JOURNAL OF MECHANICAL ENGINEERING AND SCIENCES (JMES)
ISSN: 2289-4659 e-ISSN: 2231-8380
VOL. 15, ISSUE 1, 7792 – 7806
DOI: https://doi.org/10.15282/jmes.15.1.2021.15.0615

ORIGINAL ARTICLE

Correlation of heating profile with calcination temperature for the extraction of nano hydroxyapatite (Nano-HAp) derived from bone

H.M. Rais[1], M.S.M Ghazali[2, 3], and N.F. Mohtar[1*]

[1] Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia
[*] Phone: +6096684540; Fax: +6096684949
[2] Faculty of Science and Marine Environment, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia
[3] Material Synthesis and Characterization Laboratory (MSCL), Institute of Advanced Technology, Universiti Putra Malaysia 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia

ABSTRACT – Bone is one of the major sources of hydroxyapatite (HAp) in the form of calcium phosphate. The increasing demand for HAp has brought much interest in research line to find the best method for its production either synthetically or naturally. Calcination of natural bone is a common heat treatment method for production of HAp. However, the optimum temperature of calcination depends on source and heating profile of the bone. Therefore, this study aimed to determine heating profile and the optimum calcination temperature of Nano-hyroxyapatite (Nano-HAp) extracted from spotted sardinella (*Amblygaster sirm*) bone. Characterization of Nano-HAp was carried out using thermogravimetric analysis (TGA), chromameter and X-ray diffraction (XRD) spectroscopy. The composition of protein, ash, moisture and lipid content of raw bone (RB) were 1.08%, 50.98%, 10.47% and 3.78%, respectively. Results demonstrated that the optimum temperature for extracted nano-hydroxyapatite (HAp-1) was 700°C based on yield percentage (55%) and colour changes value (ΔE=18). Heating profile of raw bone demonstrated the highest reduction of weight loss percentage (320°C to 610°C=36%); which determined the melting point of the bone that acted as a supporting analysis for calcination. XRD confirmed the crystallinity phase of HAp-1 particle similar to the standard peak of HAp, (PDF No: 1-1008) and comparable with the standard HAp. Based on the heating profile and colour changes value, the findings demonstrated that the calcination of HAp from bone of spotted sardinella (*Amblygaster sirm*) could be carried out at an optimum temperature of 700°C. Overall findings have suggested that the optimum calcination temperature obtainedmay contribute to the development of optimal method for the extraction of HAp via heat treatment process with different fish species.

ARTICLE HISTORY
Received: 04th Dec 2019
Revised: 06th July 2020
Accepted: 04th Dec 2020

KEYWORDS
Nano-Hydroxyapatite; bone; calcinations; heating profile temperature

INTRODUCTION

Bone is a major source of protein and mineral that contributes to the building block of living things. It is made up of organic collagen protein and inorganic hydroxyapatite (HAp) which gives strength to the bone structure [1]. HAp is an inorganic mineral found in bone and teeth in the form of calcium phosphate as its major element. Approximately around 60% of inorganic HAp and other organic components of bone were packed together to form the structure [2]. The appearance of HAp in the bone commonly in nano structure which enhance its property and contribute to self-healing process of the bone. It has biocompatible properties that can contribute in biomedical and biodental applications [3].

Nano-hydroxyapatite (Nano-HAp) can be used in many applications especially for crucial fields such as medical and dentistry. It can be used in medical field for bone scaffold and drug delivery system due to its osteoconductivity and adsorptive activity [4]. There are a few researches investigated on development of Nano-HAp as a coating implant for dentistry application due to its bioactivity that mimicked the enamel structure of teeth [5, 6]. The unique ability of Nano-HAp has increased the demand for obtaining the material. There are many research lines has been modified to find the best method and structure for applications. The performance of Nano-HAp is strictly depending on the structure, size and shape of the material [7]. The morphological structure of HAp influences the mechanical properties and biological behaviour of the particles. Different size of HAp has different uses and applications due to different properties. The composition, crystallinity, morphology and particle size of HAp are the important characteristics which influence its performance and application [7]. Nano-HAp has higher absorption ability and low water solubility compared to conventional-sized HAp due to the large specific surface area [8]. The average size of 20 to 50 nm of Nano-HAp crystals has similar mechanical properties with the bone structure in bioresorbability [9]. Cui et al. [10] demonstrated that Nano-HAp with 20 nm size has a greater specific surface area and absorption ability compared to larger size of 80 nm. In another view, Reichert et al. [11] specified that Nano-HAp had a high capacity of ion exchange with heavy metals and it is followed by another process called ion adsorption mechanism. Ion exchange property of Nano-HAp plays a crucial role in remineralization of teeth and removal process of heavy metal ions. Jahan et al. [8] reported that HAp had successfully

*CORRESPONDING AUTHOR | N. F. Mohtar | ✉ fazliyana@umt.edu.my

reduced fluoride and cadmium ions in aqueous solution. This phenomenon is due to the excellent ion exchange and ion absorption performance of HAp. Pore size is one of the main factors which determined the absorption efficiency of HAp [12].

The extraction process of HAp can be classified into two categories which are natural and synthetic sources. The natural sources including fish bone [13], fish scale [14], bovine bone [15] and it can be extracted via heat treatment including calcination, thermal decomposition, subcritical water and hydrothermal processes. The natural extraction process is more preferable and attracted many research lines due to low-cost production, simple and less time-consuming [16]. Such sources of HAp (bone and shell) usually contain high biological elements that are absent in most of the chemically synthesized HAp [17]. There are a few studies had been using natural sources as the main method for HAp production. Some of the methods were modified and optimized depending on the source such as species, thus increasing the possibility of obtaining pure HAp. A study from Barakat et al. [15] demonstrated extraction of HAp from bovine bone via three different methods which were thermal decomposition, subcritical water and alkaline hydrothermal processes. They found that all of the investigated methods had successfully produced pure HAp with an average yield percentage of 65%. The structure of the HAp produced from the methods are varied and influenced by many factors such as temperature and holding time. They concluded that the HAp from thermal decomposition produced a good crystallinity compared to the other methods. Another study from Rujitanapanich et al. [18] demonstrated the uses of natural oyster shell as a starting material for HAp extraction. The extraction involved calcination process of the shell that produced calcium oxide (CaO). The CaO powder was later reacted with nitric acid and di-ammonium hydrogen phosphate at various temperatures. The chemical reaction of the HAp production is shown by equations below:

$$CaCO_3 \rightarrow CaO + CO_2 \tag{1}$$

$$CaO + HNO_3 \rightarrow Ca(NO_3)_2 + H_2O \tag{2}$$

$$10Ca(NO_3)_2 + 6(NH_4)2HPO_4 + 8NH_4OH \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 20NH_4NO_3 + 6H_2O \tag{3}$$

They concluded that the obtained HAp at pH 10 had a good crystallinity compared to the other conditions. Other than that, synthetic source of extraction can be synthesized via wet chemical [19–21], sol-gel [22] and spray drying [23]. The synthesized process usually required high cost due to the uses of chemical reagents for obtaining the HAp. Some of the methods are time-consuming and complicated for controlling the rate of production. However, most of the synthetic methods can easily control the structure and shape of the synthesized HAp. The wet chemical precipitation method had been demonstrated byAbidi and Murtaza [24] who used calcium hydroxide and phosphoric acid as precursors for the chemical reaction. The synthesized HAp was obtained from the following chemical reaction:

$$CaO + H_2O \rightarrow Ca(OH)_2 \tag{4}$$

$$10Ca(OH)_2 + 6H_3PO_4 \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 18H_2O \tag{5}$$

They added a point that the crystallinity, crystallite size and calcium-to-phosphorus (Ca/P) ratio of HAp were depending on the calcination temperature.

