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Heat-deproteinated xenogeneic bone from slaughterhouse waste: Physico-chemical properties

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Abstract. **Xenogeneic bone procured from the slaughterhouse waste was deproteinated by heat treatment method intended for use as a bone substitute. The effect of heat treatment was investigated by thermal analysis and by physico-chemical methods such as X-ray powder diffraction (XRD) and Fourier transformed infrared (FTIR) spectroscopy. The heat treatment temperatures for the bovine bone samples were predetermined by thermogravimetric (TG) analysis. The XRD results revealed that the process of heat treatment promoted the crystallinity of bone samples, particularly at 700 and 900°C. There was no secondary phase transformation detected for heat-deproteinated bone except the presence of the hydroxyapatite (HA) phase, which indicated its phase purity even at a higher temperature. The FTIR spectra of raw bone and bone heated at 300°C indicated the presence of organic macromolecules whereas these disappeared in the samples heated at 500, 700 and 900°C, which suggested the removal of antigenic organic matters around 500°C. The same results were also confirmed quantitatively by calculating the amount of collagen using hydroxyproline estimation. There was no significant change in the TG-thermogram of bone heated at 500, 700 and 900°C, which indicated their thermal stability. These findings implied that the heat treated bone at 500°C had properties similar to carbonated HA with low crystallinity, while 700 and 900°C samples had the same with higher crystallinity. As low temperature treatment does not alter morphological and structural properties, we propose that the 500°C heat treated xenogeneic bone may act as an excellent osteogenic bone substitute.**

Keywords. **Xenogeneic bone; deproteination; heat treatment; hydroxyapatite; crystallinity; bone substitute.**

1. Introduction

The need to repair bone for osseous defects resulting from trauma, tumour and bone diseases has been recognized for the past few decades. Although autogeneous bone is the best candidate (Arrigton *et al* 1996; Tancred *et al* 1998), its limited volume and requisite additional surgery indicate a need for an alternative. Allogeneic bone could overcome these limitations, but it bears the risk of infections and immune response (Stevension 1999). So another possibility is to use material such as xenogeneic bone, which is morphologically and structurally similar to human bone. As compared to other methods, it is easy to obtain and has lower cost with faster recovery time. However, the problem of antigenicity remains open if xenogeneic bone is used as such, clinically (Urist *et al* 1994). To alleviate this, various chemical treatments have been developed to remove the antigenic proteins and cellular elements of xenogeneic bone (Katthagen 1986). The results show that the chemically deproteinated materials retain some antigenicity, which could evoke immunological response. Recently, heat treatment has been suggested as an alternative to obtain protein-free xenogeneic bone (Lin *et al* 1999; Joschek *et al* 2000). The heat-treated bone maintains the morphological structure of natural bone, which has an interconnective porous structure of 70% volume porosity and hence allows faster bone in-growth (Lin *et al* 1999).

The crystalline phase of heat treated xenogeneic bone resembles hydroxyapatite (HA) with composition, $Ca_{10}(PO_4)_6(OH)_2$. HA is a potential implant material as a bone substitute owing to its excellent biocompatible and osteoconductive properties (De Groot 1980; Jarcho 1981). Moreover using HA, as a bone substitute is advantageous, since it is non-inflammatory and causes no immunological, foreign body or irritating response (Paul and Sharma 1997). It is thermally stable up to 1200°C, however, the decomposition temperature may vary depending upon the heating atmosphere (Locardi *et al* 1993). In addition, functional groups of HA are also an important factor for the decomposition temperature. Hence, the influence of heat treatment on the deproteination of xenogeneic bone at low and high temperatures is of great importance. Keeping the above points in view, the present study was aimed to prepare low cost heat-

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deproteinated xenogeneic bone obtained from the slaughterhouse waste for use as a potential bone substitute. The physico-chemical properties and the effect of temperature on deproteination were analysed systematically.

2. Experimental

2.1 *Materials*

An adult bovine tibia of ~ 5–6 years old male was procured from local slaughterhouse. Sodium chloride and organic solvents used were of analytical grade (S.D. Fine Chem Ltd., India).

