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Nano-silver biosynthesis using culture supernatant of *Penicillium politans* NRC510: Optimization, characterization and its antimicrobial activity.

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Abstract : The development of microbial assisted green synthesis of nanoparticles through reliable processes is critical due to its incredible applications in most of science fields. In this manuscript, silver nanoparticles (AgNPs) were successfully synthesized using an extracellular supernatant of *Penicillium politans* NRC510 under shake culture condition. Factors affecting the reduction of silver ions (Ag^+) to silver metallic nanoparticles (Ag^0) were investigated. The resulted AgNPs were characterized by UV–visible spectrophotometry, Scanning electron Microscopy (SEM), Energy Dispersive X-ray (EDX) analysis, Transmission Electron Microscopy (TEM), and Fourier transform infrared spectroscopy (FTIR). TEM studies detected the formation of spherical shaped AgNPs in the range of 3–30 nm. The formed nanoparticles showed antimicrobial activity against *Bacillus subtilis, Bacillus pumilus, Bacillus mycoides, Staphylococcus aureus and Escherichia coli* as well as *Candida albicans.*

Keywords: Silver nanoparticles, Synthesis, Characterization, *Penicillium politans,* Antimicrobial activity.

Introduction

Nowadays, the area of nanotechnology has a great research interest for developing reliable synthesis processes and metal nanoparticles stabilization[1,2]. There is a critical need to synthesize noble metal nanoparticles using eco-friendly methods (green synthesis)[3,4], due to the interest to minimize waste and to develop sustainable processes adopted with the principles of green chemistry[2,5]. Traditional physical and chemical nanoparticle synthetic methods have a considerable environmental taxing, technically laborious and economically expensive[6,7]. The common method for metal nanoparticles preparation is through reducing the metal saltswith satisfactory reducing agents;however most cause acute environmental toxicity and biological threats[8].

To achieve the objective of developing simple and ecofriendly technology in the field of nanoparticles synthesis, researchers have buckle down to biological systems [9,10].Useof microorganisms in nanoparticles green synthesis is a new and research thrilling area. Microorganisms have been used as prospective biofactory for metallic nanoparticles synthesis [11,12].Fungi have high wall-binding specific capacity, intracellular metal uptake abilities andproduce a huge amount of enzymes which make it as robust microorganisms in metal nanoparticles biosynthesis [13,14].

Concurrent witha rapid increase in antibiotics resistant microbes, isan inevitable and urgent need for development of novel antimicrobial agents[5]. Compared with other metals, silver and its compounds are effective antimicrobial agents,exhibiting abroad spectrum against microorganisms while exhibiting a decreased toxicity toward mammalian cells[4,9]. Silver nanoparticles (AgNPs) are very important due to its cytotoxic, antimicrobial, antiseptic, and anti-inflammatory activity [15,16]. Silver particles in the nanoscale possess unique physical properties different from its ion and bulk material. Their uniqueness results from higher surface to volume ratio, large number of edges, corners, and high-energy surface defects and hence shown better antimicrobial activity[16–18]. Thus, the use of AgNPs considered one of the promising approaches for overcoming antibiotic resistance of microorganisms.[19].

Studies on AgNPs synthesis by the cell free filtrate showed color change to intense brown color which may be due to the reduction of silvernitrate to AgNPs. Synthesis may be either through intracellular or extracellular pathway. Intra-cellular pathway has some disadvantages like harvesting, product recovery and purification, all which are cumbersome and expensive techniques[12,18]. In extracellular pathway a reductase enzyme is released into the medium which is responsible for silver ion reduction[5,15].

In this study, a *Penicilliumpolitans*NRC510 cell free filtrate was utilized to synthesize AgNPs extracellulary. The AgNPs were characterized by determining the Surface Plasmon Resonance(SPR)by means of UV–visible spectrophotometry, Scanning Electron Microscope (SEM), Energy Dispersive X-ray (EDX) analysis, Transmission Electron Microscopy (TEM)andFourierTransform Infrared spectroscopy (FTIR).The antimicrobial activityagainst bacterial and yeast strains wasalsoexamined.

Materials and Methods

1. Microorganisms

*Penicilliumpolitans*NRC510 fungal strain was maintained on slants of modified solid Czapek-Dox's medium and kept at 4 °C.Representative microorganisms of Gram-positive bacteria (*Bacillus subtilis, Bacillus pumilus,Bacillus mycoides, Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*), as well as the yeast *Candida albican s*were maintained on nutrient agar slants at 4 °C, and were used to evaluate the antimicrobial activity of prepared silver nanoparticles.

2. Preparation of supernatant

*P.politans*NRC510 was grown on a liquid media containing (g/L): KH_2PO_4, 7.0: K_2HPO_4, 2.0; $MgSO_4{\cdot}7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0; yeast extract, 0.6; and glucose, 10.0; at 28°C and 100 rpm. After 72 h of incubation, the culture was filtered via Whatman No.1 filter paper. Thesupernatant was used for the extracellular formation of silver nanoparticles as the cell free filtrate (CFF). For biomass filtrate, the biomass was harvested and extensively washed with distilled water. Typically, 10 g of biomass was added to 100 mL of Milli-Q deionized water for 72 h at 28 °C and 100 rpm then the cell filtrate was assayed for nanoparticles formation [20].

