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Biosynthesis and Characterization of Zinc Nanoparticles Using Strains of Pseudomonas and Actinobacteria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Research was carried out at the Green Nanotechnology Laboratory, University of Agricultural Sciences, Dharwad, Karnataka, with a specific emphasis on the biosynthesis of zinc nanoparticles using Pseudomonas and Actinobacteria. Collection and screening of Pseudomonas and Actinobacterial isolates were done at Microbial Genetics Laboratory, Department of Agricultural Microbiology, UAS, Dharwad. The ZnNPswere biosynthesized and characterized through UV-Visible spectroscopy, Particle Size Analyzer (PSA), Scanning Electron Microscope (SEM), Energy-dispersive X-ray spectroscopy (EDS), X-Ray Diffraction (XRD) and Fourier Transform Infrared Spectroscopy (FTIR). The ZnNPs synthesized through Pseudomonas(AUDP209) and Actinobacteria(AUDT636)isolates exhibited peak values at 365 and 374 nm, with average sizes of 48.7 nm and 54.9 nm, respectively.SEM images revealed the spherical shape of the biosynthesized ZnNPs, while EDX confirmed the zinc ions presence in the biosynthesized samples and the crystalline structure of the ZnNPswas confirmed by XRD.FTIR spectroscopy confirmed the organic compounds present in the microbial extracts responsible for the capping and stabilizing agents for biosynthesis of ZnNPs.

Keywords: Biosynthesis; Zn-nanoparticles; pseudomonas; actinobacteria; characterization.

1. INTRODUCTION

“Nanotechnologies can contribute to a new technological revolution in agriculture. Several problems with conventional biofortification could potentially resolved by nanotechnology” [1]. “It is possible to produce nanofertilizers using nanomaterials because of their high surface-to-volume ratio, gradual and controlled release at target places, and other characteristics” [2]. “The encapsulation of nutrients with nanomaterials results in efficient nutrient absorption by plants, due to the gradual or controlled release of NPs and simple passage through biological barriers by NPs entering the plant vascular system” [3]. “In comparison to conventional fertilisers, long-term delivery of plants *via*nanofertilizers enables enhanced crop growth. As nanofertilizers are added in small amounts, these also prevent soil from becoming burdened with the by-products of chemical fertilisers and reduce the environmental hazards “[4] “Unlike chemical fertilisers, nanofertilizers can be synthesized and applied based on the crop's nutritional needs and the status of the soil's nutrient levels using biosensors” [5]. “Additionally, nanofertilizers, as opposed to chemical fertilisers, allow for high mineral bioavailability to plants due to their smaller size, greater reactivity, and higher surface area” [6].

Zinc is essential for the synthesis and activation of several hormones (auxin and gibberellin) and enzymes that enhance seed germination and seedling growth. Additionally, Zn plays a important role in biosynthesis of proteins, carbohydrates, lipids, and nucleic acids in [7]. The ZnNPs are among the top three most manufactured and used engineered NPs [8]. The ZnNPs, one of the best source for preventing Zn deficiency and enhancing crop quality and productivity [9]. Zn can serve as a cofactor for P-solubilizing enzymes like phosphatase and phytase, and nano-ZnO boosted their activity in the soil [10]. Zn NPs have an impact on plant metabolism at the molecular level by activating antioxidants and reductases, as well as influencing the synthesis of plant hormones [11].

Synthesis of nanoparticles involves a variety of biological, physical and chemical methods; however, only the biological methods are safe and environment friendly. Traditional chemical methods, which could be hazardous to the environment, are being replaced with biological systems, which are preferred for NP synthesis [12]. Microorganisms (fungi, viruses, bacteria, yeast, and actinomycetes) can be thought of as biological nanofactories since they can take in metal ions from the environment and change them into elemental compounds. These processes can take place as either intracellular or extracellular events, and as a result, NPs produced by bacteria are categorised as either intracellular or extracellular NPs. Additionally, bacteria are great producers of NPs it can create a variety of secondary metabolites (Rani et al*.,* 2022). Keeping the above facts in view, the present investigation is planned to study the biosynthesis of zinc nanoparticles using two different microbial strains and characterized through UV-Vis Spectroscopy, PSA, SEM, EDS, XRD and FTIR.

2. MATERIALS AND METHODS

2.1 Biosynthesis and Characterization of ZnNPs

The Pseudomonas and Actinobacterial isolates were collected from culture collection maintained at Microbial Genetics Laboratory, Department of Microbiology, UAS, Dharwad. The collected isolates were spotted and sub cultured in King's B and Starch casein agar media (pH 7) and incubated for 2 and 7 days, respectively, in incubator at 30ºC.Pseudomonas isolates: AUDP204, AUDP207, AUDP209, AUDP218, AUDP219, AUDP223, AUDP264 and AUDP265.Actinobacteria isolates: AUDT504, AUDT535, AUDT574, AUDT592, AUDT606, AUDT608, AUDT620 and AUDT636.Out of eight Pseudomonas and Actinobacterial strains AUDP209 and AUDT636 performed very well in terms of both germination percentage and seedling vigour index and was selected for synthesis of zinc nanoparticles and used in further experiments.

2.1.1 Biosynthesis of ZnNPs using pseudomonas strain AUDP209

Pseudomonas strain AUDP 209 bacterial cells were inoculated into Erlenmeyer flasks, each containing 100 ml of King's B broth. The flasks were then placed in an incubator-shaker, set at 30°Cand 150 rpm for two days to allow bacterial growth. At the end of each incubation period, the liquid medium was centrifuged at 10,000 rpm for 15 minutes to remove cell debris. Subsequently, 60 ml of a zinc acetate dihydrate stock solution was introduced into the 20 ml supernatant solution. The flasks were then placed in an incubator-shaker at 150 rpm for 48 hours at 30 ºC. Then, the pH of the culture broth was adjusted to 10-11 by adding NaOH 1%. After the addition of NaOH, the flasks were kept in autoclave for 30 minutes.After that, the pellet was collected by centrifugation process at 10000 rpm for 5 minutes, which was subsequently washed three times with deionized water. The collected pellet was then dried in a hot air oven, and the dried powder was utilized for characterization.