2.2 *Deproteination of xenogeneic bone*

The deproteination of xenogeneic bone was carried out by heat treatment process. In brief, the procured bone samples were cleaned well to remove macroscopic adhering impurities. These were dissected into smaller pieces and sliced longitudinally by cutting on a low speed saw (Buehler Ltd., USA) in running water. In order to avoid soot and crack formation during heat treatment, the bone samples were boiled in distilled water for 12 h. After boiling, the bone samples were immersed in 2% NaCl solution as a preservative for 12 h and degreased by immersing in acetone-ether mixture at a ratio of 3 : 2 for 24 h and then stored at – 20°C until assay (Raspanti *et al* 1994; Walsh *et al* 1994). The bone samples (5 g) were then pre-heated overnight at various temperatures i.e. 300, 500, 700 and 900°C. All the heat treatments were performed in a muffle furnace (Indfur, India) at 10°C/min in atmospheric pressure and ambient humidity. The amount of organic phase present in the bone samples was calculated by heating the bone samples at 300, 500, 700 and 900°C for 18 h and the weight changes of the sample were recorded at every 6 h for 3 times. The weight difference of the samples due to the variation of water content was adjusted with the average water content of all the samples used in this experiment. Finally all the samples were normalized to the bone heated at 700°C since it was reported that the heating at 700°C removed all the organic phases in 18 h (Gong *et al* 1964; Armstrong and Singer 1965).

2.3 *Physico-chemical characterization*

The thermogravimetric (TG) analysis of the bone samples during heat treatment was studied on 50 mg of powder samples using Perkin-Elmer TG analyser (PE-7 series, USA). The measurements were recorded from 50 to 900°C at a heating rate of 10°C/min in a stream of high purity nitrogen (50 cc/min). The amount of collagen present in the bone samples was calculated by indirect method using hydroxyproline estimation. For this, 100 mg of raw bone and heat treated bone samples were hydrolyzed with 6 N HCl in a sealed tube at 100°C for 18 h. The insoluble residues were removed by filtration method. The hydrolyzed solution was then diluted with known volume of distilled water and analysed for hydroxyproline content (Neuman and Logan 1950). The phase purity and crystallographic parameters of the bone samples before and after heat treatment were examined with a high resolution X-ray powder diffractometer (XRD 3000, Seifert & Co, Germany) in a Guiner geometry using monochromatic CuKα radiation at a wavelength of 1·5406 Å. The XRD patterns were recorded in steps of 0·01° intervals with 1 s counting time at each step. The functional groups were identified by Fourier transform infrared spectroscopy (FTIR Nicolet 20 DxB spectrophotometer, Madison, USA) equipped with DTGS-KBr window detector using KBr pellets at a ratio of 1 mg sample per 300 mg KBr. The transmission IR spectra were recorded over the range of 400–4000 cm^{-1} with 2 cm^{-1} resolution averaging 100 scans. The carbonate content was determined, qualitatively, by spectroscopic method (Featherstone *et al* 1984) from the infrared spectrum of the bone sample by comparing the extinction coefficient (E) of the 1450 cm^{-1} (carbonate) and 569 cm^{-1} (phosphate) peaks using the formula

$$\%CO_3 = 13{\cdot}5\ (E_{1450}/E_{569}) - 0{\cdot}2, \quad (1)$$

where

$$(E_{1450}/E_{569}) = Ei = \log\ (T_2,I/T_1,I). \quad (2)$$

Here, "i" is 1450 or 569 cm^{-1} and "T_2,I" and "T_1,I" are the transmission intensities at the peak maximum and local baseline, respectively. The carbonate content present in the prepared samples was also determined, quantitatively, as carbon using a carbon hydrogen nitrogen (CHN) analyser (Medac, UK). Morphological structure of raw bone and heat-treated bone samples was studied by scanning electron microscope (SEM) (JEOL, Model-840A, Japan). The samples for SEM analysis were prepared by placing the raw bone and heated bone (500°C) onto one side of a double adhesive tape, which was stuck to an aluminum stub. The stub was then coated with gold using a sputter coater (Polaron SC-500, UK) to a thickness of 20–30 nm and examined with an accelerating voltage of 15 kV.

