MINISTRY OF EDUCATION AND TRAINING
NHA TRANG UNIVERSITY

PHAM VIET NAM

RECOVERY OF PROTEIN HYDROLYSATE AND HYDROXYAPATITE FROM TRAFISH BY-PRODUCTS AND THEIR ORIENTED APPLICATION FOR PACIFIC WHITE SHRIMP FEED

Major: Aquatic Product Processing Technology

Code: 9540105

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Asoc. Prof. Dr. TRANG SY TRUNG
Asoc. Prof. Dr. NGUYEN VAN HOA

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NOVELTIES OF THE THESIS

Title: Recovery of protein hydrolysate and hydroxyapatite from catfish by-products and their oriented application for Pacific white shrimp feed.

Major: Seafood Processing Technology. **Code:** 9540105 **Course:** 2015 - 2019

Student: Pham Viet Nam

Supervisors: 1. Assoc. Dr. Trang Si Trung
2. Assoc. Dr. Nguyen Van Hoa

Institution: Nha Trang University

The novelties of the thesis:

1. The data on the chemical composition of catfish by-products (*Pangasius hypophthalmus*) collected at Nam Viet Seafood Processing Company, An Giang province was reported, which can be used in further studies.

2. A process was presented to recover three value-added products, including fish protein hydrolysate (FPH), hydroxyapatite (HA), and crude lipid from catfish by-products. Using alcalase, the FPH had a DH of 35%, with > 70% M_W of < 1000 Da, a nitrogen total of 11.7%, a lipid total of 10.8%. Using both alcalase and lipase, the FPH had a nitrogen total of 33.2%, a lipid total of 1.93%, and an acid amine total of 420.16 mg/g protein. The best conditions for the preparation of HA from the fishbone were reported. The prepared HA had a size of 50 – 70 nm, a Ca/P ratio of 1.83, a surface area of 2.87 m^2/g, a pore volume of 0.02 m^3/g, a pore size of 1.2 nm, and an absence of heavy metals. This is a novel approach to obtain maximum components from catfish by-products and towards a "zero-waste" process.

3. For practical application, the mixture of FPH and HA was added to the feed of the white-leg shrimp at 20-55 days. The best supplement of 5% showed that the shrimp gained a weight of 123.5% and a length of 112% compared to the control sample. The supplement of a mixture of FPH and HA from by-product Trafish in feed did not alter the water quality for shrimp culture.

On behalf of Supervisors — Student

Assoc. Dr. Trang Si Trung — Pham Viet Nam

PREFACE

1. The necessary

Catfish (*Pangasius Hypophthalmus*) is one of the leading seafood products of Vietnam. The total catfish farming area of Vietnam in 2019 was 7,127 hectares. The harvested catfish output reaches 1,519,000 tons. The total catfish export value of Vietnam in 2019 was about 2 billion USD, which contributed 23.26% to the export value of the Fisheries sector. Most of the catfish products were exported as frozen fillets. However, the fillets' volume only accounts for about 40%, so about 60% is fish by-products, including head, fins, skeleton, organs, skin, and trimmings. This is a considerable material, and if there is no proper solution, it will cause a waste of resources and environmental pollution.

In Vietnam, fish by-products are currently mainly used to produce fishmeal with simple technologies, including steaming, drying, and grinding. The obtained products are often used as animal feed of both low quality and economic value. Meanwhile, fish by-products contain many components with high nutritional and economic value, such as protein, minerals, lipid,... These compounds can be used to produce value-added products for feed, even used as food for humans. Therefore, it is necessary to recover value-added compounds from catfish by-products.

Recently, there have been many studies on recovering bioactive compounds from fish by-products by different methods. However, most of these reports often use only a portion of fish waste to obtain value-added products. Some authors focused on getting FPH and lipid from the muscle of fish by-products, neglecting the mineral part (mainly HA) from the fishbone. In contrast, some authors only obtain HA from fish bones, ignoring the protein and lipid parts in fish by-products. Therefore, these methods caused both the waste of resources and potentially polluting the environment or requiring costs of waste treatment.

