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Effect of Calcination on The Bioactivity of Hydroxyapatite (HAp) from Black Tilapia Fish Scale

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Abstract

Hydroxyapatite, HAp is extensively used for orthopaedic and dental reconstruction as implant material due to their chemical and biological similarity to human hard tissue. Recently, vigorous research efforts made to obtain HAp from an animal bone in providing alternative feedstock materials for biomedical applications. Therefore, the extraction of natural HAp from the Black Tilapia (*Oreochromis Niloticus*) fish scales was produced via a conventional heat treatment (calcination) at 1000 °C. To produce HAp fine powder, the natural HAp from the tilapia fish scale went through a grinding process before characterization and testing. The sample was characterized using powder X- rays Diffraction (XRD), Field Emission Scanning Electron Microscopy (FESEM), and Energy Dispersive X-ray spectroscopy (EDX). The bioactivity of the samples was characterized using a Simulated Body Fluid (SBF) Test, Anti-Microbial and MTT-assay using a Human Fetal Osteoblast (hFOB) 1.19 cell line. XRD result shows the crystallinity of extracted HAp is similar to the standard HAp. The FESEM image shows the particles have different morphologies. The EDX analysis shows that the Ca/P ratio is 1.69 that slightly different from the standard HAp (1.67). The SBF result shows apatite deposition on top of the pellet sample surface after immersion for 7 days. Anti-Microbial shows that there are no anti-microbial properties on the extracted HAp and the MTT-assay analysis shows that the samples were not toxic to the cell. This work shows that studies on the extraction of fish scale into high value-added product are the promising alternative to produce natural HAp that is beneficial to medical applications. The bioactivities show that the natural HAp produced is bioactive and not toxic.

1. Introduction

A well-known biomaterial, HAp ($Ca_{10}(PO_4)_6(OH)_2$), is used extensively in biomedical for products like oral pastes, bone cements, and implants [1] because of its chemical appearances, which are equivalent to those of the mineral found in mammal bones and hard tissues [2]. Generally, HAp could be gained by two main approaches, including:

(i) a synthetic method using calcium and phosphate sources [3] and (ii) an extraction method from biological resources [4]. Numerous synthetic techniques, including precipitation [5], microwave [6], ultrasonic [7], sol-gel crystallization [8], electrodeposition [9], hydrothermal treatment [10], and spray pyrolysis [11], have been developed to generate HAp in the literature. The synthetic techniques rely on a chemical exchange between phosphate and calcium ions. These methods make it simple to adjust the prepared HAp shape, crystallinity, and stoichiometric composition (1.67) [1]. But these approaches are challenging, expensive [12], time-consuming [4], and only offer HAp with minimal activity.

Over half of all fish caught worldwide are not turned into important food products and instead end up as millions of tons of useless biowaste [13]. Fish by-product waste is massive, and it has a negative effect on the ecosystem [14]. Fish scale waste has been used recently as an inexpensive source for natural HAp preparation. However, the characteristics of commercially available stoichiometric HAp and fish waste synthesized HAp are inherently different from one another. Greater biological activities were observed in HAp generated from fish waste because it contained important anions (such as Cl- and F-) and cations (such as Mg^{2+}, Al^{3+}, Sr^{2+}, Zn^{2+}, K^{+}, and Na^{+}) [15]. The presence of both ions indicated superior biomedical applications, primarily for the rapid regeneration of bone [16]. The growth and stability of teeth and bone depend more on the availability of Na+ and Mg^{2+} ions, whereas their lack or insufficient concentration might cause bone loss or weaken it.

