Screening of copper resistant microorganisms in mines and mathematical modeling of bioaccumulation and extracellular nanoparticle biosynthesis by *Bacillus cereus*

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Abstract

Some microorganisms were isolated from copper mines and recognized as copper resistant. Genotyping was conducted using 16srDNA sequencing. The bioaccumulation and extracellular nanoparticle biosynthesis were conducted for *Bacillus cereus* under the varying copper concentration range using mathematical modeling. AFM, TEM, and particle size analyzer were used for the characterization and the nanoparticle analyses. Cell toxicity was assayed against human cells. Maximum biosorption capacities were 1200 or 2500 μg g⁻¹ when 0.5 or 1 mg ml⁻¹ CuSO₄ was provided for the bacillus cells, respectively. In contrast, extracellular bioreduction kinetics revealed a threefold change from ≈250 to 700 μg ml⁻¹ in the same treatment conditions. No time shift was recorded for reaching the maximum extra/intra nano-copper synthesis in both copper concentration models. *Bacillus cereus* synthesized nano-coppers within a range of 80–150 nm. Metal nanoparticles were at least twofold less toxic than their copper sulfate on the human cells and T47D was the most resistant cell against nano-coppers. The toxicity effects were dose-dependent, time-dependent, and also organism- and heavy metal type-dependent. The results revealed that the copper-resistant *Bacillus cereus* is a robust and high-throughput microorganism for intracellular and extracellular nano-copper biosynthesis.

1. Introduction

One of the interesting approaches in nanotechnology is the biological synthesis of metal nanoparticles (NPs). NPs are low-cost, effective alternatives to parent materials with large reaction rates, superior efficiency, and a high surface/volume ratio. In recent years, NPs have been manufactured using physical and chemical processes, which had better production rates but poor biocompatibility, the need to use toxic compounds and large amounts of energy should be considered. [1–3]. Green chemistry is an eco-friendly, economical and robust method for nanometal biosynthesis. Green technology was widely accepted for bioremediation due to its non-toxic effect and clear natural strategy [4, 5]. Nanoparticle synthesis using green nanotechnology approaches was reported via various living organisms, mostly plants and microorganisms. Single-cell microorganisms, including bacteria, actinomycetes, yeasts, and fungi, were widely applied for commercial use and rapid decontamination process because of their high tolerance and reproduction potency [6, 7]. Some microorganisms could also passage the metal ions using the extracellular paths employed to a broader extent compared to the intracellular passages only. The extracellular method can produce high numbers of nanoparticles, and it contains easy processes that eliminate the different stages of production and easy
detachment and industrialization. In contrast, extra steps are involved in the recovery procedure of intracellular nanoparticles, such as collecting the cell biomass, cell lysis, and sonication to generate refined and uniform nanoparticles [8].

Nano-sized inorganic particles are produced by current approaches, form a particular set of metallic structures that possess beneficial biological, physicochemical, and mechanical properties [9]. Significant attention has been dragged towards Cu nanoparticles as they are inexpensive and have new visual, electrical, mechanical, catalytic, and heat transfer features that are different from the features of their large metals [10]. Anticancer properties have already been recorded for such a precious nanometal [11]. On the other hand, Cu nanoparticles have been employed as DNA- and protein- purification factors since they exert efficient binding capabilities and refined surface characteristics through combination with different biomolecules like proteins and enzymes. Another interesting application of Cu nanoparticles is their usage in drug delivery nanoformulations and molecular doping structures to control cancer cell progression [12–16].

Abundance makes copper more sensible option for wide verities of applications to other precious metals with analogous features like silver and gold. Although a lot of research has been conducted on biosynthesis and therapeutic utilization of gold and silver nanoparticles, investigation on copper biosynthesis through bacteria is not satisfactory and more works should be done on the therapeutic ability of biosynthesized copper nanoparticles [5, 17–19]. Therefore, the present study has focused on the screening, isolation, and characterization of copper-resistant bacteria from copper-rich soils. Subsequently, the metal nanoparticle production capacity was characterized and a comparative evaluation of the purified nano-coppers and copper sulfate was carried out for their cytotoxic activity on cancer cell lines.

