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Synthesis of Gold Nanoparticles using *Endophytic streptomyces* and its Bactericidal Activities against Enteric Bacterial Pathogens

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The present study aimed to report the rapid and one-step synthesis of gold nanoparticles (AuNPs) using the cell-free supernatant (CFS) of an endophytic *Streptomyces* sp. BDUSMP S05 isolated from *Solanum trilobatum*. The synthesized AuNPs were characterized by using UV-visible spectroscopy (UV-vis), Fourier-transform infrared spectroscopy (FTIR), energy dispersive X-ray (EDAX) spectroscopy and X-ray diffraction (XRD). The UV-visible spectroscopy (UV-vis) absorption of colloidal AuNPs displayed a surface plasma resonance (SPR) centred at 533 nm. FESEM micrograph analysis showed that synthesized particles were predominantly spherical with a 20-30 nm size range. Dynamic light scattering (DLS) analysis showed that the average particle size varied between 2-28 nm. Zeta potential analysis indicated the negative value of -35.2 mV characteristic for stable AuNPs. The synthesized AuNPs displayed significant antimicrobial activity against Gram-negative bacteria, including *Eescherichia coli*, *Salmonella typhi* and *Vibrio cholerae* compared to CFS alone. The results revealed that endophytic *Streptomyces* sp. potential to be used for the synthesis of antibacterial gold nanobased materials.

Keywords: Endophytic *Streptomyces*, Extracellular biosynthesis, Gold nanoparticles, Antibacterial activity.

INTRODUCTION

Perhaps the most worrisome event in this technological world is the global spread of antibiotic resistance in health care sectors and the community alike. Multiple drug-resistant (MDR) bacterial infections globally attribute a serious health concern to both humans and animals. Antibiotic resistance infections pose difficulties to treat and, in most cases, lead to increased mortality. Therefore, therapeutic agents that should override the existing resistance mechanisms is essential. In this context, nanotechnology has received considerable interest as new materials with the potential to develop new drugs. Recent evidences show that there have been gaining attention in studies of metal nanoparticles, biosynthesis considers a safe and environmentally friendly approach rather than the chemical method [1]. In recent years, several studies have demonstrated that the potential applications of gold nanoparticles there, such as antibacterial [2], antimalarial [3], larvicidal [4], anticancer [5], catalytic [6] and optoelectronic applications [7]. It has also been reported that gold nanoparticles are non-toxic and photostable and have a high surface volume to ratio [8,9].

Due to various applications, it is desirable to synthesize gold nanoparticles (AuNPs) at a low cost and small size for better efficiency. Most of the studies reported many microorganisms, including Gram-negative and Gram-positive bacteria, have been used as biomaterials to synthesize AuNPs [3,10,11]. Endophytic microorganisms are live inside plant tissues and do not harm to host [12]. Recently, endophytic *Streptomyces* have been attracting attention in the search for novel bioactive compounds. However, so far, *Streptomyces* isolated from plants for the synthesis of metal nanoparticles are minimal. *Solanum trilobatum* (Thuthuvalai) is known for its curative effect on respiratory ailments of cough, cold and digestive disorders in traditional Siddha medicine.

South Asian countries are known to have endemic infections of *Salmonella typhi* [13,14]. Recently Mukhopadhyay *et al.* [15] reported that the emerging *Vibrio cholerae* is a significant contribution to the epidemiological disease of cholera in Asian and African countries. Thus, the objectives of the present study were to isolate and characterize the endophytic *Streptomyces* sp. BDUSMP S05 from *S. trilobatum* and utilized for the synthesis of AuNPs by extracellular method. The prepared AuNPs were characterized with UV–visible spectroscopy, FTIR, EDAX, XRD, FE- SEM, DLS and zeta potential. Efforts were also made to identify the small molecular weight proteins in the cell-free supernatant using SDS-PAGE and finally determined the antibacterial activity against *Escherichia coli*, *Salmonella typhi* and *Vibrio cholerae* causing enteric diseases in humans.

EXPERIMENTAL

Bacterial strains: Lyophilized cultures of *Escherichia coli* (MTCC 1687), *Salmonella typhi* (MTCC 3231) and *Vibrio cholerae* (MTCC 3906) were obtained from Microbial Type Culture Collection, IMTECH, India. All the test pathogens were cultured at 37 ºC in Luria broth or on Luria agar.