Previous publications state that the synthesize of HAp from fish scales is an inexpensive and simple process. The synthesize of HAp from natural sources such cow bone [17], marine corals [18], eggshells [19], fish bones [20], and fish scales has been documented by numerous researchers recently. Kerian has shown that HAp may be made from fish scale and bones using a straightforward calcination process [21]. The HAp that has been created in this way resembles human bone in both structure and shape. Furthermore, the original bones' infections and organic components may all be eliminated by processing them at a high temperature [3]. However, HAp that is isolated from fish bones typically has traces of ions including Na^{+}, Mg_{2}^{+}, Zn_{2}^{+}, and K^{+} [15]. The natural HAp has many benefits due to the presence of these trace ions [16]. It has been claimed that these trace ions assist the processes of bone growth and regeneration.

In this study, HAp was synthesized from Black Tilapia fish scale biowaste using a straight-forward heat treatment technique called calcination. The surface morphology and chemical composition of the synthesized HAp material were characterized using various techniques including XRD, TGA, FESEM and EDX. SBF was used to conduct additional biological evaluations with the obtained HAp. Excellent biocompatibility was demonstrated by the results, indicating that HAp made from Black Tilapia fish scale biowaste could be a viable substitute biomaterial for use in biomedical applications.

2. Methodology

2.1 Sample and Preparation

Black Tilapia fish were harvested from the G3 lake inside Universiti Tun Hussein Onn Malaysia (UTHM) in Batu Pahat, Malaysia. The leftover of the Black Tilapia fish scale waste has been cleaned and isolated to produce the HAp. Fig. 1 shows the Black Tilapia fish collected from Lake G3 UTHM.

Fig. 1 *Black Tilapia harvested from lake G3*

2.2 Drying

Before the calcination process take part, the raw samples (fish scale) were boiled 3 times, for one hour, in order to eliminate the fish skin or any raw meat and foreign matter that might cause contamination during the experiment. The samples then were dried out under the sunlight for 24 hrs. After that, the drying process is carried out for two hours at 90 °C using an oven (Termoline, China). The samples will subsequently be ground into a fine powder using a mortar and pestle.

2.3 Calcination

Samples of Black Tilapia fish scales (powder form) were heated to 1000 °C with a 10 °C/min rate in a Protherm furnace. To remove the biologically harmful elements and impurity layers, a three-hour calcination process was employed during the holding period, followed by a natural cooling process. During this process, the HAp was extracted from the fish scales. To minimize water capture and avoid contamination, the HAp samples were carefully located inside a vacuum area desiccator after the calcination process.

2.4 Samples Pelleting

Following the calcination process, the HAp samples were ground into an even finer powder and then sieved again to make sure the sizes were uniform and wouldn't affect the powder's capacity to form pellets. The size of the powder is approximately 60 µm by using sieve mesh 270. The samples were produced as pellets, to ensure consistent size and weight for each experiment. For better precision, the weight of the powder will be determined at 1.1 grams using a digital scale. Next, the sample was placed within the pressure pellet mold of the hydraulic jack. Nine tones of pressure were the perfect amount to make sure the pellet wouldn't break and turn back into ashes.

2.5 Characterization

Validating the transparent structure and stage composition of the conducted experiment was a higher-resolution Bruker Advance D8 X-RD diffractometer. X-ray diffraction operating in the Bragg-Brentano configuration using Cu-Kα (λ = 1.5405 Å) emission at 40 mA current and 40 kV peak voltage. To examine the thermal stability and phase identification of the decomposition stage for both HAp samples, simultaneous thermogravimetry and differential scanning calorimetry (TG-DSC) (Linseis, STA PT 1600 Germany) were used. 15.1 mg of the powdered material was placed in an alumina crucible (cup) and heated under nitrogen at a rate of 10 °C/min from 25 °C to 1000 °C. The microstructure of the samples was confirmed through the use of field emission scanning electron microscopy (FESEM) JEOL JSM – 7600F from Japan with an accelerating voltage of 5.0 kV, 20 000 magnification, and nanoscale imaging, an energy dispersive X-ray spectroscope (EDS) (Oxford, United Kingdom) equipped with an energy up-voltage of 15 kV. The elemental composition of the specimen was validated using EDS.

