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CALCINATION TIME AND TEMPERATURE EFFECT ON NATURAL HYDROXYAPATITE OBTAINED FROM FISH BONES FOR BONE TISSUE ENGINEERING

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Abstract

This paper presented the effect of calcination time and temperature on the natural hydroxyapatite (HAp) obtained from two different fish bones types: (1) *Chirocentrus nudus*, in which commonly known as whitefin wolf herring or "ikan parang" in Malay and (2) *Scomberomorus commerson*, English named as Spanish mackerel or "ikan tenggiri" by the local in Malaysia. These two fish bones were selected as hydroxyapatite source due to its abundancy, no conflict in religion means and ease of acquiring in Malaysia. The particle structure, phase composition and crystallinity of hydroxyapatite from fish bones were analyzed. XRD results showed that higher temperature shows better crystallinity and particle structure of hydroxyapatite. However, it was found that when the calcination temperature reaches 1000°C, the hydroxyapatite crystallinity reduces. FTIR results showed the presence of phosphate, carbonate and hydroxyl groups after calcination. Besides, traces of biological elements were detected by FTIR on fish bones exposed to only elevating calcination temperature. Small traces of elements such as magnesium, sodium and such are detected in all samples. SEM results showed rod-like and circular particle shape which aids for biocompatibility and scaffold foundation. Comparing the two fish bones, whitefin wolf herring resulted in overall better hydroxyapatite properties than in Spanish mackerel. The optimum temperature and calcination holding time to obtain the desired hydroxyapatite properties from Spanish mackerel and whitefin wolf herring are both at 800°C and 4 hours. These results proved to be promising for future medical application where bone restoration is heavily involved.

Keywords: Bone scaffold, Calcination, Fish bones, Natural Hydroxyapatite (HAp).

1. Introduction

Skeletal system of the human body comprises of bones connecting one another through joints, mainly serves for support and mobility. Similar to any other organ in the human body, bones are made out of cells combining together to form tissues. In this case, they are made out of bone tissues and bone marrow. Besides, it has a unique regeneration and healing abilities once its architecture disrupted. In an event where the bone experiences huge architecture lost due to trauma, bone infection or disease or such, this healing ability can fail. As a result, it will leave a permanent bone defect. Moreover, this self-heal rate decreases with aging. In order to counter this, new medical treatments that heavily involve bone regeneration or grafting must be implemented. According to statistics, there is a high demand of bone treatments as bone is the most transplanted tissue after blood [1].

Natural hydroxyapatite (HAp) is mineral found in human bone -calcium-phosphate based compound that promotes bone regeneration. This mineral can be found in the human body and teeth. Natural HAp has been widely used for bone implantation due to similarity in composition. Unlike synthetic HAp, it contains not only calcium phosphate element but other minerals and organic compounds in small amounts - magnesium, zinc and such. Implementing natural HAp rather than synthetic HAp is desirable for implant material as it results in exceptional bioactivity and osteointegration [2, 3].

Natural HAp is found in living organisms. Fish bones and scales [4-6], sea corals [7], egg shells [8] and bovine [9, 10] are few of the sources that have been reported of natural HAp extraction. Among all these, fish bone is the ideal medium for natural HAp extraction. Acquiring fish bones to extract HAp could be economically viable - cheap source. Besides, selecting fish or any other living organism from the ocean - jellyfish, shells and such, grant easy access and comes in abundant. In addition, it brings no religious conflicts in terms of animal selection. In this research, Spanish mackerel (SM) (scientific name: *Scomberomorus commerson)* and whitefin wolf herring (WWH) (scientific name: *Chirocentrus nudus)*, locally known as "*ikan tenggiri"* and "*ikan parang"*, respectively, are chosen mainly because their availability and ease acquiring in Malaysia. SM and WWH are selected to make processed-food products, in which discarding the bones as wastes.