3. Results and discussion

Bone is composed of about 70 wt.% inorganic and 30 wt.% organic components and it is necessary to remove the unwanted organic phases for use as a bone repair and regenerative material. In the present study, all the organic impurities were eliminated by heat treatment method in order to obtain antigenic-free inorganic bone minerals. To gain information about the thermal stability of xeno-

geneic bone during heat treatment, the TG analysis of a small amount of raw bone was carried out and the thermogram is shown in figure 1. The thermogram of the raw bone shows initial weight loss up to 180°C pertaining to dehydroxylation. A continuous weight loss was observed from 300 to 480°C due to the decomposition of organic debris associated with the bone. There was no significant weight loss occurring between 500 and 900°C, which may suggest that the proteins associated with the organic phase of xenogeneic bone were removed at the temperature 500°C and above. However, a small weight loss occurred at 720°C, which may be due to the decomposition of carbonate phase present at trace level. The endothermic dissociation of carbonate is reported to occur at temperatures between 400 and 600°C in air and between 500 and 890°C in nitrogen atmosphere (Joschek *et al* 2000). Hence, heat treatments were planned at 300, 500, 700 and 900°C to study the changes observed in the HA as well as the removal of the organic debris. The average amount of organic phase removed at these temperatures during heat deproteination was calculated and listed in table 1. No organic materials were removed from the raw bone as it was not heated. In the case of bone heated at 300°C, the average percentage of organic phase removed was found to be 82·4%, whereas 100% of organic phase was removed for 500, 700 and 900°C heated samples. These results suggested that they are free from organic debris and hence may prevent immunological response upon implantation.

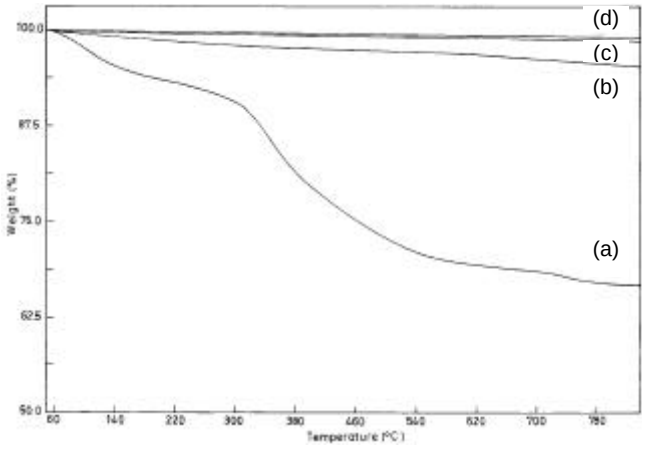

Figure 1. TG-thermogram of (a) xenogeneic raw bone and bone heated at (b) 500°C, (c) 700°C and (d) 900°C.

The amount of collagen present in the bone samples was calculated by indirect method using hydroxyproline estimation. The percentage of collagen present in each bone sample is listed in table 1. The results suggested that there is a definite decrease in the amount of collagen with increase in the temperature. The percentage of collagen present in the raw bone was found to be 18·3% whereas it was 5·2% for the bone heated at 300°C, which indicated that organic matters still existed in these samples. It was found that the collagen content was zero for 500, 700 and 900°C samples, which confirmed the complete removal of organic debris associated with the bone, leaving behind the inorganic mineral phase only. Hence, the remaining inorganic phase after heat treatment at 500°C may be absolutely free from antigenicity and may not cause any immunological reactions. The biocompatibility of the HA is well known (Muller-Mai *et al* 1995). Hence the removal of organic phases may not hinder the biocompatibility of implant materials.

The X-ray powder diffraction method is employed to assess the phase purity and the structural changes of the bone samples. Figure 2 summarizes the XRD patterns of raw bone and bone heated at different temperatures.

Table 1. Structural properties of raw and heat-treated bone samples.