2.6 Preparation of SBF Solution

Recent research has shown that the best in vitro technique for examining a material's bioactivity is to test it in simulated body fluid (SBF) [22]. Kokubo's SBF solution, which has a chemical makeup similar to human plasma, is the most often utilised of the several SBF solutions suggested in the literature. Table 1 shows the compounds used to prepare the SBF solution according to Kokubo [23]. By adding HCl solution, the pH of the stock solutions was adjusted to the physiological value of 7.4. Further information about this protocol is available in Ref. [24].

Table 1 *The chemical composition of the simulated body fluid (SBF) used [23]*

Order	Reagent	Amount
1	NaCl	8.035 g
2	$NaHCO_3$	0.355 g
3	KCl	0.225 g
4	$K_2HPO_4.3H_2O$	0.231 g
5	$MgCl_2.6H_2O$	0.311 g
6	HCl (1 M)	38 mL
7	$CaCl_2.2H_2O$	0.386 g
8	Na_2SO_4	6.118 g
9		

3. Results and Discussion

3.1 Mineralogy

Fig. 2 shows the XRD spectra of calcined fish scale HAp. For confirmation, standard HAp from JCPDS No. 00-009-0432 was implemented. One well-known technique for increasing a sample's crystallinity is calcination. Thus, in the sample of Black Tilapia fish scale HAp, prominent peak of well-crystalline HAp can be seen. The x-ray pattern of the synthesized Black Tilapia fish scale HAp; where the characteristic diffraction peaks on 2θ of 25.9°, 32.8o, 34.6°, 35.9°, 39.7°, 47.5°, 50.2°, and 65.3° corresponding to (102), (211), (112), (202), (200), (220), (213), and (323), respectively, was fully coordinated to standard HAP pattern of JCPDS No. 00-009-0432 [25]. The crystal structure was determined to be HAp, and the pattern's second phase the β-TCP was to be traced. It is due to the HAp started changing the phases at high temperature (1000°C). β-TCP will contribute to the biodegradable properties. It is important for biomedical depends on the application of the part of the hard tissue.