3. Biosynthesis of AgNPs

For synthesis of silver nanoparticles, 50 mL of *P. politans*NRC510cell free filtrate containing 1 mM silver nitrate (unless otherwise stated), was incubated in the dark at 30 °C (to avoid the photo activation of silver nitrate) at 100 rpm. *P. politans*NRC510cell free filtrate as well as silver nitrate solution (1 mM) were used as control. After incubation, the absorbance was measured using UV–visible spectrophotometer (Cary 100 UV-Vis; Agilent Technologies, Germany).The effect of the silverwas determined by varying the $AgNO_3$ concentration (0.5,1.0,1.50, 2.0, 3.0, 4.0, or 5.0mM). *P. politans*NRC510cell free filtrate concentration wasvaried (25, 50, 75 or 100%) while keepingthe $AgNO_3$ concentration at a level of 1.0mM.For pH-dependent effects, the reaction mixtures pH value was adjusted between 4.0 and 10. For the effect of temperature on nanoparticles synthesis, the reaction mixtures were incubated between 30 and 90 °C for 2 h at pH 7.0. The effect of reaction time was evaluated by incubating thereaction mixtures with optimum composition for 8, 24, 48, 72 and 120 h[7].All experiments were carried out in triplicates and the average data was presented.

4. Characterization of synthesized AgNPs

The UV-Visible spectra of AgNPs were recorded as a function of wavelength using UV/Vis spectrophotometer(Cary 100 UV-Vis; Agilent Technologies, Germany) operated at data interval of 1.0 nm. Both the scanning electron microscopic (SEM) and elemental analysis of the biosynthesized AgNPs were studied using scanning electron microscope (SEM -Quanta FEG250) operated at an accelerating voltage of 20 kV and coupled with energy dispersive X-ray analysis (EDX) for compositional analysis and the conformation of presence of elemental silver. The AgNPs solution was centrifuged for 20 min at 10,000 rpm and was drop coated on a carbon coated copper grid and dried[21]. The shape and size of AgNPs were determined by TEM. For TEM, a drop of aqueous AgNPs sample was loaded on a carboncoatedcopper grid, and it was allowed to dry at room temperature,the micrographs were obtained using TEM (JEOL JEM-HR-2100) operating at 160 kV.

For FTIR spectroscopy measurements, dry powder of the AgNPs was obtained from a 24 h reaction of the cell free filtrate of *P. politans*NRC510with silver nitrate that was centrifuged at 10,000 rpm for 15 min, and was re-dispersed in sterile distilled water. The process of centrifugation and re-dispersion was repeated four times to ensure good separation of the AgNPs from other contaminants. The obtained pellets were then dried and the powders were subjected to FTIR spectroscopy measurement. These measurements were carried out on a JASCO FTIR (Japan) instrument in the diffuse reflectance mode at a resolution of 4 cm^{-1} in KBr pellets[22].

5. Potency of the synthesized AgNPsas antimicrobial agent

The antimicrobial activity of AgNPswas investigated utilizing the agar well diffusion assay [23]. The tested microorganisms (*Bacillus subtilis, Bacillus pumilus, Bacillus mycoides, Staphylococcus aureus, Escherichia coli,* and *Candida albicans*)were seeded into nutrient agar plates, then, wells of 15 mm diameterwere made using sterile cork borer. 200 µL of AgNPs solution of variable concentrations(12.5, 25, 50 and100 µg/mL) has pipetted into the corresponding well.Control sample containing cell free filtrate (CFF) was used to assess the antimicrobialactivity. Standard antibiotic discs (7 mm)(Colstin 10 µg, Tobramycin 10 µg, Gentamicin 10 µg, Ampicillin 10 µg, Streptomycin 10 µg, and Erythromycin 15 µg)from Bioanalyse® were also used for comparison with the synthesized nanosilver particles. The plates were incubated at 37 °C for 5 hand the diameterof inhibition zone was measured.

Results and discussion

1. Visual observation and UV-visible spectroscopy

AgNPs exhibit unique optical properties because of their characteristic optical resonance, known as Surface Plasmon Resonance (SPR), which occurs due to its convergent oscillation of conduction electrons, as a consequence of nanoparticles shape and size[24,25].In this study, the results obtained from using different sources of *P. politans*NRC510filtrates from different cultural and treatment techniques indicated that optimal biosynthesis was achieved by subjecting the cell free filtrate mixture under shaking conditions (Fig 1).

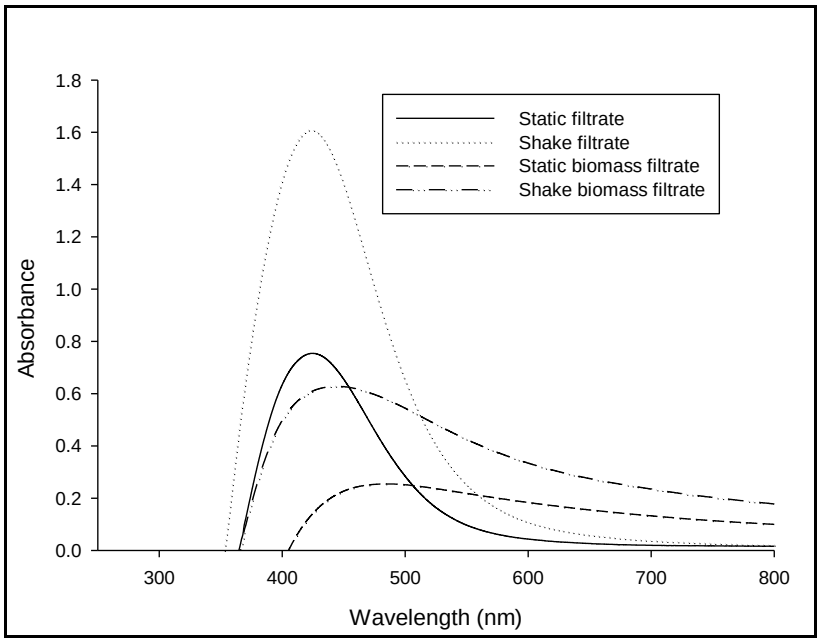


Fig 1.UV-Visible absorption spectra of synthesized AgNPs, using different filtrates of *P. politans*. The experiment was carried out in triplicates and the average data was presented.

