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Mangrove *Streptomyces* sp. BDUKAS10 as nanofactory for fabrication of bactericidal silver nanoparticles

Periyasamy Sivalingam[a], Jacob Joe Antony[b], Durairaj Siva[b], Shanmugam Achiraman[b], Kumarasamy Anbarasu[a,*]

[a] Department of Marine Biotechnology, Bharathidasan University, Tiruchirappalli 24, Tamil Nadu, India
[b] Department of Environmental Biotechnology, Bharathidasan University, Tiruchirappalli 24, Tamil Nadu, India

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ABSTRACT

Biosynthesis has led to the development of various biomimetic approaches for the fabrication of nanoscale materials. The present study reveals a unique procedure for the biosynthesis of bactericidal silver nanoparticles (AgNPs) using a novel *Streptomyces* sp. BDUKAS10, an isolate of mangrove sediment. Aqueous silver nitrate ($AgNO_3$) solution was treated with cell free supernatant (CFS) of the isolate to synthesize bactericidal silver nanoparticles. Initial characterization was performed by visual observation for color change to intense brown color. UV–visible spectrophotometry (UV–vis) for measuring surface plasmon resonance indicated a maximum absorption peak at 441 nm. Fourier Transform Infrared Spectroscopy (FTIR) analysis provides evidence for proteins as possible reducing, and capping agents. Energy dispersive X-ray (EDAX) spectroscopy analysis showed elemental silver as major signal. Transmission Electron Microscopy (TEM) study indicated spherical silver nanoparticles in the size range of 21–48 nm. Compared to the CFS, the biosynthesized AgNPs exemplified superior bactericidal efficacy towards the tested bacterial strains. Results from this study suggested that *Streptomyces* sp. BDUKAS10 can be advantageous for the synthesis of AgNPs by extracellular method in the view of sustainable and ecofriendly approach.

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1. Introduction

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, immunosuppressors and many other biologically active compounds [1]. Exploitation of *Streptomyces* in nanotechnology has recently received considerable attention [2,3]. Nanotechnology holds promising application in biosensing, drug delivery and cancer therapy [4–6]. The expensive and extensive use of toxic solvents and hazardous reducing agents in chemical procedures to synthesize nanoparticles has augmented the necessity in view of eco-friendly and green chemistry approach. Hence, a well established non-toxic and eco-friendly potent methodology for the synthesis of nanoparticles has mounted to a level of supreme importance [7]. 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 nanofactories [8–12]. Emerging multidrug resistant (MDR) bacteria has raised a demand for the urgent need to identify novel antimicrobial agents. It was reported that silver had been used as antimicrobial agents since ancient times [2]. With the advancements in nanotechnology, AgNPs have found its significant applications as antimicrobial agents, in fields of microelectronics, catalysis and biomolecular detection [13–16]. Albeit the antibacterial activity of AgNPs has been proved in the recent years, the actual mechanism of action is not yet clear. They may inactivate microorganisms by interacting with their enzymes, proteins or DNA to inhibit cell proliferation [17]. It is also evident that the increased antimicrobial activity of AgNPs may be attributed to its special characteristics of small size and high surface area to volume ratio [18]. The advantage of adapting biosynthesis of AgNPs is the simplicity of extracellular synthesis and downstream processing [19,20]. Mangrove ecosystems are highly productive than other habitats including diverse groups of microbial communities situated in the intertidal coastal regions of marine environment [21]. Hence, considering the importance of *Streptomyces* in pharma industries, in the present study, *Streptomyces* sp. BDUKAS10 was isolated from mangrove ecosystem, characterized and utilized as a nanofactory for the biosynthesis of AgNPs. UV–visible spectroscopy, FTIR, EDAX and TEM were employed to characterize the AgNPs. The

* Corresponding author. Tel.: +91 431 2407071x436; fax: +91 431 2407045.
E-mail address: anbubdu@gmail.com (K. Anbarasu).

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comparative efficacy of antimicrobial activity of CFS and CFS mediated AgNPs was evaluated against gram positive and gram negative test strains.

