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Characterization of biosynthesized gold nanoparticles from *Streptomyces misionensis* PYA9 with biomedical and environmental applications

Abirami, B.[1], Akshata, V.[2], Radhakrishnan, M.[1*], Namitha, R.[3], Govindaraju, K.[3], Gopikrishnan, V.[1] and Manigundan, K.[1]

[1]Centre for Drug Discovery and Development, Sathyabama Institute of Science and Technology, Chennai - 600119, Tamil Nadu, India; [2]Post Graduate and research Department of Biotechnology, Hindustan College of Arts and Science, Coimbatore – 641028, Tamil Nadu, India; [3] Centre for Ocean Research, Sathyabama Institute of Science and Technology, Chennai - 600119, Tamil Nadu, India.

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Abstract Result showed marine actinobacterium PYA9 isolated from the coastal sediments of Pondicherry, India effectively reduced aqueous chloroauric acid to gold nanoparticles (AuNPs). Characterization of actinobacteria mediated AuNPs was performed using UV-Vis, Particle size analyzer, TEM, FTIR, and XRD. A strong absorbance peak was observed at 539 nm in the visible region confirming the presence of AuNPs. The average size of AuNPs was found to be 61 nm. Images from TEM analysis revealed the presence of spherical and triangular nanoparticles. The biosynthesized AuNPs were screened for their antibacterial activity against *Salmonella typhi*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Escherichia coli*. Relative to the actinobacterial compounds, the synthesized AuNPs displayed highest activity. The biosynthesized AuNPs also displayed anti-biofouling property when tested against two biofouling bacteria. Taxonomy of the actinobacterial strain PYA9 was identified using 16S rRNA sequencing as *Streptomyces misionensis*. Our study showed new opportunities of utilizing actinobacterial extracts as a reducing agent for synthesizing biocompatible, large scale AuNPs with potential applications in the fields of medicine and environment.

Keywords: *Streptomyces misionensis*, Gold nanoparticles, Biosynthesis, Marine, Applications

Introduction

Extensive investigations have been executed in the branch of nanotechnology owing to its enormous applications and the progress could be attributed to the rise in the development of nanoparticles with unique characteristics and controlled morphologies (Sharma *et al.*, 2019). Broad spectrum applications of nanoparticles include drug delivery, antimicrobials, monitoring cancer cells, biological sensors, photocatalysis,

[*] **Corresponding Author**: Radhakrishnan, M.; **Email**: mrkactinos@gmail.com

water treatment, analytical probes, etc. (Salem and Fouda, 2020). Nanoparticles of diverse size, characteristics, morphology and shape are synthesized via different routes namely chemical, physical and biological. Over the years, synthesis using biological routes is preferred because of the safety, cost effectiveness, and cleaner production of the nanoparticles. Even though plants and their extracts and microbes like bacteria, yeast, and algae are utilized to biologically synthesize nanoparticles, bacteria are ideally preferred since their metal resistance mechanism aide in reduction of ionic metals to metallic nanoparticles. This adds advantage over the other routes of synthesis being more efficient, pure, affordable and eco-friendly (Hutchison, 2008; Li *et al*., 2011; Manimaran and Kannabiran, 2017; Ahmad *et al.,* 2019).

Gold (Au) as a metal is considered non-reactive or inert, while gold as nanoparticles possess numerous properties that are unique and beneficial (Menon *et al*., 2017). Biological means of gold nanoparticles (AuNPs) synthesis from chloroauric acid (HAuCl4) has been studied more due to their ability in reduction at room temperature, various geometrical shapes, cost-effectiveness and non-toxic nature (Sayed and Saad, 2021). When compared to intracellular synthesis, extracellular synthesis is prioritized since the process of nanoparticles separation, that involves removal of cell constituents and cell lysis, can be eliminated (Patil *et al*., 2019). Precipitation occurs readily upon incubation of bacterial cells with gold ions confirming the synthesis of AuNPs (Shivaji *et al*., 2014). Biomedical applications, specifically antimicrobial toxicity, prefer the use of AuNPs in comparison with other inorganic nanoparticles owing to its biocompatibility with human cells and stability of oxidation resistance. They were also found efficient against cancer cells (Składanowski *et al*., 2017).

