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Antibacterial activity and biogenesis of silver and zinc nanoparticles by *Streptomyces* sp. strain ANB 1 isolated from marine water of Port Blair, India

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The uncontrolled and random misuse of commercial antibiotics has led to the ever-increasing rise in drug resistant bacteria. There is still a lag between the development of novel drugs and the emergence of drug resistant microorganisms. Thus, multi-drug resistant microorganisms pose a serious threat to the human race. The marine environment has a great biodiversity and is largely unexplored. It has proved to be an excellent source for isolating microorganisms that produce secondary metabolites with unique properties. In the present study, the focus was to appraise the hostile behavior of marine environment and isolate actinomycetes, which are capable of having antibacterial activity and also to synthesise metallic nanoparticles. Biogenic metal nanoparticles bear immense potential as an alternative to conventional antibiotics. Out of 15 actinobacterial isolates, ANB 1 identified as *Streptomyces* sp. strain ANB 1 showed the most efficient antagonistic activity against the clinical pathogens *Klebsiella* sp., *Proteus* sp., *Escherichia coli*, *Pseudomonas* sp. and *Bacillus* sp. The inhibition zones varied in diameter from 5 – 20 mm. Further, *Streptomyces* sp. strain ANB 1 successfully synthesised silver and zinc nanoparticles, which were characterised by the formation of characteristic peaks in the UV-Vis spectrophotometer. The nanoparticles thus produced bear immense potential as an alternative to treat multi-drug resistant pathogens.

[**Keywords**: Actinomycetes, Antagonistic activity, Clinical pathogens, Multi-drug resistant pathogens, Nanoparticles]

Introduction

Pathogenic microorganisms causing various infectious diseases are slowly becoming resistant to commercially available antibiotics[1,2]. The World Health Organization (WHO) states that the inappropriate and over usage of antibiotics is responsible for the rising resistance among the pathogens. In addition, the emergence and proliferation of resistance-conferring mobile genetic elements has also sparked an increase in multi-drug resistance[3]. Multi-drug resistance is such a phenomenon where microorganisms become insensitive or develop resistance to the administered antimicrobial agents, which were previously found to be effective. These resistant microbes can counteract the given antimicrobial medicine resulting in ineffective treatment and thus, further spreading the infection. The rise of multi-drug resistance is extensive in immune compromised patients with conditions like HIV infection, diabetes, organ transplantation, and severe burns[3]. According to WHO report 2021, a priority list of 12 classes of resistant bacteria plus tuberculosis was prepared as an initiative to trigger the investigation and development of novel antibiotics. This list included bacteria such as (i) carbapenem-resistant *Acinetobacter baumannii*, (ii) fluoroquinolone-resistant *Campylobacter* spp., (iii) vancomycin-resistant *Enterococcus faecium*, (iv) & (v) carbapenem-resistant and third-generation cephalosporin-resistant Enterobacteriaceae (*Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., *Citrobacter* spp., *Morganella* spp., *Providencia* spp., *Proteus* spp., *Serratia* spp.), (vi) ampicillin-resistant *Haemophilus influenzae*, (vii) clarithromycin-resistant *Helicobacter pylori*, (viii) fluoroquinolone-resistant and third-generation cephalosporin-resistant *Neisseria gonorrhoeae*, (ix) fluoroquinolone-resistant non-typhoidal *Salmonella*, (x) carbapenem-resistant *Pseudomonas aeruginosa*, (xi) fluoroquinolone-resistant *Salmonella typhi*, (xii) fluoroquinolone-resistant *Shigella* spp., (xiii) methicillin-resistant and vancomycin-resistant *Staphylococcus aureus*, and (xiv) penicillin non-susceptible *Streptococcus pneumoniae*[4]. The rise of drug resistant pathogens is more rapid compared to the rate of novel drug discovery, and although the novel drugs bear immense potential, it will take years before the pre-clinical candidates can reach patients[5]. Therefore, this is a rising concern in the context of human healthcare and requires in-depth research on finding compounds with a broad spectrum of activity. Natural compounds and their derivatives are the predominant source of discovering novel drugs to treat numerous human diseases[6]. Biotechnology has utilised microorganisms of terrestrial origin for the production of thousands of drugs but is still lagging to meet the demands for the treatment of new infectious diseases[7,8]. The marine ecosystem thus represents a novel source of potent pharmaceutical products. The extreme physical and chemical factors of the marine ecosystem render the marine organisms with the capability to yield a vast array of biomolecules with exceptional structural properties unlikely in the terrestrial natural products. Till date, approximately 11,000 metabolites have been sequestered from marine organisms, several of which had pharmacological properties[9]. However, marine biodiversity has still not been exploited much and the identification of novel chemical compounds is in its preliminary state of research[10]. Actinomycetes present a rich source of bioactive metabolites. They have contributed almost 70 % of all known drugs, of which 75 % are used for medicinal purposes and 60 % are used for agricultural purposes[11].