2. Material and methods

2.1. Collection of soil samples and isolation of actinomycetes

Soil samples from mangrove sediment of Pitchavaram (latitude of 11.4′N–longitude of 79.8′E), Tamil Nadu, India were collected in sterile airlock polythene bags and stored at 4 °C. Selective pre-treatment was performed to increase the number of mycelium-forming actinomycetes relative to the non-actinomycetal heterotrophic microbial flora and inoculated on humic acid-vitamin agar (HV) medium [22,23] supplemented with antibiotics such as cycloheximide (40 μg/μl), nystatin (30 μg/μl) and nalidixic acid (10 μg/μl). The plates were incubated at 30 °C until the appearance of colonies with a tough leathery texture, dry or folded appearance, and branching filaments with or without aerial mycelia [24]. Pure colonies were isolated and subcultures were carried out by streaking the particular isolate directly on ISP4 agar media (HIMEDIA Labs, India).

2.2. Characterization and culture conditions

Morphological and biochemical characterization of the isolate was performed by following the method of Shirling and Gottlieb [25]. Morphology was studied in both bright field and scanning electron microscopy (SEM model, JEOL-JSM 6390, Japan) [26]. Utilization of various carbon sources was assessed on a minimal media containing M9 salts [27]. Cultural characteristics were carried out at 28 °C by methods followed by International Streptomyces project (ISP) [25]. Assessment of color pattern was done according to color chips using the ISCC-NBS Color Charts Standard No. 2106 [28]. Diaminopimelic acid in the cell wall was analyzed using a previously described method [29]. The 16S ribosomal DNA gene was amplified by PCR using the universal primer pair 533F 5′-GTGCCAGCMGCCGCGGTAA-3′ and 1492R 5′-GGTTACCTTGTTACGACTT-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 Eurofins Genomics India Pvt. Ltd., India. DNA sequence analysis was then performed by BLAST network services at the NCBI. The 16S rRNA gene sequences of the strain BDUKAS10 was aligned with reference sequences obtained from GenBank using ClustaL X 2.0.11 [30]. Phylogenetic tree was generated using the neighbor-joining method with MEGA 5 package [31,32]. The evolutionary distance matrix was derived with Jukes and Cantor model [33]. Topology of phylogenetic tree was evaluated by bootstrap analysis based on 1000 replicates [34]. The isolate BDUKAS10 was further cultured in modified ISP2 medium (pH 6.8) and grown for 80 h at 28 °C in an orbital shaker at 220 rpm. CFS was obtained by centrifugation at 4 °C, 10,000 rpm for 15 min.

2.3. Microbial synthesis of AgNPs and characterization

For the bioreduction process to occur, 95 ml of 1 mM $AgNO_3$ (Qualigens – 99.8%) was added to 5 ml CFS and incubated at 32 °C in dark for 36 h. Followed by initial observation of color change in the bioreduction process, UV–visible spectrometric measurements were performed on Hitachi double beam equipment (Model Lambda 35) in the 200–600 nm range. FTIR analysis of microbiologically synthesized AgNPs was performed on a Spectrum RX 1-One instrument in the diffuse reflectance mode at a resolution of 4 cm^{-1} in KBr pellets and the spectra were recorded in the wavelength interval of 4000 and 400 nm^{-1}. TEM measurements were carried out on a Tecnai 10 instrument operated at an accelerating voltage of 120 keV to determine the AgNPs size. Energy dispersive X-ray (EDAX) spectroscopy analysis was carried out with the SEM instrument equipped with an EDAX detector operated at an accelerating voltage of 20 keV to perform elemental analysis.

Table 1
The biochemical characteristics and carbon source utilization features of the isolated strain *Streptomyces* sp. BDUKAS10 from Pitchavaram mangroves forest.

Characteristic features	*Streptomyces* sp. BDUKAS10
Biochemical	
Gram staining	Positive
Citrate utilization	Negative
Urea hydrolysis	Positive
Gelatin hydrolysis	Negative
Starch hydrolysis	Positive
Methyl Red	Negative
Voges-Proskauer	Negative
H_2S production negative	
Nitrate reduction test	Positive
Catalase test	Positive
Utilization of carbon	
Arabinose	−
Cellulose	(+)
Dextrose	+++
Fructose	+++
Galactose	++
Lactose	++
Mannitol	−
Sucrose	(+)

+++: strong positive, utilized; ++: positive, utilized; (+): weakly positive, utilized; −: negative, not utilized.

