IRON NANOPARTICLES BIOGENIC SYNTHESIS THROUGH CELL-FREE EXTRACT OF SURFACTIN PRODUCER *BACILLUS SUBTILIS*

Isabela Karina Della-Flora[1,2*], Dachamir Hotza[1], Aldo R Boccaccini[2], Cristiano José de Andrade[1]

[1] *Department of Chemical Engineering & Food Engineering, Federal University of Santa Catarina, Florianópolis, SC 88040-900, Brazil*
[2] *Institute of Biomaterials, Friedrich-Alexander-University Erlangen-Nuremberg, Caustraße 6, 91058 Erlangen, Germany*
** Corresponding author's email address: isa_dellaflora@hotmail.com*

ABSTRACT

This study presents a method for producing iron-based NPs (FeNPs) using cell-free extracts (CFE) from *Bacillus subtilis* ATCC 6633, utilizing cassava wastewater as a low-cost medium. The synthesized FeNPs were characterized and compared to chemically synthesized lauric acid-coated iron oxide nanoparticles (SPIONs). Despite no significant antibacterial activity observed, the biosynthesized FeNPs demonstrated higher cell viability and biocompatibility than SPIONs, highlighting their potential for safer biomedical applications, The greater biocompatibility of FENPs may be a result of this new biosynthesis process. Further research should focus on direct antibacterial tests to explore the full potential of FeNPs.

Keywords: Iron nanoparticles 1. Biosynthesis 2. Bionanomaterials 3. Antibacterial activity 4. Cytotoxicity 5.

1 INTRODUCTION

Nanoparticles (NPs), defined as materials with at least one dimension between 1 and 100 nm, are utilized across various fields, including biotechnology, environmental science, materials science, and engineering [1]. Biosynthesis, or green synthesis, of NPs is a bottom-up method offering significant advantages over traditional physical and chemical methods, which often involve high temperatures, sophisticated instruments, and toxic chemical additives that pose environmental and health risks [2]. Compared to these methods, bacteria-produced NPs are generally less toxic, more biocompatible, and cost-effective [3]. Biosynthesis using bacteria can be performed through intracellular or extracellular methods, though the underlying mechanisms and processes of purification and standardization remain challenging, hindering industrial-scale production [1]. Many bacteria, such as *Bacillus* spp., have been reported in the biosynthesis of different NPs [2]. Cell-free extract (CFE) methods are particularly promising, as they simplify NP recovery and preserve crucial biomolecules responsible for the reduction, oxidation, and capping of NPs [5]. This study introduces a straightforward and cost-effective biological method to produce iron-based NPs (FeNPs) using CFE from *Bacillus subtilis* ATCC 6633, utilizing cassava wastewater as a low-cost medium.

2 MATERIAL & METHODS

The nanoparticles (NPs) were biosynthesized using the cell-free extraction (CFE) method. The extract was obtained by lysing *Bacillus subtilis* ATCC 6633 cells through autoclaving at 121 ºC for 20 minutes, followed by centrifugation at 10,000 *g* for 15 minutes. *B. subtilis* was cultivated in cassava wastewater to produce surfactin, following the process described in [6]. The CFE was then mixed with the iron precursors $FeCl_2$ and $FeCl_3$ in a 1:1 ratio. Three different concentrations of Fe^+ were tested: 1 mM (Run 7), 19 mM (Run 8), and 10 mM (Run 9). The pH of the mixture was adjusted to 7, and the synthesis was carried out at room temperature with shaking at 150 rpm for 24 h. The results presented here are part of a broader and more detailed experimental design to optimize the biosynthesis of FeNPs using CFE from *B. subtilis*. After synthesis, the NPs were characterized using UV-vis spectroscopy (200 to 700 nm), zeta size analysis, and zeta potential measurements. Their antibacterial activity was evaluated against *S. aureus* and *E. coli*, while cell viability was assessed using MG-63 cells with WST-8 as the viability indicator. Additionally, cell morphology was examined using fluorescence microscopy with phalloidin and DAPI staining. The biosynthesized FeNPs were compared to chemically synthesized lauric acid-coated iron oxide nanoparticles (SPIONs) [7].

