Effect of pretreatment processes on physicochemical properties of hydroxyapatite synthesized from *Puntius conchoni* fish scales

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Abstract. The current study emphasizes on the novel idea of synthesizing hydroxyapatite (HAp) from *Puntius conchoni* fish scales' bio-waste and study the effect of acid, alkali and acid-alkali pretreatments on its physicochemical properties. Material characterization is carried out to study the physicochemical properties of the synthesized HAp using Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), thermogravimetric analysis (TGA), scanning electron microscopy energy dispersive X-ray spectroscopy (SEM-EDX) and transmission electron microscopy (TEM) analysis. FTIR and XRD plots show peaks corresponding to natural bone apatite which confirms the formation of HAp. TG analysis shows a maximum weight loss of 2.28% indicating high thermal stability of HAp beyond 800°C. SEM micrographs illustrate highly porous morphology with interconnected pores. EDX analysis of the HAp exhibits close proximity of the calcium/phosphorous (Ca/P) ratio to natural bone apatite. TEM micrographs show the formation of polycrystalline HAp particles with size ranging from 38 ± 1.54 to 219 ± 5.88 nm. A comparative study on the physicochemical properties of HAp synthesized using three different pretreatment processes is performed. Results reveal that HAp synthesized from acid-treated fish scales show highest yield percentage, better thermal stability and highly porous morphology with a particle size of 38 ± 1.54 nm. Thus, HAp synthesized from acid-treated fish scales can be a promising candidate for the development of porous bone scaffolds.

Keywords. Fish scales; bio-waste; hydroxyapatite; biomaterials; advanced characterization.

1. Introduction

Over decades, fish has been consumed as a major food ingredient in the diet of human. Although, fish scales are rich sources of hydroxyapatite (HAp) in the form of calcium (Ca) and phosphate, it is still discarded as waste [1]. It would be sensible to utilize this bio-waste to develop value added by-products. HAp, having the stoichiometric formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH}_2)$ with a calcium/phosphorous (Ca/P) molar ratio of 1.67 [2], is one of the major constituents in the development of bone scaffolds owing to its similar composition to natural bone and teeth [3,4].

HAp is either synthesized from natural sources or from artificial derivatives of chemicals. HAp has been chemically synthesized using processes such as solid state reaction, hydrothermal reaction, co-precipitation reaction, sol-gel method, wet chemical process, microwave irradiation process etc. [3,5–7]. It can also be extracted from natural resources such as fish scales, bovine and cattle bone, egg shells, shells of garden snail, corals etc. to develop biomaterials for regeneration of bone tissues [8–12]. Prabakaran *et al* [8] synthesized HAp from egg shells and phosphoric acid through a precipitation method. Chemical analysis confirms that the Ca/P ratio of the HAp is 1.67 which is similar to pure HAp. Moreover, a total weight loss of 8.56% is observed using thermogravimetric analysis (TGA), which reveals high thermal stability of the

synthesized HAp. Krithiga *et al* [9] developed a composite from egg shell powder, bone ash and gelatin that resembles around 63.5% of the crystalline structure of HAp. Scanning electron microscopy (SEM) micrographs show porous structure of the composite with pores ranging from 50 to 150 μm suitable for bone ingrowth. The developed composite can find promising application in the treatment of maxillofacial and spinal cord bone defects with low cost bone grafting. Anjuvan Singh [10] prepared HAp from garden snail shells and observed higher bioactivity for the synthesized HAp in comparison with conventional HAp. The synthesized HAp can be a suitable bone replacement material to promote bone formation. Huang *et al* [5] extracted HAp from fish scales by enzymatic hydrolysis, while Mondal *et al* [12] adopted a thermal decomposition process. Panda *et al* [13] prepared HAp from synthetic body fluid (SBF) using the wet precipitation method and compared its characteristics with HAp synthesized from fish scales of *Labeo rohita* and *Catla catla*. The investigation performed for biocompatibility testing using MTT assay and quantitative DNA analysis confirms that HAp obtained from fish scales are physico-chemically and biologically equivalent to the HAp chemically synthesized from SBF. However, results of bioactivity test, MTT assay and ALP analysis confirmed that the HAp synthesized from *Probarbus jullieni* fish scales is biologically better than the chemically synthesized HAp due to the fact that fish

