



Thermal extraction and physicochemical characterization of nanocrystalline hydroxyapatite from bovine bone waste

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This research demonstrated the feasibility of extracting nanostructured hydroxyapatite (HAp) from bovine bone waste through controlled thermal calcination at 600, 800, and 1000 °C. Thermal analysis showed mass losses of 32.50–34.90%, reflecting progressive removal of the organic matrix. Updated XRD analysis confirmed the HAp diffraction fingerprint in all calcined samples but revealed a non-monotonic structural response: H2 (800 °C) produced the largest apparent crystallite size (100.09 nm), the highest crystallinity proxy (96.4%), and the lowest microstrain (2.207×10^{-3}), whereas H3 and H1 both showed crystallite sizes of 19.91 nm, crystallinity proxies of 92.7%, and microstrain values of 9.211×10^{-3} . The phase-purity proxy is highest for H3 and H1 (90.6%) and lower for H2 (88.2%), indicating that crystallinity and phase-purity proxies should be interpreted separately. The additional comparative curves for crystallite size, microstrain, crystallinity, and phase purity visualize these temperature-dependent differences. FTIR confirmed characteristic phosphate and structural hydroxyl bands, with H2 showing the clearest overall HAp fingerprint and H1 the lowest prominence of collagen-related bands. DLS identified a small effective hydrodynamic diameter of 15.1 nm and a low polydispersity index of 0.289 for H1, while SEM showed progressive development of granular and faceted morphologies. Overall, 800 °C favored XRD lattice ordering and FTIR definition, whereas 1000 °C favored organic removal and dispersion behavior; therefore, the optimum calcination condition depends on the targeted structural, chemical, or particulate property.

Keywords: Hydroxyapatite; Bovine bone waste; Thermal; Nanocrystalline; Biomedical.

1. INTRODUCTION

Hydroxyapatite [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$] serves as the primary inorganic constituent of human bone, rendering it an essential material for the fabrication of biocompatible scaffolds and implants in tissue engineering. Recent research has identified bovine bone waste, an abundant byproduct of the slaughterhouse industry, as a sustainable and cost-effective precursor for synthesizing high-purity hydroxyapatite [1, 2]. Due to its chemical similarity to human bone tissue—characterized by inherent bioactivity, osteoconductivity, and biocompatibility—this natural derivative is increasingly favored for orthopaedic and restorative applications [3]. However, the structural integrity and physicochemical performance of the derived bioceramic are highly sensitive to processing parameters during thermal decomposition [4]. Consequently, this study aims to systematically optimize controlled thermal calcination conditions to achieve superior phase purity and tunable nanocrystalline morphology [5, 6]. By establishing a relationship between calcination temperatures and the resulting crystallinity, this research seeks to standardize production protocols that minimize phase impurities while maximizing yield for clinical integration [7, 8]. This process specifically evaluates how specific annealing parameters effectively eliminate residual organic components, such as fats and proteins, to produce stable, high-performance powders suitable for synthetic bone grafts [9, 10]. Furthermore, this investigation characterizes the resultant material through advanced analytical techniques, including X-ray diffraction and electron microscopy, to ensure the synthesized hydroxyapatite meets the rigorous standards required for long-term physiological stability [11, 12]. This optimization process ultimately focuses on balancing the degree of thermal degradation with the preservation of structural characteristics, ensuring the material maintains the essential nanostructural integrity required for effective bone integration [13]. This investigation provides a framework for scaling the production of biogenic hydroxyapatite, thereby facilitating its adoption as an affordable and ecologically sustainable alternative to conventional synthetic powders [14]. By systematically investigating the influence of temperature on phase transformation, this study addresses the challenges inherent in converting biogenic precursors into reliable biomedical-grade scaffolds [15,16].

Hydroxyapatite is widely recognized as the most thermodynamically stable calcium phosphate phase under physiological conditions and closely resembles the mineral component of mammalian bone [18]. Because of its excellent biocompatibility, osteoconductivity, and ability to form direct bonds with regenerating bone tissue, HAp has become an important biomaterial for orthopaedic repair, prosthetic applications, scaffold fabrication, implant coatings, and drug delivery systems [19]. The extraction of hydroxyapatite from natural bovine bone waste provides several advantages over purely chemical synthesis methods, particularly because biogenic sources may retain trace minerals and ionic substitutions that improve biological recognition and support long-term osseointegration [20, 21]. In contrast, synthetic routes such as precipitation, sol-gel processing, hydrothermal synthesis, and combustion-based approaches offer controlled stoichiometry and particle morphology but often require intensive purification, chemical reagents, and additional thermal treatment to achieve the crystallographic maturity associated with natural bone-derived apatite [22, 23]. Natural extraction procedures generally involve cleaning, degreasing, thermal decomposition, and fine grinding to remove organic contaminants while preserving the inorganic apatite framework [24, 25]. Controlled calcination is especially effective because it decomposes collagenous proteins and lipids, isolates the mineral phase, and reduces the possibility of chemical residues introduced by wet synthesis methods [26]. However, the calcination temperature must be carefully optimized, since insufficient heating may leave residual organic matter, whereas excessive thermal exposure can promote grain growth, agglomeration, or phase instability [27]. Although chemically synthesized HAp can provide highly uniform particles, natural-derived hydroxyapatite often demonstrates higher crystallinity, favorable ionic complexity, and enhanced bioactivity, which are important for bone regeneration and scaffold performance [28, 29]. Therefore, comparing natural and synthetic production routes is essential for

determining the most sustainable and clinically relevant method for preparing biomedical-grade hydroxyapatite. In this context, bovine bone waste represents a valuable low-cost precursor for producing high-purity HAp with potential applications in bone grafts, implant coatings, and advanced bioceramic composites. The relevance of well-characterized HAp powders to implant-coating applications is further supported by studies showing that substrate surface roughness influences electrophoretically deposited HA/chitosan coatings on 316L stainless steel and that electrophoretic deposition can provide better HAp phase stability than thermal spraying on the same substrate.

Although several studies have reported the extraction of hydroxyapatite from bovine and other biogenic waste sources, important gaps remain regarding the systematic optimization of thermal calcination conditions and their combined influence on crystallinity, particle-size behavior, surface morphology, and functional-group evolution. Many previous investigations have focused mainly on phase identification or general material preparation, while fewer studies have directly compared untreated bovine bone with samples calcined across multiple temperatures using an integrated characterization approach that includes XRD, FTIR, DLS, and SEM. In addition, limited attention has been given to linking mass loss, organic matrix removal, nanocrystalline development, and biomedical suitability within a single controlled experimental framework. Therefore, this study addresses this gap by evaluating bovine bone-derived hydroxyapatite produced at 600 °C, 800 °C, and 1000 °C and by identifying the calcination condition that provides the most favorable balance between phase purity, structural stability, particle uniformity, chemical confirmation, and potential biomedical application.

The primary aim of this research is to develop a sustainable and cost-effective physical method for the extraction of high-purity, nanostructured hydroxyapatite (HAp) from natural bovine bone waste. To achieve this overarching goal, the study focuses on the following specific objectives:

- To investigate the influence of different calcination temperatures (600 °C, 800 °C, and 1000 °C) on the elimination of the organic matrix from bovine bone.
- To evaluate the structural evolution and crystallinity index of the extracted HAp using X-ray diffraction (XRD) analysis.
- To identify the characteristic functional groups of the extracted HAp using Fourier Transform Infrared Spectroscopy (FTIR), particularly phosphate, hydroxyl, carbonate, and residual organic bands.
- To characterize the particle size distribution and colloidal stability of the produced nanopowders via Dynamic Light Scattering (DLS).
- To examine the surface morphology and grain growth of the HAp particles at various thermal stages using Scanning Electron Microscopy (SEM).
- To determine the optimal thermal treatment conditions that yield the highest phase purity and structural integrity for potential biomedical and industrial applications.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Materials and reagents

The fundamental raw material for this study is sourced from healthy adult bovine specimens to ensure a high mineral content. Chemical reagents are primarily employed for the rigorous cleaning and organic removal phases. The specific materials and reagents used are detailed in the following table:

Table 1 Materials and chemical reagents used for the preparation and thermal extraction of hydroxyapatite from bovine bone waste.