Collection of plant material and isolation of endophytic actinomycetes: Healthy plant (stem, leaf and root parts) of *S.trilobatum* were collected in clean plastic bags from foothills of Kolli Malai Mountains, Tamil Nadu, India (Latitude: 10º12′ -11º7′ N, Longitude: 76º-77º17′ E), authenticated by the botanist, Jamal Mohamed College, Tiruchirappalli, India and finally stored at 4 ºC. Samples were processed within 24 h of collection. Surface sterilization of plant stems was performed as described by a previous method [16]. Surface sterilized stem fragments were placed on humic acid-vitamin B agar (HV) medium [17] supplemented with antibiotics cycloheximide (25 µg/mL), nystatin (25 µg/mL) and nalidixic acid (20 µg/mL) to inhibit fungal and non-filamentous bacterial growth. The inoculated plates were then incubated at 28 ºC for three weeks. Actinomycetes colonies were generally observed with features including a tough leathery texture, dry or folded appearance, branching filaments with or without aerial mycelia and presence of any spores [18]. Pure cultures were obtained by repeated streaking on ISP4 media (HI MEDIA Labs, India). The effectiveness of surface sterilization was validated according to Goudjal *et al*. [19] to ensure that the isolates were endophytic.

Identification of endophytic actinomycetes: Morphological characterization was done by the method described by Shirling & Gottlieb [20]. Visual observation of general morphology was performed by a light microscope. The ornamentation of the spore surface was analyzed by scanning electron microscopy (SEM model, JEOL-JSM 6390, Japan). The carbon and nitrogen sources was determined according to Miller [21]. Colonial features of aerial spore mass colour, substrate mycelial pigmentation and the colour of diffusible pigments were recorded on different media, including ISP2, ISP3, ISP4, ISP5, ISP6 and ISP7. All plates were incubated at 28 ºC for 14 days. Diaminopimelic acid (DAP) was determined as described elsewhere [22]. The genomic DNA of the strain was isolated according to the method described by Hopwood *et al*. [23]. The 16S rRNA gene of the isolate was amplified by PCR using a pair of universal 27f 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492 r 5-GGTTACCTTGTTACGACTT-3' primers. PCR amplifications were conducted on a thermal cycler (Eppendorf, Germany). PCR products were purified from 0.8 % agarose gel (w/v) and sequenced on an ABI 310 automatic DNA sequ-encer (Applied Biosystems). BLAST network services at the NCBI were used to analyze the resulting 16S rRNA gene sequence [24].

Multiple alignments were performed using the CLUSTAL_X version 1.8 [25]. The phylogenetic tree was inferred Neighbor-joining method with the MEGA 6.0 software package [26,27]. The unrooted phylogenetic tree topology was evaluated using the bootstrap resampling method with 1000 replicates [28].

***Streptomyces* cell free supernatant preparation and synthesis of AuNPs:** The organism was grown in a modified ISP2 medium for eight days at 28 ºC and agitated at 220 rpm. Cell-free supernatant of the isolate was obtained by centrifugation of mycelia mass at 4 ºC, 10,000 rpm for 15 min and filtered through 0.2 µm polyethersulfone (PES) syringe filters (Sartorius Minisart® high flow Syringe Filters). The least concentration (5% CFS) was assessed for the synthesis of AuNPs. For the synthesis of AuNPs, 1 mM final concentration of $HAuCl_4$ (aq.) was blended with 5 mL of CFS and incubated at 30 ºC in a dark condition for four days. The reaction mixture was adjusted to neutral pH.

Characterization of AuNPs: Preliminary characterization of AuNPs in the reaction mixture was done through visual observation for change of colour. UV-vis sp.ectroscopy (JASCO-V650) was used to analyze the reduction of Au^{3+} to Au^0 at a wavelength range between 300 and 700 nm. The spectra were recorded at 24 h and 96 h. FTIR spectra were measured on a spectrum RX 1-one instrument in the diffuse reflectance mode at a resolution of 4 cm^{-1} in KBr pellets and the wavelength in the range of 4000-400 cm^{-1}. The surface morphology of synthesized AuNPs and elemental composition were analyzed in a field emission scanning electron microscope (FESEM-ZEISS) equipped with an EDAX. The energy dispersive spectrometer spectrum was measured at 20 kV accelerating voltage. The potential of AuNPs was carried out using Zetasizer Nano-ZS (Malvern, UK). XRD pattern was recorded for AuNPs on X-ray diffractometer (XRD-600, Shimadzu, Japan) instrument operating at a voltage of 40 kV and current of 30 mA with Cu$K\alpha$ radiation to determine the crystalline phase. The DLS analysis was performed on Malvern Zetasizer Nano series compact scattering spectrometer (Malvern Instruments Ltd., Malvern, UK)