The extracellular AgNPs biosynthesized by *P. politans*NRC510broth under shake cultural condition was selected for optimization and characterization. Fig. 2 depicts nanoparticles formation by the cell free filtrate before reacting with silver ion and after 24 h of reaction, both under dark and shake conditions.CFF Ag^+ reduction, forming the AgNPs, was monitored by UV-Vis spectroscopy. The reaction mixture changed color firstly to yellowish brown and then to reddish brown which indicated the formation of silver nanoparticles.[26]. This color is attributed to theexcitation of Surface Plasmon Resonance (SPR). As shown in Fig. 3a, a characteristic andwell-defined SPR band for AgNPs was obtained at around 433 nm [7]. Controls using silver nitratesolution or CFF neither developed the reddish brown color nor the characteristic band at 433 nm, indicating that abioticreduction of silver nitrate did not occur under the experimental conditions (Fig. 2, Fig. 3a).

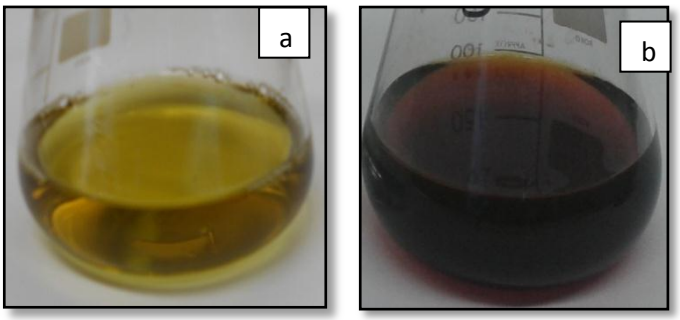


Fig. 2.Formation of AgNPs by *P. politans*NRC510cell free filtrate (a) at the beginning of the reaction and (b) after 24 h of reaction.

2. Effect of incubation period

The intensity of the characteristic color was directly proportionalto the timeof reaction mixture incubation.The rate of silver ions reduction was slow during thefirst incubation hours (8 h), as indicated by the low absorbance values at the maximum absorptionwavelength(Fig. 3b).Interestingly, an increase in the absorbance was observed after longer time periods up to 120 h, which gave the maximum reduction of silver ions. This difference in absorbance alongwith color intensity could be interpreted by the increase in AgNPs numbers in relation with time[20]. Korbekandi et al., [27]reportedthat the time required for full reduction of the metal ions through thebacterial and fungal biosynthesis of metalnanoparticles can range from 24 to 124 h. As a

function with time, the rapid formation ofnanoparticles was due to the excellent reducing potential ofthe CFF active components and theirpolymeric stabilization within a narrow size spectrum.

3. Effect of silver nitrate concentration

The biological factor and concentration of metal salt variation has been shown to affect nanoparticles synthesis effectively[28]. Here, the range of intensity of reaction mixtures colors varied from yellowish brown to light reddish brown at the lower salt concentrations (0.5 – 1.0 mM), and darker shades of reddish brown at higher silver nitrate concentrations between 1.5 and 5.0 mM. By increasing the concentration of $AgNO_3$, the absorbance intensity increased drastically up to 2.0 mM, which was the maximum obtained peak intensity (Fig. 3c). When the silver nitrate concentration increased above this value, Ag^+ was not completely reduced to Ag^0, and depended on the quantity of proteins and enzymes present in the reaction mixture. By increasing silver nitrate concentration above 2.0 mM, the enzymes and proteins were not sufficient to perform the reduction of all silver nitrate to AgNPs and to stabilize them[11].

4. Effect of CFF concentration

Reaction mixtures containing 1 mM silver nitrate and different ratios of CFF of *P. politans* NRC510(v/v) in 25 and 50% CFF developed a light reddish brown color, while those containing 75 and 100% of CFF developed darker reddish brown color. The SPR peaks intensity were directly proportional to the CFF concentration up to CFF content of 75%; above this, the SPR peak did not increase significantly (Fig. 3d). With an increase in the biological material concentration mediating the nanoparticle synthesis, an increased number of biomolecules participated in the process of metal reduction and nanoparticles formation[7].

