

**SCHOOL OF MATERIALS AND MINERAL RESOURCES ENGINEERING
UNIVERSITI SAINS MALAYSIA**

**EVALUATION OF HYDROXYAPATITE FROM FISH BONE AS
POTENTIAL PROTEIN ADSORBER AND FILLER**

By

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of the requirements for the degree of Bachelor of Engineering with Honours
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DECLARATION

I hereby declare that I have conducted, completed the research work and written the dissertation entitled “*Evaluation of Hydroxyapatite from Fishbone as Potential Protein Adsorber and Filler*“. I also declare that it has not been previously submitted for the award of any degree or diploma or other similar title of this for any other examining body or university.

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TABLE OF CONTENTS

Contents	Page
DECLARATION	i.
ACKNOWLEDGEMENT	ii.
TABLE OF CONTENTS	iii.
LIST OF FIGURES	vii.
LIST OF TABLES	x.
LIST OF ABBREVIATIONS	xi.
LIST OF SYMBOLS	xv.
ABSTRAK	xvi.
ABSTRACT	xvii.
CHAPTER 1 INTRODUCTION	1
1.1 Background	1
1.2 Problem Statement	3
1.3 Research Objectives	5
1.4 Thesis Outline	6
CHAPTER 2 LITERATURE REVIEW	7
2.1 Waste from Fish Processing Industry	7
2.1.1 The Stickwater	8
2.1.2 The Solid Waste	11
2.2 Hydroxyapatite (HA)	12
2.2.1 Sources of HA	12

2.2.2 Characteristics of HA	13
2.3 Methods of HA Synthesis	17
2.3.1 Precipitation Method	18
2.3.2 Ultrasound Irradiation Method	18
2.3.3 Electrodeposition Method	18
2.3.4 Hydrothermal Process Method	19
2.3.5 Spray Pyrolysis	19
2.3.6 Calcination and Heat Treatment Method	20
2.3.7 Reflux	22
2.3.8 Other Methods	22
2.4 Methods of Nano-size Hydroxyapatite (nHA) Synthesis	24
2.4.1 Ultrasound Irradiation	26
2.4.2 Flame Spray Pyrolysis	27
2.4.3 Alkaline Hydrolysis	27
2.4.4 Milling and Mechanical Alloying	28
2.5 Protein Recovery	30
2.5.1 Surface Modification of Nano-HA (nHA)	30
2.6 PLA and Fish Waste Protein Blends	36
CHAPTER 3 METHODOLOGY	37
3.1 Preparation of Hydroxyapatite from Fishbone by Calcination Method	38
3.1.1 Raw Materials Preparation	38
3.1.2 Calcination Step	39
3.2 Preparation of Nano-Hydroxyapatite by Planetary Milling	40

3.2.1 Procedure	40
3.3 Surface Modification of Nano-Hydroxyapatite	40
3.4 Synthesis of Ion Oxide Nanoparticles (IONP)	40
3.5 Preparation of Magnetic nHA by Electrostatic Self-Assembly	41
3.6 Application of Protein Recovery	41
3.6.1 Collection of Wastes Samples	41
3.6.2 Activation of Magnetic nHA	42
3.6.3 Recovery of Fishwaste Protein	42
3.6.4 Bradford Assay's Characterization	43
3.7 Preparation for Tensile Test	43
3.7.1 Materials	43
3.7.1.1 Polylactic acid (PLA)	43
3.7.1.2 Fish Proteins (FF)	44
3.7.1.3 Hydroxyapatite (HA) and Modified HA (mHA)	44
3.7.2 Compounding Process	44
3.7.3 Formulation of Blends	44
3.7.4 Compounding Step	45
3.7.5 Compression Molding	45
3.8 Research Flowchart	47
CHAPTER 4 RESULTS AND DISCUSSION	48
4.1 Physical Characteristics of Modified HA (Mha), IONPs and Magnetic Particles	48
4.2 Analysis of the Functional Chemical Composition by FTIR	49

4.2.1 Analysis of the Chemical Composition in Fishbone	49
4.2.2 Analysis of the Chemical Composition in Modified nHA	53
4.2.3 Analysis of the Chemical Composition in Recovered Protein	54
4.3 Surface Morphology of Hydroxyapatite Powder by SEM Analysis	55
4.4 Phase Analysis by X-Ray Diffraction (XRD)	57
4.5 Determination of Protein Recovery by Bradford Assay	59
4.6 Determination of Particle Size and Charge by Zeta Potential	60
4.7 Evaluation of Mechanical Properties	62
CHAPTER 5 CONCLUSION AND FUTURE RECOMMENDATIONS	66
5.1 Conclusion	66
5.2 Recommendation of Future Works	67
REFERENCES	68
APPENDIX	73

LIST OF FIGURES

Figure 1.1	Illustration of protein modification.	3
Figure 1.2	Separation process using magnet	3
Figure 2.1	Example of a simple rendering process	9
Figure 2.2	Structure of a fish	11
Figure 2.3	Classification of natural resources for the extraction of HA and its precursors	13
Figure 2.4	Structure of <i>lutjanus campechanus sp.</i>	13
Figure 2.5	X-ray analysis of HA refill in bone resurrection	14
Figure 2.6	The schematic diagram of a flame spray hydrolysis process	19
Figure 2.7	The illustration of mechanical milling (attrition milling)	28
Figure 2.8	Example graph of crystalline size and lattice strain variation of powder vs. milling time	30
Figure 3.1	Overall background of the study	37
Figure 3.2	The difference in water changes after cooking and boiling process	38
Figure 3.3	The structure of fishbone after manual crushing using blender	39
Figure 3.4	The colour change of fishbone (i) before calcination and (ii) after calcination.	39

Figure 3.5	IONPs nanoparticle	41
Figure 3.6	The dispersion of MnHA in each sample of protein waste	43
Figure 3.7	The appearance of plastic sheet from compression molding	46
Figure 3.8	The strip test piece prepared form tensile testing according to ASTM D882	46
Figure 3.9	The overall flowchart of research	47
Figure 4.1	The physical observation of (a) mHA (b) IONPs (c) MnHAand (d) HA when reacted with distilled water.	48
Figure 4.2	Difference in FTIR spectra of fishbone before calcination, after calcination and after modification	50
Figure 4.3(a)	FTIR spectra for fishbone before and after calcination	51
Figure 4.3(b)	FTIR spectra showing a narrow –OH bands of HA	51
Figure 4.3(c)	FTIR spectra showing triple degenerate bending of PO groups.	52
Figure 4.4	FTIR spectra for calcined HA and modified nHA	53
Figure 4.5	FTIR spectra of recovered protein by modified nano-HA	54
Figure 4.6	SEM micrographs of the HA sample; a) before calcination, b) after calcination, c) after milling for1 6 hours and d) after surface modification	55
Figure 4.7	The colour change of fishbone powder (i) before	59

calcination and (ii) after calcination

Figure 4.8	Zeta potential size distribution	62
Figure 4.9(a)	The tensile strength of PLA/FP blend with different types of fillers.	63
Figure 4.9(b)	The tensile modulus of PLA/FP blend with different types of fillers.	63
Figure 4.9(c)	The elongation at break of PLA/FP blend with different types of fillers.	64