scales' HAp shows significant increase in cell proliferation and activity of osteoblasts during cell culturing as observed by Weeraphat *et al* [14]. Likewise, bones of sword fish, tuna and cuttlefish are used to extract HAp [2,15]. HAp obtained from fish bones shows excellent biocompatibility, bioactivity, osteoconductivity, better metabolic activity and dynamic response to body environment compared to synthetic HAp [2]. Milovac *et al* [16] and Beom-Su *et al* [17] derived HAp from cuttlefish bone (CB-HA) and compared the properties with synthetic HAp. Results reveal that CB-HA is highly non-toxic and shows greater cell adhesion, proliferation and differentiation as compared to chemically synthesized HAp.

The literature reveals that fish scales of *L. rohita*, *C. catla* and *P. jullieni* have been preferred as a major ingredient in the synthesis of HAp. However, the fish scales of *Puntius conchoni* have not been exploited for the synthesis of HAp. Moreover, investigations on the effect of pretreatment processes of the fish scales for the synthesis of HAp have remained almost unexplored. In the current study, HAp is synthesized from the fish scales of *P. conchoni* and the effect of acid, alkali and acid-alkali pretreatment processes on the physicochemical characteristics of the developed HAp is examined. A comparative study is performed to investigate the yield percentage, particle size, thermal stability and crystallinity for the developed HAp amongst these three pretreatment processes.

2. Materials and methods

2.1 Synthesis of HAp from fish scales

Fish scales of *P. conchoni* (Rosy barb) are collected and washed thoroughly with water. The scales are then subjected to acid, alkali and both acid-alkali pretreatments for deproteinization of the scales. In the case of acid-treatment, the fish scales are dipped in 1 N hydrochloric acid (HCl, Merck, 35%) solution for 24 h at room temperature $28 \pm 3^\circ\text{C}$ followed by decantation and then washed with distilled water. A similar approach is followed for alkali treatment by dipping the fish scales in 1 N sodium hydroxide (NaOH) solution. In order to carry out the acid-alkali treatment, the fish scales are at first treated with 1 N HCl. The treated scales are washed with distilled water several times followed by treatment with 1 N NaOH solution for a period of 24 h. Deproteinized fish scales are dried at 50°C in a hot air oven for 10 h after thorough washing with distilled water. The dried fish scales are calcined at 900°C at a heating rate of $10^\circ\text{C min}^{-1}$ in a muffle furnace for 3 h and allowed to cool inside the furnace for 24 h to obtain HAp ceramics. The calcination temperature is chosen based on the TGA result of collected fish scales which show no degradation in the temperature zone of $800\text{--}1000^\circ\text{C}$. The corresponding actions performed during the synthesis of HAp from the fish scales of *P. conchoni* are depicted in figure 1.

2.2 Characterization techniques for the synthesized powder

Techniques used with instrument details to execute the characterization on the synthesized powder along with their pre-requisite conditions are briefed below. However, the results obtained are discussed in the subsequent section.

2.2a Fourier transform infrared spectroscopy (FTIR): Calcined fish scale powders are analysed by FTIR analysis to determine the functional groups present in it. The infrared spectra are obtained by the KBr pellet technique using Perkin Elmer Spectrum1 FTIR instrument at a range of $400\text{--}4000\text{ cm}^{-1}$.

2.2b X-ray diffraction (XRD): A fully automated computerized powder X-ray diffractometer (XRD) (model: X'Pert Pro, PANalytical) is used to analyse the crystalline phases of the synthesized powder. The scanned angular range (2θ) is adapted from 10 to 90° at a step size of 0.02° . The working voltage and current are maintained at 40 kV and 20 mA. The counting time is 2 s per step.

2.2c TGA: TGA is performed to investigate the phase changes and weight loss with respect to temperature in the fish scales to determine the calcination temperature for the synthesis of HAp. The thermal stability and degradation of the synthesized HAp is also characterized using TGA. The tests are performed with a NETZSCH STA 449 F3 Jupiter instrument under a combined atmosphere of oxygen and nitrogen at a temperature range of $30\text{--}900^\circ\text{C}$ at a heating rate of $10^\circ\text{C min}^{-1}$.

