

**SYNTHESIS AND CHARACTERIZATION OF HYDROXYAPATITE
NANOPARTICLE FROM CUTTLEFISH BONES FOR
BIOMEDICAL APPLICATIONS**

Submitted in partial fulfillment for the award
of Master of Science degree in Physics

By

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INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE INIVERSITY)

Accredited with Grade "A" by NAAC

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This is to certify that this Project Report is the bonafide work of **K.SANDHYA (Reg No.40590002)** who carried out the project entitled **“SYNTHESIS AND CHARACTERIZATION OF HYDROXAPATITE NANOPARTICLE FROM CUTTLEFISH BONES FOR BIOMEDICAL APPLICATIONS”** under my supervision from **NOVEMBER 2021 TO MARCH 2022**

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ABSTRACT

The synthesis of nano hydroxyapatite (bio-material) from cuttlefish bone has been done by precipitation method and ultrasonication method for its various biomedical applications. Cuttlebones are produced in large amounts by the marine food industry, also an inexpensive source of calcium carbonate and non-toxic as well. It can be used in the fabrication of hydroxyapatite as it is an excellent calcium carbonate precursor. This paper focuses on the synthesis of hydroxyapatite from nanosized cuttlebone powder using precipitation and ultrasonication method of different frequencies and intensities with the aim to find the conditions which allow control of the particles size. The results are differentiated and evaluated by X-ray diffraction (XRD), Scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), thermo gravimetric analysis (TGA). Particle size analysis techniques to evaluate their physicochemical properties. The studies indicate that cuttlefish bone-derived HAp, will be promising biomaterials in the field of bone tissue regeneration.

KEYWORDS:

Nano hydroxyapatite; cuttle fish bone; Precipitation; ultrasonication; bone tissue.

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CHAPTER 1

INTRODUCTION

Nanotechnology is a field of research and innovation related to the construction of "things" in general, devices and materials on the atomic and molecular scale. Nanometers are one billionth of a meter, ten times the diameter of a hydrogen atom. The average diameter of the size of human hair is 80,000 nanometers. At such scale, the usual rules of physics and chemistry applied no longer. For example: Material properties such as colour, conductivity, strength, and reactivity can vary significantly between macroscale and nanoscale.

Carbon nanotubes are hundred times stronger than steel, but 6 times lighter. Nanotechnology believed to have the potential to increase energy efficiency, purify the environment and solve major health problems. You should be able to significantly increase manufacturing production at significantly reduced costs. Nanotechnology products are smaller, cheaper, lighter, but more functional, require less energy and raw materials to manufacture, and support nanotechnology.

1.1 POTENTIAL BENEFITS OF NANO TECHNOLOGY

Nanotechnology keeps the promise of various new solutions to the arising problems that hinder the development of poor undeveloped and developed countries, especially relating to health and hygiene, sanitation, food security, and the environmental claims. In 2005, report entitled Innovation applying knowledge in development, the UN Millennium project task force on science innovation and technology has written that "Nanotechnology is likely to be the most important in the developing world, because it involves less labour, small quantity of land and less maintenance; it is in great demand, highly productive and inexpensive; and it requires only modest amount of energy and materials".

1.2 DRUG DELIVERY USING NANOTECHNOLOGY

Nanotechnology could also one day lead to cheaper, more reliable systems for drug-delivery. For example, materials that are built on the nanoscale can provide encapsulation systems that protect and secrete the enclosed drugs in a slow and controlled manner. This could be a valuable solution in countries that don't have adequate storage facilities and distribution networks, and for patients on complex drug regimens who cannot afford the time or money to travel long distances for a medical visit.

1.3 NANO MATERIAL IN MEDICAL FIELD

The development of medical drug delivery and bio imaging systems based on advanced nanomaterials, including nanoparticles, nanopores, and nanocomposite materials, has been one of the most crucial challenges to creating personalized medicine using nanotechnology. Nanomedicine has offered a wide range of innovative biological healthcare strategies, from targeted cancer therapy (chemotherapy, gene therapy, immunotherapy, phototherapy) to dynamic diagnosis, monitoring of disease treatment, and even to vaccination. This Special Issue covers the significance of advanced nanomaterials related to current medical issues, including but not limited to synthesis, characterization, and applications in imaging, diagnostics, improved therapeutics, biomaterials, stem cells and tissue engineering, nano pharmaceuticals, nano cosmetics, nano toxicology, infectious disease, vaccination, antipathogens, etc.

1.4 BIOMATERIAL

Biomaterials are understood as materials for applications in contact with the human body in the biomedical field, including materials in medicine, medical devices, biosensors, implants, scaffolds for tissue engineering, drug delivery systems, as well as antibacterial and dental materials. All material classes are

considered, including metals, ceramics, polymers, and their composites, both synthetic man-made materials and those of natural origin, e.g., materials derived from the animal and the vegetable kingdoms. The section covers all aspects of the biomaterials field with a highly interdisciplinary scope, from the synthesis, processing, characterization, and testing to the biomedical applications of biomaterials. Topics of interest for this section include but are not limited to: materials for implants and prosthetic devices for orthopedic, cardiovascular, and other applications also bioactive and cell instructive materials, nanostructured biomaterials and nanoparticles, scaffolds for tissue engineering, biomedical coatings and antibacterial surfaces, materials for drug delivery carriers, tissue or biomaterial interfaces, and studies on clinical applications of biomaterials.

1.5 BIO-COMPATIBLE NANOPARTICLE

Nanoparticles represent the key elements of nanotechnology. It can be obtained by two basic approaches: 1. the top-down approach which includes photolithography, electron beam lithography, milling techniques, anodization, ion and plasma etching. 2. The bottom-up one for self-assembly of monomer/polymer molecules, chemical or electrochemical nanostructural precipitation, sol-gel processing, laser pyrolysis, chemical vapor deposition. Nanoparticles have important applications in the field of aerospace industry, electronics, energy production and storage, food packaging, cosmetics, and also in medicine. They can be used as contrast agents in imaging, gene and drug delivery systems, as well as in tissue engineering. Their use in the medicine generates highly-sensitive diagnostic tools, facilitating advanced therapies in cancer, Alzheimer's, Parkinson's, and Huntington's disease, diabetes, and also reduces the negative effects of treatments. The interaction of engineered nanoparticles with cells, tissues, organs and systems of organs can result in different toxic effects through the generation of reactive oxygen species that can be counteracted by enzymatic and non-enzymatic anti-oxidants. When the level of reactive oxygen species is too high, oxidative stress occurs. Reactive oxygen species activate the transcription factors involved in the regulation of different signaling pathways involved in inflammation, cellular death or

proliferation. The biocompatibility of nanoparticles depends on the shape, size, and surface functionalization, as well as the time and dose exposure.

1.6 BIOMATERIAL IN TISSUE ENGINEERING

There is still a fast-growing interest in the applications of biomaterials in the field of tissue engineering as well as drug delivery systems. A lot of effort has been made in recent years in the research of polymers, metals alloys, and ceramics dedicated to tissue engineering and sustained drug delivery systems. This special Issue is focused on advances in biomaterial scaffolds formed by various techniques with potential in the aforementioned medical applications. Scaffolds' morphology and chemical and molecular structure are crucial for material–cell interactions.

1.7 HYDROXYAPATITE

Hydroxyapatite (HA) is the most major component nearly 75% of the bone mineral. Hydroxyapatite (HAP) has been widely used in bone regeneration. It is a naturally occurring form of calcium phosphate that constitutes the largest amount of inorganic components in human bones. The chemical formula of HAP is $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ with a Ca/P ratio of 1.67. It can be used in the field of orthopedic. Synthetic HAP is known to be similar to naturally occurring HAP on the basis of crystallographic and chemical studies. Because, synthetic HAP is thermodynamically stable at physiological pH5 and osteoconductive, it has been widely used in hard tissue replacement and reconstruction applications, such as implant coatings, and bone substitutes etc. Its porous character also offers high binding affinity for a variety of pharmacological substances such as antibiotics, hormones, enzymes, antibody fragments, steroids etc., This has opened the potential for using synthetic HAP to deliver pharmacological substances in many clinical applications with sustained release capacity for the treatment of osteomyelitis, osteoporosis, osseous cancers etc., in which local delivery is effective with the need to fill defects in the skeleton. New economic and versatile methods for HAP synthesis are of interest due to the importance of this material for various biomedical applications. The current communication deals with various

methodologies for the preparation of synthetic HAP with optimum properties closer to those of living hard tissues like bone and teeth, aiming at better and more effective biomedical applications.

1.7.1 CONDITIONS AFFECTING THE BONES AND JOINTS

The knowledge about the structure and chemical composition of bones was gradually acquired over time. It dates back the ancient civilizations that used bones as tools and extended until present time with the understanding of the basic unit that constitutes them. Bone is a hierarchically ordered structure having a uniform arrangement of calcium phosphate (CaP) nanocrystals with calcium deficiency in collagen fibrils, which may be settled in a lamellar or layer by layer fashion, depending on the nature of the bone. Bone tissue is a specialized form of connective tissue, metabolically active, composed by an extracellular matrix and some cell types, called osteoclasts, osteoblasts and osteocytes. These cells are immersed in the mineralized extracellular matrix, composed of organic and inorganic phases, which together give resistance, resilience and hardness to the tissue. Osteoclasts are multinucleated cells that participate in the dynamic and cyclic process of bone resorption and remodeling. They are followed by osteoblasts that deposit the organic part of the matrix and many are trapped in the extracellular matrix becoming osteocytes cells. In turn, osteocytes act in the maintenance of bone mineralized matrix. Therefore, the bone has the intrinsic capacity of regeneration and it is the only tissue that is reconstructed without scar formation. However, since bone regeneration depends on the type of defect it is necessary to analyze strategies to improve tissue repair capacity.