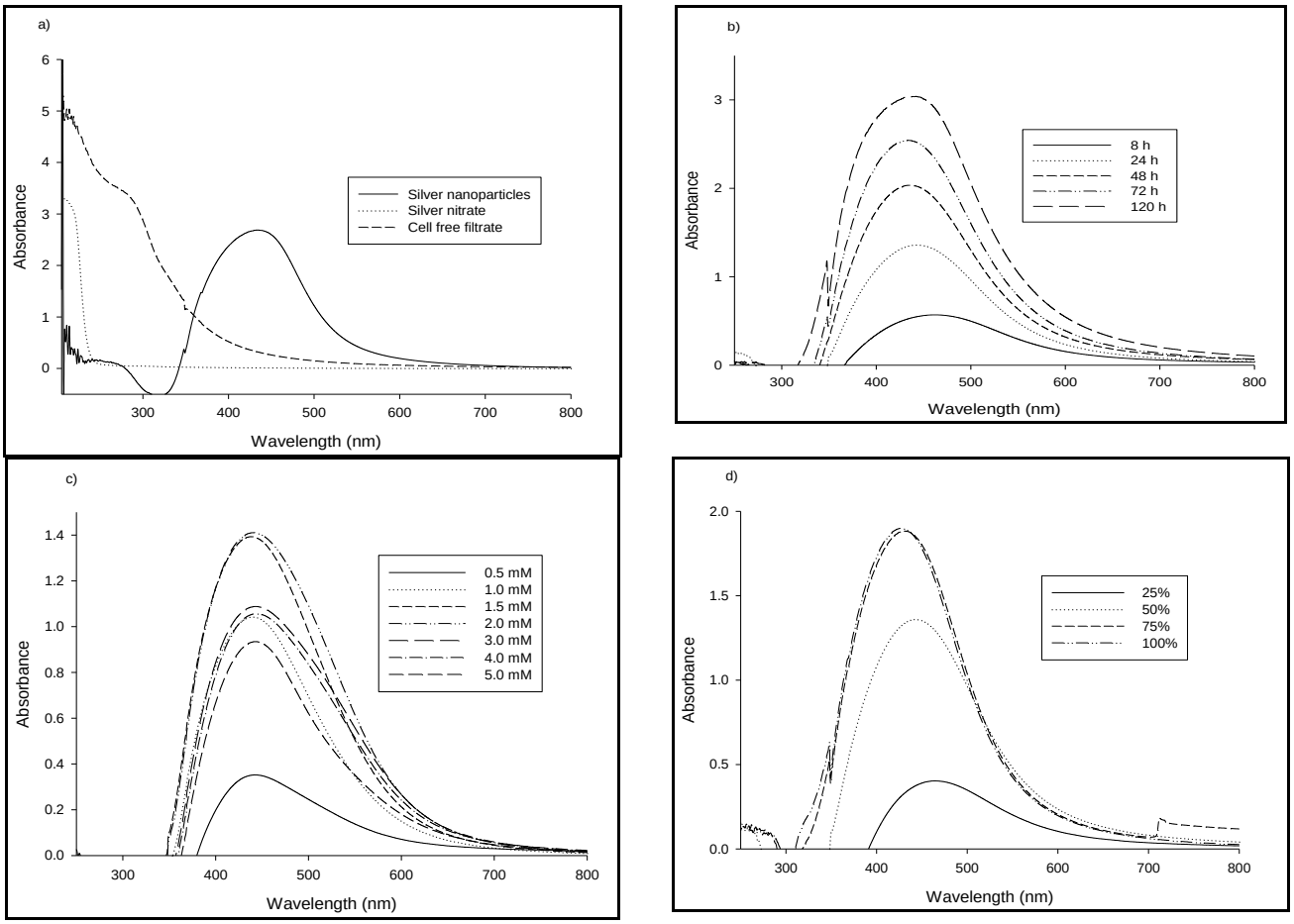


Fig.3. UV-Visible absorption spectra of: a) The extracellular biosynthesis of AgNPs using *P. politans*NRC510CFF and the controls, b) Effect of incubation period on the extracellular nanosilver formation, c) Effect of silver nitrate concentration, and d) Effect of CFF concentration. All experiments were carried out in triplicates and the average data was presented.

5. Effect of pH

The AgNPs formation and the SPR peak intensity were pH dependent. When the reaction was performed at pH 2.0 and 3.0 neither the characteristic SPR peak nor a color change were observed. By contrast, varying shades of reddish brown color were observed at pH values 5.0-8.0. The highest color intensity was obtained at pH 7.0 (Fig 4a). In agreement, Roopan et al.,[29]reported that at pH 2.0 no reaction occurred. The biomolecules that are supposed to be participated in the synthesis of biological nanoparticle, are likely to be inactivated under the extremely acidic conditions (pH 2.0) 7. Based on these findings, the proteins secreted by fungus *P. politans* NRC510 are stable in neutral pH but neither in acidic nor alkaline pH, which reveal the capability of the produced proteins and enzymes as stabilizing and reducing agent in neutral medium, in contrast to the results reported using *Fusarium solani* as a filamentous fungus[11].

6. Effect of temperature

Incubation temperature is greatly affected the silver reduction process. The incubation of reaction mixtures at 30, 40 and 50 °C gave light reddish brown color and decreased SPR peaks. However, by increasing the incubation temperature (60, 70, 80 and 90 °C) dark reddish brown color and more intense SPR peaks were obtained (Fig4b). At room temperature (30 °C) the color change took more time to develop, while by incubating the reaction mixtures at 40-90 °C the reduction process was faster and the reddish brown color was developed within 5-15 min. Maximum SPR peak intensity was detected at 90 °C; UV-Visible λmax at higher wavelength region (483 nm at 90 °C), which indicate the high intensity of nanoparticles formation. The reactants are consumed rapidly due to temperature increasing which lead to the formation of smaller nanoparticles [30]. Similarly, Fayaz et al., [12]reported that when the incubation temperature increased, the size of AgNPs produced by *Trichoderma viride* also decreased.