In Vietnam, a large number of catfish by-products are discharged from the frozen catfish fillet processing lines at the factories in some provinces in the Mekong Delta, for example, Nam Viet, Vinh Hoan, Hung Ca, ... These by-products are mainly used as

raw materials for fishmeal factories. They are used as an animal feed with low economic value and low quality. Therefore, suitable procedures need to develop for the recovery of high-value products from all the remaining by-products and at the same time. Also, the applications of obtained products in the agricultural sector in Vietnam are essential to investigate.

We proposed "Recovery of protein hydrolysate and hydroxyapatite from catfish by-products and their oriented application for Pacific white shrimp feed" from the above considerations. The study aims to simultaneously receive various value-added products such as fish protein hydrolysate (FPH), hydroxyapatite, lipids from catfish by-products. Also, it will investigate to use of a mixture of FPH solution and HA to white-leg shrimp feed. These results will improve Vietnamese catfish products' economic value, reduce the risk of environmental pollution, and provide more nutrition sources for shrimp feed.

2. Objectives

- To develop a process to recover value-added products, especially in FPH and HA, from catfish by-products.
- To investigate the use of an FPH and HA mixture for shrimp feed at the period of 20-55 days old.

3. Aims and contents

The thesis focuses on research, evaluation, and clarification of the following contents:

- To determine chemical composition, weight composition of catfish by-products.
- To determine the appropriate parameters for the hydrolysis of catfish by-products to obtain the highest DH and NR in 2 steps. Step 1: characteristics of by-products, the enzyme-substrate ratio, temperature, reaction time, and NaCl concentration. Stage 2: weight lipase and reaction time.
- To determine the appropriate parameters for recovering the nano-hydroxyapatite from fish bones, including raw material pretreatment, temperature, heating time, and heating rate.
- To determine the appropriate mixture of FPH and HA for the shrimp feed from 20 to 55 days old to evaluate shrimp's growth, the impact of the farming environment compared to the control sample.

4. Materials

Catfish by-products (*Pangasius hypophthalmus*) were collected from catfish size of 1.2 – 1.5 kg/fish at Nam Viet Seafood Processing Company, An Giang Province.

5. Scientific and practical achievement, the novelty of the thesis

- The data on the chemical composition of catfish by-products *(Pangasius hypophthalmus)* collected at Nam Viet Seafood Processing Company, An Giang province was reported, which can be used in further studies.
- A process was presented to recover three value-added products, including fish protein hydrolysate (FPH), hydroxyapatite (HA), and crude lipid from catfish by-products. This is a novel approach to obtain maximum components from catfish by-products and towards a "zero-waste" process.
- The best conditions for the recovery of the FPH from catfish by-products using alcalase and lipase were presented. Using alcalase, the FPH had a DH of 35%, with > 70% MW of < 1000 Da, a nitrogen total of 11.7%, a lipid total of 10.8%. Using both alcalase and lipase, the FPH had a nitrogen total of 33.2%, a lipid total of 0.35%, and an acid amine total of 420.16 mg/g protein.
- The best conditions for the preparation of HA from the fishbone were reported. The prepared HA had a size of 50 – 70 nm, a Ca/P ratio of 1.83, a surface area of 2.87 m^2/g, a pore volume of 0.02 m^3/g, a pore size of 1.2 nm, and an absence of heavy metals. A comparison of prepared HA and HAs from other fishbone sources was also reported.
- The chemical composition, Mw, acid amine, and fatty acid profile, reduction capacity of the obtained FPH were measured.
- For practical application, the mixture of FPH and HA was added to the feed of the white-leg shrimp at 20-55 days. The best supplement of 5% showed that the shrimp gained a weight of 123.5% and a length of 112% compared to the control sample. The supplement did not form any side effects on the water quality, indicating this mixture's potential application for the aquaculture feed.

