INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 11, No. 3, p. 20-30, 2017

RESEARCH PAPER OPEN ACCESS

# Extraction and characterization of hydroxyapatite from bovine cortical bone and effect of radiation

**Md. Masud Rana, Naznin Akhtar, Md. Shaifur Rahman, Md. Zahid Hasan, S.M. Asaduzzaman***

*Institute of Tissue Banking and Biomaterial Research, Atomic Energy Research Establishment, Dhaka, Bangladesh*

**Key words:** Bovine bone; Annealing; Hydroxyapatite; Radiation.

http://dx.doi.org/10.12692/ijb/11.3.20-30 Article published on September 14, 2017

## Abstract

The demands for the use of artificial materials in the biomedical application have increased significantly due to the limited accessibility of natural bone for grafting. To fulfill the demand, natural biomaterials play vital roles in the design and production of biocompatible matrix that is biomimetics, elucidating specific cell functions, allowing cell-cell interactions and tissue regeneration. Hydroxyapatite (HA) is a natural biomaterial, not only a biocompatible, osteoconductive, osteoinductive, non-toxic, non-inflammatory and non-immunogenic agent, but also bioactive andhas got the ability to form a direct chemical bond with living tissues and promote tissue growth. In this study, natural HA has been extracted from bovine cortical bones using a cost effective and ecofriendly way. Shortly, bovine cortical bones were annealed at different temperature ranging from 650°C to 1250°C in an air atmosphere. The powder was characterized by thermo-gravimetric analysis (TGA), x-ray diffraction (XRD), flourier transformed infrared (FTIR) spectroscopy and scanning electron microscopy (SEM) analysis. The TGA result of raw bone indicated the presence of organic compounds which was removed upon annealing above 650°C. SEM analysis demonstrated that, the average particle size of extracted HA was 255.28 ±63 nm.XRD analysis confirmed that HA was the only crystalline phase when sintered at 950°C.Above this temperature HA was decomposed into β-TCP and α-TCP. After radiation the extracted HA, it was revealed that there were not any significant alteration of the properties of HA. It may be a good complement in different biomedical application.

* **Corresponding Author:** S.M. Asaduzzaman ✉ sikderasad@yahoo.com

**Introduction**

The demands for the use of artificial materials in the biomedical application have increased significantly due to the limited accessibility of natural bone for grafting. After fracture the ability of self-repairing of bone is limited, according to the extent of the damage. Small fractures are usually able to heal perfectly, but larger fractures can leave permanent damage (Dimitriou *et al.,* 2011, Pilia*etal.,* 2013).Although autogenous bone is most preferred for the treatment of bone defects, there are disadvantages and risks involved in using autogenic bone such as limited harvest due to insufficient donors, post-operative pain, increased blood loss, secondary surgical wounds and risk of thrombosis (Gomes *etal.,* 2012; Pandit*etal.,*2012; Reichert *et al.,* 2011). Allograft bone could overcome the above limitations, but it bears the risk of transmission of infection (e.g. HIV, Hepatitis etc.) (Giedraitis *et al.,*2014;Greenwald *etal.,*2012;Ooi *et al.,*2007). Xenogenous bone is morphologically and structurally similar to human bone, another possible alternative for treatment of bone defects. It is usually derived from bovine origin and easy to obtain, lower cost and available in unlimited supply. At the material level, bovine bone is composed of organic and inorganic components. The organic part contains mainly collagen and proteins, whereas the inorganic component is mainly hydroxyapatite (HA) with a small percentage of other elements being incorporated in the structure such as carbonate, magnesium and sodium etc.(Akram*et al.,*2011;Miculescu *et al.,* 2014).HA is not only a biocompatible, osteoconductive, osteoinductive, non-toxic, non-inflammatory and non-immunogenic agent, but also bioactive, i.e. It has got the ability to form a direct chemical bond with living tissues and promote tissue growth, which makes it the material of choice in orthopedic and dental applications (Dhandayuthapani *et al.,*2011;Fahmy *et al.,*2016;Thrivikraman *et al.,*2014).

To produce biologically preferable HA and avoiding the sophisticated procedures used in the synthesizing of HA, some researchers have turned into extraction of natural HA from bio-wastes (usually via calcination). Extraction of HA from the bio-wastes is a biologically safe (no foreign chemicals are utilized) and economically desirable process especially with the increasing world demand of HA bioceramics.

HA can also be isolated from different bio-wastes, such as- bovine sources (Barakat *et al.,* 2009;Sofronia *et al.,*2014), bones and teeth of pig (Xiaoying *et al.,*2007), corals(Chattopadhyay*et al.,*2007), eggshells (Rahman *et al.,*2014, Wua *et al.,*2016), ostrich eggshells(Ferreira*et al.,*2014), fish scales (Kongsri *et al.,*2013, Panda *et al.,*2014), fish bone(Venkatesan*et al.,*2010, Venkatesan *et al.,*2011), *Latescalcarifer*(Zainon *et al.,*2012), cuttlefish bone (Kim *et al.,*2014), cat fish bone (Chattanathan *et al.,*2013)and cod fish bone(Piccirillo *et al.,*2014) etc. Nowadays,manylabs extractHA from different sources and use it for bone tissue engineering application. None of them were not study about the effect of radiation in extracted HA from bovine bone.

The main objective of our studywas to find out an available source for extraction of HA in aspect of our country and sterilization it by gamma radiation.So if we extract HA from bovine cortical bone that will be economically supportive for us. At the same time, if the properties of HA is stable in irradiation, than it will be cost effective process for sterilization of HA than other process.