Actinobacteria are filamentous, gram-positive bacteria sharing significant attributes of both eukaryotic fungi and prokaryotic bacteria. They are widely recognized for their potentiality to release secondary metabolites possessing wide range of applications as antibiotics, antioxidants, anticancer agents, immunomodulatory agents, heavy metal remediators, enzyme producers, biosurfactants etc. (Balagurunathan *et al.,* 2011; Manivasagan *et al*., 2016). Owing to the successful reports on biosynthesis of metallic nanoparticles by actinobacteria, they are considered bionanofactories (Aswani *et al*., 2019; Edison and Pradeep, 2020). Actinobacteria synthesized nanoparticles display remarkable polydispersity and stability making it more acceptable for biological and industrial applications (Bhosale *et al*., 2015). Moreover, productivity could be increased easily by optimizing the culture conditions and media compositions for better inoculums size and growth thereby enhancing the nanoparticles synthesis. Biological properties of actinobacteria synthesized metallic nanoparticles include but not limited to antibacterial, antifungal, antiparasitic, antibiofouling, anticancer, and antioxidant (Manivasagan *et al*., 2016).

There have been several studies reporting the synthesis of various metallic nanoparticles by Actinobacteria. *Streptomyces* sp., *Nocarida* sp., and *Rhodococcus* sp. are the most studied actinobacterial genera to synthesize gold nanoparticles. Actinobacteria reduces metals to nanoparticles by one or more of the following – cell wall components, extracellular enzymes, intracellular proteins, nitrate reductase and other cofactors. The mechanisms involved in detoxification of metal ions to non-toxic nanoparticles by Actinobacteria are biomineralization, extracellular precipitation or intracellular bioaccumulation (Kumari *et al*., 2020).

Many reports have suggested exploring marine sources, precisely, marine microorganisms for the biosynthesis of AuNPs (Patil *et al.,* 2019). The current study investigated biosynthesis of nanoparticles by an actinobacterial strain isolated from marine sediment with potential antimicrobial and anti-biofouling properties. Characterization studies were also performed for the actinobacteria synthesized nanoparticles.

Materials and methods

Culturing and maintenance of actinobacteria strain

Actinobacteria strain PYA9 was obtained from the culture consortia of Centre for Drug Discovery and Development, Sathyabama Institute of Science and Technology. It was previously isolated from marine sediment samples of Pondicherry, India (Lat. 11.9416℃N, Long. 79.8083℃E). The isolate was grown in ISP2 (International Streptomyces Project 2) media for 7 days at 28℃. Cultural characteristics including growth, colony consistency, substrate mycelium, aerial mycelium and reverse side pigment were recorded. The culture was stored in slants and glycerol at 4℃ and -80℃ respectively.

Biosynthesis of gold nanoparticles

Strain PYA9 was grown in 100ml of ISP2 broth for 7 days at 28℃ in a shaking incubator at 120 rpm. Mycelium was separated and discarded after centrifugation at 8000 rpm for 15 mins. The cell-free supernatant containing the bioactive metabolites was used for biosynthesis of gold nanoparticles (AuNPs). 10M stock solution of the metal precursor, Chloroauric acid (HAuCl4), was prepared. Different concentrations, namely, 0.5:4.5, 1:4, 1.5:3.5, 2:3, and 2.5:2.5, of cell-free supernatant and metal precursor were tested to determine the optimum concentration of nanoparticles biosynthesis by Actinobacteria. The solutions were incubated in a shaker for 24 hrs in dark at 120 rpm.

Characterization of nanoparticles

The reaction mixtures were observed visually for color change. Color transition from yellow to red indicates synthesis of AuNPs. After 24 hrs, the solution with optimum color change was centrifuged at 10000 rpm for 10 mins to obtain the AuNPs. The biosynthesized AuNPs were then washed thrice with sterile distilled water, dried and stored for further studies. The biosynthesized nanoparticles were characterized using the following instrumentations.

UV-Vis Spectroscopy

UV-Vis spectrophotometer (Shimadzu UV-1800, Japan) was used to measure the maximum absorption of biosynthesized AuNPs between the wavelength range 400 – 700 nm.

Particle size distribution

The size distribution of synthesized AuNPs was performed using Particle size analyzer, Horiba, Japan. The size was measured by injecting the diluted Actinobacteria synthesized AuNPs into the particle size analyzer chamber.