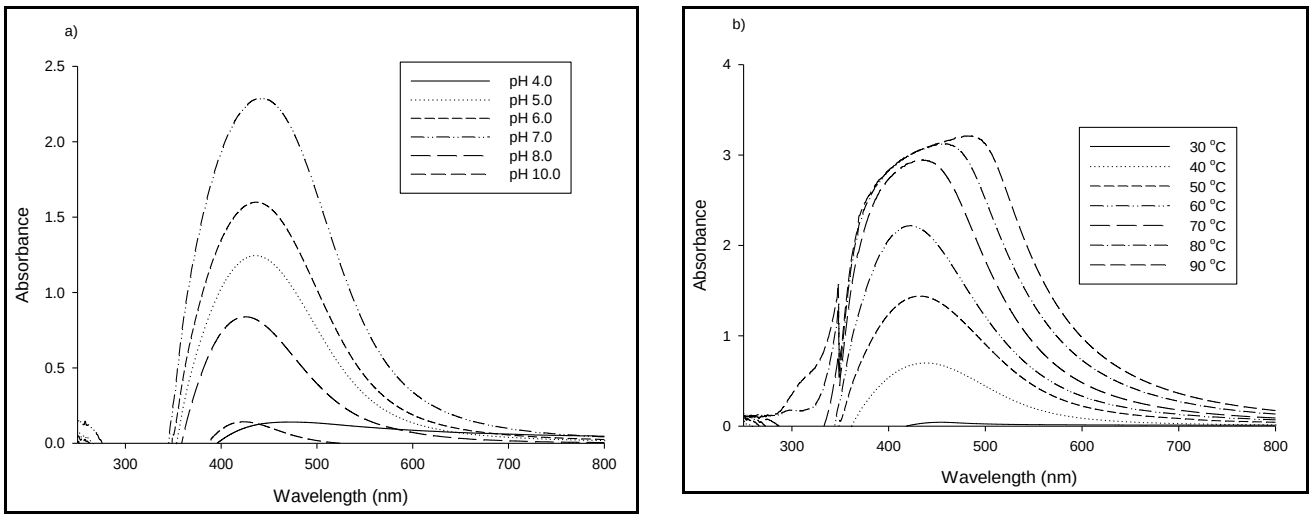


Fig.4. UV-Visible absorption spectra of: a) Effect of different pH values and b) Effect of different incubation temperatures. All experiments were carried out in triplicates and the average data was presented.

7. Characterization of silver nanoparticles

7.1. Scanning electron microscope (SEM) and EDX analysis

SEM micrographs (Fig. 5a,b) detected at high magnifications suggest that the biosynthesized AgNPs are almost spherical in structure and in the size of nanoscale range. Energy dispersive X-ray (EDX) analysis gives qualitative and quantitative estimation for elements that may be involved in nanoparticles formation. Generally, metallic silver nano-crystals showed typically higher counts at 3 keV due to their surface plasmon resonance [7,19]. The obtained EDX analysis (Fig. 5c) confirmed the presence of elemental silver, and showed strong signal energy peaks for silver particles in the range 2.5–3.5 keV, confirming the successful biosynthesis of AgNPs using *P. politans* CFF. As expected, the elemental analysis of the AgNPs also revealed highest proportion of Ag followed by Cl.

