nanoparticles combined with antibiotics against Multi-Drug resistant of pathogenic fungi.

## **2. Details experiment**

### ***2.1 Isolation and Characterization of Bacterial Strains***

The sea water samples were collected from the red sea coast at Jeddah city, KSA during February-Mai, 2015

with 1000 mL of sterile plastic container. The seawater sample was kept in ice box and transport to the laboratory. The sample was processing within 8 hours. The isolation of marine bacteria was carried from the “Serial Dilution Method” as described by [23]. One ml of each dilutions was taken and plated on marine agar medium supplemented with different concentrations (1Mm ,2Mm, 3Mm ,4Mm and 5 Mm) of silver nitrate. The plates were incubating overnight in the incubator at 37°C. Colonies survived in the plates were considered as a silver resistant strains . Then the silver resistant bacteria colonies were sub cultured on same medium to ensure their purity.

In order to screen an efficient strain for the synthesis of AgNPs, all the bacterial strains were freshly inoculated in an Erlenmeyer flask containing marine broth. The flasks were incubated at 37 °C for 24 h .After incubation, the bacterial mass was obtained by centrifugation at 7000 rpm for 15 min. The final volume concentration of 1 mM AgNO<sub>3</sub> was added into 20 ml of sterilized Q water containing 50 ml Erlenmeyer flask. The cell free supernatant without addition of AgNO<sub>3</sub> was maintained as a control. Subsequently, the bio-reduction of silver ions was monitored by the visual color change and UV-visible spectrum analysis for the reaction mixture. Based on the rapid reduction of AgNO<sub>3</sub> into AgNPs the efficient bacterial strains were selected and used for further characterization. The formation of the AgNPs was confirmed by UV-vis spectrophotometer All the experiments were carried out in triplicate and average values have been reported

## **2.2 Identification of selected bacterial isolates**

### **2.2.1 Morphological examination**

The selected strains were cultivated on marine agar plates and incubated at 30°C for 24 h. After that , the characters of the selected strains such as color , shape and colony edge were recorded .Bacterial morphology, Gram reaction ,acid fast staining ,capsule and spore formation were examined using light microscope by using oil immersion lens.

### **2.2.2 Physiological and biochemical characters**

The morphological and physiological characterization of the selected isolate will be carried out by biochemical tests using the Bergeys Manual of Determinative Bacteriology [16] . These characters including some enzyme production(Urease , Catalase and Lechithinase),Utilization of casein , production of indole and fermentation of sugar (Voges proskaure) were also recorded. Growth temperature and growth pH were also studied. In addition to, the ability of selected strains to utilize different carbon source and nitrogen source were also investigated

### **2.2.3 Molecular identification**

The nanoparticle synthesizing bacteria further identified based on morphological and physiological characterization according to the methods of Bergeys manual of determinative bacteriology followed by nuclear ribosomal DNA sequencing. Total genomic DNA was isolated from a selected strain according to standard method .The amplification of DNA was carried out by colony PCR with 27 F5’ AGAGTTTGATCCTGGCTCAG3’ and 1492R5’TACGGTTACCTTGTTACGACTT3’. Amplification includes

initial denaturation of the target DNA at 94 °C for 5 minutes, followed by 25 cycles of denaturation at 94 °C for 30 seconds, annealing temperature of primers at 53 °C for 30 seconds and extension at 72 C for 1 minute. A final extension at 72 C for 15 minutes was used. The product of sequencing reaction was analyzed by using DNA sequencer ABI PRISM 310 genetic analyzer (Perkin Elmer, USA). Data were submitted to Gen Bank database. The DNA sequence was compared to the Gen bank database in the National Center for Biotechnology Information (NCBI) using the BLAST program'

### **2.3 Biosynthesis of AgNPs**

Biosynthesis of AgNPs was carried out according to the method described previously by [17] with some modifications. To obtain stable silver nanoparticles, strains grown in a 500 mL Erlenmeyer flask containing marine broth. The flasks were incubated for 24 h in a shaker set at 120 rpm and 30 °C .After the incubation period, the culture was centrifuged at 10,000g and 2 g of biomass (wet weight) was brought into contact with 100 mL sterile Q-water for 48 h at 28 °C in an Erlenmeyer flask and was shaken at 200 rpm. After incubation, the cell filtrate was obtained by centrifugation. For synthesis of silver nanoparticles ,silver nitrate was mixed with 100 mL of cell free filtrate to obtain a final concentration of 1 mM silver ions. The extracellular synthesis of AgNPs was monitored by visual inspection of the filtrate for a change in the color of culture medium from a clear, light yellow to brown, and by measurement of the peak exhibited by silver nanoparticles in the UV–Vis spectra.

### **2.4 Optimization of different parameters**

Different reaction parameters may have variable effect on the reduction process thereby altering the final product shape and size. So Various parameters such as concentration of silver nitrate, pH, temperature and silver salt concentration on the synthesis of AgNPs was studied .

#### **2.4.1 The effect of AgNO<sub>3</sub> concentration on Silver reduction and nanoparticles formation**

This assay was carried out using supernatant and different Ag<sup>+</sup> ions concentration varying 1-5mM and pH 7.2 .The samples were incubated at 37°C for 24 h. The reduction of silver and silver nanoparticles formation were followed by visual observation and UV-Vis spectroscopy [10] .

#### **2.4.2 The effect of pH on Silver reduction and nanoparticles formation**

The effect of pH on Silver reduction and nanoparticles formation was evaluated with the silver reduction assay using specific buffer ranging from pH 3 to 10. Acetate was used for the acidic range phosphate buffer for the natural pH range and NaOH for alkaline pH range. The samples were incubated at 37°C for 24 h. The reduction of silver and silver nanoparticles formation were followed by visual observation and UV-Vis spectroscopy [10] .

#### **2.4.3 The effect of temperature on Silver reduction and nanoparticles formation**

The effect of temperature on silver reduction and nanoparticle formation was tested through incubation of

bacteria supernatant with 1 mM AgNO<sub>3</sub> at 10 °C; 25 °C; 37 °C; 40 °C; and 60 °C respectively. The colour change of the solution was monitored periodically and subjected to UV-VIS Spectrophotometric analysis.