Fourier Transform Infrared Spectroscopy (FT-IR)

The functional group characterization of the biosynthesized AuNPs was determined using FT-IR spectrometer (Bruker Alpha with ATR, Germany). The nanoparticles were dispersed in a dry KBr compressed matrix to form a disc. The spectrum was scanned at a resolution of 4 cm in the range of 450 cm^{-1} – 4000 cm^{-1}.

Transmission electron microscopy (TEM)

Sterile double distilled water was used to dilute Actinobacteria synthesized dried AuNPs and was deposited on TEM grids that are carbon coated and let to dry in room temperature. Technai 120kV TEM, Netherlands was the instrument used.

XRD

Structural determination of crystalline AuNPs and their characterization was investigated by X-ray diffraction (XRD) analysis. Powdered AuNPs was applied onto the sample holder and pressed to get a smooth plane surface. The pattern of diffraction was recorded using Rigaku miniflex 600.

Antibacterial activity

Biosynthesized AuNPs were tested against different bacterial pathogens namely, *Salmonella typhi* (ATCC 27870), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 29213) and *Escherichia coli* (ATCC 25922). Comparative study was undertaken to test the efficiency of PYA9 synthesized AuNPs by comparing the antibacterial activity of actinobacterial strain PYA9 using direct agar plug method and PYA9 cell-free culture supernatant using well diffusion method. The results were determined by evaluating the zone of inhibition as diameter in mm after an incubation time of 24 hrs.

Anti-biofouling activity

Two bio-film forming bacteria namely, *Staphylococcus* sp. KB6 and *Staphylococcus* sp. KB7 previously isolated and studied for their biofouling activity was collected from the consortia maintained at the Centre for Drug Discovery and Development, Sathyabama Institute of Science and Technology, and was used as test pathogens. Anti-biofouling activity of the biosynthesized AuNPs was compared with direct culture PYA9 and its cell-free supernatant. Zone of inhibition was recorded after 24 hrs incubation.

Antioxidant activity

Antioxidant activity of the biosynthesized AuNPs was measured using DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) radical scavenging assay (Boroumand *et al*., 2019) wherein AuNPs at different concentrations 25, 50, 75 and 100 µg/ml was added to 1 ml of 0.1 mM DPPH dissolved in methanol and incubated for 30 mins in dark. UV-Vis spectrophotometer (BioTek Epoch 2 microplate spectrophotometer) was used to measure the absorbance at 517 nm. The percentage of DPPH scavenging activity was calculated as % inhibition = $(A_0 – A_1 / A_0) \times 100$,Where, A_0 – absorbance of control reaction and A_1 – absorbance of test sample.

Characterization and identification of potential actinobacterial strain

Amplification of 16S rRNA gene of PYA9 was performed using universal primers and the amplified gene was analyzed at Bioserve Pvt. Ltd, Hyderabad using a 3730 ABI DNA Sequencer. MEGA version 11 and BLAST tool was used to construct the phylogeny. Accession number of the partial 16S rRNA gene was obtained from GenBank.

Results

Extracellular biosynthesis of AuNPs using PYA9

Reduction of chloroauric acid to gold nanoparticles resulted from the reaction with metabolites produced by actinobacterial strain PYA9. Color transition from pale yellow to brick red was observed within 24 h which indicates the extracellular biosynthesis of gold nanoparticles (Figure 1). While the remaining combinations displayed pale red coloration, combination 1:4 displayed intense brick red color and was used for further study. Centrifuged and dried AuNPs were stored in powder form.

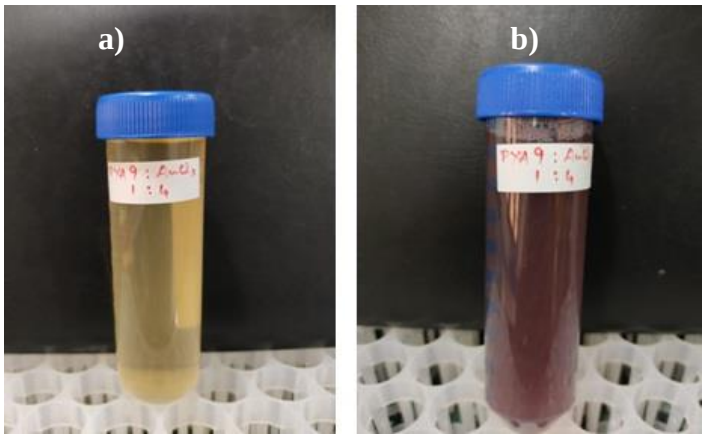

Figure 1. Synthesis of Gold nanoparticles from actinobacterial strain PYA9 (a) before synthesis (b) after synthesis