Development of different extraction methods is mainly to find the best one in terms of cost, duration and structure of HAp. The method of extraction plays a role in determining the structure and biocompatibility properties of the material. Natural extraction of HAp is commonly obtained from bone of bovine [15], fish [25] and porcine [26] origins through heat treatment. However, such sources (porcine and bovine) have an issue which decreases the interest of research compared to marine source.

The heat treatment of bone such as calcination is an effective method for the production of HAp from natural sources. The advantages of this method are less time consuming and required less chemicals with a low-cost production. Such common methods for the production of HAp (wet chemical precipitation, spray drying and sol-gel) are time-consuming and require high initial cost for the production of HAp. However, there are many considerations to be taken into account for heat treatment method such as heating profile of the raw material and optimum calcination temperature. There are many studies had been reported on the extraction of HAp using fish as an initial material [27–30], but none of the studies were carried out on the species of spotted sardinella (*Amblygastersirm*). The discovery of this new species of material for HAp extraction is needed to fulfil the gap of this research field. Besides, such heat treatment method (calcination) is a very common method for the production of HAp using biological sources [17, 31]. However, the optimization of the calcination temperature is still less discovered in terms of heating profile, yield percentage and colour. The relationship of these parameters was a main focus in this study for the optimization of the temperature. Therefore, the objectives of this present study aimed to determine the heating profile and optimize the best temperature for the extraction of Nano-HAp based on yield percentage and colour. In this paper, the extraction of Nano-HAp was carried out via calcination of fish bone (*Amblygastersirm*) using furnace (Carbolite ELF11/14B/301, UK) at different temperatures.

METHODS AND MATERIALS

Collection of Raw Materials

The raw materials were collected from the fish processing industry and identification of fish species was carried out by the help of taxonomist. A total amount of 100 kg of spotted sardinella (*Amblygaster sirm*) bone was collected and was stored in a freezer at -20°C. Standard hydroxyapatite was purchased from Sigma Aldrich (USA) and used as control.

Preparation of Raw Materials

Preparation of raw materials was carried out according to the method of Boutinguiza et al. [13]. The frozen raw materials were proceeded to bone separation process to remove the unwanted part. The raw materials were boiled for 1 hour and rinsed with tap water to remove adherent fish meat. The raw materials were dried in an oven for 24 hours at 60°C.

Extraction of Nano-hydroxyapatite

The extraction of Nano-HAp was carried out via calcination process according to the method of Boutinguiza et al. [13]. An amount of 2 g of dried bones was mounted into 50 ml porcelain crucible before preceeded to the calcination process. The bones were heated in a furnace (Carbolite ELF11/14B/301, UK) at temperatures ranged from 600 to 1000°C with a heating rate of 10°C min^{-1} for 5 hours and it was cooled isothermally for 3 hours. This part is a crucial process to ensure the reaction can occur properly for the formation of Nano-HAp. The calcined bones were stored in a desiccator containing silica gel to avoid moisture absorption into the samples. The calcined bones were proceeded to milling process to produce a fine Nano-HAp using ball-mill (Retsch PM 100, Germany). The milling method for production of Nano-HAp depends on the milling parameters, type of sample and temperature of calcination process. The milling parameters have been optimized based on the previous studies using different speed (300 rpm, 450 rpm) and different ball-to-powder ratio (6:1, 10:1, and 18:1) to obtain desired size. The milling process in this study involved the uses of 50 ml jar size and 7 units of balls (10 mm, 4 g). An amount of 4 g of calcined bones were mounted into the jar containing the balls with 28 g of total weight which is equivalent to 6:1 of ball-to-powder ratio. The jar was then placed and tightened on the jar holder of the milling instrument. The inclined guide rail was adjusted for balancing the counterweight of the milling device that suits the size of the jar. The milling parameter was set with 300 rpm of speed for 6 to 8 hours of holding time without interval. After milling, the obtained Nano-HAp was then being observed under Scanning Electron Microscope (SEM) to confirm the size. The extraction procedure of Nano-HAp is summarized in Figure 1.

Proximate Composition of Raw Bone

The proximate analysis of raw bone (RB) was conducted according to the method of AOAC [32]. It was done to determine protein, ash, moisture and lipid contents in initial RB powders. The proximate analysis was conducted for 3 replicates to get the average results.

Protein

A number of 0.2 g of RB was weighed and placed in the Kjeldahl digestion flask and was recorded as *W*. An amount of 5 ml of concentrated sulphuric acid (H_2SO_4) was mixed with a half tablet of catalyst in six test tubes except for blank. The test tube was shaken well and was placed on a test tube rack for heating before it was then be titrated with hydrochloric acid (HCL). The percentage of protein content was calculated according to the following formula (Eq.(6)):

$$Nitrogen\ (\%) = \frac{T-B\times N\times 14.007}{W\ 2}\times 100\% \quad (6)$$

where, Protein (%) = $N \times F$, F = Protein factor (6.25), T = Volume of HCL in titration (ml), N = Normality of HCL and W_2 = Weight of titrated sample (mg)

