BIOSYNTHESIS, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF IRON OXIDE NANOPARTICLES SYNTHESIZED BY FUNGI

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ABSTRACT*:*

In the present study, extracellular synthesis of iron oxide nanoparticles (FeNPs) was achieved by *Aspergillus flavus* isolate D05.The biosynthesized iron oxide nanoparticles have been widely favored because of biodegradablity, low toxicity and highly reactive surfaces. IONPs were synthesized through the reduction of aqueous Fe^{3+} ions. The obtained iron oxide nanoparticles were characterized by UV-vis spectroscopy, Fourier transforms infrared spectroscopy(FTIR), and Transmission electron microscope (TEM). TEM image of iron nanoparticles synthesized by *Aspergillus flavus sp.* showed 28-33 nm sized particles.

Keywords: nanoparticles, green synthesis, *Aspergillus flavus*, iron oxide NPs, antimicrobial activity

1. Introduction

Nanotechnology is the application of nanoscience, which is the study of nanoscale materials that exhibit remarkable properties, functionality, and phenomena due to the influence of small dimensions. Nanotechnology is based on the manipulation, control, and integration of atoms and molecules to form materials, structures, components, devices, and systems at the nanoscale **(Hornyak *et al*., 2009).**

Unlike micromaterials, nanomaterials are capable of possessing remarkable properties that deviate dramatically from the bulk of the parent material. Nanomaterials typically have high reactivity and degree of functionalization, large specific surface area, and size-dependent properties, which makes them suitable for applications like wastewater treatment, as well as for water purification **(Cloete, 2010; Abd Elmohsen *et al*., 2019; Bader *et al*., 2019)**.

Iron oxides are prevalent, widely used as they are inexpensive, and play an imperative role in many biological and geological processes. They are also extensively used by human (iron ores in thermite, catalysts, durable pigments, coatings, paints, and colored concrete, and hemoglobin **(Laurent *et al*., 2008)**. The three most common forms of iron oxides in nature are magnetite (Fe_3O_4), maghemite (g-Fe_2O_3), and hematite (α-Fe_2O_3) **(Islam *et al*., 2012)**.

Ferromagnetic nanoparticles with size <10–20 nm exhibit an inimitable form of magnetism, i.e., superparamagnetism. The ferromagnetic materials include elemental metals, alloys, oxides, and other chemical compounds that are magnetized by an external magnetic field. This is an important phenomenon normally present only in NP systems **(De and Joniau, 1988).** Due to their low toxicity, superparamagnetic properties, such as surface area and volume ratio, and simple separation methodology, magnetic iron oxide (Fe_3O_4 and g-Fe_2O_3). NPs have attracted much attention and are especially interesting in biomedical applications for protein immobilization, such as diagnostic magnetic resonance imaging (MRI), thermal therapy, and drug delivery **(Hasany *et al*., 2012).**

NPs produced by physical and chemical methods are complicated, outdated, expensive, and produce hazardous toxic wastage which is harmful to the environment as well as human health. The biological method is a better alternative to physical and chemical methods for the production of NPs **(Mohanpuria *et al*. 2008; Sharma *et al*. 2009; Yosri *et al*., 2019)**. It is not only inexpensive but it is also less complicated and time-consuming, safe, eco-friendly, and most importantly non-toxic. Moreover, it includes far less requirement for energy, less wastage of inputs, and more practical control of chemicals and reagents. An additional advantage is a fact that this is a bottom-up approach **(Schröfel *et al*. 2014; Malik *et al*. 2014; Moustafa *et al*., 2015)**.

The use of fungi in the biosynthesis of nanoparticles was discovered recently, the science of NPs synthesis by fungi is referred to as myco- nanotechnology which has a great demand and considerable potential, partly due to the wide range and diversity of fungi **(Tyagi, 2016; Sidkey *et al*., 2017).** The fungal proteins are capable of hydrolyzing and reducing metal ions. In addition to this, fungi are easy to isolate and

culture. Fungi can accumulate metal ions by physicochemical and biological mechanisms including extracellular binding by metabolites and specific polypeptides **(Alghuthaymi *et al.*, 2015)**. It is more favorable when compared to the bacterial production of NPs which involves the use of sophisticated instruments to obtain clear filtrate from the colloidal broth **(Sastry *et al*., 2003)**.

