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Isolation and characterization of hydroxyapatite powder from the scale of freshwater fish *Labeo rohita* using green technology

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The objective of the present work was to isolate and characterize hydroxyapatite (HAP) from *Labeo rohita* scale using green technology. The method used only 0.6% KOH solution for isolation, which did not generate any spent liquid for disposal to the environment. Investigation through thermogravimetric analysis, X-ray diffraction study, scanning electron microscopy, energy-dispersive X-ray spectroscopy, and particle size distribution measurement was carried out for characterization of HAP. The powder after isolation from scale contained < 7% organic matter and could be converted to thermally stable perfectly crystalline pure HAP without any organic impurity by exposition to 1000 °C for only 1 h. The heated HAP powder had a Ca:P ratio of 1.52 and indicated the grains to be in submicron size but remaining in soft agglomerated condition with 50% of the mass passing through ≈ 167 µm.

Keywords: Green technology, Hydroxyapatite, *Labeo rohita* scale, Organic matter.

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Introduction

Hydroxyapatite (HAP) is bio-compatible material valuable in biomedical applications including dental and orthopaedic fields, biosensors, and as drug carrier[1,2]. It is also important in chromatographic application and removal of pigments as well as heavy metals from waste water[2-4]. The possible use of HAP as a catalyst for the treatment of toxic gases has also been postulated[5]. The chemical composition of hydroxyapatite (HAP) may vary from stoichiometric ($[Ca_{10}(PO_4)_6(OH)_2$, Ca/P ratio 1.67]) to nonstoichiometric with a Ca/P ratio being <1.67 or >1.67. The biological properties and other applications of HAP are dependent on the Ca/P ratio[6,7]. The use of HAP in various commercial applications is gradually increasing.

To avoid the complicacy, large time-consumption, and high cost involved in the preparation of synthetic HAP, its production from biological sources is gaining importance[8]. Extraction of HAP from animal bones and teeth, as well as fish bones, has been investigated by several workers[4,9-12]. However, HAP is also a natural part of the fish scale and remains commingled with collagen protein[13,14]. Due to the increasing production of fish, the availability of scales is also progressively rising. Since the fish scale is throwaway waste material, researchers have concentrated on isolation and characterization of its HAP in the last decade[14-16]. Further, fish scale HAP is biologically better than chemically synthesized HAP[17]. An added advantage of using fish scale would be a feasible route of municipal waste management.

The main concern to recover HAP from the fish scale is the removal of collagen. Several investigations on such recovery from scales of different fishes have been reported. HAP was isolated from scales of *Tilapia nilotica*[3,4] and *Labeo rohita*[14,18,19]. In these reports, the actual deproteinization approaches were case-specific, but, in general, followed sequential treatment with widely variable concentrations of HCl (3.5-35%) and NaOH (4-50%); however, details of the solvent was not available from the publication[14]. While isolating from a mixture of *L. rohita* and *Catla catla* scales, a slight modification was done where after the HCl-NaOH treatment for deproteinization, the scales were further treated with $CaCl_2,2H_2O$[16]. Mondal *et al.*[20] and Pon-On *et al.*[17] have reported the isolation of HAP from *Oreochromis mossambicus* (Tilapia fish) and *Probarbus jullieni* by treating with only 4% solution of NaOH and HCl, respectively. In all the foregoing cases, the deproteinized scales were further subjected

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to different extents of heat treatment to meet the end purposes. Interestingly, enzymatic hydrolysis, i.e., sequential treatment with protease N and flavourzyme for protein removal from Tilapia (*Oreochromis* sp.) scale has also been reported[15]. Muhammad *et al.*[21] isolated HAP from Cyprinidae (Carp) scale by removing the organic portion through dissolution using 1-butyl-3-methylimidazolium acetate, followed by increasing the pH of the mixture to 9 by adding 2% NaOH.

Discarding the HCl and NaOH spent solutions during isolation of HAP from fish scale causes environmental pollution, which contradicts the recently evoked concept of green technology[22]. Again, to avoid global warming, it is always desirable to use thermal exposition as low as possible for the removal of organic matter. Using enzymes in the extraction process, on the other hand, makes the end product costly. Moreover, in none of the publications relating to extraction of HAP via HCl, NaOH, heat, or enzyme treatment, or their combination, simultaneous recovery of the protein part was not mentioned. Though Muhammad *et al.*[21] commented on this aspect of byproduct retrieval, there is a point regarding environmental risk from the disposal of imidazolium cation, particularly to aquatic ecosystem[23,24]. To overcome these aspects, the objective of the present study was to isolate and characterize HAP from *L. rohita* scale through a green technology that provides a scope of recovery of collagen and utilization of spent liquid as fertilizer.