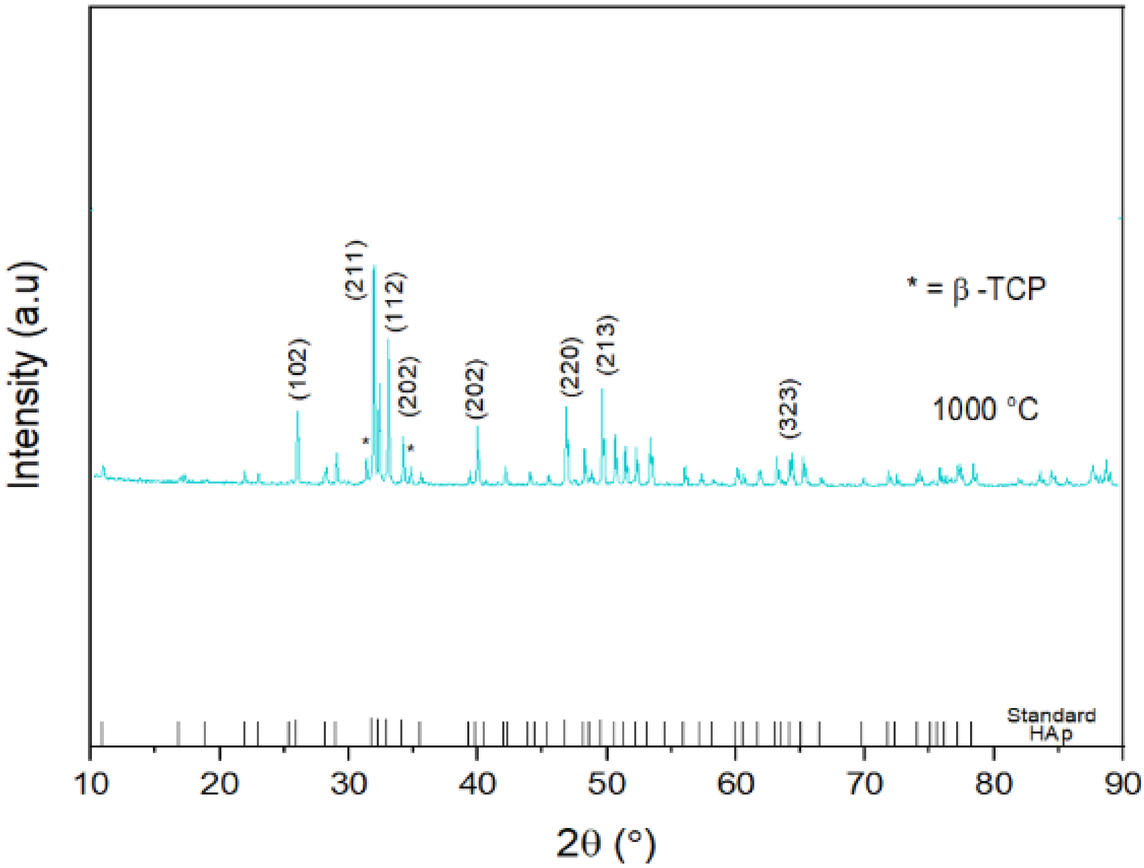

Fig. 2 *XRD spectra of calcined Black Tilapia fish scale HAp.*

3.2 Thermal Degradation

Fig. 3 shows the thermogravimetric (TGA) curve for the fish scale sample, along with the corresponding heat flow range of 25 °C to 1000 °C. There are three separate regions in the TGA curve. The first is related to the evaporation of some lattice water as well as weakly adsorbed water, and occurs in the temperature range of 25°C to 200°C with a mass loss of around 15.5%. HAp typically contains two different forms of water: lattice water and adsorbed water. Whereas the tightly bonded lattice water is permanently lost below 200°C, the adsorbed water can be withdrawn reversibly up to temperatures of bigger than 200°C. Between 200°C and 480 °C, there is a mass loss of over 30%, which may be attributed to the burning of organic materials such lipids, proteins, and collagen in addition to the removal of lattice water. A loss of roughly 3.0% in the 480 °C to 1000 °C range is indicative of both the slow dihydroxylation of HAp and the loss of OH- ions, as well as the loss of CO3 2- ions, which is mostly caused by the release of CO_2 from the apatite network as a result of carbonate breakdown [26].

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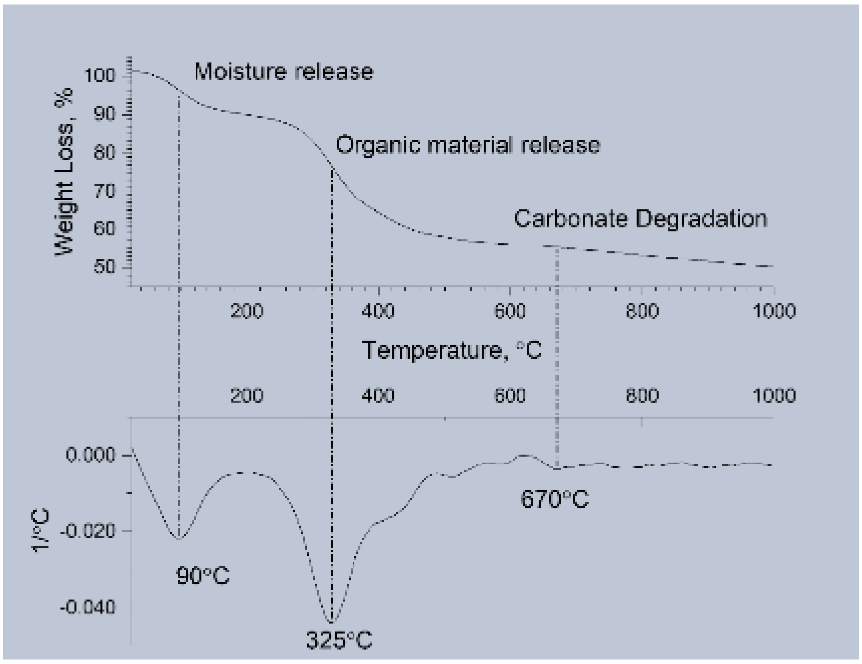