1.7.2 MINERALS FOR BONE HEALTH

The inorganic phase of the bone is composed by calcium phosphates (CaP), predominantly in the form of hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$, corresponding to 66%-70%, and bone water with 5% - 8%. The organic phase is mainly in the form of collagen, a large fibrous protein with repetitive amino acid sequences formed in triple helix structure, equivalent to the remaining portion. Collagen is responsible for

the elastic resistance of bone, acting as a matrix for the deposition and growth of mineral salts. The bone recovery process can be aided by the use of calcium phosphates such as amorphous calcium phosphates, brushite ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$), monetite (CaHPO_4), tricalcium phosphate [$\text{Ca}_3(\text{PO}_4)_2$] (TCP), α -TCP and β -TCP, and hydroxyapatite. The biological and medical interest in these phosphates is due to their biocompatibility, bioactivity, resorbability, and chemical similarity to the mineral component of mammalian bones and hard tissues. However, crystalline HAP has called the attention of researchers because of its thermodynamic stability in the body fluid. The number of routes or the production of HAP particles has increased due to its wide range of applications in the biomedical field, either as an implant material for bone tissue regeneration increasing osteogenesis or as a coating material improving implants bioactivity. Moreover, HAP is seen as a suitable material for the controlled and sustained release of drugs into particular sites, stimulating the growth of osteoblastic cells. This work briefly reviews the state of art related to HAP gathering information about the synthesis methods, material characteristics, use of doping ions, and some applications.

1.7.3 REGENERATION OF BONE, TISSUE ENGINEERING

Bone is a dynamic tissue rich in blood vessels that acts as a structural and functional support in the vertebrate's body. This specialized hard connective tissue undergoes remodeling and chemical exchange with other parts of body constantly. The majority of bone injuries go through a gradual healing process without any scar formation and with considerable resumption of bone characteristics so that eventually the newly formed bony tissue is indistinguishable from peripheral healthy bone; in extensive bone injuries like traumas, tumors and skeletal abnormalities healing is not successful because the extent of damage exceed the regenerative potential of bone. From past to now researchers have been looking for appropriate methods to potentiate and accelerate healing process or to replace the lost bone. In different clinical conditions there are several therapeutic options available in order to reconstruct the defected bone; each has advantages and disadvantages. Among these, bone grafts have been popular to researchers. Although autologous bone crafts have been mentioned as a gold standard, disadvantages of this method such

as prolonged surgery time, pain and hemorrhage has limited its application lately. Ideal bone substitutes should be non-immunogenic, bioactive, osteoconductive and osteoinductive, biodegradable, easily sterilizable, thermally non-conductive, traceable, readily available and economical.

Hydroxyapatite, the most stable forms of calcium phosphate has been utilized widely as a bone substitute in orthopedic and maxillofacial surgery in three past decades. It is a porous material that makes vascular ingrowth possible and provides oxygen and nutrients for cells. Recent days researchers are looking for natural materials considering them superior and more desirable. Here several raw materials such as eggshell and animal bone have been used to prepare hydroxyapatite; however, religious and social limitations along with disease transmission have made the use of these sources restricted. Marine sources are novel in this field. Another biologic source that has widely introduced lately is platelet rich plasma; defined as a fraction of blood which platelet concentration is above base. PRP have been applied as an economical angiogenic, orthobiological in association with ceramics such as hydroxyapatite and majority of studies reported promising results; however few found opposite in outcome so its regenerative ability is controversial still. The aim of recent study is to evaluate in-vivo effectiveness of raw Calcium bone and CBHA prepared by synthesis of hydrothermal method along with PRP in bone repair and to investigate synergism between the biomaterials.

1.7.4 CUTTLEFISH BONE

Cuttlebone is a natural mineral material possessing an exceptional combination of desirable mechanical properties of high compressive strength, high porosity and high permeability. The properties of this material are sought after both in biomimetic and in biomedical structural materials. The fundamental step in comprehending the construction of this biogenic material is to elucidate how organisms utilizing physical principles to generate the complex structures.

Researchers tried to find the source of HA which was expected to be more available characteristics that were not inferior to another source, and one of which is done by using cuttlebone. Study on Hydroxyapatite synthetic with CaCO_3 source of cuttlebone has been done by many researchers. The study yielded HAp with quite

well characteristics. Based on the preliminary studies a synthesis of HAp from cuttlebone with variations in temperature and sintering time was conducted to determine the effect of these parameters on the characteristics of hydroxyapatite which include crystallinity, morphology, compressive strength and toxicity of the material.

Aquatic living organisms such as coral, nacre and cuttlefish have been used in order to enhance bone regeneration recently. Cuttlebone is the hard internal structure of cuttlefish consists of crystals of calcium carbonate - Aragonite plays essential role in protecting organism vital organs and act as a floating tank besides. It is biocompatible, osteoconductive and has plasticity in regard to morphology and mineral composition. In addition, this structure is available in many oceans and seas worldwide with low expense. Parallel interconnected sheets give CB porosity. The pores diameter is in the range of 200-500 micrometer and therefore they are optimum for new bone formation and neovascularization.

CHAPTER 2

LITERATURE SURVEY

Faksawat, Kridsada, et al. "Preparation and characteristic of hydroxyapatite synthesized from cuttlefish bone by precipitation method." *Advanced Materials Research*. Vol. 1125. Trans Tech Publications Ltd, 2015. The purpose of this study was to Calcium phosphates was prepared and characterized for future applications on the utilization of bioactivity and resorbability of Hydroxyapatite, was synthesized by precipitation method using cuttlefish bone as the calcium (Ca) source in the form of calcium nitrate and ammonium phosphate as the phosphate (P) source.

Li, Xinnian, et al. "Synthesis of hydroxyapatite from cuttlefish bone via hydrothermal solid-state conversion." *Journal of Biomaterials and Tissue Engineering* 6.10 (2016): 775-779. This purpose of this study was to HAP were successfully prepared from cuttlefish bone (CB) powders via hydrothermal solid-state conversion without surfactants or complex-structural agents were evaluated by the X-ray diffraction (XRD), and scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), Thermal analysis (thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC)) and X-ray spectrometer (EDS).

Venkatesan, Jayachandran, et al. "Hydroxyapatite from cuttlefish bone: Isolation, characterizations, and applications." *Biotechnology and Bioprocess Engineering* 23.4 (2018): 383-393. The purpose of this study was improve the biological properties of cuttlefish bone-derived HA, bioglass, polycaprolactone, and polyvinyl alcohol were added to it, which gave better results in terms of cell proliferation and osteogenic differentiation.

Kim, Beom-Su, et al. "Enhanced bone regeneration by silicon-substituted hydroxyapatite derived from cuttlefish bone." *Clinical oral implants research* 28.1 (2017): 49-56. This purpose of this study was prepared Si-HAp from CB (Si-CB-HAp) using a hydrothermal and solvothermal method were characterized by

scanning electron microscope (SEM), X-ray diffraction (XRD), and energy dispersive X-ray spectrometer (EDS).

Rogina, Anamarija, Maja Antunović, and Dajana Milovac. "Biomimetic design of bone substitutes based on cuttlefish bone-derived hydroxyapatite and biodegradable polymers." *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 107.1 (2019): 197-204. This purpose of this study was focuses on biomimetic approach of the hydroxyapatite synthesis from the cuttlefish bone in order to preserve highly porous structure.

CHAPTER 3

SOURCES AND EXTRACTION TECHNIQUES

3.1 NATURAL HYDROXYAPATITE

Natural hydroxyapatite is usually extracted from biological sources or wastes such as mammalian bone such as bovine, camel, and horse, marine or aquatic sources (e.g. fish bone and fish scale), shell sources (e.g. cockle, clam, eggshell, and seashell), and plants and algae and also from mineral sources (e.g. limestone). Stoichiometric HAp is basically composed of calcium and phosphorus with molar ratio of Ca/P equal to 1.67. This ratio has been proven to be the most effective in promoting bone regeneration. Natural HAp is non-stoichiometric and is either deficient in calcium or phosphorus. Calcium positions are the most common AAS calcium positions, while carbonate ions can substitute for either phosphate or hydroxyl ions while fluoride ions substitute for hydroxyl ions.

The presence of trace elements in some natural HAp mimics the apatite produced from human bone. The trace elements are essential in the regeneration of the bone and accelerates the process of bone formation. Research by Balamurugan et al. proved that 3–6 mol% silicon added to synthetic hydroxyapatite increased cell growth density which enhanced osteoblast growth. Research by Capuccini et al. showed that the presence of 1–10% of strontium ions in synthetic HAp enhanced osteoblast activity and differentiation and also inhibited osteoclast proliferation and production.

Carbonate-substituted hydroxyapatite was also proven to enhance osteogenesis by enhancing bioresorption. The usage of HAp extracted from natural sources can be considered to be an environmentally friendly, sustainable, and economical process to fabricate these materials since these materials are available in large quantities. This can result in positive contributions to the economy, environment, and to general health.

3.2. EXTRACTION OF HYDROXYAPATITE (HAp) FROM NATURAL SOURCES

3.2.1. MAMMALIAN SOURCES

Among mammalian sources, the extraction of HAp from bovine bone was frequently reported in literature compared to other sources such as camel, horse and, porcine. The cortical part of the femoral bone is usually used because they are morphologically and structurally similar to human bone. Reviewing the literature shows that the properties of the extracted HAp, such as the Ca/P ratio, size, shapes and crystalline phases of Ca-P have been discussed. These properties differ with the applied extraction methods and thus the parameters such as calcination temperatures and pH. Generally, most literature have reported that pretreatment of the bone is usually done before proceeding with the extraction method. The pretreatment involves washing and removing the dirt, fats, protein, and other components such as bone marrows and soft tissues. Some literature reported the usage of boiling water to remove organic components from the bone by boiling for times of 8 h or more.

A combination of boiling and washing with solvents such as acetone and chloroform have been employed for the pretreatment of bone. Another pretreatment method that has been widely used is washing the bone alternatively with surfactant and alkali solutions to remove the soft tissues and decellularise it. The bone was also cut into smaller pieces before or after removing the organic constituents. Most majority of literature reported that the bone was cut first into smaller pieces before boiling or treated with the solvent to remove the unwanted components such as bone marrow located inside the bone. In this review, most of the methods for extraction of HAp from mammalian bones used the calcination method which is either the sole process or a combination of calcination with other methods.

