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Article

Synthesis of Chitosan from Shrimp Exoskeleton an its Characterization by SEM and DRX

Síntesis de quitosano a partir del exoesqueleto del camarón y su caracterización mediante SEM y DRX

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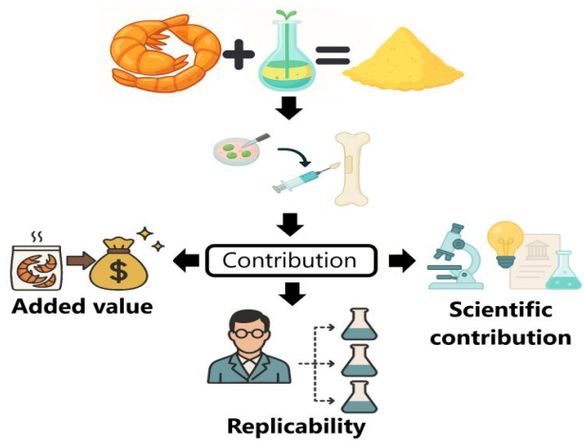
Abstract

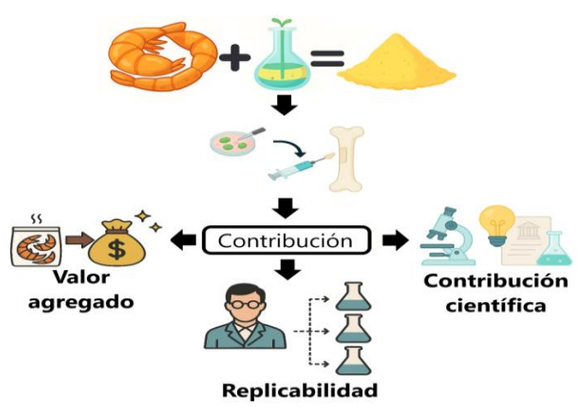
Currently, sustainable alternatives are being explored for the development of biomaterials from organic waste. In this study, **chitosan [CTS]** was extracted from shrimp shells through a process of demineralization, deproteinization, and deacetylation. The resulting material was **characterized** using SEM and DRX techniques, confirming its structure and purity. To assess its **biocompatibility**, **in vitro** assays were performed using **C6 glial cells** [from rat], applying the **Alamar Blue** method to evaluate **cell viability**. The results showed good cell tolerance to chitosan, indicating its potential as a biomaterial for **tissue engineering** applications. This approach represents an eco-friendly, feasible, and low-cost alternative for the development of functional materials derived from marine waste.



Biomaterial, Chitosan, Tissue

Resumen

Actualmente, se buscan alternativas sostenibles para desarrollar biomateriales a partir de desechos orgánicos. En esta investigación, se obtuvo **quitosano [CTS]** a partir de cáscaras de camarón mediante un proceso de desmineralización, desproteinización y desacetilación. El material obtenido fue **caracterizado** mediante técnicas como SEM y DRX, confirmando su estructura y pureza. Para evaluar su **biocompatibilidad**, se realizaron ensayos **in vitro** utilizando células **C6** [gliales de rata], aplicando el método **Alamar Blue** para determinar la **viabilidad celular**. Los resultados mostraron una buena tolerancia de las células al quitosano, lo que indica su potencial como biomaterial para aplicaciones en **ingeniería de tejidos**. Este enfoque representa una alternativa ecológica, viable y de bajo costo para el desarrollo de materiales funcionales a partir de residuos marinos.



Quitosano, Biocompatibilidad y tejidos

Area: Dissemination of and universal access to science

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Introduction

In recent decades, the increasing volume of agro-industrial and marine waste has motivated an active search for sustainable solutions that allow their reuse as raw materials for the development of new functional materials. In this context, shrimp shells, rich in chitin, represent an economical and abundant source for the production of chitosan [CTS], a versatile biopolymer with biomedical, pharmaceutical, and environmental applications [Zhou et al., 2023].

