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Biosynthesis characterization and antibacterial activity of bioiron nanoparticles synthesized by *Pseudomonas putida* strain LUA 15.1

Neha Sharma, Himani Sharma, Praveen Khatri and Poonam Shirkot

Abstract
Bioiron nanoparticles have been used as antimicrobial agents, bioremediation of waste material, effluent detoxification, decolorization of dyes and pesticides degradation. In present investigation, *in vitro* synthesis of bioiron nanoparticles by *Pseudomonas putida* LUA 15.1 was carried out by using various optimization parameters. Optimum condition parameters for maximum synthesis of bioiron nanoparticles was selected which included nutrient broth containing 5g/l peptone, 3g/l beef extract, 3g/l NaCl and 2.0mM $FeSO_4$. Nutrient broth (pH; 8.0) using optimum condition was used for *in vitro* synthesis of bioiron nanoparticles at 35°C for 24 hrs of incubation. Nanoparticles synthesis indicated by change in colour from pale yellow to orange was confirmed by UV–Vis spectroscopy with absorbance peak obtained between 300-550 nm. Characterization of bioiron nanoparticles was carried using FTIR, XRD DLS and SEM. These bioiron nanoparticles found to be monodispersed as well as in aggregates with triangular/square/rectangular/rod shaped with size range of 75nm and FTIR spectrum revealed presence of alchol-phenol, alkenes, aromatic, alkane, alkyl halide, amine, and ether biomolecules and these were tested for antibacterial activity against two bacterial plant using agar well diffusion test producing significant zones of inhibition against bacterial pathogens after 24 hrs of incubation.

Keywords: Bioiron nanoparticles, *Pseudomonas putida*, optimization and antibacterial activity

Introduction
Nanotechnology is a fastest growing technical field involving design, characterization, production and application of structures, devices and systems by controlled manipulation of size and shape at the nanometer scale that produces structures, devices and systems with at least one novel property. Nanotechnology or nanoscience is defined as, the production or use of materials and structures so that at least one of their dimensions in the range of 1–100 nm [1, 4]. Nanoparticles or nanomaterials are of interest because at this scale unique optical, magnetic, electrical, and other properties emerge. Engineered nanomaterials are resources designed at the molecular level to take advantage of their small size and novel properties which are generally not seen in their conventional bulk counterparts. The two main reasons emerged why materials at the nanoscale can have different properties are increased relative surface area and new quantum effects which can lead to greater chemical reactivity and affect their strength. The use of microorganisms to synthesize functional nanoparticles has been of great interest recently [8]. The ability of microorganisms to change oxidation state of metals and their microbial processes has opened up new opportunity to explore novel applications such as biosynthesis of metal nanomaterials. In contrast to chemical and physical methods, microbial processes for synthesizing nanomaterials can be achieved in aqueous phase under gentle and environmentally benign conditions [7]. Bioiron nanoparticles occupy an important place among nanomaterials due to their enormous applications [6] ranging from catalysis to electronics to biomedics. Among the vast range of oxide nanoparticles, bioiron oxide nanoparticles like magnetite and maghaemite are unique due to their technological interest. Magnetic bioiron oxides are a versatile class of material that enables a wide range of technologies, many of which are contingent with their distinct magnetic properties[5].These bioiron nanoparticles find applications as catalysts, sorbents, pigments, flocculants, coatings, gas sensors, ion exchangers and for lubrication [2].
Nanoparticles synthesized either biologically or chemically must be characterized in order to understand their intrinsic properties such as size, monodispersity, aqueous stability, net charge, adsorption to biomolecules, aggregation and flocculation in various media using

Scanning electron microscopy (SEM), FTIR spectroscopy, X-Ray diffraction, Dynamic light scattering, UV-Vis spectroscopy. Bioiron is a known antimicrobial agent and it has been observed that Bioiron released ions, which reacted with the thiol group (-SH) of the proteins present on the bacterial surface. Such proteins protruded through the bacterial cell membrane, allowing the transport of nutrients through the cell wall [9]. Nanomaterials inactivated the proteins, decreasing the membrane permeability and eventually causing cellular death. In the present study bioiron nanoparticles were synthesized by *Pseudomonas putida* LUA 15.1. For the synthesis of bioiron nanoparticles process, synthesis medium and cultivation conditions play critical role because these affect formation, concentration and yield of a particular end product. Therefore it was found important to consider the optimization of medium and process conditions in order to maximize the profits from fermentation process and various physical factors which include incubation time, temperature, pH, concentration of components of nutrient broth and substrate concentration.

Materials and methods

Procurement, maintenance and characterization of bacterial culture

Bacterial culture *Pseudomonas putida* LUA 15.1 was procured from laboratory of Department of Biotechnology, Dr. Y S Parmar University of Horticulture and Forestry Nauni-Solan (H.P.) which was one of the 409 bacterial isolates isolated from paddy fields and paper mills of Himachal Pradesh. The bacterial isolate was maintained using nutrient agar medium at pH 7.0 followed by its colony, microscopic, biochemical and molecular characterization.

Quantitative evaluation of *Pseudomonas putida* strain LUA 15.1 for bioiron nanoparticles synthesizing ability

Pseudomonas putida strain LUA 15.1 was assessed for its ability to synthesize bioiron nanoparticles. One percent (1%) inoculum (overnight culture) of *Pseudomonas putida* strain LUA 15.1 was inoculated into the 50 ml nutrient broth followed by incubation at 37 ºC for 24 hrs**.** Supernatant of *Pseudomonas putida* strain LUA 15.1 was collected by centrifugation at 10000 rpm for 10 mins at 4ºC (Cooling Centrifuge REMI CM-8 Plus) to investigate extracellular synthesis of bioiron nanoparticles, an aqueous solution of $FeSO_4$ ranging from 1.0-3.5 mM was treated with 50 ml of bacterial supernatant in 250 ml Erlenmeyer flask. The whole mixture was incubated at temperature ranging from 20-45 °C at 150 rpm (Incubator Shaker RIVOTEK) for 24-120 hrs. Formation of bioiron nanoparticles was indicated by the colour change of the solution followed by measuring the O.D at 300~600nm wavelength (Spectronic 20, Milton Roy Company) (Sundram *et al*., 2012).

