DISCIPLINARUM SCIENTIA *Disciplinarum Scientia.* **Série: Naturais e Tecnológicas**
Santa Maria, v. 25, n. 2, p. 303-314, 2024
Recebido em: 29.07.2024. Aprovado em: 03.10.2024.
ISSN 2176-462X | DOI: doi.org/10.37779/nt.v25i2.5038

TOWARDS SUSTAINABLE NANOMATERIALS: ZINC PHOSPHATE NANOCRYSTALS SYNTHESIZED BY *BACILLUS SUBTILIS*[1]

EM DIREÇÃO A NANOMATERIAIS SUSTENTÁVEIS: NANOCRISTAIS DE FOSFATO DE ZINCO SINTETIZADO POR BACILLUS SUBTILIS

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ABSTRACT

This study focuses on the synthesis of zinc phosphate nanocrystals ($Zn_3(PO_4)_2 \cdot 4H_2O$) using the bacterium Bacillus subtilis: a sustainable, economical and energy-efficient approach that produces biocompatible nanocrystals with controlled properties and less environmental impact. The central question investigates how biological synthesis can be a sustainable alternative to chemical and physical synthesis. The aim of this work was to demonstrate the feasibility of biological synthesis of nanocrystals, with specific objectives of evaluating their physicochemical properties, examining the efficiency of the synthesis process, analyzing the stability of the nanocrystals and exploring their potential applications in agriculture and medicine. The theoretical framework is based on the advantages of biological synthesis, including biocompatibility and sustainability. The study on the synthesis of zinc phosphate nanocrystals using the bacterium Bacillus subtilis confirmed the formation of hopeite and alpha-zinc phosphate phases, demonstrating the feasibility of biosynthesis as a green and efficient method. The physical and chemical characterization of the nanocrystals by X-ray diffractometry and scanning electron microscopy showed orthorhombic and monoclinic structures before and after calcination, respectively, with an increase in the hydrodynamic diameter of the particles. The biocompatible nanocrystals exhibited controlled physicochemical properties, highlighting their potential for applications in agriculture and medicine. The biosynthesis stood out as a sustainable, efficient approach without the use of toxic chemicals, offering an advantageous alternative to traditional methods.

Keywords: nanotechnology; biological synthesis; nanomaterials.

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RESUMO

Este estudo foca na síntese de nanocristais de fosfato de zinco ($Zn_3(PO_4)_2 \cdot 4H_2O$) utilizando a bactéria Bacillus subtilis: um método com abordagem sustentável, econômica e energeticamente eficiente que produz nanocristais biocompatíveis com propriedades controladas e menos impacto ambiental. A questão central investiga como a síntese biológica pode ser uma alternativa sustentável à síntese química e física. O objetivo deste trabalho foi demonstrar a viabilidade da síntese biológica de nanocristais, com objetivos específicos de avaliar as suas propriedades físico-químicas, examinar a eficiência do processo de síntese, analisar a estabilidade dos nanocristais e explorar suas aplicações potenciais na agricultura e medicina. O marco teórico baseia-se nas vantagens da síntese biológica, incluindo biocompatibilidade e sustentabilidade. O estudo sobre a síntese de nanocristais de fosfato de zinco utilizando a bactéria Bacillus subtilis confirmou a formação das fases hopeíta e alfa-fosfato de zinco, demonstrando a viabilidade da biossíntese como um método verde e eficiente. A caracterização física e química dos nanocristais por difratometria de raios X e microscopia eletrônica de varredura mostrou estruturas ortorrômbicas e monoclínicas antes e após a calcinação, respectivamente, com aumento do diâmetro hidrodinâmico das partículas. Os nanocristais biocompatíveis exibiram propriedades físico-químicas controladas, evidenciando seu potencial para aplicações na agricultura e medicina. A biossíntese se destacou como uma abordagem sustentável, eficiente e sem o uso de produtos químicos tóxicos, oferecendo uma alternativa vantajosa aos métodos tradicionais.

