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Mycosynthesis of iron nanoparticles by *Alternaria alternata* and its antibacterial activity

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Nanotechnology is one of the most emerging fields in the recent years. In the current investigation, we report the biosynthesis of iron nanoparticles (Fe-NPs) employing *Alternaria alternata* fungus, which is an eco-friendly process for the synthesis of metallic nanoparticles. Fe-NPs were synthesized through the reduction of aqueous Fe^{3+} ion in the dark conditions. Ultraviolet–visible spectrum of the aqueous medium containing iron ion showed a peak at 238 nm and another peak at 265 nm. The forming of nanoparticles was confirmed by transmission electron microscope, scanning electron microscope and energy-dispersive x-ray. The morphology of nanoparticles is found to be cubic shapes mostly and the average particle diameter as determined by scanning electron microscope was found to be 9±3 nm. Fe-NPs showed antibacterial activity against both Gram-positive and Gram-negative bacteria used in this study due to its oxidative damage for bacterial cell wall. Iron nanoparticles show more antimicrobial activity to *Bacillus subtilis* than *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Key words: Mycosynthesis, *Alternaria alternata*, iron nanoparticles, antibacterial.

INTRODUCTION

Nanobiotechnology is defined as the study of biological phenomenon at nanosize scale. Generally, Nanotechnology comprises the study of materials that are less than 100 nm in size. Recently, the study of nanosize particles has gained much attention due to their unique size-dependent properties and their various applications (Habeeb, 2013). The advantages in nanotechnology make us to apply the concepts in a variety of fields. The unique characteristics of nanomaterials over their macroscaled counter-parts gave them high importance in a lot of valuable applications due to their altered physical and chemical properties (Feynman, 1991). In addition to their special physical and chemical characteristics, they showed unusual optical, photoelectrochemical and electronic properties (Peto et al., 2002). The problems with the physical and chemical methods used for the production of nanoparticles such as: short time stability and safety issues can be solved by the use of other biosynthetic methods such as the use of microorganisms. Many organisms can produce either extracellular or

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intracellular inorganic substances (Mann, 1996). Magnetite nanoparticles are examples of nanoparticles produced by unicellular magnetotactic bacteria (Lovley et al., 1987). The use of fungi in biosynthesis of nanoparticles was discovered recently. Fungi are eukaryotes which are characterized by production of large amounts of enzymes. They are relatively more complicated than prokaryotes in terms of genetic manipulation when used in over expression of enzymes included in nanoparticles biosynthesis. Ultraviolet-visible spectra analysis of biologically synthesized iron nanoparticles in a culture media of *Pleurotus* sp. fungus that grow in $2x10^{-4}$ M $FeSO_4$ solutions for 72 h showed formation of nanoparticles at 226 and 276 nm wavelength. Transmission electron microscope images reflect depositions of particles in both inside as well as outside indicating the biosynthesis process. X-Ray fluorescence of control samples do not shows any iron elements but was present in treated mycelium (Mazumdar and Haloi, 2011). Gold nanoparticles were formed by *Verticillium* fungus that was exposed to 10^{-4} M $HAuCl_4$ solution which was indicated by appearance of purple colour in the fungus biomass (Mukherjee et al., 2001a).

The mechanism of gold nanoparticles biosynthesis by *Verticillium* sp. includes the electrostatic interaction between ions and enzymes carboxylate groups at first, followed by reduction of ions by the enzymes which are present in the cell wall of the fungus mycelia resulting in nuclei formation which grow and accumulate more and more forming these nanoparticles (Mukherjee et al., 2001b). The emergence of infectious diseases in general poses a serious threat to public health worldwide, especially with the emergence of antibiotic-resistant bacterial strains. Generally, both Gram-positive and Gram-negative bacterial strains are thought to present a major public health problem. Over the years, antibiotics have been used to control infections resulting from both community and hospital environments (Lowy, 1998; Komolafe, 2003; Hawkey, 2008). *Staphylococcus aureu*s is one of the most common human pathogens, and leads to many types of infection (Grinholc et al., 2008). *S. aureus* is also known to possess an increasing ability to resist antibiotics (such as penicillin, methicillin, tetracycline, erythromycin, and vancomycin) (Jevons, 1961; Hiramatsu, 2001).