Optimization of culture conditions for growth and synthesis of bioiron nanoparticles by *Pseudomonas putida* strain LUA 15.1

The culture conditions for *Pseudomonas putida* strain LUA 15.1are optimized to obtain higher yields of their important products. *Pseudomonas putida* strain LUA 15.1 depicting maximum bioiron nanoparticles synthesizing activity was further investigated to study effect of different factors such as incubation time (24-120 hrs), temperature (20-45 ^{0}C), pH (6.0-11), peptone concentration (0.0-12.5g/l), beef extract concentration (0.0-7.5g/l) NaCl concentration (0.0-10 g/l), $FeSO_4$ concentrations (1.0-5.0mM), and inoculum size (1-5%) on bioiron nanoparticles synthesis as well as growth of *Pseudomonas putida* strain LUA 15.1.

In vitro synthesis of bioiron nanoparticles by *Pseudomonas putida* strain LUA15.1

Preparation of bacterial culture

One percent concentration of inoculum (overnight culture) was inoculated into 100 ml nutrient broth and incubated at optimum temperature for optimum incubation period. The supernatant was collected by centrifuging the culture broth at 10000 rpm, 4 ºC for 10 min (Cooling Centrifuge REMI CM-8 Plus).

Bioiron nanoparticles synthesis by selected bacterial strain

Fifty ml of supernatant was mixed in 50ml of optimum concentration of $FeSO_4$ prepared with double distilled water and incubated at optimum conditions of temperature incubation period. Formation of bioiron nanoparticles was indicated by colour change of the solution, which was confirmed using spectrophotometer (Spectronic 20, Milton Roy Company). The bioiron nanoparticle suspension obtained was used for further experiments.

Lyophilization of bioiron nanoparticle suspension

For the preparation of powdered form of bioiron nanoparticles the process of lyophilization was used, 100 ml of bioiron nanoparticle suspension was prepared in a sterilized conical flask at 37 ºC for 24 hrs using various optimized conditions. The nanoparticle suspension was transferred to petriplates which was placed in the lyophlizer chamber (BIOGENTEK (I) Pvt Ltd) for subsequent lyophilization involving freezing the bioiron nanoparticle solution at -30 ºC for 48hrs and then reducing the surrounding pressure to allow the frozen water to sublime directly from liquid phase to gas phase. Thus finely powdered bioiron nanoparticles were obtained.

Characterization of bioiron nanoparticles

Bioiron nanoparticles synthesized by *Pseudomonas putida* strain LUA 15.1 culture were analyzed using various techniques such as UV- visible spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), Dynamic Light Scattering (DLS) and Scanning Electron Microscopy (SEM).

UV- visible spectroscopy

The optical characteristics of the biosynthesized bioiron nanoparticles were analysed using UV–Vis spectrophotometer. For this, bioiron nanoparticles solution was subjected to absorption analysis at 300–550 nm range using UV–Vis spectrophotometer (Perkin Elmer Lambda 2, USA).

Fourier Transform Infrared Spectroscopy

Microcup was washed with 100% absolute ethanol. Ten microlitre sample was filled in a 2 mm internal diameter microcup and loaded onto the FTIR set at 26 °C±1 °C. The sample was scanned in the range of 4,000 to 400 cm−1 using a Fourier transform infrared spectrometer (Thermo Nicolet Model 6700, Waltham, MA, USA). The spectral data obtained were compared with the reference chart to identify the functional groups present in the sample.

Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) technique has been used to determine the size of particles by measuring the intensity of peaks which depends on the size distribution of particles. The

detector was put into 90° position. Five drops of toluene were placed into the center hole of the XY-translation stage. Sample was placed in glass test tube into the center hole of the XY-translation stage. The sample was filled into test tube in a way so that the liquid fills ~75% of the test tube and spectra was taken using software.

Scanning electron microscope

Sample was prepared for SEM analysis by separating thebiomass from the liquor by centrifugation and washed twicein sterile distilled water. The sample was fixed for 1 hr in 2.5% gluteraldehyde in 0.075 M phosphate buffer (pH 7.4), followed by 3 washes in 0.075 M phosphate buffer. After a second fixation step of 1 hr in 1% osmium tetroxide, the cells were washed in distilled water. The cell pellet was subjected to dehydration with 30, 50, 70% ethanol, followed by three dehydration steps in 100% ethanol. Infiltration of the resin was carried out by placing the pellet in 30% Quetol in ethanol for 1 hr, followed by 1 hr in 60% Quetol. After centrifugation, the pellet was resuspended in 100% Quetol for 4 hrs before polymerisation at 65 °C for 24 hrs. Ultrathin sections were not stained prior to analysis, due to possible interference of the stain with the bioiron nanoparticles. SEM analysis were done on a JSM 6100. For SEM analysis of the cell-free extract, a drop of the sample was placed onto a carbon-coated coppergrid. After about a minute, the extra solution was removed using blotting paper and the grid air-dried before analysis. Elemental analysis on single particles was carried out on an air-dried, carbon coated sample using an energy dispersive spectroscopy (EDS) attachment on a Jeol 6100 LV scanning electron microscope using the following instrument conditions: accelerating voltage of 20 keV and counting time of 100 secs.

Antibacterial activity of bioiron nanoparticles preparations against bacterial pathogens.

Bioiron nanoparticles possess appreciable potential of antimicrobial activity against bacterial pathogens. The following pathogenic bacteria were obtained from the Departments of Basic Sciences, Microbiology laboratory and Plant Pathology Dr. Y.S. Parmar UHF, Nauni**,** Solan**.** Two bacterial plant pathogens procured were *Ralstonia solanacearum and Xanthomonas axonopodis,*

Standardization of optimum concentration of bioiron nanoparticles

Bioiron nanoparticles synthesized by *Pseudomonas putida* strain LUA15.1 were standardized for antibacterial activity against various plant and a human pathogens. Nutrient agar was prepared and was poured into sterile plates followed by solidification. Through spread plate technique plates bacterial culture (1.0ml) was transferred on to solidified agar plate and spread using sterilized L shaped glass rod under aseptic conditions. Wells were made in the solidified agar plate by using a sterile borer of size 250 mm. Different concentrations of bioiron nanoparticle preparation i.e. 20, 40, 60, 80 and 100 mg/ml were prepared and an aliquot of 50 µl was poured in each well with the help of sterile pipette followed by refrigeration of plates for one hour for diffusion and then incubated at 37 ^{0}C for 24 hrs. Zone of inhibition was measured in centimetres using measuring scale in each plate.