Material / Reagent	Description / Grade	Source/Manufacturer	Purpose
Bovine Bone	Femur cortical sections	Local Slaughterhouse	Natural HAp Precursor
Distilled Water	Laboratory Grade	University Laboratory	Rinsing and Boiling
Acetone	Analytical Grade (99%)	Sigma-Aldrich	Lipid and Fat Removal
Sodium Hypochlorite	Standard Solution	Local Supplier	Protein Decomposition
Ethanol	Absolute (99.8%)	Merck	Dehydration and Cleaning

2.1.2. Laboratory equipment and tools

A variety of high-precision equipment is utilized to execute the thermal treatment and subsequent characterization of the samples. The technical specifications and applications of the primary laboratory tools are summarized below:

Table 2 Laboratory equipment, technical specifications, manufacturers, and applications used for hydroxyapatite extraction and physicochemical characterization.

Equipment / Tool	Model / Specification	Manufacturer	Application
Electric Muffle Furnace	High-Temp (Up to 1200°C)	Carbolite Gero	Thermal Calcination
Electronic Balance	Precision (0.0001g)	Sartorius	Accurate Mass Measurement
Laboratory Drying Oven	Forced Convection	Memmert	Sample Dehydration
Mechanical Grinder	High-Speed Blades	IKA	Bone Powdering
Ultrasonic Cleaner	Digital Frequency Control	Branson	Deep Surface Cleaning
XRD Spectrometer	Cu-Kα Radiation	Rigaku / Bruker	Phase and Crystallinity
FTIR Spectrometer	ATR / KBr Pellet	Thermo Scientific	Functional Group Analysis
SEM-EDX System	Cold Field Emission	JEOL / Hitachi	Morphology & Composition

2.2. Methods

2.2.1. Study design

This work is structured as a controlled experimental laboratory study designed to investigate the effect of calcination temperature on the extraction, purification, and physicochemical properties of hydroxyapatite (HAp) derived from bovine bone waste. Bovine bone powder is used as the biogenic calcium phosphate precursor and is processed through a standardized sequence of cleaning, boiling, drying, crushing, milling, sieving, and thermal treatment. The principal experimental factor is calcination temperature, with three treatment levels of 600 °C, 800 °C, and 1000 °C, in addition to an untreated natural bone powder group used as the baseline control. The samples are therefore classified as HB for untreated bone, H3 for 600 °C, H2 for 800 °C, and H1 for 1000 °C. All calcined groups are subjected to the same soaking duration and post-treatment processing conditions to ensure that the observed differences are primarily attributable to the applied thermal temperature. The primary outcomes are mass-loss percentage, phase purity, crystallinity index, crystallite size, particle-size distribution, polydispersity index, surface morphology, and functional-group composition. Phase development and crystallographic parameters are assessed by X-ray diffraction (XRD), chemical

bonding and characteristic phosphate/hydroxyl vibrations are evaluated using Fourier Transform Infrared Spectroscopy (FTIR), particle-size behavior and dispersion uniformity are evaluated using dynamic light scattering (DLS), and morphological evolution is examined by scanning electron microscopy (SEM). The comparative design enabled systematic assessment of thermal decomposition, organic matrix removal, grain growth, functional-group preservation or disappearance, carbonate substitution behavior, and nanostructural development across the selected temperature range. This approach allowed identification of the calcination condition that produced the most structurally stable, highly crystalline, chemically confirmed, and potentially biomedical-grade bovine-derived hydroxyapatite.

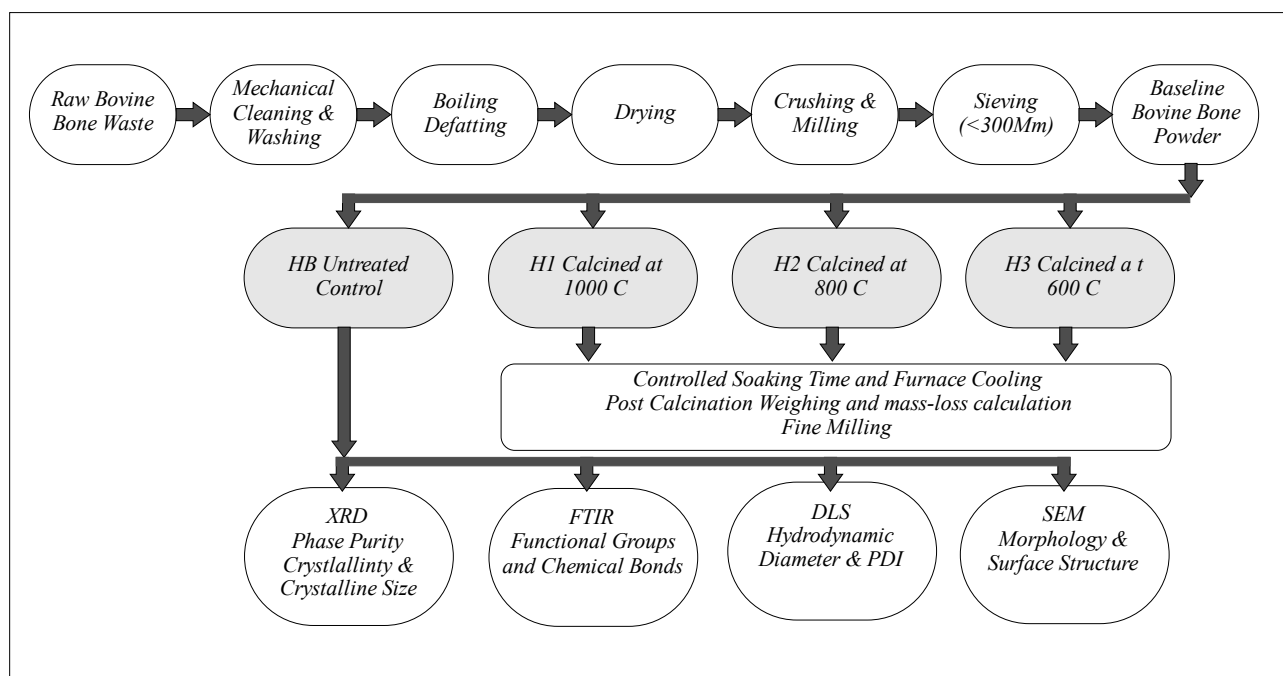


Figure 1 Experimental workflow for the preparation, thermal extraction, and physicochemical characterization of bovine bone-derived hydroxyapatite at different calcination temperatures.

2.2.2. Samples preparation

The preparation phase encompassed a rigorous sequence of physical, mechanical, and thermal treatments specifically designed to isolate the inorganic bone matrix from all biological contaminants.

2.2.2.1. Mechanical cleaning and washing

Initially, the acquired raw bovine bones are meticulously stripped of all macroscopic soft tissues, including adherent meat, cartilage, and internal marrow. This intense physical debridement is followed by extensive washing procedures to completely eliminate superficial biological residues and prepare the anatomical specimens for deeper processing.

2.2.2.2. Cutting and sectioning

To maximize the exposed surface area for optimal purification, the solid bone sections are systematically reduced in size. The tough bone structures are initially fractured and crushed into smaller, manageable fragments using a heavy-duty manual mortar and pestle. Subsequently, a

mechanical milling device is utilized to extensively grind these fragments into a highly fine powder. To guarantee a consistent microstructural profile, the resulting pulverized material is strictly passed through a specialized analytical sieve with a mesh aperture of less than 300 micrometers. Any oversized particles that failed to pass through the sieve are repeatedly isolated and re-ground until the entire batch achieved the targeted granular dimensions.

2.2.2.3. Chemical defatting and protein removal

To reduce residual fats and proteins without relying on additional organic solvents, the mechanically processed bone samples are boiled in water for one hour. This hydrothermal cleaning step removed soluble and loosely bound organic matter and prepared the mineral matrix for subsequent calcination, during which the remaining collagenous and carbonaceous components are thermally decomposed.