		Bone heated at			
Properties	Raw bone	300°C	500°C	700°C	900°C
Organic phase removed (%)	NOR*	82·4	100	100	100
Amount of organic removed (mg/g of bone sample)	NOR*	247	299	302	303
Amount of collagen (mg/g of bone tissue)**	183	52	0	0	0
Carbonate content (%) by spectroscopy method	5·4 ± 0·82	5·0 ± 0·71	4·4 ± 0·42	2·6 ± 0·64	2·3 ± 0·16
Carbonate content (%) by CHN analyser	5·5 ± 0·64	5·2 ± 0·44	4·6 ± 0·26	2·7 ± 0·36	2·2 ± 0·28
FWHM [002 peak]	0·640 ± 0·058	0·582 ± 0·042	0·465 ± 0·068	0·314 ± 0·038	0·306 ± 0·024
FWHM [211 peak]	1·268 ± 0·094	1·210 ± 0·064	0·986 ± 0·072	0·420 ± 0·041	0·412 ± 0·032
[002] peak location (2q)	26·10	26·14	25·92	26·02	25·94
[211] peak location (2q)	32·02	32·06	31·92	31·82	31·83

*No organic phase removed since, raw bone was not heated. Data given were the average of two samples and adjusted for H_2O content using a control at 50°C. Total amount of organic matters was found to be 0·3 g/g of raw bone tissue.
**Calculated by hydroxyproline estimation.

The XRD pattern of raw bone (figure 2a) showed amorphous phase as the organic materials are still within the bone mineral, whereas the bone heated at 300 and 500°C showed semi-crystalline phases (figures 2b and c, respectively). A slight qualitative change in the crystallinity between raw bone and bone heated at 300 and 500°C was observed at [0 0 2] and [2 1 1] peaks. The peak full width at half-maximum height (FWHM), an inverse measure of crystal size and crystallinity was not significantly different for the raw bone and bone heated at 300 and 500°C at either [0 0 2] or [2 1 1] peak (table 1). The XRD patterns of bone heated at 700 and 900°C (figures 2d and e, respectively) were identical and showed highly crystalline phases as compared to 300 and 500°C samples. The heat treatment at 700 and 900°C caused substantial changes in the Bragg diffracted peaks when compared to raw bone and bone heated at 300 and 500°C. The background signals were also reduced in both the cases. The results showed the step-by-step transformation of the amorphous raw bone to crystalline state with the corresponding decrease of the organic phases with increasing temperature. The high temperature heat treatment reduced the FWHM of [0 0 2] and [2 1 1] peaks to 51 and 69%, respectively, of the bone heated at 700°C and 52 and 68%, respectively for the bone heated at 900°C when compared to the raw bone. This is a quantitative evidence that the peaks of bone heated at 700 and 900°C are more distinct and sharper, which indicated that the thermal process at high temperature brings about substantial improvement in the crystallinity and crystal size. The Bragg peaks at ~ 26, 28, 29, 30–35, 39, 46, 49, and 50° (2q) observed for bone heated at 500, 700 and 900°C corresponded to the characteristic peaks of HA (JCPDS 9-432). However, there were no sharp and distinct peaks detected for the bone heated at 500°C owing to low temperature process. The peaks observed for 700 and 900°C exhibited that the heating at high temperatures (700–900°C) brought about substantial improvements in the crystallinity and hence an increase in crystal size. The results suggested that there were no secondary phases except for HA phase observed for heat treated bone samples particularly at 500, 700 and 900°C, which confirmed their phase purity. The heat treated bone at 500°C has the properties similar to carbonated hydroxyapatite with low crystallinity as indicated by FTIR analysis, whereas 700 and 900°C heated bone samples have the same properties with high crystallinity.

