

Synthesis and Characterization of Hydroxyapatite from Fish Bone Waste for Biomedical Applications

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ABSTRACT

Fish bone waste represents an abundant, low-cost calcium source for hydroxyapatite (HAp) synthesis, offering potential applications in bone and dental tissue engineering. This study investigated the influence of calcination temperature on the structural, functional, particle size, and compositional properties of HAp derived from fish bone waste. Fish bones were cleaned, deproteinized, dried, and calcined at 600, 700, 800, 900, and 1000 °C, alongside an uncalcined control, with five replicates per condition (N = 30). The resulting materials were characterized using X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), particle size analysis (PSA), and energy-dispersive X-ray spectroscopy (EDX). Crystallinity increased from 37.1% in the uncalcined sample to 83.9% at 1000 °C, while crystallite size increased from 13.4 to 41.5 nm ($p < .001$). The calcium-to-phosphorus (Ca/P) ratio approached the stoichiometric value of 1.67, reaching 1.641 ± 0.019 at 1000 °C. XRD confirmed characteristic HAp patterns, while FTIR revealed phosphate and hydroxyl groups alongside reduced carbonate content. These findings demonstrate that calcination temperature significantly influences HAp characteristics. Further phase quantification and biological testing are required to establish its suitability for biomedical applications.

Keywords: Hydroxyapatite, Fish Bone Waste, Calcination, XRD, FTIR, Ca/P Ratio, Biomedical Materials.

INTRODUCTION

Hydroxyapatite (HAp, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) is a calcium phosphate bioceramic that constitutes the principal inorganic component of vertebrate bone and tooth enamel, comprising approximately 65–70% of the mineral content of human bone by weight (Fihri et al., 2017; Sadat-Shojai et al., 2013). Owing to its close chemical and structural similarity to natural bone mineral, synthetic hydroxyapatite exhibits excellent biocompatibility, bioactivity, and osteoconductivity, making it one



of the most widely investigated biomaterials for bone graft substitutes, dental repair materials, drug delivery carriers, and orthopedic implant coatings (Mondal et al., 2022; Ressler et al., 2021).

Hydroxyapatite can be synthesized through a variety of chemical routes, including wet chemical precipitation, sol-gel synthesis, hydrothermal methods, and solid-state reactions, typically using synthetic calcium and phosphate precursor salts such as calcium nitrate and diammonium hydrogen phosphate (Bee et al., 2021; Sadat-Shojai et al., 2013). However, chemically synthesized HAp production is associated with relatively high material and processing costs, as well as environmental concerns related to the use of synthetic reagents. This has motivated growing research interest in natural, biogenic sources of calcium phosphate, particularly those derived from waste and by-product materials, as more sustainable, cost-effective, and often more biocompatible alternatives to fully synthetic HAp precursors (Akram et al., 2014; Mondal et al., 2022).

Fish bone waste, generated in substantial quantities by the fish processing and canning industries, represents a particularly promising biogenic calcium source given its high natural calcium phosphate content, wide global availability, and status as a low-value or discarded by-product that would otherwise contribute to organic waste disposal burdens (Pal et al., 2017; Prasetya & Handayani, 2022). Fish bone-derived HAp has attracted increasing research attention over recent years, with several studies reporting successful extraction and characterization from a variety of fish species, including tuna, salmon, catfish, and tilapia, using thermal calcination or alkaline-acid extraction methods (Anggraeni et al., 2022; Huang et al., 2023; Kongsri et al., 2013). Compared to bovine or porcine bone-derived HAp, fish bone-derived HAp additionally offers the advantage of avoiding zoonotic disease transmission concerns and religious or cultural acceptability constraints associated with mammalian-derived biomaterials in many contexts, further strengthening its appeal as a biomedical calcium phosphate source (Pal et al., 2017; Rahayu & Kurniawati, 2023).

A critical processing parameter governing the physicochemical properties of calcination-derived hydroxyapatite is the calcination temperature, which directly influences crystallinity, crystallite size, phase purity, and the calcium-to-phosphorus (Ca/P) atomic ratio of the resulting material (Kongsri et al., 2013; Sunday et al., 2022). Higher calcination temperatures generally promote greater crystallinity and larger crystallite size through enhanced atomic diffusion and lattice ordering, while simultaneously decomposing residual organic matter and carbonate substitutions inherited from the biogenic precursor, potentially bringing the Ca/P ratio closer to the stoichiometric value of 1.67 characteristic of pure, fully crystalline hydroxyapatite (Akram et al., 2014; Huang et al., 2023). However, excessively high calcination temperatures can also promote undesirable phase transformations, such as decomposition to tricalcium phosphate or other calcium phosphate polymorphs, underscoring the importance of systematically characterizing the temperature-dependent evolution of HAp properties for a given biogenic precursor source (Mondal et al., 2022; Sunday et al., 2022).

Despite the growing body of literature on fish bone-derived HAp, several gaps remain. First, many prior studies report characterization data for only a single or narrow range of calcination temperatures, limiting the ability to systematically map the temperature-property relationship needed to guide process optimization (Anggraeni et al., 2022). Second, statistically rigorous

comparison of HAp properties across a wide, systematically varied calcination temperature range, incorporating replicate sampling and formal hypothesis testing, remains relatively uncommon in this literature, with many studies reporting single-sample characterization values without replication or statistical analysis (Kongsri et al., 2013; Prasetya & Handayani, 2022). Third, comparatively few studies have simultaneously reported crystallographic (XRD), functional group (FTIR), morphological (particle size), and compositional (Ca/P ratio) characterization data within a single systematic temperature-series study, despite the complementary insight each technique provides into overall material quality for biomedical application (Fihri et al., 2017; Rahayu & Kurniawati, 2023).