2.1.2 Biosynthesis of ZnNPs using actinobacterial strain AUDT636

Actinobacterial strain AUDT 636 bacterial cells were inoculated into Erlenmeyer flasks, each containing 100 ml of starch casein broth. The flasks were then placed in an incubator-shaker, set at 30°Cand 150 rpm for seven days to allow bacterial growth. At the end of each incubation period, the liquid medium was centrifuged at 10,000 rpm for 15 minutes to remove cell debris.Subsequently, 85 ml of a zinc acetate dihydrate stock solution (1500 ppm) was introduced into the 15 ml supernatant solution. The flasks were then placed in an incubator-shaker at 150 rpm for 72 hours at 30 ºC. Then, the pH of the culture broth was adjusted to 10-11 by adding NaOH 1%. After the addition of NaOH, the flasks were kept in autoclave for 30 minutes.After that, the pellet was collected by centrifugation process at 10000 rpm for 5 minutes, which was subsequently washed three times with deionized water. The collected pellet was then dried in a hot air oven, and the dried powder was utilized for characterization.

2.2 Characterization of Biosynthesized ZnNPs

2.2.1 UV-Visible spectroscopy

The confirmation of nanoparticle reduction was achieved through UV-Visible spectrophotometer analysis at scanning wavelengths ranging from 200 to 700 nm, based on their optical absorbance peak. As the nanoparticles' size decreases, the band gap increases, leading to an elevation in optical absorbance compared to bulk particles. Consequently, their colour change. The biosynthesized ZnNPs initial sonication was using a solid probe ultrasonicator to ensure a uniform distribution of nanoparticles. Subsequently, the solution was diluted five times with deionized distilled water before spectrophotometric measurement, and an aliquot of the extract served as the control, with absorbance maxima recorded at room temperature.

2.2.2 Particle size analyzer

The average particle size of the NPs was measured using dynamic light scattering (DLS) in a high-performance particle-size analyzer. Before analysis, the sample was diluted and placed in disposable polystyrene cuvettes. The measurements were conducted at room temperature in triplicate, with a scattering angle of 90º.

2.2.3 Scanning electron microscope

The surface morphology of the zinc nanoparticles was analyzed through scanning electron

microscopy (SEM). The nanoparticles were affixed to an aluminum holder using carbon adhesive tape, and SEM images were captured using an EVO 18 microscope equipped with smart SEM software. Additionally, the presence of Zn ions was confirmed through energy-dispersive X-ray spectroscopy (EDS), which was attached to the EVO 18 scanning electron microscope. To examine the pellets obtained through centrifugation of the synthesized nanoparticles, the powder was evenly placed on an aluminum stub, coated with gold in a sputter coater, and observed using SEM at various voltages. The SEM was operated at a working distance of 8-9 mm and a voltage range of 5-10 kV.

2.2.4 X-ray diffraction

After reduction, the ZnNPs powder was subjected to X-ray diffraction (XRD) analysis to examine its crystal structure. The Rigaku TTR was employed for this purpose, operating at 40 KV and 150 mA, with a 2h scanning range between 10 and 90 degrees using the Cu Kα line (wavelength = 0.15406 nm). Furthermore, the particle sizes were determined using the Debye-Scherrer equation (1918).

$$D = \frac{k\lambda}{\beta cos\theta}$$

Where,

D = Average size of nano particles

k = Scherrer constant, (value 0.9)

λ= The wavelength of radiation (0.15406 nm)

β= Full width half maximum in radians (FWHM)

θ= Angle of diffraction

2.2.5 Fourier transform infrared spectroscopy

The biosynthesized ZnNPs powder was utilized for FTIR analysis using a Bruker TENSOR 27 FTIR spectrometer. To identify the existing functional groups in the samples, 25 mg of the particles were briefly dissolved and coated with KBr pellets. The spectra are recorded between 4000 and 400 cm^{-1} with a resolution of 4 cm^{-1}. The identified bands were then compared with the standard FTIR spectrum to characterize the functional components.

3. RESULTS AND DISCUSSION

3.1 Biosynthesis of ZnNPs Using Microbial Extracts

Out of eight Pseudomonas and Actinobacterial strains AUDP209 and AUDT636 performed very well in terms of both germination percentage and seedling vigour index and was selected for synthesis of zinc nanoparticles and used in further experiments.

3.1.1 Biosynthesis of ZnNPs by using microbial extract of Pseudomonasstrain AUDP209

The study involved shaking a mixture of 60 ml zinc acetate dihydrate and 20 ml AUDP209 extract in a rotary shaker for 48 hours, followed by autoclaving for 30 minutes to examine the synthesis of ZnNPs. The reduction was visually confirmed by observing the colour transformation from colourless to milky white, with the nanoparticles settling at the bottom of the flask (Fig. 1). The results are confirmed with Al-Janabi and Al-Kalifawi [13], observed the formation of a white precipitate at the bottom of a flask after incubating 50 ml of *P. aeruginosa* culture filtrate with 50 ml of 1 mM zinc sulphate at 37°C for three days. The zinc nanoparticles were biosynthesized by using microbial extracts of Pseudomonas [14].

3.1.2 Biosynthesis of ZnNPs by using microbial extract of actinobacterial strain AUDT636

ZnNPs were synthesized by shaking 85 ml of zinc acetate dihydrate and 15 ml of AUDT636 extract in a rotary shaker for 72 hours, followed by autoclaving for 30 minutes. The reduction was confirmed visually by monitoring the change in colour from colourless to milky white colour (Fig. 2) using Actinobacteria as reported by Sanjivkumar et al. [15].

