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FULL PAPER TAM MAKALE

EXTRACTION, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF HYDROXYAPATITE FROM SEABASS AND SEABREAM SCALE

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Abstract:

The present study investigates the characterization of hydroxyapatite (HAp) extracted from seabass and seabream scales as by-product. Fish scales obtained from a seafood processing company were used to extract natural HAp powder. HAp powder was extracted by alkaline heat treatment of fish scales and the synthesized HAp (FS-HAp) was extensively characterized with Fourier transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM) and X-ray diffraction (XRD) analysis. Calcium to phosphate ratio of the HAp was confirmed by inductively coupled plasma (ICP) and elemental analysis of HAp were also carried out using energy dispersive x-ray spectroscopy (EDS). The results of the characterization analysis were compared with commercial hydroxyapatite standard (CHAp) and it was clearly confirmed that the extracted FS-HAp exactly showed CHAp characteristics physicochemically which is used as biomaterial. However, well diffusion assay revealed out that synthesized hydroxyapatite showed no activity against *C. albicans*, *S. aureus* and *E. coli*. It was concluded that, instead of synthetic apatite, extracted FS-HAp presents a potential promising biomaterial as the raw materials are by-product which economically cheap and sustainable substances.

Keywords: Seafood Processing By-product, Fish Scale, Hydroxyapatite, Biomaterial

Introduction

There has been growing interest in developing bioactive synthetic ceramics that could closely mimic natural apatite characteristics. In recent years, environmental and economic conditions have raised concerns about the treatment and use of by-product. Fish by-product has tremendous unexploited potential for adding value. However, the use of fish by-product in foods and biochemical products for human consumption is still under intensive study in the aquaculture industry (Huang *et al*., 2011). Hydroxyapatite (HAP), with a chemical formula of $Ca_{10}(PO_4)_6(OH)_2$, is one of constituent that had found in either in human bone or teeth and the major elements are including calcium and phosphorous. HAp is derived from natural materials such as fish bone (Jensen *et al*., 1996; Ozawa and Kanahara, 2005) and fish scale (Mondal *et al*., 2010; Zainon *et al*., 2012), bovine sources (Sofronia *et al*., 2014). Bovine and pork origins are often associated with disease transmission and religious sentiments (Gómez-Guillén *et al*., 2011). Fish sources are presumably much safer, and the wide evolutionary gap between fish and humans suggests a low risk of disease transmission (Venkatesan *et al*., 2015). The main by-product of the seafood processing industry is fish scales with a portion of 30-40 % of the total amount and managing those by-products is causing problems for the companies (Ozawa and Suzuki, 2002; Gumisiriza *et al*., 2009). The Turkish seabass and seabream industry has been increasing production volumes recently, to the point where Turkey is now the world's major producer of seabass and also closing the gap on the Greek seabream sector (FAO, 2015). In 2015, total aquaculture amount of Turkey was 240334 tones while sea bream and sea bass production amounts were 51844 and 75164 tones, respectively (TUIK, 2015). There are lots of companies processing those species and marketing the processed fish so that the by-product amounts are gradually increase. Using by-products for creating a new biocompatible material is an economical way to reduce the waste management costs and also provide a sustainable raw material. In this sense, fish-originated ceramics have a great potential for being bioactive media as they are environment-friendly materials (Ozawa and Kanahara, 2005). Therefore, this research aims to utilize the fish scales of sea bass and sea bream which have a great import and export capacity in Turkey.

Materials and Methods

Fish scale (FS) by-products was provided from a seafood processing company located in Aydın, Turkey which process sea bass (*Dicentrarchus labrax* L. 1758) and sea bream (*Sparus aurata* L. 1758) with high import and export capacity. Commercial synthetic hydroxyapatite (CHAp) was purchased from Sigma–Aldrich (Switzerland). All other chemicals were reagent grade.

Extraction of Hydroxyapatites from Fish Scales

Scales of the fish were transferred to research laboratories and washed thoroughly in distilled water to remove the organic substances. Thereafter, scales were left to air dry in the laboratory conditions until dryness. Alkaline heat treatment method was used to obtain FS-HAp (Kongsri *et al*., 2013). Dry scales was initially deproteinized with 0.1 M HCl and washed with distilled water for several times. The remaining proteins of scales were treated with 5% (w/v) NaOH, heated and stirred at 70 °C for 5 h. The obtained precipitate was washed with distilled water and dried at 60 °C. 50% (w/v) NaOH was added into the powder, heated up to 100 °C and stirred for 1 h. FS-HAp powder was washed with deionized water and then dried at 60 °C. Treated fish scales were subjected to calcinations at 800° C (NUVE, MF 120) for 1 h to synthesize HAp ceramics. The synthesized ceramics were milled with a mortar and sieved to obtain powder.

