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Marine *Streptomyces cyaneus* Strain Alex-SK121 Mediated Eco-friendly Synthesis of Silver Nanoparticles Using Gamma Radiation

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Authors' contributions

This work was carried out in collaboration between all authors. They designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript, managed the analyses of the study and managed the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: The present study aimed to develop cost-effective, eco-friendly marine *Streptomyces cyaneus* strain Alex-SK121 mediated synthesis of silver nanoparticles (AgNPs) with

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antimicrobial, antitumor and antioxidant activities.
Methodology: Aqueous 1mM silver nitrate ($AgNO_3$) solution was treated with cell-free supernatant (CFS) of a novel *Streptomyces cyaneus* strain Alex-SK121 isolated from marine sediment samples. The prepared solution was irradiated with different doses of gamma rays ranged from 0.5 to 30.0kGy. Initial characterization of the synthesized AgNPs was performed by visual observation of color change in the prepared solution followed by analysis of UV-Visible Spectrophotometer (UV-Vis.), Fourier Transform Infrared Spectrometer (FT-IR), Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM). Evaluation of antimicrobial activity of the synthesized AgNPs against some pathogenic microorganisms was carried out. Antitumor activity of AgNPs was carried out against some human cancer cell lines using the method of Sulphorodamine B (SRB) assay, antioxidant activity of AgNPs was also studied using DPPH scavenging assay.
Results: In the present study, the cell-free supernatant of *Streptomyces cyaneus* strain Alex-SK121 isolated from sediment samples collected from Sidi Kerir region, Alexandria governorate, Egypt was found to reduce Ag^+ ions to AgNPs. Identification of the producer strain was performed according to spore morphology and cell wall chemo-type, which suggested that this strain is Streptomyces. Further cultural, physiological characteristics and analysis of the nucleotide sequence of 16S rRNA gene indicated that this strain is identical to *Streptomyces cyaneus* and then designated *Streptomyces cyaneus* strain Alex-SK121. To maximize the production of AgNPs, the tested supernatant was irradiated with different doses of gamma rays and it was found that, 15 kGy is the best applied dose induces AgNPs synthesis. The synthesized AgNPs showed the characteristic absorption spectra in UV–Vis. at 425 nm. The microbiologically synthesized AgNPs showed significant antimicrobial activity towards some pathogenic microorganisms with inhibition zone ranged from 13 up to 20 mm. Also AgNPs exhibited antitumor activity against human breast carcinoma cells and human liver carcinoma cells with IC_{50} 9.63 and 33.75 µg/ml respectively in addition to 96% antioxidant activity.
Conclusion: Gamma irradiation which induced AgNPs synthesis by cell-free supernatant of marine actinomycetes *Streptomyces cyaneus* strain Alex-SK121 with different applications is a simple, clean, economic and environmental friendly approach.

Keywords: Silver nanoparticles; Streptomyces cyaneus Alex-SK121; marine sediment; antimicrobial activity; antitumor activity; antioxidant activity and gamma irradiation.

1. INTRODUCTION

Nanotechnology is the application of science to control matter at the molecular level. It had been well known that the living cells were the best examples of machines that operate at the level of nanomaterials and perform a number of jobs ranging from generation of energy to extraction of targeted materials at very high efficiency [1].

Nanotechnology is an emerging field of science which involves synthesis and development of various nanomaterials [2]. At present, different types of metal nanomaterials had been produced using copper, zinc, titanium, magnesium, gold and silver. These nanomaterials were used in various fields such as optical devices [3], catalytic [4], bactericidal [5], electronic [6], sensor technology [7], biological labelling [8] and treatment of some cancers [9].

Nanotechnology holds promising application in bio-sensing, drug delivery and cancer therapy [10–11]. Synthesis of nanoparticles can be achieved by employing physical, chemical, and biological methods. The problems associated with the chemical synthesis are the side effects, use of toxic chemicals and hazardous by-products [1] .To overcome the problems of physical and chemical synthesis of nanoparticles biological methods can be followed. Biologically synthesized AgNPs were used as spectrally selective coatings for solar energy absorption, intercalation material for electrical batteries, optical receptors, catalysts in chemical reactions, biolabeling and antimicrobials [12,13].

Biological synthesis provides an eco-friendly and also a cost-effective method. An alternative approach for the synthesis of metal nanoparticles is to apply biomaterials such as plants, microorganisms encompassing groups such as bacteria, yeasts, fungi and actinomycetes as manufactories [14]. Actinomycetes showed considerable interest owing to their ability to produce new chemical entities with diverse pharmacological activities. Marine actinomycetes in particular had yielding numerous novel secondary metabolites [15].

Streptomyces sp. are 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, immune suppressors and many other biologically active compounds [16]. Exploitation of *Streptomyces sp.* in nanotechnology has recently received considerable attention [17,18].

Silver nanoparticles are potent and broad-spectrum antibacterial agents with activity against diverse species within both Gram-positive and Gram-negative bacteria [19]. Silver nanoparticles were found to have wide applications in various areas like optical receptors, bio-labelling [20] sensors and bio active materials [21].

Silver nanoparticles are undoubtedly the most widely used nanomaterials among all. Silver nanoparticles were used as antimicrobial agents, in textile industries, water treatment, sunscreen lotions, etc. [5,22,23].

Gamma-irradiation synthesis of metallic nanoparticles had been employed as one of the most promising method to produce AgNPs due to some important advantages. As compared to conventional chemical /photochemical techniques, the radiochemical process can be performed to reduce Ag^+ ions at the ambient temperature without producing unwanted by-products of the reductant or using excessive reducing agents. Moreover reducing agent can be uniformly distributed in the solution and AgNPs are produced in highly pure and stable form [24,25,26,27]. Several actinomycetes were found to synthesis silver nanoparticles [28].

So, the main aim of this study is to biosynthesize silver nanoparticles from marine *Streptomyces cyaneus* strain Alex-SK121 and confirming AgNPs using (UV-Vis.). Characterization studies were performed using (FT-IR), (DLS) and (TEM). The antimicrobial antitumor and antioxidant activities of the produced silver nanoparticles were checked.

2. MATERIALS AND METHODS

2.1 Chemicals

All the media components from Oxoide, Chemicals and reagents used in the following experiments were of analytical grade and used without further purification.

2.2 Irradiation Source

The process of irradiation was carried out at the National Center for Radiation Research and Technology (NCRRT) using Cobalt 60 source (Gamma cell 4000-A-India) at a dose rate of 0.919 Gy/s and a specific activity of 3496.8 Curie at the time of the experiments.

2.3 Collection of Samples and Isolation of Actinomycetes

Marine sediment samples from Lake of SidiKerir (latitude of 30º59'29.50"N-longitude of 29º38'55.30"E), Alexandria governorate, Egypt were collected in sterile airlock polythene bags and stored at 4℃. Selective pre-treatment was performed to increase the number of mycelium forming actinomycetes relative to the non-actinomycetal heterotrophic microbial flora. The collected samples were air-dried, mixed with $CaCO_3$ and incubated for five days at 35℃ then sieved to remove various unwanted contaminant materials before plating, [29]. The plates were incubated at 36℃ until the appearance of colonies with a tough leathery texture, dry or folded appearance, and branching filaments with or without aerial mycelia [30]. Pure colonies were isolated and subcultures were carried out by streaking the particular isolate directly on ISP4 agar media.

