CHARACTERIZATION OF NATURAL HYDROXYAPATITE EXTRACTED FROM PIG BONE

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TÓM TẮT

ĐÁNH GIÁ VẬT LIỆU HYDROXYAPATITE TỰ NHIÊN PHÂN TÁCH TỪ XƯƠNG HEO

Công trình này trình bày việc tách chiết vật liệu hydroxyapatite (HA) từ xương heo. Xương heo được làm sạch, sấy khô, nghiền nhỏ rồi nung nóng ở 800 ^oC trong 3 giờ. Vật liệu HA tổng hợp được đặc trưng bằng một số phương pháp hóa lý như XRD, FTIR, SEM và EDX. Kết quả phân tích thu được cho thấy HA tổng hợp thu được ở dạng đơn pha có độ kết tinh và độ tinh khiết cao. Vật liệu tổng hợp có tỷ lệ của Ca/P là 1,65, rất gần với giá trị 1,67 trong công thức HA. Hoạt tính sinh học và tính tương thích sinh học của vật liệu HA phân tách từ xương heo được đánh giá qua thực nghiệm in vitro trong dung dịch SBF và trong môi trường nuôi cấy tế bào. Kết quả khẳng định hoạt tính sinh học qua sự hình thành lớp khoáng HA mới trên nền HA cũ và tính tương thích sinh học thể hiện qua tỷ lệ sống cao của tế bào sau tiếp xúc với vật liệu.

Từ khóa*: Xương heo, hydroxyapatite, phân hủy nhiệt, hoạt tính sinh học, tương thích sinh học*.

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1. INTRODUCTION

Hydroxyapatite (HA) is a biomedical material used as an artificial bone material due to its chemical composition similar to human bones and teeth [1-2]. Hydroxyapatite can be synthesized chemically using precursors containing calcium and phosphorus [2-3]. Chemical synthesis can be performed by precipitation, sol-gel, hydrothermal, solid reaction, or a combination of these methods. Chemical methods can prepare HA materials with accurate stoichiometric coefficients, HA materials are obtained with diverse structural forms. However, the chemical process naturally requires complex synthesis techniques with control of reaction parameters and is generally expensive [2]. Therefore, the separation of HA from natural sources has received widespread research attention [4-5]. The A.M.B. Nasser's research team has

synthesized hydroxyapatite from bovine bone by different processes including thermal decomposition, and hydrothermal processes in water and alkaline environment [6]. The results show that hydroxyapatite materials are obtained with different structural morphologies. The thermal decomposition method yields pure hydroxyapatite material in a nano-rod shape at 750 ^{o}C for 6 hours. Meanwhile, the hydrothermal process in water results in flat flakes of hydroxyapatite at 275 ^{o}C within 1 hour; the hydrothermal process in an alkaline environment yields nanoparticles of hydroxyapatite at 250 ^{o}C in 5 hours. The V.N. Pham's research team has separated hydroxyapatite by the thermal decomposition of some types of fish bones at 700 °C for 2h [7]. Research results show that different types of fish bone produce HA with different morphology, porosity, and purity. The HA material prepared from seabass bone shows the most uniform structure and smallest size of 50–70 nm. The HA powder obtained from tilapia bone has the highest value of specific surface area. All HA samples isolated from fish bones have a similar composition with a Ca/P ratio of about 1.80. The thermal process combined with the chemical precipitation has been used to synthesize HA material from eggshells [8]. The powder of the eggshell was baked to obtain the CaO compound, which is used as a Ca source for the chemical precipitation with phosphoric acid. As a result, the research shows that by using thermal decomposition and chemical precipitation, HA can be synthesized from eggshell waste. The special isolation of hydroxyapatite from the stem and leaves of the Carpobrotus edulis plant is performed by thermal treatment [9]. The crushed powder of the Carpobrotus edulis plant was calcined at temperatures ranging from 600 ^{o}C to 800 ^{o}C. Physical-chemical analysis confirms that HA material is the main mineral phase obtained when thermally decomposing the stem and leaves of the Carpobrotus edulis plant. Continuing the research on separating hydroxyapatite from natural sources as listed above, this work presents the results of separating HA material from pig bone collected in Vietnam using the thermal process. The resulting material was characterized by several physical-chemical methods. The bioactivity and biocompatibility of synthetic material was also verified by the in vitro experiments.