3.2 Characterization of Biosynthesized ZnNPs

3.2.1 UV-Visible spectroscopy

The UV-visible spectrophotometer, which uses surface plasmon resonance to identify the maximum peak, is an efficient preliminary test to confirm the formation of nanoparticles (NPs). The maximum surface plasmon resonance in the

current investigation was found at 365 and 374 nm, respectively, where ZnNPs were biosynthesized through AUDP209 and AUDT636, confirming the presence of ZnO-NPs (Fig. 3). ZnNPs synthesized by *Streptomyces olivaceus* showed maximum peak between the wavelengths of 325 and 380 nm and were tested using a UV-Visible Spectrophotometer [16].

3.2.2 Particle size analyser

Particle size analyser works on the principle of DLS method. The dynamic light scattering method is a non-intrusive method for determining the size and distribution of nanoparticles dispersed in liquid. The DLS method was used to measure the hydrodynamic diameter-based time-dependent oscillation of scattered light in dispersed nanoparticles caused by Brownian motion [17]. The microbial extracts used in the study was prepared from AUDP209 and AUDT636, produced ZnNPs with mean diameters of 48.7 nm and 54.9 nm, respectively (Fig. 4). Eltarahony et al. [18] state that ZnO-NPs synthesised using the *Pseudomonas* strain have an average diameter of 48 nm. *Streptomyces plicatus* was used to biosynthesize ZnO-NPs, which were characterised by DLS method with a range of particle sizes and an average size of 22.4 nm. The size of the particles was determined by the amount of bacterial metabolites that had accumulated on the surface of the particles, which act as reducing, capping, and stabilising agents [19].

3.2.3 Scanning electron microscope

The surface morphology of NPs is determined using a SEM and the elemental composition of the NPs was also determined using EDS. In the present experiment, ZnNPs biosynthesised using AUDP209 and AUDT636 were characterised by SEM and EDS. SEM analysis showed spherical morphology (Fig. 5) and EDS showed elemental weight percentage of zinc was 70.80% and 58.20%, respectively and oxygen was 21.86% and 25.19%, respectively (Fig. 6). According to Rajivgandhi et al. [20], ZnNPs biosynthesised from actinomycetes have an irregular shape and a percentage elemental weight of 81.24% for zinc and 18.76% for oxygen. According to this EDS result, the final synthesised product is contamination free and pure. In order to synthesize ZnNPs, Wafyet al. [21] used actinomycetes, ZnONPs was spherical and uniform shape, and the Zn atom's existence was confirmed by the observed peaks at 1.03 and 8.63 KeV. The elemental analysis showed that the biosynthesized material is in its most pure form, and no evidence of contaminants were found. Jayabalan et al. [14] used *Pseudomonas putida* for the biosynthesis of spherical ZnNPs.