6. Structure of the dissertation

The thesis includes 227 pages with 130 content pages, 165 references, and 59 appendices. The content is presented in 3 chapters with 31 tables and 55 pictures, graphs, diagrams, and processes.

CHAPTER 1. INTRODUCTION

1.1. Catfish by-products

The frozen catfish fillet processing industry discharges a large amount of fish head, bones, fat, viscera, and skin. Fish waste (head, skin, bones, fins, organs, blood, fat, fish trimmings) accounts for about 60% of raw fish. According to estimates, if raw catfish output reaches one million tons per year, more than 600,000 tons of by-products will be formed. Fish by-products contain many valuable components, such as protein, lipid, minerals, enzymes, and bioactive compounds. By using these by-products, many useful products such as fish meal, fish oil, collagen, gelatin, protein hydrolysate, calcium powder, enzymes, biodiesel, and HA can be produced. Utilizing fish by-products not only contributes to limit environmental pollution but also improves the use-value of the by-products, increasing economic efficiency for seafood processing enterprises and bring significant economic benefits to the country.

1.2. Protein hydrolysate

Protein hydrolysate a product of protein hydrolysis. The main components of FPH are amino acids, peptides with different chain lengths. In addition, the FPH solution also contains a small amount of minerals and lipids. FPH solution can be recovered from catfish by-products using various methods such as chemical method, autohydrolysis method, an enzymatic method.

In the chemical method, different agents, including acids, strong alkalis or its salts (HCl, NaOH, $Na_6(PO_3)_6$, ...), organic acids (formic acid, lactic acid, ...) are used. The resulting product is primarily used as animal feed or as fertilizer. This method is easy to operate with a high recovery efficiency, fast response time, and low production cost. However, the use of chemical agents often destroys biological compounds, affects the odor of the product, and may not be safe for human use. Furthermore, the production process generates a large amount of chemical waste, which increases treatment costs and can pollute the environment.

The method of hydrolysis of fish waste using internal enzymes is also widely studied. The fish viscera contains a large number of beneficial microorganisms. At a favorable temperature and pH, the

organisms will produce the protease enzyme system to conduct protein-cleavage hydrolysis to create protein hydrolyzed products. This hydrolysis process has the advantages of simplicity, ease of implementation, low production cost. However, the hydrolysis speed is slow, with prolonged reaction time, low yield, and unstable product.

The method of adding enzymes from outside to speed hydrolysis, shortening reaction time, and increasing the yield is widely applied in many fields. The protease system is often used. Studies often use one enzyme or a mixture of enzymes such as alcalase, flavourzym, and protamex to determine the optimal conditions to produce hydrolysate from some fish by-products. In Vietnam, there are currently no systematic studies on the production and evaluation of hydrolyzate for application.

1.3. Hydroxyapatite (HA - $Ca_{10}(PO_4)_6(OH)_2$)

Canxi hydroxyapatite (also known as hydroxyapatite, HA) has the molecular formula of $Ca_5(PO_4)_3(OH)$ or $Ca_{10}(PO_4)_6(OH)_2$. HA is the main component of bones (accounting for 65-70% of the mass) and teeth (accounting for 99%). HA is widely studied due to its high activity and biocompatibility with cells and tissues, forming a direct bond with young bones, leading to rapid bone regeneration without elimination. On the other hand, HA is the most easily absorbed form of calcium phosphate for the human body with a Ca / P ratio of 1.67, like the human bones and teeth ratio. HA is white, ivory-white, light yellow, or cyan, depending on its reaction conditions, particle size, and molecular structure. The basic lattice structure of HA crystal includes Ca^{2+}, PO_4^{3-} và OH^-.