2. Material and methods

2.1. Media and chemicals

Bacterial and fungal media were obtained from Merck (Darmstadt, Germany). Human cell culture media, supplements, and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA). Primer sets were synthesized by Bioneer Company (Seoul, Korea). Copper sulfate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and all other analytical grade reagents were ordered from Sigma-Aldrich (Deisenhofen, Germany).

2.2. Screening for copper-resistant isolates and colony purification

The resources of the present soil and water samples were copper-rich soils in Zanjan’s copper mining area located in Iran’s northwest. The screening of copper-resistant bacteria/fungi in soil samples in-depth 10–20 cm was performed using the enrichment culture technique by spraying 1 mg ml⁻¹ of CuSO₄ for a month. The soil was then water extracted and cultured for bacteria, actinomycetes, or fungi isolation on nutrient agar, starch casein agar, or sabouraud dextrose agar media supplemented with 1 mg ml⁻¹ of copper sulfate. Water samples were also initially cultured onto a solid medium. Soil and water samples for actinomycete isolation were preheated at 45 °C for 10 min or pretreated with nalidixic (a naphthyridone antibiotic) and nystatin (a polyene mycostatin agent) spray before culture to reduce Gram-negative bacteria and fungi and increase the frequency of actinomycete growth. The growth of resistant colonies was correspondingly monitored for at least one week. Resistant isolates were severely subcultured in the corresponding copper-supplemented media for colony purification. CuSO₄-resistant bacterial and fungal isolates were chosen for intracellular and extracellular copper nanoparticle bio-prospecting. The enrichment of bacterial cells was conducted by growing them in LB (Luria–Bertiarii) broth at optimum growth conditions (pH = 7.2, temperature 37 °C, and mixing rate of 150 rpm for 24 h). Actinomycetes were grown in an ISP2 medium (yeast extract 4 g l⁻¹, malt extract 10 g l⁻¹, and glucose 4 g l⁻¹ at pH = 7.2) for two weeks at 28 °C. Fungi were cultured in the Erlenmeyer flask containing sabouraud dextrose broth with a pH of 5.6, at 25 °C temperature and mixing at 100 rpm for 5 days [5].

2.3. Phylogenetic analyses and gene submission

Genomic DNA was extracted from each bacterial or fungal colony using DNA extraction kits prepared from QiaGen Company. 16S rDNA was amplified by degenerate 27F-forward primer (5’-AGA TTT GAT CMT GGC TCA G-3’) and 1492R-reverse primer (5’-TAG GGY TAC CTT GTT AGC ACT T-3’) for bacterium isolates and 9F-forward primer 5’-AAG AGT TTG ATC ATG GCT CAG-3’ and 1541R-reverse primer 5’-AGG AGG TGA TCC AAC CGC A-3’ for actinomycetes. Purified fungal colonies were also DNA extracted and subjected to PCR through 18S rDNA using forward primer (nu-SSU-0817: 5’-TTA GCA TGG AAT AAT RRA ATA GGA G-3’) and one of the reverse primers (nu-SSU-1196: 5’-TCT GGA CCT GGT GAG TTT CC-3’ or nu-SSU-1536: 5’-ATT GCA ATG CYC TAT CCC CA-3’). PCR was conducted in 50 μl reaction mixture containing 1 μg genomic DNA, 1 IU Taq DNA polymerase, 10 pmol forward primer, 10 pmol reverse primer, and 5 μl of 10X buffer. PCR
was run on initial denaturation at 95 °C for 10 min, 35 cycles of denaturation at 95 °C for 60 s, primer annealing temperature (56 °C for bacterial, 55 °C actinomycete, and 56 °C for fungal primers) for 60 s, and extension at 72 °C for 90 s and a final extension at 72 °C for 10 min terminated the procedure. 5% DMSO was added to the actinomycete PCR mixture and 98 °C was used for denaturation steps since actinomycetes contain GC-rich sequences. PCR products were gel purified using a silica-membrane-based kit (QIAquick gel extraction, Qiagen, USA). Purified samples were sent for Sanger sequencing and the results were controlled on Chromas software version 4.0 and then subjected to BLAST analyses on the NCBI database. Finally, the sequences were deposited on the NCBI-GenBank using Sequin software, version 11 [20].