2.4. Antimicrobial activity of AgNPs

Antimicrobial activity was tested for microbiologically synthesized AgNPs and CFS against bacterial pathogens of gram-negative (*Pseudomonas aeruginosa*, MTCC 1688), and gram-positive (*Bacillus cereus*, MTCC 1272 and *Staphylococcus aureus*, MTCC 96) origin adapting disc diffusion method on Luria Bertani (LB) agar plates. The growth inhibition of bacterial pathogens was assessed by the corresponding zone of inhibition (ZoI) [35]. Sterile standard antibiotic disks with diameter of 6 mm were purchased from HIMEDIA Laboratories, India. Pure cultures of bacteria were grown in LB broth (HIMEDIA, India) at 37 °C in an incubator shaker at 160 rpm. 50 μl of test samples were loaded on the disc, air dried completely and 5% CFS loaded disc was taken as positive control. LB agar was spread plated with 10^6 CFU/ml of bacterial cultures, impregnated with the sample loaded disks and incubated at 37 °C for 18 h. ZoI was measured using a vernier calliper.

3. Results and discussion

3.1. Isolation and characterization of actinomycete strain

After 10 days of incubation at 28 °C, pure colonies were isolated on HV agar and subcultured on ISP4 agar medium. Aerial and substrate mycelia of the isolate were examined under bright field microscopy (data not shown). Outcomes of the biochemical characterization and carbon source utilization tests were as summarized in Table 1 [25,27]. SEM images indicated that the isolate possessed substrate mycelia and extensively branched aerial hyphae that further differentiated into smooth surfaced spores (Fig. 1). The strain exhibited superior growth on ISP 2, 3, 4, 7, moderate growth on ISP 5, and poor growth on ISP 6 (data not shown). Diffusible pigment or melanin on any of the tested media was not noticed. 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

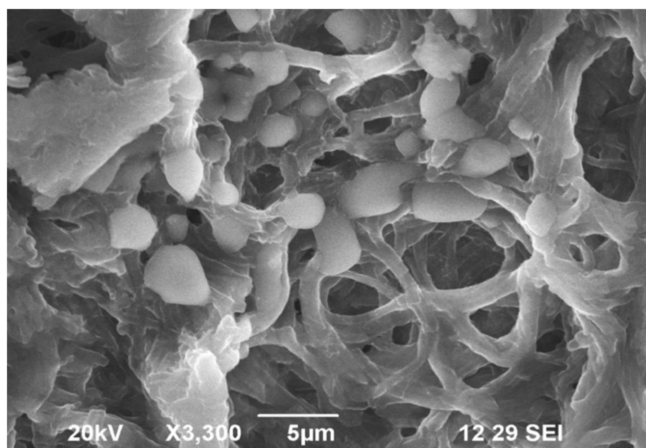

Fig. 1. Scanning electron micrograph of mycelia and spore of *Streptomyces* sp. BDUKAS10 strain grown on modified ISP2 medium for 3 days at 28 °C. Bar: 5 μm.

the genus *Streptomyces* (data not shown). Partial gene sequence (855 nucleotides) of isolate were deposited at GenBank database (NCBI) under the Accession No. JQ231271.1. Based on physiological, biochemical characterization and 16S rDNA sequence analysis the isolate was named as *Streptomyces* sp. BDUKAS10 (Fig. 2).

3.2. Microbe assisted synthesis of silver nanoparticles

In course of searching potential bioactive compound producing actinomycetes, mangrove habitat was chosen. *Streptomyces* sp. BDUKAS10 was isolated and identified to be a potent strain. In this study, we have reported the potentiality of *Streptomyces* sp. BDUKAS10 for the biosynthesis of silver nanoparticles by extracellular method. 5% of the CFS, the minimum concentration that supported the synthesis of AgNPs, was treated with 1 mm aqueous $AgNO_3$ and kept in dark at 32 °C. Interestingly, the development of brownish yellow color was observed after 16 h of incubation. Maximum color intensity (OD at 441 nm) was observed at 36 h. Color change after 16 h indicated the formation of AgNPs by the possibly available reductants in the culture supernatant. In contrast, there was no color change observed in aqueous $AgNO_3$ incubated without CFS under the same conditions (data not shown). Past studies suggest that NADH and its dependent enzymes are possibly involved in the biosynthesis of metal nanoparticles [36–38]. Chun et al. reported that NADH-dependent enzymes play a key role in these biosynthetic and biotransformation reactions as an electron carrier [39]. Yet, the mechanism underlying the biosynthesis of AgNPs by microorganisms is to be studied.