## **2.5 Characterization of AgNPs**

### **2.5.1 UV-Vis spectral analysis**

The biologically synthesized silver nanoparticles using the cell free filtrate of MB1 was characterized by UV-vis spectroscopy (Perkin Elmer, Lambda 25) scanning in the range of 200–700 nm, at a resolution of 1nm. All samples for UV-Vis spectra measurement were prepared by centrifugation of an aliquot of culture supernatant (1.5 ml) at 10,000 rpm for 10 min at 25 °C. UV-Vis spectra analyses of AgNPS produced were carried out as a function of bioreduction time at room temperature. Cell free supernatant without addition of AgNO<sub>3</sub> was used as a control through the experiment.

### **2.5.2 Transmission Electron Microscopy (TEM) Analysis**

Samples for transmission electron microscopy (TEM) analysis were prepared on carbon-coated copper TEM grids. Studies of size, morphology and composition of the nanoparticles were performed by means of transmission electron microscopy (TEM) operated at 120 kV accelerating voltage (JTEM-1230, Japan, JEOL) with selected area electron diffraction (SAED). Finally, the obtained images were processed using the software ImageJ. ImageJ developed at the National Institutes of Health (NIH), USA is a Java-based public domain image processing and analysis program [22].

### **2.5.3 Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) Analysis**

FTIR measurements were carried out using attenuated total reflection Fourier transform infrared (ATR-FTIR) Spectrometer (Bruker, Germany, Alpha-P). The instrument was configured with ATR sample cell including a diamond crystal with a scanning depth up to 2 micrometers. Sample powders were applied to the surface of the crystal then locked in place with a “clutch-type” lever before measuring transmittance. Each of the spectra was collected in the range 4,000-400 cm<sup>-1</sup> at 2 cm<sup>-1</sup> resolution. Comparing with the conventional transmission mode, the present technique is faster sampling without preparation, excellent reproducibility and simpler to use.

### **2.5.4 X-Ray Diffraction (XRD) Analysis**

X-ray diffraction (XRD) analysis was performed using an automated diffractometer (Philips type: PW1840), at a step size of 0.02, scanning rate of 2 in 2θ/min., and a 2θ range from 10° to 70°. Indexing of the powder patterns and least squares fitting of the unit cell parameters was possible using the software X'Pert High Score Plus204.s

## **2.6 Antimicrobial activity of AgNPS**

The antifungal activity of AgNPs was investigated against And pathogenic fungi such as *C. albicans* and *C. krusei* were also used in this study.

### **2.6.1 Antifungal and Synergistic activity of AgNPS**

The Disc diffusion method that described previously by [9] was followed to assay the antibacterial activity and the synergistic effect of synthesized AgNPs with some modification. Briefly, Filter paper discs (6mm diameter) were prepared using a punch machine. The discs were sterilized and kept in the refrigerator for further use. The test microorganisms were inoculated in 10 ml of sterile nutrient broth for 24 hours at 37°C. By using sterile cotton swabs, the cultures were aseptically swabbed on the surface of sterile Mellor Hinton agar plates. Then M.H.A plates were dried for 15 minutes in the laminar air cabinet. AgNPS (50 µl) were added on filter paper disc and on antibiotic disc and left for drying. The third disc contains the antibiotic alone. Three discs were placed on the plates. The plates were incubated at 37°C for 24 hours and the diameter of the inhibition zones was measured in mm.

### **2.6.2 Assay the effect of AgNPs on bacterial cell structure by Scanning Electron Microscopy (SEM)**

The interaction between pathogenic isolates and the potentially effective AgNPs was examined by Scanning electron microscopy (SEM). Muller Hinton broth was prepared and sterilized to use for preparing the inoculums. After the incubation period 100 µl of the bacterial cultures were added in sterilized tube and incubated with 100 µl of AgNPs for 2 h at 37 °C. Control group was generated without AgNPs. After that One milliliter of treated and untreated cells were added to sterile blank discs and left to dry for morphology and structure analysis by SEM according to the method that described by [14].

### **2.6.3 Antibiotic bound biogenic silver nanoparticles**

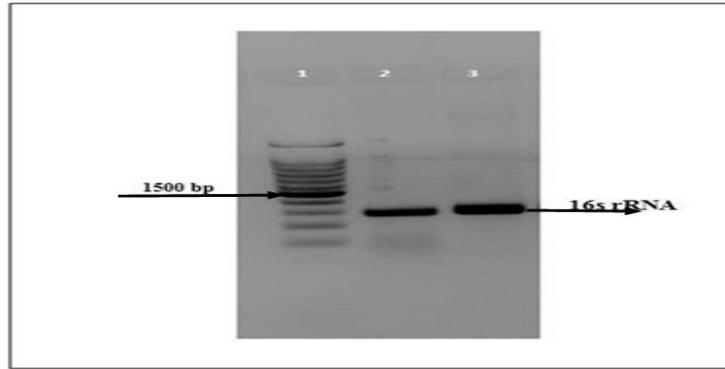
Antibiotic bound silver nanoparticles will be prepared by stirring 1mL of silver nanoparticles with 0.5mL of antibiotic (50 µg/mL concentration) for 15 min and then kept overnight at room temperature [2]. After 24 h of incubation the solution will be centrifuged and pellet will be collected for resuspension in sterile distilled water and stored in refrigerator for UV-Vis spectroscopy analysis.