2.4 Characterization and Culture Conditions

Morphological and biochemical characterization of the isolate was performed by following the method of Shirling and Gottlieb [31]. Morphology was studied by scanning electron microscopy (JEOL JSM 5300, JEOL Technics Ltd., Japan) [32]. Cultural characteristics were carried out at 36℃ for 7 daysby methods followed by International *Streptomyces* project (ISP) [31]. Assessment of color pattern was done according to color chips using the ISCC-NBS Color Charts Standard No. 2106 [33]. Diaminopimelic acid in the cell wall was analyzed using described method [34]. Various biochemical tests included melanin pigment production, starch hydrolysis, lipid hydrolysis, protein hydrolysis, tyrosine degradation, urea hydrolysis, nitrate reduction, catalase production, chitinase production, utilization of various carbon and nitrogen sources, tolerance to NaCl concentrations, growth at different temperature, pH, different growth inhibitors and resistance to antibiotics were performed [35,36].

The 16S ribosomal DNA gene was amplified by PCR using the universal primer pair F27, 5'-AGAGTTTGATCMTGGCTCAG-3' and R1492 5'-TACGGYTACCTTGTTACGACTT-3'. The amplified products were analyzed by electrophoresis in 0.7% (w/v) agarose gel and purified using DNA extraction kit (RBC, Korea). The 16S rDNA sequencing was done by ABI PRISM 377 DNA sequencer and ABI PRISM BigDye Terminator Cycle Sequencing (Perkin Elmer, Ohio, U.S.) at a sequencing facility at Cornell University in the USA. DNA sequence analysis was then performed by BLAST network services at the NCBI. The 16S rRNA gene sequences of the strain Alex-SK121 was aligned with reference sequences obtained from Gene Bank using ClustaL X 2.0.11 [37]. Phylogenetic tree was generated using the neighbor-joining method with genius pro 7.1.5 [38,39].

2.4.1 Preparation of cell-free supernatant (CFS)

The actinomycete strain Alex-SK121 was grown in Peptone yeast extract-iron medium (ISP-6) which contains (g/L): Bacto-peptone 15.0 g, Protease peptone 5.0, Ferric ammonium citrate 0.5, K_2HPO_4 1.0, Sodium thiosulphate 0.08 g, Bacto-yeast extract 1.0 g, Distilled

water up to 1000 mL, The pH value was adjusted from 7 to 7.2 before autoclaving at 36℃ for 7days at 200 rpm. At the end of incubation period, the culture broth was centrifuged at 4000 rpm for 20 min. The collected supernatant was tested for antimicrobial activity against some pathogenic microorganisms and stored for biosynthesis of silver nanoparticles.

2.4.2 Melanin estimation

Melanin pigment was estimated from peptone yeast extract iron medium according to [40] and by using UV.Vis. Spectrophotometer it had been shown that melanin pigment had O.D. at 475 nm [41].

2.5 Synthesis and Characterization of Silver Nanoparticles (AgNPs)

Cell-free supernatant produced by *Streptomyces cyaneus* Strain Alex-SK121 of optimum medium (ISP-6) was mixed with 1mM or more of $AgNO_3$ solution in ratio of (1:5)(v/v) and were irradiated at ambient temperature with different gamma irradiation doses, (un-irradiated control), 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, 10.0, 15.0, 20.0, 25.0, and 30.0 kGy). They were exposed for different times according to the dose and dose rate as mentioned in section (2.2) [24].

2.5.1 UV-Visible Spectrophotometer (UV-Vis.)

UV-Vis. Spectra of AgNPs were recorded as a function of wavelength using JASCO V-560. UV-Vis. Spectrophotometer from 200-900 nm at a resolution of 1 nm and using a filtrate (which contain melanin without silver nitrate addition) as a base line blank.

2.5.2 Dynamic Light Scattering (DLS)

Average particle size and size distribution were determined by PSS-NICOMP 380-ZLS particle sizing system St. Barbara, California, USA. Before measurements the samples were diluted 10 times with deionized water. 250µl of suspension were transferred to a disposable low volume cuvette. After equilibration to a temperature 25ºC for 2 min, five measurements were performed using 12 runs of 10 s each.

2.5.3 Fourier Transform Infrared Spectrometer (FT-IR)

FT-IR measurements were carried out in order to obtain information about chemical groups present around AgNPs for their stabilization and conclude the transformation of functional group due to reduction process. The measurements were carried out using JASCO FT-IR-3600 infra-red spectrometer by employing KBr Pellet technique.

2.5.4 Transmission Electron Microscopy (TEM)

The size and morphology of the synthesized nanoparticles were recorded by using TEM model JEOL electron microscopy JEM-100 CX. TEM studies were prepared by drop coating silver nanoparticles onto carbon-coated TEM grids. The Film on the TEM grids were allowed to dry, the extra solution was removed using a blotting paper.

2.5.5 Atomic Absorption Spectrophotometry

Silver nanoparticles concentration assessment using UNICAM939 Atomic Absorption Spectrophotometry, England, equipped with deuterium background correction. All solutions were prepared with ultra-pure water.

2.6 Chitinase Assay

The chitinase enzyme activity of actinomycete strain Alex-SK121 was determined according to the method of Miller [39] as follows: 0.025 gm of colloidal chitin, 0.5 ml of 0.05 M phosphate buffer (pH 5.5), 1 ml crude enzyme and 1ml distilled water, reaction mixture was well blended with vortex and incubated in water bath at 30℃ for 1h. The reaction was stopped by addition of 3 ml 3-5 dinitrosalicylic acid followed by heating at 100℃ for 5 min. the colored solution was centrifuged at 5000 rpm for 5 min and the absorption was measured at 575 nm using spectrophotometer against blank.

One unit of activity was defined as the amount of enzyme that is required to release 1 µM of N-acetyl glucose amine per min under the standard assay conditions.

2.7 Antimicrobial Activity of AgNPs

Antimicrobial activity of the microbiologically synthesized AgNPs was tested against microbial test strains of Gram-positive bacteria (*Bacillus subtilis* NCTC 1040, *Staphylococcus aureus* NCTC 7447) and Gram-negative bacteria (*Pseudomonas aeruginosa NCIB*-9016, *Escherichia coli* NCTC10416), also against unicellular fungi (*Candida albicans* IMRU 3669), filamentous fungi (*Aspergillus niger* IMI 31276, *Aspergillus flavus* IMI 111023) according to the method of Cappuccino and Sherman [42]. The growth inhibition of microbial pathogens was assessed by the corresponding zone of inhibition (ZOI) [24,26]. Sterile standard antibiotic disks with diameter of 6 mm were used to evaluate the activity of the synthesized AgNPs. Pure cultures of bacteria were grown in nutrient broth at 37℃ in an incubator shaker at 160 rpm. 50 µl of test samples were loaded on the disc, disks were complete lyair dried and 5% CFS loaded disc was taken as positive control. Nutrient agar was spread plated with 106 CFU/ml of bacterial cultures, impregnated with the sample loaded disks and incubated at 37℃ for 18 h. ZOI was measured using a Vernier calliper.