Palavras-chave: *nanotecnologia; síntese biológica; nanomateriais.*

INTRODUCTION

Considered the most revolutionary technology of the 21st century, nanotechnology tends to transform society through its influence on the economy and improve capacity and quality in industrial sectors, as well as contribute to the well-being of beings in general (HALEEM *et al.*, 2023), presenting possibilities of being employed in different ways, among which nanostructures stand out.

Used in various branches of science, nanomaterials have shown to be potentially applicable in medical areas, such as new diagnostic instruments, imaging and methodologies, targeted medications, pharmaceutical products, biomedical implants, and tissue engineering (HALEEM *et al.*, 2023), in food, as functional ingredients in food additives (CHEN *et al.*, 2023), and also as nanofertilizers, being an emerging field of agriculture and an alternative option to improve plant growth, replacing synthetic fertilizers (SABIR *et al.*, 2020).

In the environment, nanoparticles (NPs) have been studied for their toxic impact and, conversely, their possible beneficial effect on plants (SABIR *et al.*, 2020), using nanotechnology as an approach to improve the nutritional effectiveness of agricultural fertilizers, further reducing environmental pollution compared to fertilizer use (ANDELKOVIC *et al.*, 2018). Positive effects of NPs on growth parameters have been observed in various plants, such as tomatoes (RAHMAN *et al.*, 2021), wheat (KULIKOVA *et al.*, 2017), and soybeans (CIESCHI *et al.*, 2019), where iron-based nanomaterials improve morphological, physiological, biochemical, and plant performance characteristics.

In this scenario, nanofertilizers are an emerging field of agriculture and an alternative option to the use of synthetic fertilizers. Phosphorus nanoparticles were synthesized by *Aspergillus fumigatus*, demonstrating that commercial phosphate fertilizer could be reduced by 50% when applied in combination with nanoparticles, while also achieving a significant increase in growth parameters (YASEEN; AMIN, 2021). Zinc oxide nanoparticles (ZnO NPs), for example, can be used as a source of zinc for plants (SABIR *et al.*, 2020). Zinc is present in the structure of transferases, oxidoreductases, lyases, hydrolases, ligases, and isomerases. Furthermore, several reactions are catalyzed by Fe and Zn elements in plants (CHANDRIKA *et al.*, 2022). In particular, these particles are of great interest due to their physical and chemical properties, such as high chemical stability, including photo-stability, radiation absorption, efficient electrochemical coupling (ZHOU *et al.*, 2023), and biocompatibility (BANDEIRA *et al.*, 2020). Their use can extend to tissue regeneration, cancer therapies, antimicrobials (BANDEIRA *et al.*, 2020), biosensors, and catalysts (HAMK; AKÇAY; AVCI, 2023).

The synthesis of nanomaterials can occur through biological, chemical, and physical methods. Chemical and physical methods require reagents, often toxic, and/or energy consumption, thereby increasing their cost, risk of accidents, and environmental impact. Biological methods have been increasingly employed for their production due to their advantages (BANDEIRA *et al.*, 2020). Green synthesis, for example, mediated by plants, has been receiving increasing attention due to its excellent stability and environmental respect (ZHU *et al.*, 2023). However, its application on a large scale is still difficult (BANDEIRA *et al.*, 2020), and therefore, new studies are necessary.

Synthesis from microorganisms is even more interesting compared to synthesis by plants, as their growth rate is high and their manipulation is easy, making them true nanofactories. Typically, bacteria and fungi have the inherent ability to synthesize zinc oxide nanoparticles directed by intra or extracellular pathways (MURALI *et al.*, 2023). Researchers' preference for using bacteria in nanoparticle synthesis was reported by Murali *et al.* (2023) due to their genetic manipulation capability and ease of handling compared to other eukaryotic microorganisms.

Thus, *Bacillus* strains have shown great industrial potential, as they have the capacity to produce various metabolites and many of these strains are considered producers of zinc oxide particles, being a multitasking and considered safe (GRAS) metallic oxide (HAMK; AKÇAY; AVCI, 2023). However, due to the scarcity of studies on the synthesis of $Zn_3(PO_4)_2$ nanostructures by bacteria and the need to expand knowledge in the field, the present study presents a novel synthesis of $Zn_3(PO_4)_2 \cdot 4H_2O$ nanocrystals using *Bacillus subtilis* (a strain of relevance in the industry for the diverse production of metabolites) due to its importance in application in the agricultural sector and with the aim of complementing limited research in the area.

