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Enzyme Mediated Synthesis of Silver Nanoparticles using Marine Actinomycetes and Their Characterization

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Nanobiotechnology combines biological principles with physical and chemical procedure to generate nanosized particles with specific functions. Recently, a variety of nanoparticles with well-defined size, chemical composition and morphology have been synthesized by using different microorganisms. This work focuses on marine Actinomycetes which is a Gram positive bacteria that has been isolated from marine sediments from Kanathur East coast area, Chennai. Finally the screened strain that showed more activity for the production of oxidative enzyme was selected for the production of silver nanoparticles. The synthesis of silver nanoparticles by this novel strain has been characterized by X-ray diffraction(XRD),FT-IR, UV-Spectroscopy and scanning electron microscope(SEM).The molecular identification of this novel strain from the marine sediments was found as *S.fradiae* through 16s rRNA ribotyping. The synthesized silver nanoparticles were analysed for their inhibitory effect against *B.cerus* and *K. pneumoniae.*The synthesized nanoparticles is of high therapeutic value and can be used in drug delivery system in future work.

Key words: Silver nanoparticles, XRD, FT-IR, UV SPECTROSCOPY, SEM, 16rRNA.

Nanoparticles (NPs) are attracting increased attention in commerce and applied microbiology due to their antimicrobial activity, high electrical conductivity, and optical properties. For example, silver NPs have broad spectrum antimicrobial properties against a wide range of bacteria and fungi, making them ideal for minimizing bio fouling. By controlling the size, shape, surface, and agglomeration state of the NPs, specific ion release profiles can be developed for any given application. Biomolecules present in plant extracts can be used to reduce metal ions to nanoparticles in a single-step green synthesis process. This biogenic reduction of metal ion to base metal is quite rapid, readily conducted at room temperature and pressure, and easily scaled up.

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METHODS AND MATERIALS

Enzyme activity

In the marine environment, extracellular enzymes play a central role in the recycling of organic carbon and nitrogen compounds. High molecular weight organic compounds cannot be transported directly into bacteria to be catabolized. Thus, bacteria must hydrolyze these organic polymers to smaller molecules before they are incorporate into the cell for subsequent metabolism. This extracellular hydrolytic activity is performed by bacterial extracellular enzymes[6-7]. Fluorogenic substrates have been widely used since the early 1980s to access extracellular enzyme activity in water and sediment[1].

Screening for Enzyme Activity

The selected marine actinomycetes were screened for their ability to produce different proteolytic and oxidative enzymes using qualitative

assays. For this study, the enzyme activity was tested in the culture filtrate by qualitative assays[4]. The actinomycete strains were grown in Starch Casein Broth for 15 days. Centrifuged the culture broth (8000 rpm 4°C; 10 mins) and collected the supernatant in sterile Eppendorf. Thus obtained culture filtrate was used as source of enzyme activity.

Screening for Protease Producing Actinomycetes

Water agar medium was prepared with 0.5% of the protein substrates i.e., casein and gelatin supplemented with 50µg/ml of chloromphenicol antibiotic to avoid bacterial contamination and the medium was poured into the Petri plates and allowed to solidify. After solidification 8 mm diameter well were made using cork borer. 100 µl of the culture filtrates of actinomycetes to be tested for proteolytic enzyme activity was placed in each wells separately and incubated for 24 h at room temperature. After 24 hours of incubation period, the enzyme activity was visualized as a clear zone by flooding the plates with saturated ammonium sulphate. The diameter of zones formed was measured for all the positive strains.

Screening for Oxidative Enzymes Producing Actinomycetes

Water agar medium was prepared with 0.5% of the substrates for two oxidative enzymes Laccase (Guiacol v/v)and Tannase (Tannic Acid w/v) supplemented with 50¼g/ml of chloromphenicol antibiotic to avoid bacterial contamination and the medium was poured into the Petri plates and allowed to solidify. After solidification, 8 mm diameter well were made using cork borer. 100 µl of the culture filtrates of actinomycetes to be tested oxidative enzyme activity was placed in each wells separately and incubated for 24 h at room temperature in dark condition to avoid photo-oxidation/reduction. After 24 hours of incubation period, the enzyme activity was visualized as a brown colour zone around the wells which indicate the production of oxidative enzymes by the actinomycete. The diameter of zones formed was measured for all the positive strains.