2.7.1 Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations(MIC) determination were performed in Luria Bertani (LB) broth in duplicate using serial two-fold dilutions of AgNPs with positive control well (the microorganism and the nutrient) and negative control one (the nutrient only) [24]. The MIC was determined after 24 hrs. of incubation at 37℃ with initial inoculums of 0.1 OD at 600 nm. MIC was determined by two methods:

a) Visually by comparison with the drug free controls.
b) With ELISA plate reader at a wavelength of 620 nm.

2.8 Antitumor Activity of AgNPs

Antitumor activity of synthesized AgNPs was carried out by Sulphorodamine B (SRB) assay [43]. In this method, the monolayer cell culture was trypsinized and the cell count was

adjusted to 0.5-1.0 x 10^5 cells/ml using medium containing 10% new born sheep serum. To each well of the 96 well microtitre plates, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed once and 100 µl of different test concentrations were added to the cells in microtitre plates. The plates were then incubated at 37℃ for 72 hours in 5% CO_2 incubator, microscopic examination was carried out, and observations recorded every 24 hours. After 72 hours, 25 µl of 50% trichloroacetic acid was added to the wells gently such that it forms a thin layer over the test compounds to form overall concentration 10%. The plates were incubated at 4℃ for one hour.

The plates were flicked and washed five times with sterile water to remove traces of medium, sample and serum, and were then air-dried. The air-dried plates were stained with 100 µl SRB and kept for 30 minutes at room temperature. The unbound dye was removed by rapidly washing four times with 1% acetic acid. The plates were then air dried. 100 µl of 10 mMTr is base was then added to the wells to solubilize the dye. The plates were shaken vigorously for 5 minutes. The absorbance was measured using microplate reader at a wavelength of 540 nm [44]. The percentage growth inhibition was calculated using following formula, the percentage growth inhibition was calculated using following formula,

$$\% \text{ cell inhibition} = 100-\{(At-Ab)/ (Ac-Ab)\} \times 100.$$

Where, At= Absorbance value of test compound, Ab= Absorbance value of blank, Ac=Absorbance value of control.

2.8.1 Established cell line

Vero cells (non-tumor cells) are an African green monkey kidney continuous cell line established by [45]. It has the property of Sub cultivation, usually for more than 100 passages while the code number for human breast carcinoma cells (MCF-7) is MCF7 (ATCC HTB22) and human liver carcinoma cells (HEPG-2) is HEPG-2 (ATCC HB8065).

2.9 Antioxidant Activity Assay (Free Radical Scavenging Ability on 2, 2-diphenyl-2-picrylhydrazyl (DPPH), (*In vitro* Assay)

Antioxidant activity of the synthesized AgNPs from strain Alex-SK121 was carried out by measuring scavenging activity of 2, 2-diphenylpicrylhydrazyl (DPPH) free radicals according to [46]. In brief, 2 ml of distilled water, 1 ml of 0.1 mM DPPH solution in ethanol and 0.5 ml of the biosynthesized AgNPs were shacked vigorously and allowed to reach a steady stat for 30 min at room temperature. Decolonization of DPPH was determine by measuring the decrease in absorbance at 517nm and the DPPH radical scavenging effect was calculate according to the following equation:

$$I (\%) = 100 X (Ablank – Asample/Ablank)$$

Where I (%) is the inhibition percent, A blank is the absorbance of the control reaction (containing all reagents except the test compound) and A sample is the absorbance of the test compound. tert-Butyl hydroquinone (TBHQ) was compared as Standard.

3. RESULTS AND DISCUSSION

3.1 Isolation and Characterization of Actinomycete Strain

After 7 days of incubation at 36℃, pure colonies were isolated on starch nitrate agar medium and subculture on ISP4 agar medium (Fig. 1). The cultural characteristics of actinomycete isolate, Alex-SK121 grown on different ISP media (Table 1) exhibited that, the aerial hyphae was white therefore, it was assigned to the white vivid orange series with slight dark yellow substrate mycelium. Also, the strain was found to produce dark brown diffused pigments. The strain exhibited superior growth on ISP-4, moderate growth on ISP 2, 6, poor growth on ISP 5, 7 and good growth on ISP3. Diffusible pigment or melanin on any of the tested media was noticed on ISP 6 and ISP 7. Scanning electron microscope images indicated that, the isolate possessed substrate mycelia and extensively straight aerial hyphae that further differentiated into smooth surfaced spores (Fig. 2).

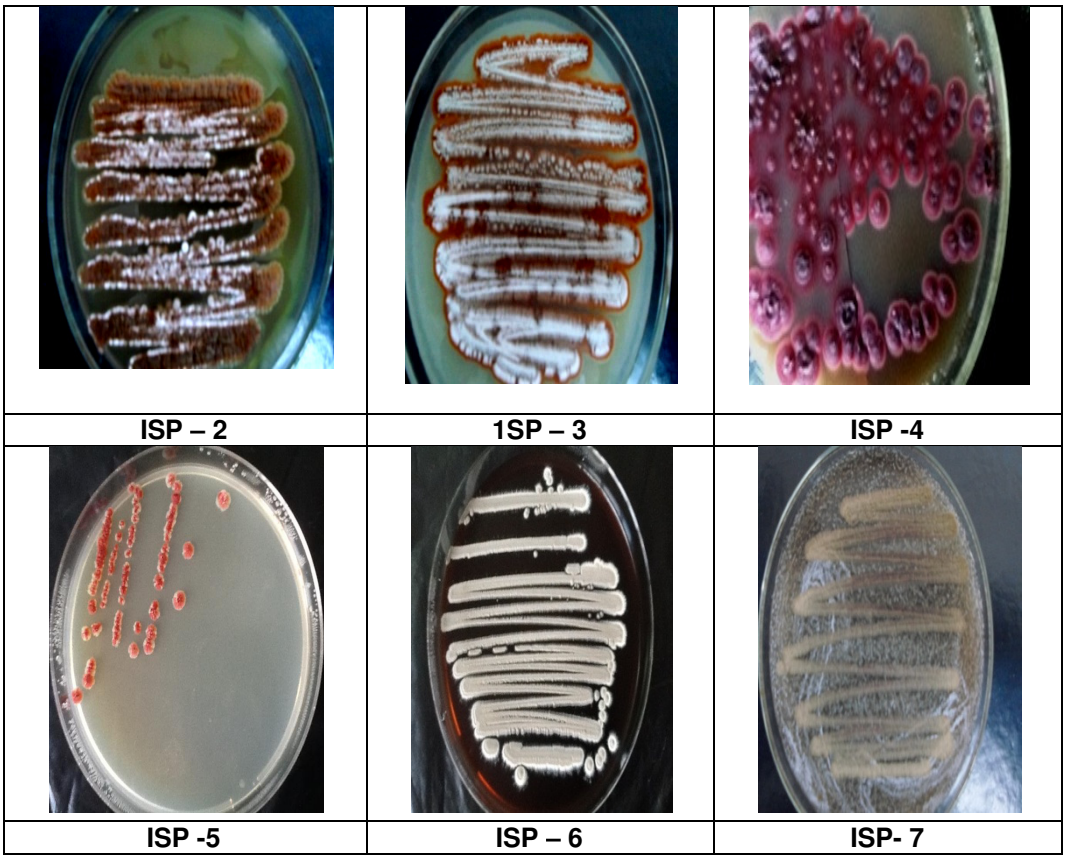

Fig. 1. Cultural characteristics of *Streptomyces cyaneus* strain Alex-SK121 grown on different ISP platesfor7days