METHODOLOGY

Pure cultures of *Bacillus subtilis* ATCC 6633 were inoculated into flasks containing BHI broth and incubated at 30 °C for 24 hours on a rotary shaker at 120 rpm. After incubation, bacterial growth was measured at 600 nm using a spectrophotometer. The culture was diluted (~2.0 abs) and allowed to grow for another 48 hours. The culture was centrifuged at 5000 rpm for 15 minutes, and the supernatant was reserved for further processing, while the pellet was discarded. 0.1 $mol \cdot L^{-1}$ zinc sulfate ($ZnSO_4$) (99.0%, Synth, Brazil) was added to an Erlenmeyer flask containing 100 mL of supernatant, followed by heating in a water bath at 80 °C for 10 minutes, until a white precipitate appeared at the bottom of the flask. The $Zn_3(PO_4)_2 \cdot 4H_2O$ crystals were be collected by centrifugation at 5000 rpm for 10 minutes, washed with deionized water, and dried at 60 °C overnight. Part of the synthesized material was separated and calcinated at 400 °C for 2 h to verify its thermal stability.

CHARACTERIZATION

The characterization of $Zn_3(PO_4)_2 \cdot 4H_2O$ nanocrystals was carried out using a Hitachi TM3000 Tabletop Scanning Electron Microscope (SEM) associated with an energy dispersive X-ray spectroscope (EDS), to obtain images and verify the elemental distribution of the sample. A Shimadzu XRD-6100 X-ray diffractometer was used to determine the crystallinity and the phases present in the nanocrystals before and after calcination. An Anton Paar Litesizer DLS 500 dynamic light scattering instrument was used to measure the hydrodynamic radius of the nanocrystals.

RESULTS AND DISCUSSION

MICROBIAL SYNTHESIS

The study on the synthesis of zinc phosphate nanocrystals ($Zn_3(PO_4)_2 \cdot 4H_2O$) using *B. subtilis* confirmed the formation of hopeite before thermal treatment and the alpha-zinc phosphate phase after calcination, demonstrating the viability of biosynthesis as an efficient and green method. The calcination step at 400 °C showed stability of the synthesized zinc phosphate, which did not undergo any decomposition, only being dehydrated at such high temperatures. The biocompatible nanocrystals, characterized by X-ray diffraction and scanning electron microscopy, exhibited controlled physicochemical properties and potential for agricultural and medical applications.

Synthesis of metallic nanoparticles by microorganisms such as bacteria, fungi, yeast, plants, and algae has been promising for the development of materials on the nanometer scale (MOHANPURIA *et al.*, 2008). One of the advantages of nanoparticle biosynthesis is that it does not

involve the use of toxic chemicals and is therefore considered a "green synthesis" method. Compared to physical and chemical methods, the biological method has the following benefits: high stability, clinical adaptability, biocompatibility, rapid synthesis and good cost effectiveness (SASTRY *et al.*, 2003). Physical methods to produce NPs require high temperatures and chemical methods require high pressure or temperature, making the process difficult. Chemically synthesized NPs generally need stabilization to avoid agglomeration, with the use of surfactants, while biologically produced NPs do not have this problem due to proteins secreted by microorganisms that aid in stabilization (GERIKE *et al.*, 2006; HULKOTI *et al.*, 2014). NPs synthesized by biological methods offer significant advantages, such as good photodegradation properties, antimicrobial activity, anticancer characteristics, and potential for drug delivery, as well as higher catalytic activity and better surface properties. Although biological in nature is slower, advances in this field can make it more attractive. Studies show that biomolecules from organisms can be used to control the growth and nucleation of inorganic structures, with the size and shape of NPs varying depending on the organism, environment, and synthesis conditions (AHMED *et al.*, 2017; TALEBI *et al.*, 2010).