Thus, it is necessary to find an alternative treatment (perhaps without the use of antibiotics) for bacterial infection and bacterial pollution. Iron nanoparticles are used recently over a wide range in different applications such as the use of metallic nanoparticles especially, as potential antimicrobials (Tran et al., 2010). This antimicrobial property of iron nanoparticles can be used in many applications such as in water treatment, food processing, textiles industry and, construction, medicine and food (Vasilache et al., 2011). Advances in nanotechnology are producing an accelerated proliferation of new nanomaterial composites that are likely to become an important source of engineered health-related products. Nanoparticles with antifungal effects are of great interest in the formulation of microbicidal materials (Nuñez-Anita et al., 2014). Metallic nanoparticles with antibacterial properties represent a promising alternative approach to antibiotics. Their complex mechanism of action influences different bacterial structures and gives them advantages compared to antibiotics with more specific mechanism of action. Targets of nanoparticles are outer and inner bacterial structures cell wall, plasma membrane, proteins, and DNA (Kon and Rai, 2013).

In this study we report the biologically synthesized iron nanoparticles by *A. alternata* fungus. The antibacterial activity of iron NPs were tested against Gram-positive and Gram-negative bacteria.

MATERIALS AND METHODS

Fungus

A. alternata (RCMB-009002) used for the synthesis of Fe-NPs, was procured from The Regional Centre for Mycology and Biotechnology, Al-Azhar University, Egypt.

Growth culture and synthesis of Fe-NPs

Fungal biomass of *A. alternata* used for biosynthetic experiments was grown in liquid medium containing (g/l): KH_2PO_4 7.0, K_2HPO_4 2.0, $MgSO_4.7H_2O$ 0.1, $(NH_4)_2SO_4$ 1.0, yeast extract 0.6, glucose 10.0. Erlenmeyer flasks were inoculated with spores and incubated at 28ºC with shaking (150 rpm) for 72 h. After the incubation, the biomass was filtered (Whatman filter paper No. 1) and then extensively washed with distilled water to remove any medium component. Fresh and clean biomass was taken into Erlenmeyer flasks containing 100 ml of Milli-Q water. The flasks were agitated at the same conditions as described above, then the biomass was filtered again and cell-free filtrate was used in experiments. Two hundred and fifty (250) millilitre of iron (III) nitrate (1 mM of final concentration) was mixed with 1 g of harvested and washed mycelia of *A. alternata* was suspended and plugged with cotton then incubated at 28°C in dark with shaking (150 rpm) for 72 h. Control (without fungus) was also run along with the experimental flasks. After incubation time fungal mycelia was removed by centrifugation at 4,000 rpm for 10 min and the resulting supernatant was used for further analytical studies for extracellular Fe-NPs (Mazumdar and Haloi, 2011).

Characterization of Fe-NPs

The preliminary detection of Fe-NPs was carried out by visual observation of colour change of supernatant. These samples were later subjected to optical measurements, which were carried out by using a UV-visible spectrophotometer (Unicam-UV2 UV/Vis Spectrometer) and scanning the spectra between 200 and 800 nm at the resolution of 1 nm. A scanning electron microscope (SEM) (JEOL JSM-5500LV) was used to characterize the structure properties of the synthesized iron nanoparticles. The element composition of the synthesized materials was identified by energy dispersive X ray microanalysis system (EDX) (Module Oxford 6587 INCA x-sigh) coupled to the SEM at 17 KV after gold coating using SPI-Module sputter coater. The morphology and particles size of

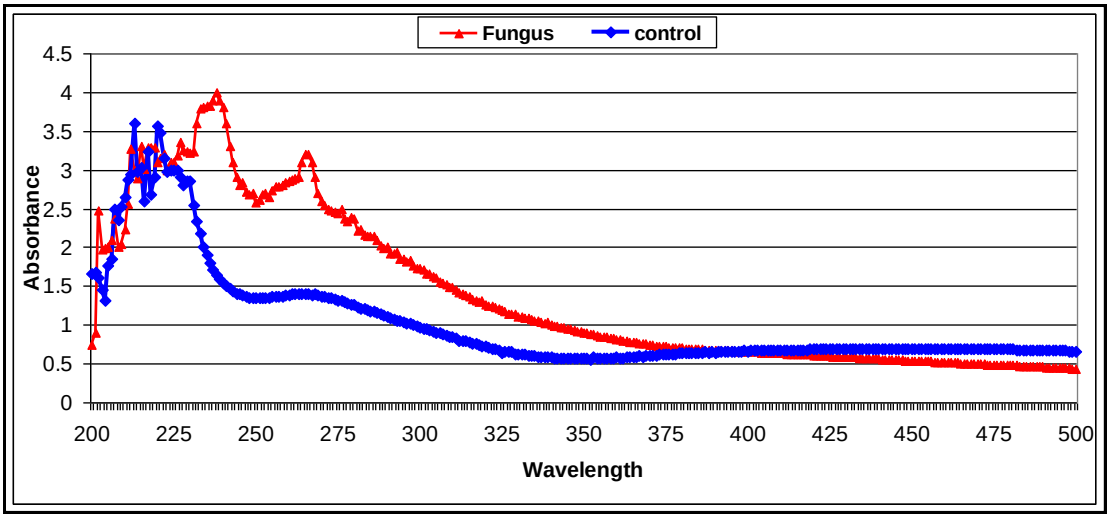

Figure 1. UV-Vis spectra of iron nanoparticles biologically synthesized by fungus.