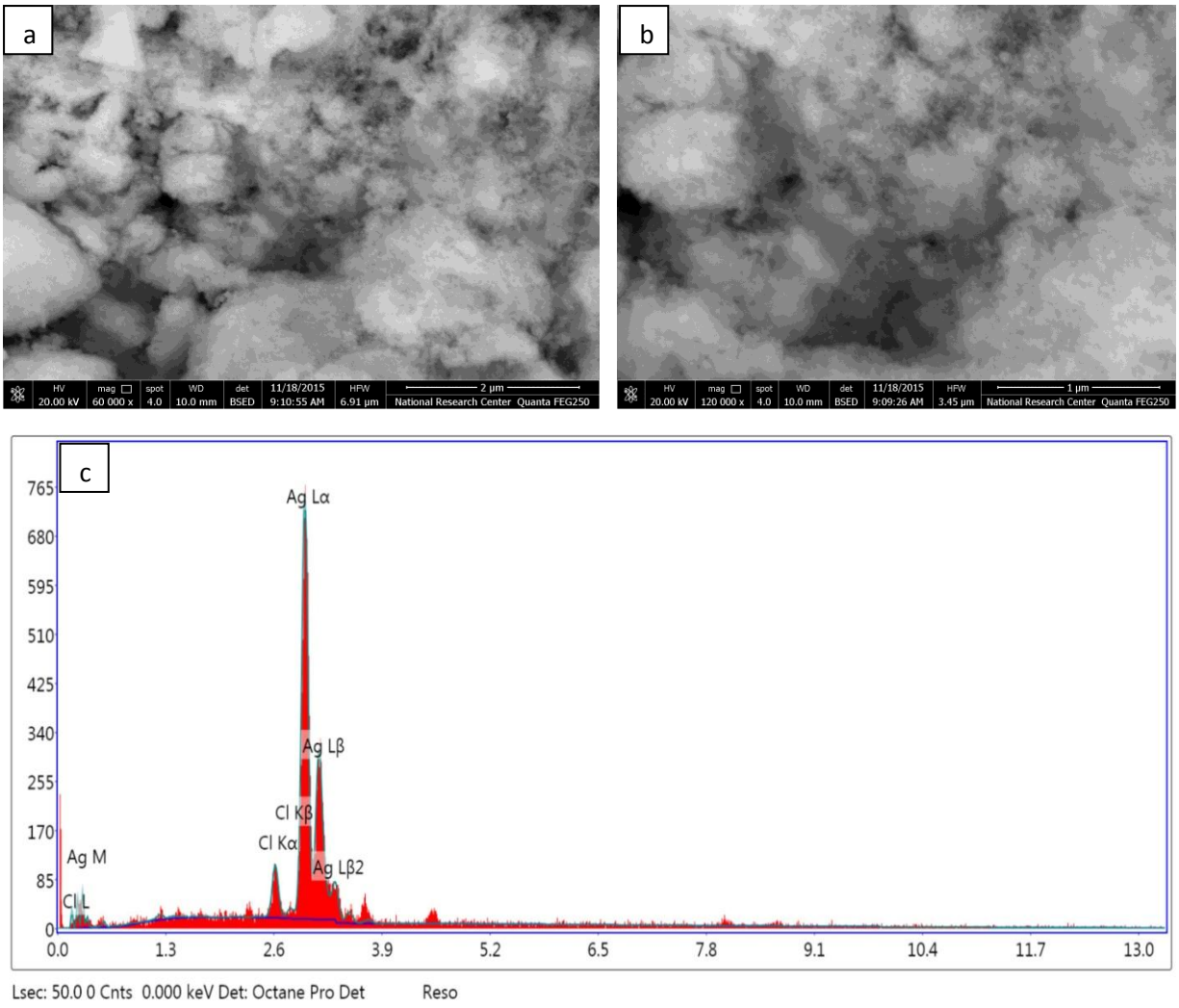


Fig. 5. SEM micrograph of the synthesized silver nanoparticles: a) magnified 60000X, b) magnified 120000X, and c) EDX profile.

7.2. Transmission electron microscopy (TEM) analysis

The morphology and particle size distribution profile of the myco-synthesized AgNPs was studied using TEM revealing that the biosynthesized AgNPsare more or less spherical (Fig. 6). Fig. 6 indicates representative TEM images obtained from drop-coated films of the AgNPs synthesized by the treatment of silver nitrate solution with *P. politans*NRC510CFF for an incubation period of 24 h. The AgNPs formed were mostly spherical and polydisperse with diameters in the range 3 to 30 nm. The pictures show individual silver particles as well as a number of aggregates in different size ranges. The nanoparticles were not in direct contact even within the aggregates,indicating stabilization of the nanoparticles by a cappingagent[19].

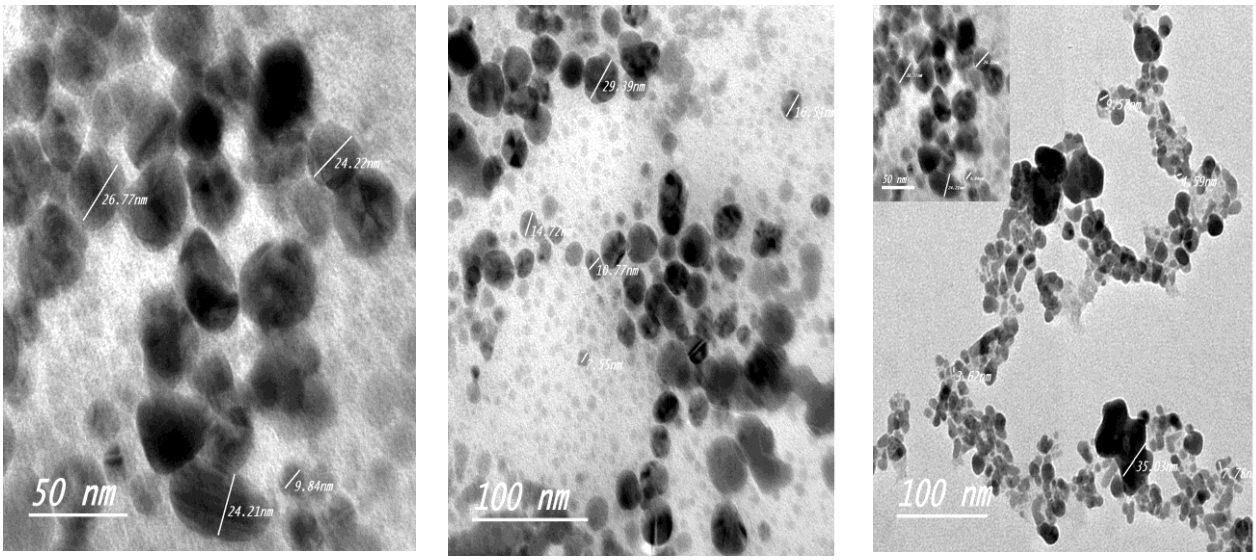


Fig. 6. TEM micrograph of the silver nanoparticles

7.3. Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopic analysis was performed to identify the major functional groups responsible for AgNPs formation, capping, and stabilization by *P. politans*NRC510 CFF. The spectrum of AgNPs was represented in Fig. 7. The absorption peaks appearing at 3919, 3762, 3691, 3417, 3233, 2926, 2859, 1642, 1426, 1184, 1097, 791, 692, 629, 531, and 431 cm^{-1}(Fig. 7), can beassigned to stretching vibration of C–N aromatic and aliphatic amines (1184, and 1097cm^{-1}), while the N–C=O amide bond of proteins due to carbonyl stretch in proteins (1642 cm^{-1}), C–H stretching vibrations of methyl, methylene and methoxy groups (2926, and 2859 cm^{-1}), and O–H stretching in alcohols, flavonoids and phenolic compounds (3762, 3691, and 3417 cm^{-1}) could be observed[31].The broad peak at 3417 cm^{-1} is thought to be overlapping –NH stretching vibration which is typical phenomenon for proteins [32].These observations imply that the proteinaceous matter may be participated Ag^{+} reduction, and where the biological components interact with Ag^{+} through these functional groups to mediate this process to nanoparticles[33].Muthukrishnan et al., [21] suggested that the asymmetric $–CH_3$bending modes of methyl groups of protein may be participatein the reduction ofsilver nitrate ions.On the other hand, Shankar et al., [22] reported that there is nopeaks in the amide region characteristicof proteins/enzymes that responsiblefor metal ions reduction when using fungi for metal nanoparticles biosynthesis.