Cell wall of the isolate composed of LL-Diaminopimelic acid (cell wall type I) as a major amino acid which confirmed the isolate belonging to the genus Streptomyces. Cell-wall composition analysis is one of the main methods that can be employed to identify the

chemotaxonomic characteristics of Streptomyces; the presence of LL-DAP in the cell wall also signifies that this strain is Streptomyces [47]. Outcomes of the biochemical and physiological characterization were as summarized in Table 2 [31,48]. Partial gene sequences (786 bp) of isolate were deposited at Gene Bank database (NCBI) under the accession no. KJ726667.1. Based on physiological, biochemical characterization and 16S rDNA sequence analysis the isolate was named as *Streptomyces cyaneus* strain Alex-SK121 (Fig. 3).

Table 1. Cultural characteristics of *Streptomyces cyaneus* strain Alex-SK121 grown on different ISP media

Medium	Growth	Substrate mycelium	Aerial Mycelium	Diffusible Pigments
Tryptone yeast extract broth (ISP–1) [49].	No growth	-	-	-
Yeast -malt extract agar (ISP–2) [50].	Moderate	Deep reddish brown (ISCC-NBS41)	Light grayish brown (ISCC- NBS60)	None
Oatmeal agar (ISP–3) [51].	Good	Dark reddish Brown (ISCC-NBS44)	Light gray (ISCC- NBS264)	None
Inorganic-trace salt-starch agar (ISP–4) [51]	Excellent	moderate reddish brown (ISCC-NBS43)	Strong purple blue (ISCC- NBS196)	None
Glycerol asparagine agar (ISP–5) [52].	weak	Light yellow (ISCC- NBS86)	Grayish yellow (ISCC- NBS90)	None
Peptone yeast extract iron agar (ISP–6) [53].	good	gray reddish brown (ISCC- NBS45)	bluish gray (ISCC- NBS191)	Light brown (ISCC- NBS 57)
Tyrosine agar (ISP–7) [54].	Moderate	Brownish black (ISCC- NBS65)	vivid reddish orange (ISCC- NBS34)	Light brown (ISCC- NBS 57)

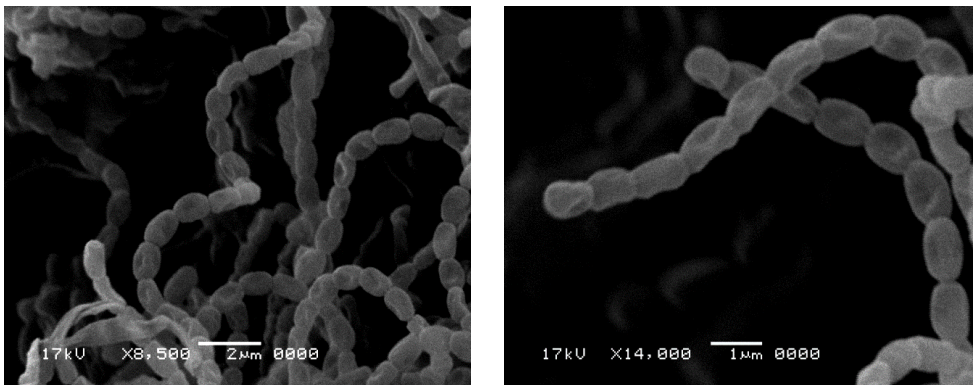

Fig. 2. Scanning electron micrograph of mycelia and spore of *Streptomyces cyaneus* strain Alex-SK121 grown on ISP-4 broth medium for 7 days. Bar: 2 µm.

Table 2. The biochemical and physiological characteristics of the isolated strain *Streptomyces cyaneus* strain Alex-SK121

Characteristic features	Results	Characteristic features	Results
1-Melanin pigment		Ammonium sulphate	++
Peptone-yeast extract iron agar	+++[a]	Urea	+++
Tyrosine agar	+++	L-Tyrosine	NG[e]
Tryptone-yeast extract broth	-[b]	L-Serine	NG[e]
2-Enzymatic activities		L-Asparagine	++
Protein	-	L-Arginine	+
Starch	+++	L-Phenyl alanine	++
Lipid	+++	L-Histidine	+
Catalase production	++[c]	**5-Tolerance to NaCl**	
H_2S production	-	1:6 %	++
Chitinase	++		
Nitrate reduction	++	7.0 %	NG[e]
Tyrosine degradation	++	**6-Growth temperature ºC**	
Urea test	-	10	+
3-Utilization of carbon sources		20:45	++
D-Glucose	++	50	NG[e]
D-Xylose	+[d]	**7-Growth pH**	
D-Fructose	NG[e]	3 :8	+++
Sucrose	+	9:11	++
Maltose	NG[e]	12:14	NG[e]
Raffinose	NG[e]	**8-Growth inhibitors**	
Dextrose	++	Crystal violet (0.0001%)	+++
D-Mannitol	++	Thallus acetate (0.001%)	NG[e]
Cellulose	++	Sodium azide (0.01%)	+
Starch	+++	Phenol (0.1%)	+
L(+)Arabinose	NG[e]	**9-Resistance to antibiotics**	
4-Utilization of nitrogen sources		Rifampicin (50 µg mL-1)	+++
Peptone	++	Penicillin (25µg mL-1)	++

[a](+++) = abundant growth, [b](-) = negative, [c](++) = good growth, [d](+) = moderate, [g](NG) = No growth.

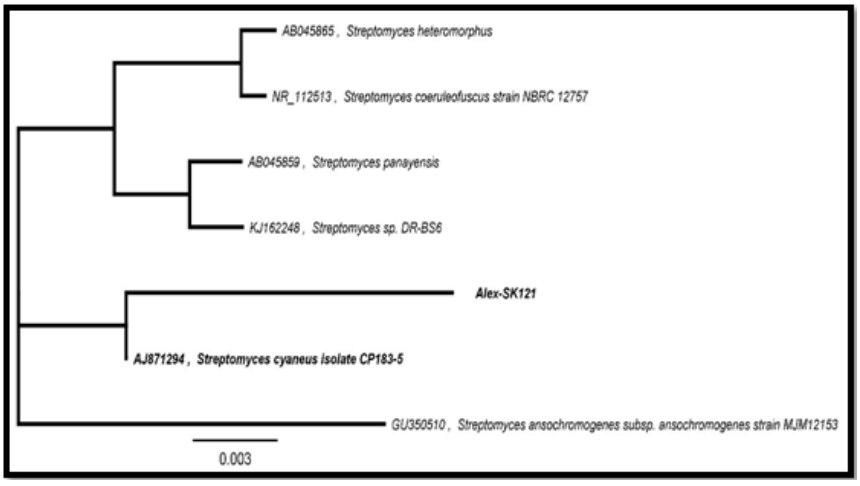

Fig. 3. The phylogenetic tree of *Streptomyces cyaneus* strain Alex-SK121 KJ726667.1 was constructed using the neighbour-joining method with aid of geneius pro 7.1.5 tree builder program. Bar 0.003 substitutions per nucleotide position

3.2 Microbial Synthesis of Silver Nanoparticles

In this study *Streptomyces cyaneus* strain Alex-SK121 was able to synthesis silver nanoparticles and this is shown when the Aqueous Ag^+ ions were reduced to AgNPs when added to the cell free supernatant of *Streptomyces cyaneus* strain Alex-SK121 and this is indicated by the color change from pale brown (due to melanin pigment) to deep brown while control dos not give any color change.