the resulting nanoparticles were analyzed using transmission electron microscope (TEM) (Jeol JEM 1200 EXII) connected to a high resolution imaging system. Samples for TEM studies were prepared by placing drops of the iron nanoparticles solutions on carbon-coated TEM copper grids.

Determination of antibacterial activity of Fe-NPs by well-diffusion method

Antimicrobial activities of the synthesized iron nanoparticles were performed against both Gram-negative (*Escherichia coli* RCMB 0100052 and *Pseudomonas aeruginosa* RCMB 0100043) and Gram-positive (*Bacillus subtilis* RCMB 010067 and *Staphylococcus aureus* RCMB 0100028) bacteria. The antibacterial activity was done by modified Kirby-Bauer well diffusion method (Azam et al., 2012). In brief, the pure cultures of organisms were subcultured in Müller-Hinton broth at 35±2°C on a rotary shaker at 160 rpm. For bacterial growth, a lawn of culture was prepared by spreading the 100 µL fresh culture having 10^6 colony-forming units (CFU)/mL of each test organism on nutrient agar plates with the help of a sterile glass-rod spreader. Plates were left standing for 10 min to let the culture get absorbed. Then 6 mm wells were punched into the nutrient agar plates for testing nanomaterial antimicrobial activity. Wells were sealed with one drop of molten agar (0.8% agar) to prevent leakage of nanomaterials from the bottom of the wells. Using a micropipette, 100 µL Fe-Nps suspension was poured onto each well on all plates. After overnight incubation at 35±2°C, the different levels of zone of inhibition were measured. Antibiotics Gentamicin and Ampicillin were used as a positive control for Gram-negative and Gram-positive bacteria respectively.

TEM observations of treated *Bacillus subtilis*

Bacterial cells of both treated and untreated samples were observed under TEM (JEOL 1010, Japan). The samples after centrifugation and washing were fixed in 25% glutaraldehyde/ paraformaldehyde in cacodilate buffer at room temperature for 1 h. Then samples were fixed in 1% osmium tetraoxide. Sample embedding was carried out using a standard protocol (Croft, 1999) and 60 nm thick slices were cut with a diamond knife (LBR ultratome III). The slices were deposited on bare 200 mesh copper grids, and stained with 2 wt% uranyl acetate for 5 min. Finally, the grids were dried in a desiccator and examined using TEM, for study biocidal action of nanoparticles and any morphological changes, and then photos were taken by special digital camera (Canon, Japan).

RESULTS AND DISCUSSION

The biosynthesis of iron nanoparticles was carried out by exposure of a precursor salt aqueous iron (III) nitrate solution of 1 mM concentration of fungal cell-free filtrate obtained by incubating the fungus *A. alternata* (RCMB-009002) in an aqueous solution. The reaction was carried out at 28°C in dark with shaking (150 rpm) for 72 h. The mycelium growth was found to be slow and there was a gradual change in colour of medium as well as the mycelium. Oxidation process of ferric (Fe^{3+}) ions may be takes place in culture solutions. Iron nanoparticle was green synthesized by fungus *Aspergillus oryzae* TFR9 using $FeCl_3$ as a precursor metal salt (Tarafdar and Raliya, 2013). The efficacy of silver synthesized biolarvicide with the help of entomopathogenic fungus, *Beauveria bassiana*, was assessed against the different larval instars of dengue vector, *Aedes aegypti* (Banu and Balasubramanian, 2014).