2.2.2.4. Drying and storage

Following the comprehensive cleaning and defatting procedures, it is scientifically imperative to completely dehydrate the samples prior to the high-temperature calcination process. The moisture-laden bone materials are transferred into a high-temperature commercial baking oven, deliberately selected for its exceptional thermal stability and heat retention capabilities. The specimens are continuously subjected to this heated environment for a total of two hours to ensure the absolute evaporation of all internal moisture. Once thoroughly desiccated, the processed bone powders are securely sealed and stored in a controlled, moisture-free environment to prevent any subsequent hydration or external contamination before the final physical extraction phase.

2.2.3. Extraction process

This critical phase of the methodology involved the precise physical transformation of the pre-treated biological matrix into highly purified inorganic bioceramics through rigorously controlled thermal degradation.

2.2.3.1. Thermal calcination protocol

To execute the thermal extraction phase, wide ceramic crucibles measuring exactly 10 cm in diameter are utilized as highly resilient heat receptacles. Prior to loading, each crucible is precisely weighed while empty, and subsequently weighed again after being filled with the processed bone powder. By subtracting the crucible's tare weight, the exact net mass of the raw biological powder is accurately determined. For this comprehensive investigation, four distinct experimental groups are established: three experimental batches subjected to varying high-temperature treatments, and a fourth unheated natural sample (designated as Sample B) which is intentionally retained as a baseline control for comparative evaluations.

2.2.3.2. Furnace parameters (heating rate and soaking time)

A highly advanced, programmable Carbolite muffle furnace is employed to administer the exact thermal conditions required for organic elimination. For the primary experimental batch, a substantial net weight of 80 grams of raw powder is introduced into the heating chamber. The thermal gradient is carefully restricted, allowing the furnace approximately one hour to steadily climb and stabilize at the absolute target temperature of 1000 °C. Once this critical thermal setpoint is achieved, a rigorous, uninterrupted soaking period of exactly four hours is maintained to guarantee the total combustion of all organic compounds. Following this protocol, identical temporal parameters are applied to the

subsequent batches, which consisted of approximately 20-gram samples processed individually at lower peak temperatures of 800 °C and 600 °C.

2.2.3.3. Cooling and sample recovery

To completely prevent internal structural fracturing or thermal shock induced by sudden temperature drops, the highly calcined specimens are allowed to undergo natural, unforced cooling entirely within the deactivated furnace cavity. Upon reaching ambient room temperature, the pure samples are meticulously recovered and immediately re-weighed to quantify the exact mass loss resulting from thermal vaporization. Detailed mathematical calculations revealed a massive weight reduction of approximately 34.9% for the 1000 °C sample. Correspondingly, the 800 °C sample yielded a final mass of 13.0463 grams, which equates to a calculated 34.76% mass dissipation. Similarly, the 600 °C sample recorded a post-calcination weight of 13.50 grams, accompanied by noticeably distinct visual color alterations that are strongly indicative of partial organic burnout stages.

2.2.3.4. Crushing and fine grinding (milling)

Following the completion of the extreme thermal extraction and cooling phases, the newly synthesized hydroxyapatite structures are occasionally observed to be slightly agglomerated into fragile clusters. To adequately prepare these pure inorganic samples for the subsequent high-resolution microstructural evaluations, a final, gentle mechanical crushing and fine milling step is cautiously executed. This final refinement strictly ensured that the ultimate bioceramic material maintained a highly uniform, ultra-fine particle distribution, which is an absolute prerequisite for accurate analytical characterizations.

2.2.4. Characterization

To rigorously evaluate the influence of varying calcination temperatures on the physicochemical and morphological properties of the isolated bioceramics, four distinct samples—treated at 1000 °C, 800 °C, 600 °C, and the untreated control (Sample B)—are subjected to a comprehensive analytical suite. In addition to XRD, DLS, and SEM analysis, FTIR spectroscopy is included to verify the chemical identity of the extracted HAp, detect phosphate and hydroxyl vibrational modes, and determine whether organic residues or carbonate-containing species remained after calcination.

2.2.4.1. X-ray diffraction (XRD) analysis

X-ray diffraction is used to identify crystalline phases and evaluate the crystallographic evolution of the extracted hydroxyapatite. The diffraction patterns are analyzed for characteristic HAp peaks and recognizable secondary phases, mainly phases of tricalcium phosphate. Measurements of crystallinity, apparent crystallite size, microstrain, and phase-purity proxies are compared for various calcination temperatures.

2.2.4.2. Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy is employed to examine the functional group composition of both the uncalcined milled bone (B) and the calcined samples at 600 °C (H3), 800 °C (H2) and 1000 °C (H1). The spectra obtained are plotted as transmittance versus wavenumber. What we observed is that the characteristic bands are examined in specific regions of interest for the different types of organic vibrations that include structural hydroxyl, phosphate, carbonate and those associated with collagen. The position of the peaks that we report here refers to the point of local minima of transmittance in each of the windows and the peaks are considered valid if the shape of the band and the neighbouring spectral features are in agreement with each other. Since the scanning stop at nearly

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600 cm⁻¹, the well-known phosphate bending peak at around 563 cm⁻¹ falls outside the range we are measuring.

2.2.4.3. *Scanning Electron Microscopy (SEM) and EDX*

The surface topography, grain morphology, and porosity of the HAp powders are scrutinized using Scanning Electron Microscopy (SEM). High-resolution SEM imaging allowed for a direct visual comparison of the particle size and shape between the natural and thermally treated groups. Simultaneously, Energy Dispersive X-ray (EDX) analysis is performed to determine the elemental composition, specifically focusing on the calcium-to-phosphorus (Ca/P) stoichiometric ratio, which is a critical indicator of the material's biological quality and purity.

3. RESULTS AND DISCUSSION

3.1.1. *Thermal decomposition and mass loss analysis*

The thermal transformation of bovine bone into hydroxyapatite is characterized by a significant reduction in mass, primarily due to the evaporation of moisture and the combustion of the organic collagenous matrix. In this section, the quantitative and qualitative changes in the samples are evaluated across three distinct calcination temperatures: 600 °C, 800 °C, and 1000 °C.

3.1.1.1. *Effect of calcination temperature on bone weight reduction*

The calcination process induced a substantial decrease in the total mass of the raw bone powder as the temperature increased from ambient levels to 1000 °C. This weight reduction is a direct indicator of the efficiency of the thermal extraction method in removing organic components, such as lipids and proteins, which constitute approximately one-third of the natural bone structure. It is observed that higher temperatures facilitate a more complete oxidation of carbonaceous residues, leading to a more purified inorganic mineral phase.

3.1.1.2. *Quantitative analysis of mass loss at 600 °C, 800 °C, and 1000 °C*

The experimental data revealed a clear correlation between the soaking temperature and the percentage of mass dissipation. For the sample treated at 1000 °C (H1), an initial mass of 80 grams is reduced to approximately 52.08 grams, representing a significant weight loss of 34.9%. This high percentage indicates the total decomposition of the collagen fibers at extreme temperatures. Similarly, the sample processed at 800 °C (H2) exhibited a mass reduction from 20 grams to 13.0463 grams, resulting in a calculated loss of 34.76%. In contrast, the 600 °C sample (H3) showed a final weight of 13.50 grams from a 20-gram starting mass. The slightly lower mass loss at 600 °C suggests that some residual carbon or organic traces may still be present within the mineral matrix compared to the 1000 °C batch.

Table 3 Quantitative mass loss data and percentage reduction for bovine bone samples.

Sample Code	Temperature (°C)	Initial Weight (g)	Final Weight (g)	Mass Loss (%)
H3	600 °C	20.00	13.50	32.50%
H2	800 °C	20.00	13.0463	34.76%
H1	1000 °C	80.00	52.08	34.90%

3.1.1.3. Physical observations and color transitions of processed samples

At first, natural bone powder had a yellowish-white color. After treatment at 600 °C the color changed showing the incompleteness of the combustion of residues of organic matter. At temperatures of 800 °C and 1000 °C the samples became chalkier white, suggesting more complete elimination of carbon-containing material. However, color cannot prove the purity of the phase or complete removal of organic matter hence color change is correlated with FTIR and XRD results. Furthermore, the sample calcined at 1000 °C looked more fragile than the sample treated at lower temperature.