Actinobacteria contain characteristics from both bacteria and fungi and were thus considered as an intermediate group initially. These are gram-positive bacteria and have high G+C content (> 55 %) DNA. High G+C content is responsible for 50 % of the bioactive metabolites produced by actinobacteria, as per the records in the dictionary of natural products[12]. Genera of actinobacteria obtained from the marine ecosystem include *Actinomadura*, *Actinoplanes*, *Marinispora*, *Nocardiopsis*, *Salinispora*, *Sachharopolyspora*, *Streptomyces*, *Amycolatopsis*, *Micromonospora*, etc. with commercial importance to the production of bioactive natural products[13,14]. *Streptomyces* is one of the predominant genera among actinomycetes, which produced approximately 7600 compounds. Approximately 289 bioactive compounds were isolated from *Streptomyces* of marine origin, as reported in the Marinlit database. They have produced a large number of metabolites with chemical structures, including peptide, macrolide, lactone, indole, terpene and quinone. These compounds are of immense industrial importance as they have activities including cytotoxic, antibacterial, antifungal, antimalarial, immunosuppressant, anticancer, antihelminthic, etc.[15].

Nanotechnology is involved with the manufacturing and stabilisation of various nanoparticles. Nowadays, the exploration of environmentally friendly processes for nanoparticle production is in consistent demand. Various synthesis methods have been devised so far, including physical and chemical methods. However, there has been a recent shift towards ‘green’ chemistry and microbial processes for the synthesis of nanoparticles as they do not use toxic chemicals and harsh reactions, which could be hazardous for the environment[16,17]. Reports have been made of magnetite nanoparticles formed by magnetotactic bacteria, and gypsum and calcium carbonate layers are produced by S-layer bacteria[18,19]. The microbial metabolism causes the precipitation of nanoparticles in the outer environment of a cell[20]. China has started the use of silver nanoparticles (Ag NPs) as antimicrobial agents in many public places, such as railway stations and elevators, and was found to be effective[21]. Zinc oxide nanomaterials (ZnO NPs) also bear the potential to be used for some next-generation biological applications as antimicrobial agents, drug delivery agents and bioimaging probes[22]. ZnO NPs have been widely researched owing to their unique antibacterial and antifungal properties. They have also demonstrated photo-oxidising and photocatalytic effects and are considered biosafe by researchers[23]. Gold nanoparticles (Au NPs) were found to be biocompatible and possessed antimicrobial activity. However, Au NPs require tagging with other biomolecules in order to exhibit their antimicrobial effect[24]. Antimicrobial activities exhibited by other biogenic metallic nanoparticles such as palladium, selenium, cerium, and tellurium comprise novel applications and show fascinating alternative to antibiotics[25,26].

Thus, the current study chose the marine environment as a source for isolating actinobacteria producing novel secondary metabolites as a potential treatment alternative to combat the problem of multi-drug resistance of bacteria.