Other methods such as alkaline heat treatment have been used to extract HAp from mammalian bone. In this method, the alkaline solution usually NaOH is used to remove the organic matter from the bone. The NaOH solution hydrolyses the organic component in the bone and the remaining calcium phosphate is rinsed and

separated using filtration. However, alkaline heat treatment produces low crystallinity HAp compared to calcination. Sun et al. revealed that the crystallinity of HAp produced using alkaline heat treatment was much lower than that seen for calcined HAp.

The morphological analyses of the HAp extracted from the mammalian bone show that the particles are mostly irregular in shape, with some studies showing the presence of rods, flakes, needles, and plate-like shapes. Thus the shape variation is believed to not be affected by the method or source. For example, calcination of same source of bone could produce the various shapes of HAp such as rod-like, spherical, and needle like. In addition, the rod shape HAp could be produced using different extraction methods such as alkaline hydrolysis and combination method. It can be concluded that, there is no relation between the morphology of HAp with the extraction method and source. The size of HAp obtained did not show any correlation with the extraction method. The use of additional milling helped to reduce the size of the HAp particles to the nanometric size which is close to that of human HAp. In addition, the nanosized particles have advantages in terms of high surface activity and ultrafine structures higher bioactivity, and better resorb ability than micron-sized particle.

3.2.2. AQUATIC AND MARINE SOURCES

The increasing consumption of fish around the world has caused significant increase in fish waste production in the form of scales and bones. The recovery of fish scales and bones allows for extraction of HAp and reduction in solid wastes in the fisheries industry. The fish bone is rich in calcium, phosphate, and carbonate which make it a great source for extraction of HAp. The pretreatment of the fish bones/scales has been done using different methods. Generally, pretreatment is done first to remove fleshy meat and unwanted debris on the bone and scale and one of the common method used is boiling.

The calcination produced HAp with larger particle sizes compared to the alkaline heat treatment method. Ozawa & Suzuki stated that using the alkaline heat treatment can preserve the size of the crystals while these tended to agglomerate in thermal calcination. In addition, Venkatesan et al. stated that the complete

removal of organic moieties by thermal calcination results in the particles growing while alkaline heat treatment does not alter the size. However, the Ca/P ratio of HAp produced from calcination was 1.65 which is near to that of stoichiometric HAp while the ratio produced by alkaline heat treatment was higher. Besides fish bone, the scales also can be used as a HAp source. Mondal et al. and Paul et al. carried out calcination using scales of fish.

The morphological structure revealed nearly spherical shapes. Panda et al. extracted HAp using a combination of alkaline heat treatment and calcination resulting in pure crystalline HAp with agglomerates of average size 76.62 nm and Ca/P ratio of 1.62.

In enzymatic hydrolysis method, the enzyme was used to hydrolyse the organic component in the scale. In order to hydrolyse the collagen, a specific enzyme must be used during the treatment. As collagen is a protein component, protease enzyme is used to hydrolyse the collagen. The enzymes usually work best at their optimal temperature and pH. As all the collagen was degraded, the mixture containing the enzyme will be subjected to high temperatures in order to deactivate the enzyme and the remaining HAp will be collected using centrifugation. Use of the hydrolysis methods and ionic pre-treatment method can allow for extraction of pure HAp without transforming the HAp to other CaP phases. The use of calcination may alter the HAp phases into β -TCP. The hydrolysis only removes the organic component of the bone or scale without altering the chemical structure of HAp. In term of crystallinity, the use of calcination produces phases of higher crystallinity compared to other methods. When analysing the morphology of the extracted HAp from aquatic and marine sources, the shapes of HAp were seen to be irregular, nearly spherical, flat plates, rod-like and so on. It can be concluded that the shape of the HAp is not influenced by the method or sources.

In addition, the elemental analysis has confirmed the presence of trace elements in the HAp extracted from fish scales and fish bones produced using calcination. Similar to that case of mammalian sources, Mg ions were present in HAp from both fish bone and scale, while trace element such as Sr, Na, and K were found in fish scale. The HAp extracted from natural sources contains trace elements that help to enhance the properties of the HAp.

In conclusion, the calcination methods may transform HAp to other calcium phosphate phases at high temperatures. However, the alkaline heat treatment method, ionic liquid pretreatment, and enzymatic hydrolysis method produced pure phase HAp but with lower crystallinity compared to calcination method. In general, most of the methods could produce nanosized HAp with different morphologies.

3.2.3. SHELL SOURCES

The shells of cockle, clam, and egg are rich with calcium carbonate (CaCO_3) which can be converted to HAp. Eggshell contains 94% of CaCO_3 which can be a source of Ca for HAp production. These shells are abundant in nature and are generally discarded. Generally, the shells were washed with distilled water to remove sand and dirt and further cleaned using bleaching agent. The shells then are calcined to form CaO which along with excess of CaCO_3 will be used for further treatment with chemicals to produce high purity HAp. These are the properties of HAp extracted from shells. Mustaffa et al. carried out calcination and sol-gel precipitation method for the extraction of HAp from cockle shells. The cleaned cockle shell was crushed and milled to form powder which was calcined at 600 C to convert the CaCO_3 to CaO. Then, the CaO powder undergoes sol-gel precipitation method to synthesise HAp.

In sol-gel technique, the CaO powder is mixed with orthophosphoric acid and then ammonia solution, NH_4OH is added to this to maintain it in alkaline condition for 24 h. The precipitate formed is rinsed and filtered before drying and further milling was done followed by sintering at 1150 C. The results showed the formation of HAp which was spherical in shape of sizes 4.03–10.4 μm and Ca/P ratio of less than 1.68.

Shavandi et al. synthesised HAp from mussel shells using rapid microwave irradiation. This method was able to produce high amounts of HAp by using less energy and at a faster rate. In summary, the shells are a rich source of CaCO_3 and can enable the extraction of HAp. However, it requires more synthesis steps to produce high purity HAp. Most of the methods described are used a combination of methods to synthesis HAp. The first step involves conversion of CaCO_3 in the shell to CaO first followed by reaction with a phosphate source such as phosphoric acid,

ammonia solution or diammonium hydrogen phosphate and then adequate treatment to convert those calcium and phosphate source into hydroxyapatite. The treatment methods includes chemical, mechanical and thermal reaction. The crucial part in order to extract HAp from this shell source is the usage of the appropriate molar ratio of phosphate source to that of the calcined shell to produce the optimal amount of HAp. Otherwise, the extracted HAp will not be pure and can contain traces of CaO during the treatment.

3.2.4. PLANT AND ALGAE SOURCES

Research had revealed that plant and algae have the potential as sources of HAp. Various studies had been intensively conducted using plant parts, wastes, effluents from fruit processing industry, and also algae to extract HAp. Although the characteristics of the obtained HAp such as morphology, size and Ca/P ratio was not studied by some of the research, however, the potential of plants to produce HAp have been proven by XRD analysis. Plant waste is one of the sources that have been used for the extraction of HAp. Orange and potato peel are fruit wastes that have been used for extraction of HAp. Nayar & Guha used papaya leaves and Calendula flower for HAp extraction. Chemical precipitation was used to extract HAp from these fruit wastes and plant parts. The fruit waste or plant part was boiled in water for 10 min and the filtrate was mixed with 0.4 M alkaline calcium nitrate tetrahydrate solution and incubated for 24 h at 30 C. After that the resulting solution was added to 0.156 M alkaline diammonium hydrogen phosphate solution, stirred and aged for one week at 30 C. Finally the precipitate was washed with dionized water and dried and the XRD results showed the formation of HAp. The morphology of the HAp particles varied with the processing method and it is clear that biomolecules in the plant waste are important in controlling the synthesis of HAp. Biomolecules such as enoic acids in potato peel extract, beta-carotene and vitamins present in the papaya leaf extract and limonene in orange peel play important roles in controlling the morphology and the size of the HAp particles. Thus, the HAp extracted from different plant wastes contain different biomolecules and consequently has different morphologies and sizes. Shaltout et al. used the

components of the leaves and stalks of selected plants to extract HAp. All the plants were calcined at temperatures of 600 C, 700 C, and 800 C.

Besides leaves and stalks, the wood part of the plant can also be used to extract HAp but this process has not been investigated in detail owing to the complexity of the process. Tampieri et al. used a combination of five steps to achieve complete phase conversion of the rattan and pine wood into pure HAp. The steps include pyrolysis, carburization, oxidation, carbonation, and phosphatization. Each step allows the phase transformation of the chemical components in the wood step by step until it produces pure phase HAp. In the first step, pyrolysis of the lignocellulose material is done to convert the organic substance into carbon. The second step which is carburisation involves transformation of carbon to calcium carbide (CaC_2). The third step is oxidation where calcium carbide is converted to calcium oxide (CaO). Then the calcium oxide is transformed into calcium carbonate (CaCO_3) by carbonation. In the last step, the calcium carbonate is converted into HAp in the phosphatization process. All in all, it requires many phase transformations to transform wood into the HAp phase.

Other than that, the abundance of phosphorus in wastewater of potato processing industry has allowed researchers the opportunity to recover phosphorus for the production of HAp. Compared to other methods of synthesising HAp, research by Monballiu et al. used the combination of biological treatment and chemical treatment to produce HAp from potato waste effluent. In the biological treatment, the effluent of the potato processing industry was added to a nitrifying sludge into a bioreactor and treated in aerobic condition. Then, the effluent from the first reactor was treated with CaCl_2 at a certain pH to initiate precipitation. The HAp was recovered using the hydrothermal mineralisation process.

During the hydrothermal mineralisation pathway, calcium hydroxide was added to achieve a molar ratio of Ca/P equal to 1.67. In addition, synthetic pure HAp was seeded to the medium to facilitate the crystallisations of the HAp. Magnesium contained in the microalgae and the HPO_4 which is the predominant phosphate in algal hydrolysate favoured whitlockite formation. Thus, the seeding of synthetic HAp is needed to facilitate HAp crystallization from the algal hydrolysate.