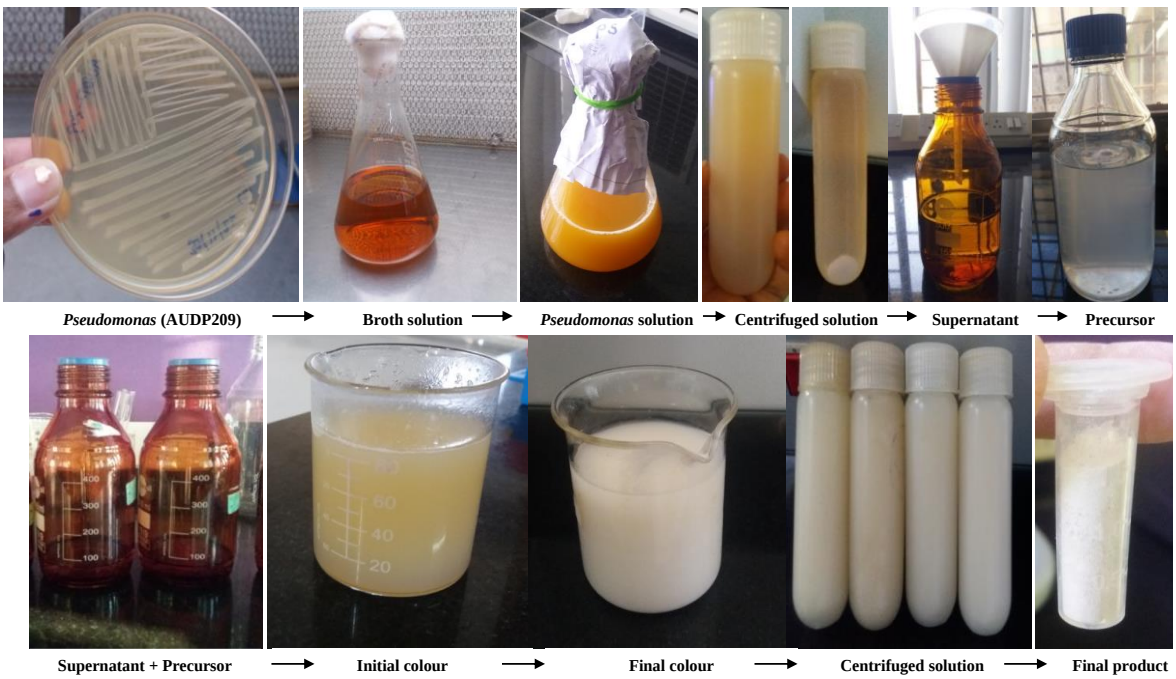


Fig. 1. Biosynthesis of zinc nanoparticles using Pseudomonas strain AUDP209

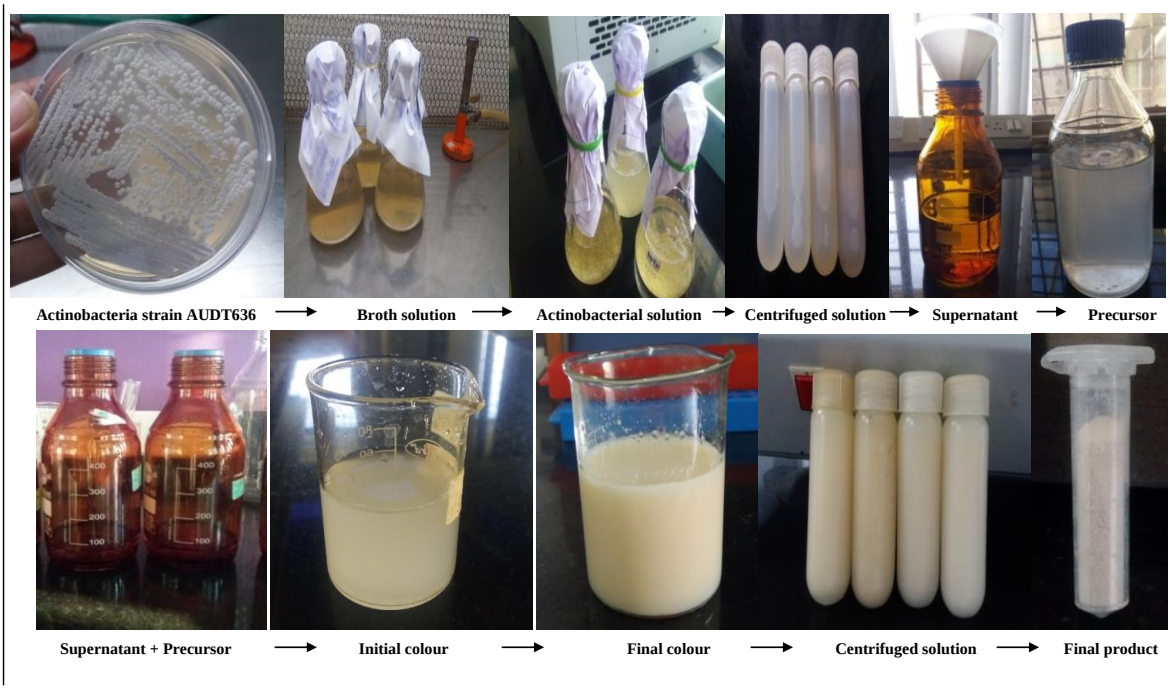


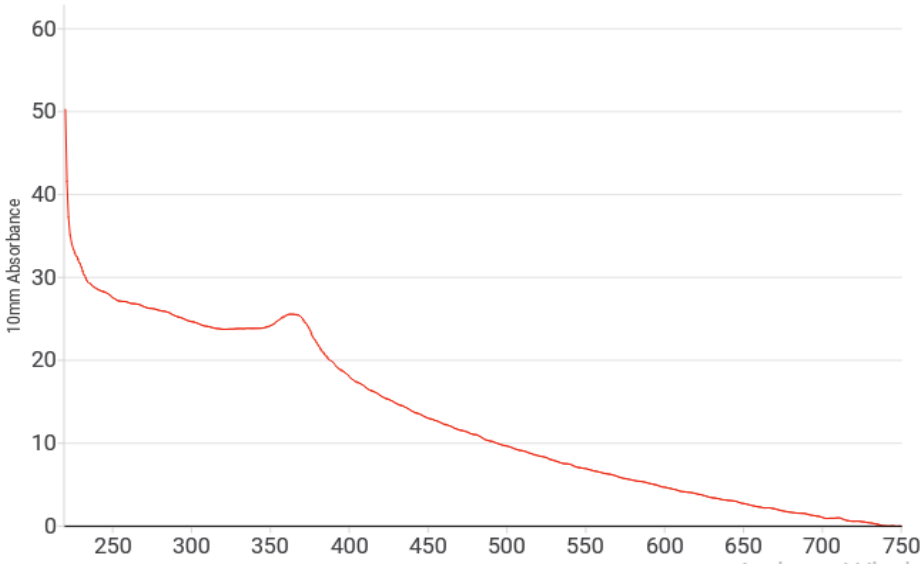
Fig. 2. Biosynthesis of zinc nanoparticles using Actinobacteria strain AUDT636