## **3. Results and Discussion**

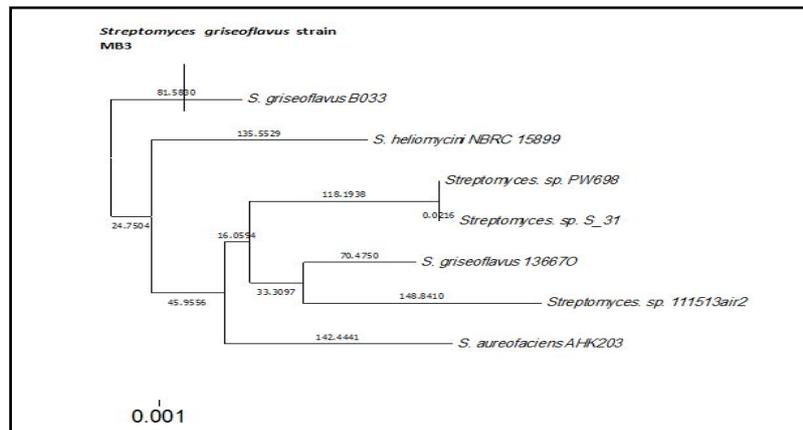
### **3.1 Identification and Characterization of Bacterial Strain**

The molecular technique was done to prove and to further confirm the identification of strain MB3. PCR product of 16s RNA gene was estimated using ethidium bromide staining of agarose /TBE (Figure. 1). Approximately 632 bp of 16s rRNA gene was sequenced and the sequence was deposited in the GenBank under the accession number (**KX180926.1**).

The target sequence was carefully posited in the BLAST program of National Center for Biotechnology Information (NCBI) in order to search for it's similar sequences inside the database. The 16s rRNA gene sequence of the isolate MB3 showed high level of sequence similarity with members of the genus *Streptomyces* such as *Streptomyces griseoflavus* (Figure.2)



**Figure 1:** PCR Amplification for partial 16s rRNA gene products of isolated strains, 10µl of PCR product visualized by ethidium bromide staining on 1.5% TBE agarose gel. The abbreviations: 1 : 1 Kb DNA leader , 2; MB3 strain



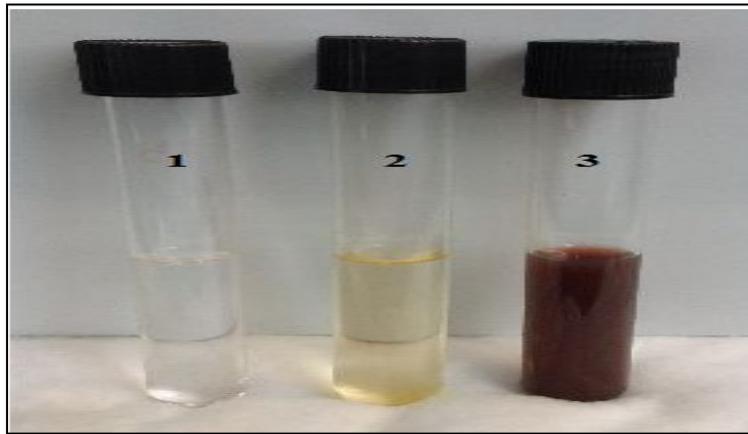
**Figure 2:** Phylogenetic tree based on 16s rRNA gene sequence comparisons of *S. griseoflavus* , using Neighbor-joining tree method, maximum sequence difference =0. 01 .

### 3.2 Characterization of AgNPs Synthesized by *Streptomyces griseoflavus*

#### 3.2.1 Visualization of color

A study on extracellular biosynthesis of AgNPs by the culture supernatant of *S. griseoflavus* was described. Visual observation of the culture supernatant incubation with silver nitrate at room temperature in dark showed a color change from pale yellow to yellowish-brown colour which is the characteristic for silver nanoparticles formation due to the excitation of surface plasmon vibrations in silver nanoparticles and provides a convenient spectroscopic signature of their formation [11] , whereas no color change could be demonstrated in culture supernatant without silver nitrate or media with silver nitrate alone (Figure. 3). This an important observation that the reduction of the  $Ag^{2+}$  ions takes place in the extracellular cell filtrate of *S. griseoflavus* rather than intracellular. Accordingly, this offers a great advantage over an intra-cellular process of synthesis from the application point of view. Since the nanoparticles formed inside the bacteria would have required additional step

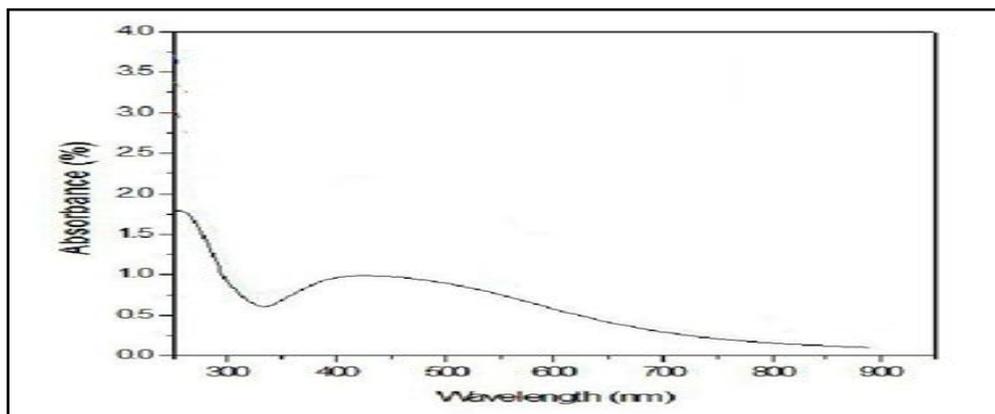
of processing for the release of the nanoparticles from the bacteria by ultrasound treatment or by reaction with suitable detergents.



**Figure 3:** Digital photograph of test tubes containing supernatant reacted with aqueous solution of 1 mM AgNO<sub>3</sub>. Blank control (tube 1) contains AgNO<sub>3</sub> only; supernatant without AgNO<sub>3</sub> (test tube 2), It is observed that the color of the solution turned from colorless to yellow brown after 12 h (tube 3) and 24 hrs respectively.

### 3.2.2 Characterization of silver nanoparticles by UV-Vis spectroscopy

The UV-Vis spectra illustrated in (Figure.4) show a well-defined absorption peak corresponds to the wavelength of the surface plasmon resonance (SPR) of AgNPs at 420 nm. Various reports have established that the resonance peak of silver nanoparticles appears around this region, but the exact position depends on a number of factors such as particles size, and the surface-adsorbed species [19].