Chitosan is a linear polysaccharide obtained by the partial deacetylation of chitin, a natural polymer mainly found in the exoskeletons of crustaceans. The transformation process of chitin into chitosan is based on three successive stages"demineralization, deproteinization, and alkaline deacetylation"in which inorganic salts, proteins, and acetyl groups are removed, respectively, generating a material with high chemical and biological functionality [Ahmed et al., 2025]. This conversion may also include a final chemical precipitation step, where the pH is adjusted to induce controlled insolubilization of the chitosan and facilitate its recovery [Olafadehan et al., 2021].

Various factors such as temperature, reagent concentration, reaction time, and the type of solvent used during the extraction process can significantly affect the characteristics of the obtained chitosan. Recent studies have reported products with a low content of impurities, such as ash [0.72%] and residual proteins [less than 0.13%], evidencing the efficiency of modern purification processes [Shahidi &Abuzaytoun, 2005].

Interest in chitosan has grown significantly due to its biodegradability, biocompatibility, antimicrobial activity, and its ability to form films, gels, micro- and nanoparticles, among other functional structures [Chen et al., 2021]. These properties make it an ideal candidate for applications in tissue engineering, where the need for biodegradable and bioactive scaffolds is essential to promote cell regeneration. In addition, its cationic nature allows the formation of electrostatic bonds with cell surfaces and materials, which further expands its functionality [Elias et al., 2022].

From a structural characterization perspective, techniques such as Scanning Electron Microscopy [SEM], which allows observing the surface morphology of the material, and X-Ray Diffraction [XRD], which provides information about its degree of crystallinity and internal structure, are commonly used [Zhong et al., 2023; Nouj et al., 2021].

Regarding its biocompatibility, chitosan has been shown to be well tolerated by various cell lines, such as fibroblasts, tumor cells, and neuronal cells. In this study, cell viability was evaluated using the C6 cell line [rat glioma], widely used as a neurotoxicity model. For this evaluation, the colorimetric Alamar Blue assay was employed, which quantifies cellular metabolic activity through the conversion of resazurin to resorufin, without generating significant cytotoxicity [Thermo Fisher Scientific]

Recent studies support the use of chitosan as a safe and efficient material in biomedical applications. For example, it has been reported that chitosan extracted from shrimp shells exhibits high compatibility with HEK 293 cells and induces a minimal inflammatory response. Furthermore, its behavior in solution and strong adsorption capacity for dyes and bioactive compounds underscore its potential not only in regenerative medicine but also in water treatment and other environmental applications. [Çelikçi et al., 2020; Mathew et al., 2020].

Specifically, it was observed that chitosan nanoparticles with silibinin [SCNP] were internalized by C6 cells and induced apoptosis by increasing Bax and caspase-3 expression, without affecting H9C2 cells [Takke et al., 2021]. In addition, chitosan-eugenol nanoparticles induce apoptosis and inhibit metastatic signals in C6 cells, confirming their antitumor potential [Li et al., 2020].

In summary, the valorization of marine waste, such as shrimp shells, for the production of chitosan represents a strategy that combines environmental sustainability with technological innovation. This biopolymer, obtained through efficient chemical methods, can be characterized and validated using structural tools and cellular assays, thereby contributing to the development of new functional biomaterials with a wide range of applications.

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The main objective of this research work was to synthesize and characterize chitosan from shrimp shells through a chemical precipitation process, using analysis techniques such as scanning electron microscopy [SEM], X-ray diffraction [XRD], and the Alamar Blue cell viability assay.

This proposal aims to add value to an agro-industrial waste by revaluing a marine residue through its transformation into a functional biomaterial with potential biomedical applications.

Through the study of C6 cells, the aim is not only to validate the biocompatibility of the obtained chitosan but also to explore its ability to support the viability, proliferation, and possible differentiation of neural-origin cells, which is key for future applications in nervous system tissue engineering, glioma therapies, or neurologically oriented drug delivery platforms.

Evaluating the interaction between chitosan and this cell line will allow identifying whether the biomaterial possesses the necessary properties to be considered a bioactive, safe, and efficient scaffold capable of functionally integrating into complex cellular systems.

2. Materials and Methods

Shrimp exoskeletons were obtained from local markets in Nicolás Romero, State of Mexico, representing an abundant and inexpensive raw material rich in chitin.

Throughout the entire procedure, analytical-grade reagents and freshly prepared solutions were employed to maintain experimental consistency and to minimize possible interferences during the structural and biological characterization of the resulting chitosan.