**Material and method**

*Material*

Cortical bone (femur, tibia, fibula, humerus, radius and ulna) of adult bovine (~ 2–3 years old) was procured from local slaughtering house (Bolivadra bazaar, Savar, Dhaka, Bangladesh). All the long bones were cleaned to remove visible adhered impurities and substances such as joint cartilage, ligaments and soft tissues stuck on the bone using bp blade no 22. Then the bones were cut at the metaphysis using electric band saw (AEW, 250) and the diaphysis of the bones was taken for HA extraction.

*Bone preparation*

Bone samples were boiled in deionized water for

about 8 hours for easy removal of the bone marrow and tendons. Then the bones were cut into small pieces (~10 mm× 5 mm×5 mm). After that the bone has been deproteinized by boiling in water. The boiled bone samples were dried overnight at 100°C in order to avoid soot formation on the surface of the material during the heating treatment.

*Thermal Decomposition*

The as received dried bone samples were annealed in an electric furnace (Borel, 1600, Swetzarland), under ambient condition at four different temperatures ranging from 650 to 1250°C, using a heating rate of 5°C/min with 3 hours holding time consequentially as multi stage sintering. The sintered product was crashed with mortar & pestle and sieved with mesh no. 200.After that samples were irradiated at 25 kGy for sterilization.

*Characterization Methods*

For characterization of as-prepared HA, phase purity analysis, particle size determination and microstructural analysis were performed using X-ray diffraction (XRD) and scanning electron microscopy (SEM) subsequently.The phase and crystallinity of HA were evaluated using X-ray diffractometer (X'Pert PRO PW 3040), Cu-Kα radiation with wave length 1.78896 Å and over a range 2θ from 10° to 70° angle, step size 0.02/s, with 40 kV voltages and 30 mA current.

The XRD pattern were analyzed and compared with "X"pert high score and "X'Pert plus" software ("XpertHighscore" File No. 01-086-0740) to identify the phase. Mass loss pattern during heating were studied by using a TGA analyzer (TGA analyzer, Model Q600, USA). The thermo-gravimetric analysis of the bone samples during heating were recorded from 30°C to 800°C at a heating rate of 10°C/min in a continuous flow of nitrogen. The stretching frequencies of samples were examined by FTIR analysis (FTIR 8400S, Shimadzu spectrophotometer, Japan) in the range 4000-400cm$^{-1}$. Morphology of HA crystals was obtained by Scanning electron microscopy (JSM 6490LA, Jeol, Japan).

**Results and discussion**

*Calcinations temperature determination*

The TGA result, Figure-01 shows that the TGA curve reaches balance at 600°C. It indicates that the organic substances in bone removed fully. Based on TGA result, 650°C was chosen as the minimum temperature to anneal the sample as multi stage annealing. At the same time, to determine the processing period weight loss, samples were annealed at different temperature ranged from 200 to 1250°C in variable interval.

**Table 1.** Residues and color of annealed Bovine Bone.

| Temperature (°C) | % of weight loss | Color |
|---|---|---|
| Raw Bone | n/a | Yellowish white |
| 200 | 13.07 | Yellow |
| 250 | 21.20 | Light yellow |
| 500 | 25.64 | Black |
| 650 | 29.52 | Gray |
| 750 | 33.51 | Off white |
| 850 | 34.71 | White |
| 950 | 34.86 | White |
| 1050 | 35.34 | White |
| 1250 | 37.14 | Snow white |

*General Observation*

In this study, HA was extracted from bovine cortical bone by multi stage annealed at different temperature sequentially. About 65% HA was extracted from this process, similar to reported studies(Bahrololoom*aet al.,* 2009). During annealed at different temperature, the percentage of weight loss and color change of bovine bone are shown in Table-01. After annealing at

different temperatures, the color of the bone was changed due to removal of organic portion. The color of the raw bovine bone was observed as yellowish white, which was consequently altered into yellow, light yellow, black and gray at 200°C, 250°C, 500°C and 650°C temperatures respectively. The color of the bovine bone was turned into white with further increase in the temperature. The different colorwas observed below 800°C, revealed the association of the organic matrix within the bone. Therefore, it can be inferred that about ~35 % of total weight loss was due to removal of water and organic substance from the bovine bone when annealed up to about 850°C.

**Table 2.** Position of XRD peaks of HA annealed at 950 °C, the results are compared with the standard HA ("XpertHighscore" File No. 01-086-0740)**.**

| Miller Indices (h k l) | d-spacing (Å°) | | 2θ | | Relative Intensity (%) | |
|---|---|---|---|---|---|---|
| | Standard | 950°C | Standard | 950°C | Standard | 950°C |
| (2 1 1) | 2.79695 | 2.79467 | 31.973 | 32.0265 | 100.0 | 100.00 |
| (3 0 0) | 2.69969 | 2.70126 | 33.157 | 33.1654 | 54.3 | 65.39 |
| (1 1 2) | 2.77146 | 2.76131 | 32.275 | 32.4240 | 43.7 | 49.61 |
| (2 1 3) | 1.83574 | 1.83373 | 49.620 | 49.7223 | 32.3 | 35.33 |
| (2 2 2) | 1.93385 | 1.93515 | 46.947 | 46.9548 | 24.8 | 33.30 |
| (2 0 2) | 2.62219 | 2.61660 | 34.167 | 34. 2711 | 24.5 | 23.01 |

*Thermo-gravimetric Analysis*

The removal of the organic portion, as well as the mass loss of annealed bovine bone was confirmed by TGA analysis (Figure-01). In the TGA curves, two inflection points were observed in the bovine bone at 100°C and 500°C which corresponds to removal of the water and organic matter.