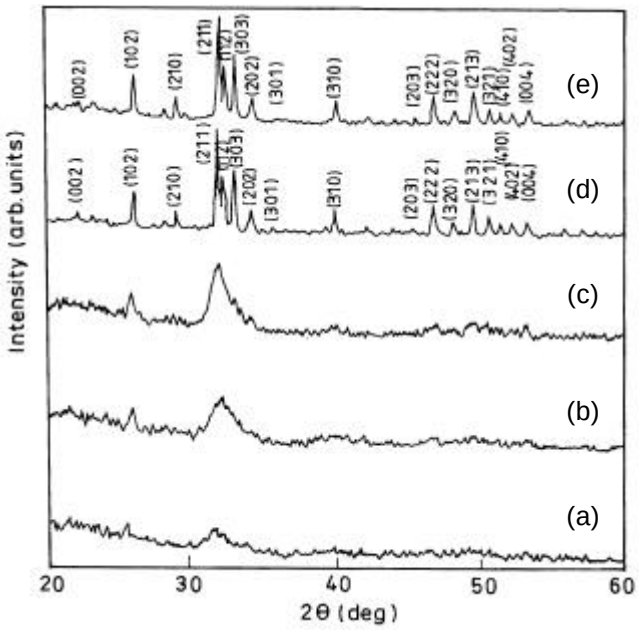

Figure 2. XRD patterns of (a) raw bone and bone heated at (b) 300°C, (c) 500°C, (d) 700°C and (e) 900°C.

The well resolved XRD peaks of bone heated at 700 and 900°C can be easily indexed on the basis of hexagonal crystal system of space group $P6_3/m$ with respect to JCPDS file no. 9-432 (figures 2d and e, respectively). The lattice cell parameters were calculated by refining the XRD data by standard least square method using the 'CELN' programme. The calculated lattice cell parameters of heat treated bone samples corresponded more to be carbonated hydroxyapatite (Sampath Kumar *et al* 2000) as listed in table 2. These results suggested that the deproteination of xenogeneic bone obtained by this simple method gave carbonated HA with variable crystallinity.

The FTIR spectra of raw bone and heat treated bones are illustrated in figure 3. It was found from the results that IR spectrum of all the samples showed a major peak at 3500 cm^{-1} due to the presence of the hydroxyl group. More absorption peaks were noted for the raw bone and bone heated at 300°C: a strong N–H stretching band around 2900 cm^{-1} and amide bands at 660 and 1550 cm^{-1}. These two peaks are pertaining to macromolecules of protein associated with xenogeneic bone. These two peaks disappeared for the bone heated at 500, 700 and 900°C, which suggested that they are free from organic impurities. There were no significant differences observed in the FTIR spectra of 500, 700 and 900°C which indicated that the heat treatment had not stimulated any secondary phase transformation. The IR spectrum of bone heated

Table 2. List of lattice cell parameters of heat-treated bone samples calculated from the XRD analysis.

	Lattice constants		
Samples	$a_0 = b_0$ (Å)	c_0 (Å)	c_0/a_0 (Å)
Bone heated at 500°C	9·435	6·902	0·732
Bone heated at 700°C	9·429	6·889	0·731
Bone heated at 900°C	9·426	6·894	0·731
CHA*	9·425	6·902	0·732
HA (JCPDS 9-432)	9·418	6·884	0·730

*Carbonated HA (Sampath Kumar *et al* 2000).

at 500, 700 and 900°C exhibited only the characteristic absorption peaks of HA (Joschek *et al* 2000; Matsumoto *et al* 2002). The FTIR spectra indicated the presence of PO_4^{3-} and OH^- ions in all the samples. The 1043 and 970 cm^{-1} bands were assigned to the stretching vibrations of PO_4^{3-} ions and 605 and 569 cm^{-1} bands were assigned to the deformation vibrations of PO_4^{3-} ions (Featherstone *et al* 1984). The bands at 3546 cm^{-1} and 640 cm^{-1}, were due to the vibration motion of the OH^- ions (Zhang and Gonsalves 1997).