The transformation of HAp ($Ca_{10}(PO_4)_6(OH)_2$) phase on heating consists of dehydroxylation, which display on Eq. 1 and 2, and decomposition of HAp and oxyapatite ($Ca_{10}(PO_4)_6O$), which display on Eq. 3 and 4:

$$Ca_{10}(PO_4)_6(OH)_2 \rightarrow Ca_{10}(PO_4)_6(OH)_{2-2x}O_xA_x + xH_2O \quad (1)$$

$$Ca_{10}(PO_4)_6(OH)_{2-2x}O_xA_x \rightarrow Ca_{10}(PO_4)_6O + (1\text{-}x)\ H_2O \quad (2)$$

$$Ca_{10}(PO_4)_6O \rightarrow 2Ca_3(PO_4)_2 + Ca_4(PO_4)_2O \quad (3)$$

$$Ca_{10}(PO_4)_6(OH)_2 \rightarrow 2Ca_3(PO_4)_2 + Ca_4(PO_4)_2O + H_2O \quad (4)$$

where A represents hydrogen vacancies and $Ca_{10}(PO_4)_6(OH)_{2-2x}O_xA_x$ is oxyhydroxyapatite (OHA) [11, 12]. Another possible decomposition of oxyapatite is shown on Eq. 5 [12].

$$Ca_{10}(PO_4)_6O \rightarrow 3Ca_3(PO_4)_2 + CaO \quad (5)$$

The objectives of this paper is to study the temperature and calcination holding time effect of natural HAp from fish bone between 600°C and 1000°C and between

2 to 6 hours. Besides, this paper aims to identify and validate the optimum temperature and calcination time for SM and WWH bones to obtain HAp.

2. Methodology

2.1. Material

SM (*Scomberomorus commerson*) and WWH (*Chirocentrus nudus)* were acquired from local morning market in Kuala Lumpur, Malaysia. The spine part of the fish bones were selected and stored in freezer for future used.

2.2. Fish bones preparation and calcination

The bones (both SM and WWH) were boiled in water for 1 hour to remove the traces of skin and meat, followed by drying at 60°C for 6 hours in oven.

The bones were placed in several ceramic crucibles and subjected to calcination process at different temperature and time between 400°C to 1000°C and 2 to 6 hours, at a heating rate of 10°C/min. The processing parameter is shown in Table 1. Once cooled, the calcined-bones were grinded to powder form for material characterization.

Table 1. Calcination process parameter.

Calcination Temperature (°C)	Time (hr)	SM samples	WWH samples
400	2	M1	WH1
600	2	M2	WH2
600	6	M3	WH3
800	2	M4	WH4
800	4	M5	WH5
800	6	M6	WH6
1000	2	M7	WH7
1000	4	M8	WH8
1000	6	M9	WH9

2.3. Samples characterization

The x-ray diffractometer (XRD, Bruker D8 Discover) is used to study the phase composition and crystallinity of the sample - verifying its purity and stability. The scanning was carried out at the angle 2θ range from 10 to 70° with step size of 0.1°. The x-ray diffractometer was operated using Cu Kα radiation (λ = 1.540 Å) with operating voltage and current of 40 kV and 30 mA.

The functional groups and chemical structure of the calcined samples were identified through Perkin Elmer Spectrum 100, a Fourier-transform infrared spectrometer (FTIR), at the condition 4 cm^{-1} resolution with frequency range set from 400 to 1400 cm^{-1}.

The surface morphology of the samples was analyzed through a scanning electron microscope (SEM, JEOL JSM-5900, Japan), equipped with energy dispersive microanalysis (EDX) to analyze the elements or composition of the

sample. The samples were observed under magnification in the range from 20 to 50k X and at the operating accelerating voltage at 15 kV.

3. Results and Discussion

The cleansed fish bones carried tinge of yellowing prior to calcination process as shown in Fig. 1, where SM's bones are yellowish-brown in nature and WWH's are yellowish-white. These colors represent the organic elements or biological composition present in and around the bones. Different species of fish may contain different elements in their respective bones.

Fig. 1. Raw SM bones (left) and WWH (right).