Using high-purity reagents—particularly hydrochloric acid and sodium hydroxide at controlled concentrations—has been recognized in previous studies as a critical factor for producing chitosan of high quality, with a uniform surface morphology and an elevated degree of deacetylation [Hisham et al., 2021].

Box 1

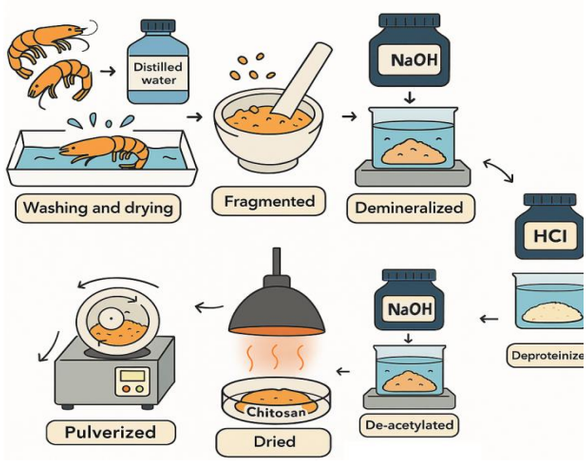

Figure 1
Methodology for obtaining chitosan

2.1. Chitosan synthesis

The process of obtaining chitin was carried out through chemical precipitation applied to shrimp exoskeletons collected from local markets in the municipality of Nicolás Romero, State of Mexico. Initially, the residues attached to the shells were removed by continuous jet washing, eliminating any superficial organic matter [Xie et al., 2021].

Subsequently, the exoskeletons were dried under an infrared lamp for one hour, simplifying water removal and facilitating the subsequent grinding process. The dried exoskeletons were subjected to primary grinding using a ceramic mortar and then pulverized in a planetary ball mill model BIC0400-0.4, in order to reduce particle size and improve the material's handling properties [Tuhua et al., 2020].

Box 2

Figure 2
Planetary mill model BIC0400-0.4

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This procedure follows efficient protocols described in the literature, which state that "shrimp shell waste is one of the main sources used for chitin preparation, with a content of approximately 15 to 40% of the dry weight of the shrimp shell" [Nanoxhitosan study, 2024]. Therefore, it is possible to obtain chitosan powder using a planetary mill. Likewise, more recent research has pointed out that particle size directly influences key characteristics of chitosan. For example, it has been found that "prolonged milling significantly reduces the molar mass of chitosan," which is a desirable property for certain applications, demonstrating that the milling operation is decisive for the final structure of the polymer [Helton et al., 2017].

Once the shrimp exoskeleton powder was pulverized, three fundamental phases were carried out to obtain chitosan: demineralization, deproteinization, and deacetylation. The first demineralization step was performed by immersing the material in a 0.6N HCl solution for 2 hours at room temperature, with the aim of eliminating calcium carbonate. This procedure coincides with that reported by Marina et al. [2022], who state that "shrimp shell waste is one of the main sources used for chitin preparation, and demineralization is essential for removing the mineral content present in the biological matrix."

Subsequently, the sample was subjected to deproteinization using a 1% NaOH solution for 24 hours under constant stirring, a process that facilitates the breaking of peptide bonds. Gao et al. [2024] note that alkaline treatment is the most effective method for removing proteins from chitinous materials without altering the integrity of the polymer.

Finally, the deacetylation of chitin was carried out to transform it into chitosan. Initially, a pretreatment with 3% acetic acid was applied, followed by filtration, and then the material was introduced into a 50% NaOH solution for 2 hours at 100°C. This protocol aims to achieve a high degree of deacetylation, a condition that is essential to improve the solubility and reactivity of chitosan. According to a study published in *Polymers* [2023], "NaOH concentrations of 40 to 50% are commonly used to induce deep deacetylation under heterogeneous conditions while maintaining the fibrous structure of chitin."

