European Journal of Biology and Biotechnology
www.ejbio.org

Biosynthesis, Characterization and Antibacterial Properties of CuO Nanoparticles of Psychrotrophic Bacteria

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ABSTRACT

Copper oxide nanoparticles have many applications in industry and medicine. Nevertheless, few studies have been conducted in relation to the biological production of these nanoparticles. In the present study, the ability of isolated Psychrotrophic (cold-resistant) prokaryotes from the Zagros highlands located in Lorestan province of Iran and some isolated Streptomyces strains from the Oman Sea has been evaluated in the synthesis of copper oxide nanoparticles. The maximum lethal concentration of copper salts was determined for the strains and the strains with the highest level of resistance were selected. The biosynthesis of copper oxide nanoparticles was done extracellularly by inoculating Psychrotrophic and Streptomyces strains in liquid TSB culture medium containing 0.01 M copper sulfate salt and keeping the culture medium in a shaker incubator at 20 °C at 150 rpm. The production of copper oxide nanoparticles was evaluated by changing the color of the reaction solution from light blue to dark green. From the 44 cold-resistant strains and the investigated Streptomyces strains, all strains were able to tolerate concentrations higher than 5 mM. Of these, the strain OSNP13 belonging to the genus *Microbacterium liquefaciens* (X77444) has been able to synthesize the highest amount of copper oxide nanoparticles. The optical absorption of the solution containing synthesized nanoparticles was determined in the range of 250-350 nm by UV-vis spectrophotometer, which had a specific peak at 288 nm. And the copper oxide nanoparticles have an average size of 63.21 nm based on DLS analysis. The crystallographic characteristics of copper oxide nanoparticles were also determined by using XRD analysis, which showed that the prepared nanoparticles had a hexagonal crystal structure with a size of 31.29 nanometers. Then, the antibacterial activity of produced nanoparticles was evaluated. The MIC values of copper oxide nanoparticles for *E. coli* and *S. aureus* bacteria were calculated as 125 and 250 micrograms per milliliter, respectively. The produced copper oxide nanoparticles have shown a good antimicrobial properties and can be suitable candidates for use as antimicrobial agents.

Keywords: Antibacterial activity, CuONPs, Psychrotrophic Bacteria, MBC, MIC.

Published Online: July 29, 2023

ISSN: 2684-5199

DOI : 10.24018/ejbio.2023.4.3.481

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I. Introduction

Nanoparticles due to their small size and high surface-to-volume ratio, have unique electrical, physical, chemical, optical, and biological properties compared to their similar bulk compounds (Singh *et al.,* 2015). Copper oxide nanoparticles are one of the most important semiconductor oxide nanoparticles that have many properties such as high electrical conductivity, thermal resistance, corrosion resistance and chemical stability, electrochemical coupling coefficient, electron mobility, high thermal and mechanical stability at room temperature (Slavin *et al.,* 2017). Having these properties, they have many uses in various industries and medicine. The applications of these nanoparticles are magnetic phase transfer, biological sensors, nanogenerators, gas sensors, luminescent materials, biosensors, optical detectors, photocatalysts, antibacterial and antifungal activity, drug delivery, wastewater treatment, ceramics and rubber (Hou *et al*., 2017 and Ojha *et al.,* 2017). One of the very important applications of copper oxide nanoparticles is its selective toxicity for bacteria and its low toxicity effects on human cells, which has made it a suitable candidate for use in the medical industry (Ravishankar *et al*., 2011). In extensive studies, the antibacterial effects of these nanoparticles on gram-positive and gram-negative bacteria as well as spore bacteria that are resistant to high temperature and high pressure have been proven (Dizaj *et al*., 2014). Nanoparticles can be synthesized by physical, chemical and biological methods. Physical and chemical methods are dangerous and toxic methods due to the need for high temperature and pressure and chemical substances that have harmful effects on the environment and limit the use of nanoparticles in pharmaceutical and medical fields (Buzea *et al*., 2007; Thirumurugan and Dhanaraju, 2011). Recently, the