Materials and Methods

Materials

Scales of freshwater fish (*L. rohita*) was procured from the Technology market, IIT Kharagpur, West Bengal, India, and washed thoroughly with potable water until all unwanted material was removed. After cleaning, the fish scale was dried at 55-60 °C for 24 hours in a hot air oven (Electronics & Electrical Engineering Co., India). The dried scale was stored in zip-lock low-density polyethylene (LDPE) pouch in ambient condition and used as raw material for extraction of HAP. All the chemicals used in the experimental work were of analar grade. The water used in the isolation and followed by purification was either potable water or distilled water (dw), as mentioned hereunder.

Extraction of crude HAP from fish scale

In the first stage (1), to 100 g of scale taken in a beaker, dw was added to make the total volume of 1000 mL. A variable amount of KOH (5/6 g) was mixed in it (to make 0.5/0.6% alkali solution) by manual stirring and the system was left undisturbed overnight at room temperature (28-30 °C). After soaking, the alkali solution was collected by decantation. In the second stage (2), the soaked scale from the stage (1) was re-soaked overnight exactly in a similar way in fresh KOH solution of the same concentration. Following this, the decanted solution from the first stage was added to the re-soaking system (2), and the mixture was boiled on a heater till the scale disintegrated. It took about 90 minutes. The liquid part (KOH solution containing the collagen) was separated and the disintegrated fish scale part was the crude HAP that contained some organic matter as an impurity. A schematic representation of the scale disintegration is shown in Fig. 1.

Purification of crude HAP

For removal of organic matter as much as possible from the crude HAP, several techniques like water wash (a), alkali wash (b), and twice alkali wash (c) were attempted. For water wash, the sample was washed five to six times with potable water, followed by washing with dw until the pH of the washing was the same as that of dw. The washed sample was dried

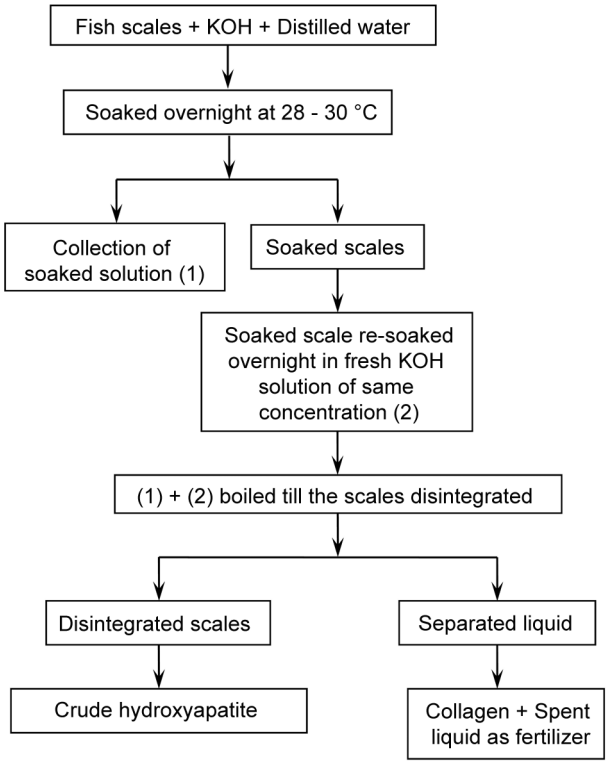

Fig. 1 — Schematic of the fish scale disintegration.

at 105 °C for 24 hours in a hot air oven. For alkali wash, the crude HAP was initially dried at 60 °C (this was required for taking a measured amount of sample). Then 10 g of this dried sample along with 0.5 g KOH was added to dw to make 100 mL. This mixture was kept for 24 hours at room temperature. After 24 hours, the liquid was decanted, and the sample was washed and dried (105 °C) in the same way as that of the water wash process. For twice alkali wash also, the crude HAP was initially dried at 60 °C. Then 10 g of this dried sample and 0.5 g KOH was added to dw, the total volume being 100 mL. This mixture was kept for 24 hours at room temperature and then the liquid was decanted. The soaking operation was repeated similarly. After discarding the soaking liquid, both washing and drying of the residue were similar as followed in case of technique (a).