2.2d Field emission scanning electron microscopy (FE-SEM): The surface morphology of the synthesized HAp is investigated using a high resolution scanning electron microscope (Quanta 200F, FEI). A thin conductive layer of gold is plated using gold sputtering machine (Cressington-108 auto, model no. 7006-8) at 230 V, 50 Hz using a high speed, direct drive two-stage vacuum pump. The gold coated samples are analysed at 20 kV at a magnification range of $150\text{--}25000\times$.

2.2e Transmission electron microscopy (TEM): The nature and size of HAp crystals formed are studied by TEM analysis using a JEM-100 CX II operated at 100 kV. TEM images provide detailed information of HAp crystals and its crystal orientation in the lattice structure. The particle sizes are determined by image analysis using ImageJ (NIH).

3. Results and discussion

3.1 FTIR analysis

The formation of apatite phase in the powder derived from alkali, acid and acid-alkali treatment of fish scales is

Figure 1. Detailed process followed for synthesizing HAp from fish scales of *P. conchoniuis*.

confirmed by FTIR analysis. The graphical interpretation of the result is represented with 02HTP, 04HTP and 05HTP for alkali, acid and acid-alkali treatments, respectively, as shown in figure 2a–c. The formation of HAp in all the treatments is indicated by a strong extensive FTIR band which is observed at $559\text{--}598\text{ cm}^{-1}$ corresponding to the ν_4 symmetric P–O stretching vibrations of the PO_4 group, at 960 and 1002 cm^{-1} corresponding to the ν_3 vibration peak which is due to the asymmetric stretching of the PO_4 group [12]. Similarly, the band at 3502 cm^{-1} is attributed to the characteristic stretching modes of hydroxyl groups (O–H), as well as the bands for O–H are also observed at 3566 , 3419 and 1647 cm^{-1} [18]. Moreover, carbonate ions are identified at 875 , 1458 , 1533 and 1652 cm^{-1} [19]. It indicates formation of B-type HAp substituting phosphate ions [20]. The formation of Ca and P is observed corresponding to the peaks of $(\text{PO}_4)^{3-}$ and $(\text{CO}_3)^{2-}$ groups. The FTIR results show the presence of sharp peaks associated with stretching vibration indicating the formation of crystalline HAp in all performed pretreatment processes [21]. Hence, it can be confirmed that the powder obtained

from calcination of fish scales is a pure form of crystalline HAp. Thus, any of the pretreatment processes are eventually capable of synthesizing HAp from fish scales.

3.2 XRD analysis

The crystalline phase analysis of the synthesized powder is carried out using XRD studies. The XRD patterns of the powder obtained through alkali, acid and acid-alkali treatments are shown in figure 3a–c. Identification of the phases is performed by comparing the experimental XRD patterns with standards complied by the International Centre for Diffraction Data (ICDD) using card no. 09-0432 for the hexagonal HAp structure. Well-resolved characteristic peaks of the highest intensity for HAp are obtained at 2θ values of 32.19 , 31.77 and 32.19° corresponding to the (112), (211) and (112) planes for alkali, acid and acid-alkali pretreatments, respectively. The phases formed are pure and matches well with the standard patterns. The standard corresponding planes for HAp (*viz.* 002, 210, 112, 300, 221, 311, 400, 222, 213, 321,

Figure 2. FTIR peaks of the powder synthesized through (a) alkali, (b) acid and (c) acid–alkali treatment of fish scales confirming the formation of HAp.

410, 004, 402, 323, 511, 513 for alkali treatment), (101, 200, 111, 002, 102, 210, 211, 112, 300, 310, 222, 312, 320, 004, 420 for acid treatment), (111, 002, 112, 300, 202, 310, 311, 222, 312, 213, 321, 410, 402, 004, 322, 510, 511 for acid–alkali treatment) are well observed in the cases of all the three pretreatment processes [22].