No significant weight loss was observed between 600°C and 800°C, indicating the complete removal of organic materials such as fat tissues, collagen, chondroitin sulfate, keratin sulfate, and lipids below 600°C in bovine bone. Different studies has shown that weight loss (~34%) due to water and organic substances removal from the bovine bone is consistent (Silva*et al.*,2004, Davim and Marques 2004), which indicate that bone is made up of about 66% of inorganic mineral crystals and about 34% corresponds to the organic phase consisting of collagen protein fibers.

**Table 3.** Peaks of infrared spectra assigned to commercial HA (cHA) and HA derived from Bovine bone (bHA).

| Name of the chemical group | Absorption band frequency ($cm^{-1}$) | | Reference |
|---|---|---|---|
| | cHA | bHA | |
| $PO_4^{3-}$ band V1 | 470 | 474 | (Destainville*et al.*, 2003; Raynaud*et al.*,2002) |
| $PO_4^{3-}$band V3 | 1058 | 1051 | (Destainville*et al.*,2003; Raynaud*et al.*,2002) |
| $PO_4^{3-}$ band V4 | 572, 603 | 570, 603 | (Destainville*et al.*,2003;Han*et al.*,2006;Mobasherpour and Heshajin 2007; Raynaud*et al.*,2002) |
| $CO_3^{2-}$ | 1462 | 1460, 1411 | (Meejoo*et al.*,2006; Ratner*et al.*,2004; Raynaud*et al.*,2002) |
| Absorbed $H_2O$ | 1633, 2854, 2924 | 1633, 2860, 2927 | (Meejoo*et al.*,2006; Xiaoying*et al.*,2007) |
| OH structural | 3452, 3782 | 3456, 3774 | (Destainville*et al.*,2003;Han*et al.*,2006;Mobasherpour and Heshajin 2007; Raynaud*et al.*,2002) |

*XRD Analysis*

The phase and purity of derived HA crystals were confirmed with XRD analysis. Figure-2 shows the XRD patterns of the powder produced after annealing bone, i.e. (a) raw bone and its heat treated forms produced at (b) 650°C, (c) 950°C, (d) 1050°C and (e) 1250°C and the XRD data of the 950°C are tabulated in Table-02 with standard HA ("XpertHighscore" File

No. 01-086-0740).

These diffraction patterns show a gradual increase in the degree of sharpness of peaks with increasing heat treatment temperature, indicating the extent of crystallinity of the HA produced at various temperatures. Further, it also shows the influence of preparing temperature to HA crystal size. The main peak of HA crystals lead to broader diffraction peaks. With the temperature increasing from 650 to 1250°C, the HA peaks become sharper and more distinct at 950°C treated sample. The sharp and distinct peaks indicated a high degree of crystallinity. It means that less than 950°C has no influence to HA crystal size. At the upper temperature HA crystal does not increase continuously with the rising temperature.

**Table 4.** Comparison of different spectrum of before and after irradiated HA derived from Bovine bone in FTIR analysis.

| Name of the chemical group | Absorption band frequency ($cm^{-1}$) | |
|---|---|---|
| | bHA | bHA/R |
| $PO_4^{3-}$ band V1 | 474 | 474 |
| $PO_4^{3-}$band V3 | 1051 | 1051 |
| $PO_4^{3-}$ band V4 | 570, 603 | 570, 603 |
| $CO_3^{2-}$ | 1460, 1411 | 1463, 1411 |
| Absorbed $H_2O$ | 1633, 2860, 2927 | 1631, 2860, 2927 |
| OH structural | 3456, 3774 | 3460, 3780 |

Table-02 represents the different prominent XRD peak position, d-spacing and relative intensity which corresponds to the plane (211), (3 0 0), (1 1 2), (2 1 3), (2 2 2) and (2 0 2) of annealed bone at 950°C and compared with the standard HA ("XpertHighscore" File No. 01-086-0740) results. The values of sample are almost same as standard HA value.

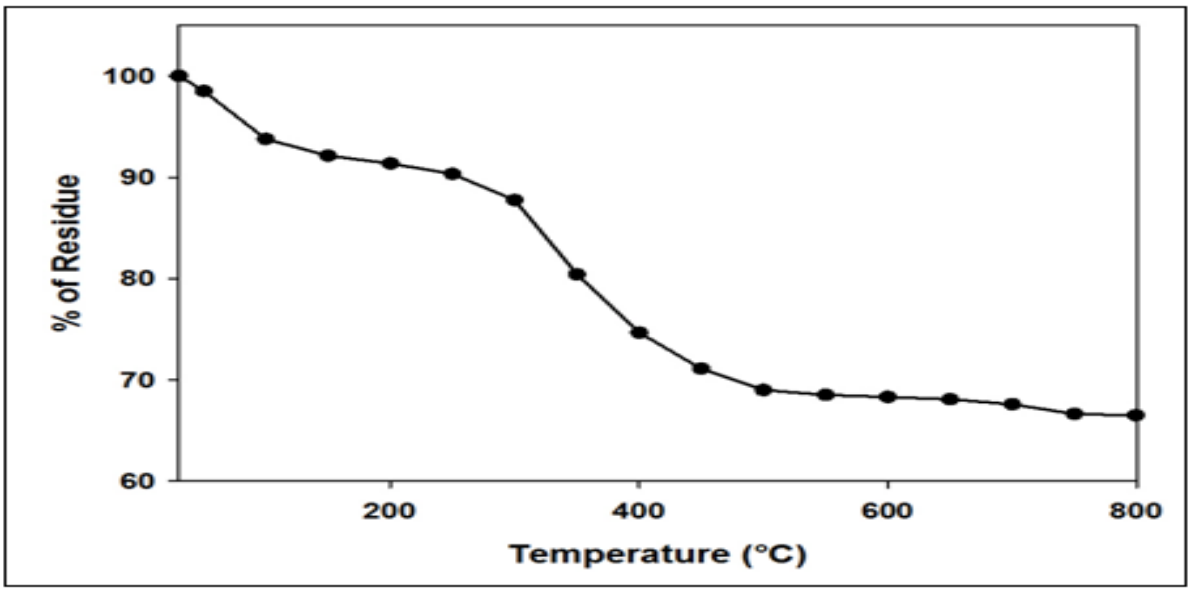


**Fig. 1.** TGA analysis of Bovine bone.

This observation is not in agreement with some of the literature, where the decomposition of HA to β-TCP has been reported to occur at a low sintering temperature below 1000°C (Jinlong*et al.*,2001, Ugarte*et al.*,2005).