However, the algae source did not allow successful conversion of all the CaCO_2 to HAp. The trace compounds are present such as whitlockite, According to Jang et al. the presence of magnesium will inhibit apatite formation and form whitlockite. Due to the presence of magnesium in the central atom of chlorophyll in the algae, and the pH used during the reaction of the algal hydrolysate, all conditions favour the formation of whitlockite. In summary, it is seen that plant and algae have some potential to be used as sources of HAp extraction. However, most of the methods required the addition of calcium or phosphate precursor because of the lack of one of these components in the plant itself. Thus, further steps are required to transform the phase into the pure HAp phase. This complicated process and the usage of additional calcium or phosphate source will make favour the use of plants for HAp extraction. For this reason, not much research has focussed on extraction of HAp from plant sources.

3.2.5. MINERAL SOURCES

Among the mineral sources that contain calcium, limestone is one of the natural minerals used for synthesising hydroxyapatite. Naturally, limestone is formed from the precipitation of the animal shells or skeletons, foraminifera or algae which are rich in CaCO_3 . The abundance of limestone makes it one of the possible calcium sources to be used for synthesising of hydroxyapatite. There are several methods that have been used to convert CaCO_3 into HAp. Most of them used combination of method. The limestone will be calcined first before another treatment such as precipitation, hydrothermal or ultrasonic irradiation is used. The purpose of calcination is to convert the CaCO_3 phase to CaO. Jamarun et al. used calcination at 900 C while Klinkaewnarong & Utara used 800 to 1100 C, both studies used 6 h.

Jamarun et al. used a combination of calcination and hydrothermal techniques to synthesis HAp. Klinkaewnarong & Utara used a combination of calcination and ultrasonic irradiation techniques. This technique uses ultrasonic waves to induce synthesis of HAp. Aquatic living organisms such as coral, nacre and cuttlefish have been used in order to enhance bone regeneration recently. Cuttlebone is the hard internal structure of cuttlefish consists of crystals of calcium carbonate - Aragonite plays essential role in protecting organism vital organs and act as a floating tank

besides. It is biocompatible, osteoconductive and has plasticity in regard to morphology and mineral composition. In addition, this structure is available in many oceans and seas worldwide with low expense. Parallel interconnected sheets give CB porosity. The pores diameter is in the range of 200-500 micrometer and therefore they are optimum for new bone formation and neovascularization. Klinkewnarong & Utara suggested that the ultrasonic irradiation affected the chemical reaction by altering the rate of formation and chemical equilibrium of HAp; thus ultrasonic irradiation method might produce more HAp monetite phase. TEM analysis revealed that with increase in irradiation time, smaller sized, needle-like nanoparticles were produced. Aquatic living organisms such as coral, nacre and cuttlefish have been used in order to enhance bone regeneration recently. Cuttlebone is the hard internal structure of cuttlefish consists of crystals of calcium carbonate - Aragonite plays essential role in protecting organism vital organs and act as a floating tank besides. It is biocompatible, osteoconductive and has plasticity in regard to morphology and mineral composition.

In addition, this structure is available in many oceans and seas worldwide with low expense. Parallel interconnected sheets give CB porosity. The pores diameter is in the range of 200-500 micrometer and therefore they are optimum for new bone formation and neovascularization. From the results, Klinkaewnarong & Utara conclude that ultrasonic waves induced HAp formation as a result of physical and chemical effects of cavitation and the combination of these effects decrease the interference from other chemical species such as Al, Si, Mg, and Fe and also accelerated the reaction of chemical species CaO , H_3PO_4 , CaHPO_4 and H_2O . According to Klinkaewnarong & Utara, the chemical reactions of the limestone to produce HAp.

All methods used calcination as one of the approaches and thus all the extracted HAp showed high crystallinity. All of the methods were seen to not produce pure HAp with traces of other calcium phosphate phases seen similar to the case of extraction from shell and algae. The morphological analysis of the extracted HAp revealed spherical, needle-like, and plate-like shape with sizes.

3.3. SUMMARY OF HYDROXYAPATITE EXTRACTION FROM ALL NATURAL SOURCES

3.3.1. PROPERTIES OF HYDROXYAPATITE

In summary, most natural sources have the potential to be a precursor for HAp production. The use of animal (mammalian and aquatic/marine), shell waste, plants, and minerals results in HAp of differing characteristics. Thus, the morphological analysis shows that the frequent shape seen for HAp is rod-like or needle like. As mentioned previously, the shape of the HAp particles was not affected by the extraction method or the source. Research is focussed on synthesising nanosize HAp owing to its superior properties and similarity in size to HAp in human bone 50–100 nm length and 1–10 nm diameter. Most methods use an additional milling step in order to achieve the nanosize of the HAp particle. However, HAp from fish scales does not require the milling process; however an alkaline hydrolysis method was used to enhance the dissolution of the scale and reduce its size. Thus, most of the extracted HAp have nanosize range.

The crystallinity of the HAp was dependent on the method of extraction. The usage of thermal method increased the crystallinity while the chemical precipitation method resulted in low crystallinity HAp. On the other hand, the results showed that the Ca/P ratio from all natural sources were non-stoichiometric. In the case of mammalian and aquatic/marine sources, the presence of trace element might increase the HAp ratio to values higher than those seen for stoichiometric HAp. For other sources like shells, algae, and minerals, the presence of other calcium species such as CaO may result in increasing Ca/P value. Lastly, that the HAp extracted from the mammalian and aquatic/marine sources contains trace elements which are not seen in the case of other sources, and these could have excellent potential for enhancing the biological properties of natural HAp. Thus, by comparing all the sources, researchers can choose the best methods for achieving the excellent

properties of HAp in term of simplicity of extraction method, high yields, and low costs.

3.3.2. OPTIMUM PROCESSING PARAMETER FOR EXTRACTION OF HAp

Various extraction method of HAp has been studied in order to extract the HAp from natural. These extraction methods result in the various properties of the extracted HAp. Out of this various technique, the calcination method, alkaline hydrolysis method and also combination method have been frequently used during the extraction process.

In calcination method, it can be seen that the pure HAp have been produced at the temperature range from 700 C to 1000 C. It showed that the calcination of mammalian bone, fish scale, and fish bone within temperature range from 700 C to 1000 C able to produce pure HAp. The calcination temperature used that successfully extract pure HAp from different sources. Instead of pure crystalline HAp, there also the traces of β -TCP formed result in the degradation of the calcium phosphate at high temperature. β -TCP are usually obtained at temperature 850 C. Although at high temperature the β -TCP could be formed, many research listed that at high temperature up to 900 and 1000 C, the pure crystalline HAp still can be produced.

Alkaline hydrolysis method also has been usually used for extraction HAp from the natural source. This method was able to extract pure HAp with low crystalline properties. Usually, the sodium hydroxide has been used to hydrolyse the organic matter in the sources and separated it from the HAp component. Various range of sodium hydroxide has been used during the extraction method. The research shows that 20–25% NaOH able to remove all the organic matter in the bovine bone and result in production of pure HAp. On the other research, they used low concentration of NaOH from 5% and the process was repeated with high NaOH 50% concentration to remove excess organic matter. Research by Venkatesan et al. was used low concentration of NaOH which is 2M (9%) NaOH for the extraction HAp from fish bone. The process was repeated until all the organic matter was completely removed. All in all, the usage of alkaline heat treatment were able to produce pure HAp without worrying of the calcium phosphate phase transformation that usually

occur during the calcination process. Some of the NaOH concentration used during the alkaline hydrolysis process.

Apart from the calcination method and alkaline hydrolysis method, combination methods have been frequently used for the extraction of pure HAp. According to Sadat-Shojai et al. the used of combination were to improve the final properties of the product which is HAp. The use of combination may enhance the properties of the extracted HAp. For example the combination of alkaline hydrolysis and calcination method will increase the crystallinity of the HAp. In addition according to Sadat Shojai et al. the used of combination calcination and rapid microwave irradiation increase the reaction kinetics and effectively reduce duration of the process and thus improved HAp properties such as reducing size, high purity, and narrower size distribution.

On the other hand, these combination methods were usually being used for production of HAp from the CaCO_3 source such as from shell source, algae and also from limestone. During the process, calcination of the CaCO_3 source were necessary before proceed with the other method that will transformed the calcium phosphate phase to HAp. After conversion of CaCO_3 to CaO , usually the CaO will be mixed with additional phosphate source using other method such as chemical precipitation, sol gel or mechanochemical method in order to complete the conversion to HAp. Thus, various chemical transformation were involved during the process depend on the type of phosphate source that have been used. Thus, this combination method was very important in order to convert the CaCO_3 source to pure HAp.

CHAPTER 4

SELECTION OF MATERIALS AND EXPERIMENTAL METHOD

4.1 PROCEDURES

Acoustic cavitation technique is being used nowadays in the synthesis of various materials, as it has been found that ultrasound waves can generate cavitation in a medium which helps in more uniform dispersion and molecular interaction. Cavitation is a technique that causes the sequential formation, subsequent growth and collapse of plenty of microscopic vapour bubbles in the liquid medium that disseminate shock waves and create very high local energy densities. These shock waves produced due to cavity collapse enable the efficient completion of chemical reactions that otherwise require rigorous stirring conditions. It was reported about the chemical and physical effects of ultrasound in a fluid medium and identified that chemical effects of ultrasound do not originate through the acoustic field with chemical species on a molecular level. Instead, it is the sonochemistry and sonoluminescence method derived mainly from acoustic cavitation that causes the formation, growth, and implosive collapse of bubbles/cavities in the liquid medium when irradiated with high-intensity ultrasound waves. With this contextual approach, the synthesis of Hydroxyapatite was investigated in the presence of ultrasound, which is likely to assist in the proper dissolution of reagents.