Materials and Methods

Isolation of actinomycetes

Marine water sample was obtained from Port Blair (11°40′06″ N; 92°44′16″ E), India, in sterile containers and stored in the laboratory at 4 °C until further use. Serial dilution of the sample was done up to 10^{-6} for the purpose of isolation. Isolation was then done in starch casein agar plates (% composition:

starch 1, casein 0.03, KNO_3 0.2, K_2HPO_4 0.2, NaCl 0.2, $MgSO_4.7H_2O$ 0.005, $CaCO_3$ 0.002, $FeSO_4.7H_2O$ 0.001, agar 1.8, pH 7.2) using spread plate method. All the agar plates were supplemented with cycloheximide to inhibit fungal growth. The colonies with powdery growth and distinct morphological features were selected for subculturing. Isolates were maintained on starch casein agar plates[27].

Clinical pathogenic indicators used

Organisms used included *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Klebsiella* sp., *Proteus* sp. and *Bacillus* sp. Pathogenic bacteria were inoculated on nutrient agar slants. Bacterial suspensions were prepared by inoculating bacteria in nutrient broth and incubating the broth at 37 °C for 24 h under shaking condition.

Preparation of actinobacterial crude extract

Actinobacterial isolates were cultured in soluble starch broth. The inoculated flasks were then kept at room temperature for 7 – 10 days in a shaker at 100 – 150 rpm. After the incubation period was over the cultured broth was centrifuged at 5000 rpm for 15 min. The supernatant thus obtained was used in further assays.

Antagonistic activity of actinobacteria

The antagonistic activity of the crude extract obtained from the isolated actinobacterial strains against a few clinical pathogens was tested using Kirby Bauer's agar well diffusion method. The assay was performed on Mueller Hinton agar. Overnight grown bacterial pathogens (1.5×10^8 CFU/mL) were swabbed onto the Mueller Hinton agar plates to prepare a lawn culture. Wells were punched into each plate, and crude actinobacterial extracts (50 µl) were given in each of the wells. Negative control for this experiment was maintained with sterile distilled water, and the plates were incubated at 37 °C for 18 – 24 h. Following the period of incubation, the plates were observed for clear zones around the wells, and the radius around each well was measured, which is considered to be the zone of inhibition.

Biosynthesis of silver and zinc nanoparticles

Microbial synthesis was done by mixing 50 % of the supernatant with 50 % of the metallic salt solution. For preparing Ag NPs, 10^{-3} M silver nitrate ($AgNO_3$) solution was prepared in deionised water. In the case of ZnO NPs formation, a Zinc Acetate solution was used. Ag NPs were incubated for 3 days, and ZnO NPs were incubated for 1 – 2 days at room temperature (28 °C) in a shaker at 100 – 150 rpm with control. After incubation, the culture broth was kept for centrifugation for 18 – 20 min at 4500 rpm, and the supernatant was taken[28,29].

Characterisation of nanoparticles

Absorbance of the biosynthesised nanoparticles was estimated with the help of a UV-Vis spectrophotometer at a wavelength range of 200 – 800 nm with 1 nm resolution and a scan speed of 1856 nm/min. The formations of characteristic peaks confirmed the production of respective nanoparticles. Broth cultures without nanoparticles were used as negative control for the experiment.

Identification of the actinobacterial isolate

Methods devised in the International *Streptomyces* Project (ISP)[30] were used for the phenotypic and biochemical characterisation of the most potent actinobacterial isolate. Phenotypic characterisation included observing the chromogenecity of the aerial mycelium (aerial mass colour); morphology of the spore chain was observed under the light microscope, and spore surface morphology was observed by using a scanning electron microscope. The biochemical tests included sodium-chloride tolerance, hydrogen sulphide production, gelatin liquefaction, starch hydrolysis, pH tolerance and lipolytic activity.

The potential isolate was further identified by 16S rRNA sequencing. At first, the 16S rRNA gene of the organism was amplified, and the sequence thus obtained was analysed with highly similar sequences at the National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST). The 16S rRNA gene was then aligned with a highly related sequence using CLUSTAL W and a phylogenetic tree was created using the software MEGA version 7. The topology of the tree was deduced using the 'neighbor-joining' method with bootstrap analysis of 1000 trees. The gene sequence data thus obtained was submitted to GenBank under the accession number MG757838.