3.3. Characterization of biosynthesized AgNPs

In accordance with a previous report, the color change in the reaction mixture due to the excitation of surface plasmon resonance (SPR) for the biosynthesized AgNPs by the reduction of $AgNO_3$ was observed [40]. The UV–visible spectra for the aqueous $AgNO_3$-culture supernatant mixture and $AgNO_3$ alone were recorded. A peak at 441 nm corresponds to the characteristic wavelength of AgNPs (Fig. 3A). In agreement with previous reports, the absorption peak at 441 nm is probably due to the excitation of longitudinal plasmon vibrations and formation of quasi-linear superstructures of nanoparticles [41]. Medina-Ramirez et al. reported that the involvement of intermolecular forces may prevent the nanoparticles from aggregation by the formation of hydrophobic–hydrophilic interactions [42]. FTIR analysis revealed intense bands at 3473 cm^{-1}, 2071 cm^{-1}, 1637 cm^{-1} and 687 cm^{-1} (Fig. 3B). The bands at 3473 cm^{-1} and 1637 cm^{-1} could be attributed to free N—H and —C=C— vibrations, respectively, which corresponds to heterocyclic compounds like proteins. This serves as support for proteins present in the CFS as capping agents for the biosynthesized AgNPs [43–46]. It has been reported that proteins may bind to the nanoparticles either with the free amine groups or cysteine residues and cap the nanoparticles [12,47–50]. Based on these earlier reports, in the present study we speculate that the proteins present in the CFS capped and stabilized the AgNPs. EDAX analysis confirmed silver as the major constituent element. The spectrum at 3 keV (Fig. 5) indicates a strong signal for silver which is characteristic to nano-sized metallic silver [51]. In addition, other peaks for Cl and O were observed which are possibly due to emissions from proteins or enzymes present in the CFS [36]. TEM analysis showed the size of AgNPs in the range of 21–48 nm (Fig. 4). The shape of AgNPs, predominantly spherical in shape is common to microbial mediated synthesis [9,52].

3.4. Antimicrobial activity of microbiologically synthesized AgNPs

After 14 h of incubation at 37 °C on LB agar plates, growth inhibition of the test strains were determined (Table 2, Fig. 6A and B). Among the bacterial pathogens tested, maximal growth inhibition was observed for *B. cereus.* A number of possible mechanisms are proposed for the antibacterial activity of AgNPs. Silver ions have been known to bind with the negatively charged bacterial cell wall resulting in the rupture and consequent denaturation of

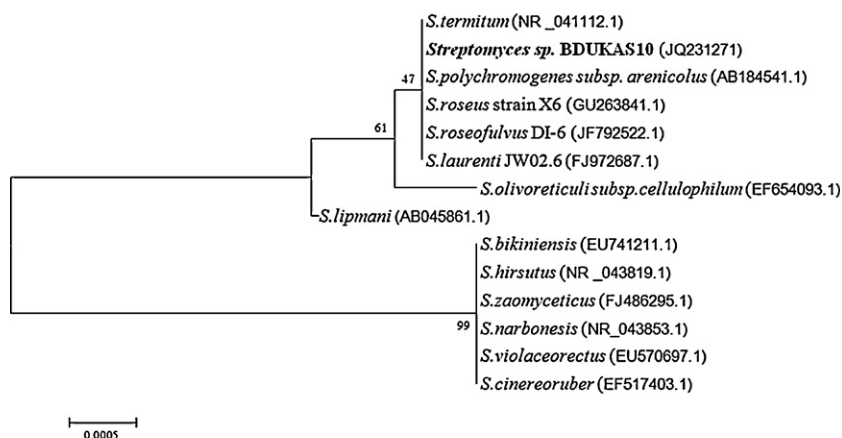

Fig. 2. The phylogenetic tree of *Streptomyces* sp. BDUKAS10 (JQ231271) was constructed using the neighbor-joining method with aid of MEGA 5.0 program. The Bootstrap values above 50%, presented as percentages of 1000 replications, are shown at the branch points. Bar 0.0005 substitutions per nucleotide position.