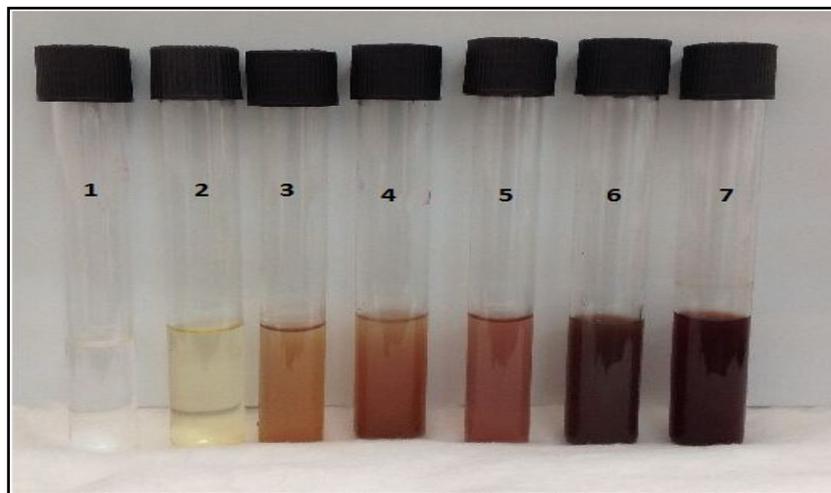


**Figure 4:** UV-Vis absorption spectra of silver nanoparticles solution prepared by resuspending the supernatant of *S. griseoflavus* in the presence of 1 mM AgNO<sub>3</sub>

### Effect of silver salt concentration on AgNPs synthesis by isolated strain

The results demonstrated that the intensity of color change enhanced with the increasing of silver ion

concentrations (Figure.5). With 1.0 mM of silver ions light brown color was developed, whereas, with 2.0mM of silver ions, dark brown color was observed then the color increased in darkness with increasing the concentration of  $\text{AgNO}_3$  (Figure. 5,tubes 5-7).



**Figure 5:** Digital photograph of test tubes(3-7) containing *S. griseoflavus* supernatant reacted with different concentrations of aqueous solution of  $\text{AgNO}_3$ (1 -5mM) Blank control (tube 1) contains  $\text{AgNO}_3$  only without supernatant and (tube 2) containing supernatant without  $\text{AgNO}_3$  .

The biosynthesized of silver nanoparticles by *S. griseoflavus* using different salt concentrations were also confirmed by UV-Vis spectroscopy. (Figure.6.A) show the spectra obtained when bacterial supernatant of *S. griseoflavus* reacted with various concentrations of  $\text{AgNO}_3$ (1-5 mM) respectively .As show in Figures,UV-Vis spectra of different reactions showed the appearance of single and strong SRP band absorption peak centered at 420 nm respectively, which indicated that the absorption spectrum of spherical AgNPs [1].Furthermore, the absorption peaks increased with the increasing of ion concentrations, indicating increasing the nanoparticles synthesis.

#### ***The effect of temperature on silver reduction and nanoparticles formation***

The effect of temperature on silver reduction and nanoparticle formation was tested through incubation *S. griseoflavus* supernatant with 1 mM  $\text{AgNO}_3$  at 10 °C; 25 °C; 37 °C; 40 °C; and 60 °C respectively, to determine the optimum temperature for nanoparticle formation with the best defined shapes. When comparing the reactions through the visual assay (Figure . 6) nanoparticle formation started at 10°C, with optimum formation at 37 °C. At 10 °C and 25 °C the supernatant reaction gave a light brown colour, signifying the formation of nanoparticles, with a clear difference in the light yellow colour of the blank reaction, indicating chemical reduction (medium only) was not formed. At 37 °C the supernatant reaction gave a slight dark brown colour, with again a clear difference in the blank reaction, proving it to be the optimum temperature to produce silver nanoparticles. This might be due to the optimum growth temperature for *S. griseoflavus* also being 37°C. At 40 °C to 60 °C the supernatant reaction gave a dark brown colour, with again a clear difference in the blank reaction. Moreover, These distinct colours indicate the variation in the type and size of the nanoparticles that

were synthesized [21] .



**Figure 6:** Digital photograph shows different colors obtained through the silver reduction assay at different temperatures , indicating silver nanoparticle formation. The tubes(3-7) represent

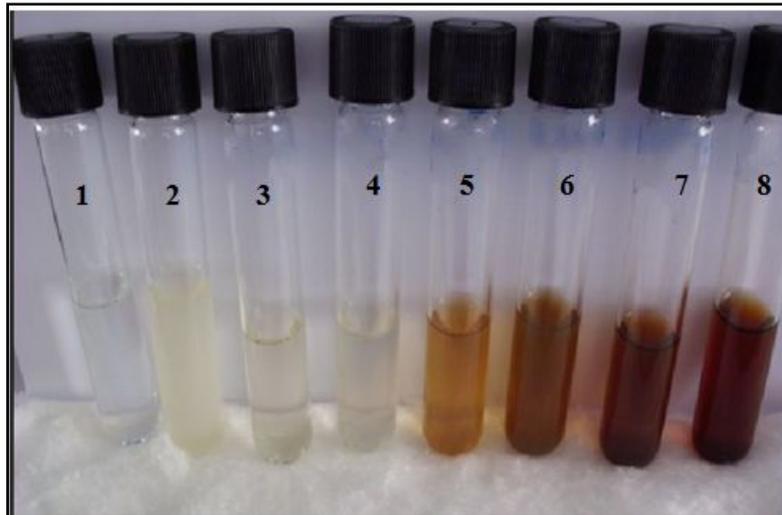
These results were also confirmed by UV–vis spectroscopy, the absorption peak shifts to 450 nm in the temperature of 25°C; while by increasing the temperature up to 60 °C, the band shifts to 410 nm, which is due to the localization of the surface plasmon resonance of the silver nanoparticles (Figure.6.B) [6] .