Figure 2 Digital photograph showing the color transition of bone powder from 600 °C to 1000 °C.

3.1.2. X-ray diffraction (XRD) analysis

X-ray diffraction (XRD) is employed as the primary analytical tool to investigate the phase composition, crystallographic integrity, and microstructural evolution of the bovine-derived material. This technique allowed for a systematic evaluation of how varying thermal treatments influence the transition from biological apatite to purified synthetic hydroxyapatite.

3.1.2.1. XRD patterns of natural bone (HB) and thermally treated samples

Figures 3–6 show the X-ray diffraction patterns of the natural bone sample and of samples H3, H2, and H1 that underwent calcination. The pattern for the natural bone sample corresponded to its amorphous nature and the apatite-like phase of the material. That is, the strongest X-ray diffraction peak appeared at 31–32° 2θ due to the crystallographic orientation of the (211) planes. The structural development of the calcined samples, however, is not unambiguous: H2 at 800 °C achieved the highest values in terms of crystallinity and crystals' dimensionality while H1 specimen at 1000 °C is confirmed to possess the properties comparable with H3.

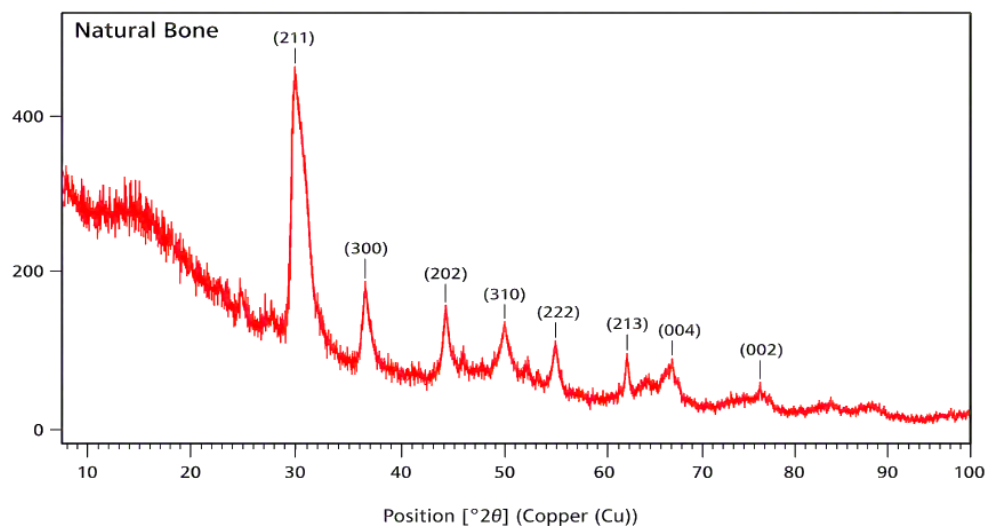


Figure 3 XRD Pattern for natural bone before heat treatment.

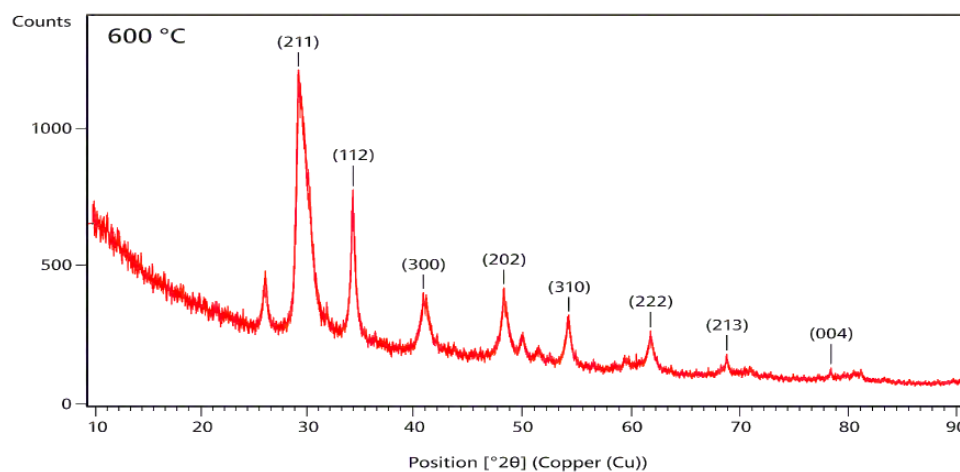


Figure 4 XRD pattern for sample H3 calcined at 600 °C.

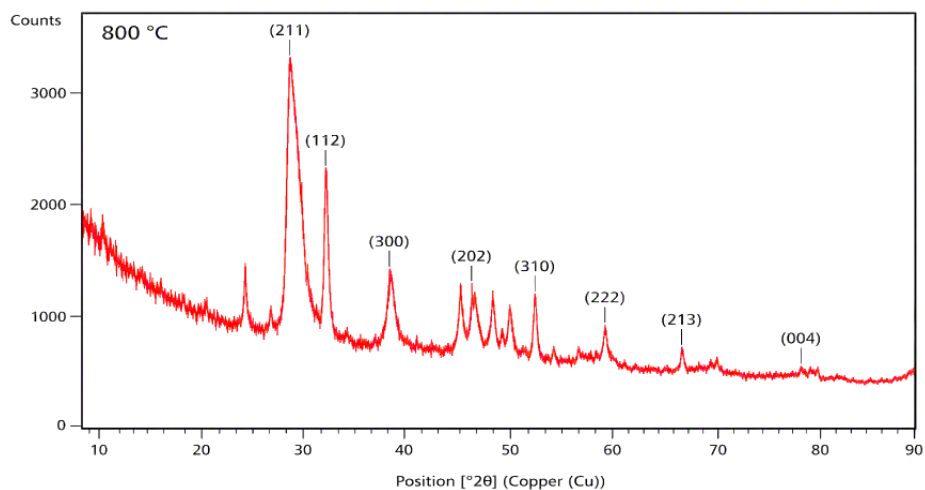


Figure 5 XRD pattern for sample H2 calcined at 800 °C.

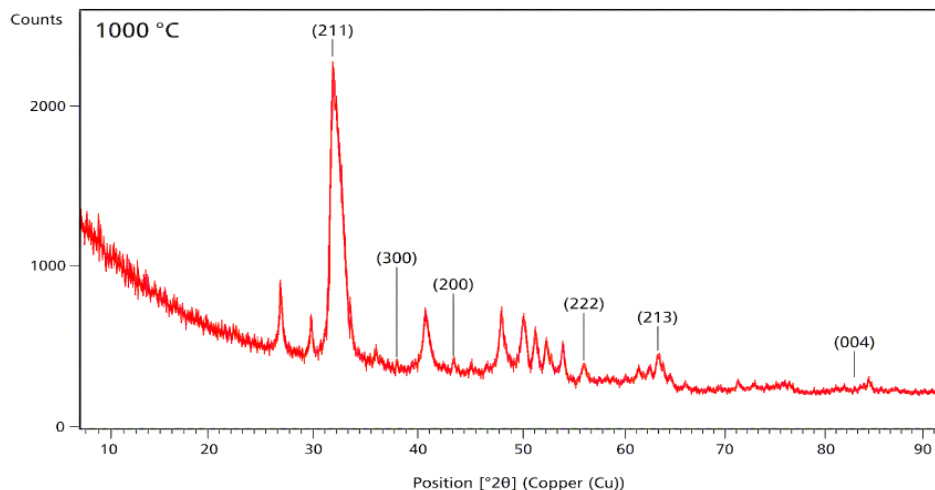


Figure 6 XRD pattern for sample H1 calcined at 1000 °C.

3.1.2.2. Assessment of phase purity and crystallinity index

The revised structural indicators in Table 4 indicate that the reaction to calcination temperature is not monotonic. H2 (800 °C) gave the most crystallinity proxy of 96.4%, while the values for H3 (600 °C) and H1 (1000 °C) are 92.7%. The phase-purity parameter behaved differently, giving values of 90.6% for both H3 and H1, while that for H2 is 88.2%. Thus, it can be said that the sample with the best apparent order in the crystal does not always give the maximum value of phase-purity parameter. Thus, the results of XRD indicate that conditions of 800 °C help to reach the maximum level of crystallinity while the phase purity parameter is slightly higher at 600 and 1000 °C.