Characterization of FS-HAp Powder

Fourier Transform Infrared (FT-IR) Spectral Analysis

FT-IR characterization of the synthesized FS-HAp powder was performed by Fourier transform infrared spectroscopy (Thermo Scientific Nicolet iS10-ATR, USA) at a resolution of 4 cm^{-1} in KBr pellets and the spectra were recorded in the wavelength interval of 4000 and 400 nm^{-1}.

Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDS)

For taking the SEM images of the FS-HAp, a piece of powder was placed on specimen stub with double-sided adhesive carbon tape. Scanning electron microscopy study was performed on a JSM 7600F Field Emission Scanning Electron Microscope (JEOL, Japan) at an accelerating voltage of 15 kV. Elemental analysis of FS-HAp was also carried

out using energy dispersive x-ray spectroscopy (EDS) (Oxford Instruments, UK) combined with SEM.

X-Ray Diffraction (XRD) Measurement

To examine the crystal structure of synthesized FS-HAp, X-ray diffraction (XRD) patterns were collected using Cu Kα monochromatic radiation (λ_{Cu} = 1.54018 Å) at 40 kV/20 mA using continuous scanning 2θ mode on a X-ray diffractometer (Rigaku-SmartLab, Japan).

ICP-OES

In order to evaluate Ca/P molar ratio, inductively coupled plasma-optical emission spectrometry (ICP-OES) analysis was performed using a Perkin Elmer Optima 8000 Spectrometer (in house method). The sample (0.1–0.5 g) was weighted and 10 mL HCl (0.1 M) was added. After microwave digestion, the volume of the solution made up to 50 mL with distilled water. This aqueous solution was used to determine the Ca and P values (mg/L). Each assay was repeated two times and the results are presented as mean values.

Antimicrobial Activity

The antimicrobial activity of synthesized hydroxyapatite was tested against *Candida albicans* ATCC 10239, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 which were provided from Culture Collection of Mugla Sitki Kocman University (MUKK). *E. coli* and *S. aureus* strains were incubated at 37±0.1°C for 24–48 h, *C. albicans* was incubated at 30±0.1°C for 24-48 h.

The antimicrobial activity of the synthesized hydroxyapatite was assayed by agar well diffusion method (NCCLS, 1993). Briefly, 20 milliliters of Mueller-Hinton Agar (Merck) sterilized and cooled to 45–50 °C. After injecting 1000 µL microorganism cultures to sterile plates, media was distributed and mixed homogenously. Wells of 6-mm diameter were made on agar plates using a cork borer. 100 µL of hydroxyapatite solution (50 mg/mL dH_2O) was injected to the wells. Antimicrobial activity was evaluated by measuring the zone of inhibition around the wells. Studies were performed in triplicate. Discs of ampicillin (10 µg), imipenem (10 µg) and nystatin (30 µg) were used as positive controls.

Results and Discussion

The obtained XRD spectra of FS-HAp have been compared with commercial HAp (CHAp). As shown in Figure 1, all the peaks corresponding to the FS-HAp (A) are close to the CHAp (B) in the spectra which emphasis that the proposed treatment process has produced clean and pure hydroxyapatite. Peaks were obtained at 2θ value of 25.9°, 31.84°, 32.98°, 34.12°, 39.84°, 46.76°, 49.54° corresponding to (002), (211), (300), (202), (310), (222), (213) planes, respectively. Well-resolved characteristic peak of highest intensity was obtained at 2θ value of 31.84° corresponding to 211 plane of HAp (Huang *et al.*, 2011; Panda *et al.*, 2014). Similar to Panda *et al.* (2014), crystallization of the FS-Hap powder has been also confirmed due to sharp peak intensity and well-resolved peaks in XRD pattern.