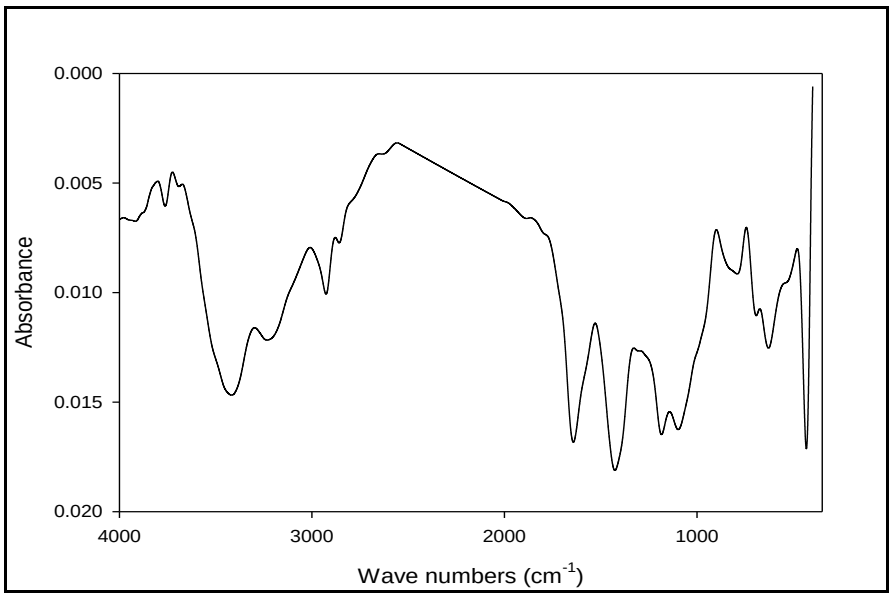


Fig.7. Fourier transform infrared (FTIR) spectroscopy

8. Antimicrobial activity of silver nanoparticles

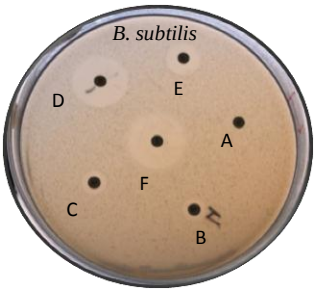
Different mechanisms of action can be employed to interpret the biocidal properties of AgNPs against different microorganisms. Firstly, AgNPs bind to the negative charge on cell surface, altering cell membrane and cell wall properties, affecting permeability, osmo-regulation, electron transport and respiration [34]. Secondly, AgNPscan interact with DNA, proteins and other cell constituents after penetrating cell wall [35]. Thirdly, silver ions released by silver nanoparticles, cause an amplified biocidal effect depending on its size and dose [34,36].

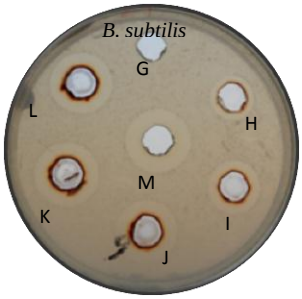
In this study, AgNPs displayed antimicrobial activity against the studied pathogenic microorganisms, which depend on the diameter of inhibition zone, while CFF didn'tshow any antimicrobial activity (Table 1). Gram negativebacteria (*Escherichia coli*) showed smaller zones of inhibitionrelative to Gram positive bacteria (*Bacillus subtilis, Bacillus pumilus, Bacillus mycoides,* and *Staphylococcus aureus*), which may due to the variation in cellwall composition. This phenomenon is contrasted to the interpretation mentioned by Shrivastava et al.,[37]where they proposed that the cell wall of Gram positive bacteria(composed of a thick peptidoglycan layer, containing polysaccharide chains cross linked by short peptides)comprises a more rigid structure resulting in difficult penetration ofthe silver nanoparticles, relative to the Gram negative bacteria cell wall has thinnerpeptidoglycan layer. The antimicrobial activity against *Candida albicans* was the lowest as indicated by the small inhibition zone diameter (Fig. 8).

The important featured applications of biosynthetic silver nanoparticles like in biosensors, catalysts, antimicrobial surfaces, and biomedical applications due to its unique features, indicate that a directed biosynthetic silver nanoparticles production is needed in the future, in which the biological system to produce biogenic silver is chosen in function of the application which exert many advantages in terms of biocompatibility and activity.