Enzyme mediated synthesis of nanoparticles

Nanoparticles (NPs) are attracting increased attention in commerce and applied microbiology due to their antimicrobial activity, high electrical conductivity, and optical properties[3]. For example, silver NPs have broad spectrum antimicrobial properties against a wide range of bacteria and fungi, making them ideal for minimizing bio fouling[2]. By controlling the size, shape, surface, and agglomeration state of the NPs, specific ion release profiles can be developed for any given application. Biomolecules present in plant extracts can be used to reduce metal ions to nanoparticles in a single-step green synthesis process. This biogenic reduction of metal ion to base metal is quite rapid, readily conducted at room temperature and pressure, and easily scaled up. Synthesis mediated by plant extracts is environmentally benign. The reducing agents involved include the various water soluble plant metabolites (e.g. alkaloids, phenolic compounds, terpenoids) and co-enzymes have utilized the bio reductive potential of *Micrococcus luteus* for the synthesis of gold nanoparticles. Biochemical and physiological analysis indicate that the biosynthesized GNPs were achieved by dual mode, involving extracellular α-amylase and cell wall teichuronic acid (TUA) of *M. luteus*[5].

Bharde *et al*. (2007) also observed that synthesis of gold nanoparticles occur with simultaneous induction of the protease enzyme secreted by the bacterium in the presence of BSA. The presence of BSA helps to enhance the rate of gold nanoparticles biosynthesis and may also impart some shape control. Controlling simple experimental conditions like incubation temperature and presence or absence of oxygen have drastic effect on the reaction rate and the morphology of the particles[9]. Various assay experiments show that the presence of enzyme protease can act as a reducing as well as shape directing agent. In this context, the present study has been undertaken with the aim to synthesize and characterize silver nanoparticles using enzyme extracts of marine actinomycetes[9-10].

Mass Culture of Selected Marine Actinomycete

One litre of Starch Casein broth was prepared in 4L conical flask and sterilized. After cooling, 100 ml of 10 days old broth culture of selected marine actinomycete culture Act11 was inoculated and the flask has been maintained at static condition in room temperature for 7 days.

Preparation of crude enzyme extract:

One litre mass culture of selected marine

actinomycete strain Act11 was centrifuged at 10000 rpm for 10 min at 4°C. The resulting cell free culture filtrate was subjected to ammonium sulphate precipitation for the extraction of crude enzymes. 85% saturation of ammonium sulphate was followed to precipitate the total proteins at room temperature with continuous stirring for 24 h. Then this material has been centrifuged at 10000 rpm for 10 min at 4°C. The resulting pellet containing crude enzymes was dialyzed against 0.1 m phosphate buffer pH 7.2 at room temperature for 24 hours with intermittent changing of buffer for every 3 h. The resulting suspension after dialysis was considered as crude enzyme extract and has been used in the further studies.

Optimization and synthesis of silver nanoparticles

Synthesis of silver nanoparticles from enzyme extract by conventional method

250 Erlenmeyer Preparation of 100 ml of MYGP medium Inoculated with 3% of UV- visible spectrophotometer at the range of 200 – 800 nm . The content seed culture Incubated at 25°C on a rotary shaker 15 rpm for 72 hours After incubation, biomass was filtrate through Whatman filter paper No.1 Washed extensively with distilled water to remove any medium components Fresh and clean biomass was taken in the in 250 ml Erlenmeyer containing 100 ml of deionized water The flask were agitated at the same condition as described above and again the biomass was filtrate thus obtained was used in nanoparticle production $AgNO_3$ (1mM) solution was mixed with cell free filtrate in 250 ml Erlenmeyer flask and agitated at 250C in the dark Control flask without silver ion was kept along with experimental flask The sample of 1 ml were taken at various times and absorbance was measured in the was centrifuged at 10,000 rpm for 15 minutes. The supernatant was used for the characterization of the silver nanoparticles or the supernatant was removed and double distilled water (2-3 ml) was added and again centrifuged at 10000 rpm 30 min the supernatant was removed and the pellets allowed to dry few drops of acetone was added mixed gently and transferred in to the Petridis for air drying after drying the particle was collected in clean vials.

Characterization of silver nanoparticles

UV-visible spectroscopy

The work described in this thesis, UV-visible spectroscopy is used for monitoring the signature of silver nanoparticles. UV-visible spectroscopy is a powerful tool for the characterization of colloidal Particles. In particle, noble metal particles are ideal candidates for study with UV- Vis spectroscopy, since they exhibit strong surface Plasmon resonance absorption in the visible region and are highly sensitive to the surface modification.

SEM Analysis

This study was undertaken to know the size and shape of the silver nanoparticles biosynthesized using scanning electron microscope.