2. EXPERIMENT AND METHODS

2.1. Processing of pig bone

Pig bones were collected at markets in Ho Chi Minh City, Vietnam. Pig bones were boiled in water to remove fat and other impurities. After that, the bones were dried, then cut into small round pieces, and ground finely. The powder of dried bone was then calcined at 800 ^{o}C for 3 hours referring to previous studies [6-7].

2.2. In vitro experiment in SBF solution

The hydroxyapatite (HA) powder separated from pig bone was soaked in SBF solution with an HA/SBF ratio of 400mg/800mL. The SBF solution (Simulated body fluid) is a synthetic solution that simulates human body fluid with an inorganic ionic composition similar to blood in the human body [10]. Chemicals (purity over 98%, Merk) to synthesize the SBF solution include $(NH_4)_2HPO_4$; $K_2HPO_4.3H_2O$; $MgCl_2.6H_2O$; HCl; NaCl; KCl; $NaHCO_3$; $CaCl_2$; $C_{14}H_{11}NO_3$. The HA powder was stirred continuously for 3 days at 37 ^{o}C under the stirring speed of 50 rpm. After finishing, the HA powder was centrifuged and washed in ethanol. The powdered material was then dried at 100 ^{o}C for 24 hours, and used to test its bioactivity.

2.3. In vitro experiment in cell culture medium

The synthetic hydroxyapatite was evaluated the biocompatibility by testing in an environment cultured with L-929 fibroblast cells [11]. The cells were cultured in a standard environment DMEM (Dulbeco's Modified Eagle Medium). The environment was kept at 37 ^{o}C for 24 hours. After exposure to HA extracts at different concentrations, the cell viability content was determined by using the colorimetric MTT method. The MTT compound (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), a yellow compound, will be converted into formazan compound with a purple color when reacting with the mitochondria in living cells. The quantification of living cells was performed by measuring the absorption wavelength of formazan at about 570 nm using a UV-Vis spectrophotometer.

2.4. Characterization

The synthetic hydroxyapatite extracted from pig bone was characterized by using several physical-chemical methods. The XRD (X-ray diffraction) was used to identify the phase composition of synthetic material. The FTIR (Fourier-transform infrared spectroscopy) was served to determine the functional groups. The morphology of synthetic material was observed by using the SEM (Scanning electron microscope). The element distribution of the synthetic sample was analyzed by the EDX (Energy-dispersive X-ray spectroscopy).

3. RESULTS AND DISCUSSION

3.1. XRD analysis

Figure 1 presents the XRD diagram of the initial dried pig bone. The diagram shows the appearance of several typical peaks for the hydroxyapatite mineral phase [JCPDS card number 9-0432]. However, the observed peaks are wide and obtuse, typical of the poor crystalline state of the measured material sample. When the bone sample was heated at 800 oC, the XRD diagram fully showed the characteristic peaks of the hydroxyapatite mineral phase (Fig. 2). On the other hand, the observed peaks are sharp with high intensity, confirming the good crystalline state of the synthetic HA material. Additionally, the XRD diagram did not show any peaks of strange phases, proving the high purity of the obtained HA material. Thus, by heating dried pig bone at 800 oC for 3 hours, HA material was successfully separated with high crystallinity and purity. The achieved result in this study is similar to previous studies [5-7], confirming the effectiveness of the thermal decomposition method in separating HA from animal bones.