Biosynthesis of NPs can be extracellular or intracellular, with extracellular methods being preferable due to ease of environmental control and potential for industrial-scale production. The exact mechanism behind the synthesis of NPs using biological agents is not yet completely understood, as it involves several biological factors in reactions with metal ions. In intracellular methods, a specific ion is transported into the cell, while in extracellular methods, the cell wall plays a crucial role in reducing metal ions to NPs through electrostatic and enzymatic interactions (MUKHERJEE *et al.*, 2001). Different microorganisms follow different mechanisms for the formation of NPs, generally involving the capture and reduction of metal ions in the presence of enzymes. Furthermore, microorganisms can influence mineral formation by changing the composition of the solution or producing organic polymers that stabilize mineral cores (ZARE *et al.*, 2017; LI, *et al.*, 2011).

Thus, in the present study, the synthesis of $Zn_3(PO_4)_2 \cdot 4H_2O$ nanocrystals by *B. subtilis* was observed based on the change in color, from yellow to whitish, and confirmed using characterization methods. It is not known for certain how the zinc phosphate biosynthesis process occurs, but, according to Beveridge *et al.*, 1996, microorganisms adopt a defense mechanism to contain the stress caused by the excess concentration of metal ions in environmental conditions. This occurs through the change in the oxidation-reduction state of metal ions through extracellular complexes, or due to intracellular precipitation of metals, often in the form of metal sulfides. Based on Hulkoti *et al.*, 2014, this process occurs through the NADH-dependent reductase enzyme, which obtains the electron from NADH and oxidizes it to NAD^+, synthesizing particles in nanometric sizes.

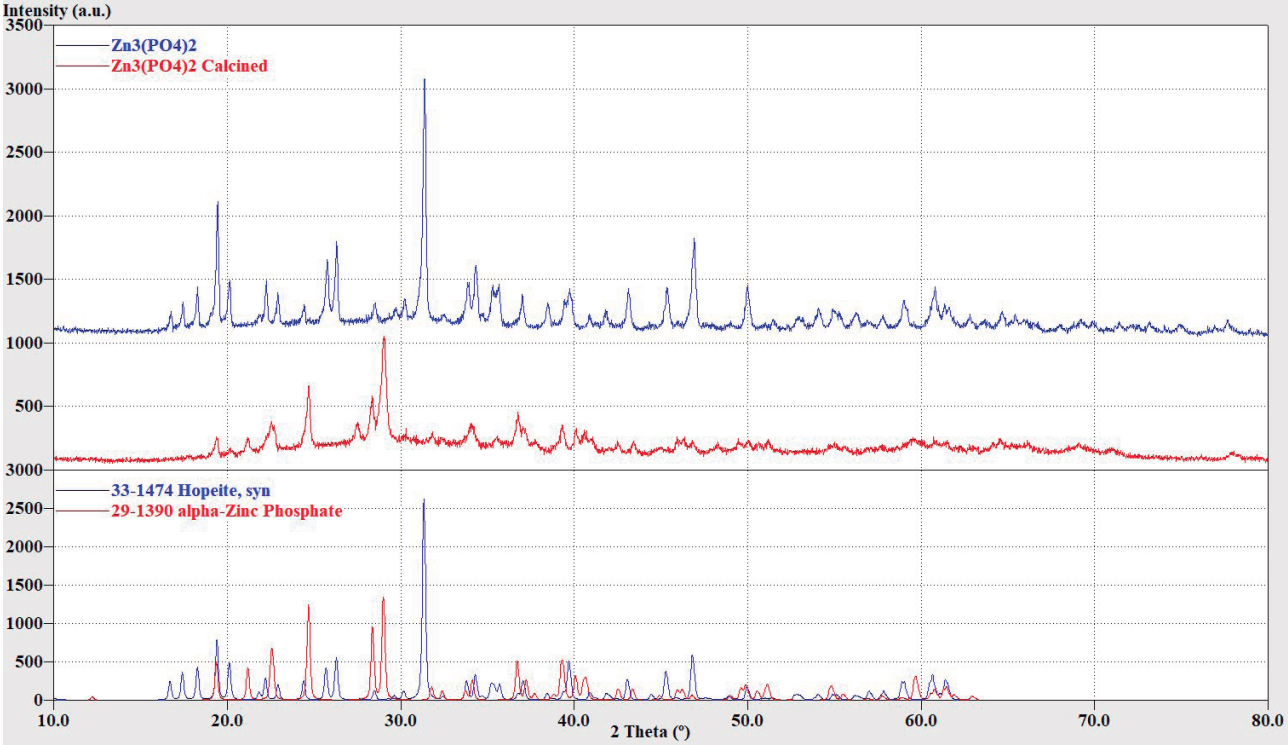
The synthesis of zinc phosphate nanocrystals using *B. subtilis* demonstrates the promising potential of nanoparticle biosynthesis, an approach considered as "green synthesis" due to the absence of toxic chemicals. Although the exact mechanism of zinc phosphate nanoparticle