3 RESULTS & DISCUSSION

The biosynthesis of FeNPs was evaluated by visual observations and UV-Vis analysis (Figure 1). During visual observations, the solutions achieved a color change from pale yellow to a reddish brown, while no color change was observed in the controls (CFE only or precursor only).

Table 1 outlines the physical and chemical properties of FeNPs synthesized via biosynthesis and chemical (SPIONs) methods. The chemical synthesis process yielded small SPIONs (41 nm), whereas the smallest size obtained through biosynthesis was 249 nm (Run 7, table 1). FeNPs biosynthesized by CFE usually achieve sizes ranging from 1 to 100 nm [2]. However, the hydrodynamic size may encompass additional biomolecules (e.g., proteins, enzymes, peptides), leading to an increase in particle size [3].This biomolecular layer, typical in the biosynthesis process, is crucial for reducing and stabilizing NPs [2]. The higher the zeta potential value (in modulus), the more stable the nanoparticle. Low-stability NPs are prone to aggregation, leading to a larger hydrodynamic size. This indicates that SPIONs, Run 7 and 8 have higher stability than those from Run 9. Other studies suggest that negative surface charges on NPs enhance stability in solution by increasing electrostatic repulsion between particles, thereby preventing agglomeration [8,9]. This may explain the smaller sizes observed for Run 7 and SPIONs.

The variation in characteristics of biosynthesized NPs can be attributed to the balance between the availability of biomolecules, which are responsible for the reduction and stabilization of NPs, and the iron precursor concentration. An insufficient quantity of

biomolecules can lead to NP aggregation, as seen in Runs 8 and 9. In contrast, Run 7 had higher concentrations of biomolecules relative to the precursor, resulting in better stabilization and less aggregation.

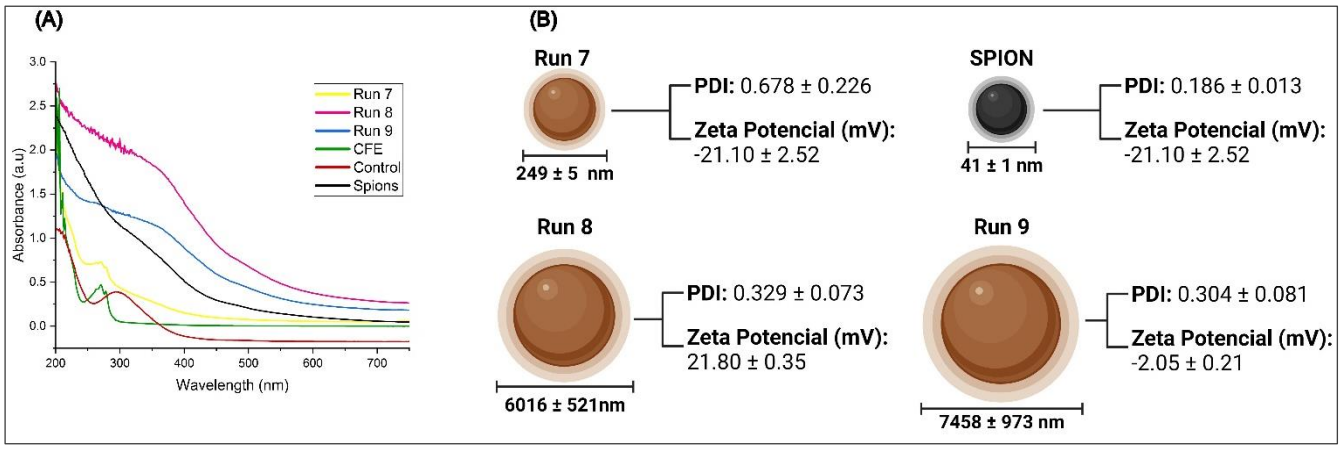

Figure 1 Nanoparticle characterization: Uv-vis spectra of FeNPs, SPIONs, CFE and control (iron precursor) (A); Hydrodynamic size (nm), PDI, and Zeta potential of FeNPs compared with SPIONs.