biological methods for production of nanoparticles has been greatly developed due to the lower production cost, greater compatibility with the environment and the creation of more stable nanoparticles. In these methods, plants and microorganisms such as fungi, yeasts, actinomycetes and bacteria are used to produce nanoparticles (Agrawal *et al.,* 2014), of these bacteria are more important than others due to the ease of growth and the possibility of genetic manipulation. (Sharma *et al.,* 2010). Considering the efficient metal resistance mechanisms and the high potential of Psychrotrophic microorganisms in the extraction of nanoparticles and the very few studies that have been carried out regarding the extraction of metal nanoparticles with these microorganisms, therefore, in the present research, the ability of new strains of the isolated cold-resistant bacteria from Zagros highlands located in Lorestan province have been taken into consideration for extracting and characterizing of copper oxide nanoparticles and investigating their intrinsic properties as antibacterial agents.

II. Research Background

Due to the wide applications of metal oxide nanoparticles in various fields of science, extensive efforts have been made in order to biological production of widely used copper oxide nanoparticles with the green and environmentally friendly methods, as well as to investigate its biological applications, which are discussed below that some of these researches have been mentioned.

Varshney *et al*. (2010), produced copper oxide nanoparticles by a non-pathogenic bacterium *Pseudomonas stutzeri* isolated from soil with an environmentally friendly method. The produced nanoparticles had a spherical structure and a size between 8-13 nm.

Singh *et al*. (2010), produced copper oxide nanoparticles in aerobic conditions of normal pH and room temperature by *E. coli* bacteria by extracellular method. The average particle size was between 10 and 40 nm and a high percentage of quasi-spherical nanoparticles.

Ramanathan *et al*. (2011), produced copper oxide nanoparticles by using the bacteria *Morganella morganii* RP42 and *Morganella psychrotolerans* by cell integrity method. The produced nanoparticles had a size in the range of 10-3 nm.

Kundu *et al*. (2014), produced zinc oxide nanoparticles by *Rhodococcus pyridinivorans* NT2 bacteria. The produced nanoparticles had an average size of 100 to 120 nm and showed good antimicrobial and anticancer properties against *Staphylococcus epidermidis* NCIM2943 and HT-29 cell line, respectively. In this research, copper sulfate was used as a precursor for the production of nanoparticles.

Jayaseelan *et al*. (2012) succeeded in producing zinc oxide nanoparticles with a spherical structure and an average size of 57 nm by *Aeromonas hydrophila* bacteria. Antibacterial properties of nanoparticles on *Pseudomonas aeruginosa* and *Aspergillus flavus* were evaluated by well diffusion method. The growth inhibition halo diameter was reported as 22 and 19 mm for these two pathogens, respectively.

Sharmila *et al*. (2018), produced zinc oxide nanoparticles using *Bauhinia tomentosa* leaf extract. The produced nanoparticles had a hexagonal structure and average particle size of 22 to 94 nm. The produced nanoparticles showed good antimicrobial properties against Gram-negative *P. aeruginosa* and *E. coli* bacteria.

III. Materials and Methods

A. Sampling and Isolation of Investigated Strains

Isolation of cold-resistant strains from Zagros highlands located in Lorestan province was done by inoculation of environmental inoculations into Tryptic Soy Broth (TSB). The plates were kept at 20°C for 48 hours. Pure strains were obtained by sequential cultivation and different types were selected based on colony morphological observations and microscopic images.

B. Investigating the Resistance of Strains to Copper Salts

In order to determine the maximum lethal concentration (MTC), 200 ml of solid culture medium (TSB) in different concentrations (2.5, 5, 10, 25 and 50 mmol/liter) of $CuSO_4.5H_2O$ and $Cu(NO_3)_2.3H_2O$ salts was prepared in separate flasks. Then, the contents of each of the jars were transferred into sterilized microbial plates and placed at 30 °C for 24 hours. Then, all the plates were divided into different parts and the plates without salt (copper) were considered as witnesses for the growth of bacteria. 5 µL of the suspension of the investigated strains (24-hour culture) with a turbidity equal to 0.5 McFarland was inoculated on each of the plate divisions. All plates were kept at 20 °C for 48 hours. A high concentration of metal in which bacteria cannot grow was introduced as MTC, and thus resistant and efficient strains were identified in the reduction of copper cations (Shakya *et al.,* 2012).