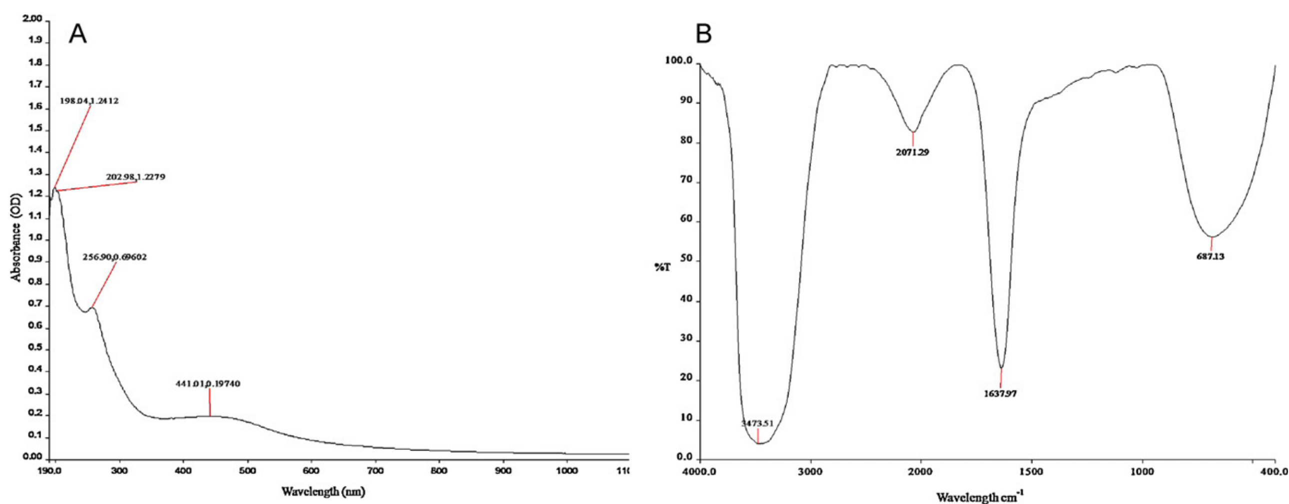

Fig. 3. (A) UV–Vis spectra of AgNPs synthesized using cell free supernatant of *Streptomyces* sp. BDUKAS10. (B) FT-IR spectrum of AgNPs synthesized by using cell free supernatant of *Streptomyces* sp. BDUKAS10.

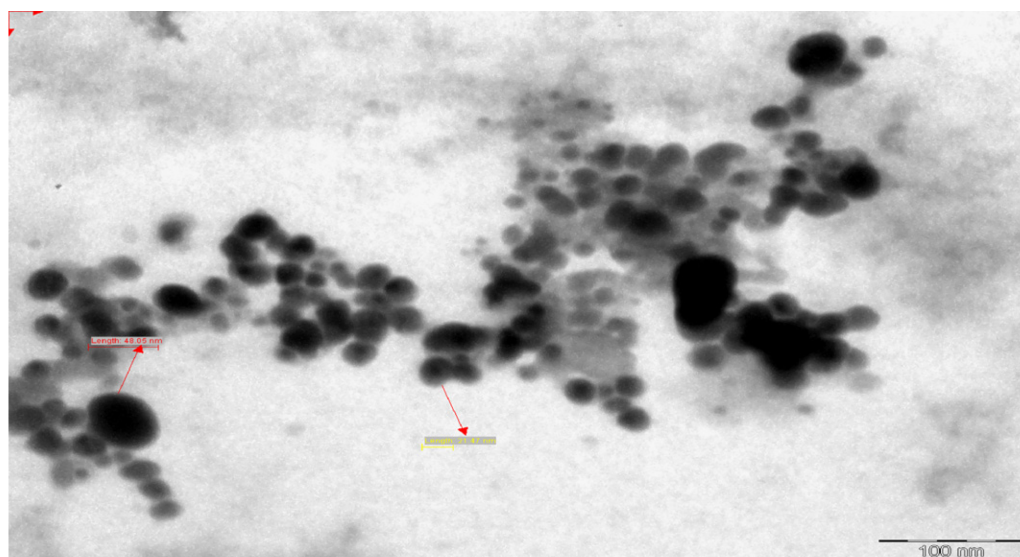

Fig. 4. TEM observation of AgNPs synthesized using cell free supernatant of *Streptomyces* sp. BDUKAS10 at a scale bar of 100 nm.