LIST OF TABLES

	Table	Page
Table 2.1	Average composition of fish waste	11
Table 2.2	Characteristics of HA	13
Table 2.3	Methods of hydroxyapatite (HA) synthesis with its reference	17
Table 2.4	Description of calcination data.	21
Table 2.5	Methods of nano-sized hydroxyapatite (nHA) synthesis with its reference	26
Table 2.6	Functional polymeric materials used to interact with protein	31
Table 3.1	The formulation of PLA and fish protein blends with different amount of additives.	44
Table 3.2	The sequence of loading materials for different sample	45
Table 4.1	The infrared absorption spectroscopy of sample calcined at 900°C compared to the reference of HA.	52
Table 4.2	Three highest peak of XRD in reference sample of hydroxyapatite	60
Table 4.3	Tabulation data obtained from spectrophotometer.	60
Table 4.4	Surface charge of HA and modified nano-HA	61

LIST OF ABBREVIATIONS

Al	Aluminium
ATRP	Atom transfer radical polymerization
BOD	Biological oxygen demand
Br	Bromide
β -TCP	Beta trichloro-phosphate
BST	$\text{Ba}_{0.6}\text{S}_{0.4}\text{TiO}_3$
Ca	Calcium
CaCl_2	Calcium chloride
CaCO_3	Calcium carbonate
Ca/P	Ratio of calcium to phosphate
$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	Hydroxyapatite
CO	Carbon monoxide
CO_3^{2-}	Carbonate ion
COD	Chemical oxygen demand
COO	Carboxylic group

COOH-NH ₂	Carboxylate – protein linkage
OH	Hydroxyl
COOH	Carboxylic acid
DC	Defluoridation capacity
DPM	Di(ethylenediamine platinum)medronate
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide
F ⁺	Flourine ion
FAO	Food and Agricultural Organization
FP	Fish waste protein
FTIR	Fourier transform infra-red spectroscopy
H ₂ O	Water
H ₃ PO ₄	Phosphoric acid
HA	Hydroxyapatite
HNO ₃	Nitric acid
HPO ₄ ²⁻	Hydrogen phosphate ion
HUP	Hot uniaxial pressing
IONP	Ion oxide nanoparticles

KOH	Potassium hydroxide
mHA/MnHAp	Modified hydroxyapatite/ Modified nano-hydroxyapatite
MNP	Magnetic nanoparticle
MWCNT	Multi-walled carbon nanotube
nHA	Nano-hydroxyapatite
Na ⁺	Sodium ion
NaBr	Sodium bromide
NaClO	Sodium hypochlorite
NaOH	Sodium hydroxide
Na ₃ PO ₄	Trisodium phosphate
NH ₂	Amine/Protein
NHS	N-hydroxysuccinimide
OH	Hydroxyl
P	Phosphorus
PBS	Phosphate buffered saline
PE	Polyethylene
PEG	Polyethylene glycol

PLA	Poly(lactic) acid
PLA/FF	Blend of PLA and FF
PMMA	Poly(methymetharcylate)
PP	Polypropylene
SEM	Scanning electron microscopy
TCP	Tricalcium phosphate
TeCP	Tetracalcium phosphate
TEM	Transmission electron microscopy
TEMPO	2,2,6,6-tetramethylpyperidine-1-oxyl
TSS	Total suspended solid
TiO ₂	Titanium dioxide
VOC	Volatile organic compound
XRD	X-ray diffraction

LIST OF SYMBOLS

β	Beta
$^{\circ}\text{C}$	Degree Celsius
%	Percent
θ	Theta
cm	centimetre
kHz	kiloHertz
MPa	MegaPascal
mg	milligrams
ml	milliliter
mm	millimeter
nm	nanometer
g	gram

PENILAIAN HYDROKSIAPATIT DARI TULANG IKAN SEBAGAI POTENSI
PENYERAPAN PROTEIN DAN PENGISI

ABSTRAK

Objektif utama kajian ini adalah untuk menunjukkan penghasilan nanopartikel hidroksiapatit magnetik dengan teknik manipulasi elektrostatik antara nanopartikel hidroksiapatit yang diubahsuai dengan ion oksida. Analisis menggunakan XRD menunjukkan kalsinasi adalah kaedah paling sesuai untuk memperoleh HA dari tulang ikan. Pemulihan protin daripada rumusan sisa telah dikaji menggunakan Bradford Assay. Melalui analisis Bradford Assay, kehadiran kepekatan protin yang tertentu mengesahkan lekatan protin ikan pada HA. Dalam analisis assay Bradford, kehadiran kepekatan protein yang tersendiri telah mengesahkan lampiran protein dari air ikan kepada hubungan-COOH HA. HA diubahsuai dicirikan oleh teknik potensi FTIR, SEM dan zeta. HA yang diubahsuai telah dikenalpasti melalui analisis FTIR, SEM dan potensi zeta. Keputusan FTIR menunjukkan puncak yang ketara pada 1414 cm^{-1} berpunca dari lekatan IONPs ke atas permukaan H. kehadiran puncak ini disebabkan oleh kehadiran asid karboksilik pada permukaan HA magnetik. Analisis potensi zeta membuktikan kejayaan lekatan IONPs pada permukaan HA. Maka, penyelidikan ini menunjukkan potensi kegunaan HA magnetik dalam pemulihan protin di mana protin yang melekat pada nanopartikel boleh dipisahkan daripada campuran dengan menggunakan magnet. Untuk menilai ciri-ciri HA sebagai bahan pengisi, morfologi HA dicirikan melalui SEM. Mesin instrin digunakan bagi mengenalpasti ciri fizikal dan mekanikal. Didapati bahawa PLA tulen dan campuran sisa ikan boleh memberikan kekuatan tegangan yang lebih tinggi berbanding penambahan HA sebagai pengisi.