Kaul *et al.* (2012) tested five different species of fungi: *Penicillium chlamydosporium*, *Aspergillus fumigates*, *Aspergillus wentii*, *Curvularia lunata*, and *Chaetomium globosum* for the production of iron oxide nanoparticles. In addition, **Sidkey *et al.* (2016a)** biosynthesized extra- and intracellular Fe(II) nanoparticles via *Aspergillus foetidus* ATCC 14916 and tested its corrosion resistance and antimicrobial activity. Iron oxide nanoparticles have received special attention because of their variety of scientific and technological applications such as biosensor **(Berry and Curtis, 2003),** antimicrobial activity **(Geffroy *et al*., 1999)**, food preservation **(Chan *et al*., 1993)**, corrosion resistance (**Sidkey *et al.*, 2016a)**, magnetic storage media, ferrofluids, magnetic refrigeration, magnetic resonance imaging, hyperthermic cancer treatments, cell sorting and targeted drug delivery **(Gupta and Gupta, 2005; Zhang *et al*., 2009).** Besides, it has also been widely used in biomedical research because of its biocompatibility and magnetic properties **(Matheson and Tratnyek, 1994)**.

The development of new resistant strains of bacteria to current antibiotics has become a serious problem in public health; therefore there is a strong incentive to develop new bacteriocides from various sources **(Abo-Shadi *et al.*, 2010; Sidkey *et al.*, 2011; Mahdy *et al*., 2012; Sidkey *et al.*, 2016b)**. Recent advancement in the field of nanotechnology has provided an attractive method for synthesizing alternative antimicrobial agents and reducing biofilm formation **(Behera *et al*., 2012; Ismail *et al.*, 2016; El-Batal *et al.*, 2016)**. The biosynthesis of nanoparticles has received increasing attention due to the growing need to develop safe, cost-effective, and environmentally friendly technologies for nano-materials synthesis (**Sagar and Ashok, 20120)**.

2. Materials and Methods

2.1. Materials

All the chemicals used in this study were analytical reagent grade from the commercial market (Al-gomhoria company). Distilled water was used for the preparation of the solutions. Ferrous sulphate ($FeSO_4.7H_2O$); iron salt, 0.1 M HCl solution, and aqueous NaOH were used to adjust the pH.

2.2. Methods

2.2.1. Isolation of fungi from the soil samples

Samples of soil were gathered from the polluted area at Belbis region, Sharkia Governorate, Egypt through July 2018. An obtained soil was transported to the laboratory in sterile polythene bags. Sterile saline solution was used to dilute the soil sample **(Deshwal *et al*., 2003)**. The fungi were isolated by plating the primary inoculum on Czapek Dox agar medium (pH 7.3±0.2) with 0.1 ml of soil samples suspension

separately after serial dilutions of collective soil samples. All the plates were incubated at 28ºC for 5-7 days. Then, the fungi were isolated **(Pitt and Hocking, 2009)**.

The plates were examined and the isolates were purified on the same previously used medium several times under the same incubation conditions to gain pure isolates.

2.2.2. Metallotolerance ability examination of the isolates

Three isolates namely F_1, F_4, and F_6 were selected for their growth in the Czapek-Dox agar medium supplemented with different concentrations (1000, 5000, 20000 & 30000 mg/l) of Ferrous sulphate heptahydrate to further determine the most efficient tolerate isolates, while the control plates were free of the metal. The plates were incubated at 28°C for at least 5 days and examined daily for the fungal growth **(Ahmad *et al*., 2006)**.

2.2.3. Molecular identification of the isolated fungus

The most promising fungal isolate showing tolerance to the metal ions was identified using molecular techniques. Molecular identification was performed in which, partial sequencing of 18S and 28SrRNA and a complete sequence of internal transcribed sequence 1, internal transcribed sequence 2 and 5.8S rRNA gene was done using universal primer according to the manufacturer protocol. Primers ITS1-F (50TCC GTA GGT GAA CCT TGC GG 30) and ITS4-B (50TCC TCC GCTTAT TGA TAT GC 30) have been used for isolation of the 18S rRNA. The genomic DNA was isolated by the CTAB extraction method suggested by **Sambrook *et al*. (1989)**. The rRNA sequence was submitted to Gene Bank of Sigma Aldrich, Cairo, Egypt.