C. Extraction, Characterization of Copper Oxide Nanoparticles and Molecular Identification of the Produced Strains

Each of the selected strains was inoculated in 400 ml of TSB medium and incubated for 48 hours in an incubator shaker at 20 °C at 150 rpm. After the growth and proliferation of the cells, the resulting supernatant was separated by centrifugation at a speed of 10000 rpm for 15 minutes. The resulting supernatant was added with copper sulfate salt with a concentration of 0.01 M in a ratio of 1:1. All the samples were placed in an autoclave with a temperature of 121 °C for 15 minutes and after 24 hours of incubation in greenhouse, the produced nanoparticles were separated by centrifugation and after washing three times with deionized water and ethanol at a temperature of 80 °C was dried up. In order to remove the biological agents on the surface of the nanoparticles, the produced nanoparticles were placed in an oven at 470 °C for 4 hours. Then, the strain with the highest amount of sediment formation and color change of the supernatant (supernatant solution) was selected for purification and characterization of the produced copper oxide nanoparticles. Purified nanoparticles were analyzed to measure optical absorption by UV-vis spectrophotometry in the range of 200-400 nm. Also, some of the sediment obtained was used for X-ray diffraction (XRD) and dynamic light scattering (DLS) analysis. Molecular identification of the selected strain was done by amplification and sequencing of the 16srRNA gene.

D. Measuring the Antibacterial Activity of Nanoparticles

For the antibacterial study of copper oxide nanoparticles, a stock of 2000 µg/ml of nanoparticles was dispersed in ammonium citrate at a ratio of 2:1 and 40% glycerol. *E. coli* and *S. aureus* bacteria were inoculated in MHB sterile medium and kept in a greenhouse at 37 °C for 18 hours. Finally, by using physiological serum (0.9% sodium chloride), the turbidity of bacteria was adjusted in McFarland's half (0.08-0.13 absorbance at 630 nm wavelength), and in this case, the number of bacteria is equal to 1.5 x 108 CFU/ml. The antibacterial activity of nanoparticles in different concentrations (4000 to 31.25 µg/ml) was used using the microdilution method (Mallick and Sahu, 2012).

IV. Results and Discussion

The resistance of 44 cold resistant strains to two salts of copper sulfate and copper nitrate in concentrations (2.5, 5, 10, 25 and 50 mM) was evaluated.

The results showed that all strains have the ability to resist concentrations higher than 5 mM. According to the initial cultivation results, four cold resistant strains were selected.

Also, bearing in mind the results of the MTC test, the resistance of these strains to copper sulfate salt was higher than copper nitrate, and therefore, concentrations of 10 mM copper sulfate per liter were used to produce nanoparticles (Fig. 1).

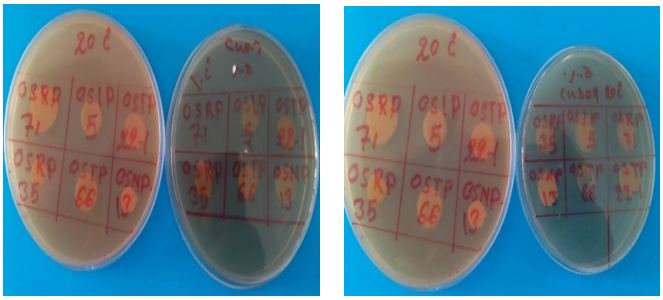

Fig. 1. Resistance of some strains to 5 mM concentration of copper sulfate and copper nitrate.