#### ***Effect of pH on silver reduction and nanoparticles formation***

The pH is one of the most important factor for nanoparticle formation. The shape and size of the nanoparticles are dependent on the pH of solution. In this study, the effect of pH on silver reduction and nanoparticle formation was tested at pH values of 5.0 :8.0; 10.0, to detect the optimum pH for nanoparticle that syntgesized by *S. griseoflavus* with the best defined shapes. When comparing the reaction through the visual assay (Fig.8) and the SPR, obtained through UV-Vis spectra. The effect of pH on the reduction of silver ions was studied by UV-Vis spectroscopy and is shown in (Figure. 6.C).

At pH 5.0, no absorption peak was observed in the range of 400– 450 nm for the colloidal suspension of all samples even after 24 h of the reaction. However, an absorption band appeared at about 440 nm when pH increased from 8 to 10 indicating the formation of AgNps, Figure 6.C.

It was observed that the absorption peak intensity increased gradually with an increase in pH, suggesting that the reduction rate of silver ions increases with an increase in pH. The formation of AgNps was suppressed by acidic conditions and enhanced by basic conditions. At lower pH (pH 5), no nanoparticles were formed, whereas, at higher pH (pH 10), smaller and highly dispersed nanoparticles formed, Figure 6. C. The results indicated that the optimum pH for synthesis of AgNPs that produced by *S. griseoflavus* is pH 8.



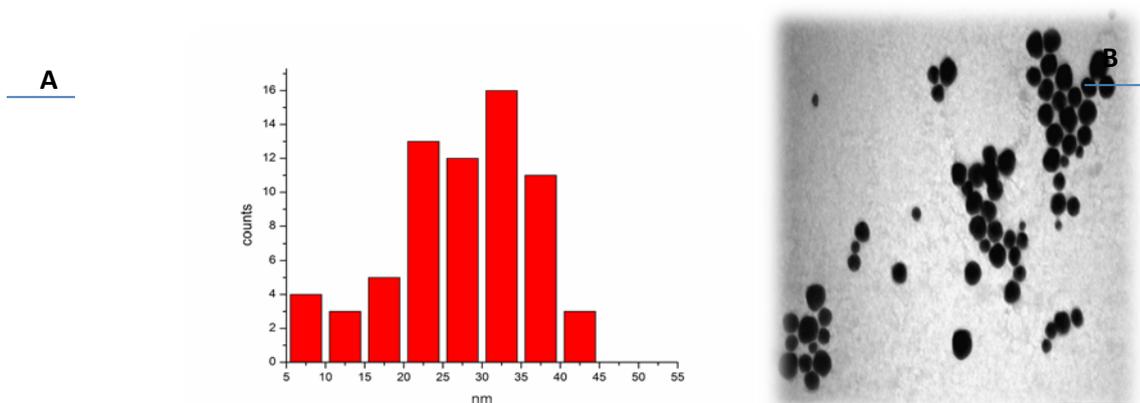
**Figure 7:** Digital photograph shows different colors obtained through the silver reduction assay at varying pH values

### 3.3 TEM Analysis

The morphology and shape of AgNPs produced by *S. griseoflavus* were determined using TEM.

TEM micro-graphs recorded from drop-coated films of the AgNPs synthesized after the reaction with silver nitrate solution for 24 h showed that the sample is composed of large quantity of AgNPs (Figure 8 a) The produced AgNPs were predominantly spherical in shape and uniformly distributed without significant agglomeration. TEM analysis in (Figure 8 a) showed presence of spherical shape and reasonably monodispersed AgNPs in size range 5-45nm and the average particles size is 27.5 nm (Figure 8 b).

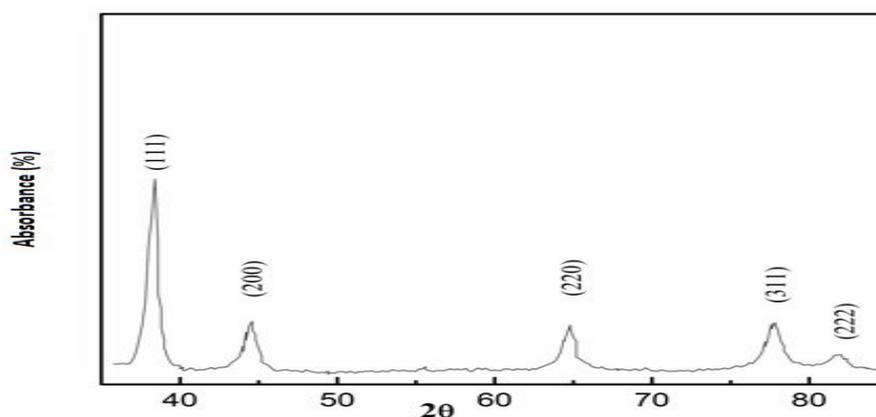
The size of nanoparticles formed by various groups of *Streptomyces* was vary. In the case of *S.griseoflavus*, it was shown that the size of silver nanoparticles formed was ranging from 5 to 45 nm [20] .



**Figure 8:** (A). TEM image of the AgNPs produced by the reaction

### 3.4 X-ray diffraction analysis

X-ray diffraction (XRD) pattern obtained for the silver nanoparticles that synthesized by *S. griseoflavus* was shown in (Figure.9). XRD shows that the crystalline structure of silver is face centered cubic. In XRD, silver has similar diffraction profile with intense peaks at  $2\theta$  of  $38.13^\circ$ ,  $44.1^\circ$ ,  $64.35^\circ$ , and  $77.88^\circ$  corresponding to the planes (111), (200), (220), and (311), respectively. This indicates that the synthesized silver nanoparticles by using *S. griseoflavus* supernatant that crystalline nature. Similar results were obtained by [24] using the *Streptomyces hygroscopicus*.