Results and discussion

Procurement, maintenance and characterization of bacterial culture

Bacterial culture was procured and maintained using nutrient agar medium (Plate-1). Morphological characterization showed bacteria was creamish in color, 0.75 mm in size, smooth texture, raised elevation, entire margin and microscopic characterization depicted Gram negative nature, rod shaped, arranged in single and clusters and nonsporulating (Plate-2). Biochemical characterization of bacterial culture revealed that bacterial strain was found to be positive for Oxidase, Citrate utilization test, Ornithine utilization test, Nitrate reductase, H2S production, Glucose, Aldonitol, Arabinose and negative for Lysine utilization test, urease, phenylalanine deaminase, lactose and Sorbitol. On the basis of morphological and biochemical characterization this bacteria isolates was confirmed to belong to the genera *Pseudomonas* as per Bergey's Manual of Systemetic Bacteriology. Molecular characterization was carried out using 16S *rrna* gene technology. Total genomic DNA of selected bacterial isolate LUA15.1 was extracted and amplified using PCR technology. Universal primer B27F and U1492R for 16S *rrna* gene was used and after 35 cycles of PCR amplification produced an amplicon of expected size of 1500 bp. To determine the nucleotide sequence of PCR product of 16S *rrna* gene of bacterial isolate LUA15.1, sequencing was carried out using same upstream and downstream primers which were used for amplification and as a result, the size of the 16S *rrna* gene sequence of selected bioiron nanoparticles synthesizing bacterial isolate LUA15.1was found to be 1476 bp. BLASTn search of selected bioiron nanoparticles synthesizing bacterial sequence with the most similar 16S *rrna* gene sequences of the Gen Bank database revealed that this bacteria showed 99% similarity with *Pseudomonas putida* strain KT2440, 16S ribosomal RNA, complete sequence. Phylogenetic tree also verified bacteria *Pseudomonas putida* as it clustered closely with *Pseudomonas putida* strain
KT2440, 16S ribosomal RNA complete sequence. Thus, based on molecular characterization, the isolate LUA15.1 was eventually confirmed as *Pseudomonas putida*.

Quantitative evaluation of *Pseudomonas putida* strain LUA15.1 for bioiron nanoparticles synthesizing ability

Pseudomonas putida strain LUA 15.1 was assessed for its ability to synthesize bioiron nanoparticles. One percent (1%) inoculum (overnight culture) of *Pseudomonas putida* strain LUA 15.1 was inoculated into the 50 ml nutrient broth followed by incubation at 37 ^{o}C for 24 hrs**.** Supernatant of *Pseudomonas putida* strain LUA 15.1 was collected by centrifugation at 10000 rpm for 10 mins at 4^{o}C and to investigate extracellular synthesis of bioiron nanoparticles, an aqueous solution of 2.0 mM $FeSO_4$ was treated with 50 ml of the supernatant. The whole mixture was incubated at 37°C at 150 rpm for 24-120 hrs. Formation of bioiron nanoparticles was indicated by the colour change of the solution from pale yellow to orange (Plate-3) which was confirmed by measuring the O.D at 350 nm with an OD value 1.417.

Optimization of culture conditions for maximum bioiron nanoparticles synthesis and growth of *Pseudomonas putida* strain LUA 15.1

Effect of incubation time

Effect of incubation time on growth of *Pseudomonas putida* strain LUA15.1 was monitored up to 120 hrs in nutrient broth and it has been observed that bacterial growth increased at fast rate depicting an OD of 1.952 at a wavelength of 540 nm during 24 hrs followed by decline in growth up to 120 hrs.

Since the bacterial isolate exhibited maximum growth OD of 1.952 at wavelength of 540 nm after 24 hrs, thus 24 hrs was selected as optimum incubation time for maximum growth of *Pseudomonas putida* strain strain LUA15.1. Effect of incubation time at different time intervals on bioiron nanoparticles synthesizing activity by *Pseudomonas putida* strain LUA15.1 was determined up to 120 hrs. It has been observed that bioiron nanoparticles synthesizing activity increased at a fast rate and maximum activity was observed at 24 hrs with an OD value of 0.859 at 350 nm and after that it was found to decrease up to 120 hrs (Fig-1a). Thus, maximum bioiron nanoparticle synthesis was achieved at an incubation time of 24 hrs and was selected as an optimum parameter for further experiments.

Effect of incubation temperature

A study on growth and bioiron nanoparticles synthesizing activity by *Pseudomonas putida* strain LUA15.1 was investigated at different temperatures ranging from 20-45 ^{o}C for 24 hrs. Bacterial growth was found to increase with increase in temperature up to 30 ^{o}C followed by decrease up to 45 ^{o}C. The bacterial strain showed a maximum growth OD value of 1.117 at 540 nm at an incubation temperature of 30 ^{o}C which was selected as an optimum incubation temperature parameter for maximum growth of *Pseudomonas putida* strain LUA15.1**.**

Bioiron nanoparticles synthesizing activity by *Pseudomonas putida* strain LUA15.1 also showed increase with increase in temperature however maximum activity with OD value of 0.788 at 350nm wavelength was obtained at 35 ^{o}C. Thus the optimum incubation temperature condition selected for maximum bioiron nanoparticles synthesis was 35 ^{o}C for 24 hrs (Fig-1b).

Effect of pH

A pH range of 6.0-11.0 of the nutrient broth was examined and persual of Table- 11 shows that growth of *Pseudomonas putida* strain LUA15.1 increased with increase in pH of the medium only up to pH 7.0 for 24 hrs at 30 ^{o}C followed by a gradual decrease up to pH 11.0. A maximum OD of 1.478 at pH 7.0 has been observed at 540 nm wavelength and thus pH 7.0 was selected as an optimum parameter for maximum growth of *Pseudomonas putida* strain LUA15.1.