biosynthesis is not yet fully understood, studies indicate that processes such as enzymatic and electrostatic interactions at the cell wall play a crucial role. The enzyme-mediated reduction of metal ions, such as by NADH reductase, exemplifies the complexity and efficiency of this method. Therefore, continuous advancements in this field can make biosynthesis even more attractive, especially for industrial and medical applications, standing out as a sustainable and efficient approach for the production of nanomaterials.

X-RAY DIFFRACTOMETRY (XRD)

The XRD diffractograms for $Zn_3(PO_4)_2 \cdot 4H_2O$ as synthesized and after the calcination process can be seen at the top part of Figure 1, along with the ICDD sheets as reference for both observed crystalline structures, hopeite (33-1474) and alpha-zinc phosphate (29-1390), at the bottom part of the figure (BOONCHOM *et al.*, 2010; LV *et al.*, 2012).

Figure 1 - XRD diffractograms for $Zn_3(PO_4)_2$ before (blue) and after (red) calcination.



Source: Authors.

As can be seen in the above figure, the bacterial synthesis led to the formation of the hopeite phase of zinc phosphate, which is a tetrahydrate phase with an orthorhombic structure. The chosen broth BHI contains phosphate in the form of Na_2HPO_4 (WEI *et al.*, 2016). In the presence of phosphate, the formation of $Zn_3(PO_4)_2$ was induced, which precipitated and formed the hopeite structure (LV *et al.*, 2012).

The evident formation of orthorhombic hopeite instead of triclinic parahopeite may be influenced by the proportion of phosphate to the excess available Zn^{2+}, which could have been the case, given the fact that all available phosphate originated from BHI and is partially consumed in cellular metabolism, while all the Zn^{2+} came from the $ZnSO_4$ solution (CHUBUKOV; SAUER, 2014; LV *et al.*, 2012).

However, after the calcination step at 400 ºC during 2 h, the material suffered a phase change, forming alpha-zinc phosphate, which is not hydrated and presents a monoclinic structure. The dehydration process is expected and discussed by previous literature, starting at 106 ºC and being completely dehydrated at 300 ºC, forming α-$Zn_3(PO_4)_2$ (BOONCHOM *et al.*, 2010).

According to previous literature, Scherrer equation was used to estimate the crystallite size for zinc phosphate samples before and after calcination, by averaging values obtained from the full-width half maximum (FWHM) value from different peaks present in each diffractogram (FATIMAH *et al.*, 2022). Results can be seen in Table 1. For $Zn_3(PO_4)_2{\cdot}4H_2O$, peaks 1, 2 and 3 correspond to 0.197, 0.322 and 0.352 rad, respectively. For α-$Zn_3(PO_4)_2$, peaks 1, 2 and 3 represent 0.197, 0.215 and 0.252 rad, respectively.