Over the past several decades, HA has been of much interest due to its excellent biological compatibility with animal cells, good interactions with bio-polymers, and compatibility with bones. Therefore, HA has been studied as materials for various biomedical applications such as bone replacement and tooth defects, dentures, bone implants, tissue engineering, drug delivery agents, osteoporosis agents, dental materials, and bioactive coating on metal bone implants. Besides, HA is increasingly being used in many other industries, such as water treatment materials contaminated with heavy metals, dyes.

Many methods have been developed for preparing HA. In general, it can be classified into two groups: (i) chemical methods; HA synthesized from inorganic salts containing Ca^{2+} and PO_4^{3-} ions, (ii) extraction methods; HA obtained from natural sources. In

chemical processes, HA was synthesized easily with the desired size and shape, a Ca/P ratio of 1.67, similar to in human bones. However, this method is very complicated, prolonged reaction time, high cost, low activity of the products. Recently, many studies reported obtaining HA from natural sources, especially from fish bones. In which raw fish bones were calcined at a high temperature of 600 - 1200^{o}C to get HA. This method is quite effective and inexpensive. The prepared HA has a nanometer size so that it can be applied in many different fields.

1.4. Feed for postlarvae shrimp

Shrimp pellets are imported into Vietnam annually at about 200,000 - 250,000 tons. Besides, a large amount of fishmeal for shrimp feed production is also imported. According to statistics, the amount of fishmeal imported from Vietnam in September 2019 reached 8.5 thousand tons ($11 million). Generally, in the first nine months of 2019, the amount of fishmeal imported was 122.1 thousand tons ($ 157.9 million).

White-leg shrimp *(Vannamei)* is an omnivore. Currently, there are three main types of food for *Vannamei* shrimp: commercial feed, natural food (phytoplankton, organic residue), homemade food (snails, trash fish, by-products from agriculture).

Postlarvae shrimp are entirely fed with commercial food, ensuring the pond's nutritional quantity and water environment. Besides, it is necessary to supplement minerals, enzymes, vitamins C, E, and squid oil. Ingredients and dietary supplements to shrimp feed include: proteins, lipids, minerals, carbohydrates, vitamins . When providing adequate nutrition, shrimp will grow fast, shrimp farming efficiency is high.

Chapter 2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Catfish by-products

Catfish by-products (*Pangasius hypophthalmus*) were collected from the fish size of 1.2 - 1.5 kg on the frozen fillet processing line of Nam Viet Seafood Processing Company, An Giang province. Fresh fish by-products (head, organs, fins, bones, skin, tail) were packed in PE bag and frozen in foam tank, transported to the laboratory. The sample is minced by a screw grinder. The size of the floor hole is 1cm, mixed and homogeneous. Each sample of 100g was put into a PE bag, tie the top, and freeze at -18^{o}C before using for the experiment.

2.1.2. Enzymes

Alcalase was purchased from Novozyme Company (Denmark). Alcalase is an endo-peptidase derived from bacteria *Bacillus licheniformis* has an activity of 2.4 AU/g, and suitable operating conditions are 55 – 70^{o}C, pH 6,5 – 8,5. Lipase was purchased from Sigma-Aldrich Company (Germany). This enzyme is extracted from pig pancreas, with an activity of 100 - 400 IU/mg, suitable operating conditions are 55 – 60^{o}C, pH 3,8 – 5,5.

2.1.3. White-leg shrimp

White-leg shrimp (12 days old, post 12) were selected at Fisheries Breeding Company, Ninh Hoa district, Khanh Hoa province. Shrimps were checked for white spot disease, EMS/AHPND disease, necrosis disease of hematopoietic organs and epithelial organ. Shrimps were raised and cared for at the Center for Breeding and Disease Research, Nha Trang University, for eight days, reaching a length of 39.45 ± 0.42 mm, weight of 0.29 ± 0.02g. The shrimps have bright colors, thin shells, well-proportioned head and tail. All shrimps are healthy, evenly distributed in the tank. The shrimp gut is full of food.