3.2.4 X-ray diffraction

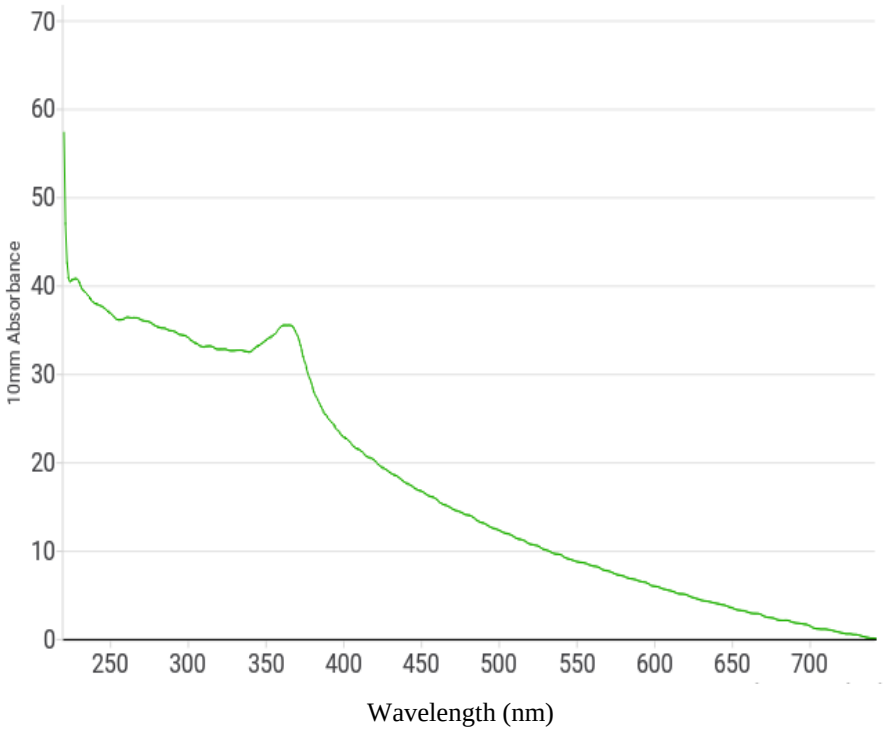
By using XRD, the crystalline nature of the synthesised ZnNPs was examined. In this study, the ZnNPs synthesised using AUDP209 and AUDT636 produced sharp peaks that were crystalline in form. Intense peaks were seen in ZnNPs synthesised from AUDP209 at 31.76°, 34.42°, 36.26°, 47.50°, 56.57°, 62.84°, 66.37°, 67.88° and 68.99° which corresponds to (100), (002), (101), (102), (110), (103), (200), (112) and (201) planes, respectively, Similarly, ZnNPs biosynthesized from AUDT636 microbial extract showed strong peaks at 31.83°, 34.48°, 36.31°, 47.65°, 56.59°, 62.88°, 66.41°, 67.97° and 69.08° corresponds to (100), (002), (101), (102), (110), 103), (200), (112) and (201) planes, respectively (Fig. 7). Abdo et al. (2021) stated that *P. aeruginosa* used to synthesise ZnNPs, and the XRD pattern showed strong diffraction peaks (100), (002), (101), (102), (110), (103), (112), and (201) at 2θ values of 31.5°, 34.2°, 36.3°, 47.3°, 56.4°, 62.7°, 67.3°, and 96.1°, respectively. XRD peaks obtained at 31.73°, 34.37°, 36.24°, 47.40°, 56.68°, 63.0° and 67.8° corresponding to the lattice plane of (100), (002), (101), (102), (110), (103) and (112) suggested the polycrystalline structure of the nanoparticles (Barsainya and Singh, 2018). Actinobacterial bioactive metabolites have an important role in the synthesis and stabilisation of ZnO-NPs. The unique XRD analysis peaks confirmed the microbial (*Streptomyces* spp.) synthesis of ZnO-NPs. Diffraction patterns were observed at the 2θ angles of 31.86°, 34.41°, 36.38°, 47.56°, 56.59°, 62.87°, 66.38°, 67.95°, and 69.15°, which were equivalent to (100), (002), (101), (102), (110), (103), (200), (112), and (201) planes, respectively Shanmugasundaram and Balagurunathan, [22].

3.2.5 Fourier transform infrared spectroscopy

The bioactive molecules responsible for reduction of Zn ions and the functional groups present in the material were identified and confirmed by FTIR ananlysis. ZnNPs biosynthesized through AUDP209 showed the absorption peaks at 2360 cm^{-1}, However, ZnNPs biosynthesized using AUDT636 showed the absorption peaks at 1581 cm^{-1}, 1419 cm^{-1}, 1014 cm^{-1}, 887 cm^{-1}, 771 cm^{-1}, 675 cm^{-1}, 648 cm^{-1} (Fig. 8). According to Singhet al. [23] the FTIR results showed peaks which were observed at 1632.67 cm^{-1} denoted the –C =O and –COO stretching vibrations of carbonyl group, respectively. Thepeaks at 800 cm^{-1} confirmed the presence of ester carbonylgroups, which was corresponded to the presence of-C-O deformationvibrations. A sharp IR band at wavenumber 1581.1 cm^{-1}, assigned to C=O stretching of the secondary amide (amide II). It clearly indicated the involvement of a protein/peptide moiety during the interaction



a) *Pseudomonas* strain AUDP209

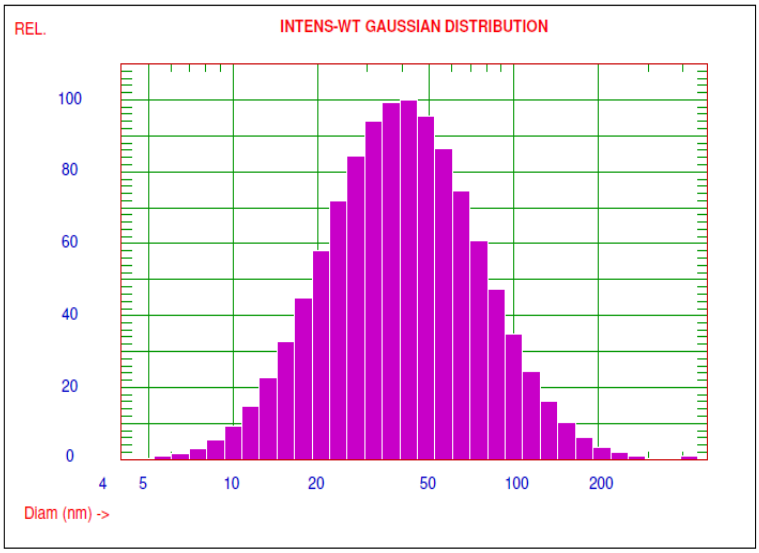


b) Actinobacteria strain AUDT636

Fig. 3. Characterization of biosynthesized ZnNPs from *Pseudomonas* strain AUDP209 and Actinobacteria strain AUDT636 by UV-Visible spectrophotometer

of pyoverdine with zinc [24]. The peaks in the range from 900 to 400 cm^{-1} canbe attributed to the ZnO stretching mode, proving the creation and purity of the ZnO structure [19]. The peaks at 1638.59 cm^{-1} confirmed the amide band led primarily to bendingvibrations of the (NH) C=O