EVALUATION OF HYDROXYAPATITE FROM FISH BONE AS POTENTIAL PROTEIN ADSORBER AND FILLER

ABSTRACT

The main objective of the current work was to demonstrate the fabrication of magnetic hydroxyapatite nanoparticles (MnHA) by the manipulation of electrostatic self-assembly technique between the modified hydroxyapatite and iron oxide nanoparticles (IONPs). Analysis by XRD revealed that calcination is the most suitable method to synthesize HA from fish bone. The application of protein recovery from waste solution was investigated by using Bradford Assay. In the Bradford assay's analysis, the presence of a distinctive protein concentration has confirmed the attachment of protein from stickwater to the $-\text{COOH}$ linkages of HA. The modified HA was characterized by FTIR, SEM and zeta potential analysis. FTIR results showed a noticeable peak at 1414 cm^{-1} due to the attachment of IONPs on the H surface. The presence of this peak might be contributed by the carboxylic group present on the surface of magnetic HA. Zeta potential analysis revealed a successful attachment of IONPs on HA surface. Therefore, this findings presents the potential application of magnetic HA for protein recovery where the protein attached to the magnetic nanoparticle can be easily separated from the complex mixture by using magnet. To evaluate the properties of HA as filler materials, the morphology of HA samples was characterized using SEM. Instron Tensile Machine were used to identify the physical and mechanical properties obtained. It was found that pure PLA and fish waste blends can provide higher tensile strength compared to the addition of HA as filler.

CHAPTER 1

INTRODUCTION

1.1 Background

Over the last 50 years, the production of seafood and fish products clearly reflected the global market demand. Fish is nutritious as it can provide the important sources of nutrients and rich in minerals, macronutrients, fatty acid and proteins. In recent years, the increasing demand of fish is not only due to the escalation in population but is also caused by the growing development of nutritional and functional food industries. The fisheries and aquaculture industry are important as it can contribute to the national food security as well as the foreign exchange for the country.

However, human activities such as industrial, agricultural and domestic pursuits had cause discharge of effluents into the riverine systems, where the aquatic resources can be found. This scenery had cause the factor of water pollution. According to a scientific article, entitled “Possible Methods for The Utilization or Disposal of Fishery Solid Wastes” by John H. Green and Joseph F. Mattick, it was mentioned that fishery wastes are different from municipal wastes. Stick water contain substantial percent of total protein as well as other compound such as fatty acids and oil. Protein, for instance, is a high value organic compound and shows an impact in economy since it has been widely applied for production of fish meal, organic soil fertilizer and dietary sources (Green and Mattick 1977).

In recent years, study on protein recovery had captured significant interest in wide research area with the introduction of various recovery techniques including membrane filtration(Pedersen, Crapo et al. 2003) coagulation and flocculation at certain

pH and adsorption using clay (Pedersen, Crapo et al. 2003). Although membrane offers great advantages over traditional method, the up-scaling process for a very large volume is still consider high and not a favorable approach (Afonso and Bórquez 2002). To date, the system design of existing waste water treatment does not consider nutrient removal. Current method to recover protein is typically done by evaporation and drying. However, this method utilize high temperature and energy intensive which is harmful to the protein structure because it would cause structural degradation that would further affect its nutritional value.

Other than stickwater, the fish bone wastes have not been used for anything. The fish bone wastes have caused several environmental problems such as clogging or organic odour. The components of fish bones are calcium phosphate, calcium carbonate, collagen fiber and hydroxyapatite (Cahyanto, Kosasih et al. 2017). The major elements in hydroxyapatite are calcium (Ca) and phosphorus (P), which can be found in the component as the bone or teeth. Besides fish bone, natural hydroxyapatite can also be synthesized from eggshells, corals, or chicken bones. The chemical formula presented by hydroxyapatite is $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. The Ca/P ratio of hydroxyapatite can determine its stability. Researchers have found that lower value of Ca/P ratio of hydroxyapatite can contribute to a greater bioactivity. A stable hydroxyapatite can be obtained by having Ca/P ratio closer to 1.67 (Mustafa, Ibrahim et al. 2014).

Studies have reported that hydroxyapatite is a molecule with adsorption ability, which is considered as an advantage. Hydroxyapatite can act as a sorbent in the wastewater purification and helps in the removal of heavy metals from aqueous solutions. This is because of the ability of hydroxyapatite itself which includes high removal capacity, low water solubility, low cost and high stability especially under oxidizing and reducing conditions. (Kongsri, Janpradit et al. 2013). However, reactive

surface of HA is covered with numerous hydroxyl (OH) groups, where the use of reactant or solvent may affect the reactivity of this group. Therefore, surface modification of HA is needed to improve the surface capability as to function as protein adsorbent.

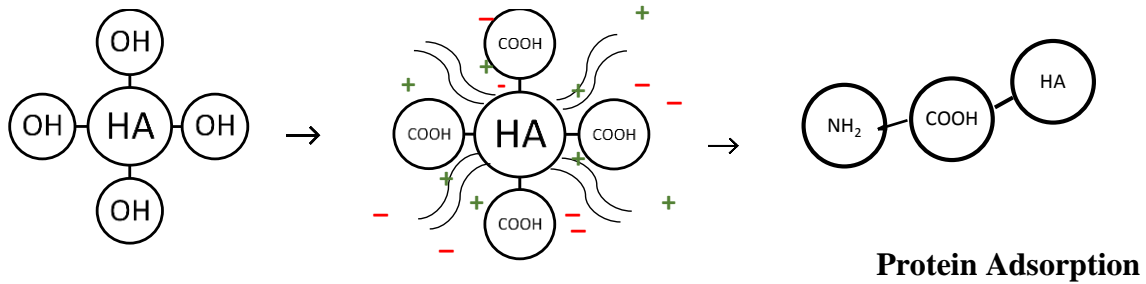


Figure 1.1: Illustration of protein modification.

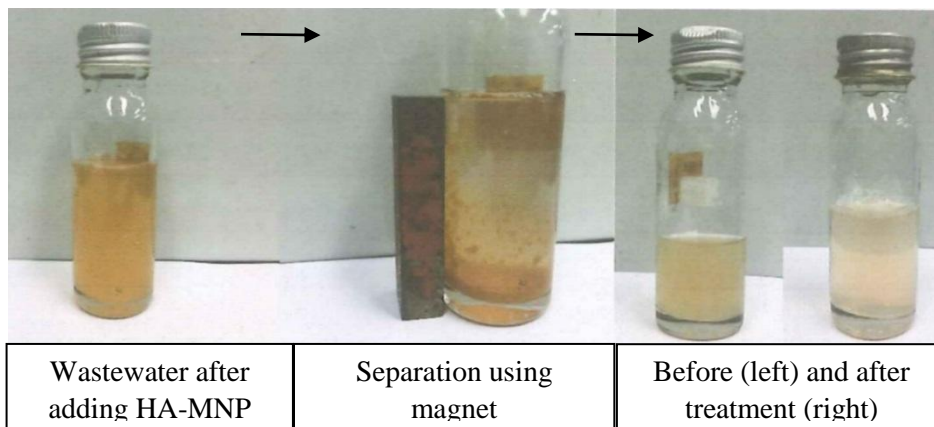


Figure 1.2: Separation process using magnet.

1.2 Problem Statement

Malaysia is a country known as a fish eating nation. The fishery products provide various sources of minerals, proteins and fatty acids. The development of aquaculture industries in Malaysia are expected to keep expanding in the future due to the growing demand of fish products. The phenomenon of fish farming or shrimp breeding are a favourable act to the government in order to export the products to other countries, at the same time offering career opportunities to the nation.