Antibacterial activity of AuNPs: Antimicrobial activity was evaluated for synthesized AuNPs and CFS against Gram-negative *Escherichia coli* MTCC 1687, *Salmonella typhi* MTCC 3231 and *Vibrio cholerae* MTCC 3906 on Luria-Bertani (LB) agar plate. Sterile standard antibiotic discs with a diameter of 6 mm were purchased from HIMEDIA Laboratories, India. Test samples of about 60 µL were loaded on the sterile disc and air-dried thoroughly before being put on the agar plate. CFS loaded disc was taken as a positive control. LB agar was sp.read plated about 10^6 CFU/mL of test pathogens, impre-

gnated with the sample loaded disks incubated at 37 ºC for overnight. The diffusion method was adapted to determine the zone of inhibition (ZOI), which indicates the growth inhibition of the tested pathogens [29]. All experiments were conducted in triplicate for each set of conditions. The data were presented as mean ± S.D. of three independent experiments using the SPSS Statistics version 20.0.

RESULTS AND DISCUSSION

Isolation and characterization of endophytic actinomycete strain: Actinomycetes were isolated from stem parts based on their typical features including filamentous growth, spore, convex and margin appearance on humic acid-vitamin agar (HV) medium [18]. Isolate treated with sterilizing solutions failed to grow on HV agar medium. Besides, surface sterilized wash solutions produced no microbial growth on the same medium [19]. The results suggest that the isolate was belonging to endophytic, not epiphytic. Light microscopy showed a branched aerial and substrate mycelium (Fig. 1a). Scanning electron microscopy revealed that the isolate forms a long chain of spores with smooth surfaces (Fig. 1b). The results of the morphological, physiological and biochemical characteristics of strain BDUSMP S05 are shown in Table-1. The cell wall of the strain found to contain LL-diaminopimelic acid (chemotype I), which is characteristic for the genus *Streptomyces* (data not shown) [30]. Phylogenetic analysis of the 16S rRNA gene sequence (1385 bp) of strain BDUSMP S05 revealed that the isolate belongs to the genus *Streptomyces* (Fig. 1c). The DNA sequence of the isolate has been deposited in the GenBank database under Accession No. KF918269. Based on morphological, physiological, biochemical characterization and 16S rDNA sequence analysis the strain was named as *Streptomyces* sp. BDUSMP S05.

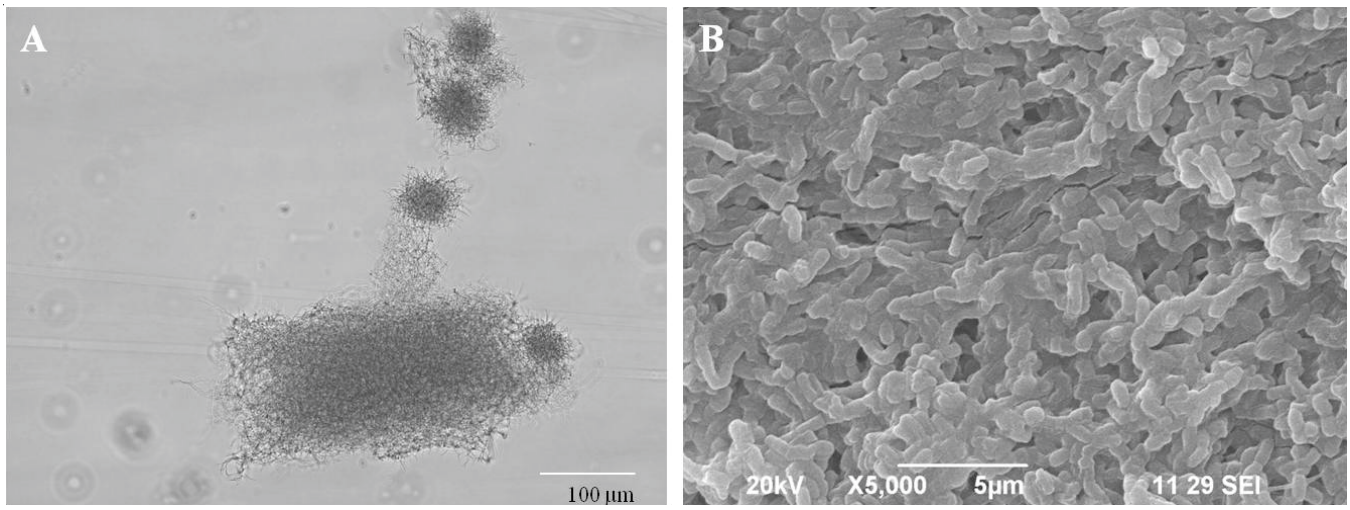

Fig. 1. (a) Morphology of filamentous *Streptomyces* sp. BDUSMP S05 in shake flask cultures; (b) Scanning electron micrograph of spore of *Streptomyces* sp. BDUSMP S05 strain grown on ISP4 media for days at 28 °C. Bar: 5 µm