UV-Vis spectroscopy

The UV-Visible spectra of the actinobacteria synthesized AuNPs is depicted in figure 2. Absorption of the brick red colored AuNPs was found to be stable at a wavelength of 539 nm. The formation of gold nanoparticles was proved by the intense and single absorption peak in the spectra.

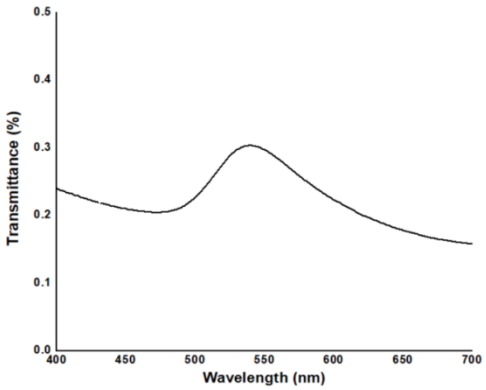

Figure 2. UV-Vis spectra of biosynthesized AuNPs from Actinobacteria

Particle size distribution

Figure 3 shows the size distribution range of AuNPs synthesized from actinobacterial strain PYA9. The average diameter of the biosynthesized nanoparticles was 61.4 nm.

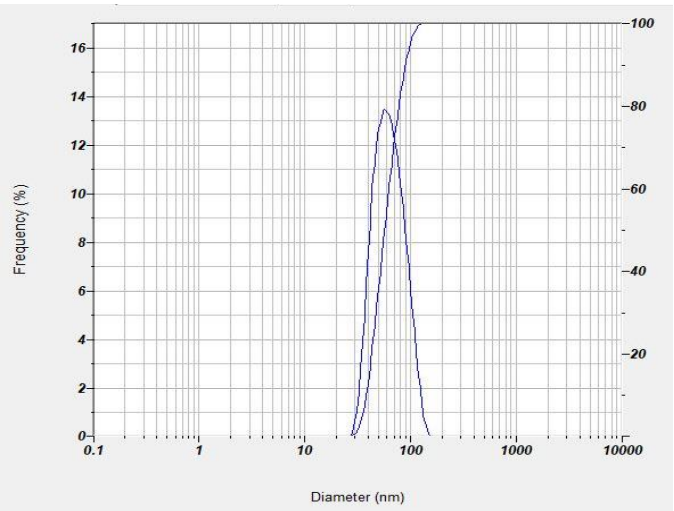

Figure 3. Particle size range of PYA9 synthesized AuNPs

FT-IR

The FT-IR spectrum obtained for the PYA9 synthesized AuNPs is given in figure 4. Different peaks were obtained for different functional groups.

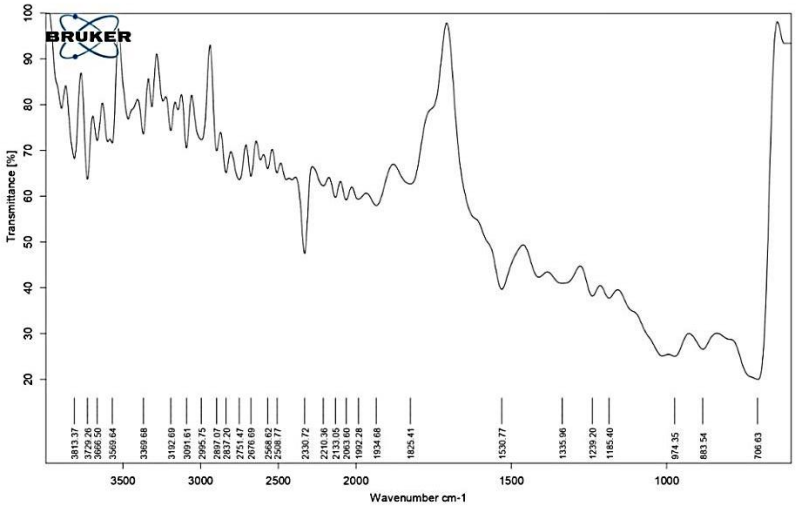

Figure 4. FT-IR spectrum of AuNPs synthesized from actinobacterial strain PYA9

TEM

Morphology of the biosynthesized AuNPs was determined using TEM analysis. TEM images obtained in the range of 38-43 nm (Figure 5). Spherical and triangular pyramid were the shapes of the dispersed AuNPs observed in the TEM images.

