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Extracellular biosynthesis of magnetic iron oxide nanoparticles by *Bacillus cereus* strain HMH1: Characterization and in vitro cytotoxicity analysis on MCF-7 and 3T3 cell lines

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ABSTRACT

Discovery of new properties and special functionalities at the nanoscale materials caused nanotechnology to become one of the leading parts in all sciences namely biology and medicine. Magnetic iron oxide nanoparticles (MIONPs) are among interesting nanomaterials in biomedical arena, which have attracted the attention of many researchers owing to their extensive capabilities. Due to the simple, cost-effective and environmentally-friendly production processes, biosynthesis is of paramount importance between different methods of nanoparticles production. In the current study, we succeeded to synthesize MIONPs using a newly extracted bacteria supernatant. Produced nanoparticles were characterized using FE-SEM, DLS, VSM, UV–vis, FT-IR and EDS spectroscopy. Analysis showed that the average particle size of very stable spherical MIONPs is about 29.3 nm. The bacteria protein profile obtained by SDS-PAGE analysis indicated induction of different proteins. In vitro cytotoxicity of nanoparticles on the viability of MCF7 and 3T3 cell lines was assessed by MTT assay. The results show that toxicity of the produced nanoparticles ($IC_{50,\ MCF\text{-}7} > 5$ mg/ml and $IC_{50,\ 3T3} > 7.5$ mg/ml) follows a concentration dependent manner.

1. Introduction

Nano-sized particles as the base and starting point of nanotechnology are required in all research of this area; therefore, their production and synthesis is of paramount importance. Biosynthesis or green synthesis of nanoparticles is among bottom-up methods for producing nanoparticles and enjoys considerable benefits in comparison to other physical and chemical methods (vapor-solid growth techniques, hydrothermal methods, co-precipitation, reverse micelle, and sol-gel) (Gao et al., 2016; Narayanaswamy et al., 2017; Zhao and Yin, 2017). Green synthesis methods are clean and safe, and due to less pollution are more environmentally consistent (Narayanan and Sakthivel, 2010; Seabra et al., 2013). In addition, the product obtained from these methods enjoys better compatibility coordination with biological systems (Faramarzi and Sadighi, 2013). It should be taken into account that biosynthesis of nanoparticles is not an ideal method and faces challenges such as time consuming processes, lack of control over-size distribution, shape and crystallization of produced nanoparticles (Narayanan and Sakthivel, 2010; Quester et al., 2013).

Metal oxides, and iron oxides among them, are of nanoparticles that considering the specific inherent characteristics have a wide range of applications in various areas. They can be used as catalysts, energy storage systems, sensors, fuel cells, drug carriers and contrast agents in imaging techniques (Saif et al., 2016; Yin et al., 2017; Zhang et al., 2017). Iron oxide nanoparticles show a good stability under different environmental conditions, they have a suitable chemical activity and significant magnetic properties (Mahmoudi et al., 2011; Yin et al., 2017). Magnetite (Fe3O4) and hematite (Fe2O3) are two well-known and important species of iron oxide which have been in the researchers' spotlight in recent years.

A variety of microorganisms are used in nanoparticle synthesis processes as biological agents including bacteria, fungi, yeast, algae and actinomycetes (Quester et al., 2013). Metallic nanoparticles synthesis by microorganisms can take place both intracellular and extracellular (Narayanan and Sakthivel, 2010). Due to availability and many known aspects of metabolism, more attention has been paid to bacteria and fungi. Bacteria are amongst the most abundant organisms on earth, they are single-celled prokaryotic which have a wide variety in terms of size, shape and methods of gaining energy and therefore, can survive in almost all environmental conditions. A variety of biological mechanisms and processes are involved in biosynthesis of magnetic iron oxide nanoparticles by bacteria. In short, all important and key processes in the

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production of metal nanoparticles are called Bio-mineralization (Nath and Banerjee, 2013). Bio-mineralization occurs either in controlled way or induced one (Perez-Gonzalez et al., 2010). Nanoparticles produced in controlled method are often produced and stored within the cell and special organelles, are in small amounts, and it is more difficult to restore and get access to them (Revati and Pandey, 2011). Therefore, the biosynthesis of nanoparticles by bacteria with induced mechanisms e.g. iron-reducing bacteria which produce nanoparticles in an extracellular way is of paramount of importance (Moon et al., 2007). Generally compared to other physical and chemical methods, iron oxide nanoparticles produced by bacteria are less toxic, more biocompatible and cost-effective (Nedyalkova et al., 2017; Saif et al., 2016).

In the current study a simple biological and cost-effective method for producing magnetic iron oxide nanoparticles using HMH1 bacteria supernatant is introduced. Unlike other proposed methods, synthesis of nanoparticles in this convenient method takes less time and considering the use of precursors in mM concentrations, is more efficient. Various effective parameters on nanoparticles formation process and probable mechanism of biosynthesis were evaluated respectively. The produced nanoparticles were investigated in terms of chemical composition, size, shape and magnetic properties using common analyses. Finally, with the aim to assess the inherent properties for biomedical applications, cytotoxicity of produced nanoparticles was measured using MTT assay. The observed low toxicity of produced nanoparticle upon 3T3 cell line suggests that obtained nanoparticles can be a good candidate for in vivo applications as drug carriers or contrast agents in MRI.