3.1.2.3. Variation of crystallite size and lattice parameters with temperature

The values from Table 4 as well as the modified comparative curves made it clear that there is a clear peak evident in the evident crystallite size at 800 °C, with H2 being 100.09 nm in size and H3 and H1 being 19.91 nm each. The microstrain value is inversely proportional to the size values found, and thus H2 is found to have the lowest microstrain value of 2.207×10^{-3} , while H3 and H1 both had a microstrain value of 9.211×10^{-3} . Thus, it is spotted that the treatment at 800 °C materialized significant growth of a coherent domain and relaxation of the lattice, however, when the temperature is increased to 1000 °C this trend is not followed. Some modifications in certain values can mean that an alteration of peak broadening, overlapping reflections, thermal alteration, or even limitations of the method used are observed, thus the methodology of grain growth process should be validated by introducing repeat fitting of the full data.

Table 4 Summary of structural parameters derived from XRD analysis.

Sample	Calcination Temperature (°C)	Crystallite Size (nm)	Crystallinity Proxy (%)	Microstrain ($\epsilon \times 10^{-3}$)	Phase Purity Proxy (%)
H3	600 °C	19.91	92.7%	9.211	90.6%
H2	800 °C	100.09	96.4%	2.207	88.2%
H1	1000 °C	19.91	92.7%	9.211	90.6%

Figures 8, 9, and 10 provide additional visualization of the updated XRD parameters. Figure 8 shows that crystallite size sharply increases at 800 °C but decreases by 1000 °C. Figure 9 indicates that this is associated with a minimum microstrain for H2, indicating good lattice relaxation at intermediate temperature. Figure 10 shows that crystallinity and phase purity proxies are not the same: H2 has the highest crystallinity proxy, while H3 and H1 have higher phase purity proxy. Thus, this series of curves shows that no temperature gives maximum value for every parameter obtained from XRD measurements.

3.1.3. Fourier Transform Infrared Spectroscopy (FTIR) analysis

In terms of FTIR spectra, all samples showed clear proof of hydroxyapatite. H2 (800 °C) showed the strongest relative peak assignable to hydroxyapatite, with several absorptions: PO₄ v3 at 1028.0 cm⁻¹ and PO₄ v1 at 962.4 cm⁻¹, as well as the OH bond stretching at 3568.1 cm⁻¹. H3 (600 °C) showed the most prominent PO₄ v3 peak at 1031.9 cm⁻¹, although the remaining bands that appeared in the organic part are more pronounced. H1 (1000 °C) showed the cleanest organic part, because it is achieved due to more intense removal of lipids and collagen. However, H2 showed more intense PO₄ peaks than H1. Sample B that is not calcined still showed the clear Amide I peak at 1652.9 cm⁻¹, attributed to collagen. The presence of carbonate peaks between 1410 and 1485 cm⁻¹ and between 850 and 885 cm⁻¹ shows that carbonate insertion took place in biological apatite.

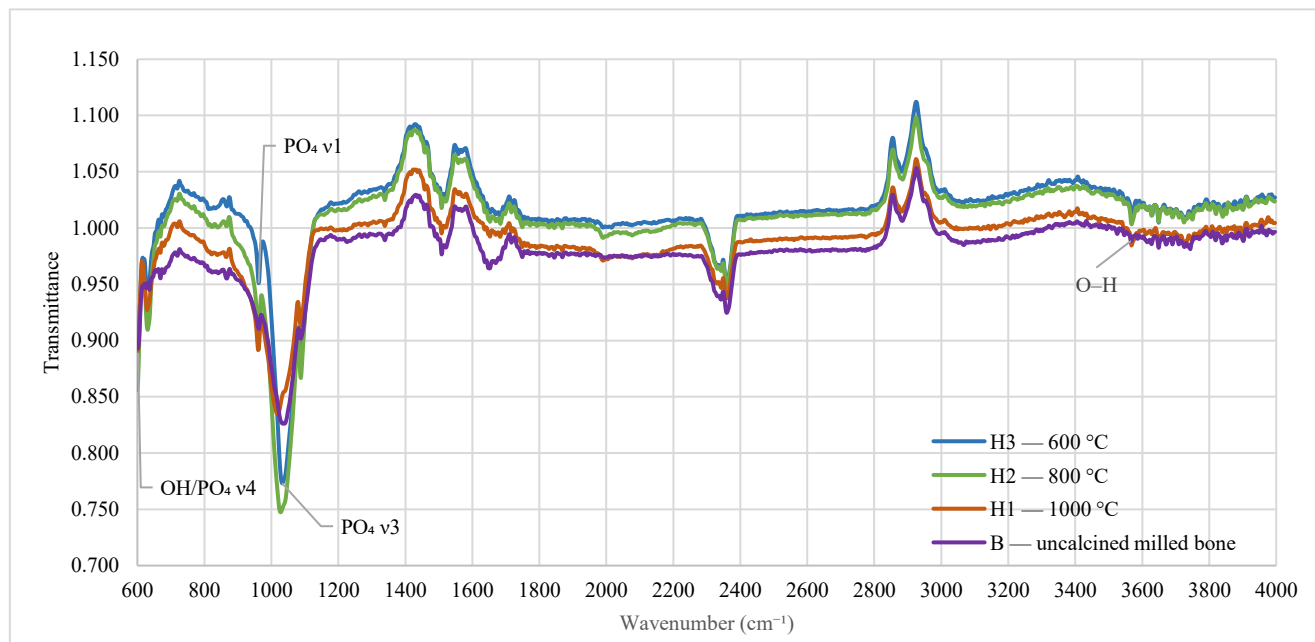


Figure 7 FTIR transmittance spectra of bone-derived samples.

Table 5 FTIR bond assignments and observed local-minimum wavenumbers for bovine bone-derived hydroxyapatite samples.

Band	Assignment	Class	Window (cm ⁻¹)	B	H1	H2	H3
OH stretch	Structural hydroxyl in HAp	HAp	3545–3590	3566.2	3568.1	3568.1	3568.1
C–H stretch	Lipid/protein aliphatic C–H	Organic	2900–2960	2900.8	2900.8	2958.7	2958.7
Amide I	Collagen peptide C=O stretch	Organic	1620–1680	1652.9	1652.9	1652.9	1670.3
Amide II	Collagen N–H bend/C–N stretch	Organic	1520–1580	1521.8	1521.8	1521.8	1521.8
CO ₃ v3	Carbonate substitution in biological apatite	Carbonated HAp	1410–1485	1483.2	1483.2	1483.2	1483.2
Amide III	Collagen C–N stretch/N–H bend	Organic	1200–1300	1226.7	1211.2	1211.2	1201.6
PO ₄ v3	Asymmetric phosphate stretch	HAp	1000–1100	1039.6	1018.4	1028.0	1031.9
PO ₄ v1	Symmetric phosphate stretch	HAp	945–975	962.4	960.5	962.4	962.4
CO ₃ v2	Carbonate out-of-plane bend	Carbonated HAp	850–885	866.0	883.4	883.4	883.4
OH/PO ₄ v4	OH libration and phosphate bending near scan limit	HAp	595–640	601.8	599.8	599.8	601.8

Note: Values for B–H3 are local-minimum wavenumbers identified within the stated windows. A local minimum supports a band assignment only when its profile and neighboring features are consistent. The scan ended near 600 cm⁻¹; therefore, the usual phosphate component near 563 cm⁻¹ is not measured.

3.1.3. Particle size analysis (DLS)

The Particle size distribution and colloidal stability of the synthesized hydroxyapatite are characterized using Dynamic Light Scattering (DLS). This technique measures the hydrodynamic diameter of the particles in suspension, providing critical information regarding the physical dimensions and the degree of agglomeration of the HAp nanoparticles.