Results

Isolation of actinomycetes

A total of 15 isolates were obtained from the marine water sample based on their distinct colony characteristics. Figure 1 provides the schematic representation of the experimental part of the current study.

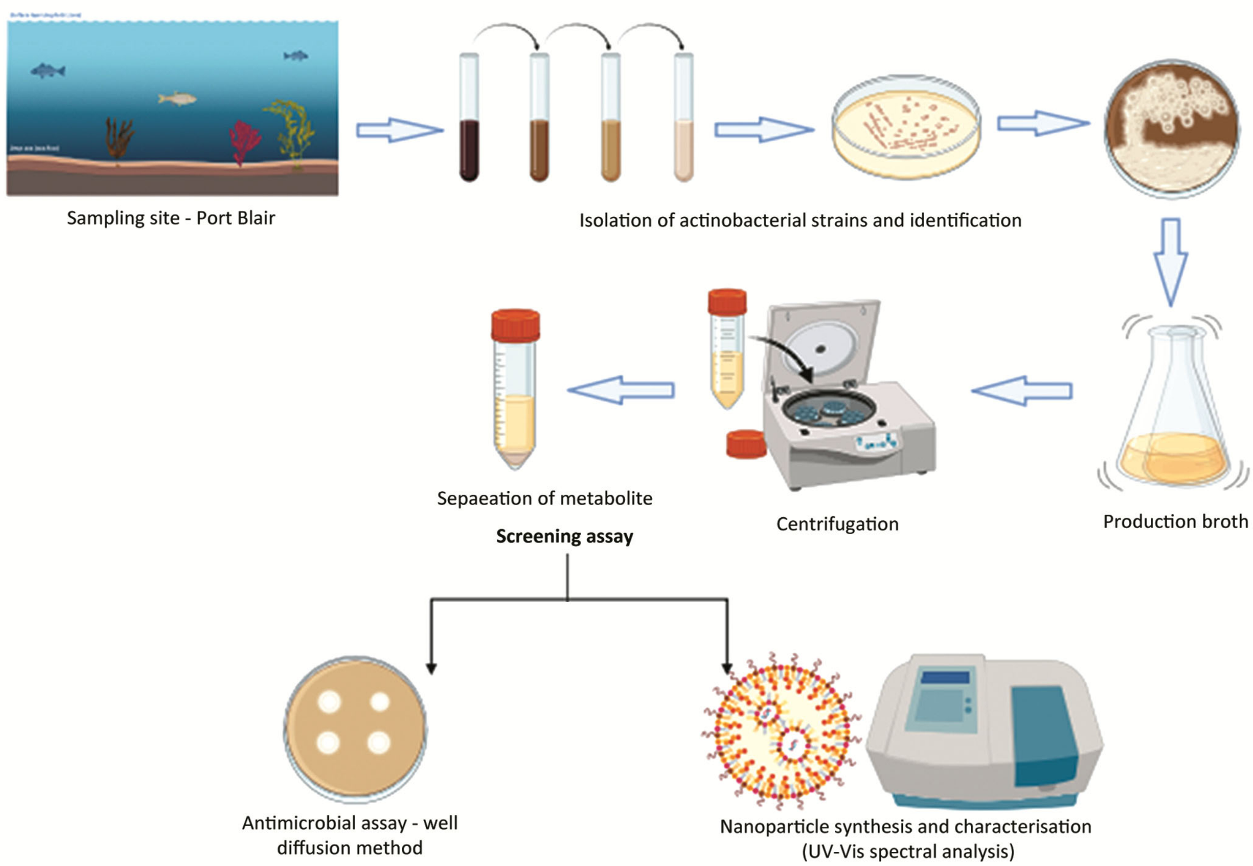

Fig. 1 — Schematic representation of the experimental part depicting isolation, their antimicrobial screening and synthesis of nanoparticles

Antagonistic activity of actinobacteria

Among the 15 actinobacterial isolates, only two isolates, ANB 1 and ANB 2 showed a prominent antibacterial activity against *Klebsiella pneumoniae* and *Proteus mirabilis*. Compared to ANB 2, ANB 1 showed better antagonistic activity against *Klebsiella pneumoniae*, *Proteus mirabilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus cereus*. The inhibition zones ranged from 5 – 20 mm. However, both the isolates were ineffective against *Salmonella typhi* (Table 1).