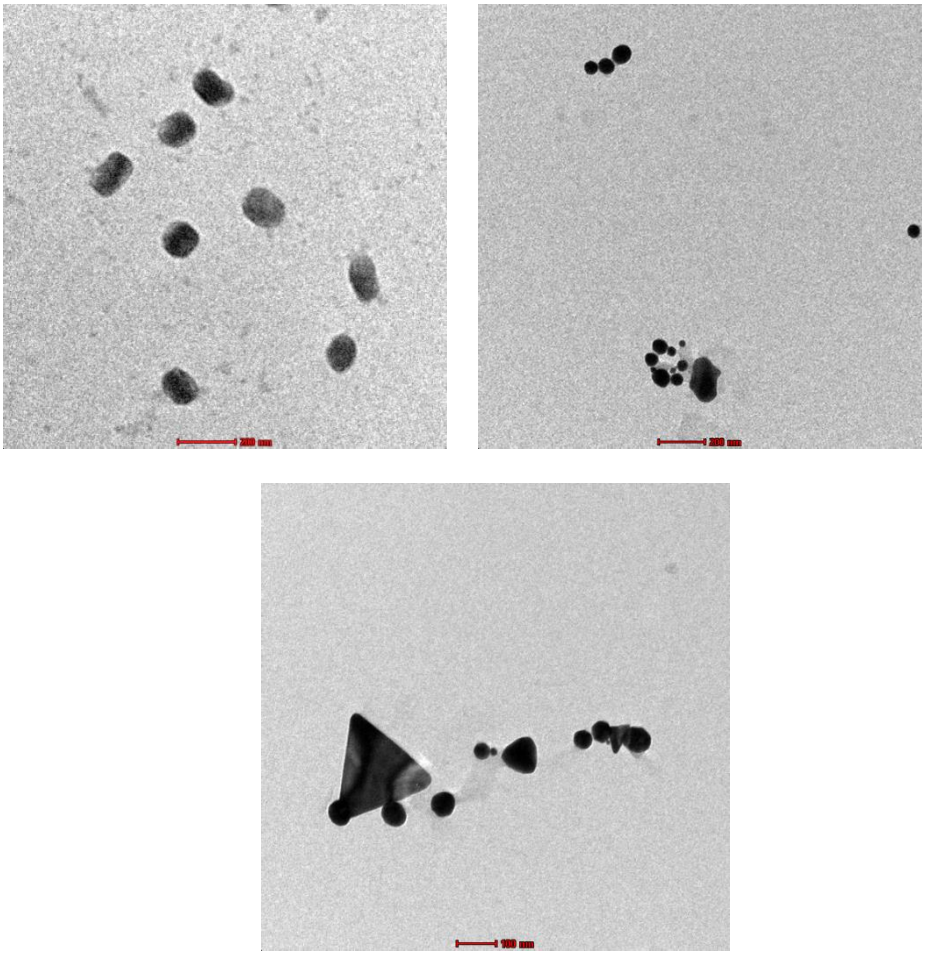

Figure 5. TEM images of PYA9 synthesized AuNPs

XRD

XRD analysis of actinobacterial strain PYA9 synthesized AuNPs is shown in Figure 6. Phase purity of the biosynthesized AuNPs and its crystalline structure are studied using X-ray diffraction analysis. The peaks detected at 2θ values of PYA9 mediated gold nanoparticles were 37.33°, 44.22 °, and 62.12 ° corresponding to the lattice (hkl) planes (111), (200), and (220) respectively.