4.2 CHEMICALS AND REAGENTS

Marine waste cuttlefish bones were collected as a source of calcium from Kasimedu fish market, located in Chennai, Tamil Nadu, India. All chemicals used for this study were of analytical grade such as diammonium hydrogen phosphate $(\text{NH}_4)_2\text{HPO}_4$ (Merck, purity – 98%, molecular weight (MW) – 132.05 g/ mol), NH_4OH (Merck, purity – 25%, MW – 35.05 g/mol), Acetone $\text{C}_3\text{H}_6\text{O}$ (Sigma-Aldrich, purity – 99.8%, MW – 58.08 g/mol) and Butyraldehyde $\text{C}_4\text{H}_8\text{O}_2$ (Sigma-Aldrich, purity –

99.9%, MW – 88.12 g/mol), Soda ash CNa_2O_3 (Sigma-Aldrich, purity – 99.6%, MW – 105.90 g/mol), and phosphate buffer saline (PBS) (pH 7.4)

4.3 PREPARATION OF CUTTLEBONE POWDER

The thick outer layer of the cuttlefish bone part, called the dorsal shield, was removed using a lancet and the inner part was washed with distilled water, acetone and ethanol to remove all the impurities. The washed bones were dried in a hot air oven at 80°C for 24 h and 50 g of cuttlefish bone pieces were taken for top down processing using high-energy ball milling for 5 h. The ground bone powder was stored in a sterile plastic container at room temperature and placed in a desiccator for the experiment.

4.4 PREPARATION OF CUTTLEBONE POWDER- Hap

4.4.1 PRECIPITATION SETUP

In the synthesis process, an oil bath was used as a temperature controller for the precipitation process and placed on magnetic stirrer. 1 molar (1.008 g) of cuttlefish bone powder was taken in a round bottom flask and 0.6 molar (0.795 g) of diammonium hydrogen phosphate was added dropwise using a burette. Then, the pH of the reaction was measured with a pH meter and was adjusted from 9 to 12 using ammonia solution. This mixture was allowed to stir for 24 h, and 48 h at 80 °C and then the final precipitate was washed with distilled water and ethanol to remove impurities. Finally, the precipitate was dried at 80 °C in a hot air oven and was crushed into uniform powder using a mortar and pestle. The resultant powder was then annealed at 700 °C for 6 h and further characterized to confirm the presence of CB-Hap. The optimization of parameters such as reaction time, pH, temperature, and concentration plays a key role in the morphology evaluation of the nanomaterials.

4.4.2 ULTRASONICATION SET-UP

Ultrasonication process was performed in a stainless steel ultrasonic bath. The bath had two transducers located at the bottom, arranged in a fixed position, and the bath was driven at 40 ± 3 kHz frequency at a power rating of 110 W. Sonication using ultrasound waves was carried out after the precipitation reaction to obtain hydroxyapatite. The temperature was maintained at 60° C with recirculation of cooling water. 1 molar (1.008 g) of cuttlefish bone powder was taken in a round bottom flask and 0.6 molar (0.795 g) of diammonium hydrogen phosphate was added drop wise using a burette. Then, the pH of the reaction was measured with a pH meter and was adjusted from 8 to 12 using ammonia solution. This mixture was allowed to stir for various reaction for 24h and 60°C. .The resultant colloidal solution was further centrifuged at 2200 rpm for 10 minutes. The precipitate obtained was washed with deionized water and ethanol, dried at 80° C for 7 h in an hot air oven and was ground to powdered form and sieved using 200-250 British Standard Sieves. The collected powder was further characterized to confirm the presence of CB-Hap.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 CRYSTALLINITY AND PHASE FORMATION ANALYSIS

The XRD spectra of the raw materials and the corresponding quantitative phase analysis in both precipitation and ultrasonication method shows that cuttlebones (CB) of 100% aragonite. The amorphous structure is revealed by the two broad bands at about 35° and 53°. The diffraction spectra of the synthesized powders confirm that all materials were converted into hydroxyapatite, with about 7 % residual calcite only in the case of CB. The width of the HAp peaks indicates that the crystals are nanosized, as confirmed also by the Rietveld analyses, which pointed out crystallite sizes of 13 nm, 20 nm and 25 nm. In addition, the high relative intensity of the (004) peak at 25° with respect to the (211) one at 31° suggests a preferential growth along the c-axis for all materials.

The products obtained at various reaction times (24 H, 48 H) by precipitation and ultrasonication method and the raw cuttlefish bone (CB) powders were characterized using powder XRD, revealing that a prolonged reaction time in both setup leads to the formation of highly crystalline Hap. It shows that the Cuttlebone powder's XRD pattern exactly matches with the aragonite crystal structure of calcium carbonate (CaCO_3). Interestingly, aragonite CaCO_3 was entirely transformed into calcite CaCO_3 after 3 h of reaction time.

In Ultrasonication setup, the reflection peaks observed at 24°, 29°, 36°, 39°, 42°, 47°, 48°, 55° and 57° correspond to the (012), (104), (111), (115), (202), (018), (116), (211), and (123) planes, which indicates the rhombohedral structure of calcite

Thus, it is observed that the reaction at basic pH conditions and a temperature of 80 °C supports the aragonite calcium carbonate bonds in the form calcite structure in 3 h.

In precipitation setup, it can be noted from the diffraction peaks that the formation of Hap crystal growth was initiated at 48 h of reaction time along with similar traces of calcite. Furthermore, the intensity of the calcite at peak positions of 24° and 29° significantly decreased with increasing reaction time. This reveals that prolonged heating of calcite crystal relaxes the lattice of calcite to facilitate the formation of Hap. Then, the calcite peak disappeared due to the complete formation of Hap at 24 h and 48 h of reaction time. The diffraction peaks were recorded at 25° , 31° , 32° , 33° , 34° , 38° , 45° , 50° and 53° , corresponding to the (002), (211), (112), (300), (202), (310), (222), (213), and (004) planes, which suggest that a hexagonal structure of Hap was formed.

Even though the XRD peaks of Hap were formed at 24 h of reaction time stable crystallinity was observed. There also exists a certain minor impurity peak at 31° , 36° , 44° and 57° , which affects the purity. Thus, the reaction time was increased to 48 h, yields a complete stable crystal of Hap and is confirmed by the XRD peaks. The average crystallite size of the CB Hap is 20.85 nm which is similar to the crystallite size of naturally occurring bone, and the lattice strain was 0.0056 nm.

Trace elements in cuttlebone of different coastal regions were detected by XRD analysis technique, which is assumed precisely by sophisticated analytical tool to estimate the elemental composition of materials. Micro- and macro elements, such as Ca, P, Mg, Sr, Cl, Na Fe, S, K, Cu and Br, were determined in cuttlefish bone samples. The dorsal and lamellar cuttlebone layers featured similar elemental compositions. Elemental composition of human bone and CB-HAp was given for comparison. The amount of phosphorus in prepared CB-HAp was found as 12% which is almost the same as in human bone 15%. Most of these elements are essential for the proper bone metabolic processes and wound injury regrowth. Trace amounts of Cu, Zn or Fe that were found in cuttlebone specimens are also beneficial for metabolic processes in a living organism. Moreover, biologically unfavourable heavy metals, such as Ag, Cd, Pb or V, were not detected in any specimen. XRD analysis data revealed the presence of trace elements in cuttlebone-derived HAp, such as Sr, Mg, Si, S, and Fe. These trace elements could influence the cartilage formation, collagen synthesis and enzymatic activity of the bone.

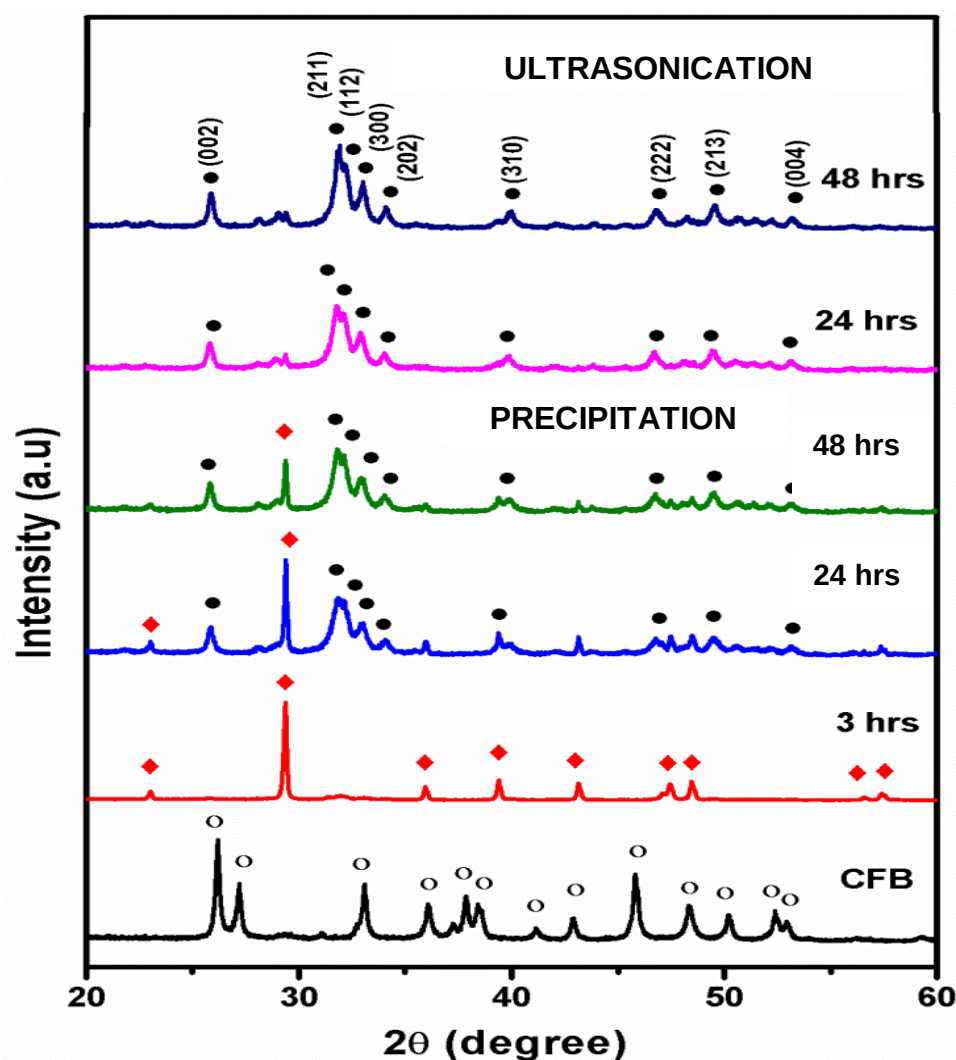


Figure 5.1 XRD spectrum of a cuttlefish bone (CB) and hydroxyapatite (HAp) prepared through precipitation and ultrasonication methods.

