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Biosynthesis and characterization of copper nanoparticles constructed by fungi isolated from several industrial centers in Isfahan

Rezvan Eftekhari¹, Monir Doudi^{*2}, Gholamreza Amiri³

1. M.Sc, Department of Microbiology, Falavarjan Branch, Islamic Azad University, Falavarjan, Isfahan, Iran.

2. Assistant Professor, Department of Microbiology, Falavarjan Branch, Islamic Azad University, Falavarjan, Isfahan, Iran.

3. Assistant Professor, Department of Biology, Falavarjan Branch, Islamic Azad University, Falavarjan, Isfahan, Iran.

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ABSTRACT

Cleanliness, high adaptability to the environmental conditions and easily manufacturing led to attention to the use of microorganisms, including fungi in nanoparticles production. The aim of this study was biosynthesis of copper nanoparticles by fungi isolated from soil and industrial wastewaters and characterization of the synthesized nanoparticles. Sampling was carried out from the soil and waste water of several coppersmiths and plating plants in Isfahan, Iran. After analysis of chemical and biological parameters of samples, Czapek Dox medium containing CuSO_4 was used for isolation of fungi. Minimum inhibitory concentration of copper ions on the selected fungi was assessed by agar diffusion method. The most resistant fungi were identified based on morphology and molecular detection. In the second step, the characteristics of copper nanoparticles produced by the selected fungi were analyzed by X-ray diffraction (XRD), UV-visible spectroscopy and transmission electron microscopy (TEM). Among the Cu-resistant fungi, *Fusarium solani* CBS490.63 and *Paecilomyces variotii* BAB-1547 possessed the highest resistance to copper with MIC of 4.5 mM and 4 mM, respectively. The results of UV-visible spectroscopy showed an absorption peak after applying 800°C heating in the wavelength of 400 nm. X-ray diffraction and TEM results showed that the copper nanoparticles were in the form of hexahedron with the diameter of 17-29 nm. The isolated fungi were efficient for biological synthesis of copper nanoparticles that according to XRD and TEM results had the single-phase formation and purification characteristics similar to nanoparticles reported in other studies with minimal environmental contamination and less toxicity.

1. Introduction

Recently, a lot of efforts have been made for metal nanoparticles production (Ahmad et al., 2003; Liu, 2006). In general, there are three

main methods for producing metal nanoparticles including physical, chemical and biological methods. In physical and chemical methods, a

*Corresponding author: Dr. Monir Doudi
Tel: 03137420134-5
E-mail address: Monirdoudi@yahoo.com

covering factor consists of a polymer, ligand, or surfactant is often required to stabilize and prevent nanoparticle accumulation. These compounds are often toxic and harmful which is from disadvantages of using physical or chemical methods for nanoparticles production (Krumov et al., 2009). Recently, biological methods that are used for synthesis of nanoparticles by plants and their extracts, yeasts, molds, algae, protozoa, and bacteria has been lowered the risks of them for humans, animals, plants, air and environment. Also the biological methods provided a low-cost and minimum time and energy consumption route for nanoparticles production (Khodashenas et al., 2015). Microorganisms feed on organic and mineral resources to carry out their metabolism and vital processes. During different processes, when these organisms exposed to metal ions, accumulate them inside cells or on their cell walls. This accumulation often leads to the generation of particles that are classified into nano-sized particles (Thirumalai et al., 2010). Microorganisms have been used for metals extraction and are now also used to synthesize metal nanoparticles (Mandal et al., 2006). Most microorganisms can survive in high concentrations of metal ions because of their ability to cope with stress conditions (Chen et al., 2003).

Intracellular or extracellular synthesis, growth temperature, synthesis duration, ease of extraction and the percentage of synthesized nanoparticles to the percentage of removed metal from each sample are important factors affect the production of biological nanoparticles (Pantidos et al., 2014). Copper nanoparticles have been highly attracted to researchers due to their unique characteristics such as high electrical conductivity and transformation capability, cost-effectiveness and more effective anti-microbial properties in contrast to other therapeutic nano-metals (Aher et al., 2017; Seyedalipour et al., 2015). The synthesis of these nanoparticles is also particularly important because of a wide range of applications in the fields of water filtration, catalyst production, medicine and electronics. The fundamental purpose for synthesis of this type of nanoparticles is the ability to control the size and shape of the atomic groups with special order (Bambo et al., 2017). The use of fungi in metal nanoparticles manufacturing has attracted much