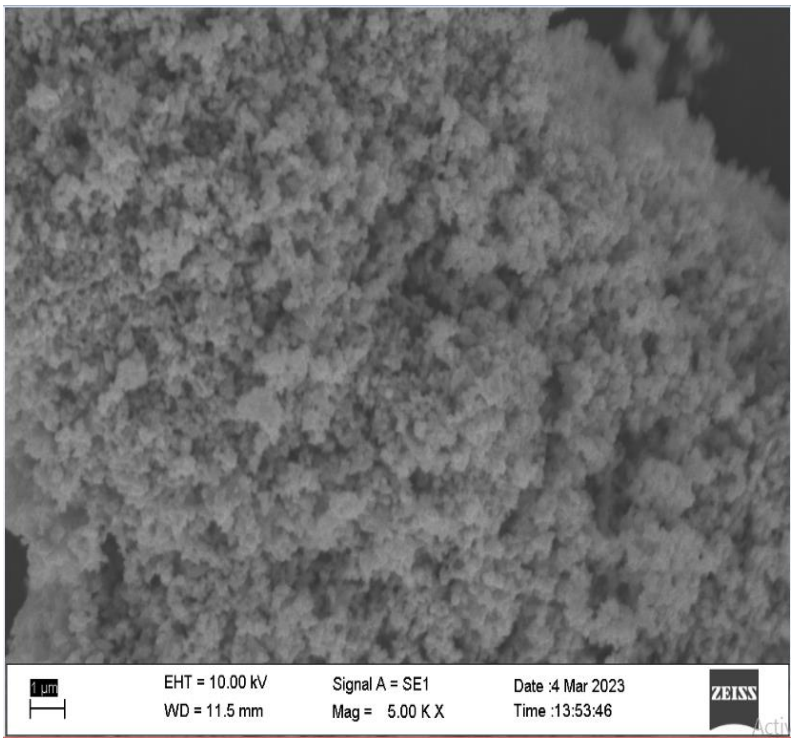
group arising due to the carbonylstretch in proteins. The peak observed at 2360.20 cm^{-1}, 2341.12 cm^{-1} and 2067 cm^{-1} which might be assigned for alkyne –C≡C or cyano–C≡N groups [25]. The FTIR spectrum of synthesized ZnONPs showed strong absorption peaks at 3740.43 which assigned to O-H stretching of alcohols and phenols. The weaker band at 1414.80 cm^{-1} corresponded to alkanes (C-H bending), this result confirms that the bioactive metabolites of actinobacteria involve formation and stabilization of ZnONPs [26]. Rajeswaran et al. [27] revealed that carbonyl, amide, alkane and alkyl groups follow the tendency to bind with metal particles. This supported in forming a layer on the metal NPs, which was ensurestheir stabilization and agglomeration. The FTIR spectrum peak at 675.09 cm^{-1} implied alkenes with C=Hstretch. In FTIR spectrum peak shown at 895 cm^{-1} might be corresponding to stretching S=O of sulfoxide and bending C=C of alkene [28]. The FTIR spectra confirmed the presence of zinc oxide formation of peak at the region between 500 and 600 $cm^{-1}$is related to the ZnO bond [29,30].



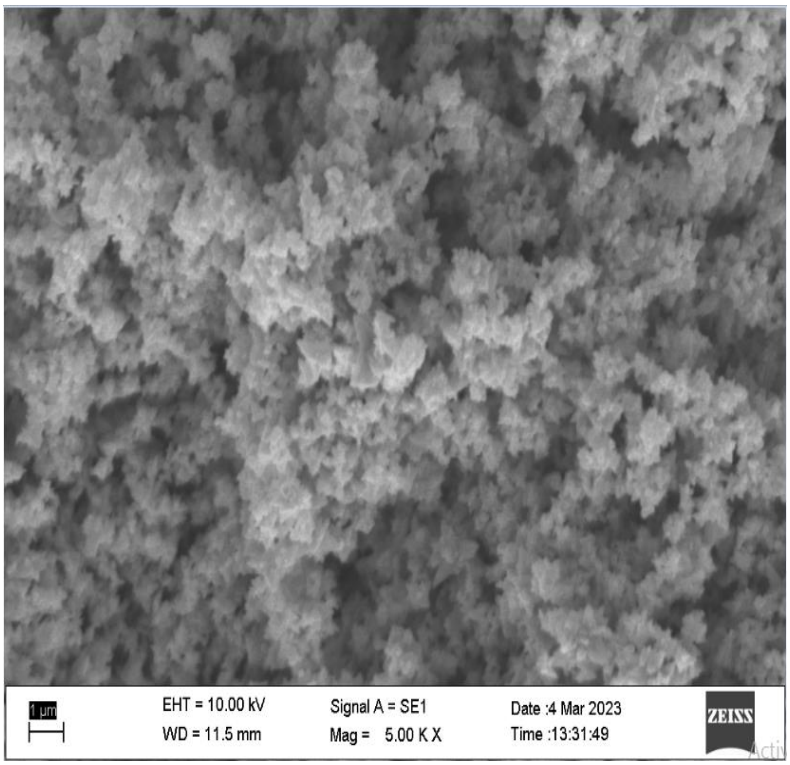
a) *Pseudomonas*strain AUDP209

b) Actinobacteria strain AUDT636

Fig. 4. Characterization of biosynthesized ZnNPs from *Pseudomonas* strain AUDP209 and Actinobacteria strain AUDT636 by particle size analyzer