**Figure 9:** XRD pattern of AgNPs synthesized by *S. griseoflavu*

### 3.5 ATR-FTIR Analysis

The functional groups that involved in the reduction of Ag ions by supernatant of *S. griseoflavus* were also predicted using FTIR spectroscopy (Figure.28). FT-IR analysis revealed intense bands at  $3058$ ,  $2875$ ,  $1633$ ,  $1516$ ,  $1403$ ,  $1340$ ,  $1106$ ,  $1028$ ,  $825$ ,  $618$ , and  $533$   $\text{cm}^{-1}$  (Fig. 10). The peaks at  $618$   $\text{cm}^{-1}$  and  $825$   $\text{cm}^{-1}$  correspond to C-S disulfide stretching vibration. Similarly, the peak at  $533$   $\text{cm}^{-1}$  showed -S-S (polysulfide) stretching vibration indicating the frequent occurrence of thiols and its substituted compounds constituting the backbone of the interacting protein. Where the bands at  $1633$   $\text{cm}^{-1}$ ,  $1516$   $\text{cm}^{-1}$  related to the band frequency of amide I, amide II and amide respectively. The amide I band results from C=O stretching mode and C-N stretching. The more complex Amide III band is located near  $1340$   $\text{cm}^{-1}$ . On addition, the infrared active modes attributed to side-chain vibrations include C-H stretching modes at  $2875$   $\text{cm}^{-1}$  and a CH<sub>2</sub>-bending mode at  $1403$   $\text{cm}^{-1}$ . The band existing at  $1028$   $\text{cm}^{-1}$  can be assigned to the C-N stretching vibrations of aliphatic amines and a CH<sub>2</sub> bending mode at  $1403$   $\text{cm}^{-1}$ . This FTIR spectrum supports the presence of protein on the surface of AgNPs. [6] confirmed that the carbonyl groups from the amino acid residues and peptides of proteins have a stronger ability to bind metal, so that the protein could form a coat over the metal nanoparticles to prevent agglomeration of the nanoparticles. With the overall observation, it can be concluded that the proteins might have formed a coating over the silver nanoparticles, which in turn supports their stabilization. Surface interactions via functional groups. From the ATR-FTIR spectra, an interaction between AgNPs and protein is further confirmed by the shift in CH<sub>2</sub> ( $1449$   $\text{cm}^{-1}$  to  $1447$   $\text{cm}^{-1}$ ) and NH<sub>2</sub> ( $1395$   $\text{cm}^{-1}$  to  $1397$   $\text{cm}^{-1}$ ) ([12,25]).

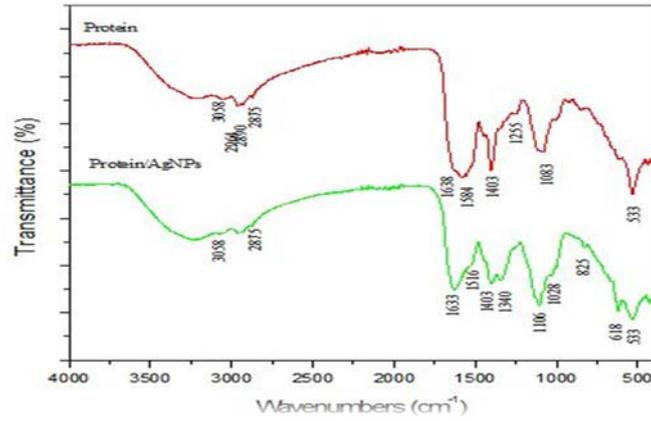
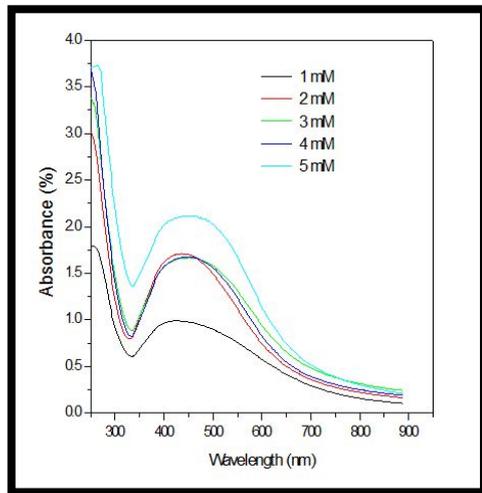
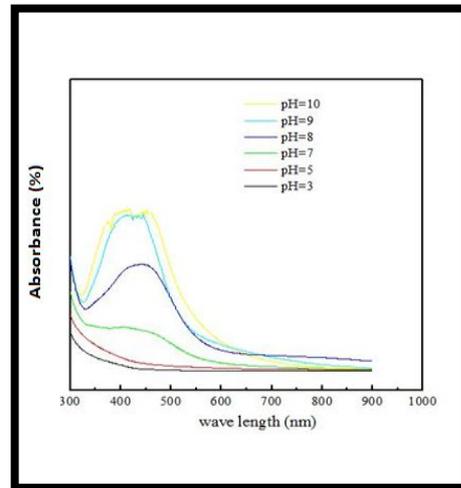


Figure 10: ATR-FTIR spectrum of AgNPs synthesized by *S. griseoflavus*

A



B



C

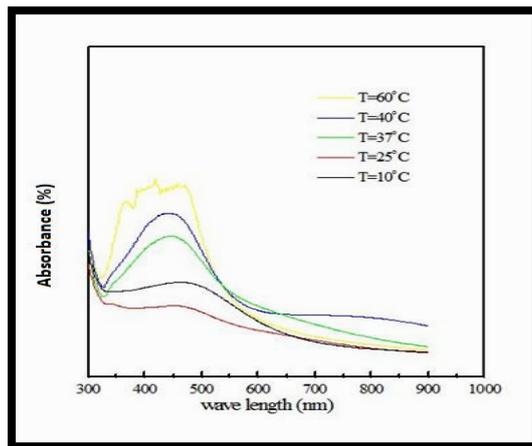
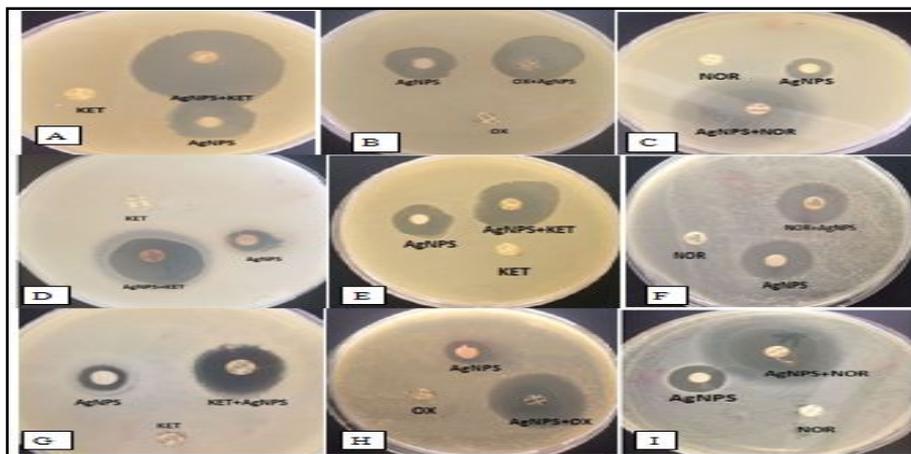