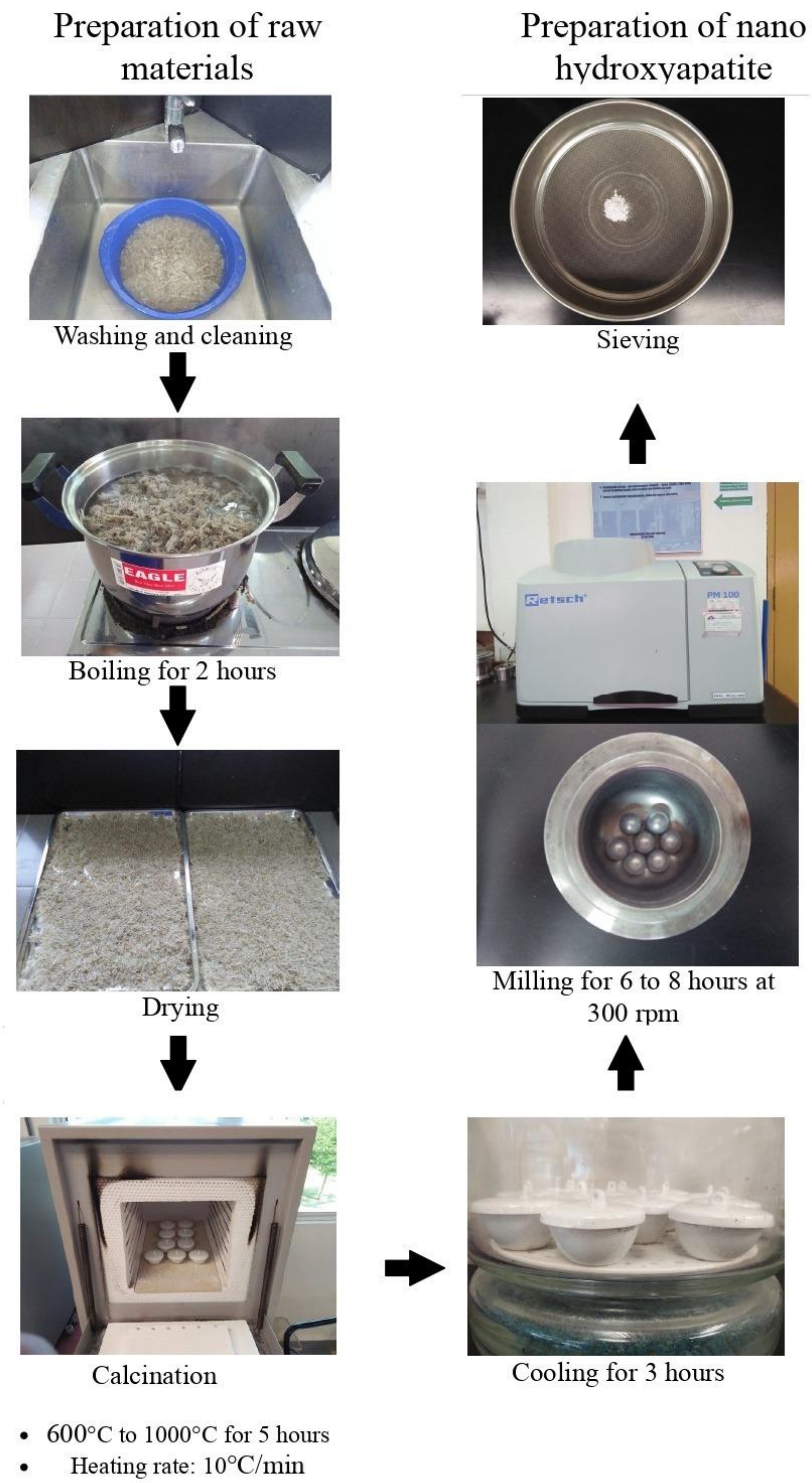

Figure 1. Summary of procedure for the extraction of Nano-HAp from spotted sardinella (*Amblygaster sirm*) bone

Ash

Crucible was dried in an oven for 1 hour at 100°C. The crucible was then being cooled in the desiccator for 30 mins. The crucible and lid were weighed to 4 decimal places and recorded as W_1. A number of 2 g samples were put into the crucible. The crucible with the sample was weighed and recorded as W_2. The sample were then heated in a muffle furnace for 3 hours at 600°C before it being cooled in dessicator. The percentage of ash was calculated based on the Eq.(7):

$$Ash\ (\%) = \frac{W3-W1}{W2-W1} \times 100\% \quad (7)$$

where, W_1 = Weight ofcrucible (g), W_2 = Weight of crucible + sample (g) and W_3 = Weight of crucible + ash (g)

Lipid

The extraction thimble was dried in an oven for 1 hour at 100°C. The extraction thimble was then cooled down in desiccator and the weight was recorded as *W1*. An amount of 2 g of RB was weighed and recorded as *W2*. The sample was placed inside the thimble before dried in oven for 2 hours at 100°C. The percentage of lipid content was calculated based on Eq.(8):

$$Lipid\ (\%) = \frac{W3-W1}{W2} \times 100\% \tag{8}$$

where, W_1 = Weight of extraction thimble (g), W_2 = Weight of the initial sample (g) and W_3 = Weight of extraction thimble + essential fats (g)

Moisture

The empty crucible and lid were dried in an oven for 1 hour at 100°C. The crucible was then cooled in desiccator and the weight was recorded as *W1*. A number of 2 g of the RB was weighed and placed inside the crucible (*W2*) before dried in oven for 6 hours. The moisture content of RB was calculated according to the Eq.(9):

$$Moisture\ (\%) = \frac{W3-W1}{W2-W1} \times 100\% \tag{9}$$

where, W_1 = Weight of crucible (g), W_2 = Weight of crucible + sample (g), W_3 = Weight of crucible + dry sample (g) and Moisture (%) = 100 - percentage of dried sample

Thermogravimetric Analysis

Thermogravimetric analysis (TGA) was carried out according to the method of Hossan et al. [33] with slight modification to determine the heating profile of RB before calcination; which measures the change in physical and chemical properties of the materials. It provides information on physical changes of RB during the heating process such as vaporization, melting point, sublimation, absorption desorption and decomposition. The heating profile information of RB is crucial for the extraction process of Nano-HAp. Thermal stability and weight loss of the bone was estimated using TGA. (Mettler Teledo TGA/ DSC 1, USA). The blank sample was run before the real analysis as a control. An amount of 40 mg of sample was mounted into tiny crucible before it was inserted into the furnace part of the TGA. The analysis was carried out for one hour with a heating rate of 20°C/ min applied in an oxygen atmosphere at 30 to 900°C. Percentage of weight loss was determined by the change of temperature. This heating profile is important to illustrate the decomposition of material and act as supporting information for calcination.

Percentage of Yield

The yield percentage (%) of Nano-HAp was calculated according to the Eq. (10) [34]:

$$Yield\ (\%) = \frac{WHAp}{WRB} \times 100\% \tag{10}$$

where, *WHAp* = Weight of extracted nano-hydroxyapatite (g) and *WRB*= Weight of raw bone (g)

Colour

The extracted Nano-HAp-1 with different temperatures was further evaluated based on colour measurement as a supporting analysis for the determination of the optimum temperature. Colour analysis of Nano-HAp was carried out to determine the whiteness level. The level of whiteness indicates the complete combustion of calcination and purity of Nano-HAp produced. The samples were mounted into a petri dish before analyzed using Chromameter (Konica Minolta CR-400, Japan). The measurement was carried out with proper equipment to avoid any possible errors. The chromameter was then firmly pressed onto the petri dish containing the samples. The data of the colour was recorded in *L a b* parameters. The reading of the colour value was taken three times for an average value. The *L a b* colour data was further calculated their whiteness level according to the following equation [35].

$$\Delta E = \sqrt{(L^* - L_0)^2 + (a^* - a_0)^2 + (b^* - b_0)^2} \tag{11}$$

Parameter L^* refers to the lightness of the sample. It ranges from black (L = 0) to white (L = 100). The negative value of parameter a^* indicates a green colour and a positive value indicates a red-purple colour. The positive value of parameter b^* indicates a yellow colour and a negative value indicates a blue colour. L_0, a_0 and b_0 indicate colour parameters of the

reference colour. White colour parameter (L^*= 100, a^*= 0, b= 0) was used as a reference to control the whiteness colour of the samples.

X-Ray Diffraction

X-ray diffraction (XRD) analysis was conducted according to the method of Bahrololoom et al. [36] with a slight modification. This analysis was carried out on RB, extracted nano-hydroxyapatite (HAp-1) and standard hydroxyapatite (HAp-2) to detect crystallinity phase and purity of HAp-1 compared to the initial RB and standard HAp-2. An amount of 0.1g of samples was mounted and flattened on a sample holder to minimize error during analysis. The samples were then placed into a diffractometer (RigakuMiniflex II) for 45 mins at 35kV. The angle (2-theta) was set with a range of 5 to 80° with a scan speed of 2.5° per mins. The raw data was stored in the computer and further analyze dusing search match software for the detection of crystallinity phase and identification of peaks formation.