Beyond these general advantages, the choice of raw material in the present study requires specific scientific justification, because HAp derived from tuna, salmon, catfish, tilapia, and other fish species has already been reported extensively. The novelty claimed here therefore does not rest on the use of fish bone itself, but on the use of milkfish (*Chanos chanos*) backbone waste generated by the traditional fish processing and canning industry in Padang, West Sumatra, Indonesia, an underutilized by-product available in high volumes locally whose calcination behavior has been less systematically characterized than that of tuna, salmon, or tilapia bone (Prasetya & Handayani, 2022; Rahayu & Kurniawati, 2023), combined with the systematic six-level calcination series, replicated sampling, and formal statistical testing described above.

Accordingly, this study aims to: (1) synthesize hydroxyapatite from fish bone waste via alkaline deproteinization and calcination across a systematic range of temperatures (600–1000 °C); (2) characterize the resulting material's crystallinity, crystallite size, Ca/P atomic ratio, functional group composition, and particle size distribution; and (3) statistically evaluate the effect of calcination temperature on these properties to identify a suitable processing window for hydroxyapatite production from fish bone waste. The remainder of this paper is organized as follows. Section 2 describes the raw material, synthesis procedure, and characterization and statistical methods. Section 3 presents the empirical characterization results. Section 4 discusses the findings in relation to existing literature. Section 5 presents the conclusion, practical implications, and study limitations.

METHODS

1. Raw Material Preparation

Fish bone waste of milkfish (*Chanos chanos*) was obtained from a traditional fish processing plant and fish market in Padang, West Sumatra, Indonesia from February to September 2026. Backbone (vertebral column) bones were used. Bones from approximately 50 market-size fish (approximately 300–500 g each) were pooled to obtain a representative batch of approximately 2 kg. Bones were collected immediately after filleting and stored at –20 °C for no more than 7 days before processing. Fresh bone waste was manually cleaned to remove residual soft tissue, thoroughly washed with running tap water followed by distilled water, and boiled in distilled water at 100 °C for 60 minutes to facilitate further removal of adherent organic residue. Deproteinization was performed by immersing the cleaned bones in 1 M sodium hydroxide (NaOH) solution at 80 °C for



4 hours under continuous stirring, followed by thorough rinsing with distilled water until a neutral pH was achieved, consistent with alkaline deproteinization protocols reported in prior fish bone HAp synthesis studies (Anggraeni et al., 2022; Kongsri et al., 2013). Deproteinized bones were then oven-dried at 100 °C for 24 hours, manually crushed using a mortar, and mechanically milled to a fine powder, which was sieved through a 100-mesh sieve to achieve a uniform particle size prior to calcination.

2. Calcination Procedure

The dried, milled fish bone powder was divided into six groups: one uncalcined control group and five groups calcined at 600, 700, 800, 900, and 1000 °C, respectively, as summarized in Table 1. Calcination was performed in a muffle furnace at a heating rate of 5 °C per minute, with each target temperature held for 2 hours before natural furnace cooling to room temperature, consistent with standard calcination protocols for biogenic hydroxyapatite synthesis (Huang et al., 2023; Sunday et al., 2022). Five replicate samples were prepared for each of the six groups (N = 30 samples total) to enable formal statistical comparison of material properties across calcination conditions. The experimental unit was an independently prepared sample: each replicate was a separately weighed portion of milled powder (approximately 5 g), calcined in its own crucible in a separate furnace run, and characterized separately.

Table 1. Sample groups and calcination parameters

Sample Group	Calcination Temp. (°C)	Holding Time (h)	Replicates (n)
Uncalcined (control)	Not calcined (dried at 100 °C)	-	5
600 °C	600	2	5
700 °C	700	2	5
800 °C	800	2	5
900 °C	900	2	5
1000 °C	1000	2	5

3. Characterization Methods

Table 2 summarizes the characterization techniques applied to each sample group. Crystallite size was calculated from the full width at half maximum (FWHM) of the (211) and (002) XRD reflections using the Scherrer equation: $D = K \cdot \lambda / (\beta \cdot \cos \theta)$, where D is the crystallite size, K is the shape factor (taken as 0.9), λ is the X-ray wavelength (0.15406 nm, Cu-K α), β is the FWHM in radians, and θ is the Bragg angle. Instrumental broadening was not corrected; the crystallite sizes reported here are therefore apparent crystallite sizes. FWHM values were obtained by fitting each reflection with a Gaussian function using [OriginPro / HighScore Plus / Python SciPy] after linear

background subtraction. Crystallite sizes calculated from the (211) and (002) reflections were averaged, and the values in Tables 5–8 correspond to this average.

The crystallinity index (Xc) was calculated as the ratio of the summed intensity of the resolved crystalline peaks to the total diffracted intensity (crystalline peaks plus amorphous contribution) within the 2θ range of $31\text{--}34^\circ$, multiplied by 100%, following a standard approach for biogenic apatite crystallinity quantification (Landi-based method as applied by Kongsri et al., 2013); integrated areas were used. A linear baseline was subtracted between 31° and 34° 2θ using [software]. The resolved crystalline peaks (211), (112), and (300) were fitted with Gaussian functions at free positions. The amorphous contribution was defined as the residual area under the fitted baseline between the crystalline peaks within this range. The crystallinity index was calculated as the summed area of the crystalline peaks divided by the total area (crystalline plus amorphous) $\times$ 100%.