However, research have found that increasing demand of seafood supply had caused problems to the environment. Human aggressive aquaculture activities had cause the discharge of effluent into the riverine which cause water pollution. The fishery waste contain nitrogen and protein. However, the design of the existing treatment systems used nowadays are unable to remove the nutrient content in the stickwater, such as the sewage treatment. A few existing traditional disposal and treatment methods have expensive operating costs such as ocean dispersion, landfill, soil disposal, screening, incineration, pyrolysis, etc. (Green and Mattick 1977)

According to a book entitled ‘Seafood Processing By-Products: Trends and Application’ in 2014 by Se-Kwon Kim, the editor stated that during fishmeal production, there are three main waste effluents that are generated. They represents the bail water, blood water and the stickwater. The stickwater generates about 60% of the processed fish waste which is considered as the highest amount of discharged. Thus, stickwater contain a variety of solid and particles, making it a complex effluent. Technically, the waste are not treated properly which allows environmental pollution(Kim 2014).

According to Md. S. Islam et al. (2004), the improper discharge of stickwater had contributed to the huge increase in biological oxygen demand (BOD), chemical oxygen demand (COD), total suspended solid (TSS) and fat-oil grease(FOG). (Islam, Khan et al. 2004) The increase in BOD and COD will lead to low oxygen level and contamination of water system with foreign particles that can pose a great threat to the aquatic residences, particularly. In addition, increase in TSS can cause reduction of water clarity, clogging and photosynthesis reaction, resulting in low water quality. Furthermore, FOG will solidify and harden in the pipeline system due to the mixture of

these substances with sanitary wastes. It would cause accumulation of this debris and contribute to a severe blockage in the pipeline system and will eventually cause flood.

Driven by the aforementioned motivation, we proposed a novel protein recovery strategy from stick water sample by using HA as adsorbent. During modification, iron oxide nanoparticles (IONPs) as the magnetic nanoparticle (MNP), which are superparamagnetic nanoparticles that allows an easy separation system by using an external magnetic field will be used. Since the MNP is in nano sized, it is important that the size of HA need to be small enough to be able to incorporate the MNP. Other than that, the preparation of MNP-HA through self-assembly technique will require electrostatic interaction between MNP and HA. Modified HA has to be negatively charge in order to recover protein which is positively charged. In this research, we tend to obtain a nano-scale of HA apparently sized below 1000 nm to be used as a filler materials in the PLA/FP waste. The use of nano-HA as filler material in this study was to observe the suitability and compatibility of HA to increase the strength of material.

1.3 Objectives

The objectives of this project are;

1. To prepare and characterize the hydroxyapatite from the bone of red snapper fish (*Lutjanus campechanus sp.*).
2. To produce nano-sized hydroxyapatite by using planetary ball milling.
3. To evaluate the performance of modified hydroxyapatite as protein adsorber by oxidation method using TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl).
4. To evaluate the use of modified hydroxyapatite as fillers in PLA and fish protein blend.

1.4 Thesis Outline

Chapter 1 represents the overview of the research and a brief literature of the previous and relevant work. It provides the introduction of dissertation including background of fish processing waste, hydroxyapatite and tissue engineering, problem statement, objectives of this study and the scope of this project.

Chapter 2 discuss about the detailed literature review of previous and relevant works about fish processing waste, natural resources of HA and the existing route for the synthesis of HA as well as presenting the available method to synthesize nano-HA. The technology regarding surface modification were also discussed.

Chapter 3 presents the raw materials, methodology and the characterization techniques used in this research.

Chapter 4 gives the interpretation and discussion of the experimental results of the experimental work.

Chapter 5 presents the significance of the results and the summary of the research study. The recommendations for the future studies in the related research are included in this chapter.

CHAPTER 2

LITERATURE REVIEW

2.1 Waste from Fish Processing Industry

Wastes from fish processing industry can be identified as solid wastes and wastewater. Improper treatment of wastes before disposal can cause water pollution and making it unsafe to use. Extreme pH change in the receiving waterbody may affect the aquatic life as well as resulting biological imbalance.

An article of “Small Fish Scale Processing” found in New Zealand Digital Library in the Community Development Library section, it was stated that there are four types of wastewater produced which can be described as follows; (Nzdl.org, 2018)

- a) Blood water and process water.
 - The blood water originates from the fish and the process water results from the washing of fish. This water may contain nutrient such as proteins.
- b) Ice melts water.
 - The ice were used to keep the fish in a fresh condition. It contain fish protein, blood and bacteria.
- c) Wash-down water.
 - This water contain larger pieces of fish and protein.
- d) Specialized wastewater.
 - This water are produced from the operations of fish processing such as the press liquor or the stickwater.

Technically, all contaminated water from fish processing operations must be treated in order to prevent further environmental pollution to the natural water sources.

For example, floating solid effluent can prevent the penetration of light to the sea bottom which cause lack of light sources can be received to the aquatic life. This will cause further threat to the marine population. Thus, it is necessary to conduct water treatment to remove the solid particles such as screening, sedimentation, centrifugal or flotation. In addition, chemical treatment is also crucial to control the pH and coagulation problems.

According to The Canadian Department of Fisheries, it was recommended that; (Nzdl.org, 2018)

The treatment of wastewater for solid removal should be equivalent to that achieved by a screen opening with size of 0.7 mm so that the receiving water received only a slight discharged wastewater.

- a) The contaminated wastewater should not be discharged directly into the receiving water. This is because the waste water such as the stickwater and blood water contain high organic materials which can affect the biological oxygen demand (BOD) in the receiving water. Therefore, it is a minimum requirement to conduct a primary treatment to these effluents such as solid removal by screening and oil removal by flotation.

2.1.1 The Stickwater

The aquatic catch were sent to the processing facilities to undergo further fish processing in order to ensure that the final product is delivered to the customers. Various types of seafood are processed in the processing facilities including fish, crabs and shrimps. As a consequence, a large number of inedible effluents are generated.

Wastes from the fish processing industry can be divided into two, which are the solid waste and the stickwater.