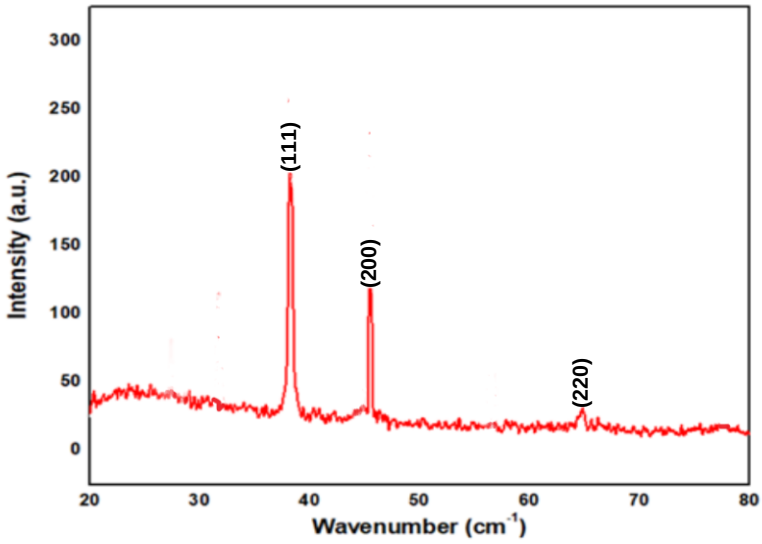

Figure 6. XRD pattern of biosynthesized AuNPs

Antibacterial activity

The antibacterial activity of the actinobacteria synthesized AuNPs was tested by comparing with gentamycin as a standard antibacterial agent. Biosynthesized AuNPs (1 mg/ml) displayed good antibacterial activity as reported in Table 1.

Table 1. Comparative antibacterial activity of PYA9 AuNPs

S. No	Test sample	Zone of inhibition (mm)			
		S. typhi	*E. faecalis*	*S. aureus*	*E. coli*
1.	Gentamycin	17	16	20	19
2.	PYA9 agar plug	9	11	12	11
3.	PYA9 cell-free supernatant	11	9	13	-
4.	PYA9 synthesized AuNPs	16	17	18	18

Anti-biofouling activity

The biosynthesized AuNPs when tested against two biofouling bacterial pathogens displayed good zone of inhibition as reported in Table 2.

Table 2. Antibiofouling activity of PYA9 synthesized AuNPs

S. No	Test sample	Zone of inhibition (mm)	
		Staphylococcus sp. KB6	*Staphylococcus sp.* KB7
1.	PYA9 agar plug	-	-
2.	PYA9 cell-free supernatant	10	11
3.	PYA9 synthesized AuNPs	13	13

Antioxidant activity

Color change from purple to yellow was quantified spectrophotometrically to determine the antioxidant activity of actinobacteria synthesized AuNPs. The scavenging of DPPH radicals tends to increase with increase in AuNP concentration as given in Figure 7.

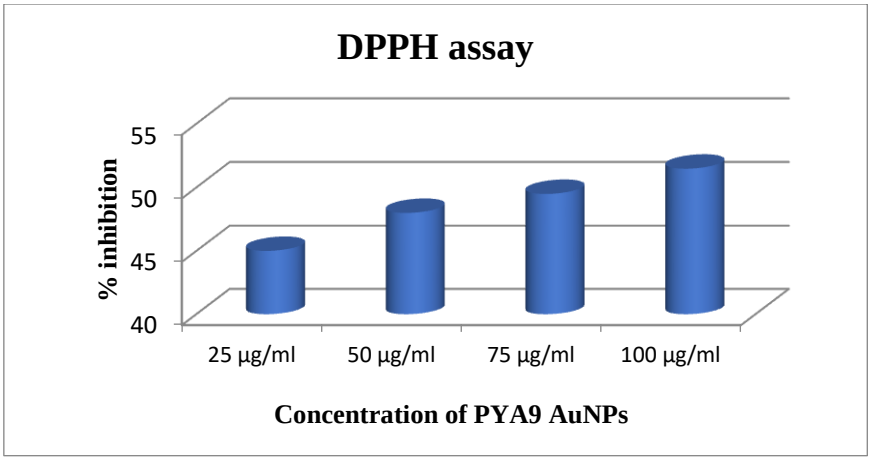

Figure 7. DPPH radical scavenging activity of PYA9 synthesized AuNPs

Taxonomy of potential strain

The morphology of the investigated strain PYA9 is given in figure 8 and its microscopic image in figure 9. The molecular sequence result from 16S rRNA sequencing identified the studied strain as *Streptomyces misionensis*. The strain PYA9 was closer to the strain *Streptomyces misionensis* strain SA20 in BLAST analysis with 99.36% similarity. Following that was *Streptomyces* sp. strain A26 and *Streptomyces* sp. strain A11. The GenBank accession number OP430798 was received from NCBI for the strain PYA9. Neighbor-joining method was used to construct a phylogenetic tree of actinobacterial strain PYA9 (Figure 10).