2. Materials and methods

2.1. Materials

In this study, iron chloride, sodium dodecyl sulfate (SDS), sodium citrate and dimethyl sulfoxide (DMSO) were supplied from Merck (Germany), nutrient broth and nutrient agar were supplied from Quelab (USA), DMEM from Biosera (UK), FBS from Biowest (France) and MTT was supplied from Atocel (Austria).

2.2. Bacterial isolation and identification

Considering the expected application, that is biological synthesis of iron-based magnetic nanoparticles, a soil sample extracted from chromite mines around Bardaskan city, Khorasan Razavi province, Iran, was chosen amongst numerous samples available in the laboratory. In order to isolate microorganisms, a small amount of the sample was solved in physiological saline (containing sodium chloride salt in distilled water at a concentration of 9 g/L) and 24 h was incubated at 37 °C in a shaking incubator at 180 rpm. The streak-plating method was performed to purify strains of isolated bacteria. To do this, 100 µl of saline solution containing the microorganism was streaked on nutrient agar medium and after 24 h of incubating at 37 °C, obtained single colonies were transferred to Erlenmeyer flasks containing nutrient broth (NB) medium following gram staining. After 18–24 h of incubation at 37 °C, the obtained strain was used for performing next steps.

In order to identify the isolated strain and its evolutionary-kinship relation with other known strains, 16S rRNA molecular method was used. First bacterial genomic DNA was extracted using phenol-chloroform method. 16S rRNA gene was amplified by designed primers based on universal primers:

Forward primer: 5'-AGTTTGATCCTGGCTCAG-3'	T_m:53/7 °C
Reverse primer: 5'-GGC/TTACCTTGTTACGACTT-3'	T_m:53/4 °C

PCR reactions were performed in accordance with the following schedule:

- Initial temperature 94 °C, for 5 min
- 30 cycle each consisting of 45 s at 94 °C, 45 s at 52 °C, and 90 s at 72 °C.
- Final amplification at 72 °C for 5 min.

After sequencing the 16S rRNA PCR product of the given strain, the multiple sequence alignment was performed based on the National Center for Biotechnology Information (NCBI) database. Finally, the phylogenetic tree of selected strain was drawn using ClustalW software, and the results were investigated to compare to other similar sequences.

2.3. Biosynthesis of magnetic iron oxide nanoparticles

In order to biosynthesis MIONPs through extracellular method, the given strain was cultured on NB culture medium for 24 h at 37 °C. Then solution of culture medium containing bacteria was centrifuged at 5000 rpm for 15 min to separate supernatant from bacterial biomass. To ensure the absence of bacterial cells and preventing contamination of the sample, supernatant was passed through a 0.2 µm sterile syringe filter. Filtered supernatant was used for nanoparticles biological production; in a way that the iron salt concentration in the desired volume of supernatant is considered to be 5 mM. Magnetic iron oxide nanoparticles biosynthesis can be determined at the room temperature (25 °C) using FeCl3·6H2O only after 5 min and using FeCl2·4H2O after 30 min, as color changes from clear golden yellow to turbid brown.

2.3.1. Isolation and concentration

The supernatant solution, to which salt had been added, was first passed through Whatman® qualitative filter paper No. 1 and then 0.2 µm sterile syringe filter into a sterile Falcon tube. Then by observing sterile conditions, obtained solution was transferred to the special rotary balloon, and was concentrated as much as possible by rotary at 20–30–rpm, steam bath temperature of 60 °C and circulator temperature of 15–10 °C. The concentrated solution including nanoparticles was poured into a sterile glass plate and was put under biological hood to be dried. The obtained powder was used in the following analysis.

2.4. Evaluation of effective parameters on nanoparticles formation

Discovering and understanding factors affecting nanoparticles formation is amongst important issues facing the biological production by bacteria. Therefore, the effect of various factors on biosynthesis of MIONPs in extracellular method was investigated using Ultraviolet and Visible Spectroscopy analysis ((UV–vis)) as follows.

2.4.1. Salt

With the aim to choose suitable precursor salt to be used in the next steps, (UV–vis) absorption comparative spectrum for FeCl3·6H2O, FeCl2·4H2O and FeSO4·7H2O salts was drawn. Other production conditions such as culture medium, temperature, pH, salt concentration and time were considered quite the same.

2.4.2. Temperature

In order to investigate the effect of temperature on magnetic iron oxide nanoparticles biosynthesis, nanoparticles synthesis process was performed at different temperatures considering the same conditions in terms of culture medium, pH, salt concentration and the time of production. Then nanoparticles biosynthesis was investigated by comparing (UV–vis) absorption spectra.

2.4.3. pH

As the amount of environmental acidity has a significant effect on the size of produced nanoparticles, by changing supernatant pH prior to salt addition, the production of nanoparticles was investigated at pH values of 3.5, 5.5, 7.4, 8.5, and 11 by (UV–vis) analysis. Other conditions were imposed quite the same.