2.2.4.Biosynthesis of iron oxide nanoparticles

2.2.4.1. Fungal biomass and mycelial cell-free filtrate (MCFF) preparation

The selected fungal isolate was grown in a 250 ml conical flask containing 100 ml sterile Sabouraud dextrose broth medium (pH 5.5) and incubated under the static condition at 28ºC for 5 days. After complete incubation, fungal mycelia were separated from the culture broth by filtration process using Whatman filter paper no. 1 (Whatman, England) under biosafety cabinet, followed by washed three times with sterile double distilled water to remove any medium components from the fungal biomass. The harvested fungal biomass was transferred to 250 ml Erlenmeyer flask containing 100 ml sterile distilled water and then incubated in a rotary shaker (150 rpm) at 28°C for 72 hr. At the end of the incubation period, it was filtered by using Whatman No.1 filter paper to obtain the cell free filtrate (CFF) **(Omran *et al*., 2018)**.

An aqueous solution of $FeSO_4.7H_2O$ (1Mm $FeSO_4$ of final concentration) was mixed with 100 ml of cell-free filtrate and the mixture was put into a shaker at 28ºC in dark with shaking (150 rpm) for 72hr. Control (without iron ions) was also run along with the experimental flasks **(Mohamed *et al.*, 2015)**

After incubation time the prepared extracellular Fe-NPs were then separated by centrifugation at 10.000 rpm for 30 minutes and then used for further studies.

2.2.6. Characterization of Iron Nanoparticles

2.2.6.1. UV-Vis spectral analysis

The formation of nanometal was monitored using UV-Visible absorption spectroscopy (UV340-111503), which is one of the important techniques to verify the formation of metal nanoparticles provided surface plasmon resonance exists for the metal **(Basavaraja *et al.*, 2008)**. The appearance of color arises from the property colored material to absorb selectively within the visible region of the electromagnetic spectrum and scanning the spectra between 200 and 600 nm at the resolution of 1nm. UV-Vis spectral analysis carried out at Desert Research Center (DRC), Cairo, Egypt.

2.2.6.2. Fourier Transform Infrared spectrophotometer (FTIR)

Analysis by Infrared spectrophotometer was used to establish the functional groups present in the prepared powders.

2.2.6.3. Transmission Electron Microscope (TEM)

The resulting nanoparticles were analyzed using a transmission electron microscope (TEM) (Joel JEM 1200 EXll) 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. Transmission Electron Microscope (TEM) measurement was carried out at the Regional Center for Mycology and Biotechnology, Al-Azhar University.

2.2.7. Assessment of antimicrobial activity of the mycosynthesized Fe-NPs

The synthesized Fe-NPs obtained from the MCFF of the selected fungal isolate were tested for their antimicrobial potency against some pathogenic microorganisms *Staphylococcus aureus* (RCM010010*), Escherichia coli* (RCMB010052) ATCC25955, *Candida albicans (*RCMB 005003) ATCC10231, and *Aspergillus Fumigatus* (RCMB 002008). The test was done with the diffusion agar technique **(Ponarulselvam *et al.*, 2012)** using nutrient agar medium in sterile petri dishes. Each strain was swabbed uniformly onto individual plates, and a concentrated solution of Fe-NPs was poured into each cup (10 mg/L per cup) on all the plates. After incubation at 37 °C or 28 °C for 24 h, the diameter of the inhibition zone was measured using a caliper. The assay was performed in triplicate and the mean value was calculated.

3. Result and discussion

3.1. Isolation and screening of fungi for the synthesis of Fe-NPs

It was found that F_1 isolate exhibited the highest iron tolerance (5000 ppm) among the isolates recovered from the soil while the other fungi (F_4 & F_6) cannot tolerate these highest concentrations. Thus, this isolate has been subjected to identification using molecular techniques.

3.2. Molecular Identification of the most Fe-tolerant F1 isolate

The most efficient fungal isolate was identified as *Aspergillus flavus* D05 and this identification was made based on molecular characterization of fungal isolate which was performed by partial sequencing of 18S and 28S rRNA and a complete sequence of internal transcribed sequence 1 (ITS-1), internal transcribed sequence 2 (ITS-2), and 5.8S rRNA gene (complex of -18S-ITS1-5.8SITS2- 28S) and has been submitted in the NCBI GenBank (accession no. MG799220.1). The sequence was compared using the Basic Local Alignment Search Tool (BLAST) of NCBI and the submitted sequence is available on a public domain http://www.ncbi.nlm.nih.gov. The phylogenetic analysis of F1 isolate using 18S-rRNA sequence data and GenBank database showed a high similarity of 100% to *Aspergillus flavus* D05 as shown in Fig. (1), so it gives the name and code *Aspergillus flavus* D05-F1**.**