Procedure for the sample preparation

After synthesis of nanoparticles, the sample was filtered through Millipore filters of 0.2 [tm pore size, to remove any contaminants interfering with the SEM images. About 25 Al of the sample was pipette out and loaded on a 'stub' provided for SEM analysis. The stub is made of copper, in the shape of a small cylinder about the size of I cm dia. One side of the stub was stuck with double sided carbon material. After loading the sample on the carbon material, the stub was fixed to a holder. The holder accommodates about 4 samples at a time.

Coating the sample with platinum

This coater (JEOL, Japan, Model No.JFC-1600), which consists of a main unit and a pump, is intended mainly to prepare specimens for SEM observation. It coats biological and other nonconductive specimens with metals, efficiently and in a short time. The cathode contains a permanent magnet to create an efficient glow discharge for sputtering. It is possible to set the chamber pressure in addition to the sputtering current. This enables the uniformity of the coating to be controlled so that a shadow-free coating can be obtained. Operation is easy, and a fine coating of platinum can be obtained in a short time. By using an optional film thickness monitor and a film thickness controller, it is possible to obtain a more accurate film thickness.

XRD Measurement

The air dried nanoparticles were coated onto XRD grid and analysed for the formation of Ag nanoparticle by Philips X-Ray Diffractometer with Philips PW 1830 X-Ray Generator operated at a voltage of 40kV and a current of 30mA with Cu

Kal radiation. The diffracted intensities were recorded from 10' to 80' of 20 angles.

FTIR Analysis

The dried Ag nanoparticles were subjected to FTIR analysis by KBr pellet (FTIR grade) method in 1: 100 ratios and spectrum was recorded in Nicolet Impact 400 FT-IR Spectrophotometer using diffuse reflectance mode operating at a resolution.

RESULTS AND DISCUSSION

Screening of culture filtrates of marine actinomycetes for proteolytic and oxidative enzymes production

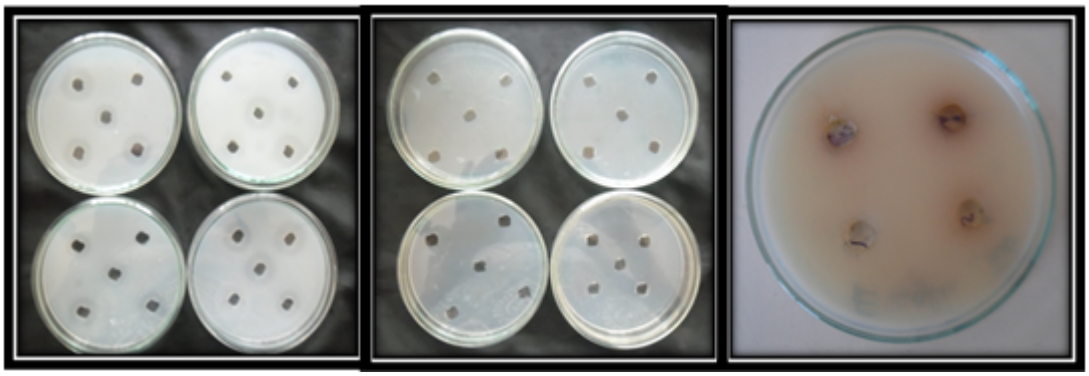
Among 16 isolates, twelve isolates showed activity for proteolytic enzyme (gelatinase) production, four isolates showed activity for oxidative enzyme (tannase) production but no isolate showed activity for laccase production. The isolates,P-311 and P-315 showed a highest zone of 16mm and a lowest zone of 11 mm was exhibited by P-314 for gelatinase production. The isolate, P-311 showed a zone of 15 mm for tannase enzyme production. (Fig 1)

Selection of potential strain

In vitro screening of all the 16 marine actinomycetes against antimicrobial activity resulted in selection of an efficient marine *Streptomyces* sp. P-311 (Fig 2). This isolate further was screened for enzyme production giving a zone of 16 mm for gelatinase enzyme and 15 mm for tannase enzyme production.