Then, the OSNP13 strain with the highest color change of the supernatant (supernatant solution) from light blue to dark green and the formation of sediment was selected for the purification analysis of copper nanoparticles, as mentioned in the development of biological methods for the and production of nanoparticles as newly emerging methods, more extensive studies are needed to discover the mechanisms involved in the processes as well as the details of the factors affecting its various aspects. Therefore, the idea of this research is to use microorganisms in which very few studies have been conducted regarding the extraction of metal nanoparticles (copper oxide) from them. According to the examination of the extraction of nanoparticles with the supernatant of microbial culture along with heating in autoclave for the strains studied in this research, based on the observation of the highest intensity of color change and the formation of precipitation of interaction mixtures after the production of nanoparticles, it is easy to achieve a high yield rate. It has been the most time-saving purification method (Fig. 2).

Therefore, there is no report about the production of copper oxide nanoparticles from cold-resistant strains with this method.

Ghanbari *et al*. (2018), succeeded in production of silver nanoparticles using the supernatant of *Aspergillus fumigatus* in autoclave conditions.

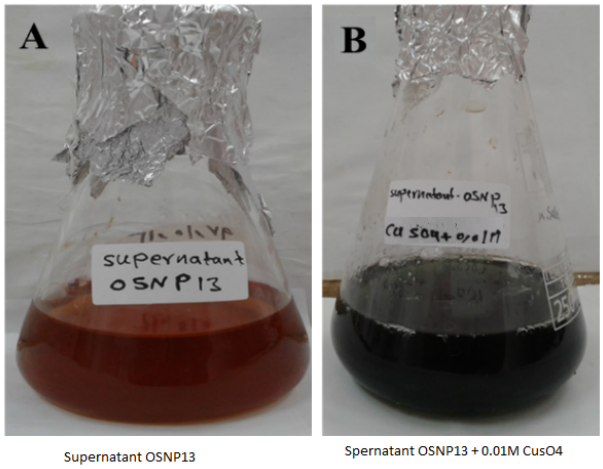

Fig. 2. Production of copper oxide nanoparticles by microbial culture supernatant method with autoclave heat. Supernatant of OSNP13 strain, supernatant after addition of copper sulfate salt and autoclaving, A and B respectively.

The results of UV-vis spectroscopy in Fig. 3 shows a specific peak between 250 and 350 nm with maximum absorption at 288 nm, which indicates the presence of copper oxide nanoparticles in the interaction solution and is caused by the surface plasmon resonance characteristic of these nanoparticles.

The presence of specific peaks at the wavelength of 288 indicates the production of copper oxide nanoparticles is by *Microbacterium* sp. OSNP13.

Mahapatra *et al.* in 2008 showed that the absorption peak for copper oxide nanoparticles is at 284 nm.

Yugandhar *et al.* (2017), produced copper oxide nanoparticles using *Syzygium alternifolium* stem extract and reported the maximum absorption peak for the produced nanoparticles 285 nm, which is consistent with the present study.

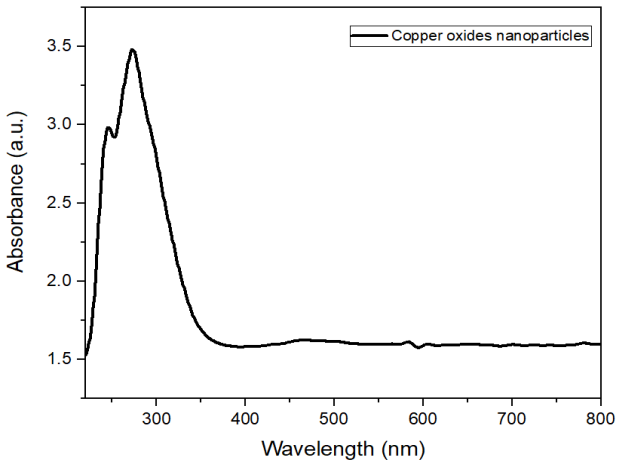

Fig. 3. Uv-vis spectroscopy chart for copper oxide nanoparticles.