Similarly synthesis of bioiron nanoparticles by *Pseudomonas putida* strain LUA15.1 was found to increase with increase in pH only up to 8.0 followed by gradual decline up to 11.0 for 24 hrs at 35 ^{o}C (Fig-1c). Maximum bioiron nanoparticles were synthesized at pH 8.0 with an OD value of 0.893. On the basis of results obtained pH 8.0 was selected as an optimum parameter to be used in further experiments.

Effect of peptone concentration

Different concentrations of peptone were investigated for growth as well as bioiron nanoparticles synthesis. Growth of *Pseudomonas putida* strain LUA15.1 was found to be maximum at peptone concentration of 5.0 g/l at 30 ^{o}C for 24 hrs and pH 7.0 with OD value of 0.924 at 540 nm wavelength. Similarly bioiron nanoparticles synthesis was also found to be maximum with 5.0 g/l of peptone at 35 ^{o}C for 24 hrs depicted by OD value of 0.678 at 350 nm (Fig-1d).

Effect of beef extract concentration

Different concentrations of beef extract were investigated for growth as well as bioiron nanoparticles synthesis. Growth of *Pseudomonas putida* strain LUA15.1 was found to be maximum when 3.0 g/l of beef extract was used, with an OD value of 1.410 at 30 ^{0}C for 24 hrs at 540 nm wavelength. Similarly bioiron nanoparticles synthesis was also found to be maximum with 3.0g/l of beef extract at 35 ^{0}C for 24 hrs depicted by OD value of 1.113 at 350 nm (Fig-1e).

Effect of NaCl Concentration

Evaluation of growth and bioiron nanoparticles synthesis was carried out at different concentrations of NaCl. Growth of *Pseudomonas putida* strain LUA15.1 was recorded at different NaCl concentration ranging from 0-10 g/l and it was found that growth of LUA15.1 depicted a maximum OD of growth 1.560 at 540 nm in nutrient broth containing 5.0 g/l NaCl at 30^{0}C for 24 hrs and further at higher concentration of NaCl the growth was found to decrease. Similar in case of bioiron nanoparticles synthesis, increase in concentration of NaCl, OD values decrease of showing maximum biosynthesis at a concentration of 5.0 g/l with OD value of 0.909 at 35 ^{0}C for 24 hrs at 350 nm (Fig-1f).

Effect of different concentration of ferrous sulfate

Effect of different concentrations of $FeSO_4$ are investigated on growth and bioiron nanoparticles synthesis. Growth of *Pseudomonas putida* strain LUA15.1 was observed at different $FeSO_4$ concentrations ranging from 1-3.5 mM at 30 ^{o}C for 24 hrs. The bacteria showed maximum growth OD of 1.499 at a wavelength of 540 nm using 2.0 mM concentration of $FeSO_4$ in nutrient broth and further at higher concentrations of $FeSO_4$, bacterial growth was found to decrease. In case of bioiron nanoaprticles synthesis, maximum activity was observed in nutrient broth containing 2.0 mM $FeSO_4$ with OD value of 1.417 at 35 ^{0}C for 24 hrs at 350 nm followed by decrease in bioiron nanoparticles activity up to 3.5 mM of $FeSO_4$ concentrations(Fig-1g).

Effect of inoculum size

Effect of inoculum size ranging from 1.0-5.0% was examined on growth and bioiron nanoparticles synthesis. Growth of *Pseudomonas putida* strain LUA15.1 increased with increase in inoculum size up to 3.0% with maximum growth OD of 1.513 at 30 ^{0}C for 24 hrs at 540 nm wavelength. Thus 3.0% inoculum size was found optimum for the maximum growth of *Pseudomonas putida* strain LUA15.1 at 35 ^{0}C for 24 hrs. Whereas in case of bioiron nanoparticles synthesis 3.0% inoculum size lead to maximum bioiron nanoparticles biosynthesis with an OD of 1.113 at 35 ^{0}C for 24 hrs at 350 nm(Fig-1h).

***In vitro* synthesis of bioiron nanoparticles by *Pseudomonas putida* LUA15.1**

Extracellular biosynthesis of bioiron nanoparticles by *Pseudomonas putida* strain LUA15.1 was carried out using the standardized and selected optimized parameters of incubation time, temperature, pH, peptone, beef and NaCl concentration, inoculum size and $FeSO_4$ concentration in the previous experiment. Three percent inoculum size was used to inoculate nutrient broth containing 5.0 g/l peptone, 3.0 g/l beef extract, 3.0 g/l NaCl, pH 7:0 for 24 hrs of incubation at 30 ^{0}C. The culture supernatant was obtained by centrifugation at 10000 rpm, 4 ^{0}C for 10 mins which was used for synthesis of bioiron nanoparticles. Ten millilitre of supernatant was mixed in 10 ml of 2.0 mM solution of $FeSO_4$ and incubated at 35 ^{0}C for 24hrs. Formation of bioiron nanoparticles was indicated by color change of the solution from pale yellow to

orange which was confirmed by measuring the O.D (1.417) at 350 nm.

Lyophilization of bioiron nanoparticle suspension

Powdered/lyophilized form of bioiron nanoparticles was obtained after subjecting 100 ml of bioiron nanoparticle suspension to a lyophlizer for lyophilization after 48 hrs of lyophilization at -30 ^{o}C powdered/ lyophilized bioiron nanoparticles were obtained. In present study 175 mg of bioiron nanoparticle powder was obtained from 100 ml of bioiron nanoparticle suspension (Plate-4).

Characterization of nanoparticles

Bioiron nanoparticles must be characterized in order to understand their intrinsic properties such as size, monodispersivity, aqueous stability, net charge, adsorption to biomolecules, aggregation and flocculation. This provides vital information in terms of application of these nanoparticles. Nanoparticles obtained were analyzed using various techniques such as UV- vis, FTIR, XRD, DLS and SEM.