In each case of water wash, alkali wash, and twice alkali wash, the sample dried at 105 °C was in powder form. These were put in a Muffle furnace (Instrumentation India, India) at 550 °C for 3 hours for ashing, i.e., for preliminary estimation of the removable organic matter that still remained after purification[25]. It was calculated as per the formula:

$$\text{Organic matter content (\%, dry basis)} = \frac{\text{mass removed during ashing}}{\text{mass of dry sample taken for ashing}} \times 100$$

The whole system of extraction-cum-purification was done in three replications. Based on the least significant difference (LSD) and t value ($t_{calculated}$) from the student's t-test, the method that retained the minimum organic matter content after purification was selected as the best method to recover HAP from the fish scale. The HAP powder prepared using the selected method was stored in a zip-lock LDPE pouch in ambient condition for further analysis.

Characterization of HAP

To assess thermal stability, the HAP was subjected to analysis using a Thermal analyzer (Perkin Elmer Pyris Diamond, USA) by thermogravimetry (TG)/derivative thermogravimetry (DTG) method over a temperature range of 50-1200 °C at a heating rate of 5 °C/min in a nitrogen atmosphere with a flow rate of 100 mL/min. The sample lost weight up to about 500 °C for the 1st phase and finally obtained thermal stability at > 800 °C. Accordingly, the HAP was passed through two heat treatments, first at 550 °C for 3 hours and second at 1000 °C for 1 hour, and was subjected to the following analyses. The phase purity and crystallinity were investigated by X-ray diffraction study (2ϵ ranging within 10 to 90° with Cu Kα radiation) using D2 Phaser (Bruker AXS, Germany). The generated peaks were matched with Joint Committee on Powder Diffraction Standards (JCPDS)-International Centre for Diffraction Data (ICDD) (PCPDFWIN v. 2.02, 1999) number for $Ca_{10}(PO_4)_6(OH)_2$. Surface morphology was studied by scanning electron microscope (Carl ZEISS Microscopy, Germany) equipped with energy-dispersive X-ray spectroscopy (Ametek EDAX, Model-Octane plus, USA). Particle size distribution was estimated through Malvern Particle Size Analyzer (Malvern Instruments, United Kingdom).

Results and Discussion

It was mentioned previously by the authors[26] that the separated KOH solution from boiling stage (as shown in Fig. 1), after neutralization with phosphoric acid was used for collagen recovery through salting out with ammonium sulfate. Also, the broth, as reported, after separating the collagen part was a good source of N, P, and K and could be applied as fertilizer. Thus, in the whole process, there was no problem with liquid waste disposal.

Organic matter content

Organic matter content after 105 °C drying of samples obtained by different purification techniques for the same amount of KOH in extraction (row-wise comparison by LSD) and by same purification technique but extracted with varying KOH concentration (column-wise comparison by t-test) are shown in Table 1.

It is seen from Table 1 that by using 5 g KOH in extraction, the organic matter remaining through different purification were significantly different (LSD, P <05) from each other following the order: water wash > alkali wash > twice alkali wash. This

Table 1 — Organic matter content[#] of different HAP samples subjected to ashing at 550 °C

KOH used in extraction, g	Organic matter content (%, db)			$LSD_{.05}$
	Water wash	Alkali wash	Twice alkali wash	
5	10.87±0.24[*]	6.48±0.25	5.71±0.36[*]	0.576
6	9.87±0.95[*]	4.98±0.07[@]	4.93±0.36[@*]	1.171
$t_{.05\ (calculated)}$	1.766	9.980	2.676	

[#]: mean of 3 replications; [@]: values in the same row are not significantly different; [*]: values in the same column are not significantly different; tabular t value at 5% level of significance is 2.776

indicates that if 5 g KOH is used, the best purification method may be taken as twice alkali wash. In the case of extraction with 6 g KOH, the organic matter decreased significantly (LSD, P <05) from water wash to alkali wash; however, further washing with alkali did not impart any extra benefit. For each purification, though the organic matter remaining was less for extraction with 6 g KOH than that for 5 g KOH, the lowering was statistically significant only for alkali wash. Thus, to provide the presence of the lowest possible organic matter content as well as avoid unnecessary wash, extraction using 6 g KOH and purification by alkali wash is recommended for the isolation of HAP from *L. rohita* scale. The yield of dry (105 °C) HAP obtained from 100 g fish scale (raw material) by the recommended method was about 26 g.

It may be noted that in the present study only KOH was used; moreover, the concentration used in extraction and purification was much less, at least 8 times, than the lowest NaOH concentration reported by previous researchers as mentioned in the introduction.