Statistical Analysis

Analysis of Variance (one way ANOVA) and multiple comparisons (Posthoc test) were applied in this study at level of significance α= 0.05 (two-sided). Statistical analyses were performed using SPSS for Windows (version 22.0).

RESULTS

Percentage of Raw Materials

The raw materials used in this study were obtained from the fish processing industry. The major fish species used in the selected processing industry is spotted sardinella species based on the species identification process in the laboratory. The information of the raw materials was directly obtained from the owner of the industry. Figure 2, shows the percentage of the raw materials used in this study for the production of the nano-hydroxyapatite (Nano-HAp).

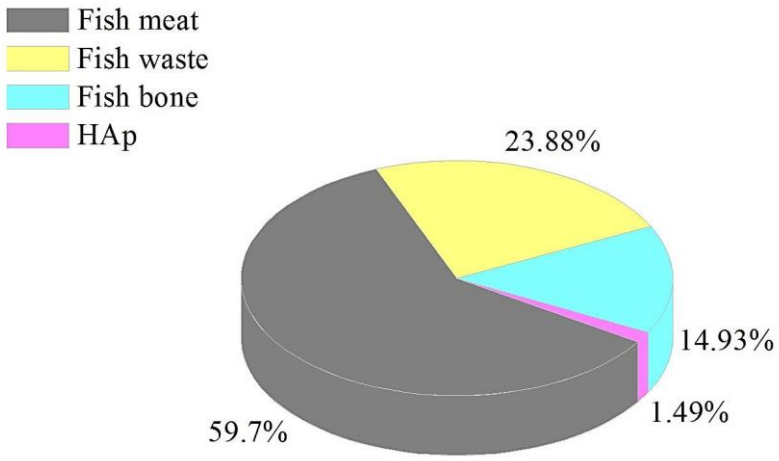

Figure 2. Percentage of the raw material used for the production of Nano-HAp from spotted sardinella (*Amblygaster sirm*) bone

Approximately 24% of fish waste was obtained from total weight of the fish. The fish waste was mixed with other parts of the fish such as skin, remaining flesh, bone, scale, fin and head. After the separation process of the unwanted parts, around 12% of fish bone was obtained and it was further cleaned, washed and boiled. This process is necessary to ensure a proper extraction process and purity of Nano-HAp. Therefore, it is approximately 1% of Nano-HAp was obtained from total weight of the fish for further analysis.

Proximate Composition of Raw Bone

Proximate content of RB was analysed to determine the protein, ash, moisture and lipid contents. Table 1 shows the percentage of proximate composition (%) of RB.

Table 1. Percentage of proximate composition (%) of RB of spotted sardinela (*Amblygaster sirm*)

Composition	RB (%)
Protein	1.08± 0.47
Ash	50.98± 0.69
Moisture	10.47±1.33
Lipid	3.78±1.04

*Values are given as mean ± SD from triplicate determinations.
*RB represents raw bone

The result demonstrated the percentage of protein, ash, moisture and lipid contents in RB. Based on the result, the protein, ash, moisture and lipid contents of RB were 1.08%, 50.98%, 10.47% and 3.78% respectively.

Thermogravimetric Analysis

Thermogravimetric analysis (TGA) was conducted to determine the heating profile of spotted sardinella (*Amblygastersirm*) bone prior to the calcination process. Figure 3 shows the percentage of weight loss of raw bone powder with an increment of temperature from 30°C to 900°C.

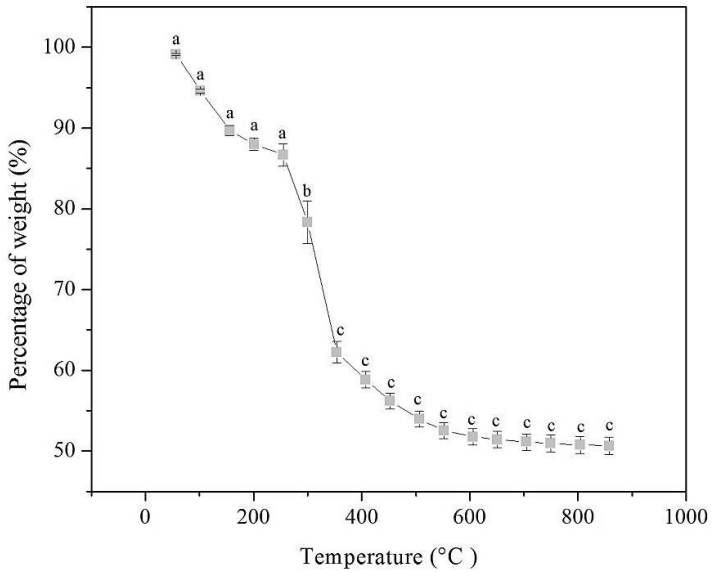

Figure 3. Percentage of weight loss (%) of raw bone (RB) with increment of temperature (°C)

*Values are given as mean ± SD for triplicate determinations.
*Values with the same superscript letters were not significantly different (p<0.05).
*RB represents raw bone

Yield

Yield percentage of extracted Nano-HAp was calculated to determine the optimum temperature for calcination. Table 2 shows the yield percentage of extracted Nano-HAp at different temperatures and colours.

Table 2. Yield percentage (%) of extracted Nano-HAp from spotted sardinella (*Amblygaster sirm*) bone obtained at different calcination temperatures (°C)

Temperature (°C)	Yield (%)	Colour
600	55.81± 0.28[a]	Yellowish white
700	55.06± 0.35[a]	White
800	53.18± 0.62[a]	Greyish white
900	52.9± 0.11[a]	Greyish white
1000	52.81± 0.39[a]	Bluish white

*Values are given as mean ± SD for triplicate determinations.
*Values with the same superscript letters were not significantly different (p<0.05).
*Nano-HAp represents nano-hydroxyapatite.

Based on the result, the average yield percentage obtained was 54.07%. The yield percentage of extracted Nano-HAp was influenced by calcination temperatures. Yield percentage decreased with the increasing of calcination temperature. Calcination temperatures at 600°C, 700°C, 800°C, 900°C and 1000°C produced yield of 55.76%, 54.95%, 53.27%, 53.30% and 53.08%, respectively.

Colour

The colour of extracted Nano-HAp was further analyzed to determine the colour parameter based on the L^* a^* b^* model. The colourcharacteristics were used to determine the quality of Nano-HAp using chromameter (Minolta CR-400, Japan). Data were stored in L^* a^* b^* model and colour change (ΔE) were calculated. The ΔE determines the whiteness of extracted Nano-HAp at different temperatures based on reference colour. The ΔE with the lowest value indicates that the colour is closer to white. Table 3 shows the colour parameters and ΔE of extracted Nano-HAp at different temperatures.

Table 3. Colour parameters and ΔE of Nano-HAp extracted from spotted sardinella (*Amblygaster sirm*) bone at different calcination temperatures

Temperature (°C)	Parameter of colour			Colour change (ΔE)
	L^*	a^*	b^*	
600	66.11 ± 1.61[a]	0.18 ± 0.24[a]	2.82 ± 0.30[a]	34.01 ± 1.59[a]
700	82.47 ± 1.53[b]	-0.23 ± 0.08[b]	2.91 ± 0.11[a]	17.94 ± 1.78[b]
800	76.93 ± 1.06[c]	0.30 ± 0.14[c]	3.27 ± 0.20[a]	23.31 ± 1.04[c]
900	73.51 ± 1.72[c]	-0.19 ± 0.12[d]	2.82 ± 0.28[a]	26.64 ± 1.73[c]

*Values are given as mean ± SD for triplicate determinations.
*Values with the same superscript letters were not significantly different (p< 0.05).
*L^*, a^* and b^* represents lightness, green-red/purple and yellow-blue respectively.
*ΔE represents colour changes.