The possiblemechanismsuggests that the radiolytic reduction of aqueous solution is carried out by organic radicals formed. *Streptomyces cyaneus* strain Alex-SK121 supernatant molecules (especially pale brown melanin pigments Fig. 4.) [55]. Play an important role in scavenging the free radicals and converted into organic radicles [56-57].

The irradiated solution at different gamma radiation doses 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, 10.0, 15.0, 20.0, 25.0, and 30.0 kGy were then characterized using UV.V is. spectrometer. So, the maximum absorption (3.61) was recorded at 15kGy as indicating in Table 3.

The appearance of deep brown color in irradiated solution at 15 kGy suggested the formation of AgNPs and the color change is attributed to the Surface Plasmon Resonance (SPR). The strong interaction of the AgNPs with light occurs because the conduction electrons on the metal surface undergo a collective oscillation when excited by light at specific wavelengths known as a Surface Plasmon Resonance (SPR) [24].

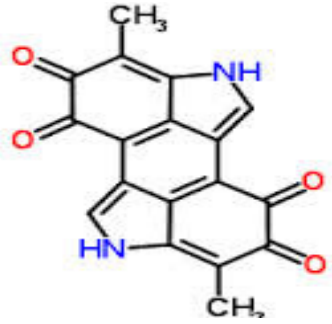

Fig. 4. Chemical structure of pale brown melanin pigment [54]

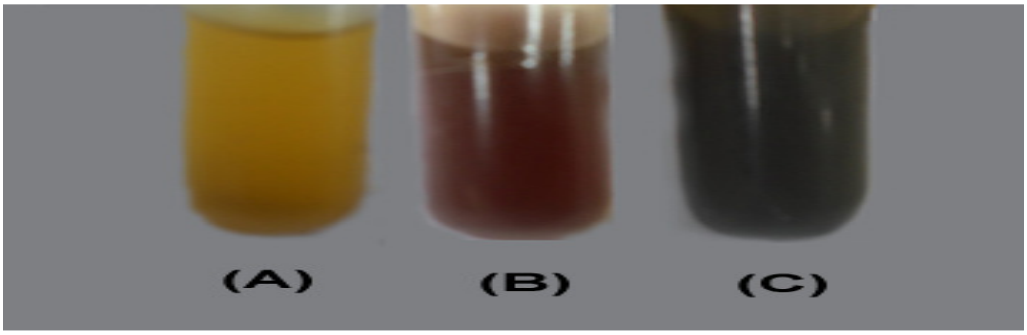

Fig. 5. Biosynthesis of silver nanoparticles color change reaction
Where (A) supernatant of organism, (B) Melanin Pigment, (C) Silver nanoparticles.

Table 3. Different gamma irradiation-induced AgNPs synthesis

Gamma irradiation doses (kGy	Wavelengths (nm)	Maximum absorption (OD)
0.5	320.00	3.198
1	380.00	3.012
1.5	385.00	3.016
2	390.00	3.065
2.5	390.00	3.170
3	385.00	3.234
5	390.00	3.583
10	405.00	3.599
15	425.00	3.616
20	390.00	3.066
25	400.00	3.002
30	405.00	3.000

When the irradiated reaction mixture was incubated under dark condition, the color of the liquid mixture changed from brown due to melanin pigment to dark brown, and then black, subsequently as shown in Figure 5., this change in color of extracellular medium was linked with the formation of AgNPs and depicts the excitation of surface Plasmon vibrations in the nanoparticles.

The following provisional suggested mechanism for reduction, which is consistent with similar studies on the irradiation reduction of AgNPs in other solutions [25].

The growth of silver nanoparticles by reduction of Ag^+ to Ag^0 is step wise show in the following Eqs. (1), (2), (3), (4) and (5).according to speculative reduction mechanisms [24,25].

$$* H_2O \xrightarrow{\text{Gamma-irradiation}} e^-_{aq}, H_3O^+, H^{\cdot}, OH^{\cdot}, H_2^{\cdot}, H_2O_2^{\cdot} \quad (1)$$

$$*C_{18}H_{10}N_2O_4\ \text{(melanin structure formula)}\ + OH^{\bullet} \longrightarrow H_2O + C^{\cdot}_{18}H_9N_2O_4 + H_2O \quad (2)$$

A secondary radical is formed **($C^{\cdot}_{18}H_9N_2O_4$)** which efficiently reduces the precursor metal ions Ag^+ to Ag^0.

$$*C^{\cdot}_{18}H_9N_2O_4 + Ag+ \longrightarrow Ag^0 + C_{18}H_8N_2O_4 \quad (3)$$

$$*C^{\cdot}_{18}H_9N_2O_4 + Ag_2^+ \longrightarrow Ag_2^0 + C_{18}H_8N_2O_4 \quad (4)$$

$$*C_{18}H_9N_2O_4 + Ag_n^{+\cdot} \longrightarrow Ag_n^0 + C_{18}H_8N_2O_4 \quad (5)$$

3.3 Characterization of the Synthesized Silver Nanoparticles (AgNPs)

Characterization of AgNPs synthesized by cell-free supernatant of *Streptomyces cyaneus* strain Alex-SK121 at 15 kGy of gamma irradiation was performed through the following analysis.

3.3.1 UV-Visible Spectrophotometer (UV-Vis.)

The dispersion of silver nanoparticles displays intense colors due to the Plasmon resonance absorption. The surface of a metal is like plasma, having free electrons in the conduction band and positively charged nuclei.

Surface Plasmon Resonance (SPR) is a collective excitation of the electrons in the conduction band; near the surface of the nanoparticles. Electrons are limited to specific vibrations modes by the particle's size and shape. Therefore, metallic nanoparticles have characteristic optical absorption spectrum in the UV-Visible region [24].

As shown in Fig. 6-A, UV-Visible spectrum of AgNPs Synthesized by gamma irradiation at 15 kGy in the presence of cell-free supernatant of Streptomyces *cyaneus strain Alex-SK121*. it worth mentioning that the specific UV.Visibe spectrum of melanin pigment at ƛ 475 nm not found in Figs. 6A and 6B because we used the filtrate as a blank as mentioned in material and methods. And Fig. 6-B.UV-Visible spectrum of AgNPs synthesized without gamma irradiation in the presence of cell-free supernatant of *Streptomyces cyaneus* strain Alex-SK121 at room temperature. Also, Table 3 exhibits the maximum absorption of prepared sliver nanoparticles in different gamma irradiation doses.