TABLE-1
CULTURAL, PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS FEATURES OF THE ISOLATED *Streptomyces sp.* BDUSMP S05 FROM *S. trilobatum*

Features	*Streptomyces sp.* BDUSMP S05					
	ISP2	ISP3	ISP4	ISP5	ISP6	ISP7
Growth	Good	Poor	Good	Moderate	Good	Moderate
Sporulation	Poor	Poor	Good	Moderate	Good	Moderate
Colour of aerial mycelium	W	Y	W/B	Y/B	W	Light Brown
Colour of substrate mycelium	LY	LY	Y	Y/B	Y	LY
Diffusible pigment	–	–	–	–	G/Y	–

Biochemical		**Utilization of carbon**		**Growth on sole nitrogen source**	
Gram staining	Positive	Arabinose	++	Alanine	–
Citrate utilization	Negative	Dextrose	+++	Arginine	++
Methyl red	Negative	Fructose	++	Asparagine	++
Voges-Proskauer	Negative	Inosittol	–	Cysteine	–
H_2S production	Negative	Lactose	+	Methionine	–
Nitrate reduction test	Positive	Mannitol	+++	Phenyl alanine	–
Catalase test	Negative	Maltose	+		
Urea hydrolysis	Positive	Sucrose	+		
Gelatin hydrolysis	Negative	Xylose	–		
Starch hydrolysis	Negative				

+++: Strong positive, utilized; ++: Positive, utilized; (+): Weakly positive, utilized; –: Negative, not utilized. W: White; Y: Yellow; W/B: Whitish Brown; Y/B: Yellowish Brown, LY: Light yellow.