Based on Table 3, calcination at 700°C shows the highest lightness (L^*) among others. Different temperature produced different lightness of Nano-HAp. There was a significant difference of L^* for Nano-HAp produced at 600°C, 700°C and 800°C. This occurrence could be due to the complete combustion of calcination at 700°C. The ΔE of Nano-HAp was calculated to determine the best temperature for calcination. Figure 4 indicates the ΔE of extracted Nano-HAp with different temperatures.

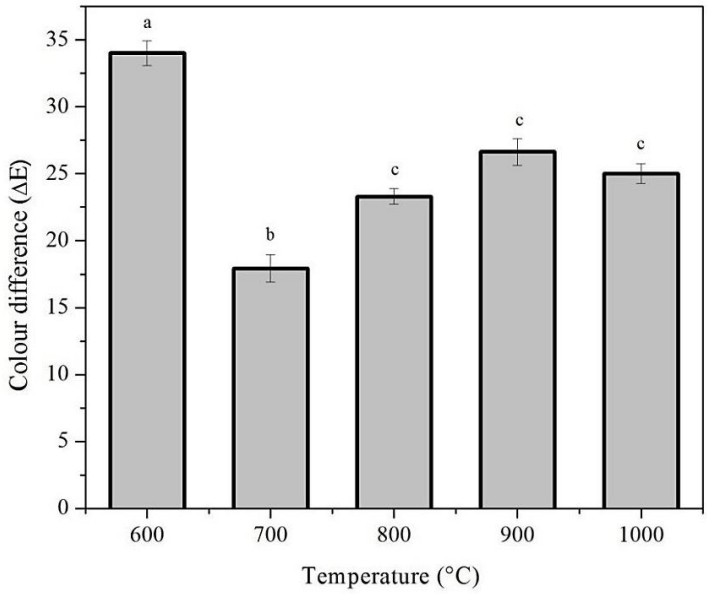

Figure 4. Colour changes (Δ*E*) of extracted Nano-HAp from spotted sardinella (*Amblygster sirm*)bone at different calcination temperatures

*Values are given as mean ± SD for triplicate determinations.
*Values with the same superscript letters were not significantly different ($p<0.05$).

Based on the result, the ΔE of extracted Nano-HAp was influenced by calcination temperatures. Calcination at 600°C, 700°C, 800°C, 900°C and 1000°C have shown the Δ*E* values of 33.96, 17.77, 23.30, 26.64 and 24.98, respectively.

X-Ray Diffraction

X-ray diffraction (XRD) was conducted to confirm the phase of crystallinity of RB, extracted Nano-hydroxyapatite (HAp-1) and standard hydroxyapatite (HAp-2). Figure 6 shows the XRD pattern of HAp-1 compared to the standard peak of HAp (PDF No: 1-1008). The crystallinity phase of the sample is an important characteristic to determinethe formation of hydroxyapatite crystal.

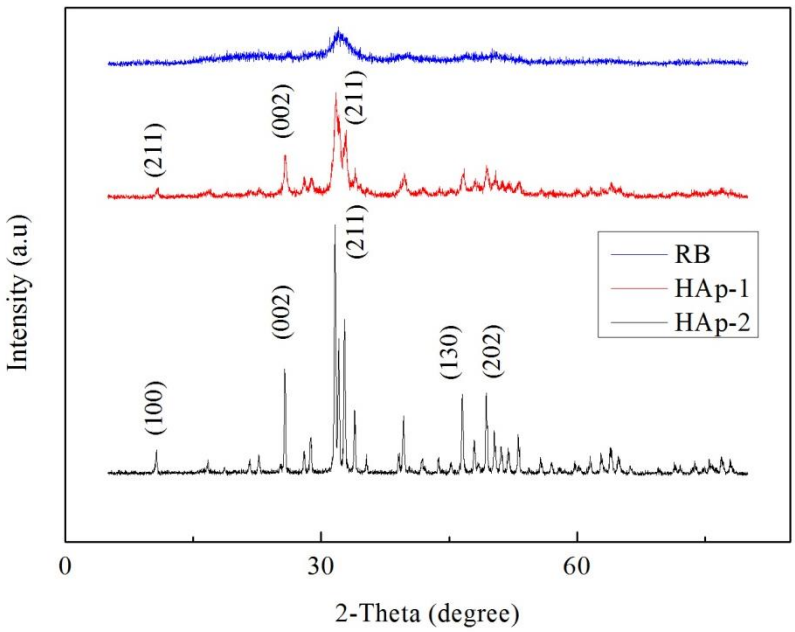

Figure 5. Comparison of XRD patterns of RB, HAp-1 and HAp-2for the confirmation of crystallinity phase of HAp

*RB represents raw bone.
*HAp-1 represents extracted Nano-hydroxyapatite.
*HAp-2 represents standard hydroxyapatite.

The plotted graph was further evaluated and compares with the standard peak of HAp using search match software for obtaining standard peak of HAp. The result demonstrated that the peak obtained at 2θ= 27.90º, 31.84º, 46.55º and 48.90 º were corresponding to the peak (002), (211); (130) and (202) respectively.The highest peak intensity demonstrated a clear resolved peak at 2θ= 31.84º which was similar to the peak of standard HAp.

DISCUSSION

Percentage of Raw Materials

The raw materials used for the extraction of Nano-HAp in this study can be considered as economical with the percentage of Nano-HAp obtained was 1%. It is in a good alignment with the section result of yield percentage of extracted Nano-HAp obtained in this study which was ranged from 52 to 55%. This finding is further supported by Muhammad et al. [37] who found that the yield percentage of extracted HAp from carp fish scale was 32% which is lower compared to this study. Therefore, the uses of spotted sardinella (*Amblygaster sirm*) bone is potentially produced a high-rate and low-cost production of Nano-HAp for many applications.

Extraction of Nano-hydroxyapatite

The extraction of Nano-HAp through heat treatment of bone was carried out at temperatures of 600, 700, 800, 900 and 1000°C for optimizing the method in obtaining pure hydroxyapatite (HAp). Such temperatures were followed based on previously described temperatures and were confirmed by thermogravimetric analysis (TGA) via heating profile result. The temperatures from 600 to 1000°C are the common range of calcination temperature for the formation of HAp with chemical formula $Ca_{10}(PO_4)_6(OH)_2$ through the decomposition of water, organic and other inorganic materials in the bone. The formation of the HAp in this study is shown by the reaction below (Eq. 12):

$$Ca_{10}(PO_4)_6(OH)_2 \leftrightarrow Ca_{10}(PO_4)_6(OH)_2 + H_2O \quad (12)$$

This formation phase of HAp can be obtained and consistent at the range of temperatures from 600 to 1000°C. The reaction changes into the formation of beta-tricalcium phosphate (ß-TCP) and tetratricalcium phosphate (TTCP) with the increase of calcination temperature from 1050°C and above [38]. The transition phase forß-TCP is shown by the following reaction (Eq. 13):

$$Ca_{10}(PO_4)_6(OH)_2 \rightarrow 2Ca_3(PO_4)_2 + 2Ca_4(PO_4)_2O + H_2O \quad (13)$$

However, the optimum temperature of the HAp extraction is varied depending on the source for obtaining the pure particles. The heat treatment process involves the removal of water, organic and inorganic materials contain in the raw bone (RB). This finding is in a good agreement with the findings in section thermogravimetric analysis (TGA) and colour which demonstrated about the hydrolysis of water. This finding is further aligned with finding from Sofronia et al. [38] who mentioned that the transformation phase of HAp from bone occurred at two stages of hydroxylation.