proteins which leads to cell death [53]. The synthesized AgNPs with smaller size can act drastically on cell membrane and further interact with DNA and causes damage [54]. 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 [55,56]. It was observed that the overall percentage fold increase for microbe assisted AgNPs was higher by 83.33% to the CFS (5%) and 19.88% to the 100% CFS as indicated by the ZoI (Fig. 6B, Table 2). 5% CFS did not indicate clear visible zones. The increased antimicrobial activity for AgNPs compared to CFS may be due to the secondary metabolites

Table 2
Mean zone of inhibition (mm) of AgNPs synthesized using CFS of *Streptomyces* sp. BDUKAS10 (5%), cell free supernatant (CFS – 100%) and the CFS (5%) against 3 different pathogenic microorganisms. Disk diameter was 6 mm.

Microorganism	Mean zone of inhibition (mm)			Percentage increase (%)	
	A	B	C	A – B	A – C
Bacillus cereus (MTCC 1272)	13	10	8	30	62.5
Pseudomonas aeruginosa (MTCC 1688)	15	13	8	15.38	87.5
Staphylococcus aureus (MTCC 96)	16	14	8	14.28	100

A – AgNPs; B – CFS; C – 5% CFS.
Percentage increase (%) of bacterial inhibition between AgNPs and CFS was calculated using the formula $(A - B)/B \times 100$.
Percentage increase (%) of bacterial inhibition between AgNPs and 5% CFS was calculated using the formula $(A - C)/C \times 100$.

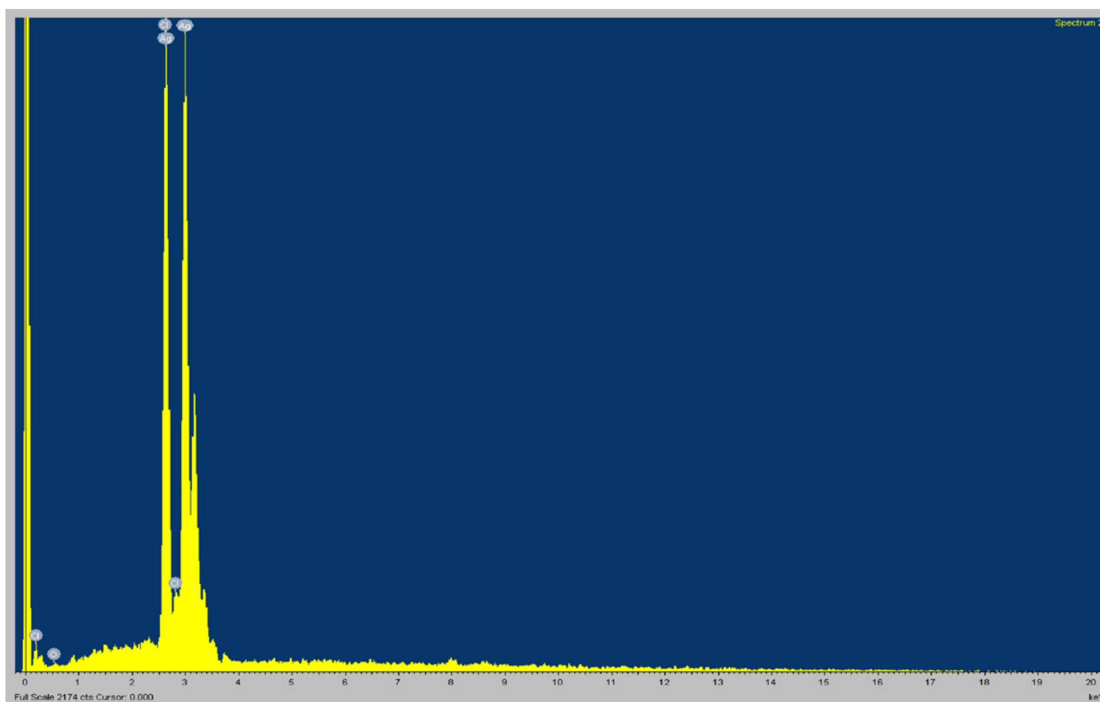

Fig. 5. EDAX analysis of AgNPs synthesized by cell free supernatant of *Streptomyces* sp. BDUKAS10.