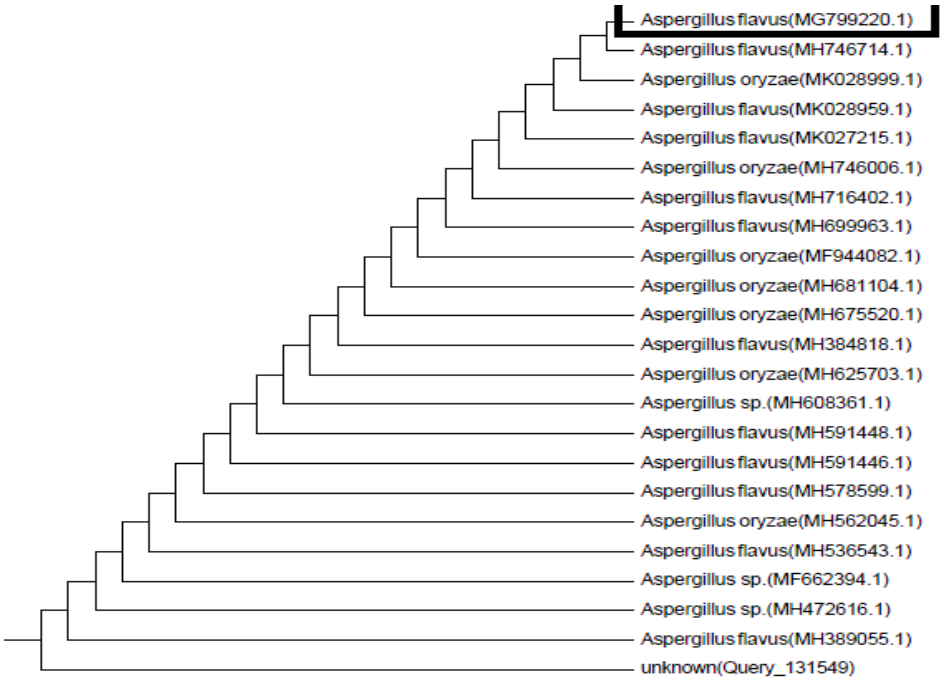


Fig. (1): Neighbor-joining phylogenetic tree derived from 18S rRNA gene sequences, showing the position of isolate *A.flavus* and phylogenetically related member of this genus

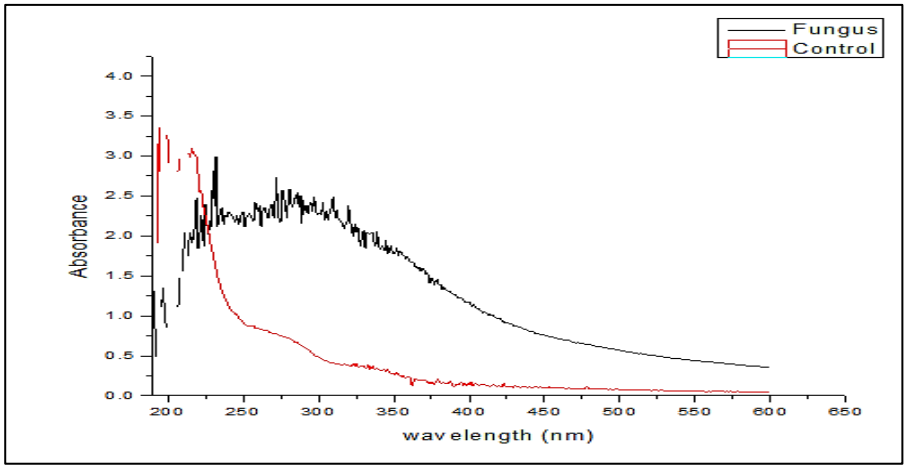
3.3. Biosynthesis of Iron Nanoparticles.