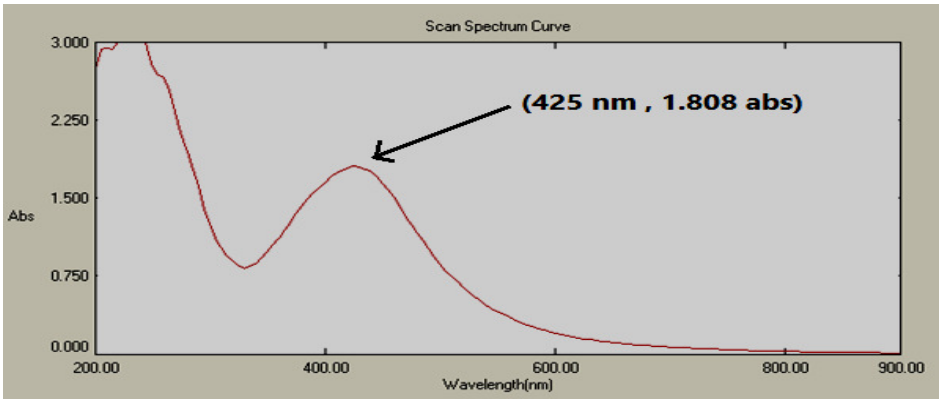

Fig. 6a. UV-Visible spectrum of AgNPs synthesized by gamma irradiation in the presence of cell-free supernatant of *Streptomyces cyaneus* strain Alex-SK121 at 15 kGy

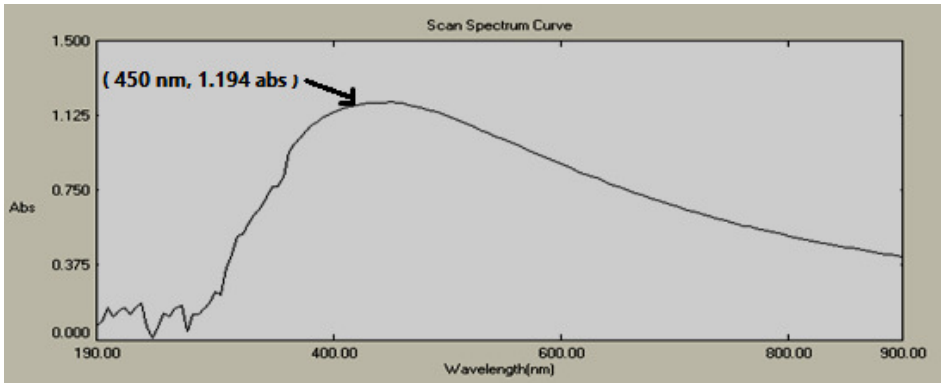

Fig. 6b. UV-Visible spectrum of AgNPs synthesized without gamma irradiation in the presence of cell-free supernatant of *Streptomyces cyaneus* strain Alex-SK121 at room temperature.

3.3.2 Dynamic Light Scattering (DLS)

The average particle size was determined by dynamic light scattering (DLS) method and found to be 19.0 nm as shown in (Fig. 7) in AgNPs synthesized by cell-free supernatant of *Streptomyces cyaneus* strain Alex-SK121 and gamma irradiated at 15 kGy.

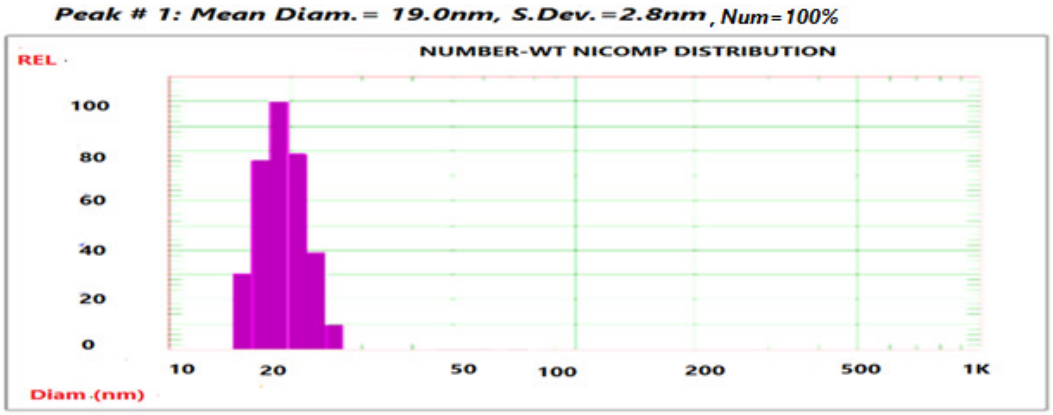

Fig. 7. DLS pattern of particle size distribution of the synthesized AgNPs in the presence of cell-free supernatant of *Streptomyces cyaneus* strain Alex-SK121 at 15 KGy

3.3.3 Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) examination of the solution containing AgNPs demonstrated spherical particles within nanoranged from 9.3nm to 20.3 nm with the main diameter of 15.76 nm as shown in Fig. 8. The particle size obtained from DLS measurement is obviously large than TEM result because DLS analyze measures the hydrodynamic radius [24].

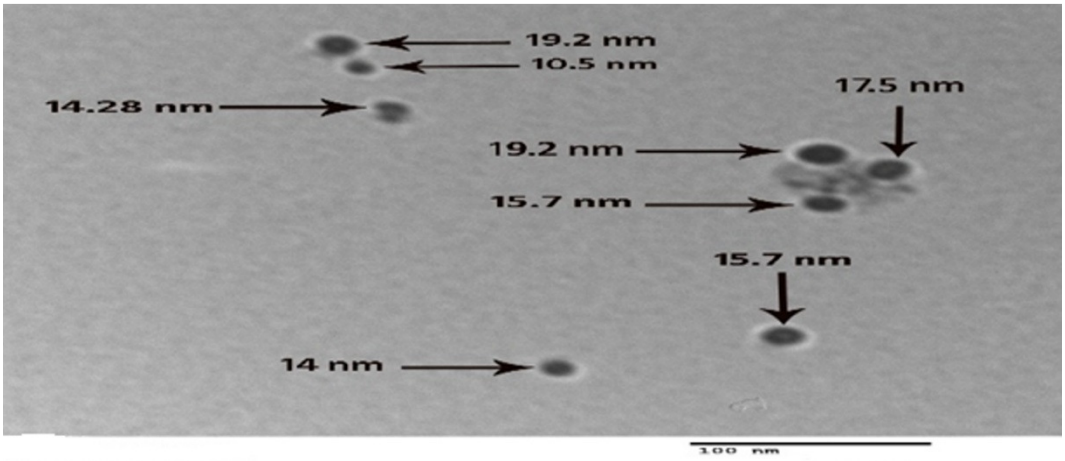

Fig. 8. TEM of the synthesized AgNPs in the presence of cell-free supernatant of *Streptomyces cyaneus* strain Alex-SK121 at 15 kGy

3.3.4 Fourier Transforms Infrared Spectrometer (FT-IR)

It was observed from the FT-IR spectrum of AgNPs which synthesized by cell-free supernatant of *Streptomyces cyaneus* strain Alex-SK12 at 15 kGy that the bands at 917.95 cm^{-1} corresponding to a primary amine (NH band), and 2321.87 cm^{-1} corresponding to a primary amine (NH stretch vibrations of the proteins) as shown in Figs. 9a and 9b and Table 4 which suggested that a broad absorption at 3823.67 cm^{-1} indicate the presence of –OH and NH_2 groups and small band at 2325.73 cm^{-1} can be assigned to stretching vibration of aliphatic C-H group. The characteristic strong band at 1002.8 cm^{-1} attributed to vibrations of aromatic ring C=C of amide I C=O and/or of COO- groups. Bands at 836.955 can be due to aliphatic C-H groups in the melanin pigment [56].