The results of the nanoparticle size distribution diagram showed that the extracted copper oxide nanoparticles have an average size of 63.21 nm. As shown in Fig. 4, the size distribution curve of nanoparticles is almost bell-shaped, which indicates the uniform ratio distribution of extracted copper oxide nanoparticles. The poly dispersity index of nanoparticles (PDI) was recorded as 0.27, which indicates the high uniformity of the colloidal solution of nanoparticles obtained in this study. The produced nanoparticles by *Microbacterium* sp. OSNP13 has an average size of 63.21 nm and a dispersion index of 0.27. In the Tiwari et al. study, copper oxide nanoparticles were produced using P. aeruginosa bacteria, and their average particle size and dispersion index were reported as 110.9 and 0.312 nm, respectively. This difference in the average size of nanoparticles is due to two different bacteria in these two studies. In the study of Kumar et al. in 2017, the average size of copper oxide nanoparticles produced by *Benth Rubus glaucus* fruit and leaf extract was reported as 43.3 and 52.5 nm, respectively, which is consistent with this research.

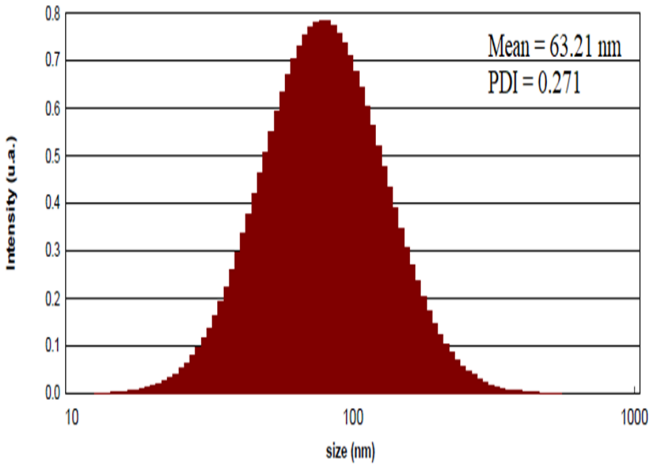

Fig. 4. Size distribution pattern of extracted copper oxide nanoparticles

In order to determine the network type and prove the crystal structure of copper oxide nanoparticles, XRD analysis was used, which shows nine specific peaks related to crystal planes 110, 002, 111, 102, 020, 202, 113, 311, 004 in the X-ray refraction pattern corresponding to Copper oxide nanoparticles are produced by *Microbacterium* sp. OSNP13. The pattern obtained from X-ray diffraction crystallography (XRD) was used at the angle of 2θ and the scanning range of 20 to 80 degrees, which is completely consistent with the standard pattern of 1548-048 too (Fig. 5), (Mallick *et al.,* 2012). Yugandhar *et* al in 2017, reported five distinct peaks corresponding to pages 110, 111, 200, 202, 020, 113, 022 in the X-ray diffraction pattern of copper oxide nanoparticles in their studies, which were obtained with the pattern consistent in this research.

The pattern observed for copper oxide nanoparticles is consistent with the studies of Ghorbani *et al.* (2015) and Kumar et *al*. in 2017. According to the calculations based on the Debye-Scherer equation, for copper oxide nanoparticles at an angle of 37.81 with the 111 crystal plane, the average size of the nanoparticles was calculated to be 31.29 nm.

The results of this section are consistent with the results of DLS analysis and indicate the smaller size of the produced copper oxide nanoparticles. Because based on reliable sources, it has been reported that a uniform colloid sample of nanoparticles, where the monodispersity of the particles is high, has a PDI between 0.01 and 0.7. Non-uniform and polydisperse samples have a PDI higher than 0.7 to 1, which represents an inappropriate sample (Honary *et al.,* 2013).

Regarding the molecular identification of the strain producing copper oxide nanoparticles, by sequencing the 16SrRNA gene and matching the results with the EZTaxon and NCBI databases, the OSNP13 strain belongs to the genus *Microbacterium liquefaciens* (X77444) with 99.73% similarity. Then, the antibacterial effects of copper oxide nanoparticles on two bacteria, *E. coli* and *S. aureus*, were investigated.