3.1.3.1. Hydrodynamic diameter distribution of hap particles

The DLS analysis confirmed that the thermal extraction process successfully yielded hydroxyapatite in the nanometric range. Specifically, for the sample calcined at 1000 °C (H1), the effective hydrodynamic diameter is recorded at approximately 15 nm. This exceptionally small particle size is highly advantageous for biomedical applications, as it increases the specific surface area and enhances bioactivity. It is observed that while the XRD crystallite size indicates the dimensions of the crystalline domains, the DLS results reflect the actual size of the particles as they exist in a dispersed state, showing a well-controlled growth even at high temperatures.

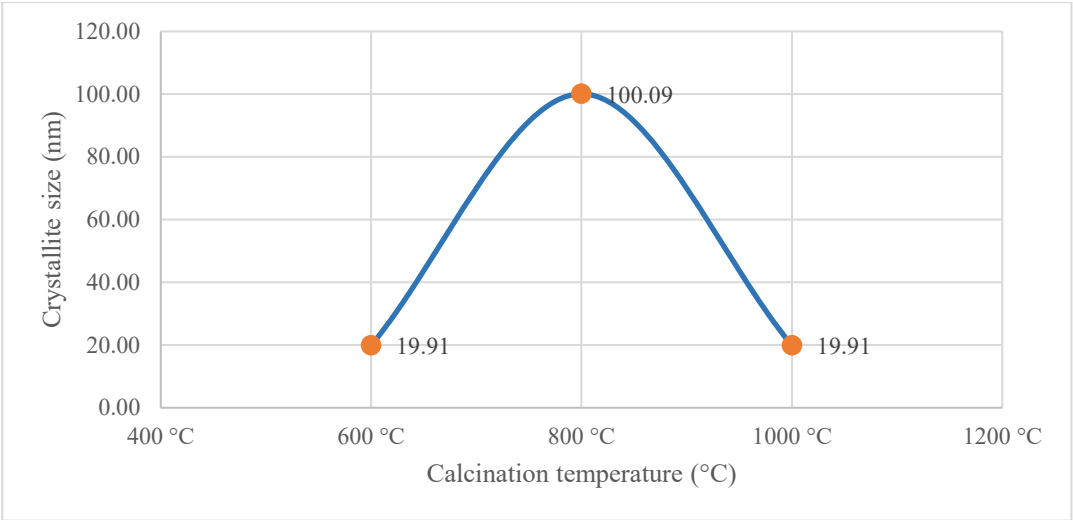


Figure 8 Crystallite size vs calcination temperature.

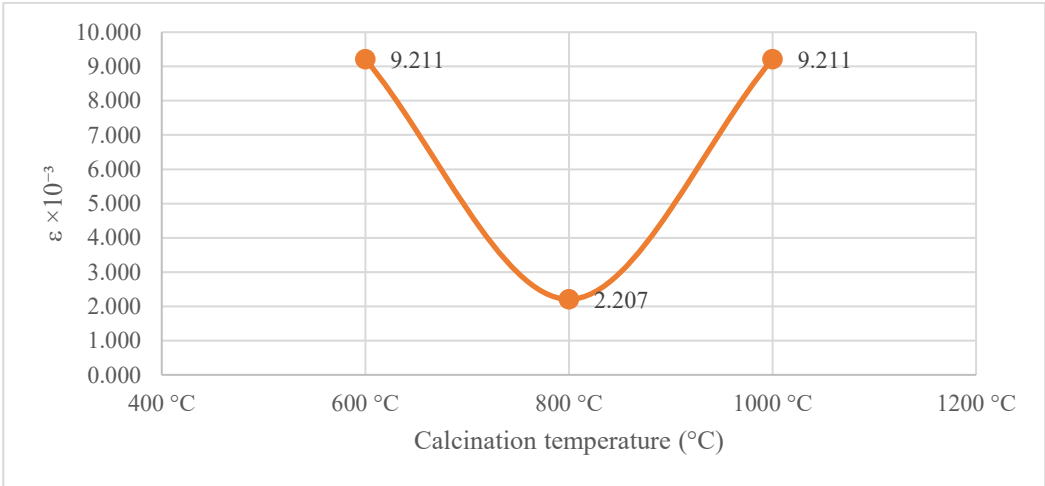


Figure 9 Microstrain vs calcination temperature.

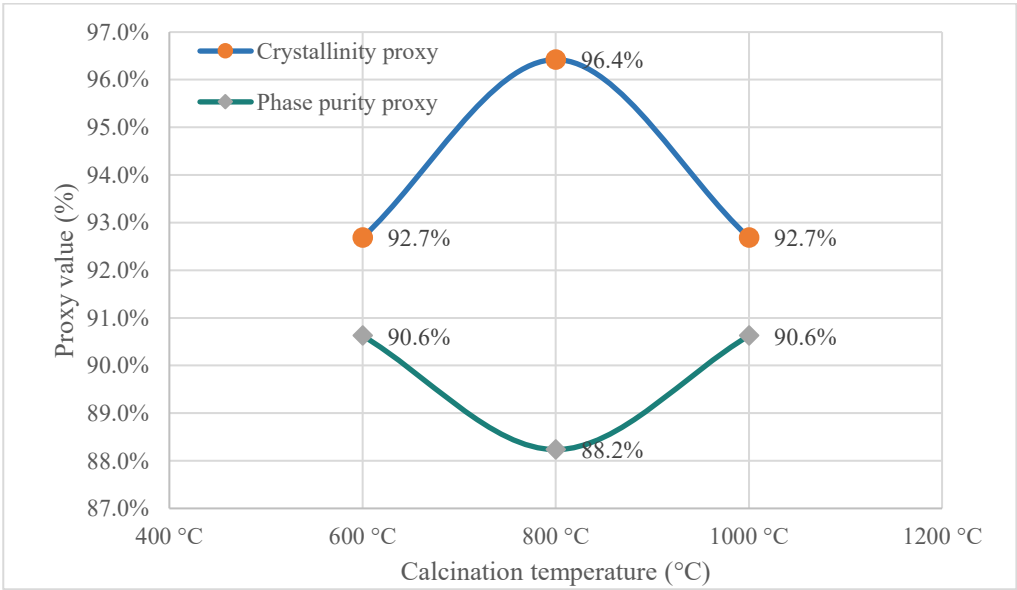


Figure 10 Crystallinity and phase purity vs calcination temperature.

3.1.3.2. Analysis of polydispersity index (pdi) and granular uniformity

The Polydispersity Index (PDI) is employed as a critical statistical parameter to assess the breadth of the particle size distribution and the degree of granular uniformity within the synthesized powders. According to the DLS data summarized in Table 6, a significant variation in PDI values is observed across the different samples, reflecting the impact of thermal treatment on particle stabilization. The natural bone sample (HB) exhibited the highest PDI of 0.634, indicating a very broad and non-uniform distribution characterized by significant particle aggregation. In contrast, the thermally treated samples H1 (1000 °C) and H3 (600 °C) displayed substantially lower PDI values of 0.289 and 0.278, respectively. These values, being below the 0.3 threshold, suggest a relatively narrow and monodisperse distribution, confirming that the combination of mechanical milling and specific calcination temperatures successfully produced a homogenous nanopowder with minimal agglomeration. Furthermore, while the H2 (800 °C) sample showed a broader distribution (PDI = 0.509) compared to H1 and H3, the overall results demonstrate that controlled thermal processing is essential for achieving the granular consistency required for predictable performance in clinical and industrial applications.

Table 6 DLS parameters for the synthesized HAp samples.