Table (1): Potency of the synthesized AgNPs as antimicrobial agent

Sample	Inhibition zone (mm)					
	Bacillus subtilis	***Bacillus pumilus***	***Bacillus mycoides***	***Escherichia coli***	***Candida albicans***	***Staphylococcus aureus***
Colstin 10 mcg	0	0	0	0	0	0
Tobramycin 10 mcg	12	11	10	13	12	12
Gentamicin 10 mcg	13	12	11	12	12	12
Ampicillin 10 mcg	28	20	12	20	14	22
Streptomycin 10 mcg	20	17	17	15	17	15
Erythromycin 15 mcg	32	25	17	29	25	25
Cell free filtrate (Blank)	0	0	0	0	0	0
AgNPs (100 µg/mL)	31	30	29	28	26	28
AgNPs (75 µg/ mL)	30	29	28	27	25	27
AgNPs (50 µg/ mL)	29	27	27	24	24	26
AgNPs (25 µg/ mL)	27	23	24	20	21	24
AgNPs (12.5 µg/ mL)	24	20	21	17	17	20
$AgNO_3$ (1 mM)	32	30	31	28	26	29





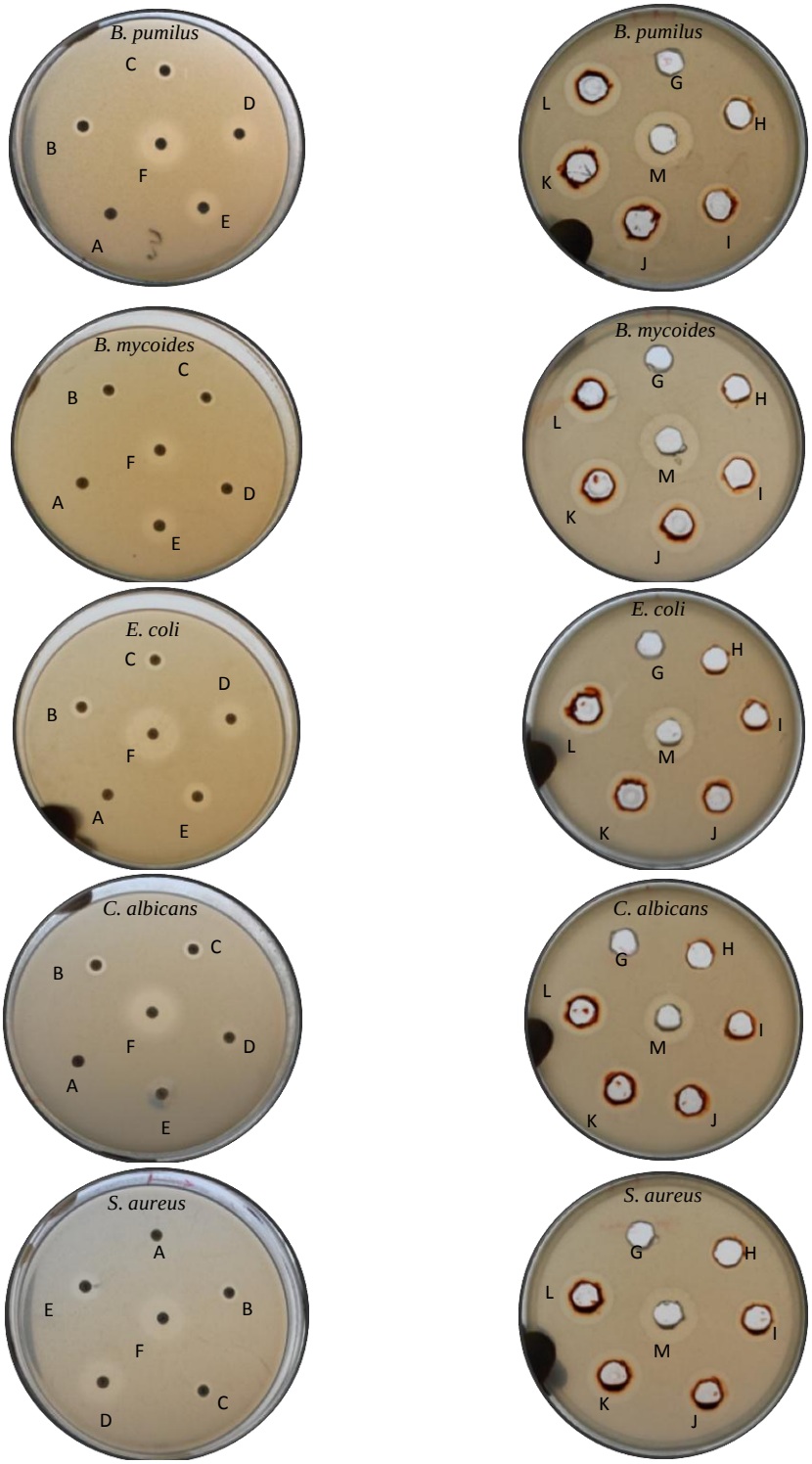


Fig.8. Potency of the synthesized AgNPs as antimicrobial agent. A) Colstin 10 mcg, B) Tobramycin 10 mcg, C) Gentamicin 10 mcg, D) Ampicillin 10 mcg, E) Streptomycin 10 mcg, F) Erythromycin 15 mcg, G) Cell free filtrate (Blank), H) AgNPs (12.5 µg/mL), I) AgNPs (25 µg/mL), J) AgNPs (50 µg/mL), K) AgNPs (75 µg/mL), L) AgNPs (100 µg/mL), and M) $AgNO_3$ (1 mM).

Conclusion

The findings of the present study leads to the conclusion that the reaction conditions like amount of CFF, $AgNO_3$ concentration, reaction time, pH, and temperature significantly affected nanoparticle yield. This synthetic approach appears to be an inexpensive, non-toxic, eco-friendly instead of the traditional chemical and physical methods, and would be convenient for biological process development for large-scale production. The

procedure applied in this work for the preparation of silver nanoparticles from *P. politans* is unique for its simplicity, low cost and time consuming from the other reported procedures as they are synthesized using culture supernatant directly.

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