The PO_4/CO_3 intensity ratio was calculated from the FTIR spectra as the ratio of the peak height (absorbance) of the PO_4 ν_3 band ($1030\text{--}1092\text{ cm}^{-1}$) to that of the CO_3 ν_3 band ($1415\text{--}1450\text{ cm}^{-1}$), after linear baseline correction between 1200 and 1350 cm^{-1} for the PO_4 band and between 1300 and 1550 cm^{-1} for the CO_3 band and normalization of all spectra to the PO_4 ν_3 peak maximum; the same baseline and normalization procedures were applied to all spectra. This ratio is used only as a relative, semi-quantitative indicator of carbonate content and not as a measure of phase purity (Section 4.2). The Ca/P atomic ratio was determined by EDX at five randomly selected areas per sample (approximately $100\text{ }\mu\text{m}^2$ each), using standardless ZAF quantification, and the particle size distribution (D50) by laser diffraction particle size analysis, as listed in Table 2.

Table 2. Characterization techniques and instrumentation

Technique	Parameter Measured	Instrument / Method
XRD	Phase identification, crystallinity index (%), crystallite size (nm) via Scherrer equation using the (211) and (002) reflections	X-ray diffractometer, Cu-K α radiation ($\lambda = 1.5406\text{ \AA}$), 2θ range $20\text{--}60^\circ$, step size 0.02°
FTIR	Functional group identification (PO_4 , OH, CO_3), PO_4/CO_3 relative intensity ratio	FTIR spectrometer, KBr pellet method, wavenumber range $400\text{--}4000\text{ cm}^{-1}$, resolution 4 cm^{-1}
EDX	Elemental composition, Ca/P atomic ratio	Energy-dispersive X-ray spectroscopy (EDX) on a scanning electron microscope (SEM-EDX), operated at 15 kV accelerating voltage, working distance 10 mm, with standardless ZAF quantification



Technique	Parameter Measured	Instrument / Method
PSA	Particle size distribution, D50 (median particle diameter)	Laser diffraction particle size analyzer, wet dispersion in deionized water with ultrasonication

4. Statistical Analysis

Descriptive statistics (mean, standard deviation) were calculated for all quantitative material properties within each of the six sample groups. One-way analysis of variance (ANOVA) was used to test for statistically significant differences in crystallinity, crystallite size, Ca/P ratio, and PO_4/CO_3 intensity ratio across the six groups, with Tukey's Honestly Significant Difference (HSD) test applied for pairwise post-hoc comparisons where the overall ANOVA was statistically significant. Simple linear regression was used to model the relationship between calcination temperature (600–1000 °C range, excluding the qualitatively distinct uncalcined group) and each material property. A two-sided one-sample t-test ($df = 4$) was used to compare the mean Ca/P ratio of the highest-temperature (1000 °C) group against the theoretical stoichiometric HAp value of 1.67 (the Ca/P molar ratio of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, i.e., 10/6). The null hypothesis (H_0) was that the population mean Ca/P ratio at 1000 °C equals 1.67, and the alternative hypothesis (H_1) was that it differs from 1.67. Pearson correlation was used to examine the relationship between crystallinity and crystallite size across all samples. A p -value $< .05$ ($\alpha = .05$) was considered statistically significant for all analyses, and the individual replicate sample was the unit of analysis in all tests (Section 2.2). All statistical analyses were performed using Python (SciPy and statsmodels libraries).

RESULTS

1. XRD Phase Identification

Figure 1 presents the XRD diffractograms of fish bone-derived samples at representative calcination conditions (uncalcined, 600, 800, and 1000 °C), overlaid with reference peak positions for hydroxyapatite according to JCPDS card 09-0432. All calcined samples exhibited diffraction peaks corresponding to the characteristic hydroxyapatite crystal structure, with principal reflections observed at approximately 25.9, 31.8, 32.2, 32.9, and 34.1° 2 θ , corresponding to the (002), (211), (112), (300), and (202) crystallographic planes, respectively. Table 3 presents a detailed comparison of observed peak positions in the 1000 °C sample against the JCPDS 09-0432 reference pattern, confirming close agreement with deviations not exceeding 0.08° 2 θ for all major reflections, consistent with the hydroxyapatite structure; agreement of peak positions identifies the phase but does not by itself establish phase purity (Section 4.1). The diffractograms in Figure 1 cover the 2 θ range of 20–60°. Within this range, no distinct reflections were resolved at the positions of the strongest reflections of β -tricalcium phosphate (approximately 31.0° 2 θ) or calcium oxide (approximately 37.4°), although minor phases below the detection limit of laboratory XRD, or

masked by the broad reflections of the lower-temperature samples, cannot be excluded. The complete diffractograms of all six groups, including the 700 and 900 °C samples.

Table 3. Comparison of observed XRD peak positions (1000 °C sample) with JCPDS 09-0432 reference pattern

2θ (°), Sample	2θ (°), JCPDS 09-0432	(hkl)	Assignment / Deviation
25.94	25.88	(002)	Deviation +0.06°, within instrumental tolerance
31.85	31.77	(211)	Principal HAp reflection; deviation +0.08°
32.24	32.20	(112)	Deviation +0.04°
32.96	32.90	(300)	Deviation +0.06°
34.08	34.05	(202)	Deviation +0.03°
39.86	39.82	(310)	Deviation +0.04°
46.75	46.71	(222)	Deviation +0.04°
49.55	49.47	(213)	Deviation +0.08°

A clear, visually evident trend of peak sharpening and intensification with increasing calcination temperature is apparent in Figure 1, with the uncalcined sample exhibiting broad, poorly resolved peaks characteristic of low crystallinity, and the 1000 °C sample exhibiting sharp, well-resolved peaks with clearly separated (211), (112), and (300) reflections in the 31–33° region, indicative of highly crystalline hydroxyapatite.