Fig. 3 *Thermogravimetric curve for the Black Tilapia fish scale sample*

3.3 Microstructure

Fig. 4 shows the FESEM micrographs of the calcined Black Tilapia fish scales HAp's surface structure. Equiaxed patterns and a morphologically uniform rod-like form are the shapes of calcined materials. There are several morphologies that range from rounded to irregular, and they typically nucleate in aggregates and agglomerates. The structure demonstrates the effectiveness of the calcination method at a low cost; the results show the HAp microstructure of the calcined scale is similar to other researchers that found in the literature and on the market. It validates the TGA analysis's conclusion that fish scale decomposes organic compounds such as protein and lipids. In order for HAp crystals to be the only components remaining in the inorganic complex.

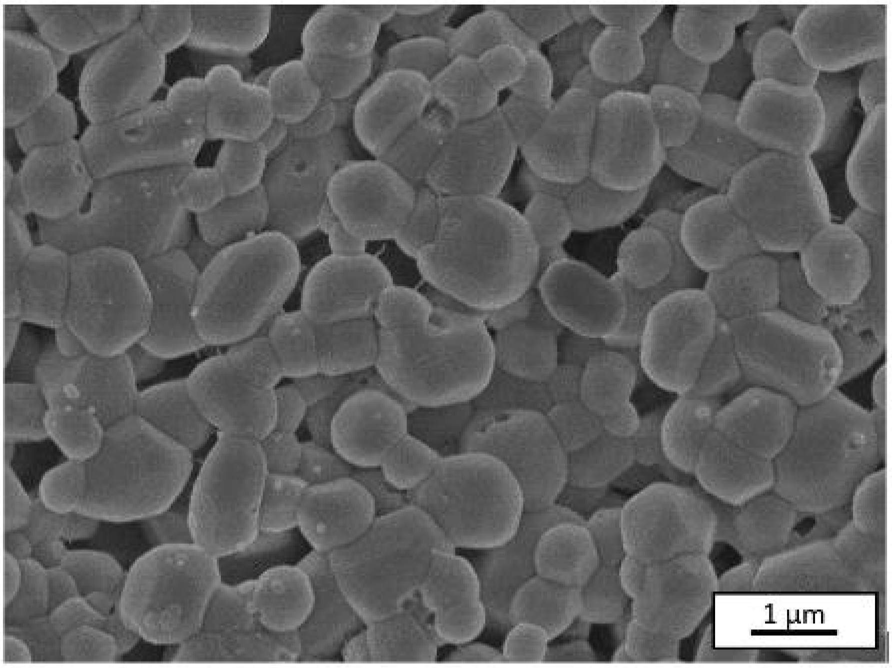

Fig. 4 *FESEM micrographs of the calcined Black Tilapia fish scales HAp's structure*

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3.4 Ca/P Molar Ratio

Table 2 demonstrate the molar ratio of Ca/P for Black Tilapia fish scale HAp was comparable to stoichiometric HAp, 1.67. Calcined fish scales HAp had a Ca/P ratio of 1.65. Phosphorus (P) and Calcium (Ca) make up the primary of the composition with trace elements of Sodium (Na) and Magnesium (Mg) also present. The presence of trace elements from this natural source could be the cause of the fluctuation of the Ca/P ratio. Mg and Na perform a significant character in tests (in-vitro and in-vivo) that imitate the natural bone growth and stimulate the development of new bone which directly enhanced osteoblast formation [27].