Box 3

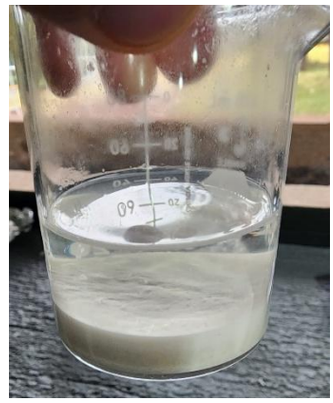

Figure 3
Chitosan production

Regarding the cell viability tests, rat glioma cells [C6 cell line] were used, which were incubated for 24 hours with chitosan at concentrations of 100 and 200 mg/mL, performing three independent replicates.

Subsequently, the Alamar Blue reagent was added, and fluorescence was measured to quantify cell viability. The data were analyzed using one-way ANOVA followed by Dunnett's post hoc test, with the aim of comparing each treatment against the control. This statistical approach is commonly used in cytotoxicity studies, including the evaluation of chitosan nanoparticle formulations on C6 cells, where the correlation between cellular metabolism and viability after 24 hours of exposure is observed.

Box 4

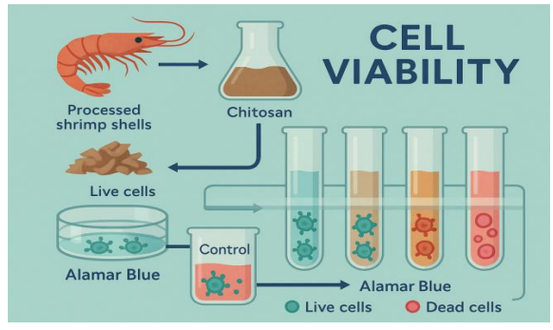

Figure 4
Cell viability test in C6 cells

3. Results

3.1 Morphological Analysis

The agglomerate obtained from shrimp shells was processed using a planetary ball mill, with the purpose of reducing the particle size to an ultrafine range.

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Box 5

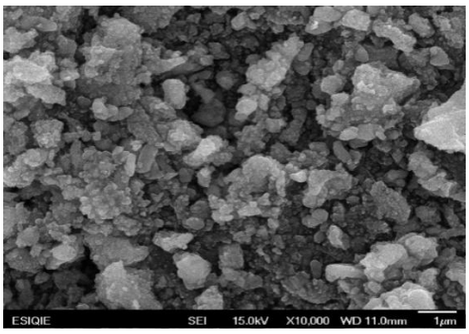

Figure 5

Chitosan scanning by scanning electron microscopy [SEM]

Subsequently, the samples were analyzed using electron scanning, and the morphology and particle size were evaluated with the Digital Micrograph software from Gatan Microscopy.

Box 6

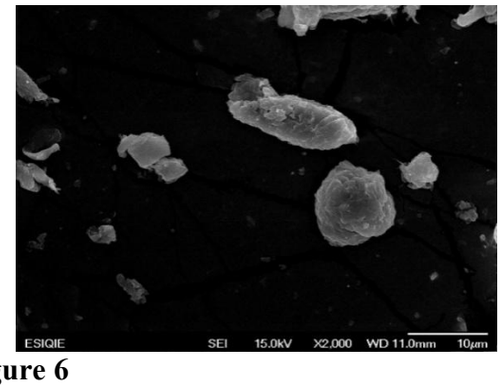

Figure 6

Scanning of a chitosan particle using

As a result of the milling process, a particle size distribution between 1.0 and 1.5 µm was obtained, with an average size of 1.3393 µm and a standard deviation of 0.5 µm.

Box 7

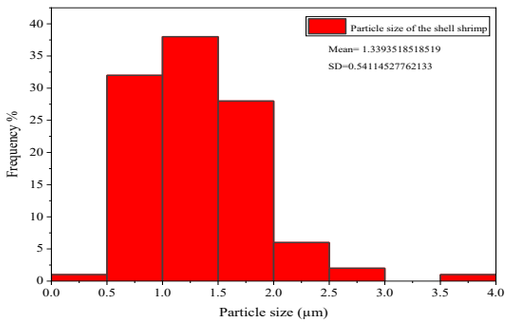

Figure 6

Determination of chitosan particle size after milling with a planetary mill

This behavior is consistent with previous studies where the use of planetary mills has made it possible to achieve fine micrometric sizes. For example, Alves et al. [2018] reported intermediate sizes [D50] around 1.3 microns when milling chitosan in a ball mill.