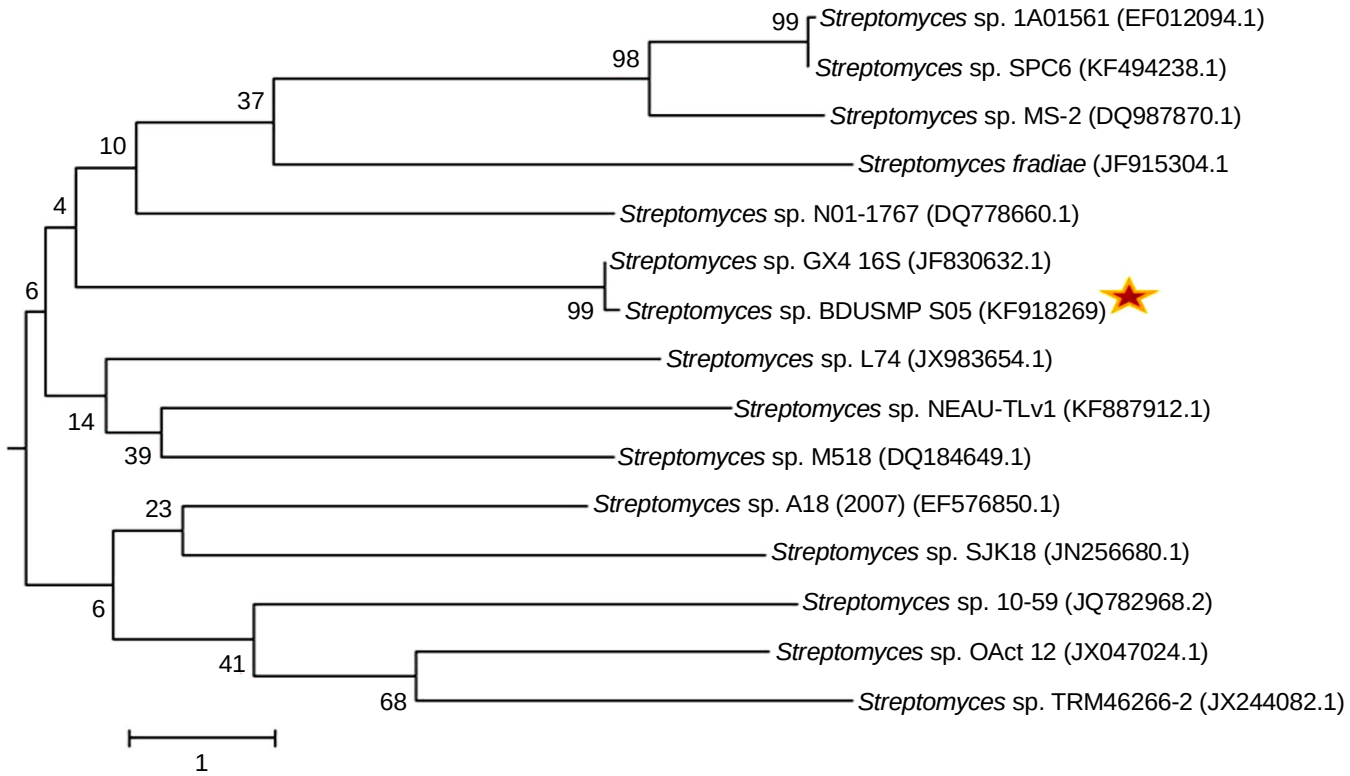

Fig. 1. (c) The phylogenetic tree of *Streptomyces* sp. BDUSMP S05 (KF918269) was constructed using the neighbour-joining method with aid of MEGA6.0 program. The Bootstrap values above 50%, presented as percentages of 1000 replications, are shown at the branch points. Bar 1 substitutions per nucleotide position

Endophytic *Streptomyces* mediated synthesis of AuNPs: In searching novel bioactive compounds from medicinal plant associated *Streptomyces* was screened them for the production of gold nanoparticles by the extracellular method. *Streptomyces* sp. BDUSMP S05 from a healthy stem was isolated and identified to be a potential strain capable of AuNPs production. The *Streptomyces* sp. BDUSMP S05 with the ability to synthesize AuNPs is the first report of gold nanoparticle producing endophytic *Streptomyces* as an antimicrobial agent against enteric bacteria. A reaction mixture of 5% CFS and $HAuCl_4$ changed from light yellow to purple, indicating the formation of AuNPs (Fig. 2a). Similar observations for colour change in the synthesis of AuNPs were also found by Aishwarya *et al.* [4]. The colour change in the reaction mixture was probably due to the surface plasmon resonance (SPR) of the synthesized gold nanoparticles [31].

On the other hand, no colour change was observed in control experiments that contained aqueous $HAuCl_4$ without CFS (Fig. 2a). The exact mechanism of biosynthesis of metal nanoparticles by microorganisms remains elusive. However, there are reports on the possible mechanism behind the formation of nanoparticles by microbes recently proposed. There may be involvement of nitrate reductases [32], NADPH-dependent reductase through electron shuttle enzymatic metal reduction process [33], small molecular weight proteins [34], and secondary metabolites [35] in the formation of metal nanoparticles by microorganisms have been reported.

Characterization of biosynthesized AuNPs: The reaction mixture containing CFS and $HAuCl_4$ was incubated at 30 ºC for four days. The UV-vis spectra were recorded at two different time intervals in the reaction mixture to study the kinetics of the bioreduction process. The UV spectra at 6 h displayed a peak around 533 nm (Fig. 2b), indicating the surface plasmon resonance (SPR) characteristic for AuNPs [31]. There was an increase in the absorption peak with time around 533 nm with no shift after 4 days of incubation in the reaction mixture. The narrow peak could be attributed to a similar size and dispersed AuNPs in the reaction mixture [36]. FTIR spectrum of AuNPs synthesized by CFS of *Streptomyces* sp. BDUSMPS05 shows intense bands at 3467, 3433, 3406, 2080, 1637, 1483, 1402, 1125, 695 and 652 cm^{-1} (Fig. 2c). The intense bands at 3467, 3433 and 3406 cm^{-1} may correspond to free N-H stretching vibration [37]. The band at 1637 cm^{-1} may be assigned to the amide I bond's -C=C- vibrations [38]. The absorption peak located at 1402 cm^{-1} may be attributed to the C-C stretching of the aromatic ring of proteins. The absorption band at 695 and 652 cm^{-1} could correspond to the bending vibration of N-H groups in the proteins [39]. FTIR analysis in this investigation indicating AuNPs was probably capped by proteins secreted by the *Streptomyces* BDUSMP S05. Thus, the result of this investigation is corroborating with the findings of Narayanan & Sakthivel [40], who proposed proteins may have involved in the capping and stabilization of the AuNPs. Similar results have also been reported by Grilal *et al.* [41], who used *Geobacillus stearothermophilus* for the biosynthesis of AuNPs. Fig. 3a shows the results of the EDAX analysis of AuNPs. The EDAX analysis displayed a strong peak at 2.0 keV and other peaks at 8.5, 9.5 and 11.5 keV, respectively, confirmed the presence of the metallic gold crystals [42].