2.4. Copper resistance of clones and growth kinetics
Pure actinomycete, bacterial, or fungal clones were cultivated for 24 h in 500 ml Erlenmeyer flasks containing 100 ml of ISP2, LB, or Sabouraud dextrose broth, respectively. Afterward, to improve logarithmic growth conditions, the cells were subcultured in fresh media. Next, different amounts of CuSO4 stock solution were added to the cell suspension to obtain final concentrations of 0.25, 0.5, 1, 3, 5, and 10 mg ml⁻¹. The incubation of these flasks was performed on an orbital shaker at the optimum temperature for several days following sampling four times each day. Maximum specific growth rates (µ_max) of all cultures were estimated regarding the linear least square method based on natural logarithms of dry cell weight values (typically measured at the beginning of exponential phases) as well as several time points. Therefore, the µ_max was represented as the slope for the best-fitted line [21].

2.5. Bioaccumulation kinetics in Bacillus cereus
Cu nanoparticles were biologically synthesized in a fresh culture containing Bacillus isolates passaging in 500 ml flat bottom flasks involving 100 ml of LB media. The filter-sterilized CuSO4 stock solution was added to the cell mixture to obtain the final concentration of either 0.5 or 1 mg ml⁻¹. The growth of negative control samples was performed onto heavy metal-free LB media. Within the duration of 96 h, the biomass sampling was performed twice a day and freeze-dried separately afterward. The microwave-assisted acid digestion of samples (0.5 g) was conducted for biological samples based on EPA-3052. Herein, 9 ml of concentrated nitric acid, besides 1 ml of fuming HCl and 2 ml of H2O2, were added to samples in PTFE vessels. These vessels were then sealed after about 1 min to allow reagents to react. Finally, tubes were heated to 180 °C in a microwave oven (Multiwave 3000™, PerkinElmer, USA) for at least 15 min. After cooling, quantification of metal contents was done by analyzing Cu content in the Triton X-114-rich phase using 797 VA Computrace polarograph (Metrohm, Herisau, Switzerland) according to the manufacturer’s protocol. Briefly, copper ions were measured using the anodic stripping voltammetry (ASV) method at ≈−0.1 V by adding 10% v/v of ammonia/ammonium acetate buffer solution (pH 4.6) to the samples. A calibration diagram was drawn using standard copper concentrations of 0, 10, 50, 100, 150, and 200 µg ml⁻¹. Extracellular nano-copper synthesis was also monitored during this period [5].

2.6. Intra/extra cellular nanoparticle purification
A freshly cultivated Bacillus isolate was tuned to OD600 = 1 and subsequently treated with 1 mg ml⁻¹ of CuSO4 for 1-day incubation. Cell harvesting was then performed through centrifuging at 5000 × g for 10 min, followed by washing the pellet twice with 100 mM phosphate buffer in the presence of 0.8% w/v NaCl. After resuspension of cell in the lysis buffer (1 mM EDTA; 100 mM NaCl; 1% w/v SDS; 0.5% v/v Triton ×100; and 10 mM Tris pH = 8), it was homogenized via the SilentCrusher™ (Heidolph, Germany). Again, the suspension was treated by centrifugation at 14000 × g for at least 15 min. The obtained metal nanoparticle-containing pellet was washed twice with 1.5 M of Tris/HCl buffer (pH 8.3) involving 1% SDS. The purification of nanoparticles was conducted three times with a two-phase extraction system containing the water/octanol mixture. At the bottom of the tube, pure nanometal sediments were washed with chloroform, ethyl alcohol, and distilled water, respectively, and then were freeze-dried. Extracellular copper nanoparticle extraction was simply conducted through the collecting of the media with paper filters after fermentation, the concentration of the flow-through using 14000 × g centrifugation forces for 20 min, and finally purification of the nanometals with the two-phase water/octanol extraction method followed by ethyl alcohol, chloroform, and distilled water polishing [21].