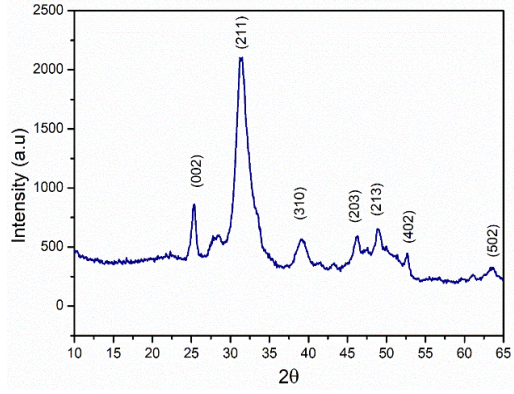

Figure 1. XRD diagram of dried pig bone

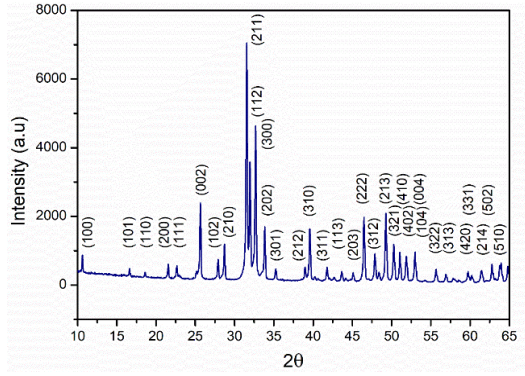

Figure 2. XRD diagram of hydroxyapatite extracted from pig bone

3.2. FTIR analysis

The FTIR spectrum of pig bone calcined at 800 ^{o}C shows the characteristic bands of hydroxyapatite material as presented in Fig. 3. The bands located at 470; 565, 604, 1033, and 1093 cm^{-1}, are characteristic of P-O vibrations from PO_4^{3-} groups [7-8]. The band at 631 and 1640 cm^{-1} corresponds to the O-H bonds in the hydroxyapatite structure [8-9]. The presence of the CO_3^{2-} group is mentioned by the characteristic bands at about 804, 875, 1415, and 1456 cm^{-1} [8-9]. Carbonate ions are common impurities in the FTIR measurements. The obtained result from FTIR analysis confirms the functional groups of the HA material extracted from pig bone.

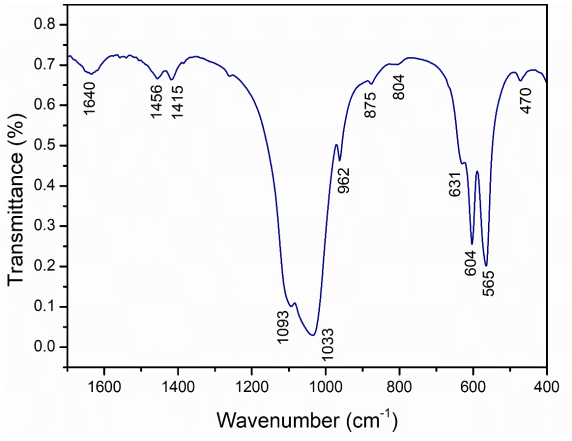

Figure 3. FTIR spectrum of hydroxyapatite extracted from pig bone

3.3. SEM and EDX analysis

The morphology of hydroxyapatite (HA) obtained from pig bone was observed by SEM images at different magnifications as presented in Fig. 4. At magnifications of 5.000 and 10.000, the surface of synthetic HA material shows the particles with various sizes and shapes such as spheres, scales, and rods. The SEM images at higher magnifications of 20.000 and 50.000 clearly show rods, scales, and pores in the structural morphology of synthesized HA. The result obtained by SEM observation is quite similar to the one reported in reference [12], in which the authors extracted HA material from bovine bone. The elemental composition of synthetic HA was analyzed by the EDX method as shown in Fig. 5. The EDX spectrum only shows the elements Ca, P, and O as in the formula of hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$, demonstrating that there are no foreign elements in the composition of the isolated HA. The calculated ratio of Ca/P is 1.65, which is very close to the value of 1.67 in the HA formula.