The positions of these bands were close to that reported formative proteins. The FT-IR results indicate that the secondary structure of proteins was not affected as a consequence of reaction with Ag^+ ions or binding with AgNPs [24].

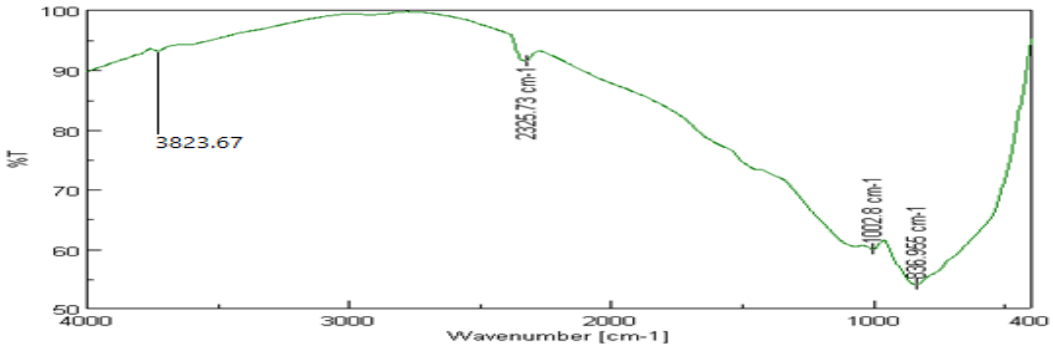

Fig. 9a. FT-IR spectrum of cell-free supernatant of *Streptomyces cyaneus* strain Alex-SK121

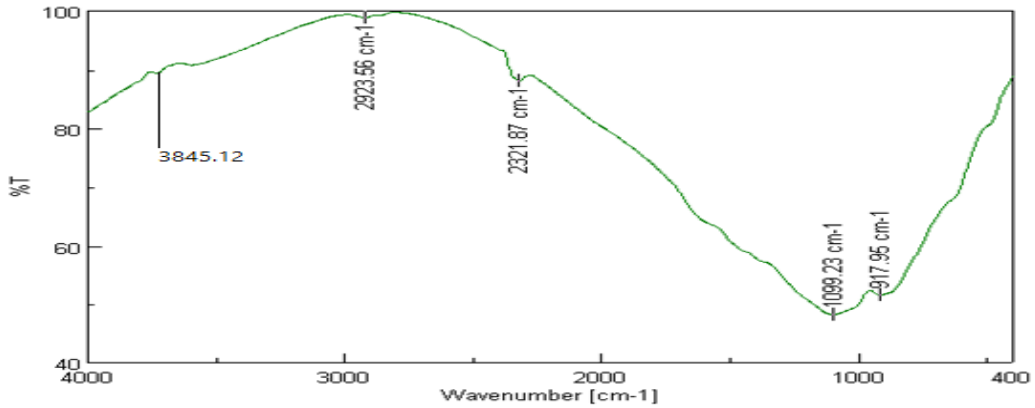

Fig. 9b. FT-IR spectrum of cell-free supernatant of *Streptomyces cyaneus*strain Alex-SK121 with silver nanoparticles

Table 4. FT-IR Wave number of characteristics bonds and corresponding assignments for *Streptomyces cyaneus* strain Alex-SK121 without and with AgNPs

Peak number	Filtrate $\bar{\lambda}(cm^{-1})$	Filtrate +AgNPs $\bar{\lambda}(cm^{-1})$	Comment
1	3823.67	3845.12	Corresponding to OH and NH- group band vibration [57].
2	-------	2923.56	The broad peaks are characteristic to the presence of–NH amino group and–OH stretching group sin alcoholic and phenolic compounds .The presence of this peak may be due to binding of Ag ions to OH group [58].
3	2325.73	2321.87	Corresponding to aliphatic C-H stretching [24] and for stretching vibration of aliphatic C-H group [56].
4	1002.80	1099.23	May be ascribed for the presence of C=O stretching group [59]. And attributed to vibrations of aromatic ring C=C of amide I C=O and/or of COO- groups [56].
5	836.95	917.95	Due to aliphatic C-H groups in the melanin pigment [56].

4. BIOLOGICAL APPLICATIONS OF THE SYNTHESIZED AgNPs

4.1 Antimicrobial Activity

The biologically synthesized AgNPs showed good antibacterial activity against Gram-positive and Gram-negative bacteria (Table 6) among the bacterial pathogens tested, maximal growth inhibition was observed for *Pseudomonas aeruginosa*. Also AgNPs showed antifungal activity against both unicellular and filamentous fungi (Table 7) and maximal growth inhibition was observed for *Candida albicans* and *Fusarium oxysporum*. The antimicrobial activity for Cell free supernatant (CFS) of *Streptomyces cyaneus* strain Alex-SK121 had been showed in Table 5. Also, MIC of the synthesized AgNPs showed in Table 8. By making comparison with the other published paper [24] it was found that the MIC for the synthesized AgNPs by *Streptomyces cyaneus* strain Alex-SK121 is lower than the synthesized by *Bacillus megaterium* [24] because of the synthesized AgNPs by *Streptomyces cyaneus* strain Alex-SK121 were larger in size than that synthesized by *Bacillus megaterium*.

Silver ions have been known to bind with the negatively charged cell wall resulting in the rupture and consequent denaturation of proteins which leads to cell death [63]. The synthesized AgNPs with smaller size can act drastically on cell membrane and further interact with DNA and causes damage [50]. Other proposed mechanisms include the AgNPs causing depletion of intracellular ATP by rupture of plasma membrane or by blocking respiration in association with oxygen and sulfhydryl (–S–H) groups on the cell wall to form R–S–S–R bonds thereby leading to cell death [60,61].