In the present work, 950°C has been identified as the optimum sintering temperature, as the sample exhibited the highest XRD diffraction intensities and sharp peaks corresponding to the HA phase (Figure 2) compared to all other sintered samples, thus

indicating that a highly crystalline HA structure was obtained at this temperature.

*FTIR Analysis*

FTIR spectrum values of HA was extracted from bovine cortical bone by annealing at 950°C as summarized in Table-03. The results are compared with commercially synthetic HA (Sigma CAS No.12167-74-7). An infrared absorption spectra of the cHA and bHA is summarized in Table-03. It shows that, a large number of bands in the spectra (470, 572, 603, 1058, 1462, 1633, 2854, 2924, 3452, 3782 $cm^{-1}$) matches the bands in the commercial HA (cHA) reference spectrum and are in close agreement with reported data on HA(Joschek*et al.,* 2009).

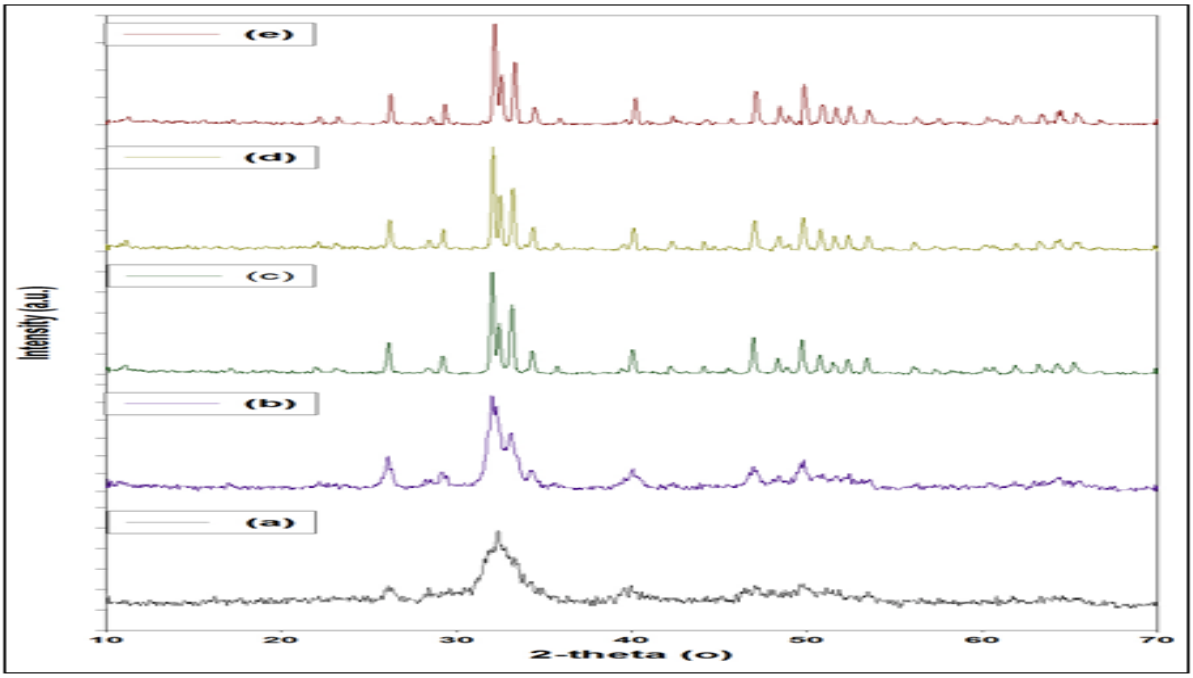


**Fig. 2.** XRD analysis of HA, extraction from bone through multi stage annealing. (a)Raw bone (b) 650°C (c) 950°C (d) 1050°C and (e) 1250°C.

The absorption bands at 3452, 3782 and 3456,3774 $cm^{-1}$ respectively correspond to the stretching and vibration of the lattice $OH^-$ ions of cHA and bHA, while the bands of absorbed water are shown at 1633, 2854,2924 $cm^{-1}$ and 1633, 2860, 2927 $cm^{-1}$.

The characteristic bands for $CO_3^{2-}$ were assigned at 1462 $cm^{-1}$ and 1411, 1460$cm^{-1}$ for cHA and bHA correspondingly. Manalu*et al.* reported that the stretching band 3420-3570 $cm^{-1}$originate by hydroxyl group(Manalu*et al.,* 2009).

Carbonate ions are a common impurity in both synthetic and natural HA(Markovic*et al.,* 2004,Haberko*et al.,* 2006). The characteristic bands for $PO_4^{3-}$ appeared at 470 and 474 $cm^{-1}$ for the v1 mode in cHA and bHA sequentially.

The signal became clearly as the hydration processing. The observation of the v3 symmetric P-O stretching vibration at 1058 and 1051 $cm^{-1}$ for cHA and bHA as a distinguishable peak, together with the bands 572/603 $cm^{-1}$ & 570/603 $cm^{-1}$ corresponding to v4 bending vibration indicates.