Characterization of HAP

Thermogravimetric analysis

Thermogravimetric analysis (TGA) of the HAP is shown in Fig. 2. It is seen that total weight loss is about 6% over the whole heating range (50-1200 °C), constituting a decreasing profile followed by an increasing tendency. The actual weight losses are observed in the temperature ranges of about 50-500 °C (≈ 5%), and 600-800 °C (≈2%), amounting to a total value of 7%. The TGA profile and % weight loss obtained by very low concentration of KOH in the present study is very much comparable to that of Mondal *et al.*[14] for *L. rohita* scale (solvent details not available) and Kongsri *et al.*[3,4] for Tilapia scale

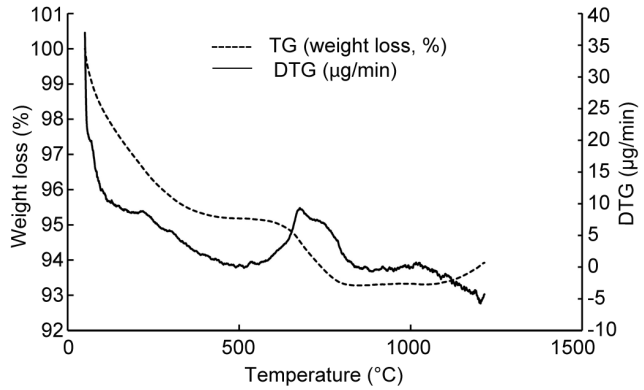

Fig. 2 — TG-DTG analysis of HAP.

(treated with 3.5% HCL, 5% NaOH, and finally 50% NaOH). Up to 500 °C, the weight loss could be due to loss of moisture and organic matter[14,27]. Further, the authors highlighted that up to 200 °C the physisorbed water is removed, whereas the chemisorbed/lattice water requires much higher energy[27]. It may be noted that HAP has a natural tendency for moisture, and the prepared sample in the present study was stored in an LDPE package at ambient conditions before the experimentation; probably during this storage, it might have absorbed some moisture. From 600 to 800 °C, the sharp decrease in weight could be due to the removal of organic matter remaining in HAP as well as possible dehydroxylation[3,4]. Ooi *et al.*[28], while annealing bovine native bone in the air, opined that all the organic material was removed at about 600-700 °C. Also, the presence of an increasing amount of gelatin in the HAP-gelation blend decreases the temperature at which thermal degradation takes place at the highest rate[29]. The DTG curve (Fig. 2) also shows that the peak rate of weight decrease occurs around 700 °C; from 800 °C onwards and up to about 1000 °C, there is no apparent change in weight, indicating thermal stability of the so long heated HAP. It may be worth mentioning that thermally stable, almost pure HAP, i.e., with no organic matter was isolated using NaOH (1%) plus acetone extraction with successive heating at 900 °C from bones of *Thunnus obesus*[1]. Interestingly, further heating from 1000 °C, in the present case, caused a low increase (<1%) in weight, which might be due to the absorption of gases from surrounding[30]. This type of weight increase in the last stage of high-temperature heating in TGA in nitrogen atmosphere has also been observed in different (not related to hydroxyapatite) case[31]. Concerning sorption isotherms, adsorption of nitrogen by HAP produced through heat treatment of Japanese sea bream bone is also available[32].

Since the loss in weight for combined removal of moisture and organic matter was 7%, the HAP prepared by the recommended method in the present study contained less than 7% organic matter.

X-ray Diffraction (XRD) analysis

It is evident from the above discussion that when heated up to ≈ 500 °C, the HAP released moisture and organic matter for the 1st phase, and ultimately attained thermal stability by 1000 °C. Fig. 3 shows the diffraction pattern of the samples heated at 550 and 1000 °C. From the figure, it is clear that the most

intense peaks appear in the range of 20-70° of 2ŧ, which matches the characteristic peaks of HAP, as indicated by JCPDS-ICDD number 74-0566. Though the diffraction angles corresponding to characteristic peaks are very much comparable for both the figures, the peaks become narrower and more intense after heating at 1000 °C. This may be due to the removal of all organic matter in the powder, and the HAP may be considered pure and completely crystalline[16].

As shown in Table 2 (discussed later), the Ca/P ratio of the sample heated at 1000 °C is less than the sample heated at 550 °C. In this context, it needs mentioning that HAP can maintain crystal structure irrespective of their Ca/P ratio[2]. A similar XRD pattern as obtained in the present study was observed by other researchers for HAP obtained from chemical synthesis[33], animal bones[10], and scales of different fishes, viz., *L. rohita*[14,18,19], different Tilapia species[3,4,15,20], and *P. jullieni*[17].