To maximize the use of aquatic resources, some processing facility use the concept of rendering in order to reduce the total solid waste. Conversion of waste materials into fish meal, known as rendering is due to the large amount of waste from seafood processing. Figure 2.1 is a simple schematic diagram of the rendering process;

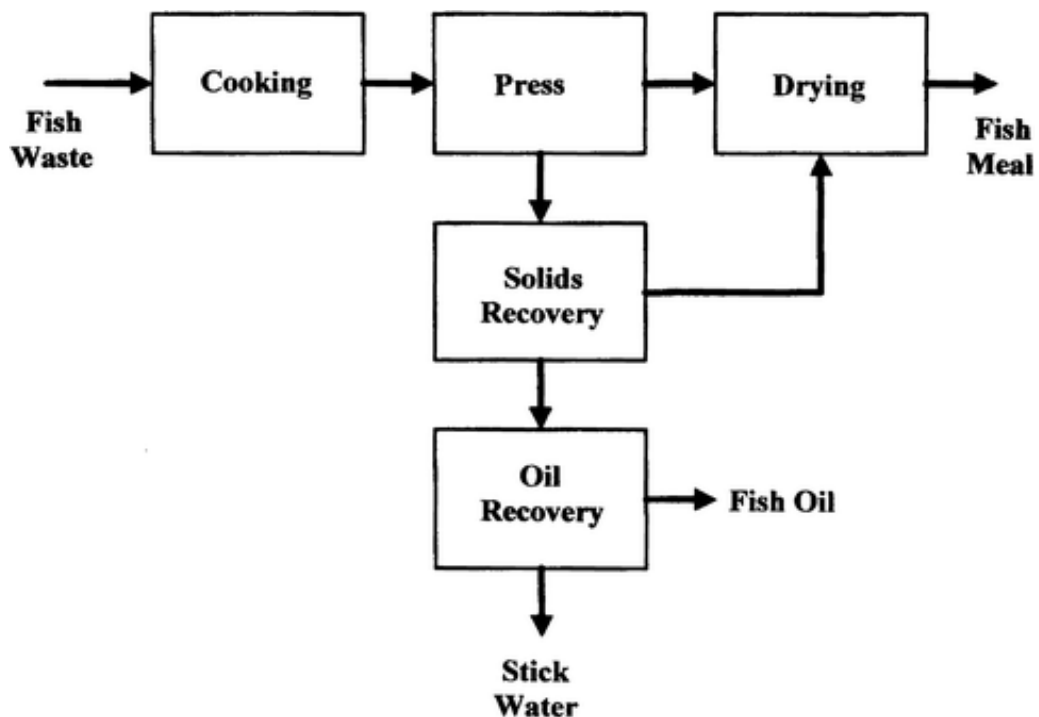


Figure 2.1: Example of a simple rendering process. (Bimbo, 2018)

Referring to a book entitled “Wastewater Treatment in The Fishery Industry” written by J.F. Gonzales in the technical paper of Food and Agriculture Organization (FAO) of the United Nations, the wastewater can be characterized by physicochemical parameters such as the pH, the solid content, temperature and odour. The decomposition of proteinaceous matter caused by the fish effluent lead to acidic properties but also close to pH 7. Extreme pH change in the receiving water body may affect the aquatic life. Besides that, he stated that the temperature of the discharged

wastewater should not affect the temperature of the receiving water. This is because, an increasing of 2 or 3⁰C of the water body can cause oxygen reduction and thus placing the aquatic wildlife at risks. On the other hand, the odour from the wastewater are due to the emitted amines and ammonia caused by the decomposition of organic matters. Technically, odours are harmless toward the human. However, it may be the source of stress or nausea.

Fat globules, inorganic ions and proteins are the example of particles that present in the stickwater. It was mentioned that there were lack of capacity for the treatment of stickwater in the industry. The possibility of getting fouled pipes may increase as the stickwater can become highly viscous once evaporated, and thus difficult to manage. The fluid movement interaction becomes limited due to the high viscosity and may cause further complications during processing. Furthermore, the traditional method of stickwater such as evaporation requires high energy evaporating systems, resulting in expensive process (Sifuentes, Aguilar et al. 2009).

Research have also found that with respect to the pollution generated by fish waste, suspended solid, total nitrogen and total phosphorus are considered as the existing nutrients in the wastewater (Turcios and Papenbrock 2014). In addition to that, the effluent from the wastewater treatment is a major contributor of phosphorus to the receiving water. In industry, phosphorus from the wastewater can be used as a raw materials for fertilizers and thus recycling them would be beneficial (De-Bashan and Bashan 2004).

2.1.2 The Solid Waste

Figure 2.2 shows the structure of a fish which is mainly composed of the meat and the bony skeleton. Other parts of fish includes operculum, gills and swim bladder.

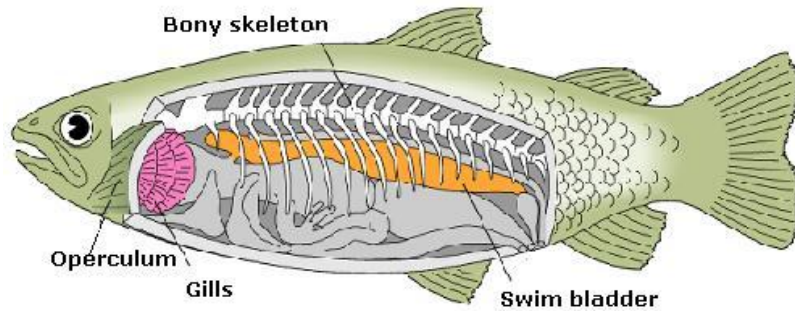


Figure 2.2: Structure of a fish

Table 2. **Error! No text of specified style in document.:** Average composition of fish waste

Component	Average Weight (%)
Head	9-20
Backbone	9-15
Viscera	12-18
Skin	1-3 3
Trimming	8-17

Synthetic hydroxyapatite require high initial cost and more complicated as it involved additional chemicals to improve its mechanical strength. Thus, to be fixed in human body, for example in bone implantation, they requires chemical involvement to stabilize the synthetic structure. Hydroxyapatite obtained from natural resources provide a better advantage compared to the synthetic hydroxyapatite. It was mentioned

that the natural hydroxyapatite have better metabolic activities and can provide dynamic response to the environment. (Mustafa, Ibrahim et al. 2014).

Generally, fish are mainly consumed by humans for their flesh. In food industries, fish are used the production in marketing, leaving the solid wastes such as the bones and scales. Major consumption of fish flesh can be seen in the filleting industry. The bones can be recycled due to the natural protein in its body. As mentioned earlier, the fish bone contains calcium phosphate, collagen fiber, calcium carbonate and hydroxyapatite. In relation to the problems stated, this research is crucial in order to identify the characteristics of hydroxyapatite and to modify it becoming magnetized nano-hydroxyapatite to be used in protein recovery.

In addition to that, it was found that several methods are used to synthesize synthetic hydroxyapatite such as hydrothermal, emulsion liquid membrane, precipitation, radio frequency thermal plasma, sol-gel, ultrasonic, etc. may lead to good results in synthetic hydroxyapatite. However, these methods are complicated and involves hazardous process such as evolution of ammonia (Venkatesan and Kim 2010).

2.2 Hydroxyapatite (HA)

2.2.1 Source of HA

A simple classification of biological resources for the extraction of HA can be referred in Figure 2.3. (Akram, Ahmed et al. 2014). In this research, we use red snapper fish bone (*lutjanus campechanus sp.*) to obtain natural HA.