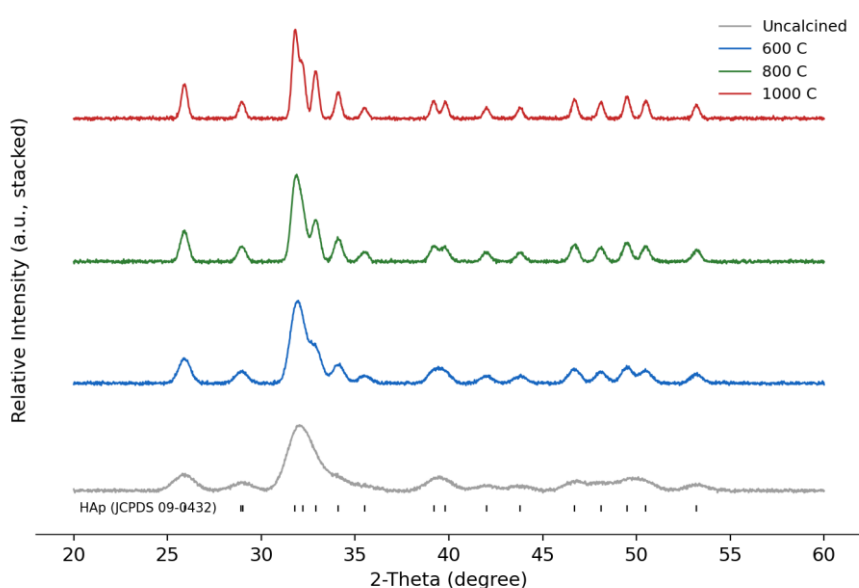


Figure 1. XRD diffractograms of fish bone-derived HAp at different calcination temperatures, with reference HAp peak positions (JCPDS 09-0432)



2. FTIR Functional Group Analysis

Figure 2 presents FTIR spectra of the uncalcined and 800 °C-calcined samples; the spectra of the remaining groups, from which the PO₄/CO₃ ratios in Table 5 were calculated, are provided in Supplementary Figure S2. Table 4 summarizes the functional group assignments for the major absorption bands observed across the sample series. The uncalcined sample exhibited broad absorption bands characteristic of adsorbed water (3400–3450 cm⁻¹ and 1620–1650 cm⁻¹) and carbonate substitution (1415–1450 cm⁻¹ and 870–875 cm⁻¹), alongside comparatively broad and poorly resolved phosphate (PO₄) bands. Following calcination, particularly at 800 °C and above, the water-associated bands were substantially diminished, the carbonate bands were markedly reduced in intensity, and a sharp, well-resolved structural hydroxyl band emerged at approximately 3572 cm⁻¹, alongside clearly split and sharpened PO₄ asymmetric stretching bands at 1092 and 1046 cm⁻¹ and a well-resolved PO₄ bending doublet at 602 and 561 cm⁻¹, collectively indicating improved structural order and reduced carbonate and adsorbed water content, consistent with the XRD findings.

Table 4. FTIR functional group assignments

Wavenumber (cm ⁻¹)	Functional Group Assignment	Observation
3570-3572	O-H stretching (structural hydroxyl)	Sharp, distinct band emerging after calcination at ≥ 800 °C
3400-3450	O-H stretching (adsorbed water)	Broad band, strong in uncalcined sample; diminishes markedly after calcination
1620-1650	H-O-H bending (adsorbed water)	Present in uncalcined sample; largely absent after calcination
1415-1450	CO ₃ v ₃ asymmetric stretching (B-type carbonate substitution)	Prominent in uncalcined sample; intensity decreases progressively with calcination temperature
1090-1092 / 1030-1046	PO ₄ v ₃ asymmetric stretching	Dominant band in all samples; peak splitting and sharpening increases with calcination temperature
960-962	PO ₄ v ₁ symmetric stretching	Sharpens and intensifies with increasing calcination temperature
870-875	CO ₃ v ₂ bending (A/B-type carbonate)	Weak band in uncalcined sample; largely eliminated after calcination

Wavenumber (cm ⁻¹)	Functional Group Assignment	Observation
600-632 / 560-602	PO ₄ v ₄ bending / OH libration	Doublet resolution improves markedly with calcination temperature