The FTIR spectra also indicated the absorption peak of carbonate ions around 1450 cm^{-1} (Jensen *et al* 1996) in all the samples. The heat treatment had little effect on the carbonate content of the bone samples. A small reduction in the amount of carbonate was observed for the bone heated at 300 and 500°C as compared to raw bone (table 1), which may be due to the presence of organic impurities associated with the bone. As expected, the bone heated at 700 and 900°C had much reduced carbonate content due to high temperature process. These results were also confirmed by calculating the amount of carbonate content using CHN analyser as given in table 1. These data suggested that the carbonate contents calculated by both the spectroscopic method and CHN elemental analyser corroborated well. The presence of carbonate in the samples suggested that the heat-deproteinated xenogeneic bone was a hydroxyl carbonate apatite. The bone mineral contains 4–8 wt.% of carbonate in human body and its presence in the apatite phase is advantageous as it increases the mechanical strength and bioactivity (Waish *et al* 1994; Mery *et al* 1998). The carbonate content in the bone heated at 500°C falls within the range of carbonate present in the human bone minerals as indicated in table 1. These findings imply that no secondary phase transformation occurred during heat treatment and the deproteinated xenogeneic bone is of hydroxyl carbonate apatite, which is beneficial for biomedical purposes due to its similarity with the bone apatite.

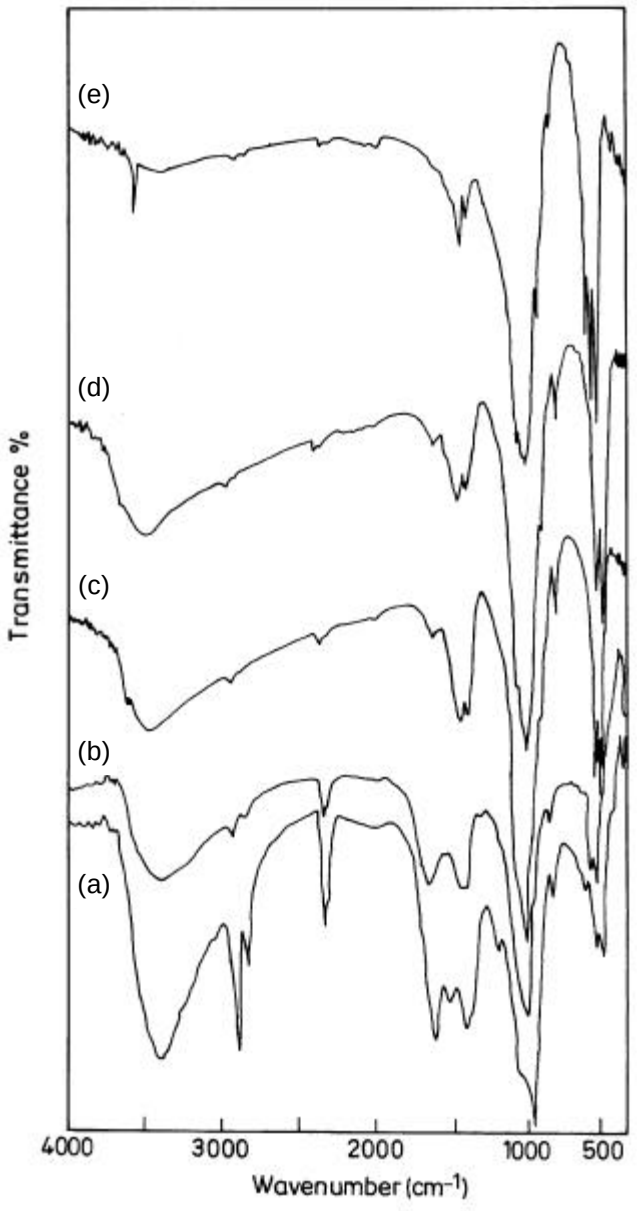

Figure 3. FTIR spectra of (a) raw bone and bone heated at (b) 300°C, (c) 500°C, (d) 700°C and (e) 900°C.

Study on thermal behaviour of HA is of great importance, when used as coatings on metallic implants (Chen *et al* 1997). The TG-thermograms, in terms of weight loss, of bone heated at different temperatures are shown in figure 1. The TG-trace of bone heated at 500, 700 and 900°C do not show the weight loss pertaining to the decomposition/evaporation of protein macromolecules, which suggested that they are absolutely free from organic debris as confirmed by FTIR qualitatively and by collagen estimation quantitatively. The observed total weight loss of about 5% for bone heated at 500°C can be due to dehydroxylation. The presence of carbonate at this temperature (500°C) was confirmed by FTIR and CHN analyses. There was no significant change observed in the thermograms of bone heated at 700 and 900°C. However, the observed small weight loss (< 2%) can be due to the release of hydroxyl ions according to

$$Ca_{10}(PO_4)_6(OH)_2 \rightarrow Ca_{10}(PO_4)_6(OH)_{2-2x}O_x + H_2O. \quad (3)$$

Further, due to very less amount of carbonate as indicated by CHN analyser, the TG-analysis do not show significant weight loss for dissociation of carbonate ions at these temperatures.