The crystallite sizes of the HAp particles are determined using Scherer's equation as depicted below:

$$D = 0.9\lambda / B \cos \theta \quad (1)$$

where D represents the average dimension of crystallites in a direction normal to the diffraction plane hkl , B stands for full width at half maximum of the peak, λ is the diffraction wavelength (0.154059 nm) and θ is the diffraction angle [23]. The Bragg reflections at five major intensity peaks are selected for calculating the crystallite size of the synthesized HAp. The crystal planes considered for alkali and acid–alkali pretreatments are (112), (300), (213), (002) and (222), and for acid pretreatment (211), (202), (102), (004) and (002). Crystallite sizes for HAp particles obtained from fish scales are

46.470 ± 1.28 , 45.877 ± 1.64 and 46.470 ± 1.28 nm for alkali, acid and acid–alkali pretreatments, respectively. The crystalline phase of the powder synthesized from the fish scales of *P. conchonius* using all the three pretreatment processes is similar to those obtained by Mondal *et al* [22]. HAp is the only phase found in all the patterns and traces of any other calcium phosphate impurities are not identified. The sharp peak intensity and well resolved peaks observed in XRD patterns confirm the complete crystallization of the synthesized powder. The crystallite size of the HAp as investigated in the current work is around 46 nm. The small dimension of the synthesized HAp crystallite makes it a promising reinforcing material for fabrication of bone scaffolds with high mechanical strength, improved bone cell attachment and growth [13]. However, among the three pretreatment processes, the HAp derived from acid-treated fish scales matches well with the high intensity peak of the standard crystalline peak of HAp.

3.3 TGA

TGA of thoroughly washed fish scales with tap water is carried out to investigate phase transformation and to determine the

Figure 3. XRD spectra of the powder synthesized through (a) alkali, (b) acid and (c) acid-alkali treatment of fish scales confirming the formation of HAp.

Figure 4. TGA of (a) intact fish scales and (b) synthesized HAp showing the thermal stability.

weight loss with respect to temperatures as shown in figure 4a. Based on that result, the calcination temperature is found which is used for the synthesis of HAp. The major constituent of fish scales are collagen, connective tissue proteins and organic protein molecules such as guanine, glycine, alanine,

hydroxyproline etc. [24]. As the thorough washing of the fish scales cannot completely remove the organic compounds, the maximum weight loss occurs mainly for these organic matters which degrade at different temperature ranges. Results presented in figure 4a show that the first phase of degradation

Figure 5. SEM micrographs and EDX analysis of HAp synthesized by (a, d) alkali, (b, e) acid and (c, f) acid-alkali treatment, respectively, showing the porous structure and the Ca/P ratio.

occurs between 100 and 300°C because of weakly entrapped water. The major weight loss is observed between 300 and 400°C due to degradation of collagen, connective tissues, proteins etc. The degradation of guanine and other organic matters take place between 500 and 700°C. Thereafter, no further degradation is observed above 800°C showing high thermal stability of the sample within the temperature range of 800–1000°C [24].

The TGA result of the synthesized crystalline HAp for three pretreatment processes is presented in figure 4b. It shows a

marginal degradation between 100 and 400°C which confirms the removal of moisture from the synthesized HAp. The major weight loss is observed in the temperature range 400–500°C which corresponds to the removal of organic substances. The synthesized HAp shows almost no degradation above 800°C. Total mass loss of 2.28% is observed for alkali-treated fish scales followed by 1.52% for acid-alkali-treated and 1.22% for acid-treated leaving 98–99% residual mass for all the pretreatments. Thus, it can be concluded that acid-treated fish scales' HAp shows the lowest amount of degradation

Figure 6. TEM micrographs of HAp synthesized by (a) alkali, (b) acid and (c) acid-alkali treatment, respectively, showing the nature and size of HAp crystals formed.

confronting a good thermal stability than the other two processes.

3.4 FE-SEM and EDX analysis

The surface morphology and pattern of particle distribution in the synthesized HAp is examined using SEM analysis and the elemental composition is studied by EDX analysis. SEM micrographs of the synthesized HAp are shown in figure 5a–c, respectively. The surface morphologies of HAp obtained from three pretreatment processes are found to have agglomerated ultrafine HAp particles [21]. SEM micrographs reveal highly porous surface making it a promising candidate for the development of bone scaffolds. HAp particles synthesized from the *P. conchoni* fish scales are almost spherical in shape.