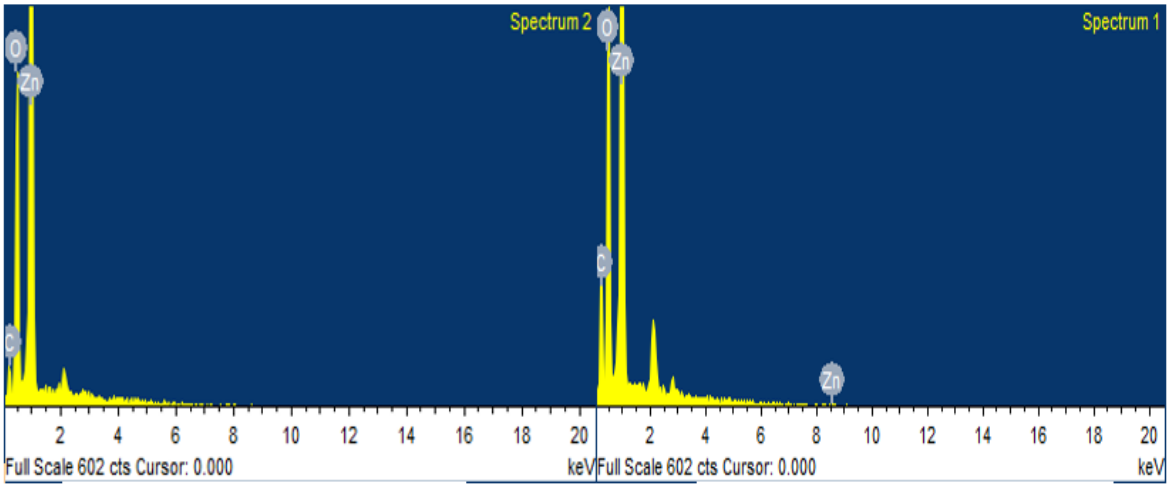


a) *Pseudomonas* strain AUDP209



b) Actinobacteria strain AUDT636

Fig. 5. Characterization of biosynthesized ZnNPs from *Pseudomonas strain* AUDP209 and Actinobacteria strain AUDT636 by scanning electron microscope



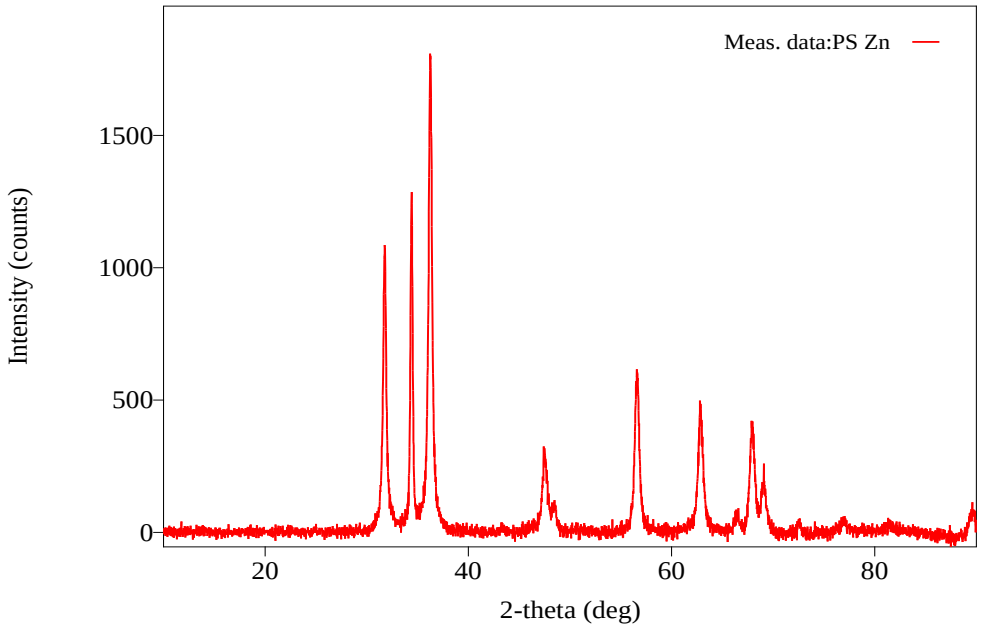
a) *Pseudomonas*strain AUDP209

Element	Weight (%)	Atomic (%)
C K	7.34	19.96
O K	21.86	44.65
Zn L	70.80	35.39

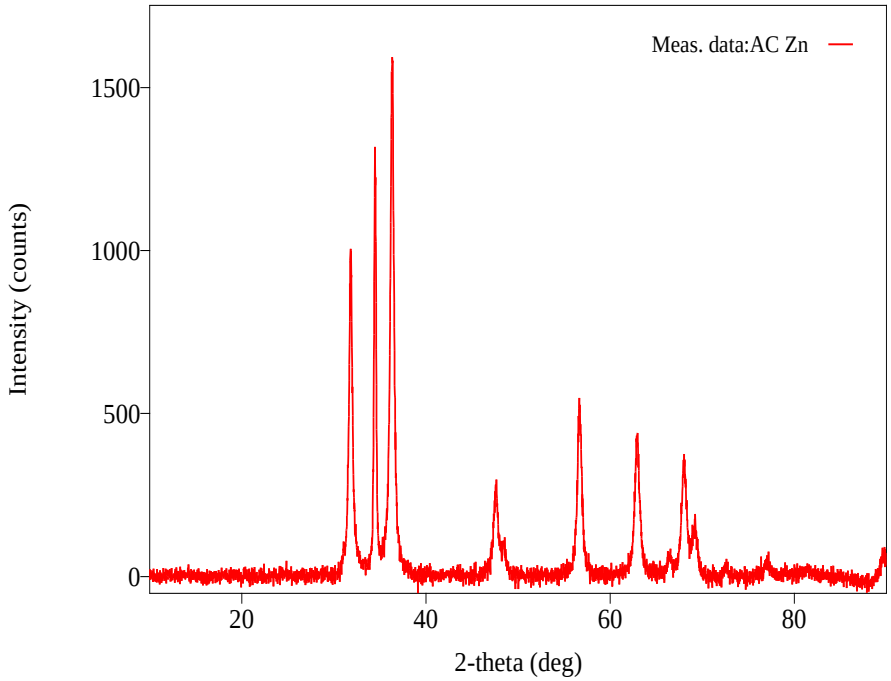
b) Actinobacteria strain AUDT636

Element	Weight (%)	Atomic (%)
C K	16.61	35.95
O K	25.19	40.91
Zn L	58.20	23.14

Fig. 6. Characterization of biosynthesized ZnNPs from *Pseudomonas*strain AUDP209 and Actinobacteria strain AUDT636 by energy-dispersive X-ray spectroscopy