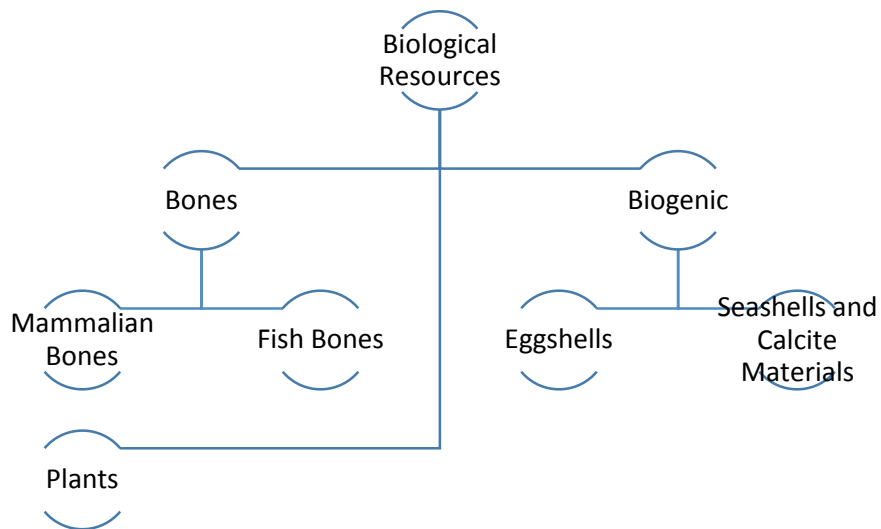


Figure 2.3: Classification of natural resources for the extraction of HA and its precursors.



Figure 2.4: Structure of *lutjanus campechanus sp.*

2.2.2 Characteristics of HA

Table 2.2: Characteristics of HA

Composition	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$
Melting Point	1670
Density	3.18
Molecular Weight	1004.69
Ca/P Ratio	1.67

Natural HA is classified as inorganic composite which can be found in human hard tissue such as teeth and bones. It has the similarity to the bones which can be explained by the wide use of hydroxyapatite in the bone reconstruction in dentistry and medicine. The biocompatibility of natural HA is similar to the bones because it has the same physical-chemical characteristics. Therefore, it is an advantage properties of the precursor materials.

In recent studies, HA have been used as clinical composites for bone substitute such as spacing or filling for bone defects. HA was applied to the defected bones that involved in curettage or in resection of tumours. Figure 2.5 shows that the tumour was completely resected and it can be seen that only HA granules filled the defected bone. This application is due to the advantages of HA which involves postoperative morphological change or decrease in volume. Other than that, HA is lack of immune-reaction, which means that this material did not elicit an immune reaction. Therefore, the bones were unaffected and this ceramic is safe to be used as filling material. To fill and reconstruct a defected bone, HA was used along with tricalcium phosphate (TCP) and tetracalcium phosphate (TeCP). The HA blocks and granules were used as spacers and fillers while the TCP and TeCP were used as collagen(Oonishi 1991).

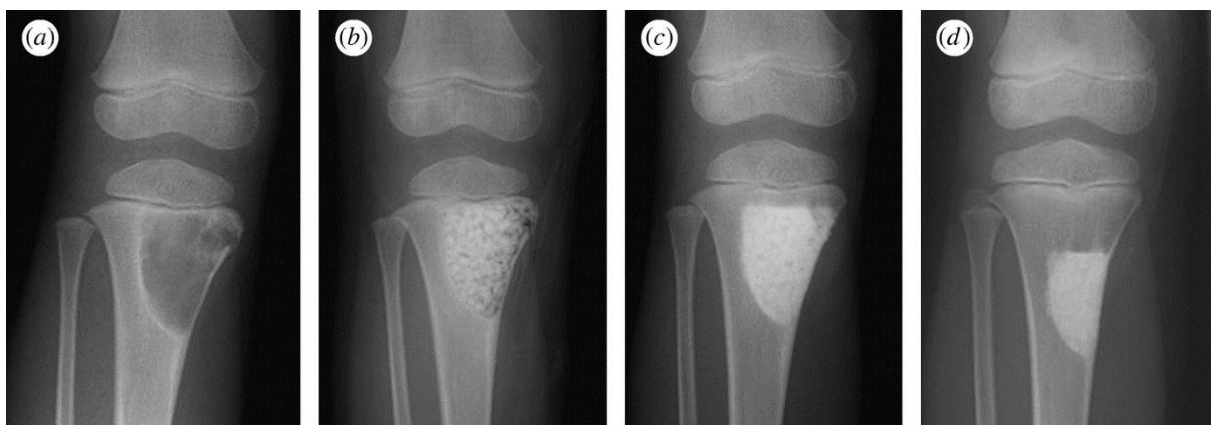


Figure 2.5: X-ray analysis of HA refill in bone resurrection.

Another study regarding bone implantation, HA were used as a potential element to develop a biomimetic apatite. HA are mainly composed of calcium phosphate, which are the physiological natural elements that exist in bones and teeth. In the human bones, the HA crystals are about 100 nm length, 20 to 30 nm width, and 3-6 nm thickness have various special properties including low crystallinity, crystalline disorder and non-stoichiometric disorder. In addition, the surface of HA can be functionalized with bioactive molecules to make them useful for applications such as information transfer or selective action on the biological environment. (Palazzo, Iafisco et al. 2007)

HA can be found in various forms including granules, powders, porous and dense blocks. It is the main minerals of a calcified tissues and have been widely used as clinical biomaterials. It has been said that due to its strong chemical similarity to the inorganic phase of human hard tissues, HA has excellent biocompatibility and bioactivity properties and thus encourage bone growth. (Ferraz, Monteiro et al. 2004). In addition, dense HA has high specific surface area with small grain size and low degree of particle agglomeration. These characteristics contributes to a good mechanical properties in bone substitution. Due to the ionic composition of HA, it can be used applications involving catalyst and ion exchangers. The low Ca/P ratio makes HA becomes sensitive to high temperature and thus suitable to be used in gas sensors and conduction of protons. (Kong, Ma et al. 2002)

Other than that, HA was also found to be used as a biocomposite in fluoride removal by synthesizing alginate bio-encapsulated nano-HA. It was mentioned that HA possesses exchangeable hydroxyl group, with high availability and eco-friendly in nature. Thus, it is an excellent biomaterial to control the water pollution problems. The use of HA in this investigation was to create a high defluoridation capacity (DC)

composite with biodegradability, biocompatibility, low cost and eco-friendly(Pandi and Viswanathan 2014).