5.2 EVALUATION OF FUNCTIONAL GROUPS

The FTIR spectra of HAp synthesised using precipitation and ultrasonication method with high crystalline structure is shown in the attached below figure. It is very essential to analyse the HAp functional groups. In precipitation setup the FTIR spectral bands were recorded at 710, 860, 1486, 2522, and 2924 cm^{-1} for Hydroxyapatite from CB powder which indicates the aragonite carbonates existence. The characteristic peak present at 3424 cm^{-1} is due to the vibration of O–H stretching, indicating the presence of alcoholic functional groups. In addition,

the IR bands were recorded for the CB-Hap sample at 1038 and 569 cm^{-1} revealing the presence of functional symmetric and asymmetric PO_4^{3-} stretching groups respectively, which in turn helps in confirming the presence of particles, CB-HAp formation. Previous reports suggested that there are two classes of carbonate substitutions on Hap, such as A-type, in which the carbonates are substituted at the site of O–H vibrations, and B-type, in which the substitution takes place at phosphate vibrational sites. Hence, the band at 1458 cm^{-1} which indicates the occupancy of B-type carbonates and the carbonated n-Hap are highly beneficial in biomedical applications and activities, especially in hard tissue repair.

The FTIR spectra presented below provides further confirmation about the apatite nature of the hydrothermally synthesized samples in the presence of OH^- ions. All n-HAp powders exhibit characteristic bands due to PO_4^{3-} ions (966 cm^{-1} , 574 cm^{-1} and 610 cm^{-1} , 139 cm^{-1}) which tend to coincide with the results of the previous study, with the strong PO stretching bands at about 1100 and 600 cm^{-1} being particularly well noticed. The presence of hydroxyl group, characteristic of the standard HAp, could be confirmed in this case of n-HAp powder synthesised in the presence of the non-ionic surfactant from the stretching vibration at 3572 cm^{-1} and liberation mode at 632 cm^{-1} . The peak at 3572 cm^{-1} is attributed to stretching vibration of OH^- ions in the HAp lattice. However, it is difficult to explain why the presence of hydroxyl group is not apparent in the n-HAp powders synthesised in the presence of the other surfactants. The highly sensitive FTIR results indicated that no molecules eventually absorbed in this, as synthesized powders remained in the samples after the complete treatment.

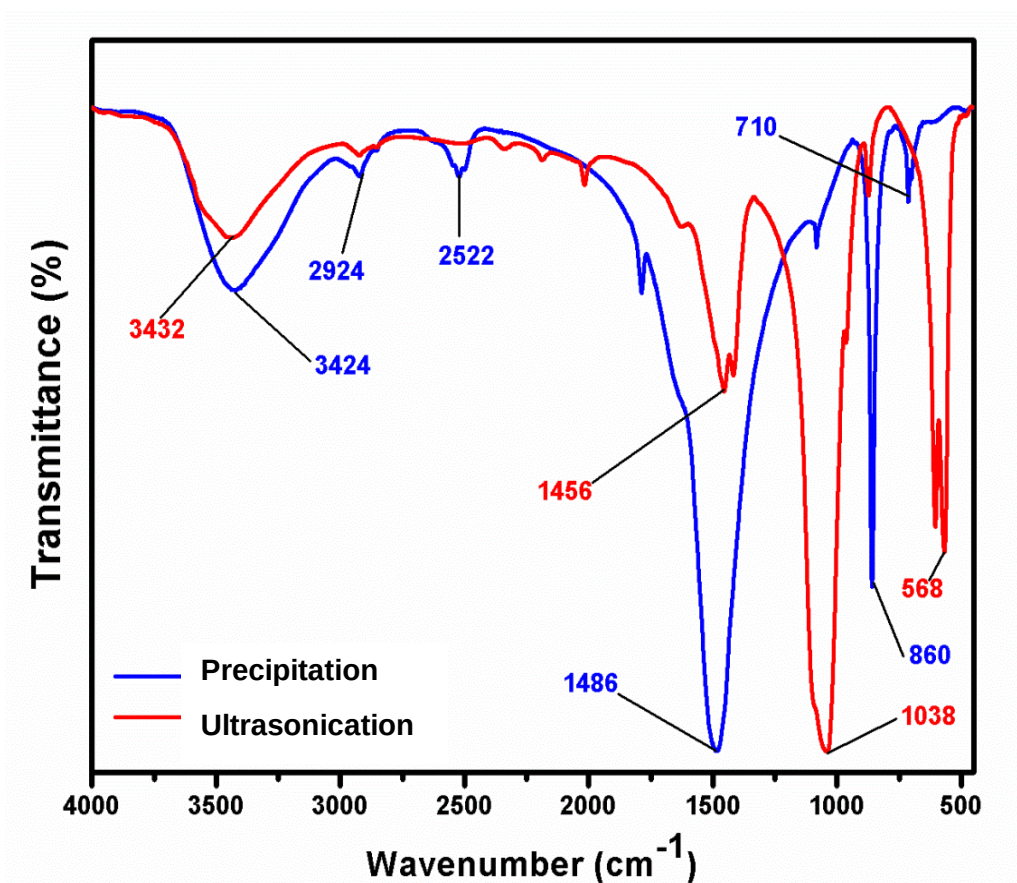


Figure 5.2 FTIR spectra of hydroxyapatite (HAp) prepared through precipitation and ultrasonication methods.

Wave number (cm-1)	Functional Group
1486,1457, 855	Carbonates
568, 1041	PO4
3430	Hydroxyl

5.3 THERMOGRAVIMETRIC ANALYSIS

The thermo gravimetric analysis (TGA) of CB-Hap nanopowder synthesized by two different methods (precipitation and ultrasonication method) are shown in the below figure. Both CB-Hap powder shows similar gradual weight loss of 8% from 39 to 590 °C due to the evaporation of water and other organic substances present in the cuttlefish bones. A gradual weight loss of 23% was observed from 585 to 690 °C for CB due to the decomposition of CaCO_3 , still no weight loss was observed for CB-HAp due to their thermal stability. Furthermore, the study revealed that the high temperature facilitates aggregation and increases the crystal size of the nanoparticles. On the other side, that a high annealing temperature will affect the porous structure of Hap nanoparticles. In the recent study, the thermo gravimetric analysis (TGA) shows 2% weight loss at 600 °C to form CB-Hap nano powder due to the complete removal of water and organic substances. Finally, 700 °C is selected as the optimum annealing temperature for the formation of Hap was suggested from previous studies.

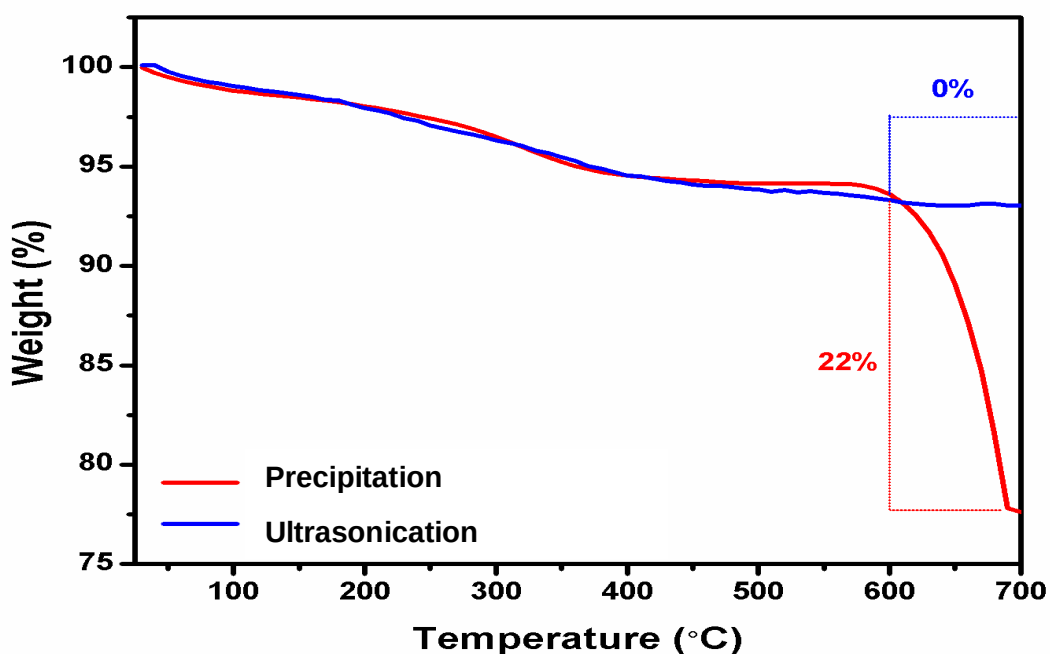


Figure 5.3 Thermogravimetric analysis (TGA) of Hydroxyapatite (HAp) prepared through precipitation and ultrasonication methods.

5.4 PARTICLE SIZE ANALYSIS

The presence of two particle size ranges in case of HAP powders possibly reflects the aggregation in the sub micrometer sizes range, yielding the so-called primary agglomerates (the first peak). These agglomerates continue to aggregate by forming interparticle bonds until they reach the micrometer size range, the second peak (>1 μm).

The average of particle sizes of the hydroxyapatite synthesized by precipitation were about 70 to 130 nm but, the average particle sized of those sample were in the range of 60 to 70 nm. Moreover, nanoparticle in sample from precipitation was dispersed more than a nanoparticle in hydroxyapatite from Ultrasonication technique. However, nanoparticles of the both samples had interconnected and porosity.