Morphology

Fig. 4 represents the surface morphology of HAP powder by scanning electron microscopy at various stages of preparation. It is seen from Fig. 4a (100X)

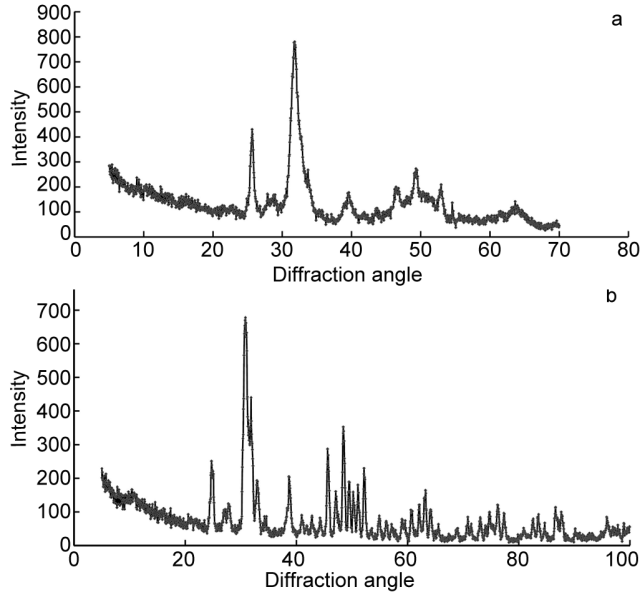

Fig. 3 — XRD analysis of HAP, a) after heating at 550 °C and, b) after heating at 1000 °C.

Table 2 — Ca:P ratio of HAP heated at different temperature

Sample	Ca (%)	P (%)	Ca:P
HAP (550 °C)	29.13	15.93	1.82
HAP (1000 °C)	20.97	13.78	1.52

that thermally stable powder particles, as obtained with presently used technology at 1000 °C, are of heterogeneous shapes with surface roughness, edges, and corners. Irregular particles shape of HAP powder was also documented by different researchers using their case-specific technology from, viz., Tilapia scale[4,15], Cyprinidae (carp fish) scale[18,21], Tuna fish bone[1], Salmon fish bone[34], and bovine bone[35].

L. rohita scale consists of two layers, a surface layer containing bony ridges of hydroxyapatite (HAP) that appear in concentric rings with alternate groove-like depressions embedded in an organic framework of randomly arranged collagen fibres; the inner fibrous layer is composed mainly of collagen[36]. This indicates that the original morphology (Fig. 4a) of the HAP ridges in scale is maintained even after isolation from the native resource. Such type of maintenance was reported for HAP isolated from marine algae[5] and cuttlefish bone[37].

Microstructure with higher magnification (10000X) as shown in Fig. 4b-d better reflects the removal of organic matter from HAP with different extents of heating during its preparation. For the sample dried at 105 °C (Fig. 4b), i.e., before subjecting to a higher temperature, the surface is highly dense[38], while the compactness decreases with heating at 550 °C (Fig. 4c). This is, perhaps, because of the removal of organic matter at a higher temperature. Heated at 1000 °C (Fig. 4d), the HAP loses all organic materials, and the structure is homogeneous, arising from interconnected grains of a single component of HAP. If further magnified at 25000X (Fig. 4e) for the HAP heated at 1000 °C, the micrograph reveals that the grains are of submicron size with a shape ranging from spherical to oval, and indicating a tendency of agglomeration but maintaining sufficient porosity. While working on bovine bone, Barakat *et al.*[11] opined that the morphology of HAP depends on the method of isolation. In this context, it may be noted that similar grain morphology, as seen in the present work, was reported for HAP prepared by chemical synthesis[39], and from *Puntius conchonius* scale by using 35% HCl and calcination at 900 °C[40].

Energy-dispersive X-ray study (EDX)

The EDX of HAP is presented in Fig. 5, while the atomic percentage of Ca and P, and their ratio are shown in Table 2. Thus, the ratios slightly deviate from the stoichiometric ratio. The sample after 550 °C heating appears to be Ca-rich, while after heating at 1000 °C it turns to be Ca deficient. In the case of