The cell viability test (Figure 2) assesses the safety of using nanoparticles for biomedical applications. Indirect cellular tests showed satisfactory results, above 70% cell viability, for all tested conditions of biosynthesized NPs and incubation times, which means that, indirectly, the biosynthesized FeNPs were not toxic for MG-63 cells. However, after 7 days of incubation, SPIONs exhibited significant ($p<0.05$) toxicity at a concentration of 1 mg L^{-1}, with cell viability dropping below 40%. Similar behavior is observed on the fluorescence microscopy images (Fig 3), showing a lower cell density for the SPIONs (1mg L^{-1}) than the biosynthesized FeNPs and the control. Studies show that biosynthesized iron nanoparticles are less toxic and more biocompatible than their chemically synthesized counterparts [2]. This is attributed to the absence of toxic substances in their synthesis and stabilization processes and the presence of a biocompatible biomolecule corona.

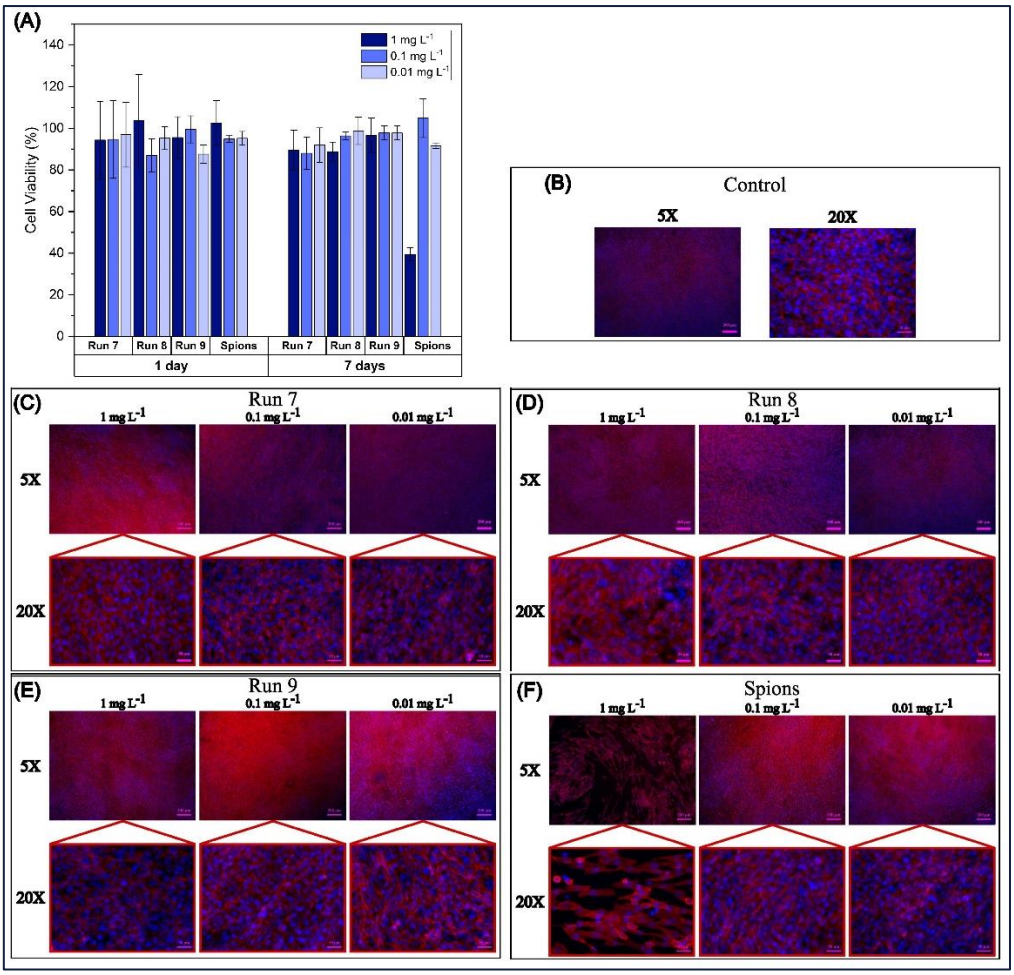

Figure 3 Indirect cell viability test (A) of MG-63 cells with FeNPs at 1 mg L^{-1}, 0.1 mg L^{-1}, and 0.01 mg L^{-1}, over 1 and 7 days of incubation; Fluorescent microscopy images stained with DAPI and rhodamine phalloidin of MG-63 cells with FeNPs (Control (B), Run 7 (C), Run 8 (D), Run 9 (E), SPIONs (F)) at 1 mg L^{-1}, 0.1 mg L^{-1}, and 0.01 mg L^{-1} after 7 days of incubation