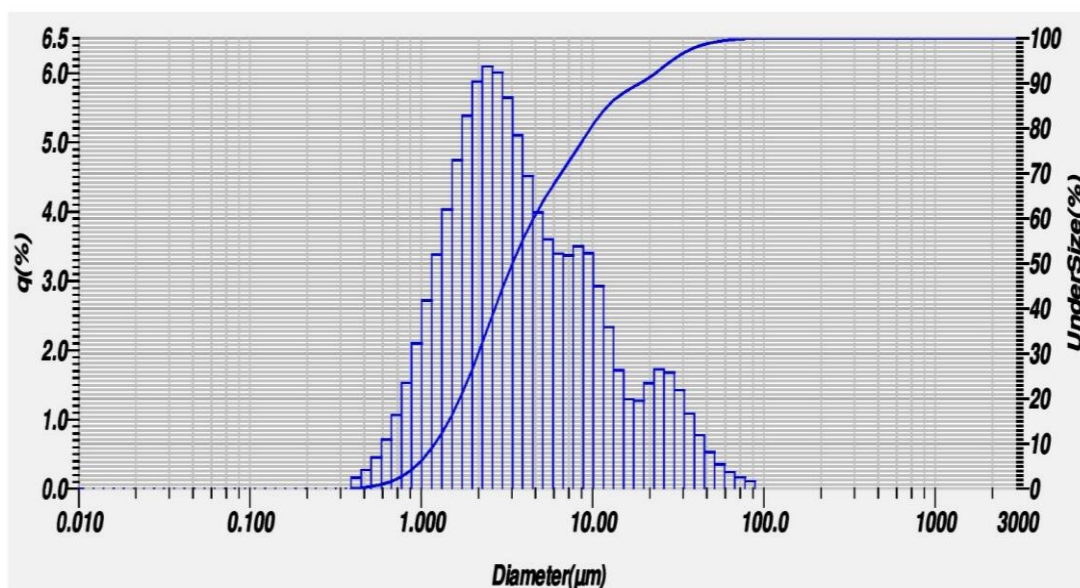


Figure 5.4 Particle size distribution of Hydroxyapatite (HAp) prepared through precipitation method.

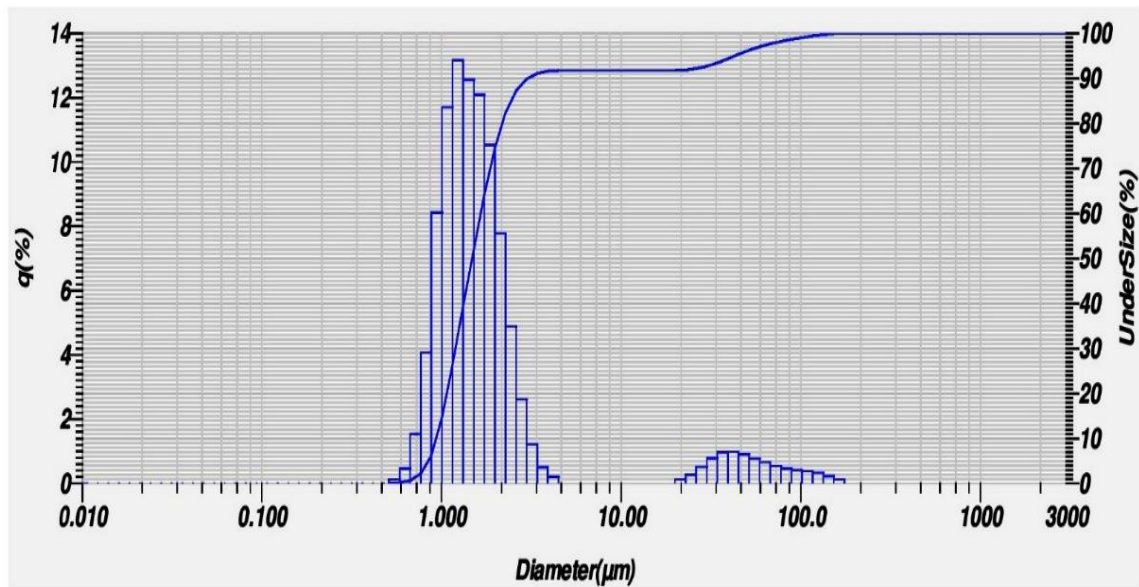


Figure 5.5 Particle size distribution of Hydroxyapatite (HAp) prepared through ultrasonication method.

5.5 SCANNING ELECTRON MICROGRAPHS

Understanding the morphological behavior of hydroxyapatite powders is important, because this allows to design ceramics with controlled grain growth, microstructure and elemental properties. In the present study, the effect of powder characteristics on densification, microstructural development and elemental properties of HAp were studied.

The microstructure of cuttlefish bones consists of two regions: a thick external wall or dorsal shield and internal regions with lamellae, which are separated by numerous pillars. SEM micrographs shows that the general image of cuttlefish bones was preserved after precipitation and ultrasonication treatment, the cuttlefish bones retained its simpler form with the same channel size ($\sim 80 \times 9300 \mu\text{m}$). The enlarged view of transformed HAp indicates the existence of many uniform, dandelion-like HAp nanostructures with diameter from 3 to 8 μm , formed on the surface of lamellae and pillars.

With the prolonged precipitation treatment, the dandelion-like structures are transformed into various nanostructure branches, with an average diameter of about 500 nm and an average length of about 5–10 μm .

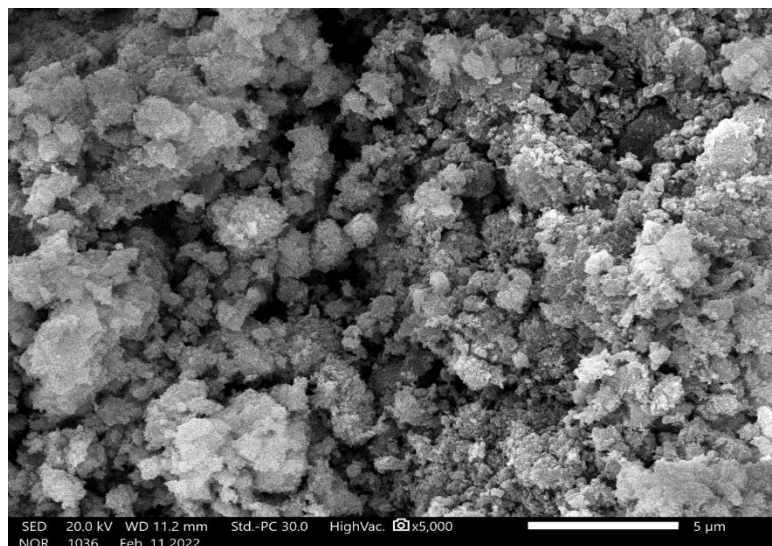


Figure 5.6 SEM of Hydroxyapatite (HAp) prepared through precipitation method (5 micrometer).

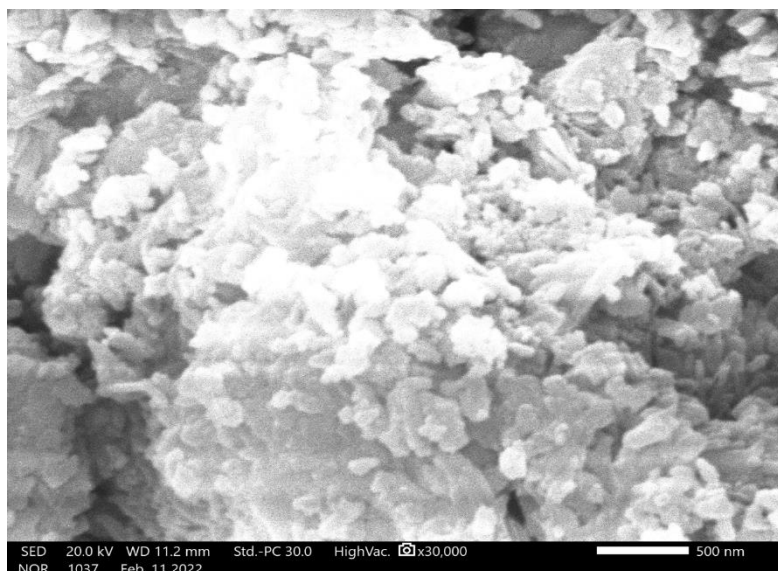


Figure 5.7 SEM of Hydroxyapatite (HAp) prepared through precipitation method (500 nanometer).