The development of air purification using alumina (Al)/titanium dioxide (TiO₂)/HA interface as a composite filters results in an efficient absorption and decomposition of organic species. The emitted gas from vehicles contain various toxic elements including nitrogen oxides, sulfur dioxide, sulfur trioxide, etc. which contribute to air pollution issues. HA was used as an environmental protection as it is able to interact with the volatile organic compounds (VOC). It was showed that the carbon monoxide(CO)gas can be removed by absorption mechanism using this composite filter. Even though HA possess the ability of adsorption properties, it decompose at a slow rate, causing the saturation time to be much longer. Therefore, TiO₂ was used to decompose the organic substance with the help of HA as a coating layer to cover the surface of TiO₂ that functions to adsorb the organic substance(Nasr-Esfahani and Fekri 2012).

Having a good biocompatibility of a pure HA is important to be used at a desired conditions. Due to this ability, HA as bone substitution is considered as interesting materials in many fields such as plastic surgeries, dental and orthopaedics. The bioactivity, biocompatibility and the spontaneous osteointegration of HA as biomaterials had cause a rapid growth and improvement of research regarding this materials.

HA are used in toothpaste or gels. It was found that HA can treat hypersensitivity problems in dentin. Human tooth enamel are roughly composed of 97% HA, while the dentin are composed of 70% HA. This results in a bright white enamel, at the same time prevents diffuse reflectivity of light as in encloses the small

pores of the enamel. The application of HA in toothpaste and gels helps to prevent surface demineralization of the enamel by restoring its mineral density. Therefore, the surface smoothness can be improved and the tooth decay can be prevented. In addition, the exposed surface of dentinal tubules can be occluded by nano-HA complexes.(Ohta, Kawamata et al. 2007)

HA with a chemical formula as above may have bioactivity that can be achieved by having a certain value of pH and Ca/P ratio. In order to achieve phosphate stabilization, the pH value of HA must be above 4.2 with Ca/P ratio of 1.67. In addition to that, HA can be found in both dense and porous structure.

2.3 Methods of HA Synthesis

Hydroxyapatite can be synthesized by a few methods such as listed in Table 2.3;

Table 2.3: Methods of hydroxyapatite (HA) synthesis with its reference.

Methods of HA synthesis	Reference
1. Precipitation	(Santos, Oliveira et al. 2004)
2. Ultrasound Irradiation	(Kojima, Kitazawa et al. 2012)
3. Electrodeposition	(Shirkhazadeh 1998)
4. Hydrothermal Process	(Suchanek and Riman 2006)
5. Spray Pyrolysis	(Cho and Kang 2008)
6. Calcination	(Mustafa, Ibrahim et al. 2014), (Akram, Ahmed et al. 2014)
7. Heat Treatment	(Ozawa and Suzuki 2002; Juraida, Sontang et al. 2011)
8. Reflux	(Cahyanto, Kosasih et al. 2017)

2.3.1 Precipitation Method

It was found that obtaining HA by wet-precipitation method is possible. This method use reagents such as ammonium phosphate, calcium hydroxide, calcium phosphate and phosphoric acid. It is crucial to control the important parameters during the synthesis stage such as pH, temperature, crystal size and morphology, precursor reagents and impurity contents in order to maintain the bioactivity of Ca/P in HA. Moreover, HA can be produced at room temperature and wet-precipitation method can be done in various ways depending on the end product to be used. It was mentioned that wet-precipitation method can increase the amount of HA produced even at a reasonable cost compared to the thermal treatment(Santos, Oliveira et al. 2004).

2.3.2 Ultrasound Irradiation Method

It was found that ultrasound irradiation is effective to synthesize HA by inducing contact between ions in a solution. A frequency of 20 kHz results in high collision and vibration which increase the contacts between ions in the liquid. In this method, 0.1 to 0.2 M CaCl_2 was irradiated with 20 kHz or 40 kHz ultrasound frequency along with the addition of 0.1 M Na_3PO_4 . This method shows an increase in temperature of the solution, where higher temperature results from 40 kHz vibrations compared to 20 kHz. The resulting Ca/P ratio was 2.00 compared to the stoichiometric HA. (Kojima, Kitazawa et al. 2012)

2.3.3 Electrodeposition Method

Formation of HA can be done by electro-crystallization from dilute electrolytes. This method was found to results in ultrafine-grained HA with comparable pH values to the biological HA. Electrodeposition method was found to produce nanophase HA using low concentration of calcium and phosphate ions. Without the formation of

precursor phase, HA can be deposited on the cathodically polarized electrodes(Shirkhazadeh 1998).

2.3.4 Hydrothermal Process Method

The morphology, size and degree of agglomeration of HA can be controlled by hydrothermal synthesis. This method involves single or heterogeneous phase reactions at elevated temperature and pressure. This was done to crystallize the hydroxyapatite directly from solutions. It also consider various thermodynamic variables including the temperature and type of reaction as well as the concentration of reactants. The kinetic variables such as the stirring speed and time were also considered to control the process. This process also shows the formation of solid solution and therefore it was said to be easier to determine the chemical composition of the powders. Hydrothermal synthesis was done at 150°C for 2 hours by using an electric oven. In addition, this method results in the ability to produce scale-up production (Suchanek and Riman 2006).

2.3.5 Spray Pyrolysis

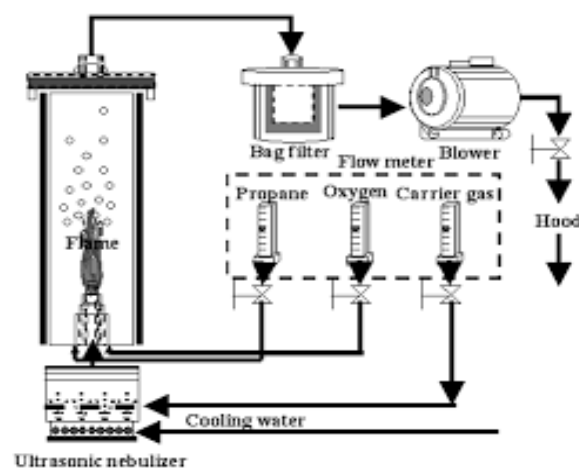


Figure 2.6: The schematic diagram of a flame spray pyrolysis process (Cho and Kang 2008)

Figure 2.6 shows the illustration of a flame spray pyrolysis process. Previous study have found that high temperature flame spray pyrolysis produce nanophase HA powders with high crystallinity. With polyethylene glycol (PEG) as spray solution, it was found that the Ca/P ratio produced was 1.69. The concentration of PEG solution and the temperature of post-treatment influence the size of HA powder formed. It was mentioned that spray pyrolysis method able to control the composition of multi-oxide powders and their morphology (Cho and Kang 2008).