Proximate Composition

The proximate composition of RB in this study was found lower than the previous study by Petenuci et al. [39] who conducted the proximate analysis on tilapia fish bone. The differences might due to the different nutrient content of fish. The proximate composition is varied depending on species and degradation of nutrients. This finding is in good agreement with the heating profile of RB mention in TGA section, which demonstrated that percentage of weight loss at the first inflection point was 11.5% due to moisture content, while the percentage of weight loss of RB at the second inflection point was 36.5% due to the degradation of organic materials including protein, ash and lipid.

Thermogravimertric Analysis

Based on the TGA result, two inflection points were observed. The first inflection point was found from 110°C to 320°C with a percentage weight loss of 11.5%. This may be due to the removal of excessive water contained in the raw bone [14, 33]. This finding is supported by Sutapa et al. [40] who found out that the first inflection point of tuna bone was due to the incomplete drying of water. This finding is further supported by Mondal et al. [41] who demonstrated that the first inflection point of roholabeo fish scale was due to the further degradation of water content.

The second inflection point was found from 320°C to 610°C with a percentage of weight loss of 36.5%. This could be due to the complete combustion and decomposition of other organic materials in the bone [38]. This finding is agreed by Sutapa et al. [40] who found a similar result on the final inflection point of tuna bone powder between temperatures of 300°C to 500°C. This indicates the removal of organic materials such as collagen, protein and fat. Such finding is similarly reported by Mondal et al. [41] who stated that the second and third inflection points of fish scale were due to

the burning of organic materials. The percentage of weight loss of raw bone did not significantly change at temperatures ranged from 600°C to 1000°C. The phenomenon indicates that the thermal stability and complete decomposition point of the bone [38, 42, 43]. The high heating temperature had caused denaturation of some organic materials including protein and collagen, thus decomposed the structure due to the breaking of the hydrogen bond. The finding suggested that the decomposition of organic materials in RB of spotted sardinella (*Amblygaster sirm*) occurred at a temperature of 600°C based on the heating profile. Therefore, this analysis provided supporting data for conducting the calcination process of the bone at temperature ranged from 600°C to 1000°C. This finding is in good agreement with the result section of yield percentage which demonstrated that there is no significant difference in yield percentage of extracted nano-hydroxyapatite (HAp-1) for all temperatures. It indicates that the calcination temperatures were stable with a minor decomposition of materials. The heating profile of RB indicates the relevant range of temperature and acts as a supporting analysis for calcination.

Yield

The result shows that yield percentage of extracted Nano-HAp obtained has lower yield compared to the bovine bone (65%) [15]. However, it was found to have higher yield compared to tuna bone hydroxyapatite (HAp) which ranged from 41.84% to 47.59% with different temperatures [40]. Calcination process at 600°C produced the highest yield (55.76%) which turned the colour to greyish white. This phenomenon could be due to the incomplete removal of organic materials and low purity of HAp produced.

The decrease of yield percentage was influenced by the removal of water and other organic materials during the calcination [40]. Based on the statistical analysis (ANOVA, α= 0.05 and Turkey's test α= 0.05) showed that the calcination temperatures did not statistically decrease the obtained yield percentage of extracted Nano-HAp. There is no significant yield percentage observed between calcination temperatures of 600°C to 1000°C due to the complete removal of excess water and other organic materials below 600°C. HAp was the only compound left at calcination temperature of 600°C. The spotted sardinella (*Amblygaster sirm*) bone had achieved thermal stability at temperature ranged from 600°C up to 1000°C. This finding is supported by Prasad et al. [14] who stated that the weight loss of HAp did not significantly decreased between temperature of 430°C to 1000°C due to complete removal of organic materials and thermal stability. Therefore, calcination of spotted sardinella (*Amblygaster sirm*) bone between 600°C up to 1000°C did not significantly decrease the yield percentage of extracted Nano-HAp. The high yield production of HAp may be due to the high composition of calcium (Ca) and phosphorus (P) elements and low protein in the raw materials. The finding on the protein content was in good alignment with the previously mentioned in result section of proximate for RB which shows the percentage of proximate content was found to be lower than the previous study conducted by Petenuci et al. [39]. The composition of organic and inorganic elements is the key characteristics for the extraction of HAp for determination of optimum temperature. This finding indicates that the yield percentage of Nano-HAp extracted at the optimum temperature (700°C) is still economical compared to the other previous studies.

Colour

Based on the result, calcination at 700°C produced the lowest ΔE (17.77) which indicates the colour is closer to white. The calcination temperatures had significantly decreased the ΔE of extracted Nano-HAp. The value of ΔE were varied significantly depending on temperatures at a critical value, $F(4, 10) = 44.345$ at $p < 0.05$. The mean difference between the five temperatures was significantly different for the ΔE. This phenomenon could be due to the different elemental content on each temperature. The extracted Nano-HAp with slight grey colour of ΔE (34.01) indicates a small amount of other organic matters content in the bone. This indicates that the organic matters were not completely removed at calcination temperature of 600°C. Similar study conducted on tuna bone HAp that was calcined at 600°C demonstrated gray in colour and low purity [40]. Extracted Nano-HAp obtained at 700°C depicted the high level of white colour compared to others with ΔE (17.94). This finding suggested that the complete removal of organic materials and the optimum temperature had been achieved. Figure 5 shows the comparison of colour of bones heated at different temperatures.

Figure 5. Comparison of colour of spotted sardinella (*Amblygaster sirm*) bone heated at different calcination temperatures

*A= Heated at 600°C, B= Heated at 700°C, C= Heated at 800°C, D= Heated at 900°C, E= Heated at 1000°C.

The extraction of Nano-HAp involved heat treatment at a high temperature which commonly produces a lighter colour of the heating product due to the absorption of light. This phenomenon is due to the condensation and degradation of the colour during the heating process. However, the heat treatment process of material at high temperature in oxygen atmosphere had caused the hydrolysis reaction occurred which produced a yellow colour [44]. The result indicated that the colour of extracted Nano-HAp varied depending on the calcination temperature. This finding is similar to the variation of the colour for the exracted Nano-HAp which variants from yellow, grey to white colour. The hydrolysis reaction of material is in good alignment with the results previously mentioned in proximate and TGA sections; which show the decomposition of moisture of raw bone (RB).