Sample	Effective diameter (nm)	Polydispersity	Mean diameter (nm)	Comment
HB	154.0	0.634	1164.4	Very broad, aggregated
H1	15.1	0.289	33.1	Small, relatively narrow
H2	44.6	0.509	91.7	Larger, broad distribution
H3	26.4	0.278	50.4	Intermediate, relatively narrow

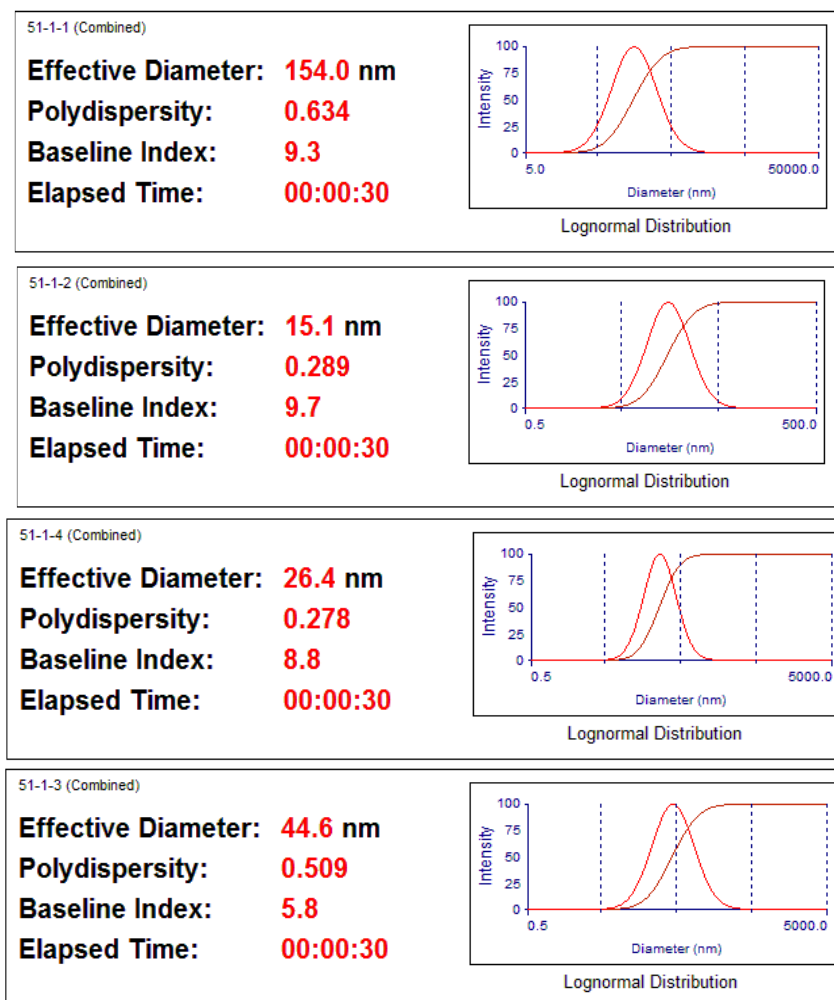


Figure 11 DLS particle-size analysis of (a) natural bone, (b) H3 at 600 °C, (c) H2 at 800 °C, and (d) H1 at 1000 °C.

3.1.4. Scanning electron microscopy (SEM) characterization

Scanning Electron Microscopy (SEM) is utilized to provide high-resolution topographical imaging of the natural and thermally treated bone samples. This morphological assessment is crucial for understanding the structural evolution of hydroxyapatite at the micro and nano-scales, as well as the impact of calcination on particle shape and connectivity.

3.1.4.1. Surface Morphology and Microstructural

Evolution The SEM micrographs revealed a significant transformation in the surface architecture of the samples as the organic matrix is progressively eliminated. The natural bone sample exhibited a dense, fibrous, and irregular surface dominated by the presence of collagenous networks and biological lipids. Upon thermal treatment at 600 °C (H3) and 800 °C (H2), the samples displayed a transition towards a more porous and granular structure, indicating the successful removal of organic matter. The particles in these stages appeared as relatively small, semi-spherical aggregates, reflecting the early stages of mineral consolidation and the exposure of the underlying inorganic framework. Formation at High Temperatures At the highest calcination temperature of 1000 °C (H1), the SEM analysis demonstrated a distinct evolution in particle morphology characterized by the formation of irregular, faceted nanoparticles. The thermal energy at 1000 °C promoted morphological consolidation and well-

defined particle boundaries. Unlike the lower-temperature samples, the H1 particles exhibited angular and faceted geometries that may provide anchoring sites for protein adsorption and mechanical interlocking when incorporated into polymer or ceramic matrices. These morphological observations are presented in Figure 12 and should be considered alongside the updated XRD result, which identifies H2 rather than H1 as the sample with the largest apparent crystallite size and lowest microstrain.

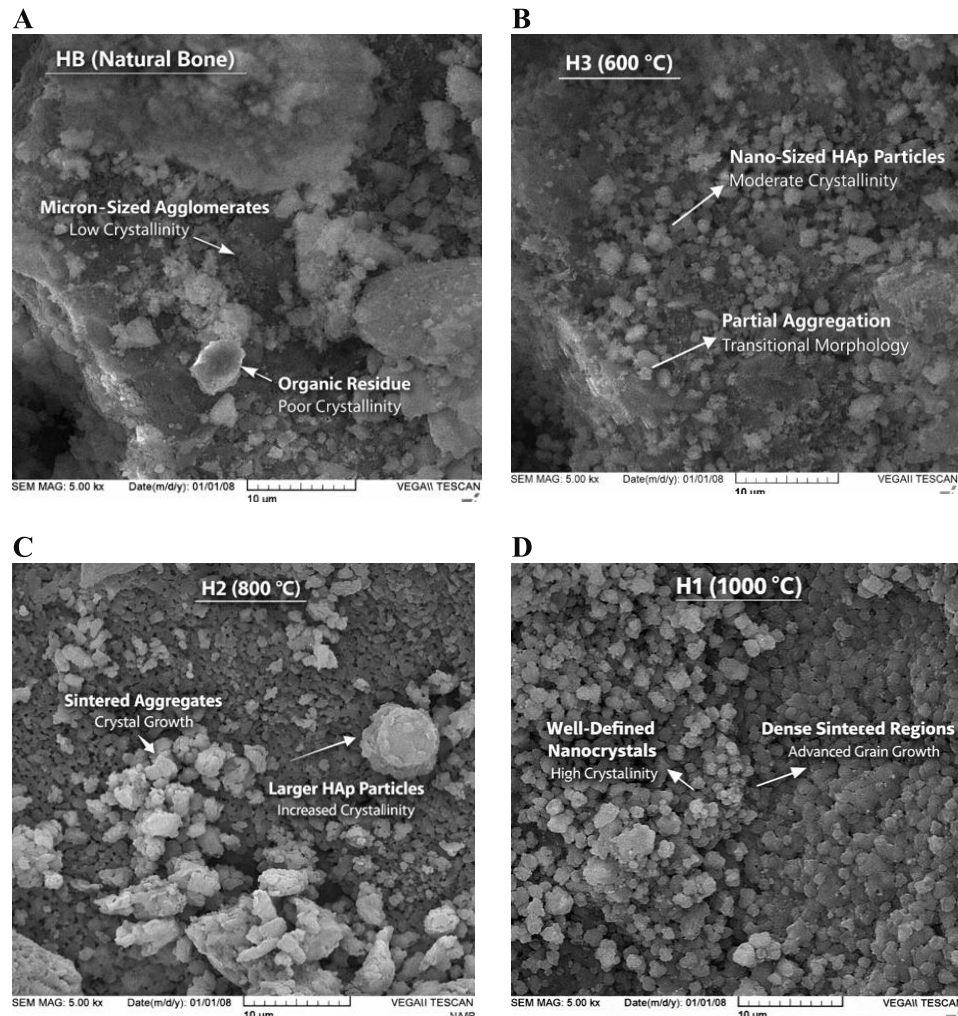


Figure 12 SEM micrographs showing the surface morphology of (a) natural bone, (b) H3 at 600 °C, (c) H2 at 800 °C, and (d) H1 at 1000 °C.

3.1.5. Thermal decomposition and mass loss analysis

The results of the current study indicated that the percentage of mass loss in bovine bone samples is directly proportional to the increase in calcination temperature, where the loss at 1000 °C is greater than at 800 °C and 600 °C. The mechanical basis for this result is attributed to the multi-stage thermal degradation of the bone matrix; initially, the loss involves the evaporation of absorbed water, followed by the complete oxidation of the organic phase, which consists of lipids and collagenous proteins. As the temperature rises, the combustion of carbonaceous residues leads to the transition of the bone ash from a black or greyish state to a pure white powder, signifying the total removal of organic components. This finding is consistent with previous thermal analyses showing that organic compounds are completely removed from the bovine matrix at temperatures above 600 °C, leaving the

inorganic HAp phase stable. Furthermore, these results agree with observations that while the organic component is eliminated at 600 °C, higher temperatures are necessary to reduce carbonate residues and achieve the structural stability observed in the 900 °C to 1000 °C range.