The absorption in the visible range directly affects the perceived colour of the chemicals involved. In this region of the electromagnetic spectrum, molecules undergo electronic transitions. The UV-visible spectrum of Fe-NPs in fungus media supernatant is shown in Figure 1. The two absorption peaks at wavelengths of 238 and 265 nm indicate the formation of iron nanoparticles. Iron oxide nanoparticle shows the peak at 222 nm (Pal, 2014). The analysis of iron nanoparticles that forming by *Pleurotus* sp. under UV-vis spectrophotometer showed nearly peaks at wavelengths 226 and 276 nm (Mazumdar and

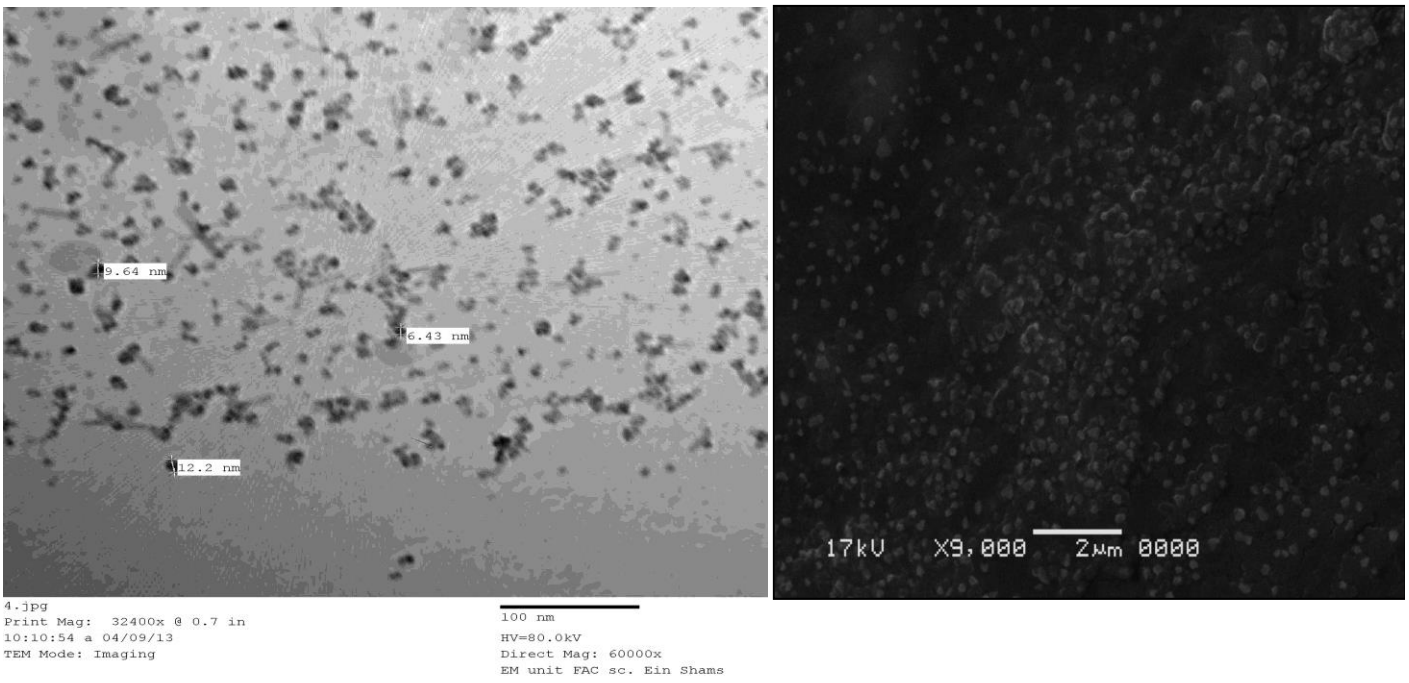

Figure 2. TEM and SEM images for synthesized iron nanoparticles.

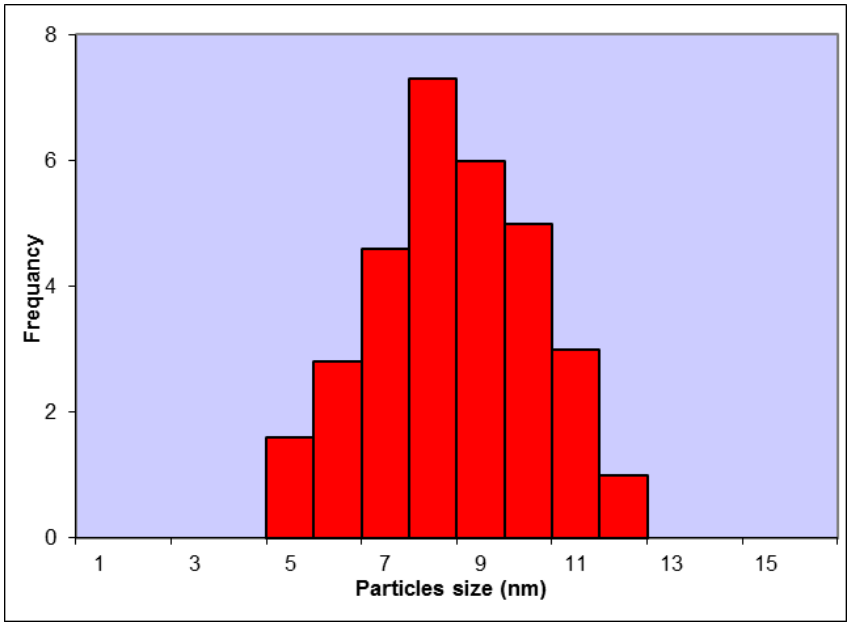

Figure 3. Particles size distribution of synthesized iron nanoparticles.

