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Research Article

Antibacterial Activity of Copper Oxide (CuO) Nanoparticles Biosynthesized by *Bacillus* sp. FU4: Optimization of Experiment Design

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ABSTRACT

Background: There are several methods for synthesis of metallic nanoparticles (NPs) including chemical, physical and biological process. In this study, Bacillus sp. FU4 was used as biological source for biosynthesis of CuO NPs.
Methods: CuO NPs have been prepared by copper sulfate ($CuSO_4$). CuO NPs were formed after oxidation of Cu NPs. Design and analysis of Taguchi experiments (an orthogonal assay and analysis of variance (ANOVA)) carried out by the Qualitek-4 software. Average effect of $CuSO_4$ concentration (0.1, 0.01 and 0.001 M), incubation and culturing time (48, 72, 96 hours) as three controllable factors with three levels were evaluated in CuO NPs biosynthesis. Characterization of CuO NPs was determined by UV-Vis spectroscopy, X-ray diffraction (XRD), Fourier transform infra-red (FT-IR) spectroscopy and scanning electron microscopy (SEM). Also, the antimicrobial properties of CuO NPs were investigated using *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 43300 as multidrug resistant (MDR) bacteria.
Results: Results: It was evaluated that, NPs size distributions were in the range of 2-41 nm with spherical shapes. The anti-bacterial activities of CuO NPs were measured based on diameter of inhibition zone in disk diffusion tests of NPs dispersed in batch cultures. Two levels of $CuSO_4$ concentrations (0.1 and 0.01M) had antibacterial effect on E.coli (33±0.57 and 6 ±2mm). In the case of S. aureus, there was surprisingly no sign of growth.
Conclusion: CuO NPs have antibacterial activity that can be benefit in medicinal aspect for fighting against prominent pathogen bacteria such as E.coli ATCC 25922 and S.aureus ATCC 43300.

Introduction

There are several chemical and organic compounds with antibacterial activity such as, penicillins (β-lactams group) and natural products which kill bacterial or slow down their growth.[1] Among them, nanoparticles (metallic and semiconductor) have recently obtained more attention.[2] Reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (HO●) and organic hydro peroxides (OHP), NPs deposition on the surface of microorganisms and NPs accumulation in the cytoplasm/periplasmic region of bacteria, can be lead to microorganisms death.[3] In the case of bacteria, ROS can resulted in damage of cellular constituents including lipids, peptidoglycan, proteins and DNA through generating of ROS by NPs and subsequently physical disruption.[4] Metallic and semiconductor NPs are considerable materials for investigation in nano-medicine field. This interest is related to size and shape based on physicochemical properties. Surface area to volume ratio of NPs is important factor for these properties.[5-7] In this case, one of the important applied materials in the industry is copper oxide (CuO) and its alloy in nanometers scale.[8-11] Also, These metallic NPs can be utilized as an alternative for silver and gold NPs.[12,13]

There are several methods for production of CuO NPs; specifically characterized as a chemical, physical and biological process.[14,15] For instance, proton irradiation as physical methods and vacuum vapor deposition (VVD) are able for synthesis a wide range of metallic NPs.[16,17] These methods have several disadvantages. As, the costs of these methods are higher and there is not approach of environment protection. Therefore, eco-friendly view of point is essential for production of metal NPs using biological systems.[18,19]

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Plants, algae, yeasts, fungi and bacteria can be applied as green approach for biosynthesis of metal NPs.[20-22] It is well known that bacteria have mechanisms to survive in hard conditions such as high amount of toxic metal by transforming toxic metal ions into their corresponding non-toxic forms (metal sulfide/oxides).[22-30] It is noteworthy that these mechanisms have prominent role in NPs biosynthesis. Also, NPs biosynthesis by bacteria than to other organisms, have several advantages such as simple culturing, extracellular NPs fabrication in mild conditions of experiment (temperature, pH) and be affordable and not time consuming.[31,32]

In this study, *Bacillus* sp. FU4 was used as biological source for biosynthesis of CuO NPs; in addition, XRD, FT-IR spectroscopy and SEM were applied as benefitted technique for the characterization of CuO NPs. In the end, the antibacterial activity of these NPs on two pathogenic species with multidrug resistance characteristic, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 43300, was evaluated.