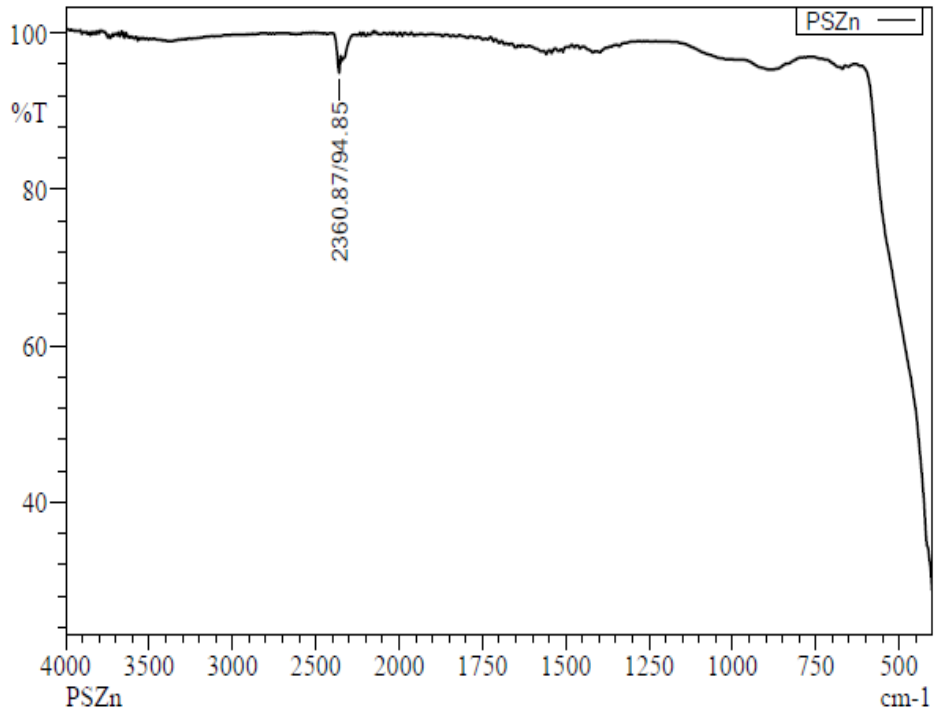


a) *Pseudomonas*strain AUDP209

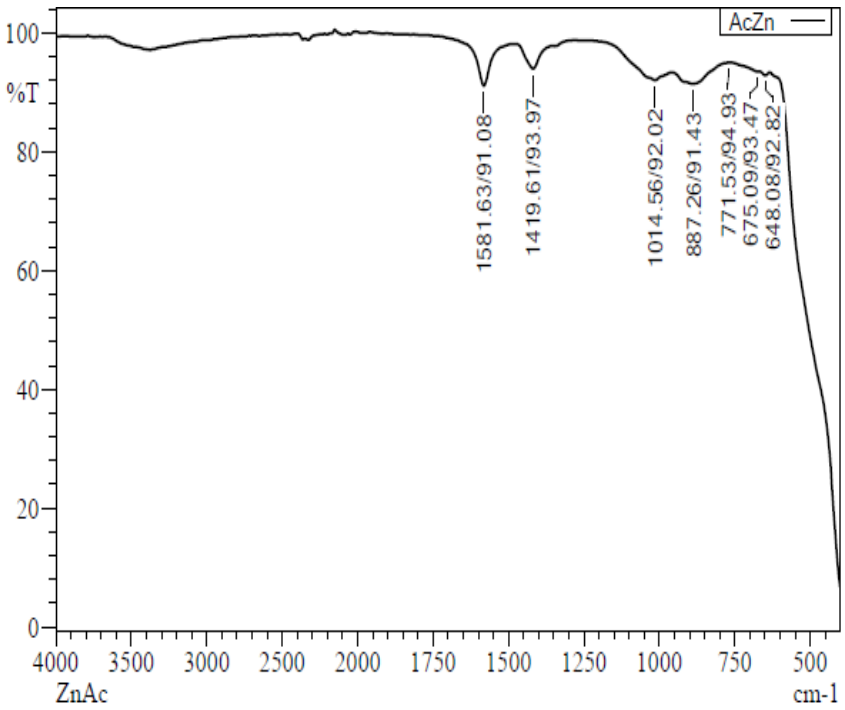


b) Actinobacteria strain AUDT636

Fig. 7. Characterization of biosynthesized ZnNPs from *Pseudomonas*strain AUDP209 and Actinobacteria strain AUDT636 by X-ray diffraction



a) *Pseudomonas* strain AUDP209



b) Actinobacteria strain AUDT636

Fig. 8. Characterization of biosynthesized ZnNPs from *Pseudomonas* strain AUDP209 and Actinobacteria strain AUDT636 by fourier transform infrared spectroscopy

4. CONCLUSION

Biosynthesis of NPs using microorganisms is considered to be an environmentally friendly approach. The organic compounds present in the microbial extracts of actinobacteria and *Pseudomonas* act as both capping and stabilizing agents for synthesis of ZnNPs. The formation of the ZnNPs was preliminary confirmed by the change in the colour of the reactant mixture, Later the synthesized samples were subjected to the UV-Visible Spectroscopy, where the ZnNPs biosynthesized through *Pseudomonas* strain AUDP209 and Actinobacteria strain AUDT636 produced a strong absorption peak at 365 and 374 nm, respectively, Particle size analyser revealed the average size of the zinc nanoparticles biosynthesized through *Pseudomonas* strain AUDP209 and actinobacteria strain AUDT636, to be 48.7 nm and 54.9 nm, Respectively, SEM analysis revealed a spherical morphology of ZnNPs biosynthesised from AUDP209 and AUDT636. In addition, EDS showed that the elemental weight percentage of zinc was 70.80% and 58.20%, respectively, and that of oxygen was 21.86% and 25.19%, respectively. The sharp peaks obtained in the XRD diagram of the ZnNPs revealed the crystalline nature of these NPs. Finally, FTIR spectroscopy confirmed the organic compounds present in the microbial extracts responsible for the encapsulation and stabilisation of these biosynthesised ZnNPs.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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