Table 1 - Crystallite sizes for non-calcined and calcined zinc phosphate samples

Sample	Peak 1 crystallite size (nm)	Peak 2 crystallite size (nm)	Peak 3 crystallite size (nm)	Average crystallite size (nm)
$Zn_3(PO_4)_2{\cdot}4H_2O$	60.9 ± 2.1	46.2 ± 1.6	35.1 ± 1.2	47.4 ± 1.6
α-$Zn_3(PO_4)_2$	74.3 ± 2.5	60.3 ± 2.2	45.0 ± 1.7	59.9 ± 2.1

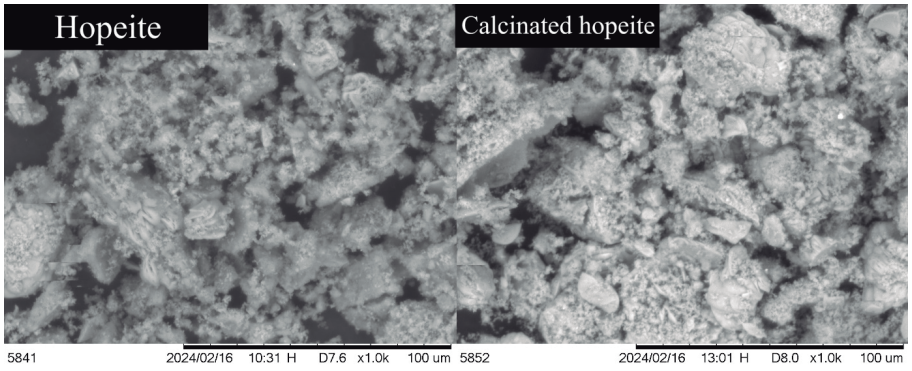
Source: Authors.

As can be seen in Table 1, calcination led to an increase in average estimated crystallite size, which is in accordance with previous literature, likely resulting from the sintering effect from the elevated temperature (CARDENAS-FLECHAS *et al.*, 2021; LEE *et al.*, 2023).

SCANNING ELECTRON MICROSCOPY (SEM)

In Figure 2, scanning electron microscopy images are presented for the samples of hopeite ($Zn_3(PO_4)_2{\cdot}4H_2O$) and the calcinated hopeite (α-$Zn_3(PO_4)_2$).

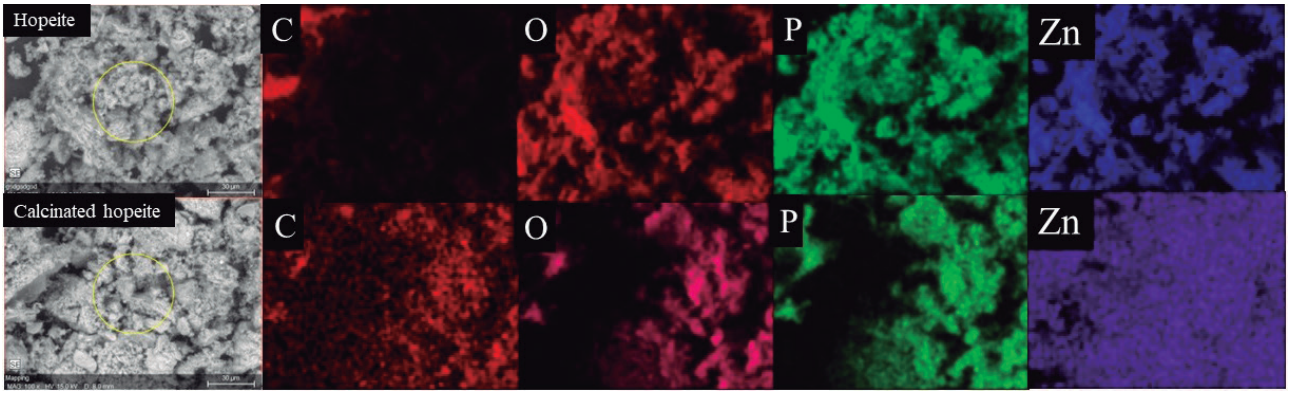
Figure 2 - SEM images for synthesized $Zn_3(PO_4)_2 \cdot 4H_2O$ and α-$Zn_3(PO_4)_2$.



Source: Authors.

As can be seen in the above figure, agglomeration is noticeable in both samples. These results agree with previous works, which demonstrate high agglomeration for zinc phosphate in the absence of surfactants such as oleic acid (NING *et al.*, 2013). In figure 3, the same SEM images are shown, along with the distribution map of the relevant chemical elements present in the sample as characterized by energy dispersive X-ray spectroscopy, such as carbon, oxygen, phosphorus, and zinc, represented by C, O, P, and Zn, respectively. Below, alpha-zinc phosphate (calcined hopeite) characterized under the same conditions is shown for comparison purposes.