The data in Figure 2 show the results of the antibacterial activity test. No significant trend ($p > 0.05$) in antibacterial activity was observed, even in treatments with higher concentrations of nanoparticles (NPs). One of the bactericidal mechanisms of iron NPs involves attachment to and penetration of bacterial cell walls, leading to structural changes and cellular stress. Also, generating reactive oxygen species (ROS) is crucial in damaging bacterial proteins and DNA and disrupting defense mechanisms. However, this mechanism may not have occurred because this study used an indirect test. Direct tests should be performed to test this hypothesis. It can be concluded that neither the biosynthesized nanoparticles nor the SPIONs released antibacterial substances.

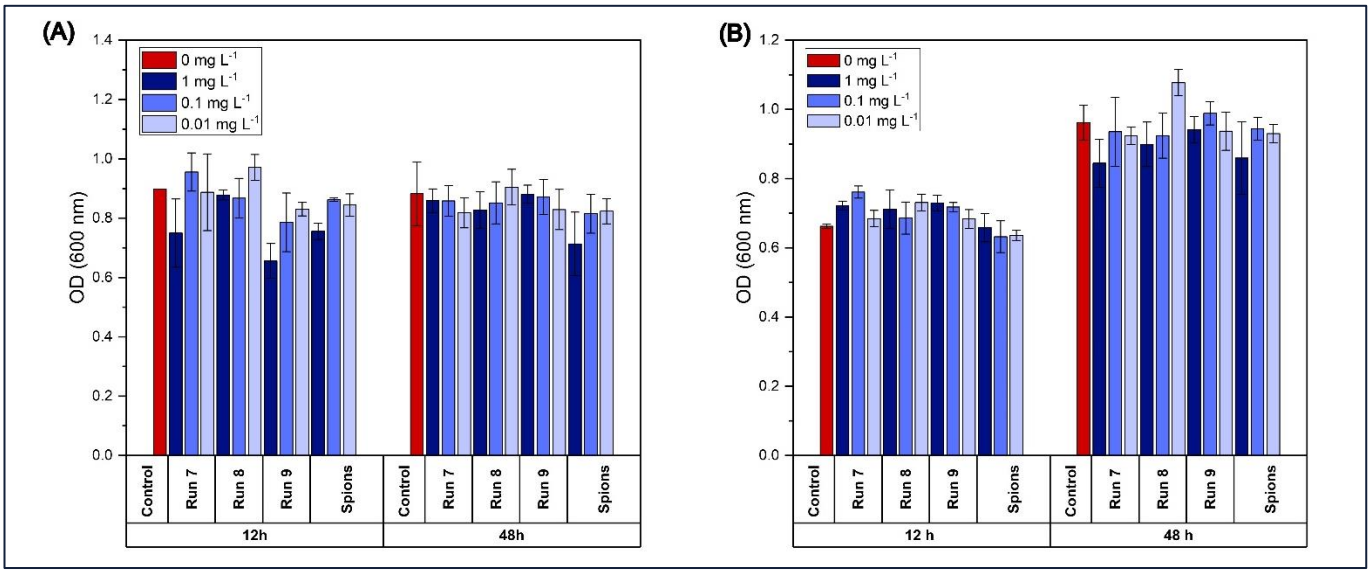

Figure 2 Antibacterial activity of FeNPs at varying concentrations (1 mg L^{-1}, 0.1 mg L^{-1}, 0.01 mg L^{-1}) against (A) *E. coli* and (B) *S. aureus* over 12 h and 48 h of incubation.

4 CONCLUSION

This study presents an innovative method for producing iron-based nanoparticles using cell-free extracts from *B. subtilis*. The biosynthesized FeNPs were thoroughly characterized and compared to chemically synthesized SPIONs. While the antibacterial activity tests did not show significant results, the biosynthesized FeNPs demonstrated higher cell viability and biocompatibility than SPIONs, indicating their potential for safer biomedical applications. These findings suggest that biosynthesized FeNPs, with their reduced toxicity and enhanced biocompatibility, are promising candidates for further development in medical applications. Future research should focus on direct antibacterial and cytotoxicity tests and physicochemical characterizations to fully explore the potential of FeNPs.

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