Materials and Methods

Taguchi methodology experimental design

In order to optimization of experimental conditions, all the combination experiments using the assigned parameter values were conducted. The Qualitek-4 software was used to design and analysis of Taguchi experiments as statistical method.[33-35] Table 1 shows three controllable factors ($CuSO_4$ concentration, incubation time and culturing time) and their levels in design of experiment.

Materials

Copper(II) sulfate pentahydrate, 98% (CuSO4.5H2O) and nutrient agar were purchased from Sigma-Aldrich and applied respectively for CuO NPs synthesis and antibacterial activity measurement without any further purification.

CuO NPs biosynthesis and preparation of supernatant

Bacteria *Bacillus* sp. FU4 was obtained from bacterial archive, Razi University, Kermanshah. Growth conditions were simple, growth in 0.5 nutrient broth (NB) medium at 37 ˚C for three levels of time culturing (48, 72, 96 hours). After growth of bacteria in these times, culture mediums with bacteria were centrifuged at 5000 for 5 minutes. Then 5cc of supernatant was added to $CuSO_4$ by three levels of concentrations (0.1, 0.01, 0.001M) at Erlenmeyer flask at three replicates for each concentration level. Afterwards, these solutions were incubated at under agitation (100 rpm) for three time levels of incubation (48, 72, 96 hours).[36]

Characterization

Structure, morphology and elemental composition of the prepared annealed samples were characterized for by XRD and SEM analysis tools. Crystallographic study was carried out using EQUNIOX 3000, diffractometer in the scanning range of 20˚ - 70˚ (2θ) using Cu K_a radiations of wavelength 1.5406 Å. A scanning electron microscope (model XL30, Philips, Eindhoven) was used to study the morphology of morphology and size of NPs. The intensity of absorption peaks of NPs was examined by UV-Vis spectrophotometer (Tomas, UV 331) from 400 to 800nm. Also, Fourier transform infrared spectroscopy measurements were done by (Germany, Bruker, Model:ALPHA) spectrophotometer.[37]

Antibacterial Effects

Escherichia coli ATCC 25922 and *Staphylococcus aureus* ATCC 45500 as multi drug resistant bacteria were utilized for measurement the effect of antibacterial properties of CuO NPs by modified agar disc diffusion.

Table 1. Biosynthesis parameters and their levels.

Symbol	Parameters	Unit	Level 1	Level 2	Level 3
A	$CuSO_4$ concentration	M	0.1	0.01	0.001
B	Incubation time	Hour	48	72	96
C	Culturing time	Hour	48	72	96

Table 2. Orthogonal array of Taguchi experimental design for biological synthesis of copper oxide NPs.

Trail No.	A (M)	B (hour)	C (hour)	Optical Density (OD)
1	0.1	48	48	0.565
2	0.1	72	72	0.308
3	0.1	96	96	0.987
4	0.01	48	72	0.988
5	0.01	72	96	1.185
6	0.01	96	48	0.790
7	0.001	48	96	0.011
8	0.001	72	48	0.064
9	0.001	96	72	0.199

MDR bacteria were cultivated on Muller Hinton agar (MHA) plates; 5 mm diameter paper discs were prepared with the help of a sterilized steel cork borer.

Afterwards, different concentrations of CuO NPs (three levels with 0.1, 0.01 and 0.001M concentrations) were loaded in paper discs followed by placing the discs on agar. Then, the plates were incubated at 37℃ for 48 hours.[38]

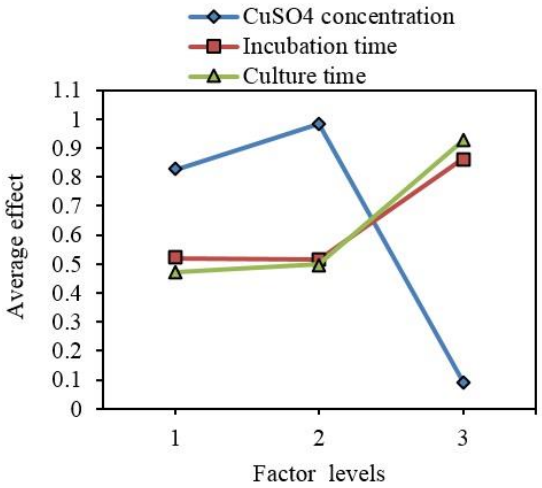

Figure 1. Taguchi results of average effect of $CuSO_4$, incubation time and culture time.