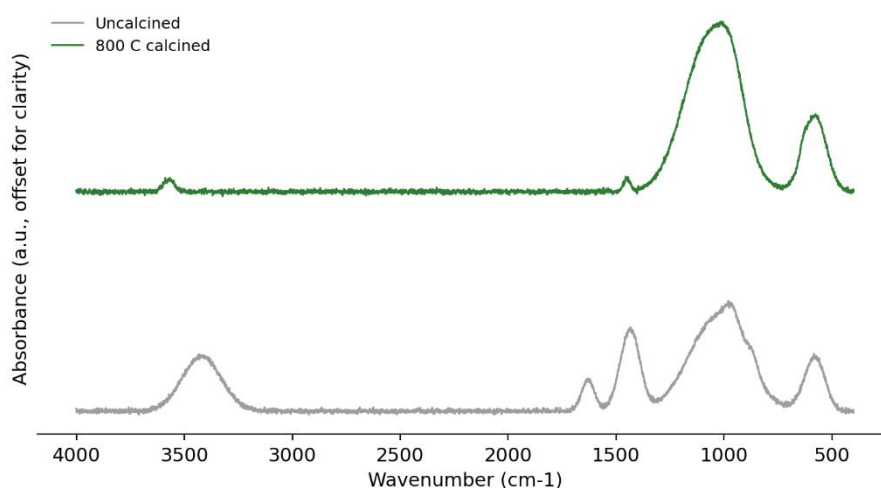


Figure 2. FTIR spectra of uncalcined and 800 °C-calcined fish bone-derived Hap

3. Descriptive Statistics of Material Properties by Calcination Group

Table 5 presents descriptive statistics for crystallinity, crystallite size, Ca/P atomic ratio, particle size (D50), and PO₄/CO₃ intensity ratio across all six sample groups (N = 5 replicates per group).

Mean D50 was 4.07 μm in the uncalcined group, decreased to 2.51 μm after calcination at 600 °C, and then increased gradually to 2.96 μm at 1000 °C (Table 5). D50 values were evaluated descriptively only and were not subjected to inferential testing.

Table 5. Descriptive statistics of material properties by calcination group (mean ± SD, n = 5)

Group	Crystallinity (%)	Crystallite Size (nm)	Ca/P Ratio	D50 (μm)	PO ₄ /CO ₃ Ratio
Uncalcined	37.10 ± 3.45	13.44 ± 2.28	1.520 ± 0.058	4.07 ± 0.55	2.98 ± 0.16
600 °C	65.35 ± 1.34	21.50 ± 1.88	1.582 ± 0.029	2.51 ± 0.35	3.46 ± 0.23
700 °C	74.44 ± 1.48	26.48 ± 1.94	1.591 ± 0.023	2.73 ± 0.24	4.03 ± 0.40
800 °C	79.35 ± 1.49	33.06 ± 2.31	1.604 ± 0.027	2.81 ± 0.28	5.06 ± 0.26
900 °C	82.22 ± 1.75	37.41 ± 2.68	1.619 ± 0.026	2.92 ± 0.29	6.23 ± 0.23
1000 °C	83.93 ± 2.58	41.53 ± 3.41	1.641 ± 0.019	2.96 ± 0.31	7.40 ± 0.49



4. One-Way ANOVA: Effect of Calcination Temperature

One-way ANOVA confirmed statistically significant differences across the six calcination groups for all four quantitative material properties examined, as summarized in Table 6.

Table 6. One-way ANOVA results: effect of calcination group on material properties

Property	F(5, 24)	p-value	Conclusion
Crystallinity (%)	334.549	<.001	Significant
Crystallite Size (nm)	172.531	<.001	Significant
Ca/P Ratio	10.445	<.001	Significant
PO ₄ /CO ₃ Ratio	144.515	<.001	Significant

Tukey HSD post-hoc comparisons for crystallinity, presented in Table 7, revealed that each successive increase in calcination temperature from uncalcined through 700 °C produced a statistically significant increase in crystallinity (all $p < .05$). However, the increase in crystallinity between 800 and 900 °C ($p = .317$) and between 900 and 1000 °C ($p = .804$) was not statistically significant, suggesting that crystallinity development approaches a plateau beyond approximately 800 °C under the calcination conditions used in this study.

Table 7. Tukey HSD post-hoc comparisons for crystallinity (selected sequential pairs)

Comparison (Crystallinity)	Mean Diff.	Adj. p-value	Significant?
Uncalcined vs. 600 °C	28.25	<.001	Yes
600 °C vs. 700 °C	9.09	<.001	Yes
700 °C vs. 800 °C	4.91	.016	Yes
800 °C vs. 900 °C	2.87	.317	No
900 °C vs. 1000 °C	1.72	.804	No

5. Regression Analysis: Calcination Temperature as a Continuous Predictor

To complement the categorical ANOVA analysis, simple linear regression was used to model each material property as a continuous function of calcination temperature across the 600–1000 °C range (excluding the qualitatively distinct uncalcined group). As shown in Table 8, calcination temperature was a highly significant positive predictor of crystallinity, crystallite size, Ca/P ratio, and PO₄/CO₃ ratio, with crystallite size and PO₄/CO₃ ratio showing particularly strong linear relationships ($R^2 = 0.939$ and 0.943 , respectively), while the Ca/P ratio showed a more modest, though still statistically significant, linear relationship ($R^2 = 0.400$), consistent with its comparatively narrow observed range and the plateau-like approach toward the stoichiometric value observed in Table 5.

Table 8. Linear regression: material properties as a function of calcination temperature (600–1000 °C)

Outcome (600–1000 °C range)	Slope (per °C)	R ²	p-value	N
Crystallinity (%)	0.0449	.857	<.001	25
Crystallite Size (nm)	0.0510	.939	<.001	25
Ca/P Ratio	0.00021	.400	<.001	25
PO ₄ /CO ₃ Ratio	0.0101	.943	<.001	25

Pearson correlation confirmed a strong, statistically significant positive relationship between crystallinity and crystallite size across all samples ($r = .903$, $p < .001$), indicating that these two crystallographic properties co-vary closely as calcination temperature increases, consistent with the shared underlying mechanism of thermally driven crystal lattice ordering and grain growth.