2.7. Physical characterization of the copper nanoparticles
UV-visible spectroscopy was used to monitor the consumption of extracellular copper ions and the formation of extracellular nano-coppers. In this regard, after the centrifugation of the culture medium at 5000 rpm for 15 min at the temperature of 4 °C, cell-free supernatant was collected to measure its absorbance within the range 300–960 nm with the UV–Visible spectrophotometer (UVD–2950 LABOMED, USA) operating at the resolution of 1 nm. Using the AFM (JPK, Deutschland), 10 µl of Cu nanoparticle colloidal suspensions were mounted on
AFM stubs after spreading on the surface of dust-free coverslips and getting dried at room temperature. Nanocopper size and shape were also evaluated using a transmission electron microscope (TEM, EM10C-100KV, Zeiss, Germany). The size evaluation of Cu nanoparticles and the thickness measurement were conducted applying the package of particle analysis function within the software based on standard protocols. Employing a particle size analyzer (Malvern Zetasizer nanosizer), particle size determination was possible after the production of pure nanometals [21].

2.8. Cell culture kinetics and cytotoxicity assays

MCF7 (Michigan Cancer Foundation-7) and T47D (human epithelial breast cancer) cell lines were generously provided by Professor Herman Lage (Molecular Pathology Department, University of Medicine Berlin, Germany). For the kinetic studies on the cell growth, RPMI medium was used involving 10% (v/v) FBS, 2 mM L-glutamine, 100 mg ml\(^{-1}\) streptomycin, and 100 IU/ml penicillin at the steady temperature of 37 °C in a humidified CO\(_2\) incubator. Cell growth was monitored for 7 days using the MTT assay. For cell toxicity assay, 1000 cells per well were seeded in 96-well tissue culture plates. Cells were treated with serially diluted metal ion concentrations (0–1000 μM) or nanoparticles (0–10000 μM) for 5 days. Subsequently, MTT was served to the cells (5 mg ml\(^{-1}\)) at 37 °C for 3 h. Formazan crystals were dissolved in DMSO and using a TECAN microplate reader (Infinite \(e\)-M200; Grödig, Austria); formazan color intensity was recorded at 570 nm after subtracting the plastic absorbance at 630 nm. The IC\(_{50}\) value was determined as the metal concentration that can reduce the surviving fraction of cells by 50% compared to untreated cells. The least-square method was selected to define the accurate IC\(_{50}\) and a trend line was fitted to surviving fraction to metal concentration [22, 23].

2.9. Statistical analysis

Three times repetitions for all experiments were performed independently. The results were expressed in the form of the mean values ± SE. The student’s t-test was conducted for data comparison. Moreover, significant parameters were statistically considered to have P-values less than 0.05.