Haloi, 2011). On the other hand, two absorption peaks of Fe-NPs that synthesized by *Sargassum muticum* aqueous extract were introduced at wavelengths 402 nm and 415 nm (Mahdavi et al., 2013) this shifts in peaks of nanoparticles may be due to size of particles.

The TEM and SEM images for produced Fe-NPs showed smaller particles in form of cubic shapes, while the average particle diameter as determined by TEM was found to be 9±3 nm, where particles size ranged from 5.4 to 12.1 nm (Figures 2 and 3). However, the mean diameter average size of iron oxide nanoparticles synthesized by bioreduction of ferric chloride solution with

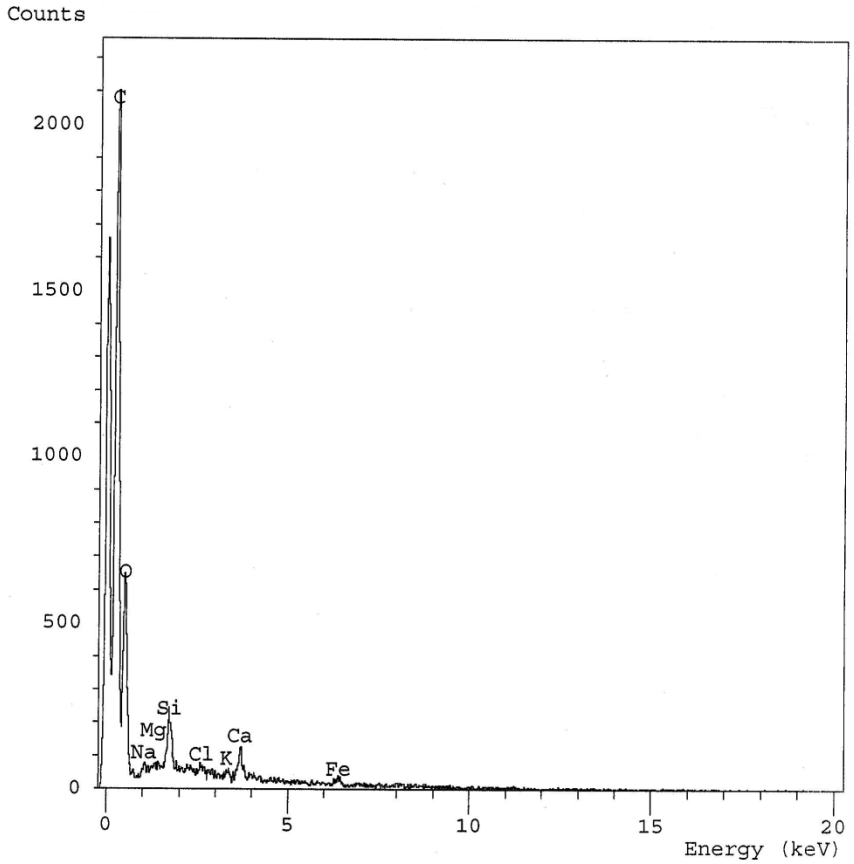

Figure 4. Spectrum of iron nanoparticles obtained by EDX microanalysis.

a green method using brown seaweed (*Sargassum muticum*) aqueous extract contain sulphated polysaccharides as the reducing agent was 18±4 nm and cubic shapes (Mahdavi et al., 2013). Moreover, iron oxide nanoparticles biologically synthesized using an aqueous extract of *Passiflora tripartita mollissima* fruit showed average particle size of spherical 22.3±3 nm (Kumar, et al., 2014).

In the EDX spectra pattern of filtered fungus growth media (Figure 4), the peaks around 0.1, 1.8, and 4.9 kV are related to the binding energies of Fe. Therefore, the pattern showed diffraction narrow peaks in the figure confirm the crystalline structure of iron nanoparticles without any impurity peaks. However, the results of XRD analysis for the synthesized nanoparticles that were obtained with different initial concentrations of $FeCl_3$ indicated that the particles consist of γ-Fe_2O_3 and α-Fe_2O_3. It also revealed that a concentration of 0.02 M of $FeCl_3$ resulted in the particles with the smallest Z-average diameter (Cheng et al., 2012).