Table 5. Antimicrobial activity for Cell free supernatant (CFS) of *Streptomyces cyaneus* strain Alex-SK121

Microorganism	Zone of inhibition (mm)		
	Cell free supernatant (CFS).	Standard antibacterial agents (Tetracyclines 100ppm)	Standard antifungal agents (Amphotericin B 100ppm)
Bacillus subtilis NCTC1040	0.0	18.0± 2.0	0.0
Staphylococcus aurous NCTC7447	0.0	29.0± 1.8	0.0
Escherichia coli NCTC10416	0.0	32.0± 1.5	0.0
Pseudomonas aeruginosa NCIB9016	0.0	20.0± 2.3	0.0
*Candida albicans*IMRU3669	17.0±2.0	0.0	19.0±2.3
Aspergillus niger IMI31276	16.0±1.8	0.0	16.0± 1.8
Aspergillus flavus IMI111023	15.0±2.1	0.0	17.0± 2.0
Fusarium oxysporum RCMB008002	25.0±1.5	0.0	18.0± 2.5
Trichoderma viride RCMB008002	18.0±2.0	0.0	15.0±2.0

Table 6. Antibacterial activity of AgNPs against some bacterial test strains

Microorganism	zone of inhibition (mm)	
	AgNPs (105 ppm)	Antibacterial agents (Tetracyclines 100ppm)
Bacillus subtilis NCTC1040	13±2.0	18±1.3
Staphylococcus aurous NCTC7447	13±2.0	29±2.3
Escherichia coli NCTC10416	13±1.8	32±2.0
Pseudomonas aeruginosa NCIB9016	16±1.5	20±1.8

Table 7. Antifungal activity of AgNPs against fungal test strains

Microorganism	Zone of inhibition (mm)	
	AgNPs (105 ppm)	Antifungal Agents (Amphotericin B 100ppm)
Candida albicans IMRU3669	20±2.1	19±2.0
Aspergillus niger IMI31276	17±1.6	16±1.8
Aspergillus flavus IMI111023	16±2.0	17±2.0
Fusarium oxysporum RCMB008002	20±1.8	18±1.4
Trichoderma viride RCMB008002	17±1.1	15±1.8

The last result in Table 6 suggests that the release of extracellular protein such as chitinase enzymes in the presence of reducing agent such as melanin pigment could possibly perform the formation and stabilization of AgNPs in aqueous medium.

Table 8. Antimicrobial Activity as MICS (µg/ml) of Synthesized AgNPs against some pathogenic microbes

Microbial test strains	Minimum Inhibitory Concentration (MIC) of synthesized AgNPs by *Streptomyces cyaneus* strain Alex-SK121	Minimum Inhibitory Concentration (MIC) of synthesized AgNPs by *Bacillus megaterium* [24].
Bacillus subtilis (NCTC 10400)	20 µg/ml	15.63 µg/ml [24].
Staphylococcus aureus (NCTC7447)	59.5 µg/ml	----------
Escherichia coli (NCTC 10416)	125 µg/ml	125 µg/ml [24].
Pseudomonas aeruginosa (NCIB9016)	140 µg/ml	_____
Candida albicans (IMRY 3669)	50 µg/ml	_____
Aspergillus niger IMI31276	55 µg/ml	_____
Aspergillus flavus IMI111023	60 µg/ml	_____
Fusarium oxysporum RCMB008002	68 µg/ml	_____
Trichoderma viride RCMB008002	62 µg/ml	_____

4.2 Antitumor Activities

The biosynthesized silver nanoparticles (AgNPs) exhibited antitumor activity against human breast carcinoma cells MCF7 (ATCC HTB22) and human liver carcinoma cells HEPG-2 (ATCC HB8065) with IC_{50} 9.63 and 33.75µg/ml, data are represented in (Table 9 for MCF7 (ATCC HTB22) cell line). and (Table 10 for HEPG-2 (ATCC HB8065) cell line. While the cytotoxicity biosynthesized silver nanoparticles (AgNPs) on non-tumor cells have IC_{50} 13.12 µg/ml.

Table 9. Antitumor activity of synthesized AgNPs against MCF7 (ATCC HTB22) cell line

Concentration (ppm)	Absorbance (540 nm)			Mean	Cell viability	IC_{50} µg/ml
0.0	0.324	0.325	0.333	0.327333333	100	
6.56	0.3079	0.313	0.306	0.308966667	94.38900204	
13.12	0.237	0.259	0.222	0.239333333	73.11608961	9.6303
26.25	0.294	0.122	0.249	0.221666667	67.71894094	
52.5	0.218	0.182	0.225	0.208333333	63.64562118	
105	0.117	0.291	0.200	0.202666667	61.91446029	

Although the use of colloidal silver as an antimicrobial agent is recognized [62], there are scarce reports on its use as antitumor agent; among these, there is recent report on the anti-proliferative effect of silver nanoparticles on human glioblastoma cells (U251) [63]. In the present study, we showed that MCF-7 breast cancer cells treated with colloidal silver, significantly reduced the dehydrogenase activity, resulting in decreased NADH/NAD^+, which in turn induces cell death due to decreased mitochondrial membrane potential. Death cell

can also be produced by ROI (Reactive Oxygen Intermediates), and RNI (Reactive Nitrogen Intermediate) metabolites [64].

Table 10. Antitumor activity of synthesized AgNPs against HEPG-2 (ATCC HB8065) cell line

Concentration (ppm)	Absorbance (540 nm)			Mean	Cell viability	IC_{50} µg/ml
0.0	2.032	2.094	2.017	2.047666667	100	33.7577
6.56	1.989	1.813	1.825	1.875666667	91.60019534	
13.12	1.916	1.776	1.716	1.802666667	88.03516197	
26.25	1.826	1.815	1.683	1.774666667	86.66775191	
52.5	1.630	1.894	1.788	1.770666667	86.66775191	
105	1.643	1.549	1.571	1.587666667	77.53540615	

4.3 Antioxidant Activity

The antioxidant activity of the synthesized AgNPs was evaluated using DPPH scavenging assay. As shown in Table 11, a significant difference was observed among the respective values obtained. The DPPH values were increased in a dose dependent manner. Silver nanoparticles (AgNPs) are one of the most commonly used nanomaterials. AgNPs are known to have antioxidant and antimicrobial properties [65]. Silver nanoparticles possessing antioxidant activity against various in vitro antioxidant systems. The free radical scavenging activity of AgNPs was found to be higher than that standard confirmed in the present investigation. From the above assays, the possible mechanism of antioxidant activity of AgNPs includes reductive ability, electron donating ability and scavengers of radicals [66].

Table 11. DPPH Free radical scavenging activity of biosynthesized Silver Nanoparticles and Tert-Butyl hydroquinone (TBHQ) (Stander)

Concentration AgNPs/ TBHQ (ppm)	Absorbance (517nm)		scavenging activity %	
	AgNPs	TBHQ	AgNPs	TBHQ
6.56	0.24	0.30	66	70
13.12	0.18	0.21	75	79
26.25	0.12	0.14	83	86
52.5	0.07	0.11	90	89
105	0.03	0.02	96	98

5. CONCLUSION

Marine ecosystem is still an unexplored estuarine habitat of its rich microbial diversity. There are huge possibilities for the occurrence of potential microbes to withstand metal stress in its nutrient rich habitat. With this background, we have isolated a unique *Streptomyces cyaneus* strain Alex-SK121 and studied its capability to synthesize AgNPs. The synthesized silver nanoparticles were characterized by UV–Visible spectroscopy, FTIR, DLS and TEM and the different biological activities of the AgNPs were evaluated. The synthesized silver nanoparticles may be advantageous as antimicrobial agent against a range of pathogenic

bacteria and fungi. The AgNPs also show promise as antitumor agents containing some level of antioxidant activity.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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