attention because they provide specific benefits in contrast to the use of bacteria. The presence of vegetative and reproductive organs in the growth surface, high biomass and enzyme production, and ease of manipulation are from the most important advantages of fungi for production of nanoparticles (Pantidos et al., 2014; Ashengroph et al., 2014). Fungi such as *Fusarium*, *Verticillium*, *Penicillium* and *Aspergillus* have been used for the production of nanoparticles from metals such as copper, cadmium, silver and gold (Mukherjee et al., 2002).

Generally, antimicrobial activity is dependent on the size and shape of nanoparticles, which affects surface contact and interaction with the cell membrane of pathogenic microorganisms (Camacho-Flores et al., 2015). High energy levels of copper nanoparticles have made them suitable for decontamination of sewages and marshes (Paisand Jones, 1997). The use of heavy metals and their compounds in various industrial processes leads to the accumulation of these metals in the environment by their waste waters (Alboghobeys et al., 2013; Anand et al., 2006). There have recently been a lot of methods to cleanse soils and waste waters from heavy metals. Biodegradation is from these methods in which the removal potential of microorganisms such as algae, molds, yeast, and bacteria are used as a biological appeal to eliminate and absorb heavy metals (Sun et al., 2010; Anand et al., 2006). The aim of this study was biosynthesis of copper nanoparticles by the fungal isolates from soils and industrial wastewaters in the city of Isfahan and characterization of the synthesized nanoparticles.

2. Materials and methods

2.1. Samples

Map sampling was done from copper contaminated soils and waste waters of several coppersmiths and plating in Isfahan. The samples were transferred to research laboratory of Falavarjan Branch, Islamic Azad University. physicochemical factors including temperature, biological oxygen demand (BOD), chemical oxygen demand (COD) and pH in waste water, and temperature, pH and electrical conductivity (EC) in soil samples were measured. Also heavy metal contents including Al, As, Ag, Cd, Cu, Cr,

Mn, Pb, Sn, Zn and Ni were detected in all samples using inductively coupled plasma-optical emission spectrometry (ICP-OES) by Elmer-800Perkin, American apparatus.

2.2. Isolation and identification of copper resistant fungi

Copper resistant fungi were isolated in Czapek Dox agar medium containing CuSO₄ (0.5 mM). The solution of copper was sterilized by Millipore filter (0.22 nm) and then added to the sterilized Czapek Dox medium. Different dilutions of samples were inoculated to the media and incubated at 25-30°C for 3-7 days. Then the isolated fungi were counted and purified on sabouraud dextrose agar medium (Alboghobeysh et al., 2013). The selected fungi were initially identified based on macroscopic (colony morphology), and microscopic (vegetative and reproductive organs in slide cultured colonies) characteristics. Then molecular detection was carried out based on the sequences of amplified 16SrRNA gene by ITS1 and ITS4 universal primers. The sequences of amplified fragments were reviewed in BLAST server and the phylogenetic trees were plotted using MEGA 6.6.0 software.

2.3. Determination of minimum inhibitory concentration (MIC) of copper

For this purpose, agar dilution method was used. The desired colonies were selected and then the suspension containing 1.5×10^5 fungal spores were cultured in the Czapek Dox agar media containing different concentrations of CuSO₄ (0.5, 1, 2, 2.5, 3, 3.5, 4, 4.5 and 5 mM). The inoculated media were incubated at 25-30 °C for 3-7 days. Then the colony formation was observed and the lowest concentration of copper in which fungal growth were eliminated detected as minimum inhibitory concentration (Alboghobeysh et al., 2013).

2.4. Fungal biomass preparation and evaluation of fungi for nanoparticle formation

After the screening of copper-resistant fungi, the selected isolates were evaluated by a qualitative/quantitative method for detection of the production of copper nanoparticles. First, for biomass production, the colonies of copper-

resistant fungi were transferred to Czapek medium containing 0.5 mM CuSO₄ and incubated for 7 days at 25-30°C with agitation in 150 rpm. Then the biomass was separated by centrifugation and washed 3 times and transferred to the stock solution of CuSO₄ with the concentration of 1 mM and incubated for 72 hrs at 25-30°C with agitation in 150 rpm since the color of solution changed to green. Finally the biomass was separated again and used for further analysis (Jehad et al., 2012).