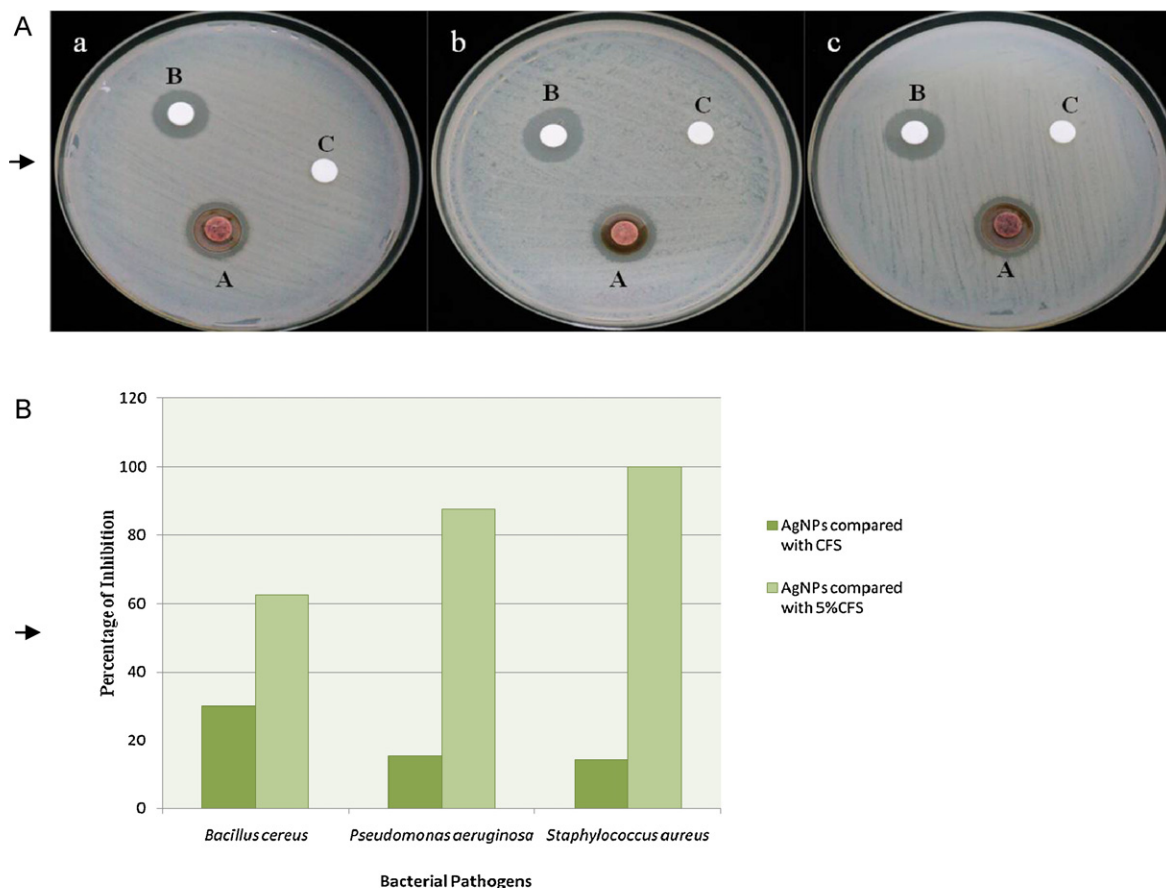

Fig. 6. (A) Antimicrobial activity of AgNPs and CFS against test strains (a) *Bacillus cereus*, (b) *Pseudomonas aeruginosa* and (c) *Staphylococcus aureus*. A – AgNPs; B – 100% CFS; C – 5% CFS. (B) Percentage of inhibition of bacterial growth by AgNPs compared to CFS (100%) and CFS (5%).

of the microbe probably capped on the surface of the nanoparticles [57].

4. Conclusion

Mangrove 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* sp. BDUKAS10 and studied its capability to biosynthesize the antibacterial AgNPs by an extracellular method. The biosynthesized nanoparticles were characterized by UV–visible spectroscopy, FTIR, EDAX and TEM and the antimicrobial activity of the AgNPs were evaluated against bacterial strains. The outcomes indicated an increased antimicrobial activity for the biosynthesized AgNPs compared to the CFS alone. With the availability of well established genetic manipulation procedures for *Streptomyces* sp., the organism can be further exploited for the identification of gene which is responsible for the synthesis of enzyme which may act as reductant. This can boost the future prospects of nanotechnology in pharmaceutical industries.

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