3. Results

3.1. Resistant clone screening, purification, and molecular identification

Various bacteria and fungal clones with copper resistance ability and copper reduction performance were subcultured multiple steps to be purified (figure 1 and supplementary figure S1 (available online at stacks.iop.org/MRX/8/095402/mediala)). The organisms were cultivated in the liquid medium to the early logarithmic phase. After harvesting, the DNA was extracted and subjected to PCR. The final PCR product size was about 1450 bp and 1500 bp for bacterial and actinomycete species, respectively. Fungal amplicons were either around 420 or 760 bp depending on using the nu-SSU-1196 or nu-SSU-1536 reverse primer, respectively. More than 85% of fungal clones were amplified with both primer pairs (supplementary figure S2). All amplicons were consistent with the sequence information recorded on NCBI. QIAquick Gel Extraction kit was used for the purification of PCR products from Gel. After cutting the DNA bands from agarose 1%, the products were subject to Sanger sequencing using BigDye\textsuperscript{TM} terminator technology on an Applied Biosystem Prism\textsuperscript{TM} apparatus (supplementary figure S3). Analyzed sequences were deposited on the National Center of Biotechnology Information (NCBI) at http://ncbi.nlm.nih.gov/nuccore/?term=elahian (supplementary figure S4, S5). Phylogenetic analyses identified Bacillus cereus, Bacillus subtilis, Lysinibacillus fusiformis, Streptomyces sp., Penicillium chrysogenum, Aspergillus niger. Clones were also biochemically and morphologically verified.

3.2. Mathematical kinetics for intra/extracellular nanometal biosynthesis

It was observed that the separated bacteria from mines were able to resist the concentrations up to 1 mg ml\(^{-1}\), while purified fungi even showed metal tolerance of more than 3 mg ml\(^{-1}\). Bacillus cereus was found to be the best extra/intracellular producer, having the highest nanoparticle production rate among all the species here. Moreover, these bacteria showed quite a favorable growth rate comparing to other bacteria. Therefore most kinetic studies were devoted to this microorganism. According to Monod growth model (\(\ln(x) = \ln(x_0) + \mu t\)) in the logarithmic phase, isolated Bacillus cereus clone grew well in the presence of 0.5 mg ml\(^{-1}\) copper sulfate with natural logarithmic pattern with \(\ln(x) = -1.41 + 0.0225 \times t\) model. This model revealed a doubling time of 30.81 h. On the other hand, these bacteria grew much slower in the 1.0 mg ml\(^{-1}\) copper sulfate with a doubling time of 42.52 h with \(\ln(x) = -1.38 + 0.0163 \times t\) mathematical model (P < 0.001); While this clone needed only 5.94 h for doubling in the same batch culture conditions. Intercellular nanoparticle yield was recorded after digesting the samples using a microwave digester; the amount of available nano-copper was recorded through changes in copper-specific voltage (figure 2). A standard curve was used for data analysis.
Biosorption kinetic followed from $Y = 0.0056 t^3 - 1.15 t^2 + 61.30 t + 219.81$ and $Y = 0.0112 t^3 - 2.50 t^2 + 141.90 t + 78.77$ mathematical models for 0.5 and 1.0 mg ml$^{-1}$ copper inoculation, respectively ($t$ is the elapsing time). Derivatives of the polynomial equations reached the times with maximum production yield, 36.29 h, and 38.09 h and $\approx 1200 \mu g g^{-1}$ and 2500 $\mu g g^{-1}$ as the maximum biosorption capacity. On the other hand, *Bacillus cereus* resulted in an efficient extracellular nano-copper production. Seven-successive fermentation days were consequenced to extracellular nano-copper production besides the copper bioaccumulation. Extracellular kinetics was depicted as $Y = -0.0004 t^3 + 0.0251 t^2 + 3.5674 t + 8.2032$ and $Y = -0.0012 t^3 + 0.0913 t^2 + 8.3747 t + 67.336$ mathematical model for 0.5 and 1.0 mg ml$^{-1}$ copper inoculation. Maximum production happened at 79.29 and 79.87 h with a capacity of $\approx 250$ and 700 $\mu g ml^{-1}$ (supplementary figures S7 and S8).

### 3.3. Nanoparticle dimension and shape evaluation

Purified nanoparticles were used for the evaluation of nanoparticle dispersion. Particle size analyses revealed the presence of uniform and homogenous nanoparticles with an average size of 90 nm for intracellular biosynthesis in bacteria. Extracellular biosynthesis of nano-copper synthesis was recorded around 100–150 nm in *Bacillus cereus*. On the contrary, the streptomyces genus and the fungi synthesized coagulated intracellular nano-coppers with bigger and broad sizes, approximately 200 and 500 nm, respectively (Table 1, supplementary figure S9). The
AFM and TEM images resultant for intra/extracellular copper nanoparticles were observed as amorphous and spherical in shapes shown in figure 3.