UV- visible spectroscopy

Optical characteristics of the biosynthesized bioiron nanoparticles were analyzed using UV–vis spectrophotometer. Addition of 2.0 mM $FeSO_4$ to the supernatant of centrifuged aqueous solution led to the development of a orange solution after 24 hrs of reaction, indicating the formation of bioiron nanoparticles as shown in the UV-vis absorption spectrum. Ultraviolet spectroscopy confirmed reduction of Fe^{2+} to Fe^0, bioiron nanoparticles that can be identified from the peaks obtained between 300-550 nm and maximum absorbance has been observed at 350nm with value 5.5, which is the signature for the bioiron nanoparticle formation, apart from the color change(Fig-2a).

Fourier transform infrared spectroscopy (FTIR)

FTIR analysis of the reaction mixture has depicted about nature of biomolecules involved in formation of bioiron nanoparticles. FTIR spectrogram of reaction mixture has shown presence of eight bands, first one at a wavelength of 3287.66 cm^{-1} can be assigned for O-H stretching, depicting presence of alcohols and phenols, second stretching at a wavelength of 1655.09 cm^{-1} indicating C=C stretching of alkenes, third at 1547.97 cm^{-1} wavelength which can be assigned to C-C aromatic bending, fourth at 1450.03 cm^{-1} wavelength depicts C-H alkane bending whereas fifth at 1335.94 cm^{-1} can assigned to C-F alkyl halide bending, and sixth one at 1243.71 cm^{-1} wavelength can be assigned to C-N amine bending. Seventh at a wavelength 1027.34 cm^{-1} can be assigned to C-O ether bending and eighth at a wavelength of 552.56 can be assigned to C-Br alkyl-halide which is supposed to be involved in reduction of Fe^{2+} to Fe^0 (Fig-2b).

Powder X-ray diffraction

XRD study revealed crystalline nature of bioiron nanoparticles. The XRD pattern clearly showed that extracellular synthesis of bioiron nanoparticles formed by reduction of ferrous sulfate ions using *Pseudomonas putida* strain LUA15.1. Bioiron nanoparticles exhibited four prominent Bragg reflections at around 24.95^0, 32.75^0, 45.10^0 and 56.45^0. The fraction between the intensity of the (111), (400), and (422) diffraction pattern was found to be much lower, suggesting that the plane (220) is in predominant orientation. The XRD facets of the bioiron nanoparticles match with standard bioiron published by Joint Committee on Powder Diffraction Standards (JCPDS) (Fig-2c).

Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) technique has been used to determine the hydrodynamic diameter of bioiron nanoparticles by measuring intensity of peaks which depends on the size distribution of particles in aqueous phase. DLS analysis revealed that maximum bioiron nanoparticles size distribution was 75 nm with an intensity of 30% (Fig-2d).

Scanning electron microscope

The sample of bioiron nanoparticles synthesized by *Pseudomonas putida* strain LUA15.1 was examined using scanning electron microscopy to study morphology of biosynthesized bioiron nanoparticles. Scanning electron micrograph revealed that bioiron nanoparticles of *Pseudomonas putida* strain LUA15.1 were found to monodispersed as well as in aggregates with triangular/square/rectangular/rod shaped (Fig-2e).

Antibacterial activity of bioiron nanoparticles synthesized by *Pseudomonas putida* LUA15.1 against bacterial plant pathogens

Bioiron nanoparticles showed variable zones of inhibition against two bacterial pathogens after 24 hrs of incubation. In case of *Ralstonia solanacearum*, bioiron nanoparticles solution preparation showed 1.5 cm zone of inhibition whereas 0.68cm zone of clearance was observed against *Xanthomonas axonopodis* (Plate5-a,b).

Plate 1: Procured culture of *Pseudomonas putida* LUA 15.1

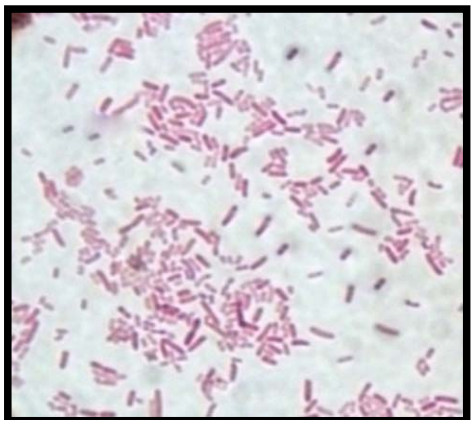
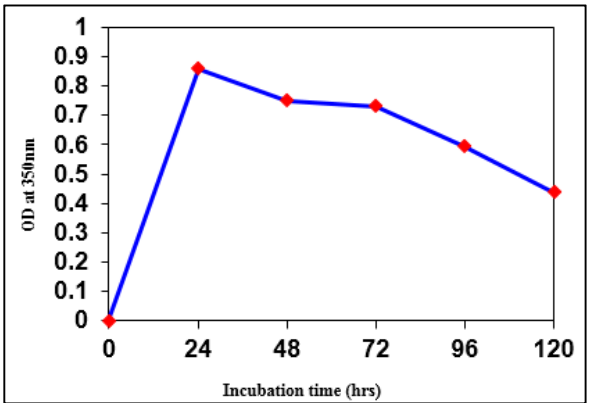


Plate 2: Microscopic characteristics of *Pseudomonas putida* LUA 15.1

Plate 3: Colour change from pale yellow to orange
(A) Control **(B)** Iron nanoaparticles solution

Plate 4: Lyophilized bioiron nanoparticle powder



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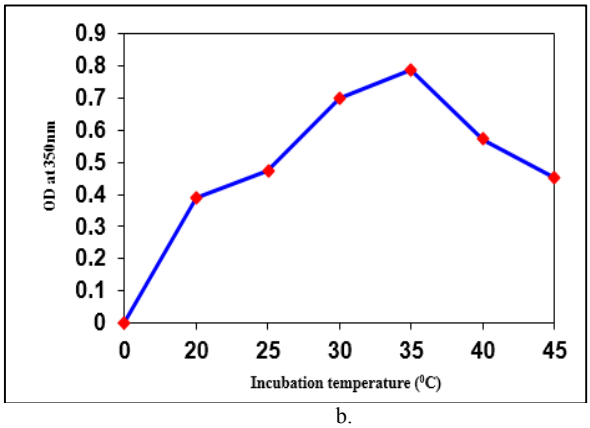