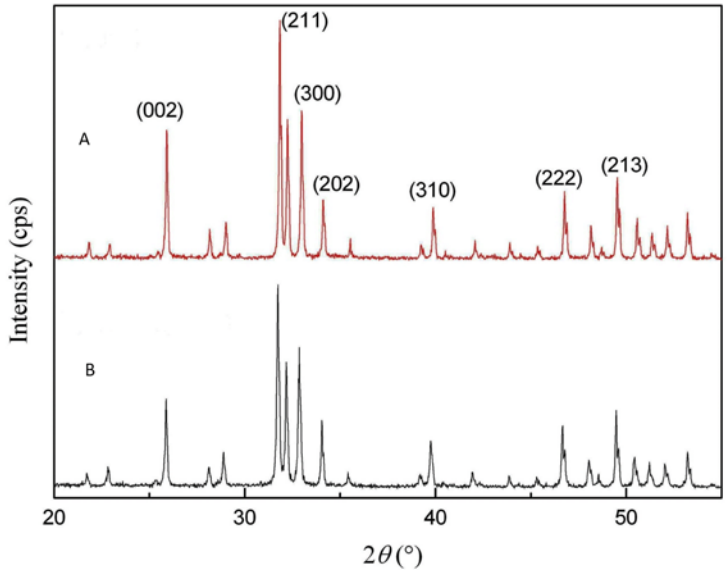


Figure 1. X-ray diffraction pattern of synthesized hydroxyapatite; A) FS-HAp and B) CHAp.

The broad patterns around (211) and (002) indicate low crystalline HAp phase (Sanosh *et al*., 2009). This indicates a good result because low crystalline components may present an improved biodegradation behavior and are expected to be more metabolically active than the crystalline HAp (Bardhan *et al*., 2011).

Formation of hydroxyapatite from fish scales are also confirmed by FT-IR spectroscopy. To make a comparative evaluation, FS-Hap and CHAp results are given in the same spectrogram in Figure 2. For FS-Hap, the bands located at 628, 599 and 567 cm^{-1} corresponds to asymmetric bending vibration of P-O band (Prabakaran *et al*., 2005; Bardhan *et al*., 2011). The strong peaks around 1043 cm^{-1} belong to asymmetric stretching mode of vibration of P-O bands of PO_4 tetrahedral (Mondal *et al*., 2010). The medium sharp peak around 628 cm^{-1} is due to O-H bending deformation mode. Carbonate group (CO_3^{2-}) in the FTIR spectra was identified by intense bands at 1464 cm^{-1} (Stoch *et al*., 2000). The band at approximately 3400 cm^{-1} corresponds to the O–H stretching of nHA (Panda *et al*., 2014). The band of carbonated group was at 1464 cm^{-1}. Similarly, there was a difference between HA Sigma and nHA salmon that carbonated groups were absent in the CHAp spectrogram (Venkatesan *et al*., 2015).

SEM Images of the Synthesized HAp

The morphology of the obtained FS-Hap was checked by FE-SEM instrument (Figure 3). Images showed that the HAp particle size is <1 µm. The surface of the FS-HAp looks to be rough and exhibits a regular microscale spherical morphology. Similar observations were reported by Pon-On *et al.* (2016) and Muhammad *et al.* (2016) who also extracted HAp from fish scale.

The Ca/P molar ratio obtained by EDS is 1.70 for FS-HAp (Figure 4) and 1.61 for CHAp, which closely resembles the theoretical value of 1.67 (Mostafa, 2005). The high amount of carbon (C) is probably came from the double sided carbon tape and sodium (Na) is came from NaOH used for alkaline treatment.