Figure 8. Morphology of PYA9 on ISP2 media

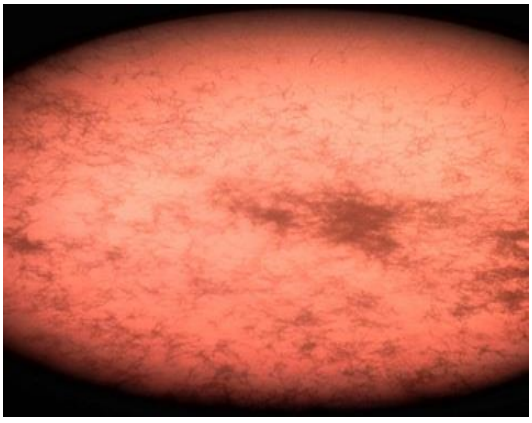

Figure 9. Micromorphology of actinobacterial strain PYA9

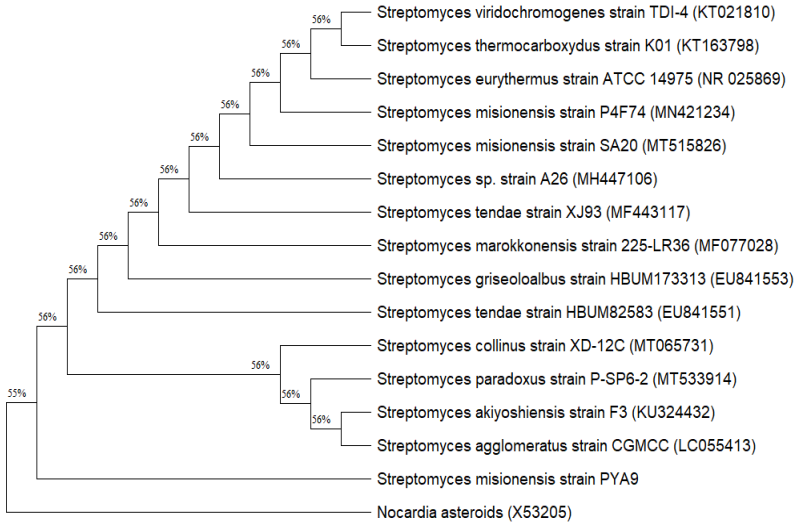

Figure 10. Phylogenic tree of *Streptomyces misionensis* PYA9

Discussion

The actinobacterial strain PYA9 displayed good growth on ISP2 media with both aerial and substrate mycelium. The aerial mass color was white with powdery consistency. When grown in ISP2 broth, the strain PYA9 showed good growth with sufficient biomass. In this study, we've utilized the cell-free supernatant containing the actinobacterial metabolites to synthesize gold nanoparticles extracellularly. Previously, Ranjitha and Rai (2017) have obtained AuNPs from *Streptomyces griseoruber* using extracellular synthesis. It was reported that color transition from yellow to red indicate formation of AuNPs (Merza *et al*., 2012; Ramakrishna *et al*., 2016). Brick red coloration of the actinobacteria – metal solution after incubation of 24 hrs can be considered efficient synthesis of nanoparticles. The absorption of the biosynthesized AuNPs was observed between 525 and 575 nm with the peak at 539 nm in the UV-Vis spectrum. The presence of a single absorption peak and absence of additional peaks confirms that there are no un-reacted metallic gold in the solution. Metallic gold nanoparticle synthesis by Ravindra (2009) and Zonooz *et al.* (2012) observed an absorption peak at 536 nm and 540 nm respectively. The strain PYA9 synthesized AuNPs were found to be different sizes from 30 nm to 100 nm. The mean size of the metallic gold nanoparticles was computed as 61 nm using the particle size analyzer. Studies by Ghosh *et al.* (2011) and Khadivi Derakhshan *et al.* (2012), showed particle size distribution of 15-60 nm and 40-60 nm respectively. FTIR peaks at 3729 cm^{-1} and 3812 cm^{-1} might be interpreted as O-H stretching, while the peak at 2330 cm^{-1} is CH_2 stretching (He and Yang, 2013; Mohanapriya *et al.,* 2016). The peaks between 1239 cm^{-1} and 1530 cm^{-1} might probably be C-N and N=O bonds which is the characteristic for amine and nitro groups (Nagajyothi *et al.,* 2013). The images from transmission electron microscope, obtained in the range of 38-43 nm, reveal that the nanoparticles did not aggregate. Also, the nanoparticles were mostly of spherical structure with a few triangular pyramids. This depicts the dispersive nature of the AuNPs synthesized from actinobacteria. Studies by Menon *et al*. (2017) and Firdhouse and Lalitha (2022), also mentioned similar results.