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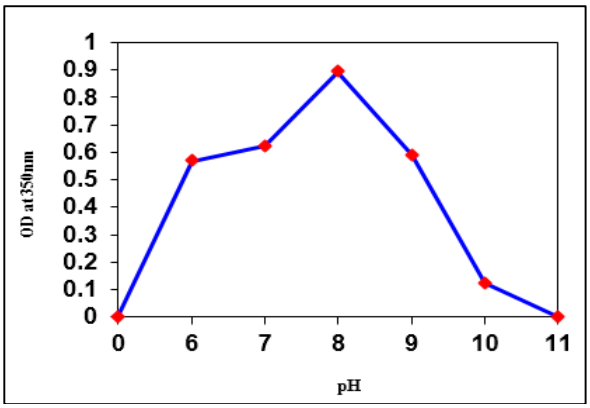
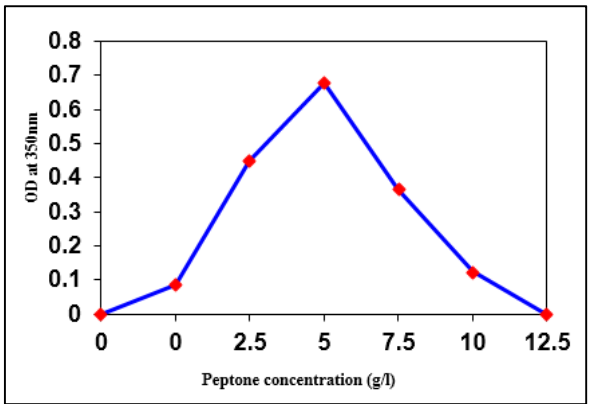
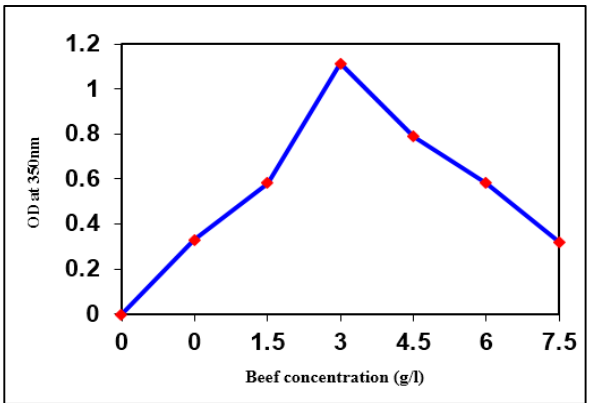


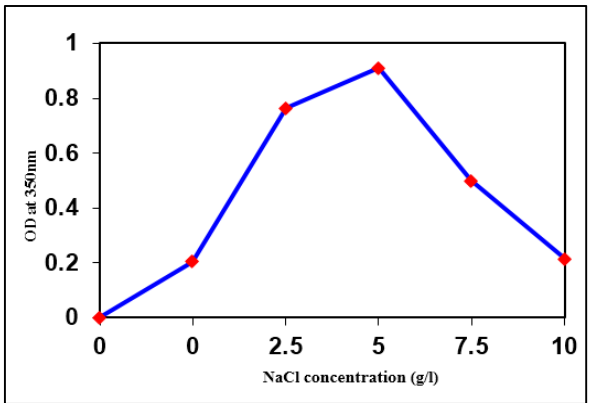
c.



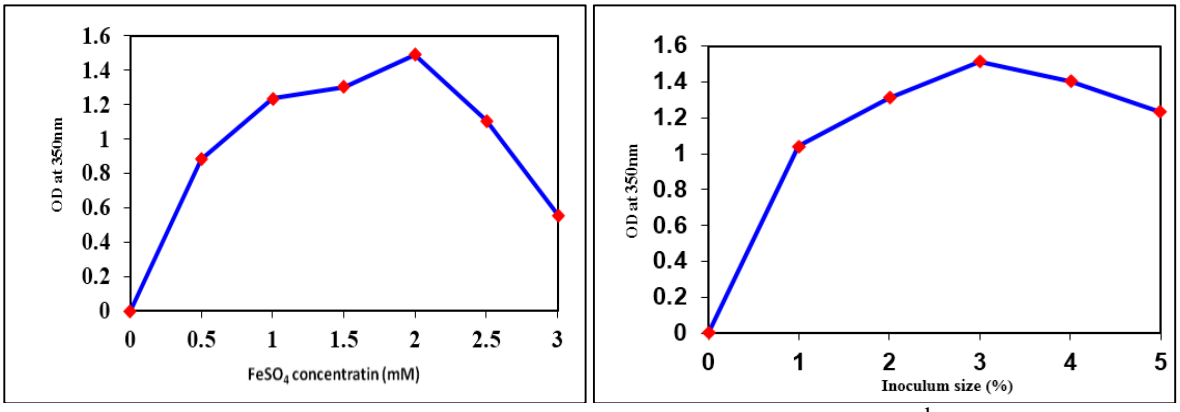
d.



e.

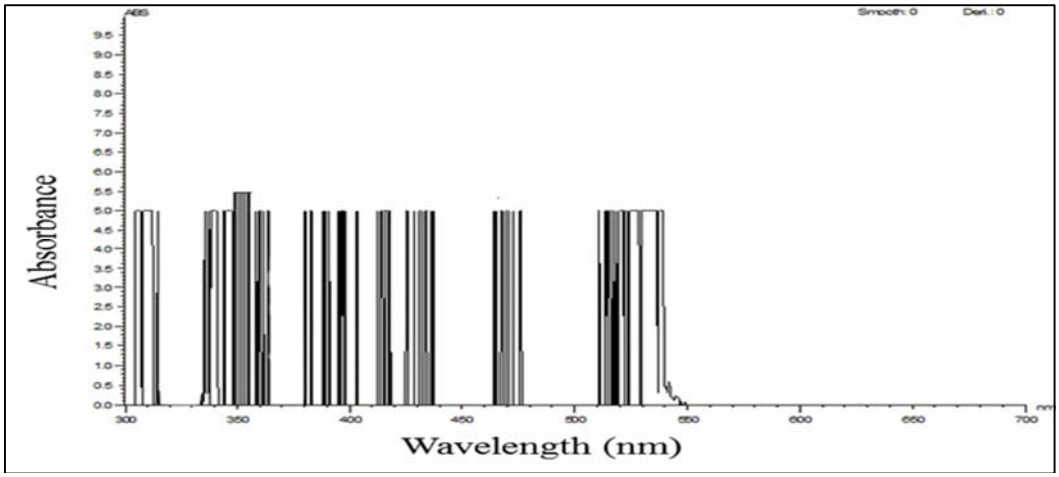


f.