The mechanism of Fe-Nps formation by fungi was discussed by some authors (Figure 5), AuNPs formation can occur either in the intracellular or extracellular space. Extracellular AuNPs formation is commonly reported for fungi when Au^{3+} ions are trapped and reduced by proteins in the cell wall. Previous work with the fungus *Verticillium* sp. ruled out the possibility that reduced sugars in the cell wall are responsible for the reduction of Au^{3+} ions and suggested adsorption of $AuCl^{4-}$ ions on the cell-wall enzymes by electrostatic interaction with positively charged groups (for example, lysine) (Mukherjee et al., 2002; Duran et al., 2011; Das et al., 2012).

Antibacterial activity results revealed that iron nanoparticles (synthesized from iron (III) nitrate 1 mM) acted as excellent antibacterial agents against both Gram-positive and Gram-negative bacteria. Table 1 and Figures 6 and 7 show the zone of inhibition produced by iron nanoparticles against both Gram-positive and Gram-negative bacterial strains. Fe-NPs exhibited maximum (16.4 mm) bacterial growth inhibition against *B. subtilis*, in the form of zone-of-inhibition studies, where diffusion of nanoparticles on nutrient agar plates inhibits growth. In contrast, Fe-NPs showed zones of inhibition of 13.2, 12.3 and 10.5 mm, respectively, against *E. coli, S. aureus* and *P. aeruginosa.* In the presence of iron oxide nanoparticles growth of both *B. subtilis* and *E. coli* strain is inhibited. Iron oxide nanoparticles show more antimicrobial activity to *B. subtilis* than *E. coli* (Pal, 2014). Moreover, iron oxide nanoparticles have excellent

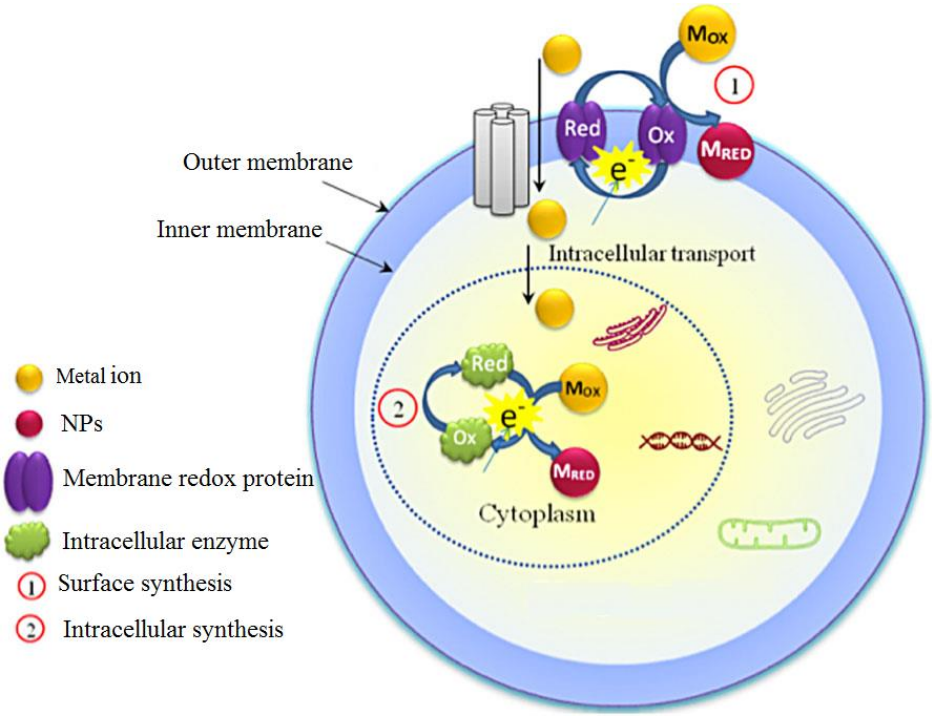

Figure 5. Schematic diagram of a proposed mechanism of metal nanoparticles biosynthesis in cell fungus (Das et al., 2012).

Table 1. Inhibition zones of iron nanoparticles compared with antibiotics.