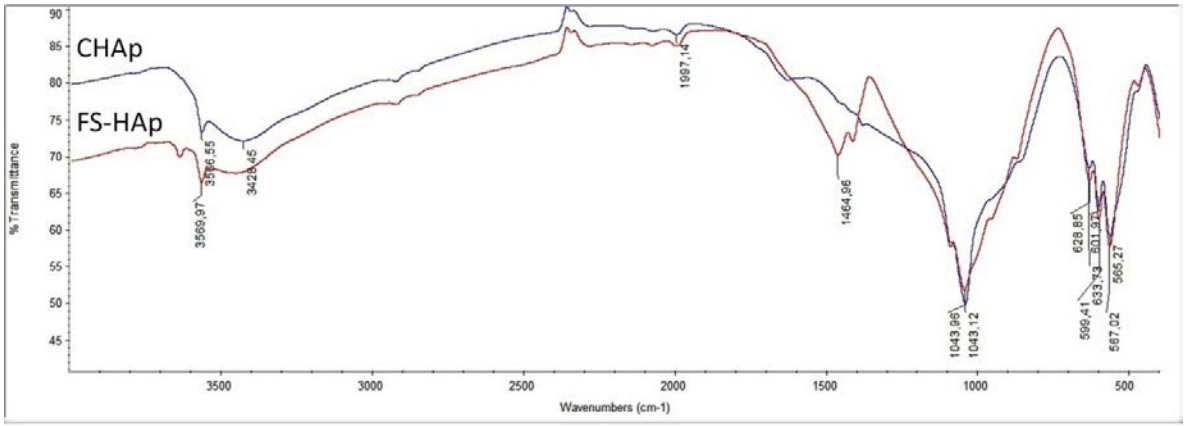


Figure 2. The fourier transform infrared spectroscopy (FT-IR) spectra of FS-Hap (red line) and CHAp (Black line).

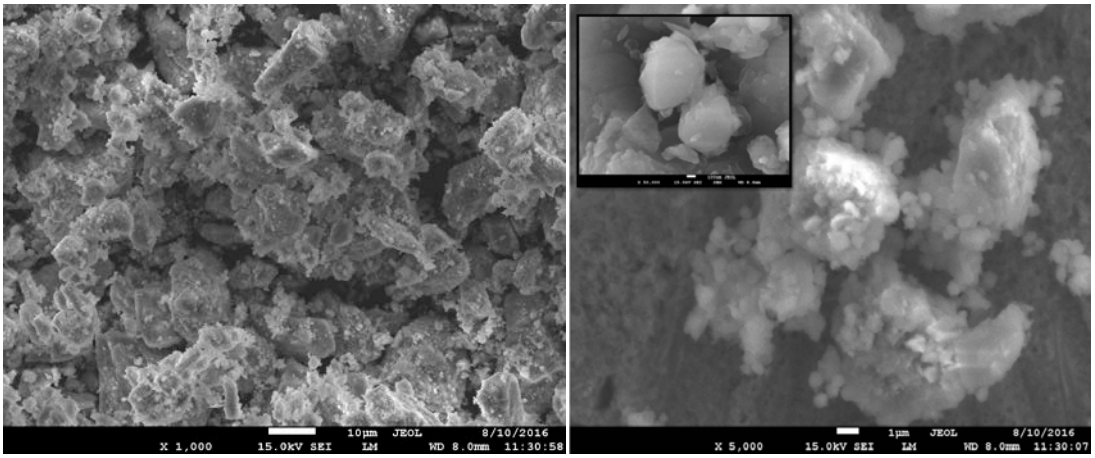
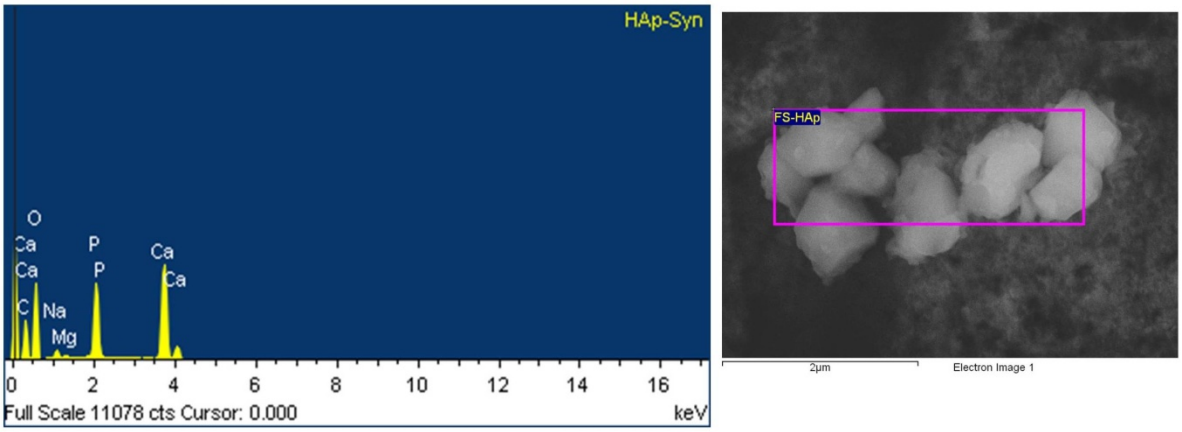