The acid-treated fish scale-derived HAp shows better porous morphology with interconnected pores as shown in figure 5b. EDX analysis reveals specific constituents and impurities in single HAp particles. The results shown in figure 5d–f for EDX analysis confirm formation of Ca and P in the calcined powder. The synthesized powder shows a high amount of Ca, P and oxygen content mostly in the form of carbonate and phosphate. The results conclude formation of HAp in all the three pretreatment processes. The ratio of Ca/P for alkali treatment, acid treatment and acid-alkali treatment is found to be 1.81, 1.75 and 1.79, respectively, which is slightly higher than the stoichiometric ratio of 1.67 as observed in the apatite of human bone [1]. The high Ca/P ratio can be accredited to the replacement of phosphate by carbonate ions in the apatite structure forming B-type carbonate HAp [14].

Table 1. Comparative analysis of the properties of HAp synthesized by three different pretreatment processes.

Sl no.	Pretreatment processes	Thermal stability (residue mass) (%)	Crystallite size (nm)	Particle size (nm)	Ca/P ratio
1	Alkali	97.72	46.470 ± 1.28	161 ± 7.25	1.81
2	Acid	98.78	45.877 ± 1.64	38 ± 1.54	1.75
3	Acid–alkali	98.48	46.470 ± 1.28	219 ± 5.88	1.79

Table 2. Comparative analysis of the yield percentage of the HAp synthesized by three different pretreatment processes.

Sl no.	Pretreatment processes	Weight before pretreatment (g)	Weight after pretreatment (g)	Weight after calcination (HAp obtained) (g)	Yield (%)
1	Alkali	30	17.947	4.817	16.05
2	Acid	30	19.22	5.159	17.19
3	Acid–alkali	30	13.35	3.583	11.94

3.5 TEM analysis

The crystalline structure of the synthesized HAp is investigated using TEM. Figure 6a–c shows TEM micrographs and selected area electron diffraction (SAED) pattern of HAp synthesized from alkali, acid and acid–alkali-pretreated fish scales, respectively. The figure indicates highly ordered dense agglomerated HAp particles. The particle size of the HAp ceramic observed from TEM images was calculated by averaging five different measurements for a single particle using ImageJ software. The particle sizes for the HAp synthesized from alkali, acid and acid–alkali-pretreated fish scales are 161 ± 7.25 , 38 ± 1.54 and 219 ± 5.88 nm, respectively. The SAED pattern represents discrete rings showing that the diffraction consists of a large number of ultra-fine crystalline powder particles which confirm polycrystalline structure of the synthesized HAp [25].

A comparative study is performed based on the physicochemical properties of the HAp synthesized from *P. conchoni* fish scales using three different pretreatment processes. It is observed that acid-treated fish scales show better characteristics in terms of thermal stability, crystallite size, particle size and Ca/P ratio than the others. It is attributed to the fact that acid treatment removes a high amount of organic substances present in the fish scales ensuring better properties of the synthesized HAp. The results of the comparative analysis are listed in table 1. Moreover, the yield percentage is calculated for the HAp synthesized using the three pretreatment processes which further shows that the acid treatment gives better yield percentage compared to the other two processes. The results of the yield obtained from the three pretreatment processes are provided in table 2.

4. Conclusions

The current research work reveals that HAp can be synthesized from the fish scales of *P. conchoni* using alkali, acid

and acid–alkali treatments. The results from FTIR and XRD analysis confirm the formation of crystalline HAp considering purity of phase for all the three pretreatment processes. TG analysis of all the treated samples indicates that the synthesized HAp is stable above 800°C which indicates its high thermal stability. SEM micrographs illustrate porous structure of HAp with interconnected pores. The key findings obtained from the current study can be summarized as follows:

- There is a degradation of 1.22% of mass for acid-treated fish scales' HAp indicating better thermal stability compared to the other two processes.
- The HAp derived from acid-treated fish scales shows uniform porous morphology with a particle size of 38 ± 1.54 nm.
- The acid treatment shows better yield with 17.19% compared to the other two pretreatment processes.

Thus, the synthesized HAp may find promising applications in the development of bone scaffolds for treatment of segmental bone defects.

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