2.5. Morphology and physicochemical analysis of synthesized copper nanoparticles

Maximum absorption of synthesized copper nanoparticles was evaluated by UV visible spectrophotometer (Shimadzu, UV-2600 TCC240A, Japan) at the wavelength of 200 -600 nm. Then the structure and the shape of produced nanoparticles was determined by X-ray diffraction (XRD) apparatus (Philips1800, Germany), and transmission electron microscope (TEM, Philips 100 KW, Germany). Finally the size of particles was calculated by Debye-Scherrer formula: $\Delta = 0.9 \lambda / \text{FWHM} \cos \theta$ peak (Manikandan et al., 2015).

2.6. The effect of temperature in single phase changing

The samples were set at 800°C for 3 hrs. Then was washed with distilled water and transferred to an oven set at 50°C and sent to the central laboratory of Isfahan University for XRD analysis (Camacho-Flores et al., 2015).

2.7. Statistical analysis:

The data was analyzed by SPSS 20 software and analysis of variance (ANOVA).

3. Results

3.1. Chemical and biological analysis of the samples

The results of the measurements of chemical and biological of factors in soil and waste water samples as well as the concentration of heavy metals in them are shown in Tables 1 and 2.

Table 1. The chemical and biological of factors in the soils and waste waters samples.

Sample	Sample code	Temperature (°C)	pH	EC (ds/m)	COD (mg/l)	BOD (mg/l)
Coppersmith soil (1)	Cu1	20	8.6	4.8	-	-
Coppersmith soil (2)	Cu2	15	6.3	0.665	-	-
Coppersmith soil (3)	Cu3	15.7	8.1	4.9	-	-
Copper plating waste water (1)	Cu4	17.2	7.5	-	6480	120
Copper plating waste water (2)	Cu5	18	8	-	430	100

Table 2. The concentration of heavy metals in the soils and waste waters samples.

Sample	Sample code	Ni mg/l	Zn mg/l	Sn mg/l	Pb mg/l	Mn mg/l	Cu mg/l	Cr mg/l	Cd mg/l	As mg/l	Al mg/l	Ag mg/l
Coppersmith soil (1)	Cu1	2.3	13.7	1.5	5.4	1.4	38.5	2.3	0.2	3	26	9
Coppersmith soil (2)	Cu2	5.8	7.2	0.7	26.1	0.9	36.6	3.3	0.9	2.5	31	16
Coppersmith soil (3)	Cu3	1.5	16.5	0.2	4.4	0.6	44.4	1.6	0.3	8	14	7
Copper plating waste water (1)	Cu4	1.45	14.2	3.3	2.1	2.7	6.3	3.7	0.2	2.9	46	42
Copper plating waste water (2)	Cu5	0.06	0.08	0.05	0.05	0.5	5.2	0.05	0.02	1.8	0.02	0.05

3.2. Copper resistance

The amounts of minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of the nanoparticles are presented in table 3. The highest amount of MIC was 4.5 mM and was belonged to a coppersmiths soil isolate, and the lowest MIC amount was 0.5 mM, which was belonged to a copper plating waste water isolate. The latest isolate was not entered to the molecular identification step. In order to identify the rest four copper resistant isolates, molecular identification was done after initial identification based on morphological characterization.

3.3. Isolates identification

Figure 1 shows the results from agarose gel electrophoresis of the PCR products with the length of 600 bp obtained by amplification of

16SrRNA gene by ITS1 and ITS4 universal primers.

The results from sequence analysis and phylogenetic identification of copper-resistant isolates are shown in the tree illustrated in figure 2. Table 4 shows the microscopic morphology and isolation region of each copper-resistant strain.

3.4. Copper nanoparticles production and evaluation

Among the copper-resistant strains that isolated from coppersmiths soil, *Fusarium solani* CBS490.63 with MIC=4.5mM and among the copper plating waste water strains, *Paecilomyces varioti* BAB-1547 with MIC=4 mM were able to convert the blue color of copper sulfate solution into blue green. The results of the color change are presented in Figure 3.