The bone started with its raw color and as calcination temperature and time increases, the bones change its color as observed in the physical appearances in Fig. 2. It turned to black/grey at 400°C, which indicate the organic compound residues due to incomplete combustion shown in the FTIR (Fig. 3) peak at small intensity with bands of 2356.64 cm^{-1}. In general, as temperature increases, the bones turn grey at 600°C, white at 800°C and lastly, blueish-white (1000°C) that shows the minerals compound in the bones. Slight differences between the two different bones were observed: WWH resulted pure white appearance after between 600°C, 800°C and blue-tinge white at 1000 °C, regardless of the calcination time; but at 600°C, SM bone contained patches of grey and white. This suggested that organic elements or biological composition was still present. Besides, comparing the two bone types, SM contained more organic elements than in WWH, since pure white color appearance was detected at lower calcination temperature of 600°C.

Both fish bones displayed similar peak trend in Fig. 3. Narrow bands suggested good crystallization of samples [13, 14]. Phosphate (PO_4^{3-}) group was detected between bands of 900 cm^{-1} and 1100 cm^{-1}. Carbonate (CO_3^{2-}) group was detected with small intensity at bands between 1300 cm^{-1} and 1660 cm^{-1}. Like carbonate group, hydroxyl (OH^-) group was detected with small intensity between bands of 3500 cm^{-1} and 3900 cm^{-1}. The results displayed on Fig. 4 showed similar trend to its lower temperature calcined samples with minor differences. At higher temperature and longer holding time, phosphate group resulted in higher intensity with sharper peak, suggesting higher crystallinity. Organic elements have been decomposed resulting in smaller and broader bands at around 2000 cm^{-1}. Nevertheless, hydroxyl and carbonate groups can be detected but in much smaller and broader intensities, comparing to the preliminary samples.

Fig. 2. Changes of bones color due to calcination temperature and time for (a) WWH and (b) SM from temperature (left to right): 400°C, 2 hrs; 600°C, 2 hrs; 800°C, 4 hrs and 1000°C, 6 hrs.

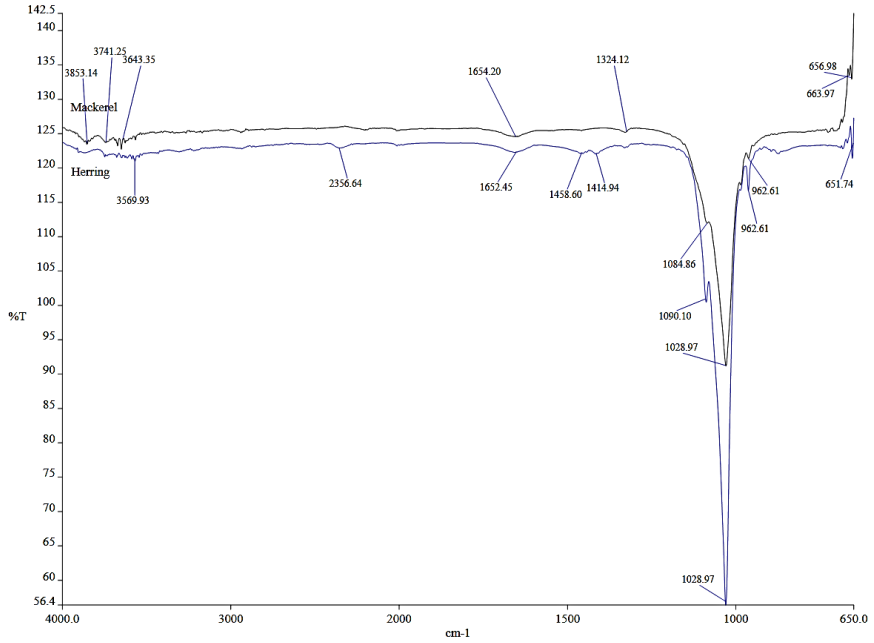


Fig. 3. FTIR analysis of fish bones at 400°C for 2 hours.