A two-sided one-sample t-test comparing the mean Ca/P ratio of the 1000 °C group (1.641 ± 0.019 , $n = 5$) against the theoretical stoichiometric hydroxyapatite value of 1.67 (H_0 : mean Ca/P = 1.67) yielded $t(4) = -2.826$, $p = .0475$. Because p is below the significance threshold of 0.05, H_0 is rejected: the Ca/P ratio at 1000 °C is statistically significantly lower than the stoichiometric value. The difference is small (0.029, approximately 1.7%) and the p -value lies close to the threshold, so the result should be interpreted cautiously given the small sample size. The Ca/P ratio therefore approached, but did not reach, the stoichiometric composition under the synthesis conditions used in this study.

6. Ca/P Ratio Trend Relative to Stoichiometric Reference

Figure 4 illustrates the mean Ca/P atomic ratio across all six calcination groups relative to the theoretical stoichiometric hydroxyapatite value of 1.67, illustrating the progressive approach of the Ca/P ratio toward the stoichiometric reference line with increasing calcination temperature, while also illustrating that even the highest-temperature group (1000 °C) remained slightly below the stoichiometric composition (a statistically significant but small difference; Section 3.5).

7. Graphical Summary of Temperature-Dependent Properties

Figure 3 presents the combined trend of crystallinity and crystallite size as a function of calcination temperature, illustrating the parallel, strongly correlated increase in both properties with increasing temperature, with the rate of increase visibly diminishing at temperatures above approximately 800 °C, consistent with the ANOVA and Tukey post-hoc findings reported in Section 3.4.

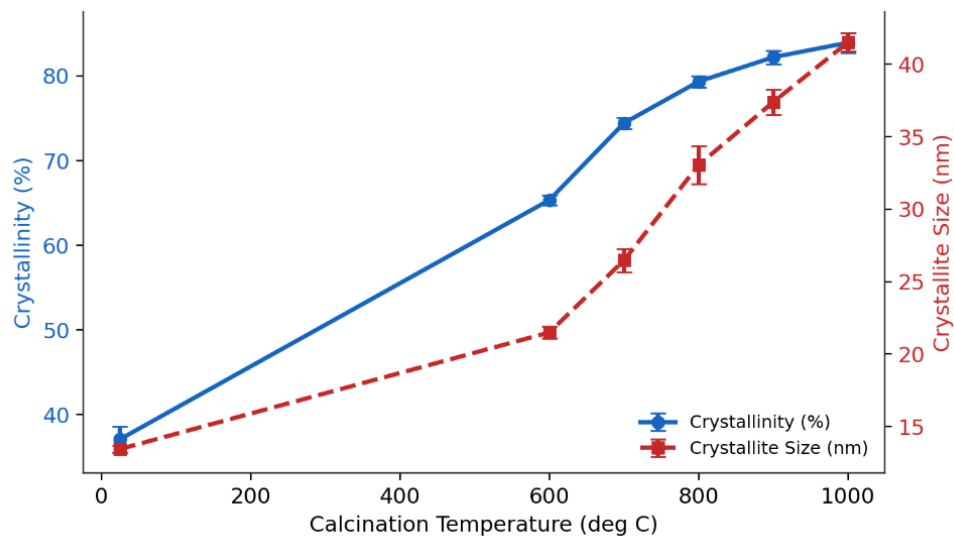


Figure 3. Crystallinity and crystallite size as a function of calcination temperature (mean $\pm$ SEM, $n = 5$)

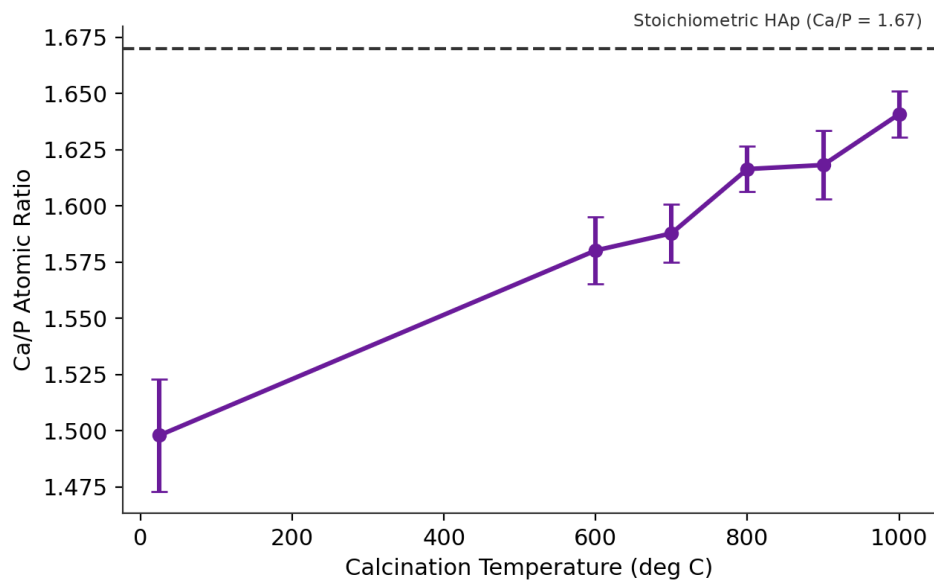


Figure 4. Ca/P atomic ratio of fish bone-derived HAp as a function of calcination temperature, relative to the stoichiometric reference value (1.67)