Different types of chemical bonds which are present in various components of bone at given infrared absorption bands. Changes in the environment of molecules cause shifts in the intensities and positions of their corresponding absorption bands.

The bands associated with the amide groups of proteins (1250 cm−1, 1560 cm−1 and 1650 cm−1) which are observed on the spectrum of bone do not exist on the FTIR spectrum of bone ash prepared in

this investigation. On the other hand, the bands for phosphate and carbonate groups in bone ash occur at the same wave numbers as those reported for bone(Boskey and Camacho 2007). The FTIR spectrum of the sample which was annealed at 950°C (Figure 3) is in good agreement with the spectra reported by Markovic*et al.* for a HA-synthetic reference material (Markovic*et al.*, 2004 ).

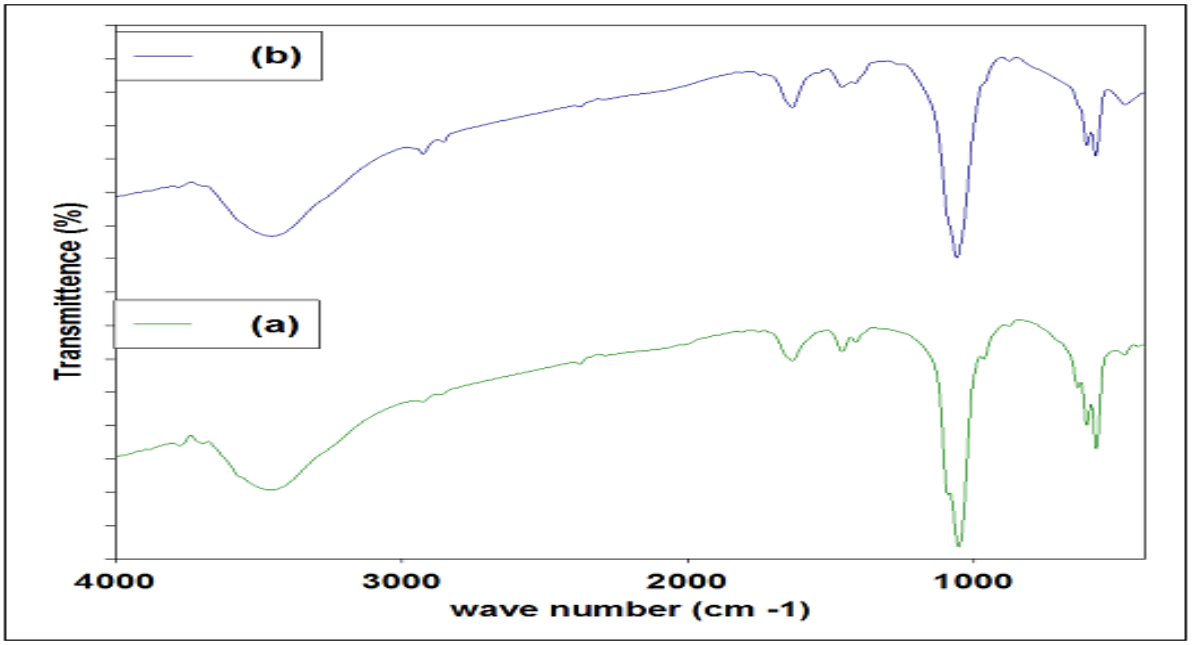


**Fig. 3.** FTIR results of - (a) HA derived from bovine bone by annealing at 950°C (bHA) and (b) Commercial HA (cHA).

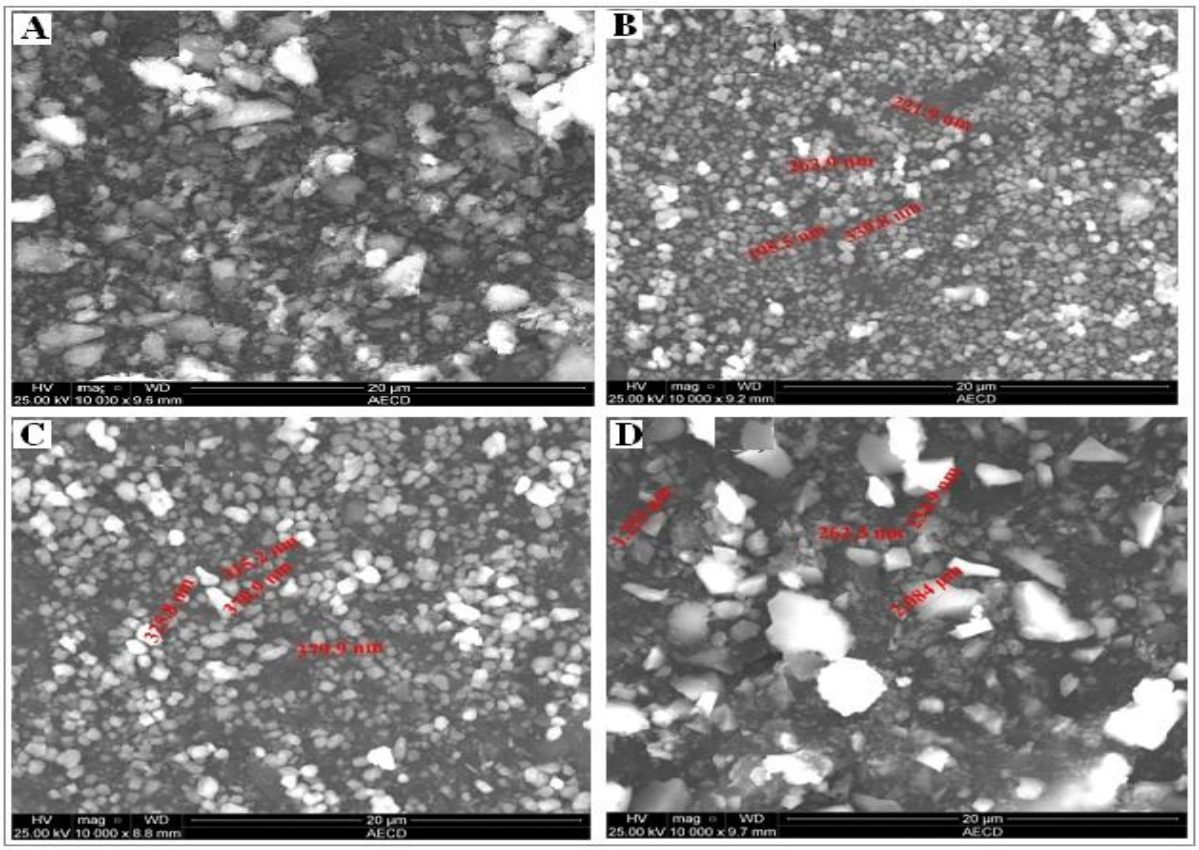


**Fig. 4.** SEM image of bovine bone (bHA) sintered at (a) 650°C, (b) 950°C (c) 1050°C and (d) 1250°C.

*SEM Analysis*

The surface morphology and crystal size of the extracted HA were determined under SEM analysis. The Scanning electron microscopy (SEM) micrograph of the samples obtained from bovine bone at 650-1250°C is shown in Figure-04. After annealing of bovine bone (bHA) at 950°C, the particle sizes were 195.5-339.8 