Figure 11: UV-Vis absorption spectra of Silver nanoparticles solutions after different reactions

### 3.6 Antimicrobial activity of AgNPS

### 3.6.1 Synergistic antibacterial activity of the selected six antibiotics in combination with AgNPs against pathogenic *Candida sp*

The resistance of fungal infection has emerged in recent years and is a major health problem. For that, the synthesized AgNPs and their combination with different commercial antibiotic were tested against *C. albicans* and *C.krusei*. In current work, The antifungal ketoconazole, that is widely used against many fungal infection beside antibacterial agents such as Norfloxacin and Oxacillin, were used as a positive control. The results in table(1),demonstrated that AgNPs synthesized by *S. griseoflavus* at concentration of 70µl was found to be effective against the examined fungi. It showed the maximum inhibition against *C.albicans*, (20mm), (Figure. 11.E) whereas less effect was observed on *C.krusei* (10 mm) (Fig 11.G) Interestingly, the combination of Ketoconazole with 70µl AgNPs exhibited maximum inhibition against *C. albicans*, (28mm) and *C.krusei* 30mm). These results indicate that antifungal activity of Ketoconazole was enhanced against the tested fungi in combined with AgNPs. These findings are in agreement with the results obtained by [1], whom studied the synergistic effect of AgNPs and Fluconazole against *C.albicans*, *P. glomerata* and *Trichoderma sp*. Their results showed the antifungal activity of fluconazole was enhanced against the test fungi in the presence of AgNPs. Fluconazole in combination with AgNPs showed the maximum inhibition against *C.albicans*, (26mm) followed *P. glomerata* (22mm) and *Trichoderma sp* (20 mm). Furthermore, In our study the activity of antifungal/or antibacterial agents were enhanced against the examined fungi in the presence of increasing concentration of AgNPs, For example combination of Norfloxacin with 100 µl of AgNPs exhibited maximum inhibition zone against *C.krusei* (45mm) and *C. albicans* (50mm)(Figure. 11,C and D) whereas The antifungal effect was found to be (58mm) (Figure. 11.A),(60mm) for *C. albicans* and *C.krusei* when ketoconazole conjugated with 100 µl AgNPs .

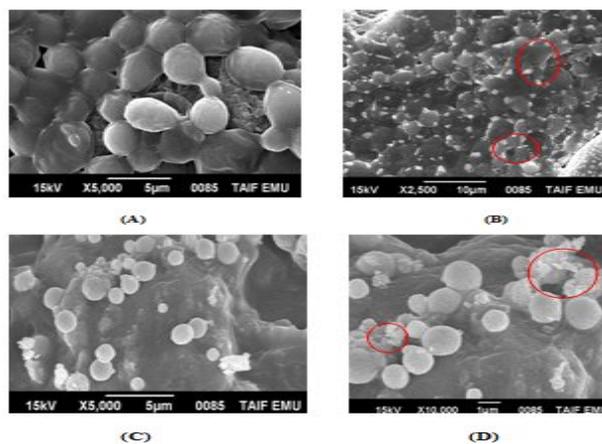


**Figure 12:** Antifungal activity of different concentration silver nanoparticles (Ag-NPs) that synthesized by *V. alginolyticus* against tested *Candida* with different antibiotics .The abbreviations: NOR= Norfloxacin, and KET= Ketoconazole ,A,B, E F and I= (*C.albicans*), C, D,G and H= (*C.krusei*)

### 3.6.2 Effect of AgNPs on bacterial cell structure by Scanning Electron Microscopy(SEM)

The morphological changes of *C. albicans* and *C.krusei* by Ag-NPs were also observed with a scanning electron microscope (SEM) untreated cells were with spherical shape and smooth cell wall and completely cell

membrane(Figure. 12 and C) but when it was treated with AgNPs we observed membrane damage and some pits that have been created cause inter cellular components leakage and finally cell death.(Figure.12 B and D). This result is in agreement with the investigation that done by [20] whom studied the change in structure and morphology of the examined yeasts such as *C. albicans*, *S.cerevisiae* that treated with 4 mg/ml (MIC90) concentration of Ag-NPs.The results showed damage in membrane and some pits that have been created cause intercellular components leakage and finally cell death.



**Figure 13:** SEM micrograph of *C.krusei* untreated (A) and treated with AgNPs ( B) and *C.albican* untreated(C) and treated with AgNPs ( D) for two h at 28°C and pH 7.0 .