Table 3. The results of MIC and MFC evaluation of CuSO₄ on the selected copper resistant isolates.

Isolate no.	Cu1	Cu2	Cu3	Cu4	Cu5
MIC	2	1	4.5	4	0.5
MFC	2.5	1.5	5	4.5	1

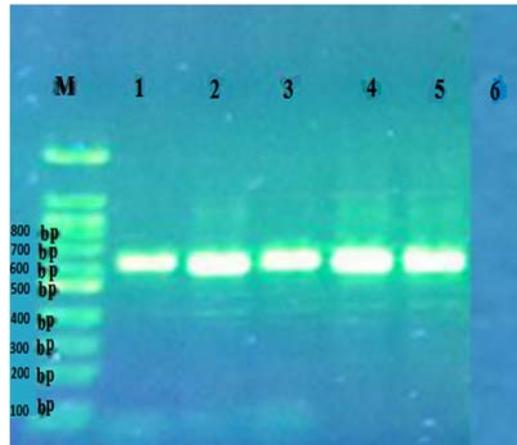
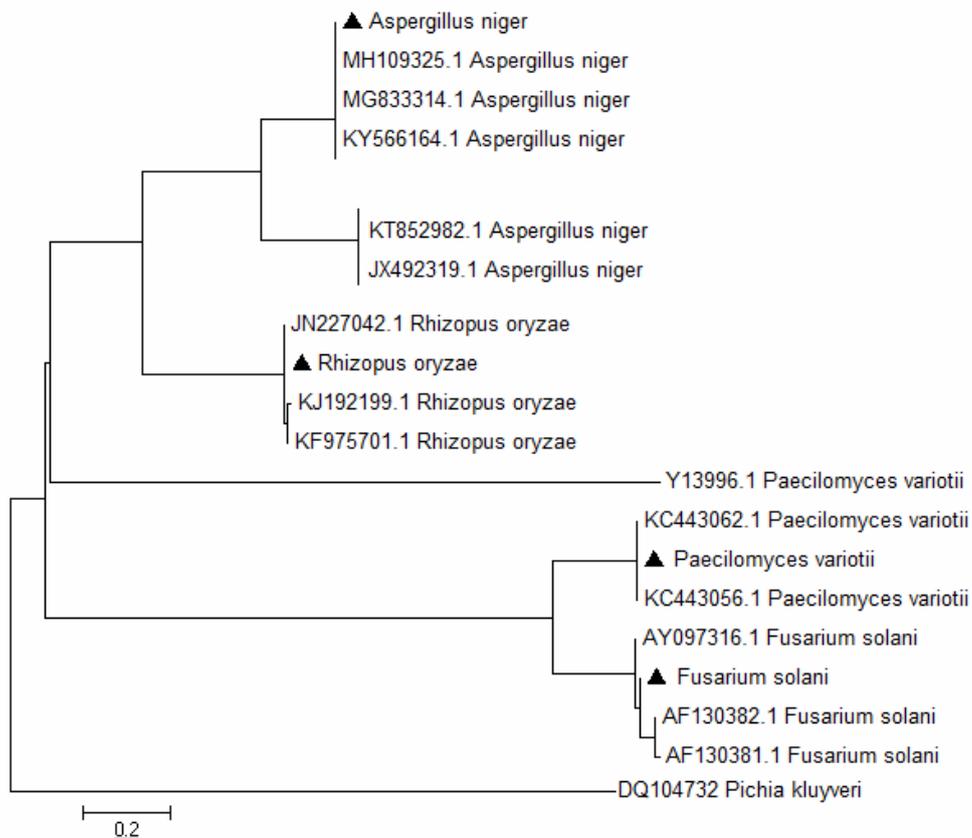
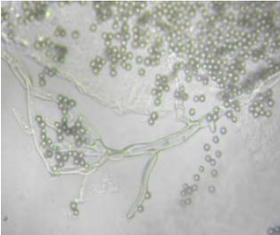
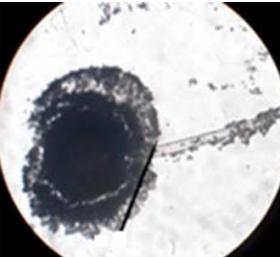
**Fig 1.** Agarose gel electrophoresis of PCR products of 16SrRNA gene in the copper resistant fungi. Lane M: 100 bp DNA size marker, Lane 1 to 4: Cu1, Cu2, Cu3 and Cu4 isolates respectively, Lane 5: positive control (*Aspergillus fumigates* PTCC5009), and lane 6: negative control.**Fig 2.** Phylogenetic tree of 4 selected copper-resistant strains. The isolates situations are shown by ▲ marker.