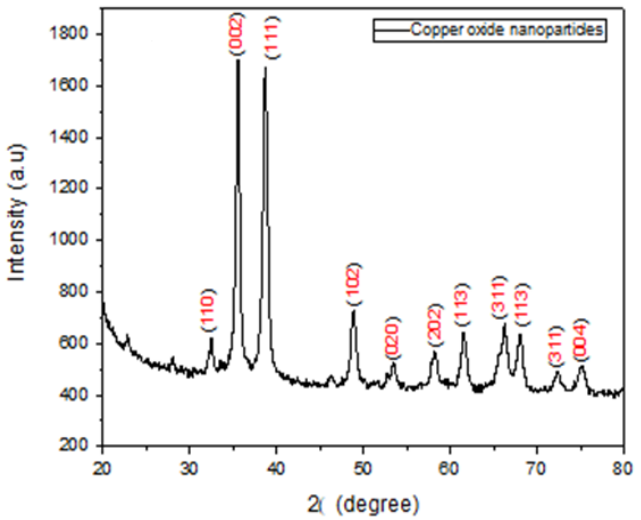

Fig. 5. X-ray diffraction analysis of extracted copper oxide nanoparticles.

MIC of copper oxide nanoparticles was calculated as 125 µg/ml for *E. coli* and 250 µg/ml for *S. aureus* bacteria, which is equal to its MBC values. During various studies, metal oxide nanoparticles, including copper oxide, show good antimicrobial activity, which mainly depends on the small size of these nanoparticles. Metal oxide nanoparticles are attached to the bacterial surface or the bacterial nuclear region and exert their antimicrobial effects. Metal oxide nanoparticles with different functions such as the production of reactive oxygen species (ROS) damage the cell wall. These nanoparticles penetrate the cell wall and cause structural damage to the cell wall and cell death. During the destruction of the cell wall, the contents of the cell are secreted out, which is associated with the expression of oxidative stress genes, which result in the inhibition of cell growth and cell death (Sirelkhatim *et al.,* 2015). Therefore, the investigation of the antimicrobial effect of nanoparticles on *E. coli* and *S. aureus* bacteria showed that, in general, the said nanoparticles have significant antibacterial properties, which is a good advantage for its use in medicine. In 2012, Azam *et al.* reported the MIC values of copper oxide nanoparticles for *E. coli* and *S. aureus* as 103 and 120 µg/ml, respectively, and this difference is due to the smaller size of the produced nanoparticles compared to this research. Of course, in terms of comparing the nanoparticles used in this research, it has shown moderate inhibitory and lethal effects compared to previous studies (Table I).

TABLE I: MIC and MBC Values of Copper Oxide Nanoparticles

Nanoparticles	Bacteria	MIC(µG/ML)	MBC(µg/ml)
CuO NPs	*E. coli*	125	125
	S. aurous	250	250

Fig. 6 shows the inhibition percentage of the growth of pathogenic bacteria by copper oxide nanoparticles. Although at higher concentrations, the growth inhibitory effect did not show a significant difference between the two pathogen strains, but at a concentration of 31.25, the inhibitory effect of copper oxide nanoparticles on *E. coli* bacteria was significantly higher.

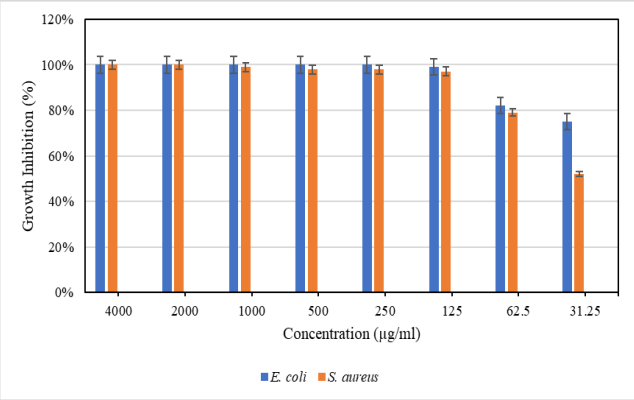

Fig. 6. The inhibition percentage of the growth of pathogenic bacteria in different concentrations of copper oxide nanoparticles

The results showed that the minimum lethal concentration of copper oxide nanoparticles produced in this study for two bacteria *E. coli* and *S. aureus* is equal to its minimum inhibitory concentration (Fig. 7).