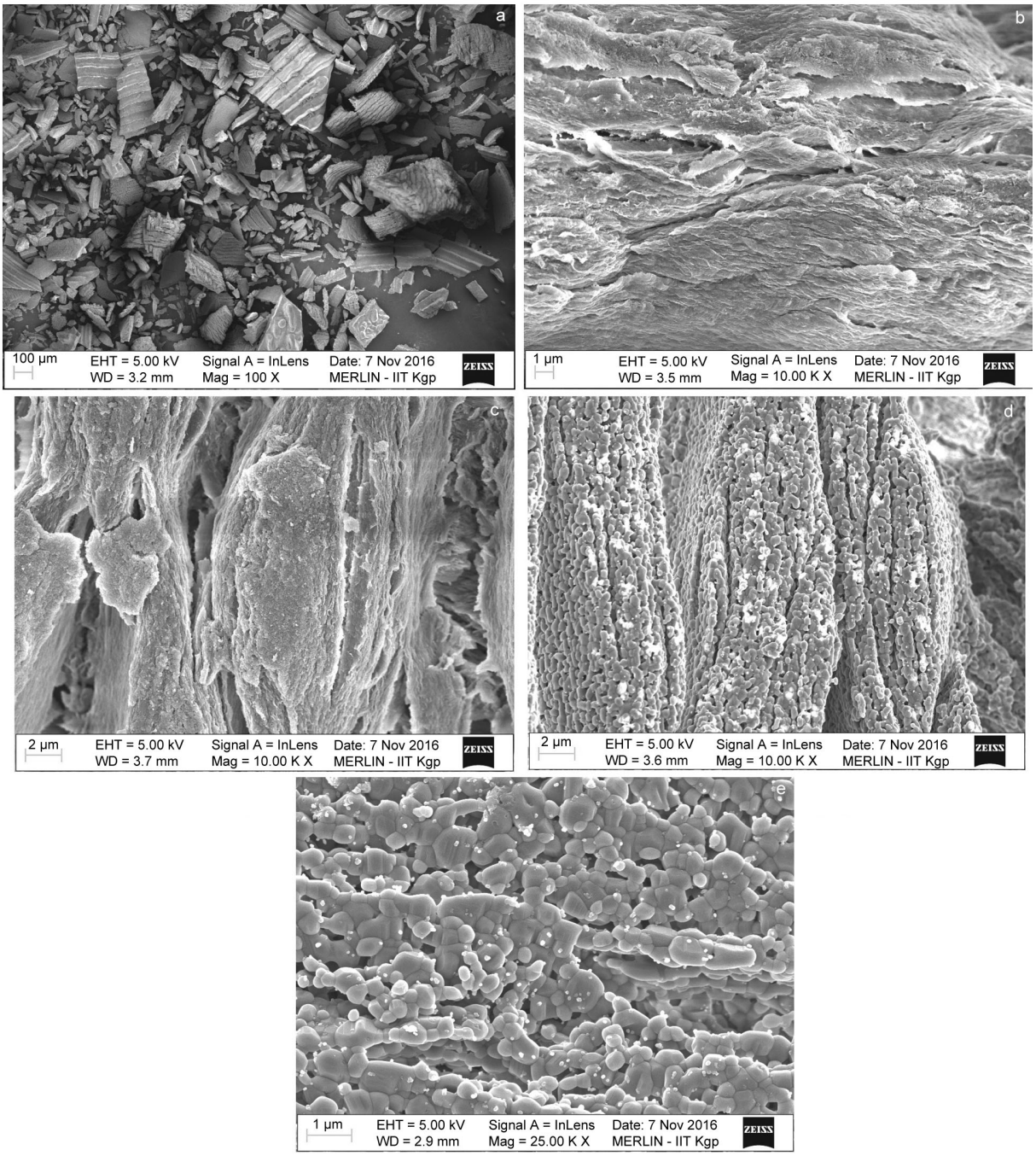

Fig. 4 — SEM image of HAP after heating at, a) 1000 °C with 100X magnification, b) 105 °C with 10000X magnification, c) 550 °C with 10000X magnification, d) 1000 °C with 10000X magnification and, e) 1000 °C with 25000X magnification.

bovine bone-derived HAP, Ooi *et al.*[28] also stated that the Ca/P ratio of 2.31 of raw bone annealed at 400 °C decreased to 1.85 with further heating up to 1200 °C. However, the obtained ratios in the present study are within the acceptable range for hydroxyapatite, as opined by several researchers[7,11]. According to Felício-Fernandes and Laranjeira[5], stoichiometric HAP is more stable but the Ca deficient HAP is of greater biological value than the stoichiometric one. It was commented that human bones are made of nonstoichiometric HAP[5].

Regarding the stability of Ca-rich HAP, Ramesh *et al.*[33] found synthetically prepared HAP with a Ca/P ratio of 1.87 to be thermally unstable