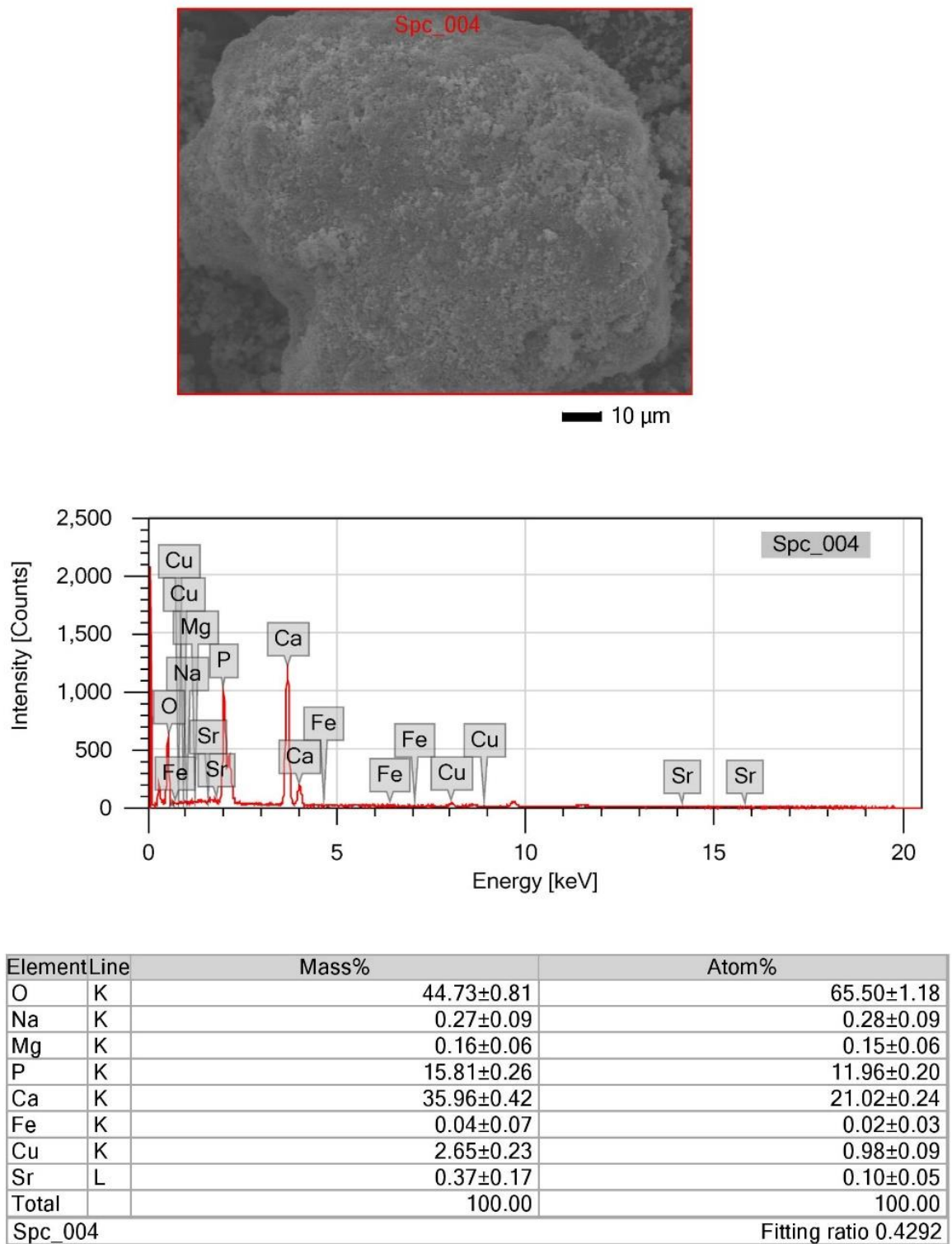


Figure 5.8 SEM with EDS of Hydroxyapatite (HAp) prepared through precipitation method.

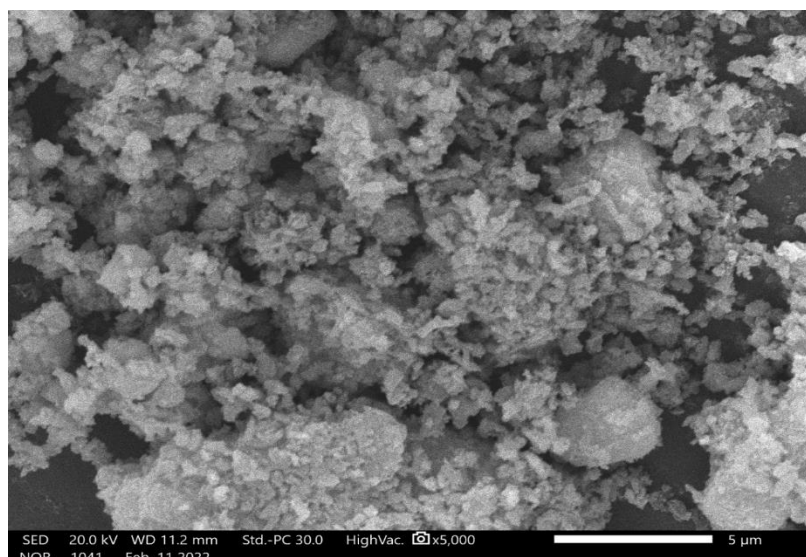


Figure 5.9 SEM of Hydroxyapatite (HAp) prepared through ultrasonication method (5 micrometer).

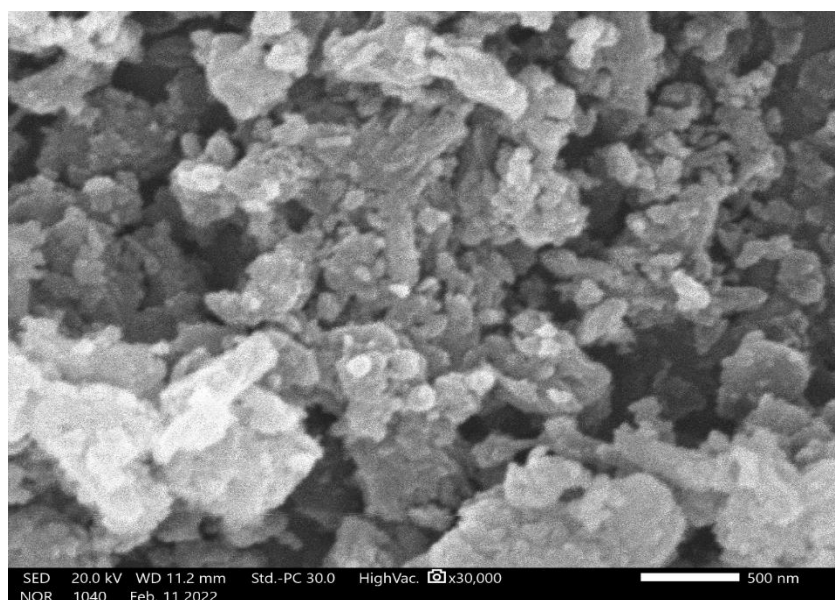


Figure 5.10 SEM of Hydroxyapatite (HAp) prepared through ultrasonication method (500 nanometer).

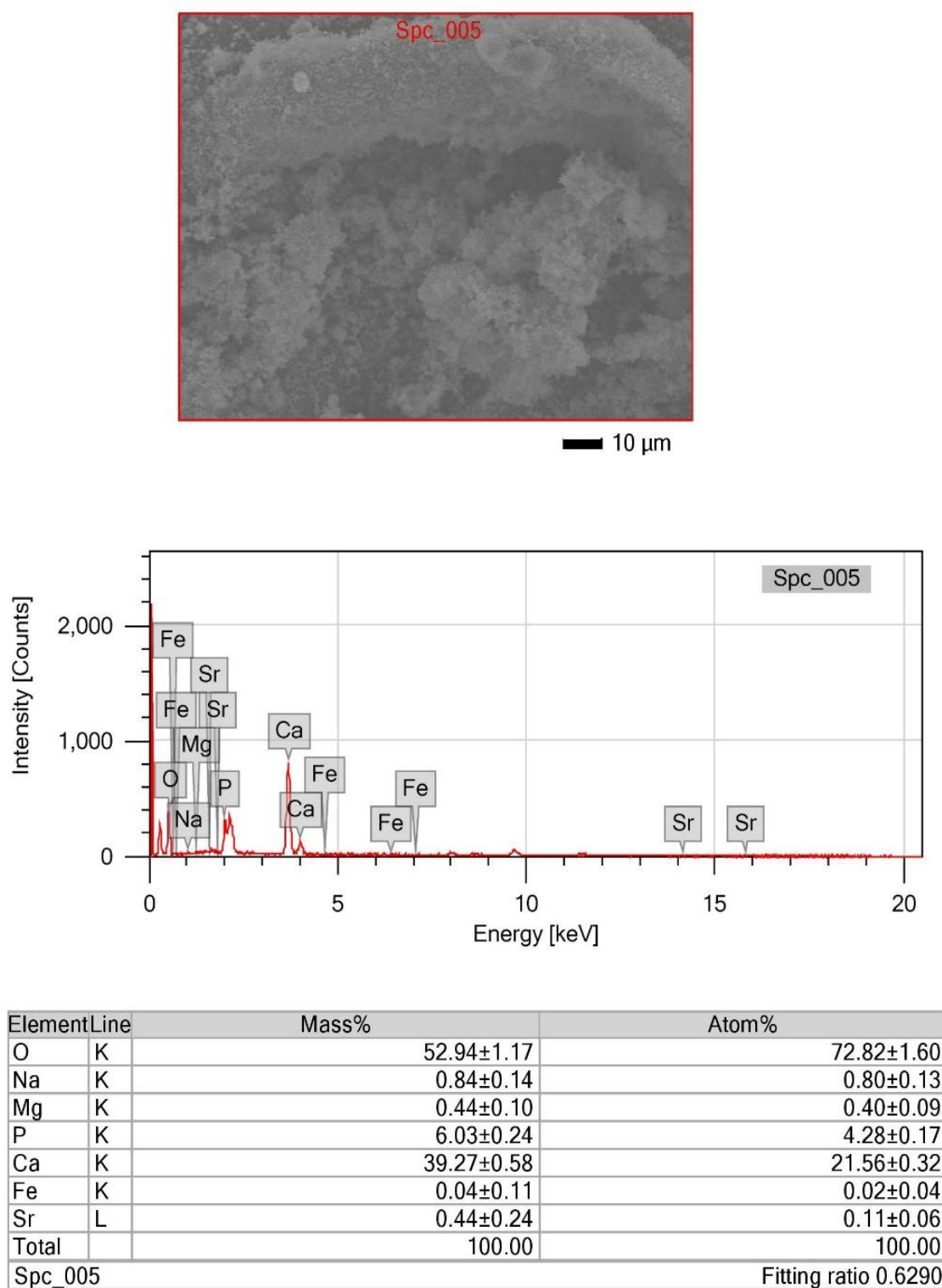


Figure 5.11 SEM with EDS of Hydroxyapatite (HAp) prepared through ultrasonication method.

CHAPTER 6

CONCLUSION

This study is a pioneering work in the preparation of hydroxyapatite using marine waste cuttlefish bones using precipitation and ultrasonication method. The resultant powder from the precipitation and ultrasonication were investigated via systematic characterization methods to confirm the physicochemical properties of the cuttlefish bone-derived HAp.

The present work deals with the synthesis and characterization of the powder. The significant findings of this work are:

XRD results indicate there is an improvement in crystallinity of HAp after calcination. Thermal stability is there upto 1300°C and there is no weight loss. The densification of HAP was the result of volume diffusion and grain boundary diffusion. There is a presence of two particle size ranges in case of HAp powders. The XRD data revealed the mechanism behind the transformation of aragonite to calcite and then to HAp of 20.86 nm to be related to crystallite size. The average length and width of the Hap was determined as 79.05 and 219.66 nm using SEM analysis and the elemental analysis showed the Ca/P molar ratio of 1.6 (1 mole of calcium per 0.6 mole of phosphorus). Both the crystallite size and elemental analysis showed that the HAp are similar to natural human bone and can be used as an implant material in hard tissue treatments. FTIR data further confirms the presence of phosphate groups and TGA data showed that the CB-HAp possess significant thermal stability.

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