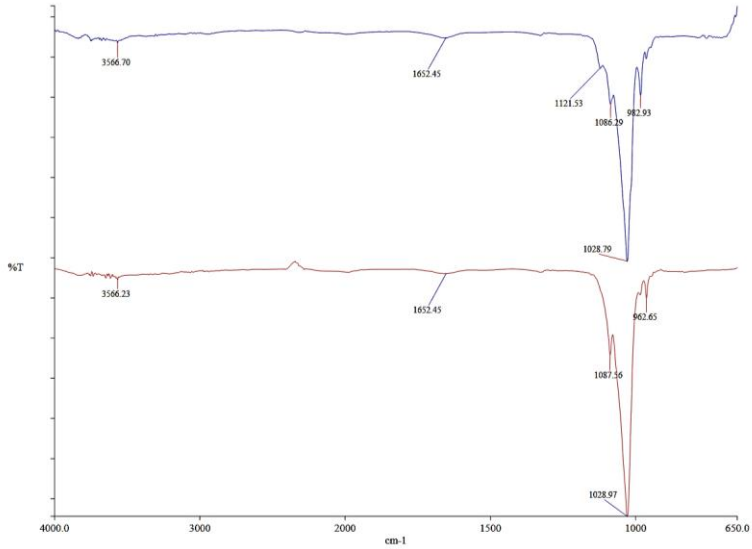


Fig. 4. FTIR analysis of SM (blue) and WWH (red) at 1000 °C for 6 hours.

The peaks around 1650 cm^{-1} were associated with carbonate ions while peaks between 1400 and 1460 cm^{-1} were associated with β-type HAp due to substitution of phosphate and carbonate groups [14, 15]. The carbonate groups between 1400 and 1460 cm^{-1} decreases or removed as calcination temperature elevates due to decomposition to carbon dioxide at temperature above 600°C, regardless of the holding time [16]. Bands around 960 cm^{-1} indicated the presence of β-type tricalcium phosphate (TCP) [4]. On the other hand, hydroxyl group band intensity resulted smaller and broader as temperature elevates due to weaken of intermolecular hydrogen bonding of water molecules. β-type TCP and HAp has been reported as appropriate compound for bone regeneration as it provides permanent scaffold for bone formation via osteo-conductivity and speeds up the regeneration through resorption [17, 18].

The crystallinity phase and purity of HAp derived from fish bones were confirmed with XRD analysis. Traces of HAp and other minerals are shown in the peak in Fig. 5. Figures 6 and 7 show the XRD patterns of different various calcination processes, where the patterns displayed are similar for both fish bones and no new peaks are generated. The XRD pattern indicates that the HAp structure was not affected, resulting in matrix stability and enhancement of crystallinity [13]. Based on the results, both SM and WWH resulted in same XRD trend. The intensity increases with decrease peak width as calcination temperature and holding time elevates. Besides, highly intense and sharp peaks suggest the removal of organic matters present in the fish bones [4]. This also indicates that the fish bones were made of organic matters or biological content. Minor presence of organic matters in both SM and WWH resulted on XRD patterns which is excellent for bioactivity, biocompatibility and osteo-conductivity, increasing the osteoblast functions [14]. Both SM and WWH contains minor of β-TCP at temperatures 800°C and 1000°C which agrees with previous authors, regardless of fish type [5, 6, 14]. The major peaks located between 30° and 35° defined as HAp. However, XRD pattern also detected other compounds that are similar to HAp such as fluorapatite and chloroapatite. This improves the bioapatite for biocompatibility unlike synthetic which contains pure HAp with limited or minimum compounds to enhance bone formation [14].

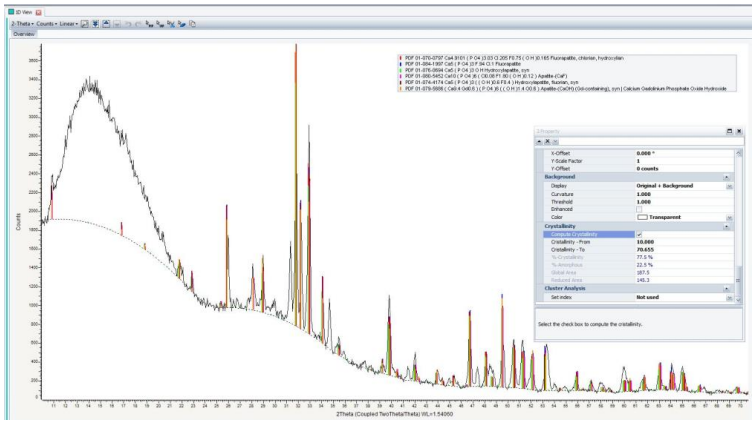


Fig. 5. X-ray diffractometer results for WWH bone at 1000 °C, 6 hrs with traces of HAp peaks.