DISCUSSION

1. Phase Confirmation and Crystallographic Evolution

The close agreement between observed XRD peak positions and the JCPDS 09-0432 reference pattern (Table 3) supports the identification of the crystalline phase formed after calcination as hydroxyapatite. In the diffractograms shown in Figure 1 ($2\theta = 20\text{--}60^\circ$), no distinct reflections attributable to secondary calcium phosphate phases such as beta-tricalcium phosphate (strongest reflection at approximately 31.0°) or to calcium oxide (approximately 37.4°) were resolved, which is in line with reports for other fish species processed under comparable thermal conditions (Anggraeni et al., 2022; Kongsri et al., 2013). This observation, however, does not by itself establish

phase purity. Agreement of peak positions identifies the dominant phase only; minor phases below the detection limit of laboratory XRD (typically a few weight percent), amorphous or poorly crystalline calcium phosphate, and phases whose reflections are masked by broad hydroxyapatite reflections (especially at lower calcination temperatures) cannot be excluded, and no quantitative phase analysis (e.g., Rietveld refinement) was performed. The calcined materials are therefore described as hydroxyapatite-dominant with no secondary phases detected within the resolution of the present XRD data, rather than as phase-pure. The slightly sub-stoichiometric Ca/P ratio at 1000 °C (Section 4.3) likewise indicates a calcium-deficient apatite rather than stoichiometric hydroxyapatite. The progressive sharpening and intensification of XRD peaks with increasing calcination temperature, and the corresponding statistically significant increase in crystallinity from 37.1% (uncalcined) to 83.9% (1000 °C), is consistent with the well-established thermally driven crystallization mechanism whereby elevated temperature promotes atomic diffusion, lattice defect annihilation, and grain growth within the hydroxyapatite crystal structure (Sadat-Shojai et al., 2013; Sunday et al., 2022).

The observed plateau in crystallinity development beyond approximately 800 °C, evidenced by the non-significant Tukey post-hoc comparisons between the 800-900 and 900–1000 °C groups (Table 7), aligns with similar plateau patterns reported in prior biogenic HAp calcination studies, which attribute this phenomenon to the approach of a thermodynamically limiting crystallinity ceiling under a given calcination duration, beyond which further temperature increases yield diminishing returns unless holding time is also extended (Huang et al., 2023; Mondal et al., 2022). This finding has direct practical relevance for process optimization, suggesting that calcination at approximately 800 °C may represent a reasonable energy-efficient operating point that captures the majority of achievable crystallinity gains without the additional energy cost associated with higher-temperature processing.

2. Functional Group Evolution and Carbonate Content

The FTIR results (Figure 2, Table 4) provide complementary evidence of the calcination-driven reduction in carbonate and adsorbed water content that accompanies the increase in crystallinity observed in the XRD data. The marked reduction in carbonate-associated bands (1415-1450 cm^{-1} and 870-875 cm^{-1}) following calcination reflects the thermal decomposition of B-type carbonate substitutions naturally present in biogenic bone apatite, a transformation consistent with previous reports on thermally processed biogenic hydroxyapatite from both fish and mammalian bone sources (Fihri et al., 2017; Rahayu & Kurniawati, 2023). The emergence of a sharp, well-resolved structural hydroxyl band at approximately 3572 cm^{-1} in calcined samples, essentially absent in the uncalcined sample, further corroborates the XRD-based crystallinity findings, as this sharp OH band is characteristic of well-ordered, highly crystalline hydroxyapatite, in contrast to the broad, poorly resolved hydroxyl-region absorption typical of amorphous or poorly crystalline calcium phosphate (Bee et al., 2021).

The progressive increase in the PO_4/CO_3 intensity ratio with calcination temperature (from 2.98 in the uncalcined sample to 7.40 at 1000 °C, $R^2 = 0.943$ in linear regression over 600–1000 °C) is



interpreted here as a relative, semi-quantitative indicator of decreasing carbonate content (Section 2.3), and is consistent with prior findings that the carbonate content of biogenic hydroxyapatite decreases with increasing calcination temperature (Akram et al., 2014; Pal et al., 2017). The ratio has important limitations. It depends on the choice of bands, baseline correction, and spectral normalization; the $\text{PO}_4 \nu_3$ band overlaps with other absorptions; and only two of the spectra from which it was calculated (uncalcined and 800 °C) are shown in Figure 2. More fundamentally, a reduction in carbonate-associated absorption indicates lower carbonate substitution but does not independently establish phase purity, because secondary phases (e.g., tricalcium phosphate or calcium oxide) or amorphous calcium phosphate may be present without affecting the carbonate bands. Likewise, the high R^2 of the regression describes the monotonic trend of the ratio within the tested temperature range and does not by itself validate the ratio as a purity measure. Phase purity is therefore not inferred from the FTIR data. A lower carbonate content is nevertheless relevant for biomedical use, as carbonate substitution, while present in natural bone mineral and sometimes considered beneficial for bioresorbability, can also reduce the mechanical stability and alter the dissolution kinetics of synthetic HAp in a manner that may be undesirable for certain load-bearing biomedical applications, underscoring the importance of calcination temperature as a tunable parameter for application-specific material design (Ressler et al., 2021).