nm. The average particle size of HA was 255.28 ±63 nm. When the samples were sintered at 1050°C and 1250°C, the particle sizes were 315.2-379.9 nm and 234.9 nm- 2.084 μm in diameter, respectively. SEM micrograph of the powder shows that, the particles had irregular shapes, including small spheres, agglomerated together in some parts. It is may be happened due to the inter link up of HA particleeach another.

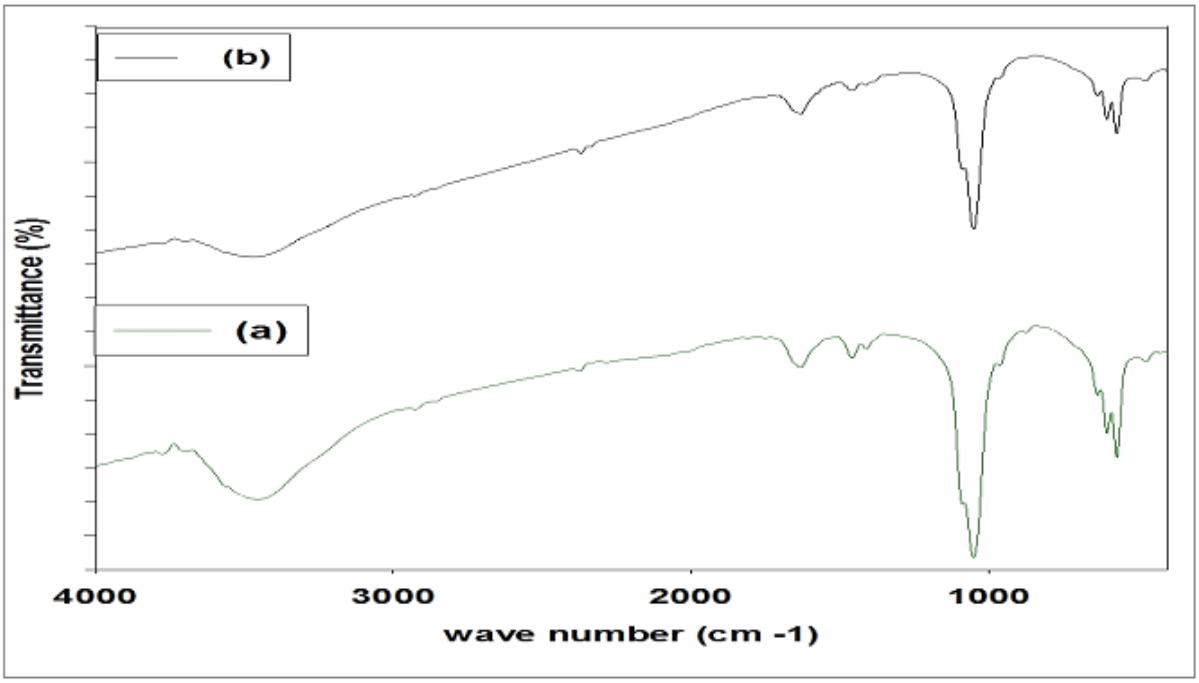


**Fig. 5.** FTIR results of - (a) Without radiated HA (bHA) and (b) Irradiated HA (bHA/R).

Accordingly, the morphology of the HA produced from bovine bone in the present study also might be influenced by the type, age and nutrition of that particular animal from which the bone for this study was obtained. For the production of HA among the bones of different animals (chicken, pig, sheep and bovine), bovine bone had yielded the highest amount of HA, after burning was chosen as the best starting natural source to get HA. Unfortunately, there was no control on the age and nutrition of the bovine from which the bone was obtained. Thus, further study is necessary to find out at the influence of these biological factors on the morphology of HA.

*3.7 Effect of Radiation in HA*

For sterilization of HA the sample were exposed in 25 kGy. The result was revealed that there were not any significant changed between initial and irradiated samples. The FTIR results of samples are illustrated figure-05.