The difference between SM and WWH is their respective intensities in the XRD results. Comparing Figs. 6 and 7, WWH shows overall higher peak intensities than in SM, in which concluded in better HAp crystallinity. Similar peak intensities are resulted in both calcination of 1000°C. However, WWH, with holding time of 6 hour of 1000°C calcination temperature, resulted in better overall peak intensities than in SM. HAp property gives high result in temperature of 800°C with 4 hours of holding time as it displays the highest intensity counts. This suggests that lower temperatures preserve better overall HAp properties than intense temperatures such as 1000°C [14]. The crystallinity of HAp structure was reported to be one of the important traits for good bioactivity and flexible structure [19, 20]. However, calcination temperature must be controlled since recrystallization can occur that leads to matrix alteration which would not match with the original body tissue architecture [14]. Calcination temperature of 800°C with 4 hours of holding time would preserve better overall HAp property.

Minor presence of β-TCP was detected in XRD patterns which verified the FTIR results. High temperatures led to decomposition of HAp into β-TCP. Even though it is essential to obtained β-TCP but high decomposition of HAp would lead to recrystallization. This is another reason where calcination temperature and holding time must be controlled. Minor presence of sodium, magnesium, potassium and other compounds is essential for biocompatibility as it contributes to metabolism in human body. Sodium and magnesium are responsible for bone metabolism but also result in osteoporosis - bone fragile [21, 22]. Magnesium is responsible for bone proliferation and function to enhance synthesis of protein. Strontium is responsible for reducing bone resorption and enhances bone formation to prevent fractures.

Three samples have been selected for FE-SEM analysis; SM calcined at 600°C for 2 hours and 800°C for 4 hours and WWH calcined at 800°C for 4 hours. SM and WWH calcined at 800°C for 4 hours were selected as it resulted in overall good crystalline structure in previous XRD analysis. On the other hand, SM calcined at 600°C was selected to analyse the surface morphology when its XRD patterns resulted in short intensity with wider peaks. Besides, it resulted in white-grey appearance rather than total white indicating higher organic content than ones calcined at 800°C. This was to analyse whether higher organic compound would affect the surface morphology of the sample.

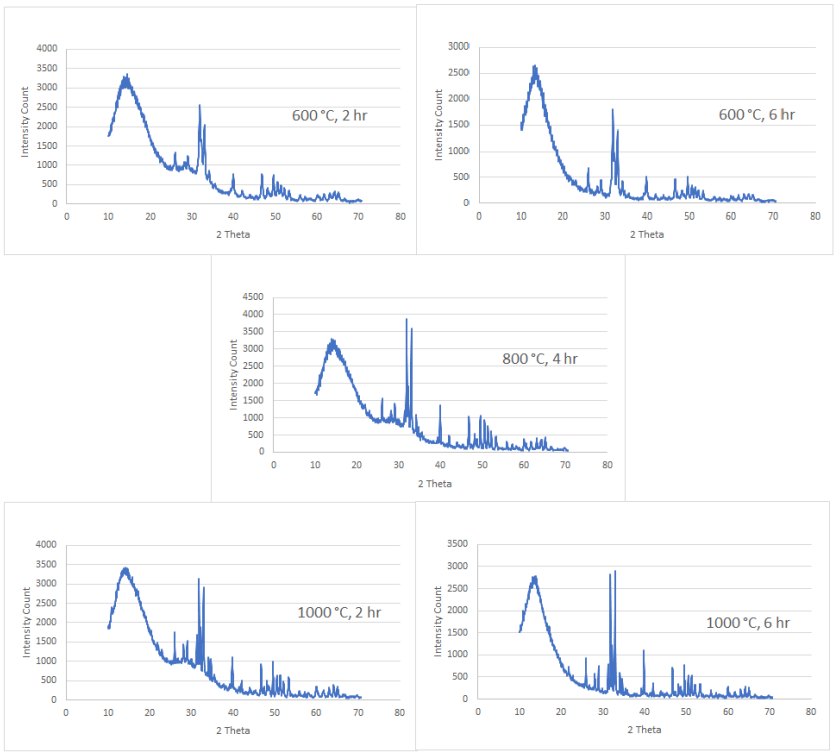


Fig. 6. X-ray diffractometer results for WWH.