Table 3 shows effects of three different factors on the copper oxide NPs biosynthesis by *Bacillus* sp. FU4. As illustrated in this Table, $CuSO_4$ concentration in level 2 (0.984), incubation time in level 3 (0.864) and culturing time in level 3 (0.93) had higher effect on the CuO NPs biosynthesis. Similarly, Taguchi results of average effect of $CuSO_4$, incubation time and culture time are presented in Figure 1.

Effective factors in copper oxide NPs biosynthesis by *Bacillus* sp. FU4 are demonstrated by variance analysis (ANOVA) (Table 4).

Table 3. Effects of three different factors on the CuO NPs biosynthesis.

Factors	Level 1	Level 2	Level 3
A	0.826	0.984	0.091
B	0.521	0.515	0.864
C	0.473	0.498	0.93

Final column determines effect percentage of each factors which major factor is $CuSO_4$ concentration with value of 40.116%. Therefore, this result illustrates higher importance of $CuSO_4$ concentration parameter than other parameter in copper oxide NPs biosynthesis by *Bacillus* sp. FU4.[11,19,39]

In addition, optimum conditions for biosynthesis of affected CuO NPs by three factors are shown in Table 5. Expected result at optimum condition was value of 1.509% that is relatively suitable result of biosynthesis by this bacterium.

UV-Vis analysis of CuO NPs biosynthesis

When cell free supernatant of *Bacillus* sp. FU4 was added to $CuSO_4$ solution and incubated for three times of incubation (48, 72, 96), the mixture's colour reaction altered from blue to light green (Figure 2). Spectrum absorption of UV-Vis spectroscopy for this solution illustrated a distinct absorption peak in the region of 700-800nm (Figure 3).

Figure 2. Changes of reaction mixture's colour from light green (left) to light green (right) after supernatant addition.

Table 4. Analysis of variance (ANOVA) for CuO NPs biosynthesis.

Factors	DOF(f)	Sum of Sqrs.	Variance	F-Ratio (F)	Pure Sum (S′)	Percent (%)
A	2	1.362	0.681	3.404	0.962	40.116
B	2	0.239	0.119	0.599	0	0
C	2	0.396	0.198	0.99	0	0

Table 5. Optimum conditions of CuO NPs biosynthesis by bacterium.

Factors	Levels	Contribution
A	2	0.35
B	3	0.23
C	3	0.296
Total contribution from all factors	-	0.876
Current grand average of performance	-	0.633
Expected result at optimum condition	-	1.509

X-ray diffraction analysis

A high crystalline level of the CuO NPs sample can be seen with diffraction angles of 21.05, 36.2 and 43.74, 50.69, 74.2 which corresponds to the characteristic face centered cubic (fcc) of copper lines indexed at (111), (200) and (220), respectively (Figure 4). Impurities such as Cu_2O may be effective on absence of any noticeable peaks in pattern.[40]

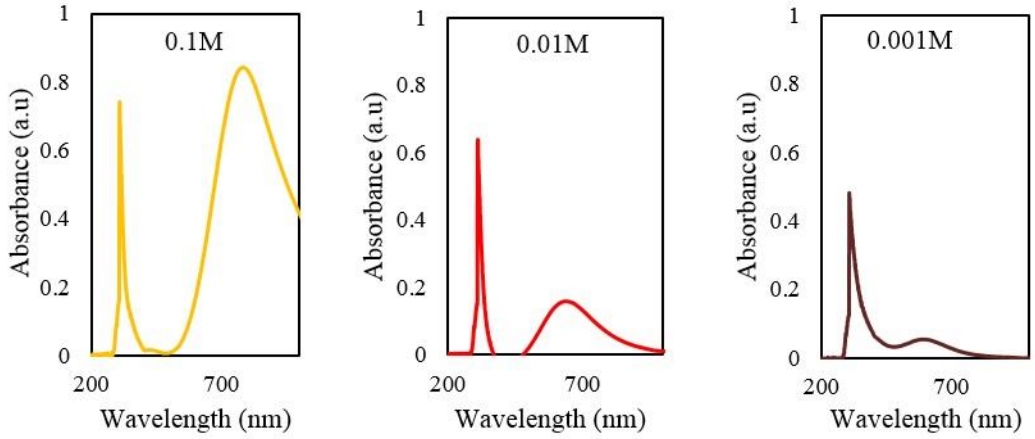

Figure 3. UV-Vis spectrum of CuO NPs produced by *Bacillus* sp. FU4 in three level of concentration 0.1, 0.01 and 0.001.