3. Ca/P Ratio and Biomedical Relevance

The progressive increase in Ca/P atomic ratio toward the stoichiometric value of 1.67 with increasing calcination temperature is consistent with the interpretation that calcination promotes the decomposition and volatilization of non-apatitic calcium and phosphate-containing impurity phases inherited from the biogenic precursor, allowing the remaining crystalline phase to more closely approach the ideal hydroxyapatite stoichiometry (Akram et al., 2014; Huang et al., 2023). The finding that even the highest-temperature group (1000 °C) remained marginally, though statistically detectably, below the fully stoichiometric value (mean 1.641 vs. 1.67, $p = .0475$) suggests that some degree of calcium deficiency or minor secondary phase content may persist even at high calcination temperatures under the specific synthesis protocol used in this study, a finding also reported in several comparable biogenic HAp studies and generally attributed to trace calcium loss during the alkaline deproteinization step or minor calcium volatilization during high-temperature calcination (Kongsri et al., 2013; Sunday et al., 2022).

From a biomedical application perspective, a Ca/P ratio close to, but marginally below, the stoichiometric value is not necessarily disadvantageous, as slightly calcium-deficient hydroxyapatite has been reported in some studies to exhibit enhanced bioresorbability and osteoconductive properties compared to fully stoichiometric HAp, owing to its closer compositional resemblance to natural, partially substituted bone mineral (Mondal et al., 2022; Ressler et al., 2021). This suggests that the compositional profile of the material produced at 900–1000 °C may be compatible with bone graft substitute applications, but this remains a hypothesis that requires quantitative phase analysis and in vitro and in vivo biological evaluation, which were not performed in this study (Section 4.5).

4. Practical and Application Implications

Collectively, these findings indicate that fish bone waste, an abundant and low-cost by-product of the fish processing industry, can be effectively converted into crystalline, near-stoichiometric, hydroxyapatite-dominant apatite through a relatively simple alkaline deproteinization and calcination process. The systematic characterization of the temperature-property relationship provided in this study offers practical process guidance: applications requiring maximal crystallinity, such as dense bioceramic implant components, may favor calcination at 900–1000 °C (subject to confirmation of phase purity and biological performance), while applications benefiting from smaller crystallite size and potentially enhanced bioresorbability, such as injectable bone graft substitutes or scaffold coatings, may favor calcination in the 700–800 °C range, where substantial crystallinity has been achieved without the larger crystallite sizes observed at higher temperatures (Prasetya & Handayani, 2022; Rahayu & Kurniawati, 2023).

The utilization of fish bone waste for hydroxyapatite production also carries broader environmental and economic significance, offering a pathway for valorizing an otherwise discarded fisheries industry by-product into a high-value biomedical material, consistent with circular economy principles increasingly emphasized in sustainable materials research (Pal et al., 2017; Prasetya & Handayani, 2022).

5. Study Limitations

This study has several limitations. First, characterization was limited to XRD, FTIR, and compositional (Ca/P ratio) and particle size analysis; direct in vitro bioactivity assessment, such as simulated body fluid (SBF) immersion testing to evaluate apatite-forming ability, and in vitro cytocompatibility testing using osteoblast-lineage cell cultures, were not performed in this study and would be valuable additions for more directly substantiating the biomedical suitability of the synthesized material. Second, this study focused on a single fish species and a single alkaline deproteinization protocol; given that prior literature indicates some variation in HAp properties across different fish species and extraction protocols, the generalizability of the specific quantitative property values reported here to other fish bone sources should be approached with appropriate caution, although the qualitative temperature-property trends are expected to be broadly generalizable. Third, mechanical property characterization, such as compressive strength or hardness testing, was not performed, which would be relevant for applications involving load-bearing bone graft substitutes. Fourth, phase purity was assessed only by comparing XRD peak positions with the JCPDS 09-0432 reference pattern and by inspecting the diffractograms for secondary-phase reflections; quantitative phase analysis (e.g., Rietveld refinement) was not performed, so minor or amorphous secondary phases cannot be excluded. Fifth, the PO_4/CO_3 intensity ratio is a relative, semi-quantitative index of carbonate content and not a direct measure of purity.



CONCLUSIONS

This study synthesized hydroxyapatite from fish bone waste through alkaline deproteinization followed by calcination across a systematic temperature range (600–1000 °C), and characterized the resulting material's crystallographic, functional group, particle size, and compositional properties. XRD analysis showed patterns consistent with the hydroxyapatite reference pattern JCPDS 09-0432, with no secondary phases detected within the resolution of the data (phase purity was not quantitatively established), and with crystallinity increasing significantly from 37.1% (uncalcined) to 83.9% (1000 °C) and reaching a statistical plateau beyond approximately 800 °C. FTIR analysis showed the expected calcination-driven reduction in carbonate and adsorbed water content alongside sharpening of characteristic phosphate and hydroxyl functional group bands. The Ca/P atomic ratio increased progressively with calcination temperature, approaching, though not fully reaching, the theoretical stoichiometric value of 1.67 at the highest temperature tested (1.641 ± 0.019 at 1000 °C, significantly lower than 1.67; $p = .0475$).

These findings demonstrate that calcination temperature is a critical, statistically significant, and practically tunable process parameter governing the crystallinity, crystallite size, and carbonate content of fish bone-derived hydroxyapatite, providing a process framework for tailoring material properties; suitability for specific biomedical applications remains to be established. Given the abundance and low cost of fish bone waste as a raw material, these findings support the continued investigation of biogenic, waste-valorized hydroxyapatite as a sustainable alternative source of calcium phosphate, whose biomedical suitability must still be confirmed. Future research incorporating in vitro bioactivity and cytocompatibility testing, mechanical property characterization, and evaluation across additional fish species and extraction protocols is recommended to further substantiate and extend the biomedical application potential of fish bone-derived hydroxyapatite.

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