X-Ray Diffraction

Low crystalline of HAp phase produced at the peak of (211) and (002) that indicated polycrystallite of HAp particle [41].This finding is further supported by Venkatesan and Kim [45], who found similar sharp peak intensity and clear resolved peaks show the crystalline phase of HAp. Besides, the increase of peak intensity was influenced by the temperature used during calcination which involves the removal of other crystalline composition, thus increase the crystallinity phase of HAp. The peak pattern of XRD is determined by the diffraction pattern of the incident beam at the crystal plane of materials which then transmitted and recorded by the means of peak [46]. The peak pattern of XRD is commonly used to identify the crystal structure of materials such as HAp whether amorphous, polycrystallite or crystallite. This finding is in a good alignment with previous study reported by Ahmed et al. [31]who demonstrated that the crystallinity phase of the HAp produced at peak (211) and (002). They added a point that the crystallinity of the HAp increase with the increasing of calcination temperature due to the crystal growth. A lower crystallinity peak of HAp-1 may be influenced by the calcination temperature. This finding indicates the confirmation for the formation of HAp crystalline phase on the HAp-1. The XRD pattern of HAp-1 in this study was in appropriate and comparable with the international standard (ISO 13779-3, ISO 13175-3) [20, 47, 48].

CONCLUSIONS

The proposed method for the extraction of nano-hydroxyapatite (Nano-HAp) from spotted sardinella (*Amblygaster sirm*) bone has been successfully conducted at the optimum temperature based on heating profile, yield and colour analyses. The present study concluded that:

1. The decomposition of water occurred at the inflection point of 110°C to 320°C, while the denaturation of inorganic protein and decomposition of lipid occurred at the inflection point of 320°C to 610°C which determined by heat profile of the raw bone (RB).
2. The yield percentage of Nano-Hap extracted at 700°C was 55% with colour change (ΔE= 18) that indicate the optimum calcination temperature.

3. Based on the heating profile and colour changes value (ΔE), calcination process of spotted sardinella (*Amblygaster sirm*) bone can be conducted at temperature ranging from 600°C to 1000°C due to its melting point.
4. The optimum temperature (700°C) could be used for further study and contributes to the development method of hydroxyapatite (HAp).

ACKNOWLEDGMENTS

The authors would like to acknowledge the Talent and Publication Enhancement Research Grant (TAPE-RG) [Vot. No 55273] for funding the research. The authors would also like to extend the acknowledgment to the Faculty of Fisheries and Food Science, Faculty of Ocean Engineering Technology and Informatics, Institute Oceanography and Environment and Centre of Research and Field Work Services of Universiti Malaysia Terengganu (UMT) for providing access to facilities and materials for completing the experiments.

REFERENCES

[1] S. Dorozhkin. "Self-setting calcium orthophosphate formulations. Journal of Functional Biomaterials", vol 4, no. 4, pp 209–311. 2013.

[2] X. Feng. "Chemical and biochemical basis of cell-bone matrix interaction in health and disease". Current Chemical Biology. vol 3, no. 2, pp 189–196, 2009.

[3] SMM, Rafie, and D. Nordin. "Synthesis and characterization of hydroxyapatite nanoparticle". Malaysian Journal of Analytical Sciences, vol 21, no. 1, pp 136 – 148, 2017.

[4] K. Loku. "Hydroxyapatite ceramics for medical application prepared by hydrothemal method". Phosphorus Research Bulletin, vol 23 pp 25-30, 2019.

[5] J-H. Jung, S-Y. Kim, Y-J. Yie, B-K. Lee and Y-K, Kim. "Hydroxyapatite-coated implant: Clinical prognosis assessment via a retrospective follow-up study for the average of 3 years". Journal of Advanced Prosthodontics, vol 10, no. 2, pp 85–92, 2018.

[6] K. Kuroda, and M. Okido. "Hydroxyapatite coating of titanium implants using hydroprocessing and evaluation of their osteoconductivity", Bioinorganic Chemistry and Applications, no. 12, pp 730-693, 2012.

[7] RX. Sun, Y. Lv, YR. Niu XH. Zhao DS. Cao J. Tang and KZ. Chen. "Physicochemical and biological properties of bovine-derived porous hydroxyapatite/collagen composite and its hydroxyapatite powders", Ceramics International, vol 43 no. 18 pp 16792–16798, 2017.

[8] AS. Jahan, MYA. Mollah, S. Ahmed, and AH. Susan. "Nano-hydroxyapatite prepared from eggshell-derived calcium-precursor using reverse microemulsions as nanoreactor". Materials Today: Proceedings, vol 4, no. 4, pp 5497–5506, 2017.

[9] Z. Shi, X. Huang, Y. Cai, R. Tang and D. Yang. "Size effect of hydroxyapatite nanoparticles on proliferation and apoptosis of osteoblast-like cells", ActaBiomaterialia, vol 5, no. 1, pp 338–345, 2009.

[10] X. Cui, T. Liang, C. Liu, Y. Yuan, and J. Qian. "Correlation of particle properties with cytotoxicity and cellular uptake of hydroxyapatite nanoparticles in human gastric cancer cells", Material Science Engineering C, vol 67, pp 453–460, 2016.

[11] J. Reichert and JGP. Binner. "An evaluation of hydroxyapatite-based filters for removal of heavy metal ions from aqueous solutions", Journal of Materials Science, vol 31, pp 1231-1241, 1996.

[12] T. Nagasaki, F. Nagata, M. Sakurai and K. Kato. "Effects of pore distribution of hydroxyapatite particles on their protein adsorption behavior", Journal of Asian Ceramic Societies, vol 5, no. 2, 88–93, 2017.

[13] M. Boutinguiza, J. Pou, R. Comesaña, F. Lusquiños, A. de Carlos and León B. "Biological hydroxyapatite obtained from fish bone", Materials Science and Engineering, vol 32, no. 3, pp 478–486, 2012.

[14] A. Prasad, SM. Bhasney, MR. Sankar and V. Katiyar. "Fish scale derived hydroxyapatite reinforced poly (lactic acid) polymeric bio-films: possibilities for sealing/locking the internal fixation devices", Materials Today: Proceedings, vol 4, no. 2, pp 1340–1349, 2017.

[15] NAM. Barakat, MS. Khil, AM. Omran, FA. Sheikh and HY. Kim. "Extraction of pure natural hydroxyapatite from the bovine bones bio waste by three different methods", Journal of Materials Processing Technology, vol 209, no. 7, pp 3408–3415, 2009.

[16] MD. Adak and KM. Purohit, "Synthesis of nano-crystalline hydroxyapatite from dead snail shells for biological implantation", Trends in Biomaterials and Artificial Organs, vol 25, no. 3, 101–106, 2011.

[17] W. Khoo, FM. Noor, H. Ardhyananta and D. Kurniawan. "Preparation of natural hydroxyapatite from bovine femur bones using calcination at various temperatures", Second International Materials, Industrial, and Manufacturing Engineering Conference, vol 2, pp 196 – 201, 2015.

[18] S. Rujitanapanicha, P. Kumpapan and P. Wanjanoi. "Synthesis of hydroxyapatite from oyster shell via precipitation", Energy Procedia, vol 56, 112-117, 2014.

[19] A. Zanotto, ML. Saladin, DC. Martino and E.Caponetti. "Influence of temperature on calcium hydroxyapatite nanopowders". Advances in Nanoparticles, vol 1, no. 3, 21–28, 2012.

[20] J. Kmaieniak, E. Bernalte, C. Foster, A. Doyle, P. Kelly and C. Banks. "High yield synthesis of hydroxyapatite (HAp) and palladium doped HAp via a wet chemical synthetic route", Catalysts, vol 6, no. 8, pp 119-134, 2016.