Table 1 — Antagonistic activity of actinobacterial isolates (ANB 1 and ANB 2) against different pathogenic bacteria

Pathogens	Zones of inhibition (mm)	
	ANB 1	ANB 2
Escherichia coli	7	5
Salmonella typhi	nil	nil
Pseudomonas aeruginosa	5	3
Klebsiella pneumoniae	20	15
Proteus mirabilis	10	8
Bacillus cereus	5	7

Biosynthesis and characterisation of silver and zinc nanoparticles

Isolate ANB 1 showed potent antimicrobial activity and was chosen for silver and zinc nanoparticle synthesis. After the incubation period, the culture supernatant turned light yellow to dark brown in color. The colour change was further confirmed by a peak which was observed under UV-Vis spectroscopy at 410 nm in the case of Ag NPs, which is a typical property of Ag NP synthesis (Figs. 2a & 3a). Otari *et al*.[31] also reported similar results on the biogeneic synthesis of Ag NPs by *Rhodococcus* sp., where there was a similar color change from colorless to dark brown and UV-Vis spectra showed Surface Plasmon Resonance (SPR) at 405 nm, which is a typical property of Ag NP synthesis. This is in line with the previous reports of synthesised Ag NPs showing an absorption band between 400 – 420 nm[32]. For instance, in a study, Ag NP was synthesised by *Streptomyces hirutus*, and the peak was observed at 418 nm[33]. Similarly, Ag NP was synthesised from polysaccharide-based bioflocculant obtained from *Streptomyces* sp. MBRC-91 exhibited SPR at 420 nm[34].

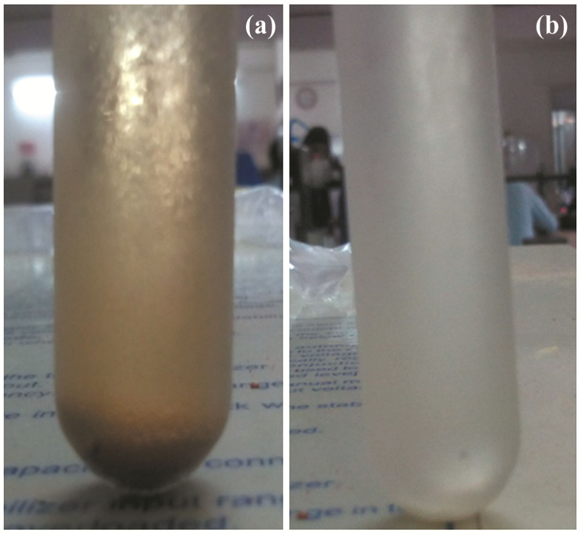

Fig. 2 — Biogenesis of nanoparticles by actinobacterial isolate ANB 1: (a) Synthesis of Ag NPs; and (b) Synthesis of ZnO NPs

While milky white color coalescent clusters to a transparent solution was observed for ZnO NPs synthesis, and the peak was observed at 390 nm under UV-Vis spectroscopy (Figs. 2b & 3b), which is similar to the results reported previously from *Pseudomonas putida* strain with the peak at 360 nm[35]. In addition to microorganisms, several plant extracts have been used effectively in the green synthesis of ZnO NP, showing similar results[36-38]. The qualitative evaluation of the respective peaks confirmed the biogenesis of silver and zinc nanoparticles.

Identification of the actinobacterial isolate

Isolate ANB 1 was identified at the species level as it was the most potent isolate. Phenotypic characterisation revealed that ANB 1 was a gram-positive, aerobic actinomycete. The aerial mass was white in color, and a Spiral Spore chain (SS) was observed under the light microscope (Fig. 4a).