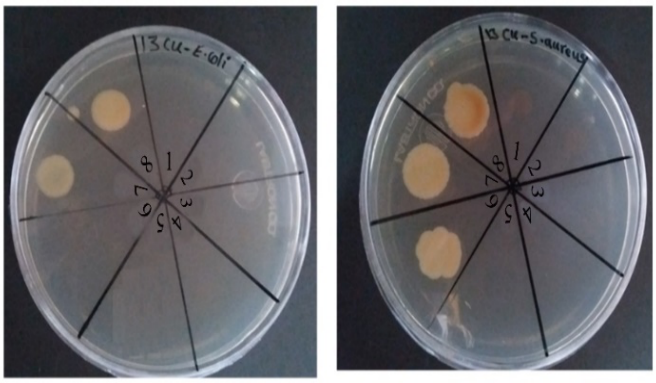

Fig. 7. MBC test results for copper oxide nanoparticles after 18 hours of keeping in greenhouse.

According to the MIC and MBC values obtained for two pathogenic strains of bacteria, the inhibitory and lethality of copper oxide nanoparticles on E. coli bacteria is higher than on S. aureus bacteria. This can be due to the thinner walls of Gram-negative bacteria than Gram-positive bacteria.

V. Conclusion

In recent years, nanotechnology has become one of the most important fields in modern science. This science examines the structure and properties of matter in the dimensions of 1-100 nanometers, and this very small size creates new properties and functions in materials.

Synthesis of nanoparticles, investigation of their properties and applications, are considered as one of the most important goals in nanotechnology, which has received attention in many fields today. Copper oxide nanoparticles, due to their unique physical, chemical and biological properties, have many applications in various medical sciences, pharmaceuticals, agricultural industries, cosmetics and electronic industries. These nanoparticles have many and significant applications, including antimicrobial, anticancer effects, use as biological sensors and drug delivery, which adds to the importance of the synthesis of these nanoparticles.

In this research, the ability of cold-resistant bacteria was used in the biological extraction of copper oxide nanoparticles. The production of copper oxide nanoparticles was evaluated by the selected strain of *Microbacterium* sp. OSNP13 using the microbial culture supernatant method along with heating in autoclave conditions. The structure and characteristics of the produced nanoparticles were characterized by using UV-Vis, DLS and XRD analyses. The characterization results have shown the accuracy of the production of these nanoparticles. According to DLS analysis, the average size of produced copper oxide nanoparticles is 63.21 nm. Also, the dispersion index (PDI) was calculated for copper oxide nanoparticles at the physiological pH of 0.27, which indicates the relatively suitable uniformity of these nanoparticles. According to the calculations based on the Debye-Scherer equation, for copper oxide nanoparticles at an angle of 37.81 with the 111 crystal plane, the average size of the nanoparticles was calculated to be 31.29 nm. The results of this section are consistent with the results of DLS analysis and indicate the smaller size of the produced copper oxide nanoparticles.

The antimicrobial effect of copper oxide nanoparticles was investigated on *E. coli* and *S. aureus* bacteria. MIC of copper oxide nanoparticles was calculated as 125 µg/ml for *E. coli* and 250 µg/ml for *S. aureus* bacteria, which is equal to its MBC values. The results showed that copper oxide nanoparticles had a relatively good antibacterial effect against both bacteria, but this effect was greater against *E. coli* bacteria than *S. aureus*, which is probably due to the lower thickness of the *E. coli* cell wall. According to the results of this research, it was found that the *Microbacterium* sp. OSNP13 strain has a high ability to produce copper oxide nanoparticles and can be used as a biological system for the production of nanoparticles. The nanoparticles produced in this research can be used as a suitable antimicrobial agent in many different fields, including medical equipment, antibacterial surfaces, etc.

Acknowledgement

Authors are thankful to the Deputy of Research, Ferdowsi University, Mashhad, Iran.

Conflict of Interest

Authors declare that they do not have any conflict of interest.

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