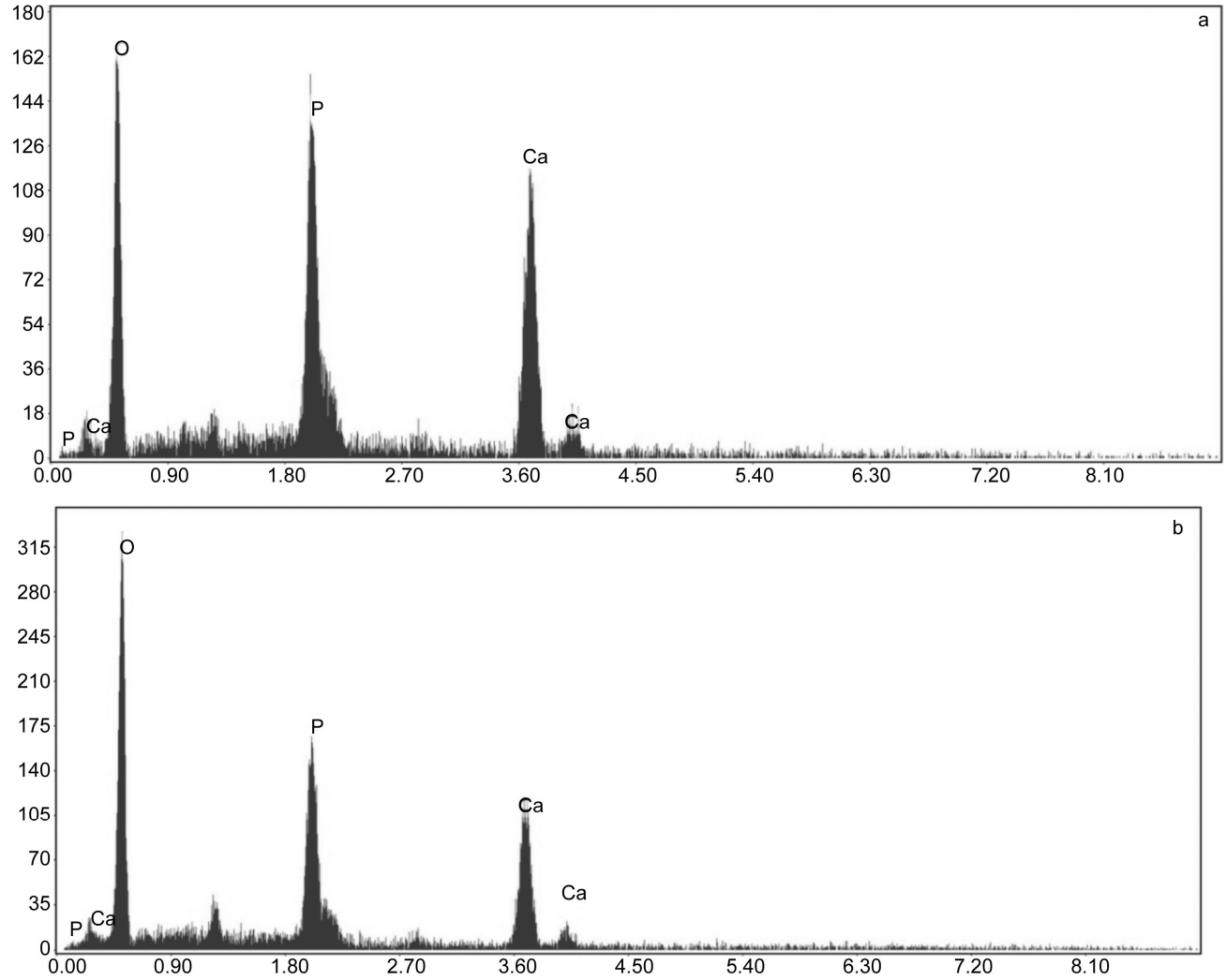

Fig. 5 — EDX of HAP, a) after heating at 550 °C and, b) after heating at 1000 °C.

when heated to 1350 °C. About HAP isolated from scales of different fishes, Ca/P ratio of 1.78 was reported for red Tilapia (*Oreochromis* spp.)[15], 2.01 for *P. jullieni*[17], and 1.71 for *L. rohita*[14,19]. On the other hand, a Ca/P ratio of 1.61 was reported for Tilapia (*Oreochromis mossambicus*) scale by Mondal *et al.*[20], and 1.6 for a mixture of *L. rohita* and *C. catla* scales by Panda *et al.*[16]. However, HAP has been targeted as the most suitable ceramic material in hard tissue replacement, and it was pointed out that heated HAP is suitable for tissue engineering[41].