3.4. Cell culture kinetics and cellular toxicity

Initially, cells were seeded onto the 96-well plate with a concentration of 2000 cells per well to evaluate the growth course for 7 days. The $\ln(X) = 0.65 \times T + 7.94$ and $\ln(X) = 0.3202 \times T + 7.93$ were respectively the best equations represented MCF7 and T47D growth kinetics in optimum conditions where X and T are cell count and time (supplementary figure S10). It appeared that MCF7 cells have a faster growth kinetic with a doubling time of 25.56 h. In contrast, T47D cells doubled every 51.95 h. Copper nanoparticle and copper sulfate toxicity were calculated for each cell after 5 days using the regression method and the results were reported as Mean ± SD (figure 4 and supplementary table S1). The most toxic copper form was seen in copper sulfate on the

Table 1. Size and PDI of nanoparticles synthesized by microorganisms.

| Nano-copper                          | PDI  | Size ± SD     |
|--------------------------------------|------|---------------|
| Bacillus cereus intracellular nano-coppers | 0.26 | 81 ± 23 nm    |
| Bacillus cereus extracellular nano-coppers | 0.28 | 132 ± 36 nm   |
| Streptomyces nano-coppers             | 0.39 | 197 ± 41 nm   |
| Fungi nano-coppers                    | 0.63 | 483 ± 88 nm   |

Figure 2. Kinetic studies on the Bacillus cereus in the presence of 0.5 mg ml$^{-1}$ (A) or 1 mg ml$^{-1}$ CuSO$_4$ (B). Growth performance, biosorption capacity, and extracellular nanoparticle potency of Bacillus cereus were measured in the presence of 0.5 mg ml$^{-1}$ or 1 mg ml$^{-1}$ CuSO$_4$. Data represent the means of three independent experiments (Mean ± Standard Error).
T47D cell line (IC50 = 485.47 ± 34.31 and a P-value < 0.005). On the contrary, the least toxic agent was zero-valent copper nanoparticles and the most resistant cell was T47D. Results represent dose-dependent and cell-specific toxicity phenomena.

4. Discussion

Copper nanoparticles have been widely used in medical sciences for their anticancer, antibacterial, and antifungal actions [24]. Some research has been conducted on the isolation of Cu-resistant microorganisms from the environment to increase Cu accumulation and copper nanoparticles' green production [25]. Metal reduction is mostly facilitated by a network of periplasmic and/or outer membrane c-type cytochromes in metal-reducing bacteria. One of the major routes for metal reduction is the Mtr pathway, an electron transport chain composed of c-type cytochromes which transfer electrons from the cytoplasm to the outer membrane. There are several processes that might explain the amount of copper nanometals found in the cytoplasm and periplasm: (A) Cu(II) ions are able to enter the cytoplasm and periplasm where they are reduced and precipitated by unidentified reductases; (B) nanocopper precipitates can be transported through the cytoplasmic membrane, presumably, Cu toxicity on the membrane may facilitate transportation; (C) Cu(II) reduction in the cytoplasm to Cu(I), followed by Cu(I) export and finally disproportionation to form Cu(0) or Cu(II) [26, 27].

In this study, several microorganisms were purified from copper mines that were at least resistant to 1 mg ml⁻¹ copper sulfate. Most of them were from the bacillus genus. rDNA analyses revealed that most of these colonies belong to the *cereus* species (figure 1 and supplementary figures S1, S3, and S5). Some recent investigations also introduced *Bacillus cereus* as a persistent microorganism to copper and an efficient copper biosorber [5, 28]. Even though in this study, several other microorganisms from kingdom fungi and empire Prokaryota were isolated and proved for higher copper resistance, *Bacillus cereus* was selected for the kinetics study because of several favored aspects, including (A) higher specific growth rate, (B) high extracellular production rate, (C) homogenous small-type nano-copper particles, and (D) higher production yields (supplementary figures S5, S8 and S9).