Table 1. Effect of silver nanoparticles against bacterial pathogens

S. No	Organisms	Zone of inhibition (mm)		
		Nanoparticle	Antibiotic	Nanoparticle + Antibiotic
1	*K. pneumonia*	14	25	27
2.	*B. cereus*	10	16	17



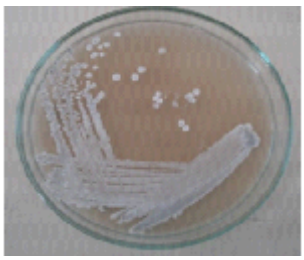
Protease production Laccase production Tannase production

Fig. 1. Screening of culture filtrate for enzyme production

Characterization of silver nanoparticles

UV-VIS spectral analysis

The bio-reduction of Ag+ in the filtrate reaction solution was monitored by periodic sampling of the reaction mixture at regular intervals by using UV-Vis spectroscopy. Control flasks maintained with silver nitrate solution (without culture filtrates) did not show any change of colour and its absorbance maximum was found to be at



P-311

Fig. 2. Potential strain of actinomycetes

340 nm, which is specific for silver nitrate solution. Whereas the reaction mixture consisted of culture filtrate with silver nitrate (Fig 3) showed a strong characteristic absorbance peak at around 430 nm. Analysis by spectrophotometer was made up to 8hrs.

SEM Analysis

Scanning electron microscopic analysis of the silver nitrate solution (Control) and reduced form of silver nitrate solution are clearly distinguishable owing to their size difference. Silver particles in the bio reduced colloidal suspensions were measured 100-200 nm in size. Fig-4 is the SEM image of bio reduced silver nanoparticles.

XRD Analysis

XRD analysis showed three distinct diffraction peaks at 28.18° 32.18°, 38.18°, 47.1° and can be indexed the angle values of (111), (200), (220), (240) crystalline planes of cubic Ag. This analysis revealed that nanoparticles are in orthorhombic crystals. The high peaks in the analysis indicate the active silver composition with the indexing (Fig 5).