The biosynthesis of iron nanoparticles was carried out by exposure of a precursor aqueous salt $FeSO_4$ solution (1Mm $FeSO_4$ of final concentration) to fungal cell-free filtrate obtained by incubating the fungus *Aspergillus flavus* D05-F1 in an aqueous solution. The reaction was carried out at 28ºC in dark under shaking (150 rpm) for 72hr. The synthesis of Fe-NPs was confirmed by the characteristic change of the mixture color to dark yellowish-brown. This indicated the reduction of aqueous iron ions to Fe-NPs when added to fungal cell-free filtrate of *A.flavus* D05-F1 and the color reaction is the result of excitation of surface plasmon vibration in the metal nanoparticles **(Shahverdi *et al*., 2007).**

3.4. Characterization of Iron Nanoparticles

3.4.1. UV-Vis spectral analysis

The bioreduction of Fe ions in aqueous solutions was monitored by measuring UV/Vis spectra. UV/Vis spectral analysis was done at a wavelength range of 200-600 nm to



study the absorption spectra of green synthesized Fe-NPs and the absorption peaks were observed at 250-350 nm ranges due to the excitation of surface plasmon vibrations in FeNPs as has been reported earlier **(Tran *et al*., 2010).** The characteristics peaks of iron oxide. nanoparticles were observed at 230,272 nm as presented in Fig. (2), which is due to charge transfer spectra.

Fig. (2): UV-Visible absorption spectroscopy showed peaks of FeNPs at 230,272 compared with a bioactive supernatant fraction as a control.

In view of the findings of other investigators, iron nanoparticles that forming by *Pleurotus sp.* under the Uv-vis spectrophotometer showed nearly peaks at wavelength 226 and 276 nm **(Mazumdar and Haloi, 2011).** While, the analysis of iron nanoparticles using fungus *Alternaria alternate* showed a peak at 238 nm and another peak at 265 nm (**Mohamed *et al*., 2015)**.

Saranya *et al*. (2017) stated that, UV/Vis absorption showed a characteristic absorption peak of iron oxide nanoparticles at 310 nm. On the other hand, **Mahdavi *et***

***al*. (2013)** reported that two absorption peaks of Fe-NPs that synthesized by *Sargassum muticum* aqueous extract were introduced at wavelengths 402 nm and 415 nm.

3.4.2. Fourier transform infra-red spectrophotometric (FTIR) analysis of FeNPs:

FTIR spectrum of nano green-synthesized FeNPs displayed stretching vibrations as illustrated in Fig. (3) at 2603cm^{-1} for alkyne group,1689.94 cm^{-1} for C=C, 1025.8cm^{-1} for C–O–C,2064 cm-1for N=C=S (iso-thiocyanate)group these adsorption peaks supports the presence of protein and other bioactive compounds on the surface of bio-synthesized FeNPs, confirming that metabolically produced bioactive compounds act as capping agent during production and prevent the reduced iron particles agglomeration. In addition, O–H absorption peak of 402.7,474.71 cm^{-1} refer to Fe-O stretches of Fe_3O_4 and Fe_2O_3, confirming the formation of biosynthesized iron nanoparticles.

Manivasagan *et al*. (2015) and **Ahmed (2017)** have reported that, bonds functional groups such as –C-O-C-,-C-O-,-C=C- are derived from heterocyclic compounds like proteins, which may be the capping ligands of AgNPs.

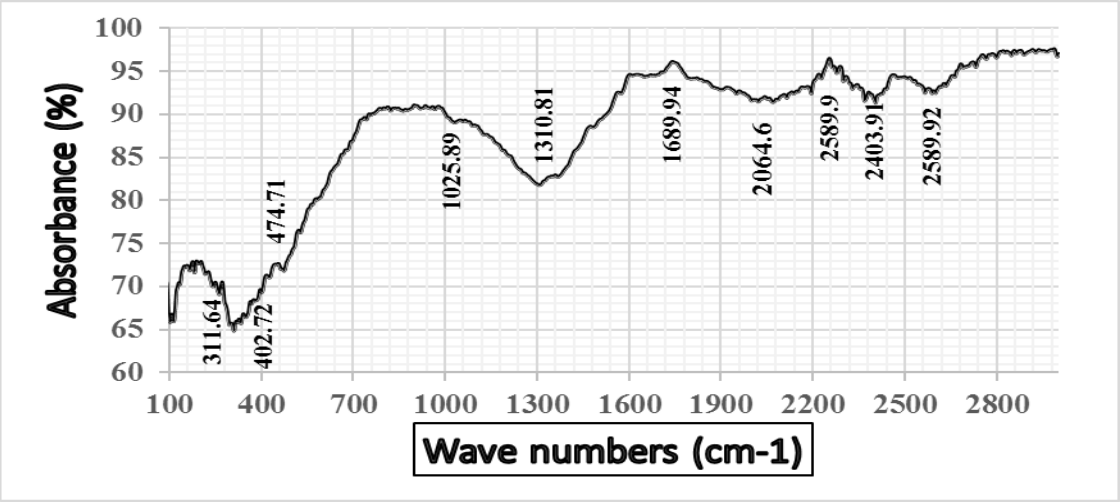


Fig. (3): FTIR spectrums supporting the presence of protein and bioactive compounds on the surface of biosynthesized FeNPs.