Bacterial species	Inhibition zone (mm)		
	Fe-NPs	Ampicillin	Gentamicin
Bacillus Subtilis	16.4±0.7	27.4±0.2	-
Staphylococcus aureus	12.3±0.5	32.4±0.1	-
Escherichia coli	13.2±0.6	-	22.3±0.2
Pseudomonas aeruginosa	10.5±0.3	-	17.3±0.1

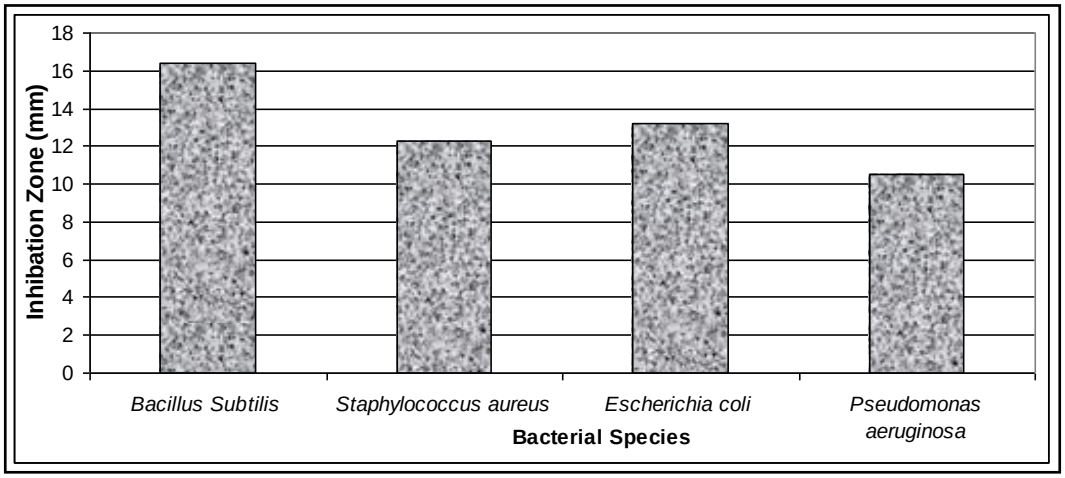

Figure 6. Inhibition zones of Fe-NPs for treated Gram-positive and Gram-negative bacteria.

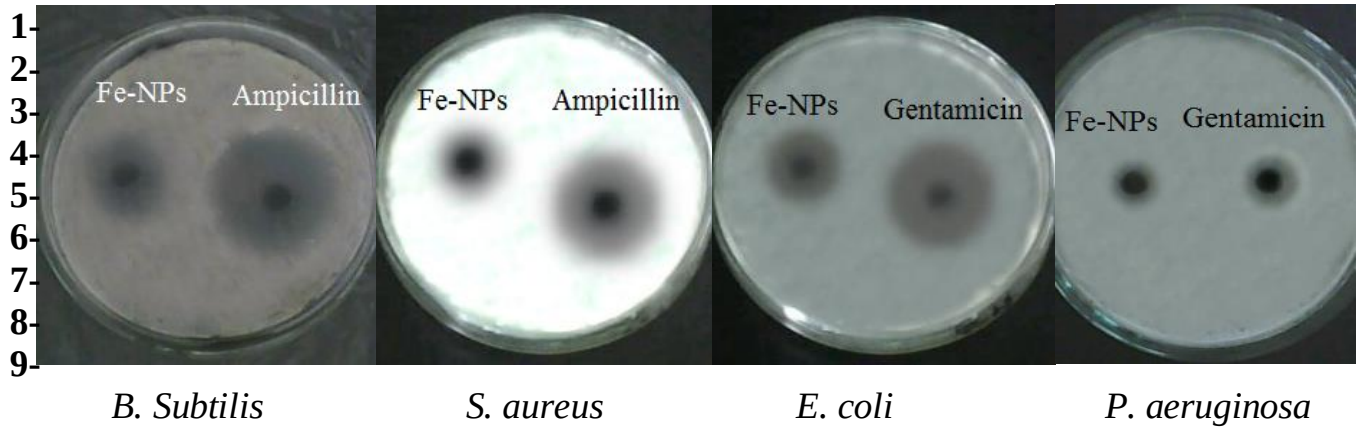

Figure 7. Inhibition zones of Fe-NPs against treated bacterial species.