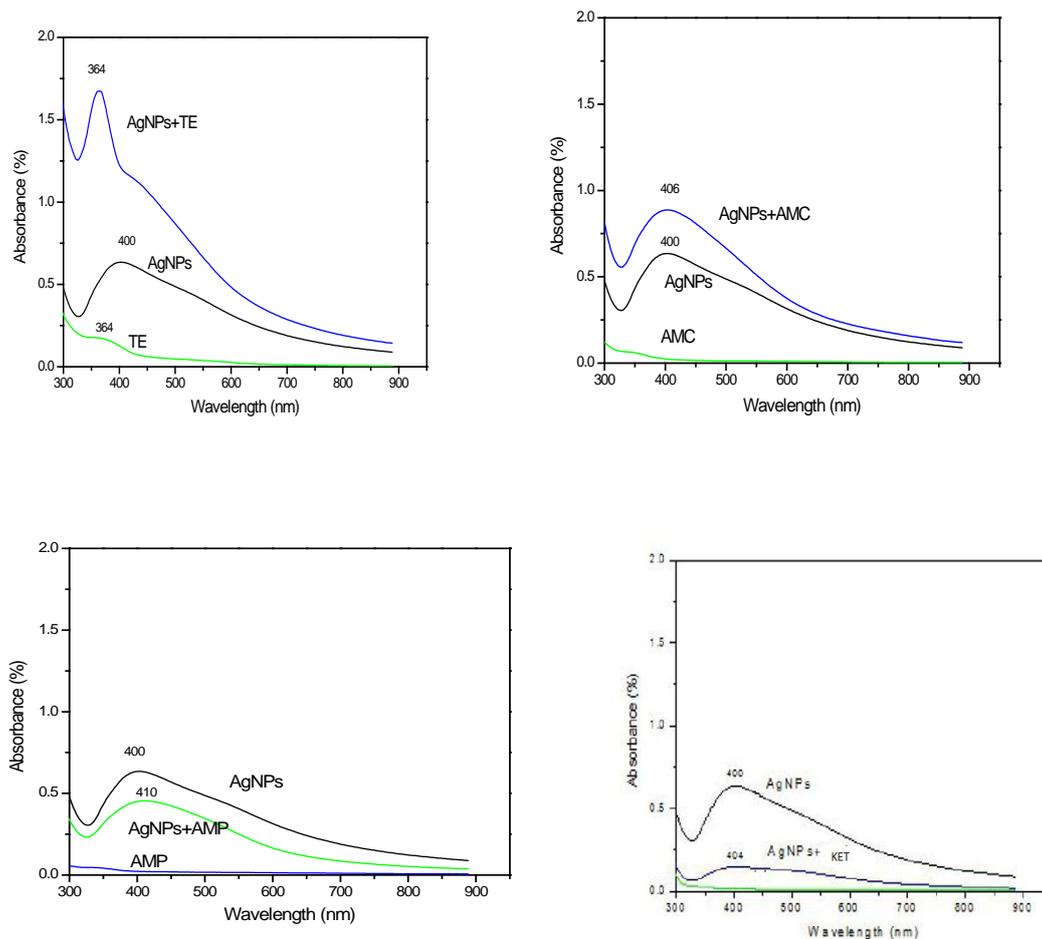
**Table 1:** Inhibition zone(mm)of different antibiotics( with and without AgNPS that synthesized by *S. griseoflavu*) against Pathogenic *Candida* sp

yeast	Different concentration of AgNO <sub>3</sub>			Antibiotics			AgNPS + Antibiotics					
	AgNO <sub>3</sub>	70 µl	100 µl	KET	NOR	OX	70 µl			100 µl		
							KET	NOR	OX	KET	NOR	OX
<i>C. albicans</i>	2	15	17	R	R	R	25	43	28	35	53	48
<i>C.krusei</i>	2	8	9	R	R	R	28	55	37	50	60	45

### 3.6.3 Antibiotic bound biogenic silver nanoparticles

UV-Vis absorption spectra of AgNPs alone, some antibiotics alone, and AgNPs-antibiotic combinations are shown in ( Figure .42). AgNPs have a characteristic extinction at around 400-410 nm . AMC (Amoxicillin ), AMP (Ampicillin) and KET (Ketozone) have no absorption in the range of 300-700 nm. Tetracycline has an absorption extending to 364 nm, which is distinguishable from the peak of AgNPs at 400 nm. Adding Tetracycline to an AgNPs solution causes a increase of tetracycline extinction at 364 nm while another broad band at 430 nm appear, which is attributed to forms a complex with AgNPs (Figure .13 A). When Amoxicillin added to the AgNPs, the AgNPs’ extinction at 400 nm increase sharply and the absorption peak was shifted to

406 nm (Figure .12.B). This demonstrates that Amoxicillin (AMC) forms a complex with AgNPs leading to enhance the activity of antibiotic against pathogenic bacteria. Similar phenomena are observed when Ampicillin is added to AgNPs solutions ,the SPR of AgNP shifted from 400 to 410 nm (Figure .13 C).Our obtained results are in agreement with results observed by [2] whom combination of the AgNPs with Ampicillin, the SPR of AgNP shifted from 390 to 405 nm. Upon addition of Ketoconazole , the extinction at 4004 nm of AgNPs slightly decreases and was shifted to 400 nm (Figure .13 D). This means that all these five antibiotics form complexes with AgNPs that cause enhancement of their antimicrobial against tested strains. It should be noted that.Tetracycline (TET) , Ampicillin (AMP), Amoxicillin (AMC) . and Ketoconazole (KET) are also the ones, which have synergistic antibacterial activity when combined with AgNPs. These results clearly point out that the synergistic effect of AgNPs-antibiotic combination against pathogenic bacteria is mediated by complexes that are produced from the interaction between AgNPs and the antibiotics, instead of individual or additive action of AgNPs or antibiotic In other words, the bacterial cells are killed more effectively by “AgNPs-antibiotic complexes”. However, the nature of the interaction between the AgNPs and the antibiotics needs further investigation .



**Figure 14:** UV-Vis spectra of AgNPs in the absence and presence of Ampicillin (AMP), Amoxicillin (AMC) , Tetracycline (TET), and Ketoconazole (KET).

#### 4. Conclusions

This study demonstrated that marine bacteria can be used for AgNP biosynthesis. The biosynthesized AgNPs were adequate for size, shape and stability, obtained considerable antifungal activity on *C. albicans* and *C. kruzei*. Therefore, the ability to biosynthesize AgNPs using marine bacteria is highly promising as a simple and reproducible process of sustainable green synthesis.

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