Table 4. Microscopic morphology and isolation region of molecular identified copper-resistant strains.

Sample	Isolate number	Microscopic morphology	Strain
Coppersmith soil (3)	Cu3		<i>Fusarium solani</i> CBS490.63
Coppersmith soil (2)	Cu2		<i>Rhizopus oryzae</i> JBM-56
Copper plating waste water (1)	Cu4		<i>Paecilomyces varioti</i> BAB-1547
Coppersmith soil (1)	Cu1		<i>Aspergillus niger</i> sp.

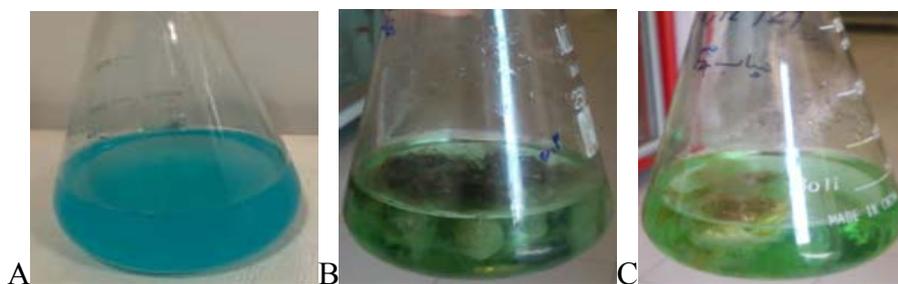


Fig 3. Color changing of copper sulfate solution by 2 strains of fungi. A: control stock without inoculation of fungi, B: *Fusarium solani* isolated from Coppersmith soil, C: *Paecilomyces varioti* BAB-1547 isolated from Copper plating waste water.

The results of UV-visible analysis of nanoparticles synthesized by *Fusarium solani* CBS490.63 is shown in figure 4. As can be seen, a certain absorption peak was obtained after treatment with the temperature of 800°C at the wavelength of 440 nm.

The study of the crystalline lattice structure and the size of copper oxide particles as well as their single phase situation were investigated by x-ray crystallography, and are presented in Figures 5 and 6. Debye-Scherrer formula agreed the formation of nanoparticles in copper

solution. According to XRD pattern, Miller's indices at the levels of -110, 002, 111, -112, -202, 020, 202, -113, 022, -311, 113 and 311 were related to 2θ equal to 32, 35, 38, 46, 48, 53, 58, 61, 65, 66, 68 and 72 angles respectively. The size of nanocrystals calculated as 29 nm with respect to copper nanocrystals reference card. The results of the analysis of TEM images showed that the produced nanoparticle was in the hexahedron shape with the size of 17 (Figure 6).

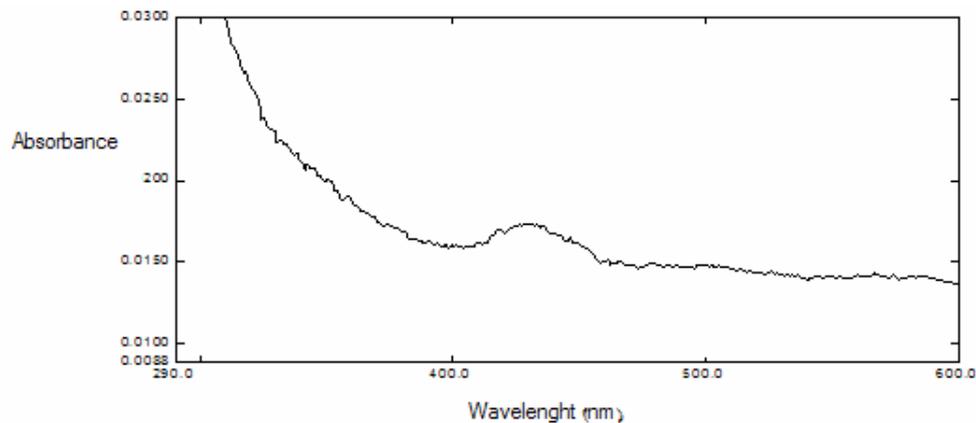


Fig 4. The absorbance peak of copper nanoparticles synthesized by *Fusarium solani* CBS490.63 in the presence of 0.05 mM CuSO_4 at the temperature of 800°C.