The planetary mill operates through centrifugal forces combined with repetitive impacts between the balls and the material inside the container. These impacts cause fractures and exfoliation of the polymeric structure, reducing the size and potentially decreasing the molar mass [depolymerization] without significantly altering the degree of deacetylation.

Box 8

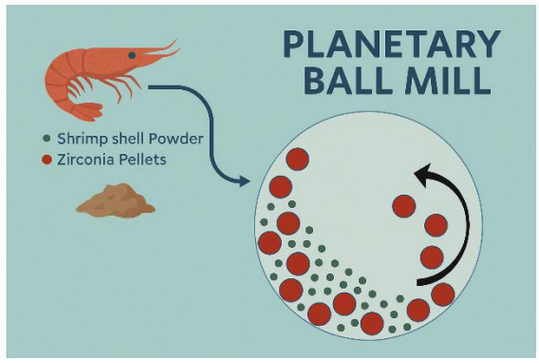

Figure 8

Internal operation of a planetary mill

3.2. Characterization by XRD

The results of the X-ray diffraction [XRD] analysis for the chitin samples extracted from shrimp shells are presented in the following figure [Figure 9].

In the spectrum, two high-intensity peaks were observed at 0° and 19.45° [2θ], as well as secondary peaks of lower intensity at 22.95°, 26.01°, 32.90°, and 39.22°. These diffraction patterns are characteristic of the crystalline structure of α-chitin, which is widely reported in the literature as the main allotrope of chitin present in crustacean exoskeletons.

The peaks of highest intensity around 9°–10° and 19°–20° correspond to the crystallographic planes [020] and [110], respectively, which reflect the orthorhombic conformation typical of α-chitin [Liu et al., 2012; Ravindra et al., 2014].

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Meanwhile, the minor signals observed at 22.95°, 26.01°, 32.90°, and 39.22° have also been reported in previous studies and are attributed to less intense reflections characteristic of the laminar organization of partially ordered chitin [Younes &Rinaudo, 2015].

Box 9

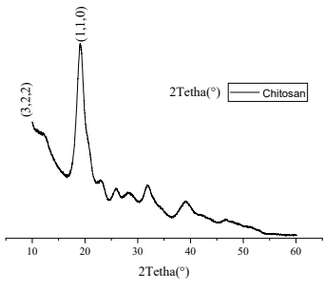

Figure 9

X-ray diffraction spectroscopy of chitosan

Although slight variations in peak position and intensity may occur due to factors such as the degree of deacetylation, moisture content, or the presence of residual impurities, the results obtained show a high degree of similarity with the patterns reported by other authors for chitins extracted from crustaceans, which validates the efficiency of the extraction process used.

3.3. Viability with C6 Cells

In the interpretation of cell viability performed with the C6 cell line using the Alamar Blue assay, treatments with chitosan were applied on different plates [1–4]. According to the statistical analyses performed [ANOVA and Dunnett's multiple comparison test], slight differences were observed compared to the control group.

The concentrations analyzed were 100 mg/mL and 200 mg/mL. As shown in the following figure, the concentration of 100 mg/mL resulted in a slight increase in cell viability, reaching approximately 101% compared to the control group, which suggests a possible metabolic stimulation without evidence of cytotoxicity. On the other hand, the concentration of 200 mg/mL showed a slight decrease in viability, recording a value close to 99% compared to the control.

Although both variations are subtle, these results indicate that chitosan, at the evaluated concentrations, does not exhibit marked cytotoxic effects, and its impact on cell viability may depend on the dose.

Box 10

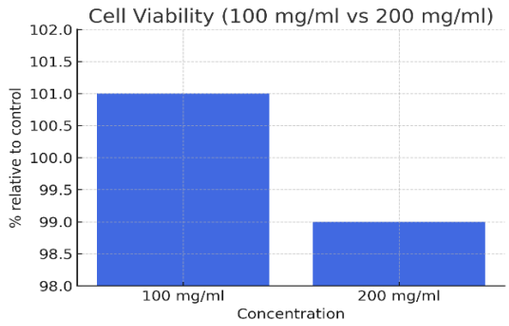

Figure 10

Cell viability of chitosan compared to the control in C6 cells

Box 11

Table 1

Cytotoxicity analysis of chitosan at doses of 100mg/ml and 200mg/ml in plates with C6 cells

Compound	Concentration	Plate 1	Plate 2	Plate 3	Plate 4
Chitosan	T1 100 mg/mL	–	–	Yes [+]	–
Chitosan	T1 200 mg/mL	–	–	Yes [+]	–

4. Discussion

During this research study, the synthesis of chitosan was carried out using the chemical precipitation method, employing biological residues from shrimp exoskeletons as raw material, with the aim of adding value to this type of marine waste.