AuNPs synthesized from strain PYA9 showed excellent potential against bacterial pathogens. The activity was comparable with that obtained from gentamycin control. Moreover, as reported from previous investigations (Składanowski *et al.,* 2017; Balagurunathan *et al*., 2011) we tested the potential of actinobacterial strain PYA9 against the bacterial pathogens. Even though direct agar plug and cell-free supernatant of PYA9 displayed activity against the pathogens, it was minimal when compared with the zones of inhibition obtained by PYA9 synthesized AuNPs. Study by Balagurunathan *et al*. (2011) showed antibacterial zones of 20 and 14 mm against *E. coli* and *S. aureus* while our study reports antibacterial zones

of 16 mm against *Salmonella typhi*, 17 mm against *Enterococcus faecalis*, 18 mm against *Staphylococcus aureus* and 18 mm against *Escherichia coli*. Biofilm forming bacteria causes biofouling in marine environment which negatively impacts the ecology and harms the marine biodiversity. Two strains of *Staphylococcus* namely KB6 and KB7 was previously isolated from Kovalam, Chennai and tested for their fouling property at the Centre for Drug Discovery and Development. These strains were used to test the potential of PYA9 synthesized AuNPs. Similar to the antibacterial study, we compared the efficacy of PYA9 agar, its cell-free supernatant and the biosynthesized AuNPs. With no zone observed on agar plug, minimal zone was observed on cell-free supernatant while 13 mm zone of inhibition was observed on PYA9 synthesized AuNPs. This suggests the use of marine actinobacteria synthesized AuNPs as potential candidates against bacterial and biofouling pathogens. Other studies by Shanmugasundaram *et al*. (2013) and Kabiri *et al.* (2022) also confirm the use of actinobacteria mediated AuNPs against biofilm forming bacterial pathogens. Moderate scavenging activity was observed in the DPPH assay to test the antioxidant activity of biosynthesized AuNPs. Percentage inhibition increased with increasing concentration. While 25 µg/ml showed 45% inhibition, 100 µg/ml showed ~52% inhibition. This increase in scavenging activity on a dose-dependent manner was also reported by El-Batal *et al*. (2015).

As suggested by the cultural and micromorphology, the strain PYA9 confirmed to be of genus *Streptomyces* in the 16S rRNA sequence results. The phylogeny showed similarity of the strain PYA9 with *Streptomyces misionensis* sp. strain SA20. Neighbor-joining method was used to construct a phylogentic tree of actinobacterial strain PYA9 with its close relatives including genera *Streptomyces eurythermus*, *Streptomyces thermocarboxydus* and *Streptomyces viridochromogenes*. To the best of our knowledge, this is the first report on synthesis of gold nanoparticles from *Streptomyces misionensis*. Nanoparticle synthesis by biological means prove to be an economical, efficient, highly productive, eco-friendly and a simple process gaining ease in scale-up. Significant purity was achieved in this study involving gold nanoparticles synthesis by actinobacterial strain, *Streptomyces misionensis* sp. strain PYA9. The biosynthesized AuNPs prove to be effective against bacterial and bio-film forming pathogens. Along with their antioxidant activity, the characterized PYA9-AuNPs could be employed as a potential candidate for diverse biomedical and environmental applications.

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