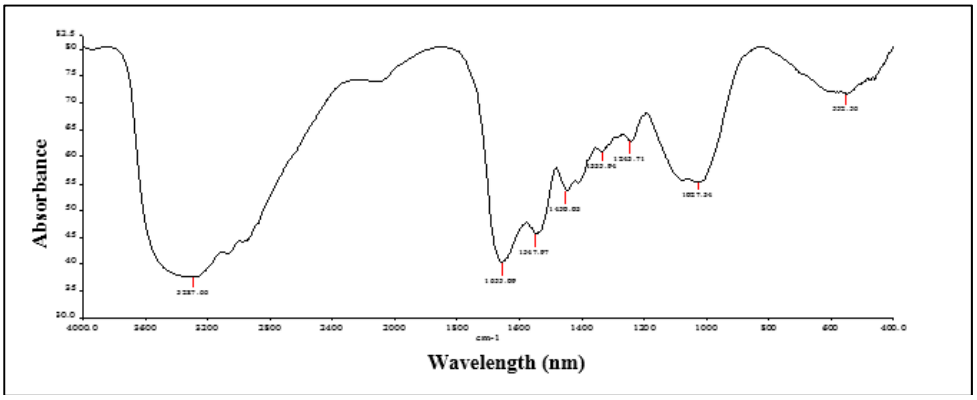


g. h.

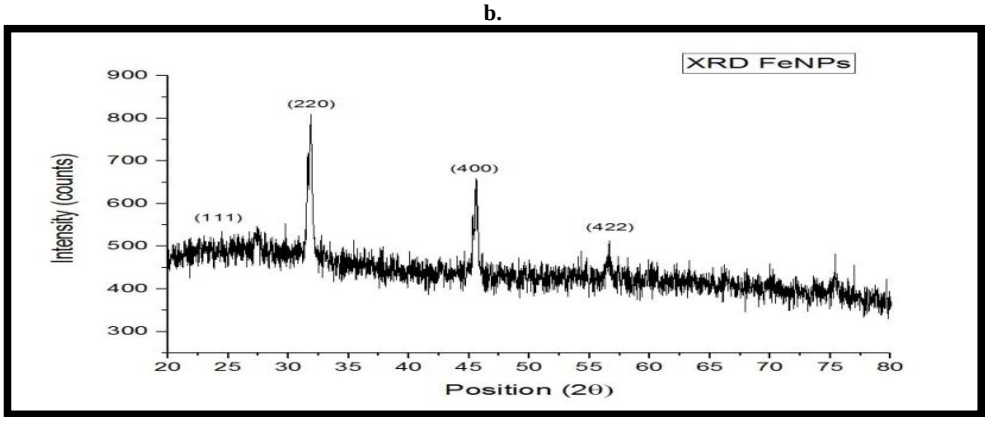
Fig 1: Effect of (a) Incubation time (b) Temperature (c) pH (d) peptone concentration (e) Beef extract concentration (f) NaCl concentration (g) $FeSO_4$ concentration (h) Inoculum size on bioiron nanoparticles synthesis by *Pseudomonas putida* LUA 15.1. Inoculum size



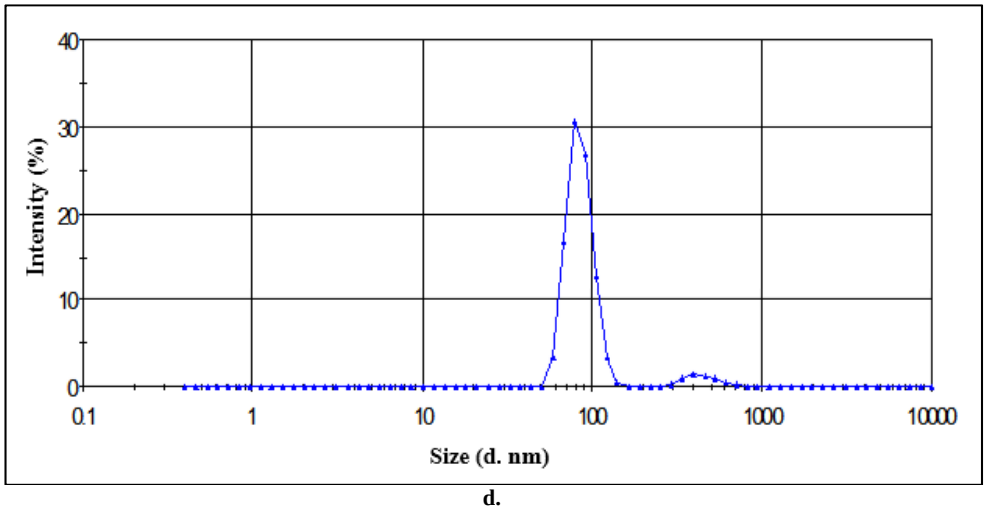
a.