Figure 3 - SEM images and EDS elemental distribution for uncalcined and calcined hopeite.



Source: Authors.

It can be observed that the hopeite sample before calcination exhibits a more individualized structure, with some aggregation between the particles. Regarding elemental distribution, it is noticeable that there is little carbon distribution over the analyzed sample, with oxygen, phosphorus, and zinc being present in a more uniformly distributed manner, as expected for the hopeite structure (DAS; SHUKLA, 2019, 2021).

In comparison with the calcined sample, it is noted that the particles appear to be somewhat more united and aggregated. This is an expected process, given that after calcination, sintering can occur, where smaller particles end up forming larger agglomerates due to heating

(NAMKUNG *et al.*, 2016). The calcined hopeite, unlike the non-calcined hopeite, showed a greater distribution of carbon and zinc, as can be observed in Figure 3. This may have been caused by the reorganization of elements during the heating process, leading to a non-uniform elemental distribution (JI *et al.*, 2019).

DYNAMIC LIGHT SCATTERING (DLS)

In Table 2, the results of the DLS characterization are shown for non-calcined and calcined samples.

Table 2 - Results of Dynamic Light Scattering (DLS) Analysis

	$Zn_3(PO_4)_2 \cdot 4H_2O$	α-$Zn_3(PO_4)_2$
Hydrodynamic diameter (nm)	1648 ± 103	2184 ± 373
Polydispersity (%)	289.0	14.99
Diffusion coefficient (μm²/s)	0.2977	0.2246

Source: Authors.

The hydrodynamic diameter can be associated with the average diameter of the particles in suspension (GUO *et al.*, 2009; STETEFELD; MCKENNA; PATEL, 2016). It is observed that after calcination, the value increased, which can be attributed to the greater agglomeration of particles due to their diffusion at high temperatures (LEITE *et al.*, 1997). This phenomenon suggests that thermal treatment promotes the coalescence of particles, resulting in an increase in average size.

Polydispersity, which is correlated with the size distribution of particles in suspension, is a crucial parameter for understanding the homogeneity of the samples (GUO *et al.*, 2009; STETEFELD; MCKENNA; PATEL, 2016). Higher polydispersity values indicate a greater variation in particle size. Notably, after calcination, the samples showed a significant decrease in polydispersity, indicating that they became more homogeneous compared to their state before calcination. This result suggests that the thermal process contributed to a more uniform particle size distribution.

The diffusion coefficient is directly related to the average speed of the particles in suspension (GUO *et al.*, 2009; STETEFELD; MCKENNA; PATEL, 2016). After calcination, a decrease in the diffusion coefficient was observed concomitant with an increase in the hydrodynamic diameter. This behavior indicates an inverse correlation between the diffusion coefficient and particle size, corroborating the hypothesis that larger particles, due to their greater total mass, tend to move more slowly. This phenomenon is consistent with the theory that particle mobility is inversely proportional to their size, which directly impacts the dynamics of the suspension.

CONCLUSIONS

This study highlights the promising potential of the biological synthesis of zinc phosphate nanocrystals ($Zn_3(PO_4)_2 \cdot 4H_2O$) using *Bacillus subtilis* strain. The research demonstrated that biosynthesis is a viable and sustainable alternative compared to traditional chemical and physical methods, offering significant advantages such as biocompatibility and the absence of toxic chemicals. The characterization of the nanocrystals confirmed the formation of hopeite and alpha-zinc phosphate phases, proving the method's efficiency. Furthermore, the stability and physicochemical properties of the synthesized nanocrystals open new possibilities for their applications in agriculture and medicine, such as using the zinc phosphate nanocrystals as a stable zinc source for seed germination due to its nanoscale crystals.

However, despite the advances presented, continued research is necessary to fully understand the mechanisms involved in biosynthesis and to enable the scalability of the process. This study significantly contributes to the field of green nanotechnology and establishes a solid foundation for future investigations and industrial applications of biosynthesized nanomaterials.

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