Growth pattern analyses in the present study revealed that *B. cereus* was more sensitive to copper in the presence of 1 mg ml⁻¹ than 0.5 mg ml⁻¹ CuSO₄. The outcome of this study suggests that metal ions deplete the
growth rate in a concentration- and time-dependent manner. The toxicity effects are also organism- and heavy metal type-dependent (figure 2 and supplementary figures S7 and S8). The inhibitory effects of heavy metals, including Ag, Au, Cd, Fe, and Se, were frequently reported on the growth response of microorganisms. Copper also demonstrated high toxic effects on growth performance because Cu ions can gain/transfer electrons from molecular oxygen to form ROS, making this metal potentially toxic [29, 30]. Extracted microorganisms were resistant to CuSO4 due to high concentration of cupric sulfate in the mine, on the other hand there are some reports that prove other cupric salts are oxidizing and irritant agents. These indicate that CuSO4 contamination in the environment is mostly susceptible to bioremediation. Therefore, the aim of this study was focused to the kinetic models for copper sulfate biotransformation since CuSO4 is less toxic [31, 32].

Bioreduction is a process for metal nanoparticle biosynthesis by microorganisms. Wide varieties of proteins, polysaccharides, and other biomolecules are involved in the microbial bioreduction process. According to the place of nanoparticles synthesis, bioremediation methods can be categorized into intracellular and extracellular production. In the intracellular approach, ions are transported into the microbial cytoplasm or any cellular compartments and then transformed into the corresponding nanoparticles in the presence of enzymes. On the other hand, in the extracellular nanoparticle biosynthesis, the metal ions can be reduced by secreted enzymes in the media solution or via entrapment on the cells’ surface. Nanoparticles are formed on cell wall surfaces, and the trapping of metal ions on this surface is the initial step in bioreduction. This is probably due to the
electrostatic interaction between metal ions and negatively charged moieties of enzymes found on the cell wall [26, 27].

In this study, a higher amount of copper was accumulated inside the cell during the exponential growth phase. The maximum amount of the metal bioaccumulation during the first 38 h of incubation may be attributed to the higher stage of metabolism activity, higher production of oxidoreductase enzymes, increasing the internal detoxification of copper through complexation, and precipitation [33]. Interestingly, a two-fold increment in copper concentration led to a doubling of maximum biosorption capacity from 1200 to 2500 μg g⁻¹, but a two-hour delay was recorded for such bioaccumulation yield. This may be dominantly due to increment in growth lag phase, adaptation time of the cells to the higher toxic conditions, and decreasing the specific growth rate. Literature has classified bioaccumulation to either adsorption related to cell surface adsorption, containing complexation, ion exchange, and surface precipitation, or intracellular accumulation associated with enzymatic detoxification, cytoplasmic dissociation, and trafficking to efflux systems. Researchers demonstrated that heavy metal bioaccumulation (Cu²⁺, Cd²⁺, Pb²⁺, and Zn²⁺) increases by a growing organism under specific culture conditions. Results showed that metal removal occurs efficiently at low concentrations of metals. The low removal efficiency or the metal accumulation at higher concentrations in some other research could be described through the low ratio of adsorptive surface to the total metal ions accessible at high metal concentrations or associated with the target cell’s low metabolic activity [29, 34]. The decreasing phase at the fermentation end suggested that intracellular bioaccumulation is a possible mechanism for metal uptake during bacterial growth. It seems that reduced metal accumulation during the stationary phase of growth could be due to a rapid decrease of pH of the cultures during the stationary phase and may probably due to a shift of the bacterial metabolism towards less efficient, fermentative growth, leading to an increase in the organic acid formation and even activation of metal-efflux systems [33].