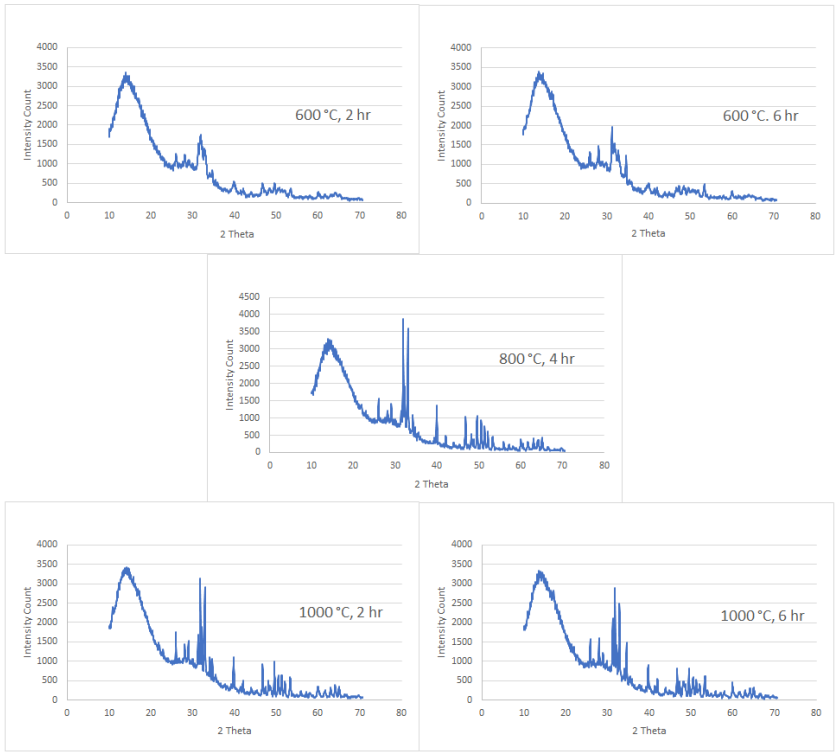
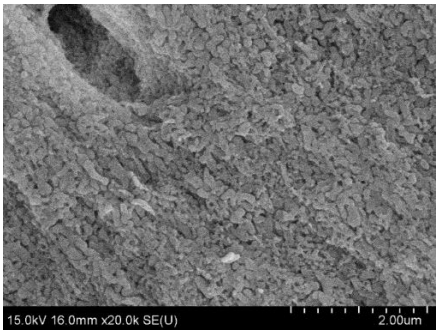
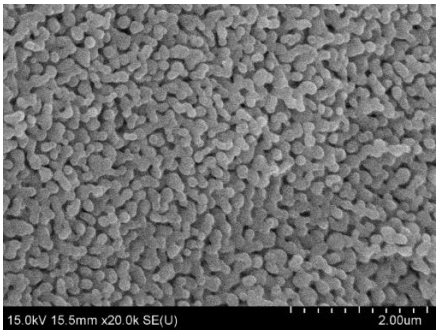


Fig. 7. X-ray diffractometer results for SM.

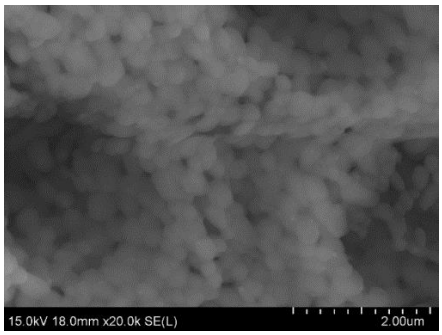
Figure 8 displays FE-SEM picture of three fish bone samples with magnification of 20kX. FE-SEM analysis indicated the samples composed of rod-like and spherical shape particles. The morphology of the samples changes according to calcination temperature, as the crystallinity of the bone structure enhanced as the calcination temperature increases. Figures 8(b) and (c) demonstrated the different structure of the bones, as WWH shows larger porosity of the bone structure. As can be observed from the FE-SEM figures, the particles were resulted in rod-like shape which changed from acicular shape due to stoichiometric apatite attributed to the coupled for ions - carbonate for phosphate and sodium for calcium, substitutions. It was found that higher temperature enhance the crystallite size, improving to nanoparticles, which is an important trait for bioactivity and flexible structure [4, 23].