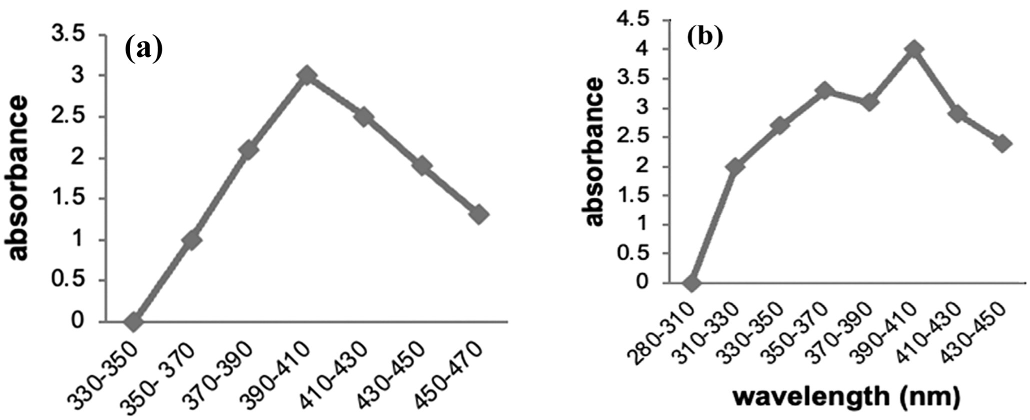

Fig. 3 — Qualitative analysis by UV-Vis spectroscopy: (a) Characteristic peak of Ag NPs; and (b) Characteristic peak of ZnO NPs

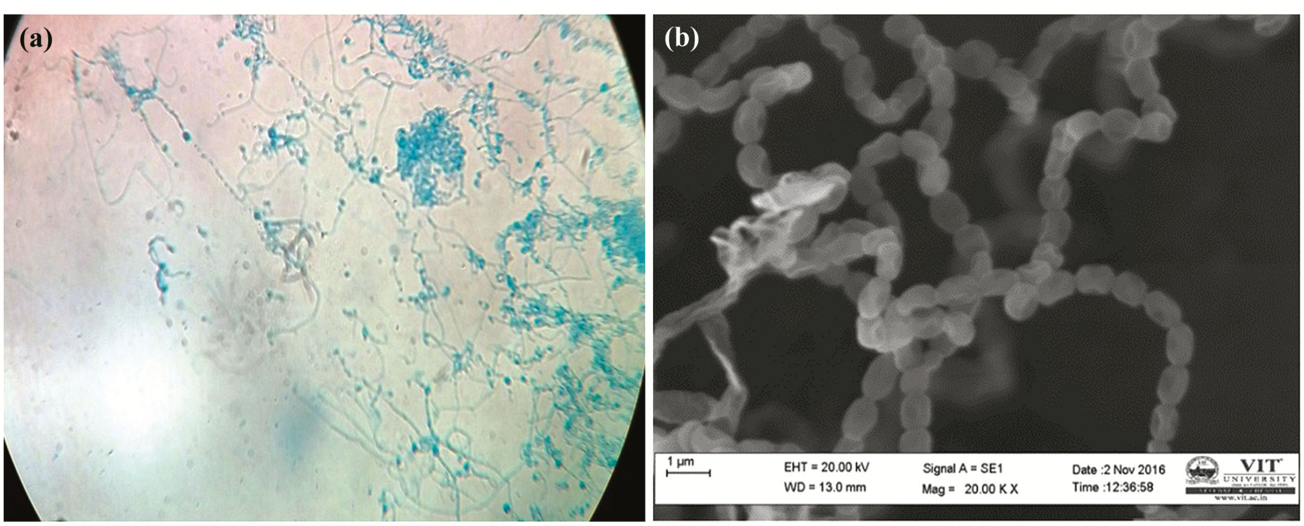

Fig. 4 — Microscopic observations of isolate ANB 1: (a) Spore chain morphology under light microscope; and (b) Spore surface morphology under Scanning Electron Microscope

Further, under a scanning electron microscope, spore surface morphology appeared to be smooth (Fig. 4b). Biochemical characteristics were also performed for the isolate. Isolate ANB 1 showed 5 % tolerance to sodium chloride, was able to survive under acidic, neutral as well as alkaline pH, produced hydrogen sulfide, and enzyme amylase (Table 2).