Figure 4 shows SEM observations of raw bovine bone and 500°C heated bone. The microstructure of raw bone (before deproteination) was highly dense due to the presence of organic substances impregnated with inorganic minerals associated with the xenogeneic bone (figure 4a). Figure 4b revealed that the heat treated bone at 500°C contains numerous pores. It may be suggested that the porous architecture had been created by the removal of

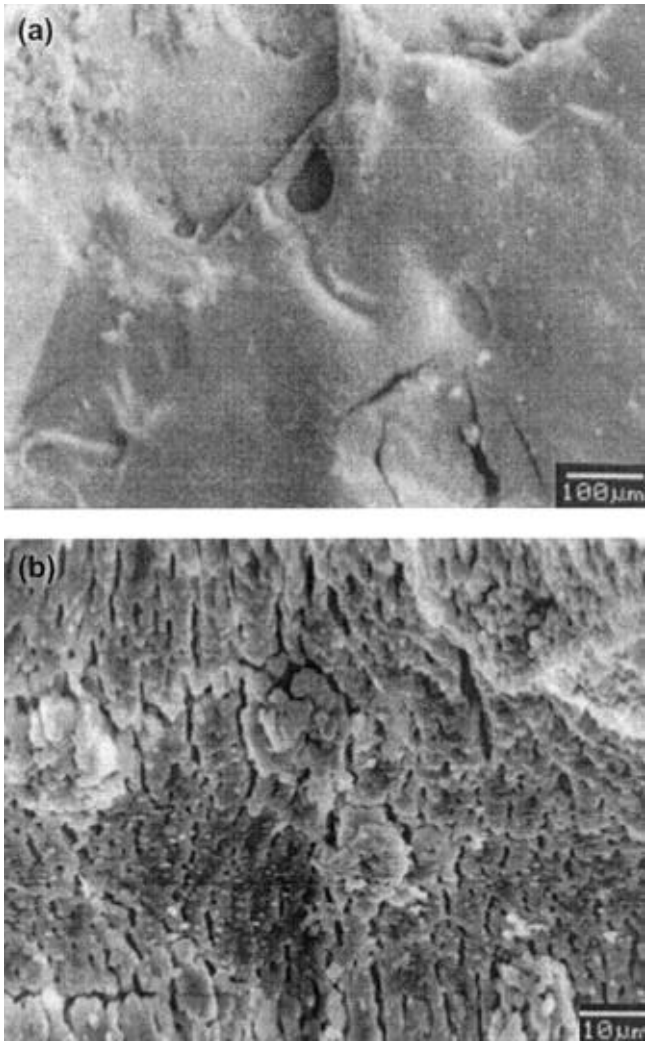

Figure 4. SEM photographs of (**a**) raw bone and (**b**) bone heated at 500°C.

organic phases by heat treatment. The obtained porous architecture resembles that of human cancellous bone and hence it may be expected that the porous feature of hydroxyl carbonate apatite would be beneficial for the bone in-growth upon implantation.

4. Conclusions

The present study showed the possibility of preparing protein-free inorganic minerals of xenogeneic bone by a simple thermal process. The heat deproteinated xenogeneic bone corresponded to hydroxyl carbonate apatite phase. The phase purity was confirmed by XRD and FTIR analyses. The effect of heat treatment in addition promoted the crystallinity of the bone samples. The FTIR revealed that the organic macromolecules were eliminated at 500°C. So, we propose that the bone heated at a low temperature of 500°C can be used as a potential bone filling material since it has similar properties of carbonated crystalline HA. Hence this study paved the way to make value added healthcare material like HA from the slaughterhouse waste at economical limits.

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