3.1.6. X-ray diffraction (XRD) and crystallographic evolution

The updated XRD analysis indicates a non-monotonic crystallographic response to calcination. H2 (800 °C) combined the largest apparent crystallite size (100.09 nm), highest crystallinity proxy (96.4%), and lowest microstrain (2.207×10^{-3}), indicating enhanced coherent-domain growth and lattice relaxation at the intermediate temperature. On the contrary, while H3 and H1 recorded the lowest crystallite size parameters and crystallinity proxy, they also had higher microstrain values than in H2, although the former's phase-purity proxy (90.6%) is higher than that of the latter (88.2%). The extra curves of Figures 8-10 reveal this discrepancy and justify the rejection of crystallinity, microstrain, crystallite size and phase-purity proxies as interchangeable parameters. The increase in visible crystallite sizes and the decrease in microstrain at 1000 °C can be caused by thermal reconstruction, overlapping of peaks or errors in peak calculation; therefore, repeat peak fitting or full pattern refinement is needed for improved interpretation. Overall, the XRD analysis shows that 800 °C is the most effective condition for lattice ordering, whereas 600 and 1000 °C show a little higher phase-purity proxy value.

3.2.3. FTIR functional-group evolution

FTIR results validate the findings of XRD by showing that the apatite structure remained unchanged in both unprocessed and calcined samples as the dominance of minerals and compounds changed with temperature. Strong PO_4 ν_3 , PO_4 ν_1 , OH, and OH/ PO_4 ν_4 vibrations of H2 show the clarity of the entire spectrum of hydroxyapatite after calcining at a temperature of 800 °C. The lowest PO_4 ν_3 peak in H3 shows that the removal of organic material at 600 °C is not as effective as in the case of H2. On the contrary, H1 has the cleanest spectrum in the organic region as the material is heated up to 1000 °C. But the intensity of the phosphate signal in H1 still proves that the temperature of calcination that is most effective for producing hydroxyapatite does not always give a clearly defined vibrational signal. This is an important factor to consider when choosing the way of processing samples, as both H2 and H1 show effective results. Also, the presence of the CO_3 ν_3 and CO_3 ν_2 bands suggests that CO_3 is incorporated into the structure of the biologically active apatite. These bands cannot be interpreted as the traces of organic matter left in the sample. Lastly, the analysis of the low-frequency phosphate bands is limited in this case due to the scanning limits that did not cover the region of 563 cm^{-1} .

H2 - 800 °C: The spectrum indicates clear structural O–H bond at 3568.1 cm^{-1} , strong PO_4 bond at 1028.0 cm^{-1} , the most intense PO_4 bond at 962.4 cm^{-1} , and a distinguishable OH/ PO_4 bond at 599.8 cm^{-1} . Organic matter present decreased compared to H3 and H4, while the carbonate bonds continued to be similar to the ones present in the apatite containing carbonate. Therefore, H2 displayed very clear and well-defined features of HAp making it to have the highest quality result in FTIR.

H3 - 600 °C: H3 featured the clearest peak of PO_4 bond at 1031.9 cm^{-1} , and also the bond at 962.4 cm^{-1} and the bond between OH and PO_4 at 601.8 cm^{-1} . But the presence of amide and C–H connections is way stronger suggesting the presence of more residual organic matter.

H1 — 1000 °C: Structural O–H at 3568.1 cm^{-1} and phosphate bands at 1018.4 , 960.5 , and 599.8 cm^{-1} confirmed the retention of HAp. H1 had the lowest prominence of collagen-related organic bands among the samples, giving the cleanest organic region, although its phosphate fingerprint is weaker than those of H2 and H3.

B — uncalcined: Phosphate bands at 1039.6, 962.4, and 601.8 cm^{-1} demonstrated the mineral HAp component of natural bone. The strongest Amide I/collagen signature and other organic bands are retained, while carbonate is expected as a component of biological apatite. Thus, B contained HAp but had the greatest organic residue and is the least pure HAp sample according to FTIR.

3.1.7. H2 has the clearest hydroxyapatite pattern, as it contains high, well-defined phosphate bands as well as hydroxyl structures, while also having low intensity of organic bands overall. Although H3 has the strongest individual PO_4 ν_3 absorption, H1 has the cleanest organic absorption area, while the detection of phosphate ions is weaker than in the previously analyzed samples. Of the uncalcined B sample, the largest amount of collagen-related matter is observed. Therefore, FTIR data support the obtained results; however, verification of phase purity and crystallinity must be performed by using XRD analysis and, if required, additional compositional studies.

3.1.8. Particle size distribution and polydispersity index (PDI)

According to the DLS results, thermally treated samples are found to have less hydrodynamic effective diameters than the bones that are not calcined. H1 had an effective diameter of 15.1 nm and a PDI of 0.289 and H3 had an effective diameter of 26.4 nm and a PDI of 0.278. These measurements reflect equivalent sizes on a diffusion basis while the results are affected by agglomeration, dispersion surroundings, and weighting system in DLS. Thus, the PDI of H1 and H3 being lower indicates narrower distributions than those of HB and H2 in the experiments.

3.1.9. Morphological characterization (SEM)

The SEM micrographs demonstrated that the surface morphology of the hydroxyapatite particles transformed from irregular, fibrous clusters in the natural bone (HB) to well-defined, faceted nanoparticles in the sample calcined at 1000 °C (H1). The underlying mechanism for this morphological evolution is the thermally driven coalescence and grain growth process; as the temperature increases, the high surface energy of the nanocrystals promotes the fusion of adjacent grains and the formation of distinct crystallographic facets. This process is facilitated by the total removal of the collagenous matrix, which otherwise acts as a barrier preventing the mineral crystals from achieving structural maturity. These results are consistent with reports that increasing calcination temperature leads to larger, more regular crystalline grains with clearer boundaries due to the sintering effect. Furthermore, the observation of faceted geometries in the H1 sample agrees with evidence that high-temperature treatment is important for developing stable angular particle shapes suitable for improving mechanical interlocking in composite scaffolds.

3.2. Limitations of the study

This study has several limitations that should be considered when interpreting the findings. First, the investigation is limited to bovine bone as a single natural precursor source, and variations related to animal age, bone type, anatomical region, and pretreatment history are not extensively evaluated. In addition, the calcination is only evaluated at three temperatures (600 °C, 800 °C and 1000 °C) with no intermediate temperatures or alternative soaking times being studied. Third, the indices of XRD crystallinity, crystallite-size, microstrain and phase-purity are indirect indicators of the results that could benefit from repeat peak fitting or full-pattern refinement especially because the result at 1000 °C does not follow a monotonous thermal pattern. Fourth, the FTIR analysis did not go further than around 600 cm^{-1} , meaning that it did not detect the standard phosphate peak appearing at the wavelength of approximately 563 cm^{-1} . Finally, cytotoxicity, antimicrobial, degradation, mechanical and scaffold formation tests are not performed.

4. CONCLUSIONS

1. The process known as controlled calcination was able to produce hydroxyapatite from cow bones, but the best temperature to use varies in relation to the specific expected outcome.
2. The amount of mass lost is dependent on temperature as well, since all organic collagen and carbon residue is eliminated during calcination.
3. The new XRD analysis results indicate that H2 sample (800 °C) had the largest crystallite size (100.09 nm), highest crystallinity index (96.4%) and lowest microstrain (2.207×10^{-3}), while H1 and H3 samples had the highest phase purity (90.6%).
4. According to the DLS analysis conducted on all samples and confirming the thermal treatment method used in this study, the nanoparticles produced had a very small hydrodynamic diameter (around 15 nm).
5. The results of FTIR analysis confirmed that all samples had characteristic phosphate and hydroxyl peaks with the H2 sample (800 °C) providing the best qualitative results overall, while H evidence with respect to its varnish properties was received from H1 sample (1000 °C), which allowed better removal of organic components. The formation of faceted nanoparticle morphologies at high temperatures provides a superior surface structure suitable for enhanced bioactivity and mechanical interlocking.

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