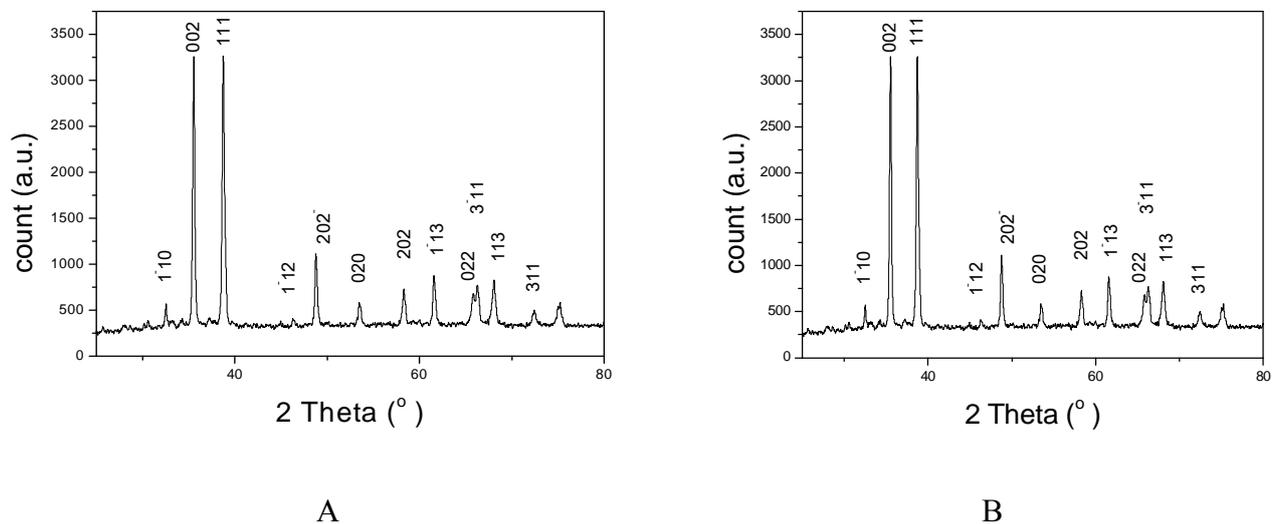


Fig 5. The X-ray christalograph of synthesized copper nanoparticles. A: nanoparticles synthesized by *Fusarium solani* CBS490.63. B: nanoparticles synthesized by *Paecilomyces varioti* BAB-1547.

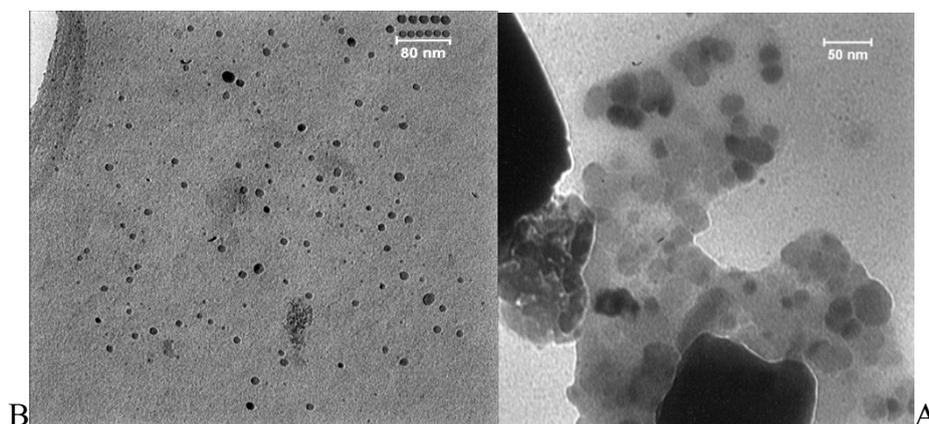


Fig 6. The TEM images of synthesized copper nanoparticles. A: nanoparticles synthesized by *Fusarium solani* CBS490.63. B: nanoparticles synthesized by *Paecilomyces varioti* BAB-1547.