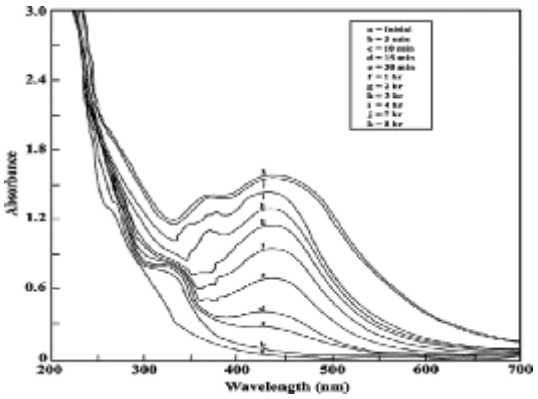


Fig. 3. UV spectrum analysis of silver nanoparticles of S. sp P-311

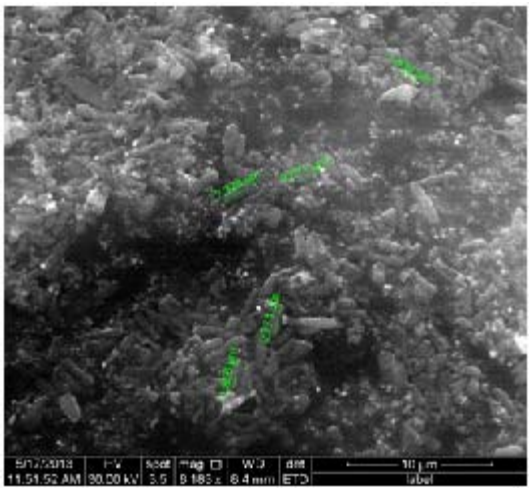


Fig. 4. SEM image of silver nanoparticles of S. sp P-311

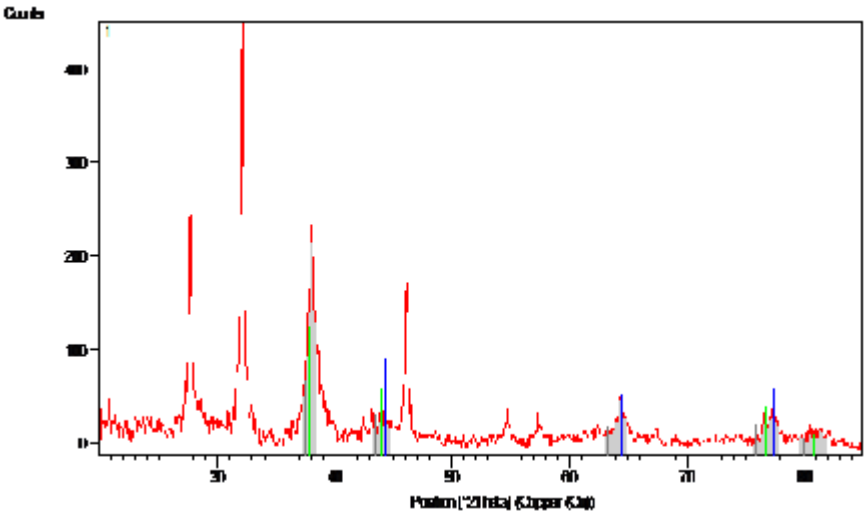


Fig. 5. XRD analysis of silver nanoparticles of S. sp P-311

FTIR Analysis

The presence of some functional group as revealed by IR spectral is shown in Fig 6. The FTIR Spectral analyses of silver nanoparticles show certain common absorption band at 3299 cm-1 is a characteristic of hydroxyl Å (O-H) and Å (N-H) vibrational frequency which are interchangeable. A common 3 vibrational peak between 1649 and 2924 cm-1 are characteristic oa Å (C-H) symmetrical vibration of saturated hydrocarbon. The vibrational frequency Å (C-O) was observed in the spectra of the extracts at 1068 and 1537 cm-1. Deviation from this region to a higher wave number was observed which is indicative of a secondary amide. These peaks were sharper than the Å (O-H) peaks due to reduction in hydrogen bonds which increases with electronegativity. Vibrational peaks at 1649 cm-1 in extract signify the possibility of an aromatic compound. Based on the physical state (oily) of the extracts and the characteristic features of the infrared vibrational peaks in the spectra, terpenoids, long chain fatty acids and secondary amine derivatives are possible compounds in the Particles.

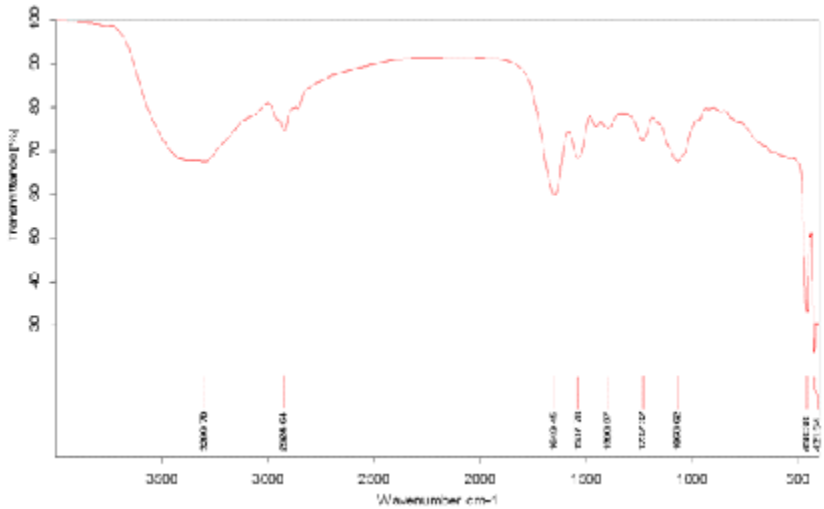
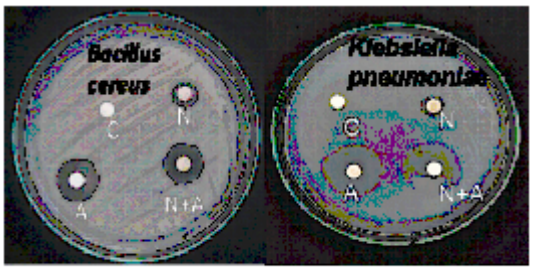


Fig. 6. FTIR spectrum of silver nanoparticles of S. sp P-311

Antibacterial activity of silver nanoparticles synthesized from *Streptomyces* sp P-311.

Disc diffusion assay

The synthesized silver nanoparticles were analyzed for their inhibitory effect against *B.cerus*and *K. pneumoniae*.The data obtained explains that the AgNPs possess considerable inhibitory effect on the tested pathogen with MIC value of 100µg (Fig7). The Zone of Inhibition (ZOI) for 100µg AgNPs was10mm and 14 mm respectively.While that for a standard antibiotic was found to be 16 mm and 25 mm respectively. In addition, the inhibitory effect of AgNP + antibiotic was recorded as 17 mmand 27 mm respectively (Fig: 7; Table 1).



C- Control N- Nanoparticle A- Antibiotic

Fig. 7. Antibacterial activity of *Streptomyces* sp

CONCLUSION

Thus among the 16 marine actinomycetes collected from the marine sediments, four strains produced proteolytic enzymes and oxidative enzymes in their culture filtrates. Among the four strains, one potential strain has been selected and has been used for the synthesis of silver nanoparticles**.** The synthesized Silver nanoparticles was characterized by UV-VIS spectroscopy, XRD, FTIR and SEM analysis. The

synthesized silver nanoparticles were analyzed for their inhibitory effect against B.*cerus* and K. *pneumonia*, which showed positive results.

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