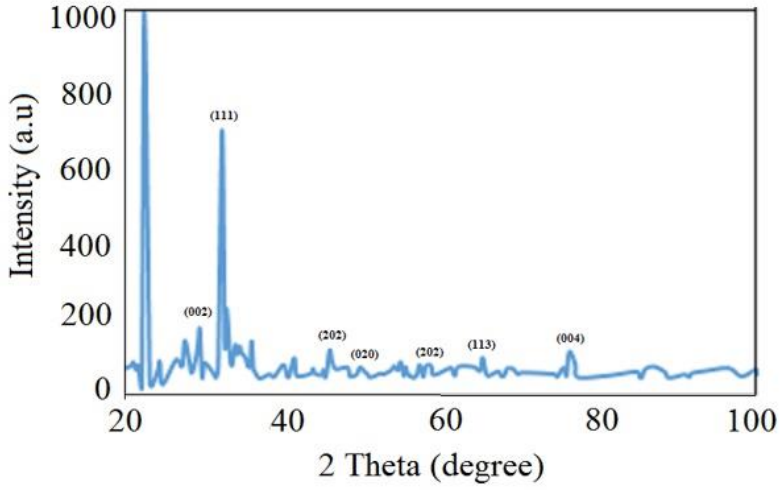

Figure 4. X-ray diffraction spectrum of CuO NPs synthesized from 0.1 M of $CuSO_4$ treated *Bacillus* sp. FU4 cell free supernatant at 28˚C.

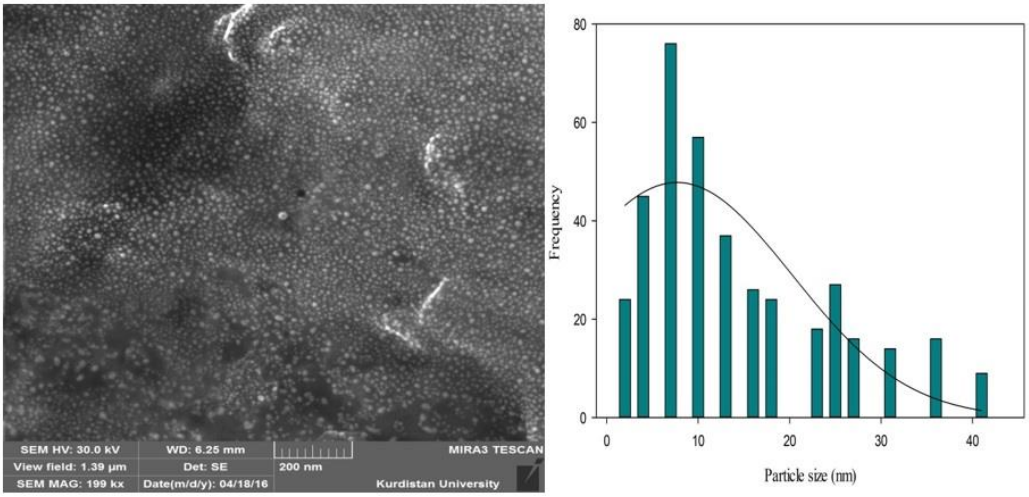

Figure 5. Scanning electron microscopy (SEM) image and particle size distributions of CuO NPs produced by culture supernatant of *Bacillus* sp. FU4.

In addition, the average grain size of NPs was 64.97 nm using Debye-Scherer Eq.(1):

$$\tau = \frac{k\lambda}{\beta cos\theta} \quad \text{Eq.(1)}$$

where K, known as Scherer's constant, ranges from 0.9 to 1.0, λ is 1.5418 Å, which is the wavelength of the X-Ray radiation source, β1/2 is the width of the XRD peak at half height and θ is the Bragg angle (Figure 4). However, XRD measurement revealed that the NPs initially formed as colloids tend to grow and react with environment oxygen.[41] Also, as shown in Figure 5, SEM images, demonstrated that CuO NPs were formed as aggregates particles with spherical shape. Particle size distributions were in the size range of 2-41nm.