Table-04 shows that, a large number of bands in the spectra (474, 570, 603, 1051, 1411, 1633, 2860, 2927, 3456, 3774 $cm^{-1}$) of non-radiated HA (bHA) matches the bands in the radiated HA (bHA/R).

It is also illustrated that, there are not any significant changed within the different groups of HA, before and after radiation. So radiation will be a good sterilization process of HA to use it maxillofacial filler and other biomedical application.

**Conclusion**

This study shows that the annealing method with high temperature is effective and feasible for preparation of natural HA from bovine bones, and 950°C is a

suitable preparing temperature. The results of XRD and FTIR show that the chemical components of the prepared materials are HA and the chemical composition revealed that there are small amount of other inorganic compounds such as $CO_3^{2-}$ that is preferable for biomedical application in bone tissue engineering. SEM result revealed that the crystals exhibit a nano-structure. Based on the results of this study, it is envisaged that with proper control of annealing temperature, HA produced from bovine bone has great potential to be used as a viable and economical graft material for orthopedic application as well as may reduce the environmental effect of byproducts from the bovine bone derived bio-waste in the country while efficiently safeguarding environmental pollution and waste management.

**Acknowledgements**

The authors would like to thank the International Atomic Energy Agency (IAEA) for their support to carry out this work as a part of Coordinated Research Project.

**Conflict of Interest**

The authors declare that there is no conflict of interest regarding the publication of this paper.

**Reference**

**Akram M, Ahmed R, Shakir I, Aini W, Ibrahim W, Hussain R.** 2014. Extracting hydroxyapatite and its precursors from natural Resources. Journal of Materials Science, **49,** 1461–1475.

**Bahrololooma ME, Javidi M, Javadpoura S, Ma J.** 2009. Characterisation of natural hydroxyapatite extracted from bovine cortical bone ash. Journal of Ceramic Processing Research, **10**, 129–138.

**Barakat NA, Khil MS, Omran A, Sheikh FA, Kim HY.** 2009. Extraction of pure natural hydroxyapatite from the bovine bones bio waste by three different methods. Journal of Materials Processing Technology **209,** 3408–3415.

**Boskey A, Camacho NP.** 2007. FT-IR imaging of native and tissue-engineered bone and cartilage. Biomaterials **28,** 2465-2478, .

**Chattanathan S, Clement T, Kanel S, Barnett M, Chatakondi N.** 2013. Remediation of uranium contaminated groundwater by sorption onto hydroxyapatite derived from catfish bones.Water Air and Soil Pollution. **224,** 1–9.

**Chattopadhyay P, Pal S,Wahi AK, Singh L, Verma A.** 2007. Synthesis of Crystalline Hydroxyapatite from Coral (Gergonaceasp) and Cytotoxicity Evaluation.Trends in Biomaterials and Artificial Organs, **20(2),** 142-144.

**Davim JP, Marques N.** 2004. Dynamical experimental study of friction and wear behaviour of bovine cancellous bone sliding against a metallic counter face in a water lubricated environment.Journal of Materials Processing Technology, **15,** 124–128.

**Destainville A, Champion E, Assollante DB, Laborde E.** 2003. Synthesis, characterization and thermal behaviour of apatite tricalcium phosphate. Materials Chemistry and Physics **80,** 269 – 277.

**Dhandayuthapani B, Yoshida Y, Maekawa T, Kumar DS.** 2011. Polymeric Scaffolds in Tissue Engineering Application: A Review.International Journal of Polymer Science, **2011**, Article ID 290602.

**Dimitriou R, Jones E, Mc Gonagle D, Giannoudis PV.** 2011. Bone regeneration: current concepts and future directions. BMC Medicine **9,** 66–76.

**Fahmy MD, Jazayeri HE, Razavi M, Masri R, Tayebi L.** 2016. Three-Dimensional Bioprinting Materials with Potential Application in Preprosthetic Surgery.Journal of Prosthodontics **25**, 310–318.

**Ferreira JRM, da Rocha DN, Louro LHL, da Silva MHP.** 2014. Phosphating of Calcium

Carbonate for Obtaining Hydroxyapatite from the Ostrich Egg Shell. Key Engineering Materials **587,** 69-73.

**Giedraitis A, Arnoczky SP, Bedi A.** 2014. Allografts in Soft Tissue Reconstructive Procedures: Important Considerations.Sports Health, **6**(3), 256-264.

**Gomes S, Leonor IB, Mano JF, Reis RL, Kaplan DL.** 2012. Natural and genetically engineered proteins for tissue engineering.Progress in Polymer Science, **37**, 1–17.

**Greenwald MA, Kuehnert MJ, Fishman JA.** 2012. Infectious Disease Transmission during Organ and Tissue Transplantation.Emerging Infectious Diseases **18(8).**

**Haberko K, Bucko MM, Brzezinska-Miecznik J, Haberko M, Mozgawa W, Panz T, PydaA, Zarebski J.** 2006. Natural hydroxyapatite—its behaviour during heat treatment. Journal of the European Ceramic Society **26,** 537-542.

**Han JK, Song HY, Saito F, Lee BT.** 2006.Synthesis of height purity nano-sized hydroxyapatite powder by microwave-hydrothermal method. Materials Chemistry and Physics **99,** 235 – 239.

**Jinlong N, Zhenxi Z, Dazong J.** 2001. Investigation of phase evolution during the thermochemical synthesis of tri-calcium phosphate. Journal of Materials Synthesis and Processing **9,** 235–240.

**Joschek S, Nies B, Krotz R, Gofpferich A.** 2000. Chemical and physicochemical characterization of porous hydroxyapatite ceramics made of natural bone. Biomaterials **21,** 1645–1658.