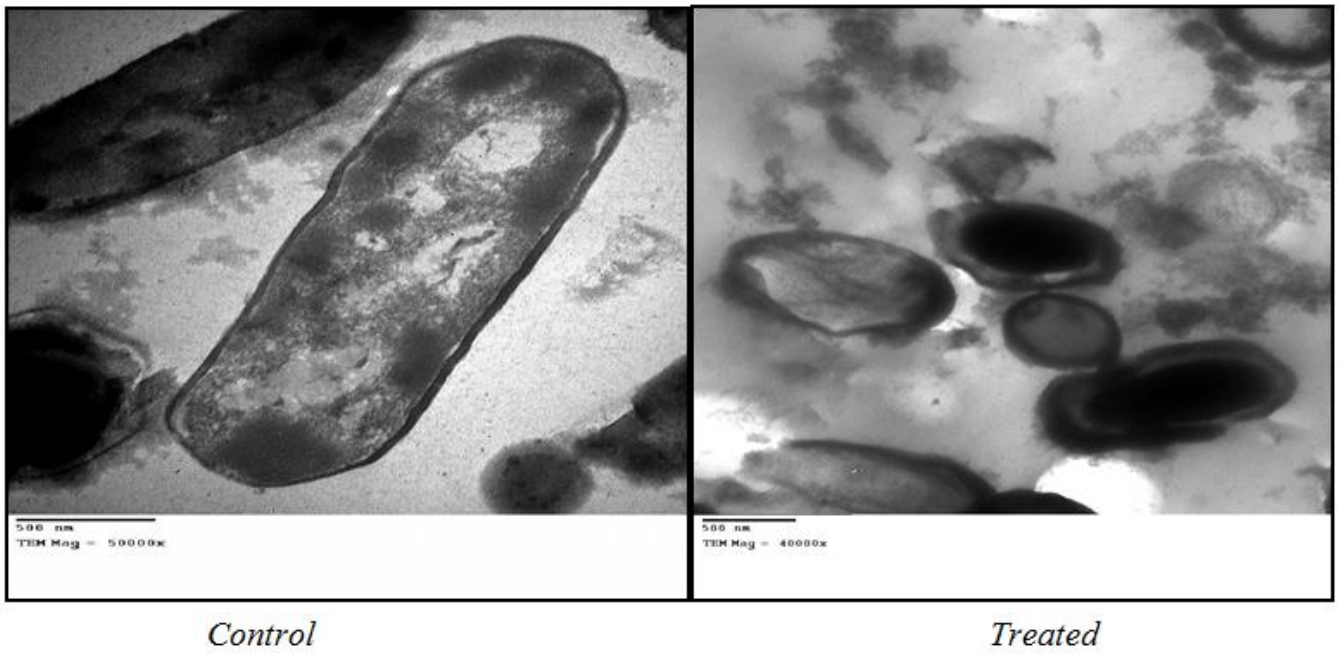

Figure 8. TEM micrographs of treated and untreated *Bacillus subtilis* bacteria with Fe-NPs.

antibacterial activity against *S. aureus* and promising Sono-Fenton catalytic ability for dye discoloration (Harifia and Montazer, 2014). The antibacterial activity of ZnO NPs was 72, 80, 88 and 84% more effective than Fe_2O_3 NPs, while 28, 31, 27, 50 and 40% more bactericidal than CuO NPs against *E. coli*, *S. aureus*, *P. aeruginosa*, and *B. subtilis*, respectively (Azam et al., 2012).

Transmission electron microscopy showed morphological changes of *B. subtilis* bacterial cells (Figure 8), Fe-NPs might cause oxidative stress via reactive oxygen species generation and the Fenton reaction. Oxidative stress in *B. subtilis* can result from disturbance of the electronic and/or ionic transport chains due to the strong affinity of the nanoparticles for the cell membrane. Similar results of zero-valent iron NPs toxicity on *E. coli* have been detected, where iron, as a strong reductant, might induce the decomposition of functional groups in membrane proteins and lipopolysaccharides, or Fe-NPs could be oxidized by intracellular oxygen, leading to oxidative damage via the fenton reaction. When Fe-NPs penetrate cells through disrupted membranes, it causes further physical damage and death (Lee et al., 2008).

Conclusions

Study report the biosynthesis of iron nanoparticles (Fe-NPs) employing *A. alternate* fungus, which is an eco-

friendly process for the synthesis of iron nanoparticles. Fe-NPs were synthesized through the reduction of aqueous iron (III) nitrate solution of 1 mM concentration in the dark conditions. The forming of nanoparticles was confirmed by UV-visible spectrum, TEM, SEM and EDX. The morphology of nanoparticles is found to be cubic shapes mostly and the mean size was 9±3 nm. Fe-NPs showed antibacterial activity against both Gram-positive and Gram-negative bacteria, but iron nanoparticles show more antimicrobial activity to *B. subtilis* than *E. coli, S. aureus* and *P. aeruginosa*.

Conflict of interests

The authors did not declare any conflict of interest.

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