Figure 3. SEM images of FSHAp a) x1000, b) x5000 (small image on the left has x50.000 magnification)

The ICP-OES analysis demonstrated a Ca/P molar ratio of 2.18±0.03 for FSHAp and 2.00±0.02 for CHAp. On contrary to this work, Ca/P molar ratio obtained from ICP-OES is different from the EDS analysis results (Kongsri *et al*., 2013). As the ratio is also high for CHAp, it can be concluded that ICP-OES analysis for this study does not clearly demonstrate the optimum Ca/P ratio. This might be caused by the different preparation technique for ICP-OES analysis, experience of the analyst or the microwave digestion conditions.

Biomaterials for medical applications should be evaluated with *in vitro* investigations of antimicrobial properties before clinical trials (Alt *et al*., 2004). The antimicrobial activity of the synthesized hydroxyapatite was assessed by the well diffusion assay against human pathogenic strains including yeast, Gram-positive and Gram-negative bacteria. No zones of inhibition were observed for tested microorganisms (Figure 5).



Element	Weight%	Atomic%
C K	21.14	32.29
O K	43.05	49.37
Na K	1.24	0.99
Mg K	0.27	0.21
P K	10.69	6.33
Ca K	23.61	10.81
Totals	100.00	

Figure 4. SEM-EDS results of FS-Hap

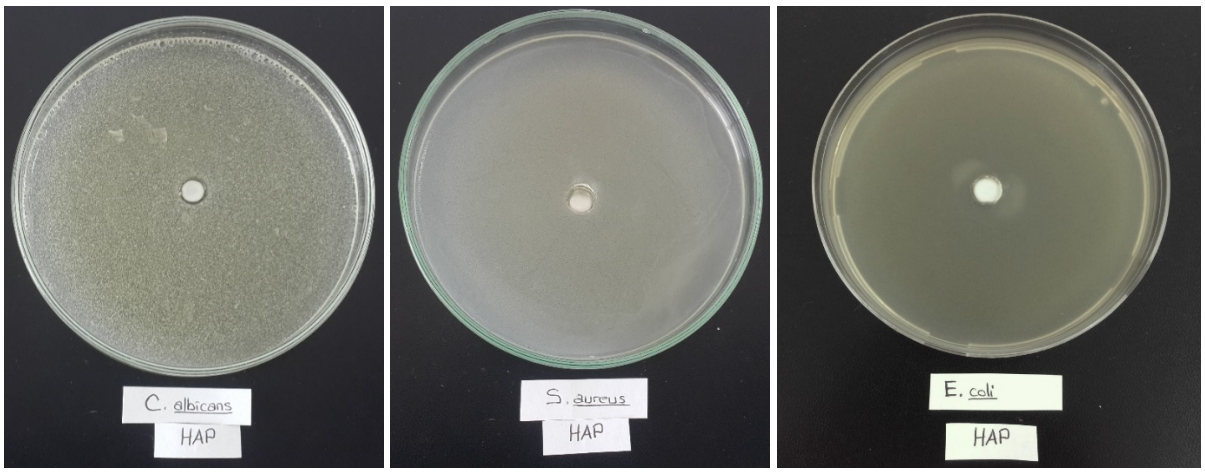


Figure 5. Agar well diffusion assay of fish scale-derived HAp against *C. albicans* (a), *S. aureus* (b) and *E. coli* (c).

Conclusions

In this study, the physicochemical characterization of HAp derived from sea bass and sea bream scale was investigated. The results of the obtained HAp were compared to synthetic HA which is commercially available. The characterizations reveal that the HAp extracted from fish scale has quite similarity to that synthetic HAp by its physicochemical features. Fourier transform infrared spectroscopy, XRD and EDS analyses indicated the crystalline and phase purity and of the obtained HAp powder. The SEM images of the fish scale-derived HAp show that microstructure form of the powder uniformly distributed and the particles have sub micrometer sizes. All characterization results compared with synthetic standard HAp and found to be nearly equivalent. As synthesized Hap exhibited no antimicrobial activity, it can be suggested that improving the antimicrobial activity of hydroxyapatite with new antibiotics or other antimicrobial agents will be useful for biomedical applications. It can be concluded that fish scales which are a high amount by-product of seafood processing companies may be regarded as an economical and accessible raw material for obtaining high quality HAp.

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