4. Discussion

Generally, all microorganisms, including fungi, are exposed to the environmental conditions such as pH that affect the growth, proliferation and death of fungi. Although fungi prefer low pH for growth, many metal compounds are converted to dissolved mode in low pH amounts and the presence of them in the environment influences the degree of resistance of the microorganisms (Kuhn and Pfister, 1990). In this study, the pH of coppersmith soils 2 and 4 were neutral and the pH of coppersmith soils 1 and 3 were basic. Also pH of copper plating waste waters 1 and 2 were neutral to slightly basic. The special electrical conductivity of coppersmith soils 1, 2 and 3 were 4.8, 0.665 and 4.9 ds/m, respectively which were in normal range according to the global standards (Alboghobeys et al., 2013). The BOD of copper plating waste waters 1 and 2 were 120 and 100 mg/l, respectively and according to the standards of the pollution control committee, both samples were relatively high contaminated (Mishra et al., 2015). The COD of copper plating waste waters 1 and 2 were 6480 and 430 mg/l, respectively that showed high chemical contamination of them especially in copper plating waste water 1. The concentrations of copper in coppersmith soils 1, 2 and 3 were 38.5, 36.6 and 44.4 mg/l, respectively and were 6.3 and 0.23 in copper plating waste waters 1 and 2, respectively that are upper than global standards (Alboghobeys et al., 2013) except in copper plating waste water 2. Therefore according to the standards of the pollution

control committee, copper plating waste water 2 was not environmentally pollutant and all 3 coppersmith soils as well as copper plating waste water 1 were from highly pollutants for the environment (Manikandan and Sathiyabama, 2015). Although Habibi et al. (2017) showed an increase in the population of fungi along with the rise of BOD and COD amounts, the effect of selective factors such as neutral to basic pH, temperature lower than 25°C and existence of different heavy metals probably caused the removal of metal sensitive fungi from the sampled environments.

Among the isolated copper-resistant fungi, four isolates including *Fusarium solani* CBS490.63, *Rhizopus oryzae* JBM-56, *Paecilomyces varioti* BAB-1547 and *Aspergillus niger* sp. were selected because of the resistant to copper ions. The Cu3 isolate (*Fusarium solani* CBS490.63) showed the highest resistance (MIC=4.5 mM) and the Cu2 isolate (*Rhizopus oryzae* JBM-56) showed the highest resistance (MIC=1 mM). Jehad et al. (2012) reported the resistant of *Aspergillus niger* to 10 mM zinc oxide nanoparticles. Naghsh et al. (2013) detected the MIC of silver nanoparticles on *Aspergillus fumigatus* as 31.25 mg/ml.

In the present study, *Fusarium solani* CBS490.63 isolated from coppersmith soil and *Paecilomyces varioti* BAB-1547 isolated from copper plating waste water were able to change the color of copper solution and production of hexahedron nanoparticles with the size of 17 nm. These particles showed a distinct absorbance peak at the wave length of 440 nm in the of the wavelength range of 320-450 nm

which also has been reported by Shahverdi et al. (2007). In contrast, Hoseini et al. (2013) has been reported the production of copper nanoparticles with the absorbance peak at the wave length of 259 nm by *Fusarium oxysporum*. The results of x-ray diffraction analysis in this study confirmed the presence of copper nanoparticles with the crystal size of 29 nm with the composition of CuO, while increasing temperature could be resulted in change to single-phase formation and purification of the synthesized nanoparticles. This phenomenon also has been reported by Magdassi et al. (2010).

Conclusion

The results of this study showed that the isolated *Fusarium solani* CBS490.63 and *Paecilomyces varioti* BAB-1547 were able to produce nanoparticles through a biological synthesis manner. This biological synthesis process can be proposed as an efficient method for industrial manufacturing of nanoparticles. This method can be less toxic and contaminant in contrast to synthesizing of nanoparticles by physical and chemical methods.

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Refereces

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