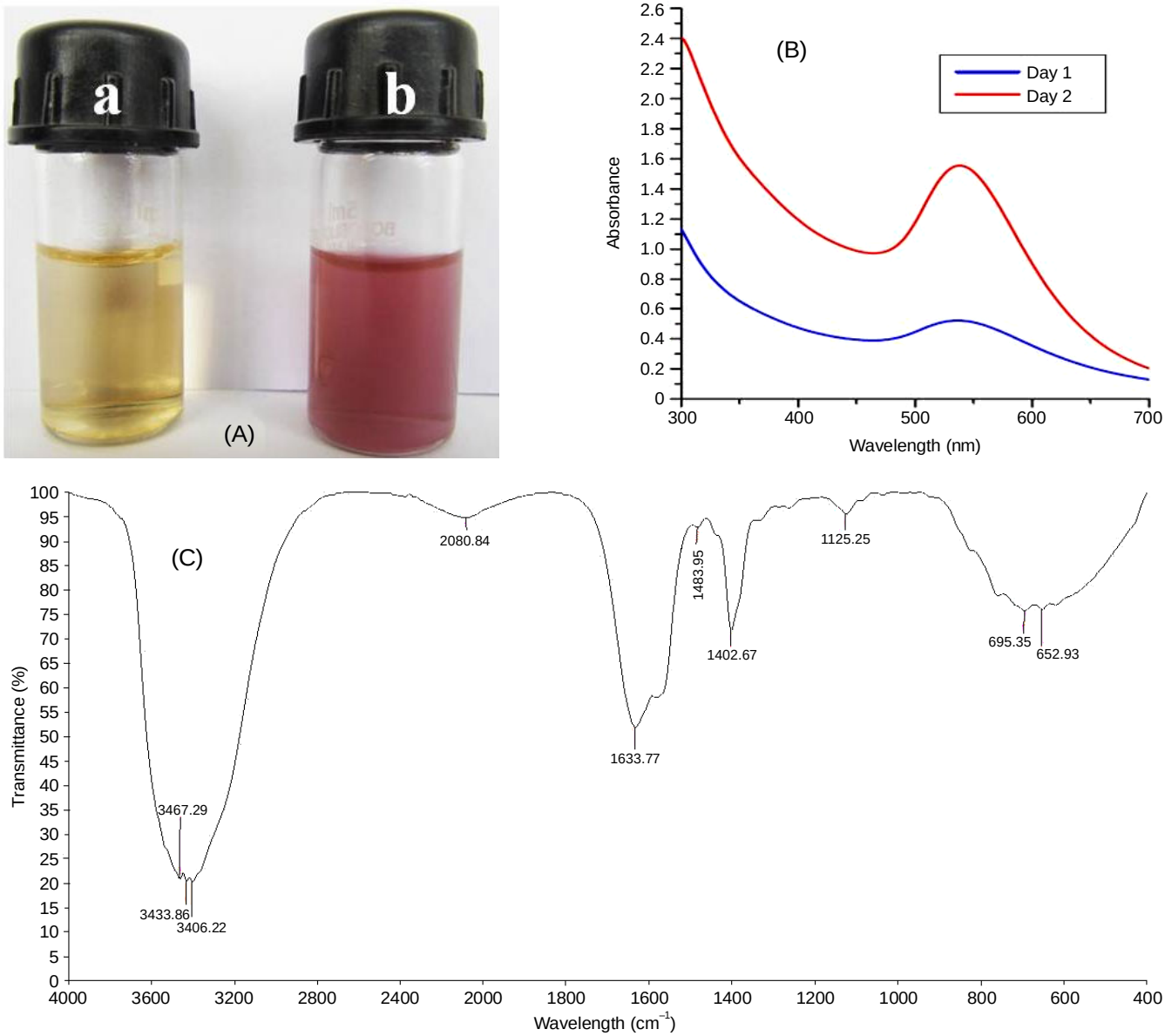

Fig. 2. (A) Color change profile of biosynthesised AuNPs. (a) control (No reductant); (b) 1 mM auric chloride and 5% cell free extract (contain reductant) (B) UV-vis spectra of biosynthesized AuNPs; (C) FT-IR spectrum of biosynthesized AuNPs using CFS (5%) of *Streptomyces* sp. BDUSMP S05