Table 2 *The Ca/P molar ratio for calcined Black Tilapia fish scale*

Sample	Elemental Composition (at%)				Ca/P
	Ca	P	Mg	Na	
Fish Scale	31.00	17.94	0.40	0.35	1.65

3.5 Biological Activity

Fig. 5 shows the FESEM image of the Black Tilapia fish scale HAp samples after soaking in SBF solution at pH 7.4 and room temperature 36 °C for 14 days. Fig. 5 illustrates the resorbability of Black Tilapia fish scale HAp and its reaction towards Ca rich SBF solution. β-TCP potentially enhanced the growth of natural bone due to resorbable properties. In addition, the medium's pH is rather alkaline due to the apatite production. It shows that Black Tilapia fish scale HAp was physiologically stable during the research period. According to the results, calcium ions were liberated from the prepared Black Tilapia fish scale, which could be because the HAp is breaking down more quickly. The calcium ion release pattern of biological apatite and the amount of calcium ion release from the HAp samples are highly compatible. Thus, it can be said that HAp dissolves in an ionic manner similarly to natural bone mineral. Given that the solubility is extremely dependent on the apatite samples' structural and chemical properties.

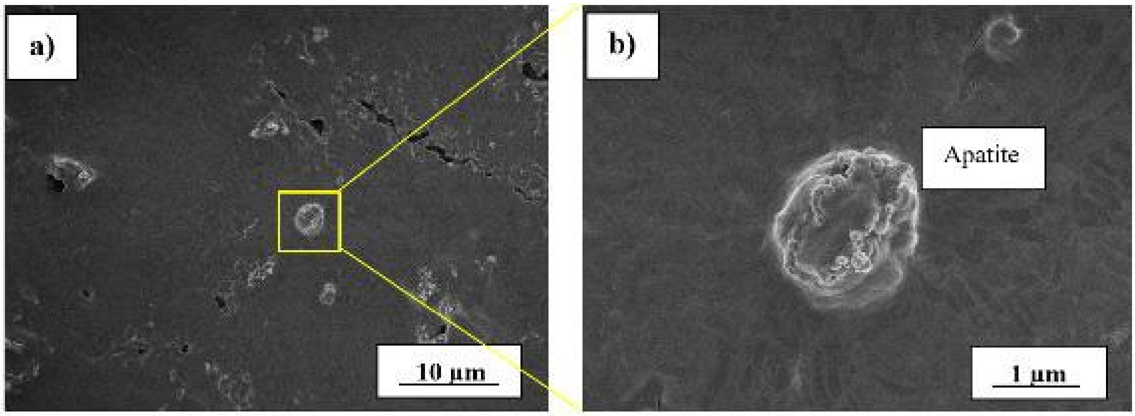

Fig. 5 *FESEM micrographs of apatite formation on top of HAp surface after 14 days of soaking in SBF solution (a) 10 µm; (b) 1 µm*

4. Conclusions

Using the calcination technique, high crystalline HAp was produced from the waste of black tilapia fish scales according to the XRD graph. According to the TGA results, the removal of organic material caused a three-stage weight reduction during the operation. The material was confirmed to be HAp with a Ca/p Molar ratio of 1.65 by FESEM and EDX results and the apatite formation on top of the HAp pellet surface was also revealed by the SBF study. In conclusion, the Black Tilapia fish scale HAp was bioactive and suitable for biomedical application.

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Conflict of Interest

Authors declare that there is no conflict of interests regarding the publication of the paper.

Author Contribution

The authors confirm contribution to the paper as follows: ***study conception, design, data collection and draft manuscript preparation:*** *Noor Hakim Rafai[1], Muhamad Zaki Jaffri;* ***analysis and interpretation of results:*** *Noor Hakim Rafai, Muhamad Zaki Jaffri, Hasan Zuhudi Abdullah, Maizlinda Izwana Idris. All authors reviewed the results and approved the final version of the manuscript.*

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