3.4.3. Transmission electron microscope (TEM)

TEM is a powerful analysis to determine the morphology and particle size of the resulting nanoparticles **(Telleria and Tibayrenc, 2017)**. In the present study, the electron microscope micrographs of the biosynthesized FeONPs revealed the formation of extracellular spherical and irregular nanoparticles, while the average particle diameter as determined by TEM was found to be 30±3 nm and a size range was 28.6-33.8 nm as shown in Fig. (4).

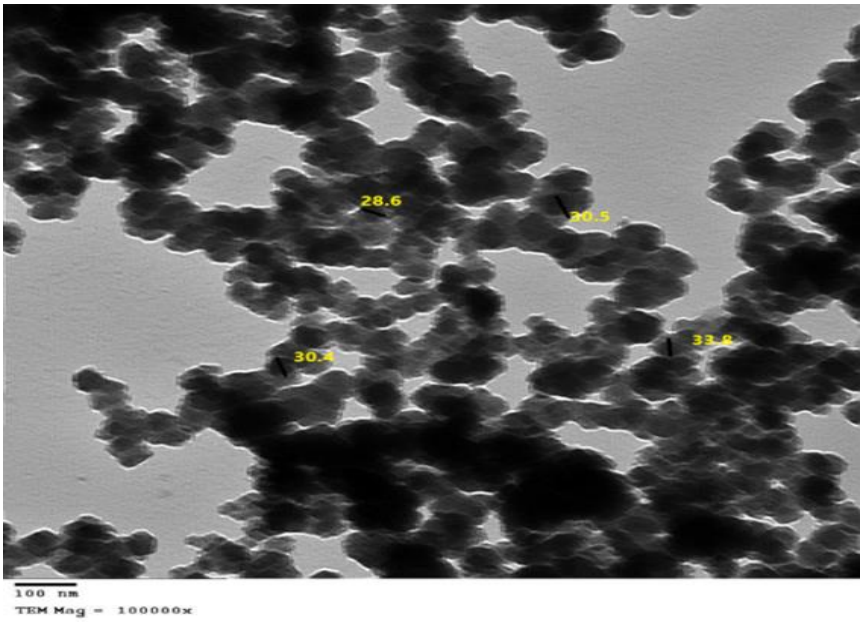


Fig. (4): TEM image of biosynthesized IONPs using cell-free filtrate of *Aspergillus flavus* D05-F1

Tarafdar and Raliya (2013) stated that, the TEM micrograph of iron nanoparticles synthesized from *Aspergillus oryzae* TFR9 showed well-dispersed nanoparticles and spherical in shape. **Parveen *et al.* (2018)** reported that, TEM micrograph of iron oxide nanoparticles was fabricated using green approach using tannic acid as capping agent revealing that these nanoparticles are not agglomerated, somewhat circular in shape with particle size approximately varies between 10 and 30 nm. While, **Sidkey *et al.* (2016b)** indicated that, the shape of the intracellular Fe+2 nanoparticles of the isolate *Aspergillus foetidus* ATCC 14916F2 Fe/S was spherical. The size of the particles with respect to the spherical form ranges from 31.53 nm to 61.94 nm. Also, **Devi *et al.* (2019)** concluded that particles of iron using *Platanus orientalis* leaf extract exhibit a spherical shape with an average diameter of 38 nm.