The 16S rRNA gene sequence analysis revealed strain ANB 1 as *Streptomyces* sp. Strain ANB 1. Further, the BLAST result showed that the strain ANB 1 has high sequence similarity values (99 %) with *Streptomyces* sp. (Fig. 5).

Table 2 — Observations on phenotypic and biochemical characteristics of strain ANB 1

Phenotypic and biochemical characterisation	Isolate ANB 1
Aerial mass color	White
Spore chain morphology	Spira spore chain
Spore surface morphology	Smooth
Sodium chloride tolerance test	
i. 5 %	+
ii. 15 %	-
iii. 25 %	-
iv. 35 %	-
Hydrogen sulfide production test	+
Gelatin liquefaction	-
Starch hydrolysis	+
pH tolerance test	
i. 5	+
ii. 7	+
iii. 9	+
Lipolytic activity	-

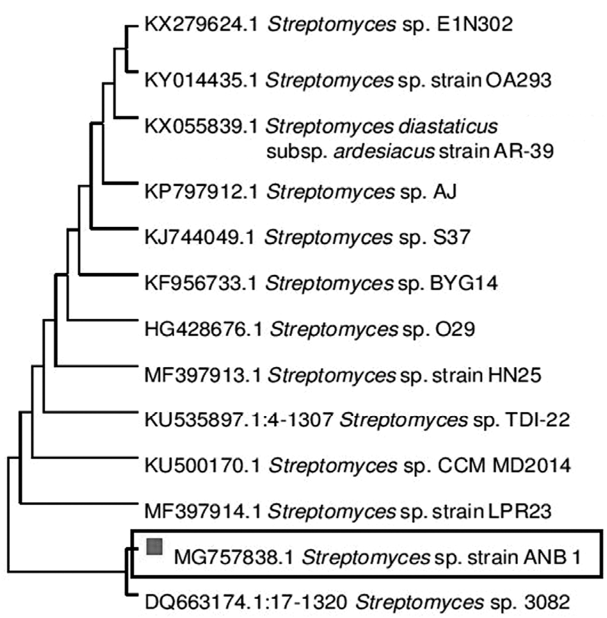

Fig. 5 — Phylogenetic tree of *Streptomyces* sp. Strain ANB 1

Discussion

Marine actinobacterial diversity from the Port Blair region is largely unexplored. Various reports are available on the antibacterial activity of plants, seaweed, microalgae and also on the mangrove sediment samples[39-42]. However, only a few reports are there on marine actinomycetes of the Port Blair region producing antibacterial metabolites. Hence, in the present study, the Port Blair water sample was used for actinobacterial isolation and screened for their antibacterial potential. A similar study by Meena *et al.*[43] isolated 26 actinomycetal isolates from Port Blair and assessed their antimicrobial activity. In another study, 23 actinobacterial colonies possessing antibacterial activity were isolated from different zones of the mangrove ecosystem of the South Andaman region[44]. Actinobacteria from marine environments have immense potential for producing bioactive metabolites as reported in several studies[44,45]. Marine actinomycetes isolated from the Thoothukudi coastal ecosystem, India, showed antibacterial activity against pathogens such as *Bacillus cereus*, *Erwinia carotovora*, *Serratia* sp., *Flavobacterium* sp., and *Pseudomonas fluorescens* with inhibition zones in the range of 2 to > 10 mm[45]. Eight actinobacterial isolates of the genera *Dietzia*, *Kocuria*, *Nocardiopsis*, and *Streptomyces* exhibited antibacterial activity against bacteria such as *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*[6]. Moreover, in 2016, Abd-Elnaby *et al.*[15] isolated 17 actinomycetal isolates from the Suez Bay, Egypt. Among the isolated actinomycetes *Streptomyces parvus* inhibited the pathogens *Aeromonas hydrophilla*, *Pseudomonas aeruginosa* ATCC 6739, *Escherichia coli*, and *Staphylococcus aureus* ATCC 6538 producing inhibition zones in the range of 14 – 20 mm. In the present study, similar results were obtained by *Streptomyces* sp. strain ANB 1, which produced inhibition zones in the range of 5 – 20 mm against pathogens *Klebsiella* sp., *Proteus* sp., *E. coli*, *Pseudomonas* sp., and *Bacillus* sp. With the rising emergence of drug resistance among the various pathogens, it is crucial to continuously research antimicrobial compounds with unique properties from organisms of unexplored environmental niches.