The process focused on the extraction of chitin and its subsequent deacetylation to obtain chitosan, a biopolymer with well-known bioactive properties and potential applications in the biomedical and pharmaceutical fields.

For the characterization of the synthesized materials, three main analytical techniques were employed: scanning electron microscopy [SEM], X-ray diffraction [DRX], and the Alamar Blue cell viability assay, which

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allowed the evaluation of the biological behavior of chitosan in contact with C6 cells.

The DRX analysis enabled the identification of the crystalline structure of the extracted material. In the diffraction pattern, two high-intensity peaks were observed at 2θ angles of 10° and 19.45°, accompanied by lower-intensity peaks at 22.95°, 26.01°, 32.90°, and 39.22°.

According to the consulted literature, also reported by Kaya et al. [2014] and Alma et al. [2019], the main peaks in the range of 9° to 19° correspond to the crystallographic planes [020] and [110], which are characteristic of the orthorhombic crystal lattice that defines α-chitin. These results confirm that the structure of the biopolymer was preserved after the extraction and modification process.

On the other hand, the morphological analysis using SEM allowed the observation of the shape and size of the chitosan particles, which were previously optimized by milling in a planetary ball mill.

This mechanical size reduction technique proved to be effective, as it allowed the production of particles with sizes ranging between 1.0 and 1.5 µm, with an average size of 1.3393 µm. This reduction in particle size is relevant, as it improves the surface reactivity of chitosan and its performance in applications involving cellular interaction, encapsulation, or controlled release.

Finally, the results obtained from the cell viability assay using Alamar Blue, applied to C6 cell lines treated with different chitosan concentrations, indicate that this biopolymer does not exert significant cytotoxic effects under the evaluated conditions.

The slight increase in viability observed at 100 mg/mL [≈101 %] could be related to a mild stimulation of cellular metabolic activity, which has been previously reported in studies where moderate chitosan concentrations promote cellular proliferation or activation processes. In contrast, the reduction observed at 200 mg/mL [≈99 %] suggests that, at higher concentrations, chitosan could begin to generate an inhibitory cellular response, although not enough to be considered cytotoxic.

The absence of statistically significant differences compared to the control group indicates that, within the studied concentration range, chitosan exhibits a biocompatible profile in the C6 cell line. This finding is consistent with reports in the literature, which recognize that cellular response to chitosan may vary depending on its concentration, molecular weight, degree of deacetylation, and physical form [soluble or particulate].

In conclusion, the chitosan synthesized from shrimp exoskeletons shows safe behavior in terms of cell viability in C6 cells at concentrations of up to 200 mg/mL. These results support its potential as a biocompatible material for biomedical applications, although additional studies will be necessary to evaluate its performance in other cell types, as well as its influence on processes such as proliferation, differentiation, or apoptosis.

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Declarations

Conflict of Interest

The authors declare that they have no conflicts of interest. They have no known competing financial interests or personal relationships that could appear to influence the work reported in this article.

Authors' Contribution

*Fuentes-Romero, María Teresa***:** Contributed to the project idea, research method, and technique. Supported the design and concept of this research. Helped guide the study through previous investigations carried out by her.

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*Bermúdez, Jesús Nicolás***:** Contributed to the research design, type of study, methodology, and the writing and editing of the article, as well as providing suggestions for the synthesis of this research.

*Medina-Mendoza, Manuel***:** Contributed to data analysis for the characterization of the equipment used, such as X-ray diffraction spectroscopy analysis, scanning electron microscopy, energy-dispersive spectroscopy, and article revisions related to technical processes.

*Maldonado-Mondragón, Erick Antonio***:** Contributed to experimental design, optimization of the synthesis process, and prior research for the production of hydroxyapatite, as well as in writing the article.

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Differences

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