2.3.6 Calcination and Heat Treatment Method

Synthesizing HA by heat treatment at 600°C was found to produce B type HA. On the other hand, heating at 950°C forms non-resorbable HA with biphasic materials known as β -TCP, which is resorbable. It was found that this method produced higher Ca/P ratio compared to the stoichiometric HA. This is due to the presence of B type carbonate HA, where the phosphate ions was substituted by the carbonate ions. Other than that, decrease in intensity of CO_3^{2-} after calcination at 950°C was due to the high temperature used. Therefore, it was adviced that calcination at lower temperature is more appropriate to conserve the carbonate content in HA. Besides that, it was also found that calcination at temperature of 900°C produce HA composed of crystalline phase with crystallite size is almost near to the stoichiometric HA. While calcination at higher temperature results in increase of the crystallite size. (Boutinguiza, Pou et al. 2012)

Calcination of fishbone at different time duration results in different properties of HA. It was found that, calcination at 900°C for 8 to 10 hours can reduce the particle size, therefore increasing the surface area. However, calcination for longer than 8 hours results in high absorption of water. This can be explained by a sharp peak in the FTIR

results which is either H₂O or free OH molecules. Therefore, 8 hours of calcination time was found to be enough for structure stabilization. (Coelho, Nogueira et al. 2006)

It was mentioned that the microcrystals of HA in natural bones are very small in which the apparent fibrils manage to form a long fiber. It was found that the fibrils partially remains even after calcination. This shows that sintering between the microcrystallite occur through the burning of collagen matrix. Thus, the /resultant fiber formed is highly crystalline. (Akram, Ahmed et al. 2014). This was supported by (Santos, Oliveira et al. 2004) who mentions that low crystallinity can be reduced by heat treatment.

Previous research have chose to perform calcination at temperature ranged between 800°C to 1000°C. This was due to the calcination at this range led to the formation of pure HA. Besides that, calcination at high temperature results in observable colour changes in the HA powder. Calcination at 800°C to 900°C results in white colour of powders. At temperature above 900°C, the powder turns to a soft blue colour (Mustafa, Ibrahim et al. 2014).

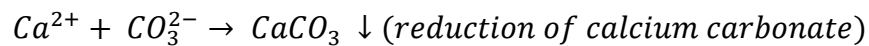
Table 2.4: Description of calcination data. (Mustafa, Ibrahim et al. 2014)

List of Samples	Calcination Temperature (°C)	Calcination Period (h)	Initial Weight (g)	After Calcination (g)	Residue (%)	Colour Descriptions
Raw fish powder	-	-	-	-	-	Yellow
1	800	5	2.100	1.230	58.571	White
2	900	5	2.016	1.170	58.036	White
3	1000	5	2.002	1.176	58.741	Soft blue

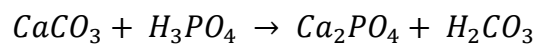
2.3.7 Reflux

The reflux method to produce HA involve KOH and H₃PO₄ solutions. Reflux method begins with deproteinization stage at 130°C for 18 hours in which KOH solution was used in the as a strong base solution. Therefore, the calcium and protein from the fishbone can be hydrolysed. After that, refluxing process was done at 130°C for 12 hours by using H₃PO₄ as weak acid due to the source of PO₄³⁻ as an ion donor to synthesise HA. Then, calcination was done at 900°C for 8 hours. It was found that the sample in this method were contaminated by excess H₃PO₄, where the colour changes from white to grey. From this method, it was found that no CaCO₃ elements exist in the XRD analysis, and thus it was an effective route to synthesise HA. (Cahyanto, Kosasih et al. 2017)

Deproteinization stage;



Reflux stage;



The byproducts were H₂O and CO₃ ↑

2.3.8 Other methods

HA can also be synthesised by microwave processing. This process is a fast route and requires suitable starting materials to control a certain conditions such as the pH as this method does not require the addition of ammonium hydroxide. In this method, 0.5 M calcium hydroxide and 0.4 M di-ammonium hydrogen phosphate was used. They were stirred at high speed at a controlled pH 11 and was immediately subjected to microwave irradiation. It was found that the mixing time, HA

crystallization and its aging occurs in a short period. Than that, the HA produced was nanocrystalline where the estimated crystal size were 28 nm and 56 nm. The varying irradiation time was found to produce medium and high crystallinity of HA. (Rameshbabu, Rao et al. 2005)

Based on an observation under Scanning Electron Microscopy (SEM), the microstructure of raw powder particle is spherical shaped with smaller size compared to after calcination process. X-ray Diffraction (XRD) shows that the peak of intensity increased with the increment of calcination temperature (due to the removal of organic portion and substances) (Coelho, Nogueira et al. 2006).

Although there are various modern technologies can be used to derive HA, it was found that the most desirable method to obtain HA is by calcination method. This is because obtaining HA by calcination requires low cost and uncomplicated method. Previously, it was stated that calcination can be done at different temperature from 800°C to 1000°C. It showed that 900°C is the most effective temperature to be used for this process. Thus, it was found that this temperature able to gain Ca/P ratio closest to 1.67 which indicate the most stable phase of HA. In addition to that, calcination at 800°C was undesirable due to the impurities and other organic elements that is not diminished at this stage. As mentioned before, by increasing the calcination temperature can reduce the particle size, which led to an improvement in the crystalline structure. Calcination at 6 hours showed that the undesirable elements presents in HA, while above 8 hours results in high water absorption (Mustafa, Ibrahim et al. 2014).

2.4 Methods of Nano-size Hydroxyapatite (nHA) Synthesis

In the last few years, science has been redirected towards other technologies, especially in nanoscale. Nanoscale material is very important in the development of

advanced materials, the controlled synthesis of macromolecules and the productions of more efficient and safer medicines. The grain sizes are very small, which gives them a very large surface/volume ratio, making them more effective in reaction processes compared to larger particles (Coelho, Nogueira et al. 2006).

Science and research has been redirecting towards the nanoscale technologies. Basically, the term 'nano' refers to a measure of 10^{-9} U (one rack unit). In the concept of nanosize for materials, the unit refers to dimensions rather than measurement. Technically, nanoscale can be defined as having an order of 100nm or less. Nanotechnology can provide various benefits in the research sectors such as making materials to be stronger, lighter or more reactive. It can be said to improve and revolutionize many sectors in industry including military, food safety, environmental science, medicine, cosmetics and homeland security. Materials at nanoscale possess a very small dimension, having a large surface-to-volume ratio.

For example, cosmetics manufacturers commonly use nanoscale zinc oxide and titanium dioxide for the base creams and sunscreen lotions. Some paints contain nanoscale titanium dioxide to have reflective properties. Plus, in order to increase durability, some marine ships use nanostructured cement carbide as coatings. Thus, it can be said that nanomaterials and its properties will continue to improve its performance. Materials at nanoscale behave chemically and physically different because there is an increase in surface area as the particle size gets smaller.

Bioactive HA is a material that can stimulate beneficial responses from the body such as bonding to the host tissue. It enables the interaction with the surrounding biological tissues and thus promoting faster bone formation. The biological and chemical properties of HA can be related to their properties in nanoscale dimensions.