[21] A. Yelten-Yilmaz and S. Yilmaz. "Wet chemical precipitation synthesis of hydroxyapatite (HA) powders", Ceramics International, vol 44, no. 8, pp 9703–9710, 2018.

[22] K. Agrawal, G. Singh, D. Puri and S. Prakash. "Synthesis and characterization of hydroxyapatite powder by sol-gel method for biomedical application", Journal of Minerals and Materials Characterization and Engineering, vol 10, no. 8, 727-734, 2011.

[23] G, Ruphy, A. Saralegi, JC. Lopez, MM. Dias, MF. Barreiro. (2016). "Spray drying as a viable process to produce nano-hydroxyapatite/chitosan (n-HAp/CS) hybrid microparticles mimicking bone composition", Advanced Powder Technology, vol 27, no. 2, pp 575–583, 2011.

[24] SS.Abidi, and Murtaza Q. "Synthesis and characterization of nano- hydroxyapatite powder using wet chemical precipitation reaction", U.P.B. Scientific Bulletin Series B, vol 75, no. 3, pp 3-12, 2014.

[25] J. Venkatesan, B. Lowe, P. Manivasagan, KH, Kang, EP. Chalisserry, S, Anil and SK. Kim. "Isolation and characterization of nano- hydroxyapatite from salmon fish bone", Materials, vol 8, no. 8, pp 5426–5439, 2015.

[26] PK,. Wlodarski, K. Haberko, M. Haberko, A. Pyda and KH. Wlodarski. "Implantation of natural hydroxyapatite from porcine bone into soft tissues in mice", Folia biologica, vol 53, no. 3-4, pp 183-187, 2005.

[27] B. Komur, E. Altun, MO. Aydogdu, D. Bilgiç, H. Gokce, N. Ekren, S. Salman, AT. Inan, FN. Oktar and O. Gunduz . "Hydroxyapatite synthesis from fish bones: atlantic salmon (Salmon salar)", ActaPhysicaPolonica Series a, vol 131, no. 3, pp 400-403, 2017.

[28] SMH. Dabiri, AA. Rezaie, M. Moghimi and H. Rezaie. "Extraction of hydroxyapatite from fish bones and its application in nickel adsorption", BioNanoScience, vol 8, no. 3, pp 823–834, 2018.

[29] S. Hamzah, NI. Yatim, M. Alias, A. Ali, N, Rasit and A. Abuhabib. "Extraction of hydroxyapatite from fish scales and its integration with rice husk for ammonia removal in aquaculture wastewater", Indonesian Journal of Chemistry, vol 19, no. 4, pp 1019 – 1030, 2019.

[30] SA. Karim, ASM. Asri, S. Mamat, M. Mohamed, NAM. Shohaimi, AZA. Halim, NM. Shukri and NH. Abdullah. "Synthesis and characterization of hydroxyapatite powder from fish bones and scales using calcination method", International Journal of Advanced Science and Technology, vol 28, no. 18, pp 82-87, 2019.

[31] YMZ. Ahmed, SM. El-Sheikh and ZI. Zaki. "Changes in hydroxyapatite powder properties via heat treatment", Bulletin of Materials Science, vol 38, no. 7, pp 1807–1819, 2015.

[32] AOAC. "Official methods of analysis" (17th edition) 2000. Washington, DC: Association of Official Analytical Chemists.

[33] J. Hossan, MA. Gafur, MR. Kadir and M. Mainul. "Preparation and characterization of gelatin- hydroxyapatite composite for bone tissue engineering.", International Journal of Engineering and Technology, vol 14, no. 1, pp 24-32, 2014.

[34] I. Ratnasari and Firlianty. "Physico-chemical characterization and skin gelatin rheology of four freshwater fish as alternative gelatin source", International Journal of Bioflux, vol 9, no. 6, pp 1196-1207, 2016.

[35] PB. Pathare, UL. Opara and FAJ. Al-Said. "Colour measurement and analysis in fresh and processed foods: A Review", Food and Bioprocess Technology, vol 6, pp 36–60, 2013.

[36] ME, Bahrololoom, M. Javidi, S. Javadpour and J. Ma. "Characterisation of natural hydroxyapatite extracted from bovine cortical bone ash", Journal of Ceramic Processing Research, vol 10, no. 2, pp 129–138, 2009.

[37] N. Muhammad, Y. Gao, F. Iqbal, P. Ahmad, U. Nishan, A. Rahim, G. Gonfa and Z. Ullah. "Extraction of biocompatible hydroxyapatite from fish scales using novel approach of ionic liquid pretreatment", Separation and Purification Technology, vol, 161, pp 129- 135, 2016.

[38] AM. Sofronia, R. Baies, EM. Anghel, CA. Marinescu and S. Tanasescu. "Thermal and structural characterization of synthetic and natural nanocrystalline hydroxyapatite", Materials Science andEngineering C, vol 43, pp 153–163, 2014.

[39] ME. Petenuci, FB. Stevanato, JEL. Visentainer and M. Matsushita. "Fatty acid concentration, proximate composition, and mineral composition in fishbone flour of nile tilapia", Archivos Latinoamericanos De Nutricion, vol 58, no. 1, pp 87-90, 2008.

[40] IW. Sutapa, Romawaty and A. Bandjar. "Synthesis Ca3(PO4)2 from tuna fish bone and potential as a catalyst in the transesterification reaction for biodiesel production I", Journal of Chemical and Pharmaceutical Research, vol 8, no. 8, pp 596–604, 2016.

[41] S. Mondal, B. Mondal, A. Dey and SS. Mukhopadhyay. "Studies on processing and characterization of hydroxyapatite biomaterials from different bio wastes", Journal of Minerals and Materials Characterization and Engineering, vol 11, no. 1, pp 55–67, 2012.

[42] A. Pal, S. Paul, A. Roy, V. Krishna, M. Das and A. Sinha. "Synthesis of hydroxyapatite from Lates calcarifer fish bone for biomedical applications", Materials Letters, vol 203, pp 1–4, 2017.

[43] VR. Sivaperumal, R. Mani, MS. Nachiappan and K. Arumugam. "Direct hydrothermal synthesis of hydroxyapatite/aluminananocomposite", Materials characterization, vol 134, 416–421, 2017.

[44] Y, Chen, Y. Fan, J. Gao and NM. Stark. "The effect of heat treatment on the chemical and color change of black locust (Robiniapseudoacacia) wood flour", Bioresources, vol 7, no. 1, pp 1157-1170, 2012.

[45] J. Venkatesan and SK, Kim. "Effect of temperature on isolation and characterization of hydroxyapatite from tuna (Thunnusobesus) bone", Materials, vol 3, no. 10, pp 4761-4772, 2010.

[46] AA. Bunaciu, EG. Udriştioiu and HY. Aboul-Enein. "X-ray diffraction: Instrumentation and applications", Critical Reviews In Analytical Chemistry, vol 45, no. 4, pp 289–299, 2015.

[47] KA. Gross, CC. Berndt, P. Stephens and R. Dinnebier. “Oxyapatite in hydroxyapatite coatings”, Journal of Materials Science, vol 33, pp 3985–3991, 1998.

[48] M. Sari and Y. Yusuf. “Synthesis and characterization of hydroxyapatite based on green mussel shells (Perna viridis) with calcination temperature variation using the precipitation method”, International Journal of Nanoelectronics and Materials, vol 11, no. 3, pp 357-370, 2018.