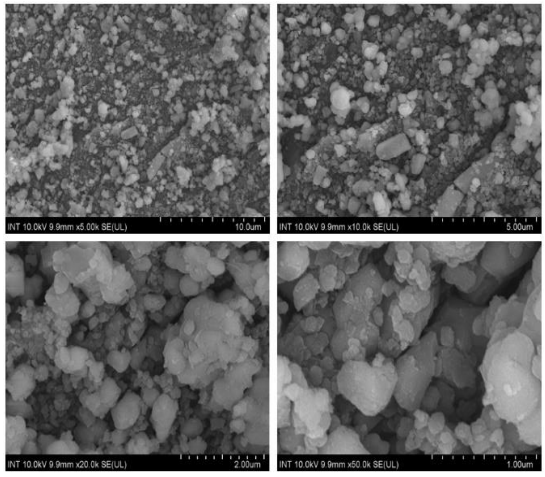

Figure 4. SEM observation of hydroxyapatite extracted from pig bone at different magnifications

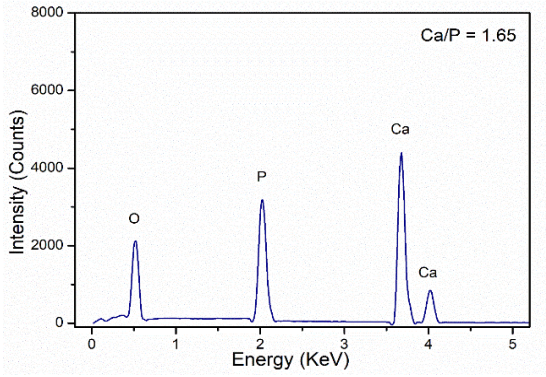

Figure 5. EDX analysis of hydroxyapatite extracted from pig bone

3.4. Microbiological testing

The HA material powder separated from pig bone was tested for the safety against microorganisms at the Center Analytical Services and experimentation HCMC, in

Vietnam (Sample code: BN 18091498). Three types of pathogenic microorganisms (Coliforms, Escherichia coli, Staphylococcus) were tested on the synthetic HA sample. The test report shows that the synthetic HA in this study is completely safe against pathogenic microorganisms according to current standards (Tab. 1).

Table 1. Result of detecting pathogenic microorganisms in synthetic HA sample

No	Parameters	Unit	Results	Test method
1	Coliforms	CFU/g	Not detected	ISO 4832:2006
2	Escherichia coli	CFU/g	Not detected	ISO 16649-3:2015
3	Staphylococcus aureus	CFU/g	Not detected	ISO 6888-3:2003

Bioactivity

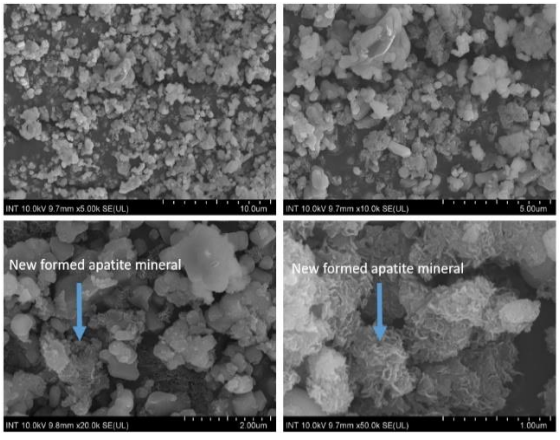

Figure 6. SEM observation of hydroxyapatite after 3 days of immersion in SBF solution at different magnifications

Figure 6 presents the SEM image of the HA sample separated from pig bone soaked in SBF solution for 3 days. The observation clearly shows the formation of new mineral crystal flakes coated on the surface or interwoven into the pores of the synthetic HA after 3 days of immersion in SBF. The interaction of HA and SBF solution leads to the formation of a new mineral layer, confirming the bioactivity of the HA material in this study.

3.5. Biocompatibility

The synthetic HA material was tested for its biocompatibility with the fibroblast-like cells (L-929 fibroblast). Different extracts of HA material were directly exposed to the cell culture environment for 24 h. Cell viability without exposure to HA material was chosen as the control (100%) [11]. The cell viability is determined as a percentage compared to the control sample. The cell survival rate is above 70%, the material is considered biocompatible. The obtained results mentioned that cell viability was 130%, 125%, 114%, and 110% for the HA extracts of 12.5%, 25%, 50% and 100%, respectively. The cell viability values for synthetic HA material in this study confirm good biocompatibility in cellular-cultured environments even in high-concentrated extracts.