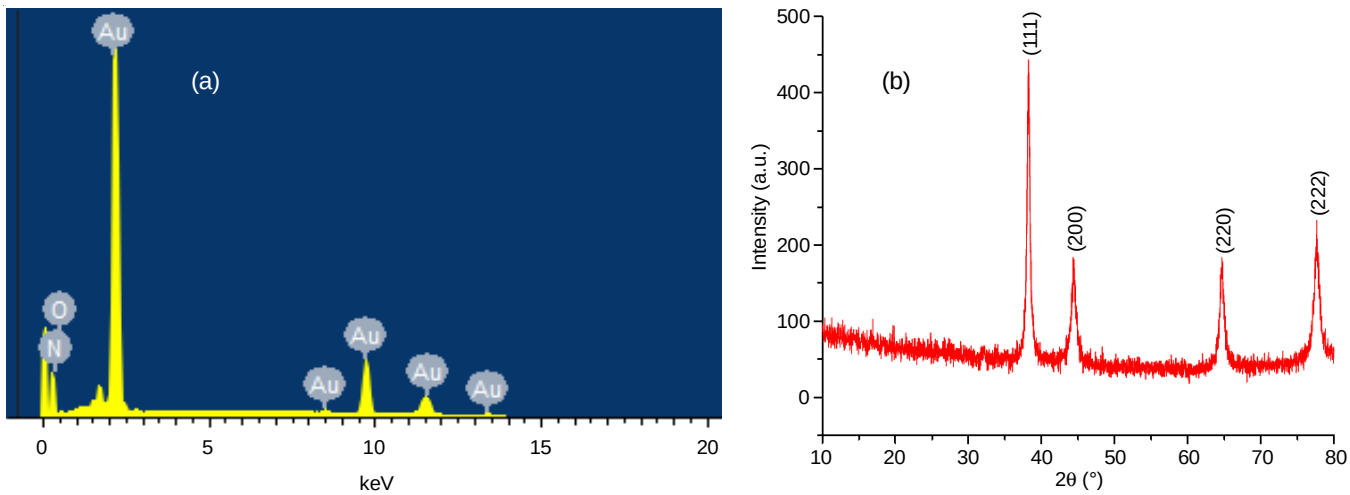

Fig. 3. (a) EDAX, (b) XRD analysis of biosynthesized AuNPs using CFS (5%) of *Streptomyces* sp. BDUSMP S05

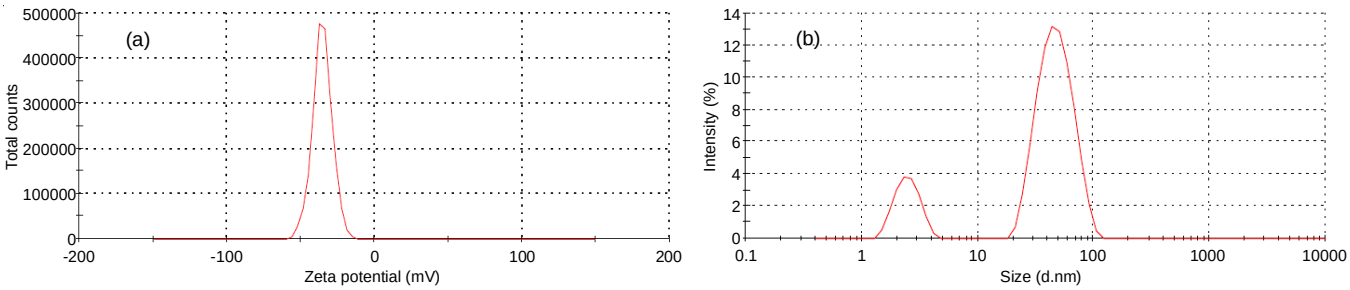

Fig. 4. (a) Zeta potential (b) DLS of AuNPs synthesized using CFS (5%) of *Streptomyces* sp. BUDSMP S05

The dynamic light scattering (DLS) analysis showed that the average particle size varied between 2-48 nm (Fig. 4a). The negative zeta potential value of –35.2 mV (Fig. 4b) suggesting the negatively charged AuNPs. Eby *et al*. [43] reported that the value of zeta (ζ) potential at ± 30 mV as theoretical limit or greater than ± 30 mV attributed to the stability of nanoparticles in the colloidal suspension. Similarly, the results indicate that more stable AuNPs with no aggregation are due to electrostatic repulsive forces [44]. Fig. 5 presents the FESEM image of gold nanoparticles. The FESEM micrograph showed that the AuNPs were predominantly spherical with a size range of 27-28 nm. According to Srivastava & Mukhopadhyay [45], the FESEM image in this investigation showed a grape bunch-like structure attributed to AuNPs. The XRD analysis showed clear peaks at 38.2 (111), 44.3 (200), 64.6 (220) and 77.6 (222) (Fig. 3b). These peaks agreed with the face-centred cubic phase of AuNPs (JCPDS card No. 04-0784). Thus, the XRD analysis proved the presence of nanocrystalline gold particles in favour of the UV-vis spectra, EDAX and FESEM analysis.