FTIR analysis

FT-IR was performed in order to evaluate the molecular interactions between the CuO NPs and the media. Figure 6 demonstrates a C=O vibration band at 1098.17cm^{-1}. In addition, the spectrum illustrates at 3381.31cm^{-1} to N-H stretching, 2038.58cm^{-1} to N=C=S stretching, 1631.89 cm^{-1} to C=C stretching, 1417.22 cm^{-1} to O-H bending, 1211.90 cm^{-1} to C-O stretching, 1098.17 cm^{-1} to C-O stretching, 776.87 cm^{-1} to C-H bending.

Antibacterial activity

Antibacterial activity is defined as killing bacteria or reducing their growth without general toxic to surrounding tissue of body.[42] There are several reports about antibacterial properties of NPs.[43-45] In this study, antibacterial activity of CuO NPs was indicated by disc diffusion assay (Figure 7). Due to evaluating of antibacterial effects, maximum zone of inhibition, two important multidrug resistant pathogenesis bacteria, *E. coli* ATCC 25922 and *S. aureus* ATCC 43300 were used. Results show that two levels of CuSO4 concentrations (0.1 and 0.01M) had respectively antibacterial activity on E. coli ATCC 25922 as inhibition zone diameter of 33±0.57 and 6 ±2mm. In the case of *S. aureus* ATCC 43300, there was surprisingly no sign of obvious growth. Green synthesis of CuO NPs (5-10 nm, spherical shape) by *Gloriosa superba* illustrated antibacterial activity against *S. aureus* and *Klebsiella aeroegenes*.[46]

Discussion

Taguchi method was used to optimize the setting of the process factors values for enhancement quality properties and to identify the product factor values under the optimal process factor values.[47] In this case, Taguchi method utilizes orthogonal arrays as a specific design to investigate the entire factors space with a minimum cost of experiments.[48] In order to analyze the quality characteristics, Taguchi method applies three types of the signal/noise (S/N) ratio. In this study, we used the higher-the-better type of S/N. Three parameters ($CuSO_4$ concentration, incubation time and culturing time) and their three levels were used for design experiment. Results show higher effect of $CuSO_4$ concentrations than other parameters which can be comparative with previous reports.[49,50] This colour change illustrates the formation and oxidation of CuO NPs. In this case, Shantkriti and Rani reported similar appearance of green colour solution with addition of $CuSO_4$ to a flask containing *Pseudomonas fluorescens*.[51] Based on special particles properties such as size, shape and capping agents, the precise position of SPR band may shift.[52] As, copper oxide NPs synthesized from *Aspergillus clavatus* species confirmed the presence of copper oxide NPs at 300 nm.[53]

Also, compared to previous studies, green synthesis of CuO NPs by *Malva sylvestris* and *Phyllantus amarus* plant leaves had spherical shape.[54,55]