In contrast, extracellular nano-copper biosynthesis was escalated in the idiophase. Interestingly, increasing a two-fold copper concentration increased a three-fold extracellular nano-copper biosynthesis capacity (from 250 and 700 μg ml⁻¹) with no change in the production time (both in 79 h), according to the mathematical model. Many reports suggested that intracellular accumulation plays a more notable role under lower metal ion exposure, but higher ions are dominantly detoxified via extracellular adsorption. It is also suggested that the presence of a metal-efflux system removes heavy metal ions from the cytoplasm at the same rate as it is taken up. Actually, the contribution of such systems in this B. cereus strain has been proven previously as detoxifying mechanisms. In addition, metabolic activities (energy-requiring processes) are probably repressed through intracellular metal accumulation and it is toxic for growing cells. These responses were generally considered essential self-protection microbial cells’ approaches versus the concentration of heavy metals at high levels [29, 35]. Apart from these reasons, it is reported that in B. cereus, under heavy metal stress releases high amounts of cations into the medium in significant order of Na⁺ > K⁺ > Ca²⁺ > Mg²⁺. Therefore, copper homeostasis is also associated with such cations and they are essential for normal metabolic activities in the cell at higher exposure [29, 36].

Nano-coppers were spherical, highly monodispersed, uniform, stable, negatively charged on the surface, and crystalline in nature. Negatively charged particles inferred that the biotransformed CuNPs are very stable. The electrostatic repulsive force between the NPs depends on the charge that resides on the surface of nanoparticles. When the charge of the nanoparticles is negative, it does not cause the aggregation of these nanoparticles, causing long-term stability [37]. The size of nanoparticles is generally reported as a range, and this can depend on several factors such as fermentation conditions, media compositions, temperature, pH, metal ion concentration, incubation time, cofactor availability, and harvesting procedures. Many reports provided larger extracellular nanoparticles than intracellular. Particle size and monodispersity are more controllable intracellularly and extracellular particles are mostly larger due to enough space for aggregation, aggregate media and protein components outside, and less-controlled bioremediation [31, 38].

Finally, the result showed that copper nanoparticles were less toxic than their conventional ionic form (CuSO₄) against the cells. Due to less availability of big zerovalent coppers to the cells, it may be known to cause cytotoxicity. Like other studies, the cytotoxic effects of our nano-coppers were more variable compared with copper ions [5, 39]. It should be noted that mitoxantrone has significantly (P < 0.001) higher toxicity on these lines. Overall, results represent dose-dependent and cell-specific toxicity phenomena (figure 4 and supplementary table S1).

5. Conclusion

Green biosynthesis of nanoparticles is attractive from two potential viewpoints, eco-friendliness and cost-effectiveness. The isolated copper-resistant Bacillus cereus is a potential candidate for nanoparticle synthesis according to the mathematical kinetics of intra/extracellular nano-copper biosynthesis. Such biosynthesis yield
may be affected via the Cu-efflux system, metabolic activity, detoxification mechanisms, and cation release. Finally, the results showed that copper nanoparticles are homogenous and less toxic than conventional copper sulfate against human cells.

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Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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Author contributions

S A Mirzaei and F Elahian coordinated the study, designed the experiments, and critically revised the final manuscript. M Mohseni and H Valinejad performed the analytical quantification of copper. M Safaei, V Afshari, M Bagheri, and F Khanduzi isolated the microorganisms, performed the biosorption experiments, participated in the data analyses and intellectual discussions of the data, and manuscript writing. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

Not applicable.

Competing interest

This paper has been read and approved by all the authors and all of them declared no conflicts of interest.

Ethical statement

The authors have read and have abided by the statement of ethical standards for manuscripts submitted to the journal. This article does not contain any studies with human participants or animals performed by any of the authors. We declare that the submitted manuscript does not contain previously published materials and is not considered for publication elsewhere. All the authors have made a substantial contribution to conception and design, collection, analysis and interpretation of data, writing or revising the manuscript, or providing guidance on the execution of the research.

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