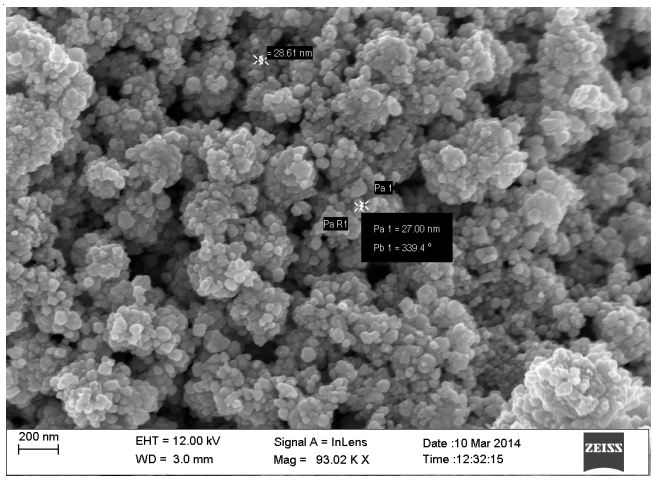

Fig. 5. FESEM observation of biosynthesized AuNPs using CFS (5%) of *Streptomyces* sp. BUDSMP S05 at a scale bar of 200 nm

Antibacterial activity of AuNPs: In this investigation, biosynthesized AuNPs was used to evaluate the antibacterial activity against three different gram-negative enteric pathogens of *Escherichia coli* (MTCC 1687), *Salmonella typhi* (MTCC 3231) and *Vibrio cholerae* (MTCC 3906) using the disc diffusion method. The AuNPs synthesized from CFS (5%) displayed a significant antibacterial activity as compared to CFS. The comparative analysis of the overall zone of inhibition indicates a percentage increase of 27.98 % between the AuNPs and CFS (Table-2). It should be noted that CFS (5%) did not show any clear visible zones of inhibition at the same concentration used for synthesis. The result suggests that the AuNPs could act as a vehicle to conjugate the secondary metabolites from CFS to penetrate the bacterial membrane and cause better antibacterial activity. The results were similar to those reported by Burygin *et al*. [46], they found that antibiotics conjugated with AuNPs showed the significant antibacterial activity. The antimicrobial activity of AuNPs could be due to the disruption of the membrane, binding with DNA whereby inhibit the uncoiling and transcription of DNA [7], inhibiting the binding of tRNA to the subunit of the ribosome [7] and high surface area to interact with bacterial membrane [47].

Conclusion

A potentially safe, eco-friendly and cost-effective methodology is reported to synthesize protein capped, uniform size gold nanoparticles using an endophytic *Streptomyces* sp. BDUSMP S05. The synthesized AuNPs were characterized using Uv-vis spectroscopy, FTIR, EDAX, DLS, zeta potential, FESEM and XRD. Antibacterial activity of fabricated AuNPs was evaluated against gram-negative enteric pathogens. The biosynthesized gold nanoparticles were appeared to be predominantly spherical morphology with an average size of 2-28 nm. The results showed significant antibacterial activity against the tested bacterial pathogens. Present study suggests that non-pathogenic industrially relevant endophytic *Streptomyces* sp. may be a potent strain to produce new gold nano-

TABLE-2
SENSITIVITIES OF GRAM-NEGATIVE ENTERIC PATHOGENS TO GOLD NANOPARTICLES

Organism	AuNPs	Coefficient of mean deviation	Increase in activity (%)
Escherichia coli MTCC 1687	17.06 ± 0.31	0.0966	30.00
Vibrio cholerea MTCC 3906	16.07 ± 0.17	0.0111	23.00
Salmonella typhi MTCC 3231	16.97 ± 0.25	0.0111	30.94

Mean zone of inhibition (mm) of AuNPs synthesized using cell-free supernatant of *Streptomyces* sp. BDUSMP S05 (5%) against three different pathogens. The disc diameter was 6 mm. The average percentage increase in antibacterial activity among the three tested pathogens was 27.98%. The coefficient of mean deviation was calculated by dividing the mean deviation using the average.

materials for antimicrobial applications against enteric bacterial pathogens.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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