Different metal nanoparticles synthesised by actinomycetes so far have been reported to have potential antibacterial, antifungal and antiparasitic activity. Majority of the studies have utilised actinomycetes for the production of Ag NPs as antimicrobial agents. Actinomycetes belonging to the genera *Streptomyces* and *Rhodococcus* are reported to produce Ag NPs, having both antifungal and antibacterial activity[46-52]. Another study synthesized silver nanoparticles from *Nocardiopsis valliformis* with resistance against clinical pathogens such as *Staphylococcus aureus*, *Klebsiella pneumonia*, *E. coli*, *P. aeruginosa*, and *Bacillus subtilis*[53]. Similarly, Rajamanickam *et al*.[54] and Usha *et al*.[55] synthesised Zn NPs using *Streptomyces*, which exhibited antibacterial and antifungal activity. Further, Shanmugasundaram & Balagurunathan[56] successfully synthesized ZnO NPs from *Streptomyces* sp. The produced nanoparticles inhibited two gram-positive (*S. aureus* and *B. subtilis*) and five gram-negative pathogens (*E. coli*, *K. pneumonia*, *Salmonella typhi*, *Proteus vulgaris*, and *P. aeroginosa*). Nanobiomedicine is a beaming new field of research for treating different human diseases and provides potential alternatives to efficiently counteract diseases. Thus, the *Streptomyces* sp. strain ANB 1 examined in this study proves to be a potential organism having pathogenic inhibition properties. Future, compound purification and identification can provide a potential alternative to treat pathogenic diseases. Its ability to produce silver and zinc nanoparticles further increases its efficiency of treatment.

Conclusion

The current study explored the marine waters of Port Blair, India, which are underexplored regions to isolate actinobacterial strains having the potential of producing novel secondary metabolites contributing towards a wide range of applications. Among all the isolated isolates, ANB 1 was found most effective against the clinical pathogens like *Klebsiella* sp., *Proteus* sp., *E. coli*, *Pseudomonas* sp. and *Bacillus* sp. Further, the isolate ANB 1 was induced to synthesise silver and zinc nanoparticles and the nanoparticles thus produced were characterised preliminarily by UV-Vis spectroscopy to confirm the presence of nanoparticles. The absorbance at 410 nm and 390 nm validated the presence of Ag and ZnO nanoparticles, respectively. Isolate ANB 1 was further identified as *Streptomyces* sp. strain ANB 1 according to their phenotypic, biochemical characteristics and molecular studies. Furthermore, other characterisation studies, including Scanning Electron Microscopy (SEM), X-Ray Diffraction (XRD), Atomic Force Microscopy (AFM), etc. need to be performed to characterise the synthesised nanoparticles. This biogenic approach in the synthesis of the nanoparticles is much more beneficial, cost effective and reliable than other physical and chemical methods of synthesis and also hold many advantages to the conventional methods. Future prospects of this study include purification and characterisation of the secondary metabolites produced by strain ANB 1. Thus, *Streptomyces* sp. strain ANB 1 bear immense potential to be used for treating multi-drug resistant pathogens and in the field of nanobiotechnology and nanomedicine.

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Conflict of Interest

The authors of this research paper have no conflict of interest.

Authors Contributions

NH: Investigation, collecting resources, carrying out formal analysis, and writing the original draft; AK: Formal analysis and writing the original draft; PS: Conceptualisation of the research, supervision of the analysis, and reviewing and editing of the research article; and KVBR: Supervision of the entire work.

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