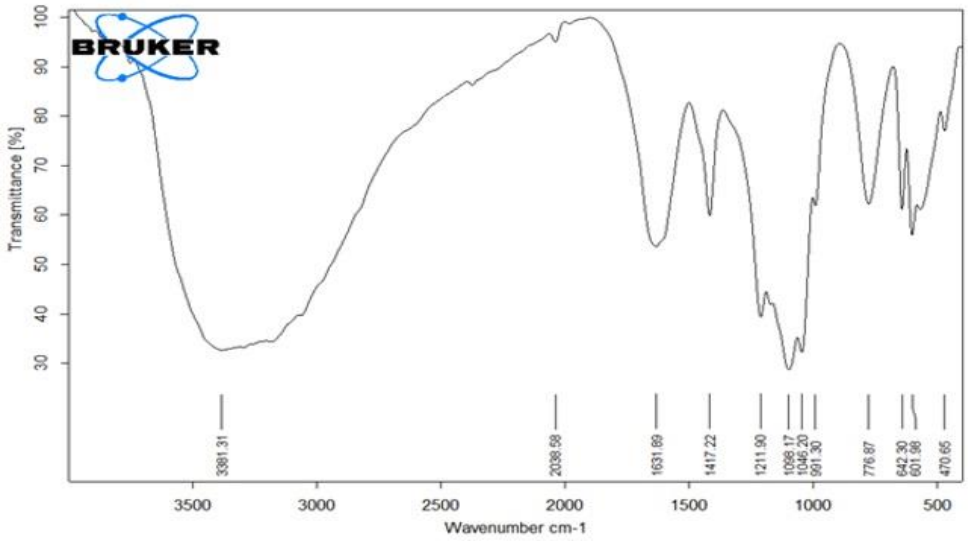

Figure 6. FTIR peaks of CuO NPs green synthesized by *Bacillus* sp. FU4.

Figure 7. Bactericidal activity of CuO NPs on *E.coli* ATCC 25922 and *S.aureus* ATCC 43300.

The interaction between the Cu-NPs and media is the cause of the corresponding vibration bond, which demonstrates a reaction between Cu-NP surface and carbonyl and hydroxyl functional groups.[56] In this case, green synthesis of copper oxide nanoparticles by *gum karaya* as a biotemplate had similar functional groups.[57] As demonstrated in similar investigation, these functional groups can contribute in NPs biosynthesis by their capping role.[58-60] The grain size of CuO NPs (64.97nm) was calculated by Scherrer formula. Similarly, Sonia et al (2016) reported the grain size of 50nm for Copper oxide NPs.[61] As shown in Figure 5, the quality and composition of biosynthesized CuO NPs were indicated by Fourier Transform Infrared spectroscopy (FTIR) in the range of 400-4000 cm^{-1}. Similar investigations illustrated Cu-O bond in CuO NPs at 430, 507, and 606 cm^{-1}.[61]

Four types of bacteria: *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Proteus vulgaris* were used to evaluate antibacterial activity of CuO NPs. Results of this study demonstrated that *E. coli* was more sensitive than other bacteria species at a highest concentration of CuO NPs (1000 µg mL) with a inhibition zone of 26.0 ± 1.00 mm.[62] Sonia and coworkers (2016) investigated the antibacterial activity of CuO NPs in three different concentrations (12.5 µg/ml, 25 µg/ml and 50 µg/ml) by agar diffusion method against four pathogenic bacteria species: *Serratia marcescens*, *Streptococcus pneumonia*, *Staphylococcus aureus*, and *Salmonella typhimurium*.[61] The minimum inhibitory concentration (MIC) for all nanostructures was in the range of 12.5 µg/ml to 50 µg/ml. Also, antibacterial effect against *E. coli*, *S. aureus* and *B. subtilis* was confirmed through green synthesis of Ag NPs.[30,63] This activity may be resulted from the attaching of copper ions (released by the NPs) to the negatively charged bacterial cell membrane.[42]

In the case of antibacterial effect mechanisms, physical disruption and oxidative stress are major cause of NPs toxicity.[64] Reactive oxygen species (ROS) including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($HO^\bullet$) and organic hydro peroxides (OHP), NPs deposition on the surface of bacteria and NPs accumulation in the cytoplasm/periplasmic region can be resulted in bacterial death[3]. ROS can resulted in damage of cellular constituents (lipids, peptidoglycan, proteins and DNA) through releasing from NPs and subsequently penetration into bacteria.[4]

Conclusion

In this study, CuO NPs with spherical shapes and average mean sizes in the range of 2-41 nm and an fcc crystal structure were synthesized in a green method by *Bacillus* sp. FU4. UV-Vis, XRD, FT-IR were used to characterization of NPs. There are many studies about biosynthesis of NPs by plant, fungi, and bacteria. Based on this study, green method is easy and eco-friendly way for CuO NPs synthesis with relative purity of NPs. Also, copper oxide NPs synthesized by *Bacillus* sp. FU4 extracellular and stabilizing of CuO NPs were possible without using any capping agents which are toxic. Also, these NPs have antibacterial effect that can be usable in medicinal aspect for fighting against prominent pathogen bacteria such as *E.coli* ATCC 25922 and *S.aureus* ATCC 43300. Generally, this study presents simple, low expensive, eco-friendly and high productivity in fabrication of CuO NPs.

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Conflict of interests

The authors claim that there is no conflict of interest.

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