3.5. Assessment of antimicrobial activity of the mycosynthesized Fe-NPs

Antibacterial activity of FeNPs

The antibacterial activity results of FeNPs as shown in Table (1) revealed that iron nanoparticles acted as good antibacterial agents against Gram-positive bacteria but not effective against Gram-negative bacteria. Fe-NPs exhibited maximum (10 mm) bacterial growth inhibition against *S. aureus*. Iron nanoparticles showed zones of inhibition of 12.3 against *S.aureus* and 10.5 against *P. aeruginosa* **(Mohamed *et al.*, 2015).** The main mechanism by which these particles showed antibacterial activity might be via oxidative stress generated by ROS **(Tran *et al.*, 2010; Mahdy *et al.*, 2012)**. ROS, including superoxide radicals (O^{2-}), hydroxyl radicals (–OH), hydrogen peroxide (H_2O_2), and singlet oxygen (1 O_2), can cause damage to proteins and DNA in bacteria. In the present study, metal oxide (FeO) could be the source that created ROS leading to the inhibition of most of the pathogenic bacteria including *Staphylococcus aureus*. A

similar process was also described by **Kim *et al*. (2007)** in which Fe^{2+} reacted with oxygen to create hydrogen peroxide (H_2O_2). This H_2O_2 consequently reacted with ferrous irons via the Fenton reaction and produced hydroxyl radicals that are known to damage biological macromolecules **(Touati, 2000)**.

Antifungal activity of FeNPs

The biosynthesized FeNPs inhibited the growth of two different pathogenic fungi as shown in Table (1), including *Aspergillus fumigatus* and *Candida albicans*. According to antifungal activity results of the Fe-NPs exhibited maximum inhibition zone of 10 and 11mm against *Aspergillus fumigatus* and *Candida albicans,* respectively. Thus, FeNPs could be considered as excellent broad-spectrum antifungal agents. Since the biosynthesized FeNPs showed considerable antifungal activity, they could potentially be used widely in clinical applications.

In view of the findings of other investigators, spherical iron oxide NPs of 30–40 nm have been used against fungal infection caused by *Candida* species by Nazanin **(Devi *et al.*, 2019).** Inactivation of fungus by iron oxide NPs involves the direct interaction between NPs and cell surfaces, which affects the permeability of membranes where NPs enter and induce oxidative stress in fungus cells, subsequently resulting in the inhibition of cell growth and eventually cell death **(Xie *et al.*, 2011).**

Table (1): Inhibition zones of iron nanoparticles compared with Gentamycin as bacteriocidal and Ketoconazole as a fungicidal

Tested microorganisms	**Inhibition zone(mm)**	
Bacterial species	**Fe-NPs (100mg/ml)**	**Gentamycin (4µg/ml)**
Staphylococcus aureus (RCM010010)	10	24
Escherichia coli (RCMB010052)	-	30
Fungal species	**Fe-NPs (10mg/ml)**	**Ketoconazole (10µg/ml)**
*Candida albicans (*RCMB 005003) ATCC10231	11	20
Aspergillus Fumigatus (RCMB 002008)	10	17

Conclusion

Nanoparticles can be produced by physical-chemical methods but it requires the involvement of hazardous chemicals and many sophisticated techniques that are not easy. On the other hand, the biosynthesis of nanoparticles by microorganisms is quick, consumes less time, it provides satisfactory biosynthesis of nanoparticles and the whole process is very cheap and effective without the involvement of hazardous chemicals. In the present study, *Aspergillus flavus* D05 was exploited to biosynthesize iron nanoparticles by reducing ferrous sulphate.

These applications of Iron Oxide nanoparticle show better bactericidal activity in Gram-positive bacteria as compared to Gram-negative bacteria. Also iron oxide NPs show antifungal activity against *Aspergillus fumigatus* and *Candida albicans*. The application of iron oxide NPs in the field of antimicrobial activity is still in its infancy.

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تخليق جزيئات أكسيد الحديد النانوية بيولوجيا بواسطة الفطريات وتوصيفها واختبار النشاط الضد ميكروبي لها

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في هذه الدراسة " تم تصنيع جسيمات الحديد النانومترية بواسطة عزلة فطر أسبرجلس فلافز عن طريق اختزال ايونات الحديد، وتتميز جسميات الحديد النانوية بأسطحها شديدة التفاعل وانخفاض السمية وقابليتها للتحلل البيولوجي .تم توصيف جسميات الحديد النانوية عن طريق التحليل الطيفي للاشعة فوق البنفسجية وتحليل الطيف بالاشعة تحت الحمراء وبالمجهر الالكتروني للارسال والذي اظهر الجسميات بحجم 28-33 نانوميتر وتبين ان لهذه الجسيمات نشاطا مضادا لميكروبات المكورات العنكودية الذهبية والأسبرجلس فلافز والكاندا ألبيكانس.

الكلمات المفتاحية: الجسيمات النانوية، التكوين البيولوجي، أسبرجلس فلافز، أكسيد الحديد النانوية، النشاط الضد الميكروبي.