(a)



(b)



(c)

Fig. 8. SM bone calcined at (a) 600°C for 2 hours; (b) 800 °C for 4 hours and (c) WWH bone at 800°C for 4 hours.

With the same analysis area and magnification, EDX was performed to analyse the chemical composition of the sample. Chemical composition, along with calcium to phosphorus ratio (Ca/P), of three samples, same as FE-SEM analysis, was analysed and summarized in Table 2.

All three samples contained similar chemical composition with carbon, oxygen, sodium, magnesium, potassium identified and analyzed. Calcium and phosphorus are the two main elements that defined HAp. The weight percentage of three different samples results in no significant difference to one another. However, increasing of calcination temperature showed slight increase of calcium and phosphorus content. Comparing with synthetic HAp (Ca/P = 1.67), Ca/P ratio in SM for both calcination conditions is lower. However, SM calcined at 800 °C for 4 hours have Ca/P ratio close to synthetic HAp. On the other hand, WWH calcined at 800 °C for 4 hours resulted 1.70 Ca/P ratio, higher than in synthetic HAp. Other elements such as carbon, oxygen, sodium, magnesium and potassium were present in smaller amount.

Table 2. Elemental composition by three fish bones with respective calcination conditions.

Fish Type	**SM**	**SM**	**WWH**
Calcination Condition	**600°C 2 hrs**	**800°C 4 hrs**	**800°C 4 hrs**
C (wt%)	4.43	4.7	6.79
O (wt%)	47.47	39.9	33.98
Na (wt%)	0.93	0.67	0.94
Mg (wt%)	1.13	1.29	1.66
P (wt%)	18.11	19.74	20.44
K (wt%)	0.21	1.05	1.47
Ca (wt%)	27.53	32.64	34.73
Ca/P (wt %)	1.52	1.65	1.7

Higher Ca/P ratio compared to synthetic HAp was resulted due to presence of carbonate ions substituting phosphate, indicating B-type HAp presence - mineral phase of biological apatites [14, 24]. Other authors who implemented other animal bones resulted with higher Ca/P ratio than in synthetic HAp [10, 25]. The substitution of carbonate ions for phosphate is favored because it has been reported that B-type HAp is more favored than A-type - higher affinity of osteoblasts (bone cells) for cell adhesion [26, 27]. Presence of B-type HAp have been confirmed from FTIR analysis. Besides, the EDX analysis is coincident with XRD analysis claiming the presence of minor elements after calcination process.

4. Conclusions

In conclusion, natural HAp was obtained from fish bones, SM and WWH, available as food waste. The results coincident with initial hypothesis and objectives are met. Higher calcination temperature and longer holding time resulted in enhancement of overall HAp properties. However, if calcination conditions are extreme - above 1000°C and longer holding time, natural HAp properties alters and will not match with human bone matrix. It was found that calcination temperature of 800°C and holding time of 4 hours resulted in best overall better HAp in both SM and WWH. Comparing between these 2 species, WWH resulted in better overall HAp properties. From characterization results, B-type HAp and β-tricalcium phosphate were detected

in minor amount. Besides, minor presence of elements such as magnesium, sodium, potassium and such were detected as well. These fish-derived HAps have been present a promising future because raw materials are food wastes, obtained from cheap and sustainable source. Furthermore, based on characterization results, use of natural HAp containing other elements for biocompatibility would be beneficial for bone regeneration.

Abbreviations

EDX	Electron Dispersive X-Ray
FE-SEM	Field-Emission Scanning Electron Microscope
FTIR	Fourier-Transform Infrared Spectrometer
HAp	Hydroxyapatite
OHA	Oxyhydroxyapatite
SM	Spanish mackerel Fish
TCP	Tricalcium phosphate
WWH	Whitefin wolf herring fish
XRD	X-Ray Diffractometer

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