Particle size distribution

Particles size distribution of HAP powder is shown in Fig. 6. The d values i.e. 0.1, 0.5, 0.8, and 0.9 indicate that 10, 50, 80, and 90% of the particles were less than or equal to the size (volume-based diameter) stated in Table 3. Thus, 50% of the particles passed through 155.772 and 167.071 µm, respectively, for samples heated at 550 and 1000 °C. The specific surface area, surface-weighted mean diameter, and volume-weighted mean diameter were found to be 0.097 m^2/g, 61.828 µm and 199.738 µm for sample heated at 550 °C and 0.0884 m^2/g, 67.846 µm and 217.976 µm for the sample heated at 1000 °C. It is noteworthy to state that the prepared samples in the present study were not subjected to any size reduction process. This indicates that exposure to 1000 °C slightly increases the agglomeration with the increase in the particle size. An increase in the size of HAP powder due to high-temperature heating was reported by Ahmed *et al.*[42]. It was also reported that the size of chemically synthesized HAP particles may vary from nano to micron level depending on the method and

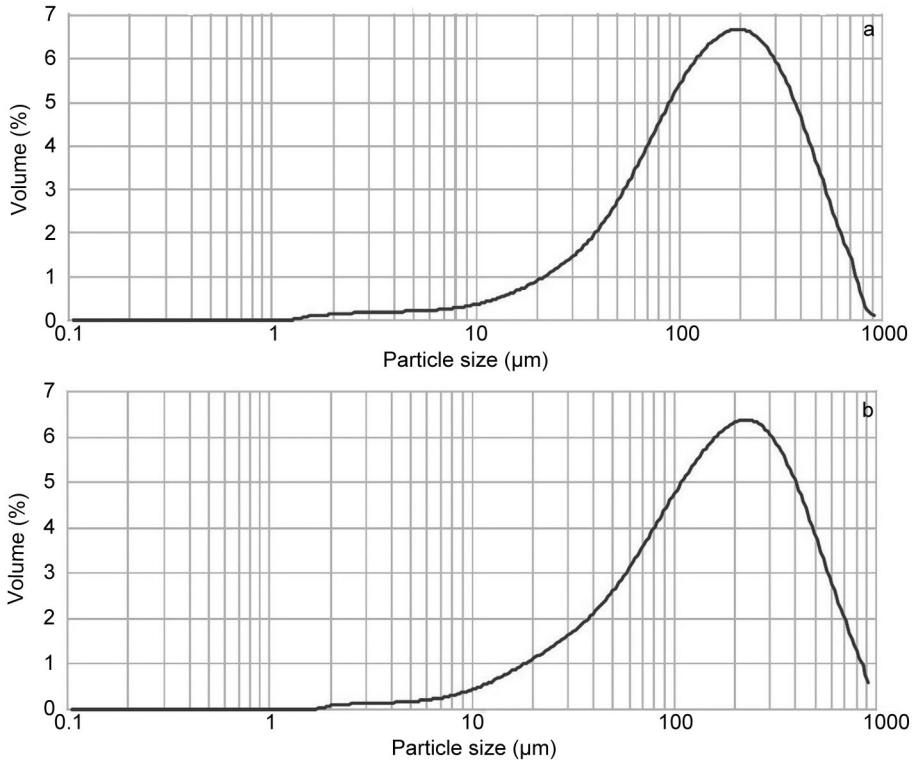

Fig. 6 — Differential analysis of HAP particles, a) after heating at 550 °C and, b) after heating at 1000 °C.

Table 3 — Particle size distribution of hydroxyapatite heated at 550 °C and 1000 °C

Percentile	Diameter	
	550 °C	1000 °C
d (0.1)	36.784 μm	33.756 μm
d (0.5)	155.772 μm	167.071 μm
d (0.8)	317.436 μm	351.168 μm
d (0.9)	429.898 μm	479.966 μm

condition of preparation[43]. Compared to other reports, it may be noted that Giraldo-Betuncar *et al.*[38] quoted five commercial HAPs to be more than 149 μm, whereas Rincón-López *et al.*[35] prepared bovine HAP powder of which 90% was below 32 μm. The size of HAPs to range within 1-2.9 μm, and less than 60 μm obtained through extraction from Cyprinidae (carp) scale[21] and calcination of bone sludge[7], respectively, are also available. On the other hand, a lower average particle size of 182 nm was documented for Tilapia scale derived HAP powder[20].

Conclusion

The method recommended for hydroxyapatite production from *Labeo rohita* scale used a very low concentration (0.6%) of KOH and provided simultaneous recovery of the protein part. After the separation of hydroxyapatite and protein, the spent liquid was a good fertilizer. Thus the question of the disposal problem was not there. The hydroxyapatite powder thus produced contained <7% organic matter, which was removed by thermal exposure at 1000 °C for 1 hour only. Thus, the technology followed could be considered green technology. The heated (1000 °C) HAP was perfectly crystalline with a Ca:P ratio of 1.52. Morphologically, the HAP comprised of grains of the submicron size that remained in soft agglomerated condition. The particle size distribution indicated that 50% of the mass passed through ≈ 167 μm. As HAP and collagen, in general, are natural constituents of fish scales, principles of this green technology may apply to scales of other fishes.

Conflict of interest

The authors declare that there is no conflict of interest.

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