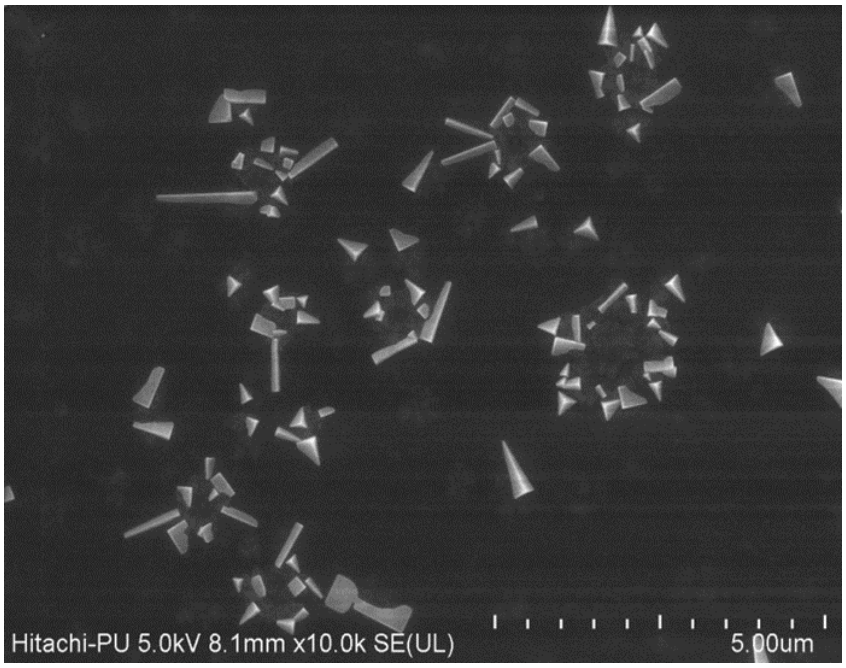
b.



c.

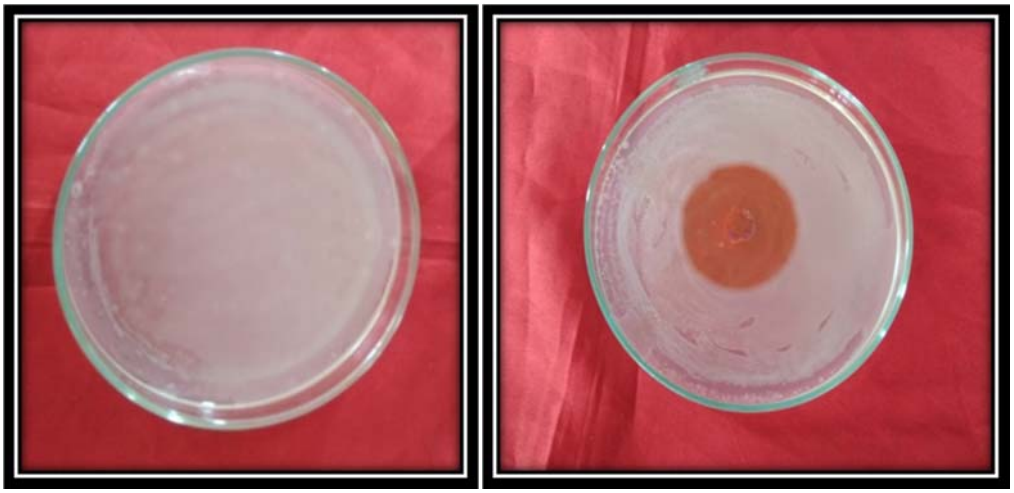


d.



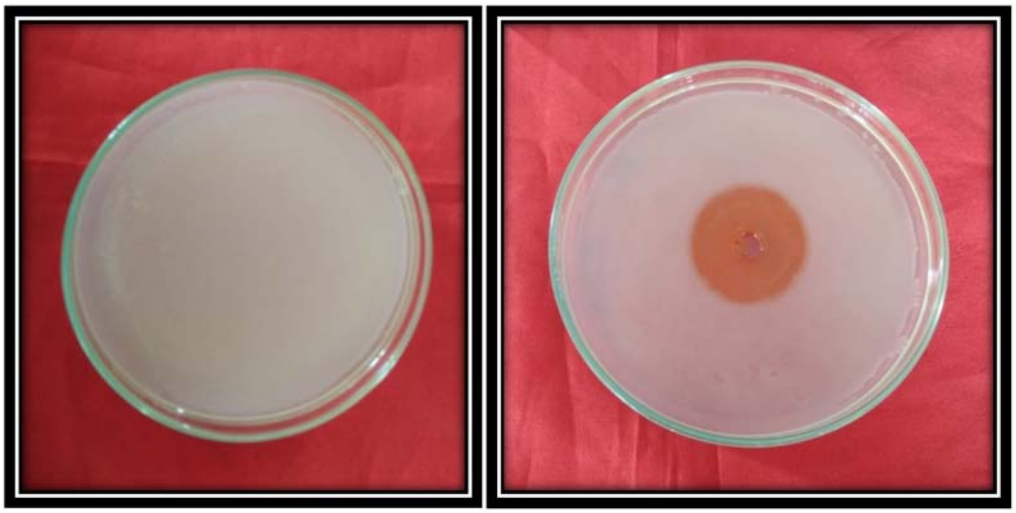
e.

Fig 2: Characterization of bioiron nanoparticles synthesized by *Pseudomonas putida* LUA 15.1 (a) UV- Vis spectroscopy (b) FTIR (c) XRD (d) DLS (e) SEM



Control **Treatment**

A



B

Plate 5: Antibacterial activity of bioiron nanoparticles synthesized by *Pseudomonas putida* LUA 15.1 against various bacteria using agar well diffusion method (a) *Ralstonia solanacearum* (b) *Xanthomonas axonopodis*

Conclusion

In the present study monodispersed as well as in aggregates with triangular/square/rectangular/rod shaped spherical bioiron nanoparticles of 75nm size synthesized by *Pseudomonas putida* LUA15.1 and maximum bioiron nanoparticles was achieved at 35^0C, pH 8.0 after 24 hrs of incubation with 2.0mM Ferrous Sulfate, 5.0% peptone, 3% beef extract and 3% inoculum size. Bioiron nanoparticles has emerged as potential agent able to inhibit important plant pathogenic bacteria *viz., Ralstonia solanacearum and Xanthomonas axonopodis,* using agar well diffusion method. After 24 hrs of incubation bioiron nanoparticles showed maximum zone of inhibition against *Ralstonia solanacearum* which was found to be 1.5 cm under *in vitro* condition, though further evaluation is required to be done under *in vivo* condition.

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