**Kim BS, Kang HJ, Yang SS, Lee J.** 2014.Comparison of in vitro and in vivo bioactivity: Cuttlefish-bone-derived hydroxyapatite and synthetic hydroxyapatite granules as a bone graft substitute. Biomedical Materials **9**.

**Kongsri S, Janpradit K, Buapa K, Techawongstien S, Chanthai S.** 2013. Nanocrystalline hydroxyapatite from fish scale waste: Preparation, characterization and application for selenium adsorption in aqueous solution.Chemical Engineering Journal **215**, 522–532.

**Manalu JL, Soegijono B, Indrani DJ.** 2015. Characterization of Hydroxyapatite Derived from Bovine Bone. Asian Journal of Applied Sciences **3(4).**

**Markovic M, Fowler BO, Tung MS.** 2004. Preparation and comprehensive characterization of a calcium hydroxyapatite reference material. Journal of research of the National Institute of Standards and Technology**109,** 553-568.

**Meejoo S, Maneeprakorn W, Winotai P.** 2006. Phase and thermal stability of nanocrystalline hydroxyapatite prepared via microwave heating. Thermochimica Acta, **447,** 115–120.

**Miculescu F, Ciocan LT, Miculescu M, Ernuteanu A.** 2011. Effect of heating process on micro structure level of cortical bone prepared for compositional analysis.Digest Journal of Nanomaterials and Biostructure, **6(1),** 225 – 233.

**Mobasherpour I, Heshajin M.** 2007.Synthesis of nanocrystalline hydroxyapatite by using precipitation method. Journal of Alloys and Compounds, **430**, 330 – 333.

**Ooi CY, Hamdi M, Ramesh S.** 2007. Properties of hydroxyapatite produced by annealing of bovine bone. Ceramics International **33,** 1171–1177.

**Panda NN, Pramanik K, Sukla LB.** 2014. Extraction and characterization of biocompatible hydroxyapatite from fresh water fish scales for tissue engineering scaffold. Bioprocess Biosystem Engineering, **37,** 433–440.

**Pandit N, Pandit IK, Malik R, Bali D, Jindal S.** 2012.Autogenous bone block in the treatment of teeth with hopeless prognosis.Contemporary Clinical Dentistry **3(4),** 437-442.

**Piccirillo C, Silva M, Pullar R, da CruzIB, Jorge R, Pintado M, Castro PM.** 2013. Extraction and characterisation of apatite-and tricalcium phosphate-based materials from cod fish bones. Material Science Engineering C, **33,** 103–110.

**Pilia M, Guda T, Appleford M.** 2013. Development of composite scaffolds for load-bearing segmental bone defects. Biomed Research International, **2013,** 1–15.

**RahmanIA,Tijani HI, Mohammed BA, Saidu H, Yusuf H, Jibrin MN, Mohammed S.** 2014. From garbage to biomaterials: an overview on egg shell based hydroxyapatite. Journal of Materials, **2014**.

**Ratner BD, Hoffman AS, Schoen FJ, Lemons JE.** 2004. Biomaterials Science. An Introduction to Materials in Medicine. Second Edition, Academic Press, **851**.

**Raynaud S, Champion E, Assollant DB, Thomas P.** 2002. Calcium phosphate apatite with variable Ca/P atomic ratio I. Synthesis, characterisation and thermal stability of powders. Biomaterials, **23,** 1065–1072.

**Reichert JC, Wullschleger ME, Cipitria A, Lienau J, Hutmacher DW.** 2011. Custom-made composite scaffolds for segmental defect repair in long bones.International Orthopaedics, **35,** 1229–1236.

**Silva RV, Camilli JA, Bertran CA, Moreira NH.** 2004. The use of hydroxyapatite and autogenouscancellous bone grafts to repair bone defects in rats. International Journal of Oral and Maxillofacial Surgery **10,** 1–7.

**Sofronia AM, Baies R, Anghel EM, Marinescu CA, Tanasescu S.** 2014. Thermal and structural characterization of synthetic and natural nanocrystalline hydroxyapatite", Materials Science and Engineering: C, **43,** 153–163.

**Thrivikraman G, Madras G, Basu B.** 2014.In vitro/ In vivo assessment and mechanisms of toxicity of bioceramic materials and its wear particulates.RSC Advances **4,** article id. 12763.

**Ugarte JFDO, Sena LÁD, Pérez CADC, Aguiar PFD, Rossi AM, Soares GA.** 2005. Influence of processing parameters on structural characteristics of porous calcium phosphate samples: A study using an experimental design method. Material Research **8,** 71–76.

**Venkatesan J, Kim SK.** 2010. Effect of temperature on isolation and characterization of hydroxyapatite from tuna (thunnusobesus) bone. Materials **3,** 4761–4772.

**Venkatesan J, Qian ZJ, Ryu B, Thomas NV, Kim SK.** 2011. A comparative study of thermal calcination and an alkaline hydrolysis method in the isolation of hydroxyapatite from Thunnusobesus bone. Biomedical Materials **6**, 2011.

**Wua SC, Hsua HC, Hsua SK, Changb YC, Ho WF.** 2016. Synthesis of hydroxyapatite from eggshell powders through ball milling and heat treatment. Journal of Asian Ceramic Societies, **4(1),** 85–90.

**Xiaoying L, Yongbin F, Dachun G, Wei C.** 2007. Preparation and Characterization of Natural Hydroxyapatite from Animal Hard Tissues.Key Engineering Materials, **342-343**, 213-216.

**Zainon I, Alwi N, Abidin M, Haniza H, Ahmad M, Ramli A.** 2012. Physicochemical properties of hydroxyapatite extracted from fish scales. Advance Material Research **545,** 235–239.