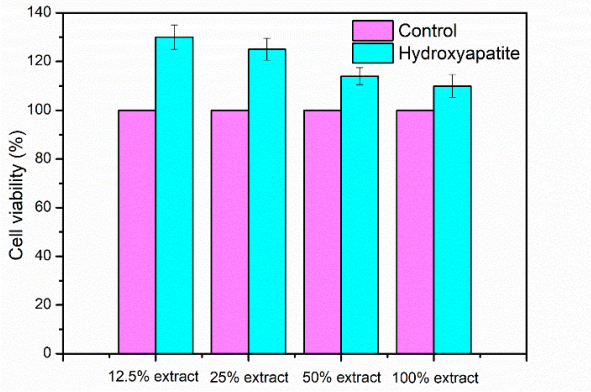

*Figure 7. Cell viability of hydroxyapatite at different extracts*CONCLUSION

The hydroxyapatite material was successfully separated from pig bone using the thermal-treated process. The analysis data highlighted that the obtained HA material is pure with high crystallinity. The XRD and FTIR results only show typical peaks and functional

groups of hydroxyapatite material. Synthetic HA material is also safe against pathogenic microorganisms such as Coliforms, Escherichia coli, and Staphylococcus. The in vitro experiment in SBF solution confirms the bioactivity of synthetic HA through the formation of new mineral crystals on the surface after 3 days of immersion. The good biocompatibility of synthetic HA with L-929 fibroblast cells is also verified by the in vitro experiment in a cellular-cultured environment.

Declaration: The authors declare that this is the work of our research group, and this content has not been submitted to any journal.

REFERENCES

[1] Mythili P, Janis L, Kristine SA, Dagnija L, Alain L, Liga BC, (2015). Fabrication, Properties and Applications of Dense Hydroxyapatite: A Review. *Journal Functional Biomaterial*, **6(4)**, 1099–1140.

[2] Mohd PNAS, Abdul HRH, Noh HM, Abdullah HZ, Idris MI, Lee TC, (2020). Synthesis method of hydroxyapatite: A review. *Materials Today: Proceedings*, **29(1)**, 233-239.

[3] Ritesh K, Smita M, (2022). Hydroxyapatite: A Versatile Bioceramic for Tissue Engineering Application. *Journal of Inorganic and Organometallic Polymers and Materials*, **32**, 4461–4477.

[4] Mohd PNAS, Koshy P, Abdullah HZ, Idris MI, Lee TC, (2019). Syntheses of hydroxyapatite from natural sources. *Heliyon*, **5(5)**, e01588.

[5] Mohamed SFH, Hasan ZA, Maizlinda II, Mohd AAW, (2022). Extraction of natural hydroxyapatite for biomedical applications—A review. *Heliyon*, **8(8)**, e10356.

[6] Nasser AMB, Myung SK, Omran AM, Faheem AS, Hak YK, (2009). Extraction of pure natural hydroxyapatite from the bovine bones bio waste by three different methods. *Journal of Materials Processing technology*, **209**, 3408-3415.

[7] Pham VN, Nguyen VH, Trang ST, (2019). Properties of hydroxyapatites prepared from different fish bones: A comparative study. *Ceramics International*, **45(16)**, 20141-20147.

[8] Mohd PNAS, Alipal J, Abdullah HZ, Idris MI, Lee TC, (2021). Synthesis of eggshell derived hydroxyapatite via chemical precipitation and calcination method. *Materials Today: Proceedings*, **42(1)**, 172-177.

[9] Abdallah AS, Moussa AA, Mohamed AM, (2011). FTIR spectroscopic, thermal and XRD characterization of hydroxyapatite from new natural sources. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, **83(1)**, 56-60.

[10] Kokubo T, Takadama H, (2006). How useful is SBF in predicting in vivo bone bioactivity. *Biomaterials*, **27(15)**, 2907-2915.

[11] Mosmann TJ, (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, **65**, 55–63.

[12] Ramesh S, (2018). Characterization of biogenic hydroxyapatite derived from animal bones for biomedical applications. *Ceramic International*, **44**, 10525- 10530.