2.1.4. Chemicals

All chemicals are of pure analytical grade, purchased from Mecrk Company (Germany). The chemicals are stored in proper conditions before use.

2.2. Methods

A diagram of the research method is shown in Figure 2.1

- *Pretreatment of raw materials:* To select the hydrolysis conditions, fish by-products (head, skeleton, internal organs, skin, fins, ...) were analyzed in terms of weight and chemical composition. Based on the results, the proper pretreatment of fish waste with and without lipid separation will be chosen, such as (i) cooking and pressing, (ii) without lipid separation.

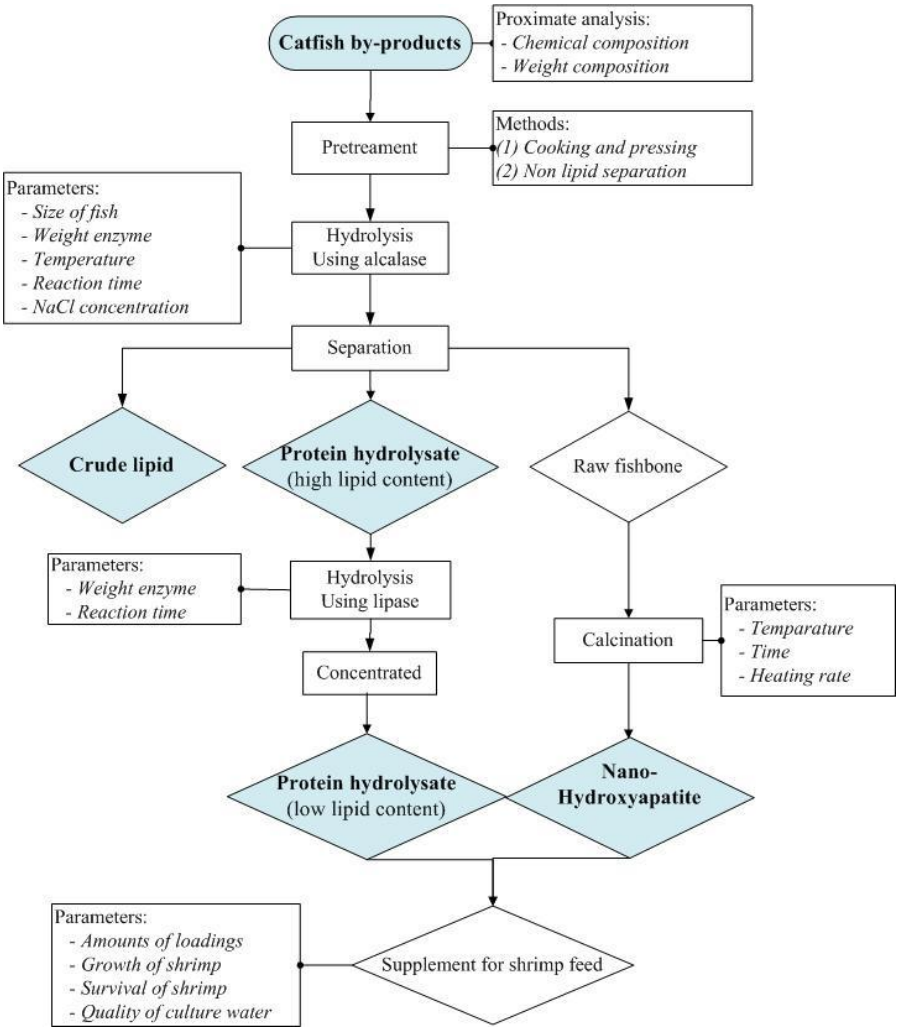

Figure 2.1. Diagram of the research method.

- *Recovery of FPH from the fish by-products using alcalase*. To determine the most suitable hydrolysis conditions, investigating the effects of parameters: material size, enzyme–substrate ratio, temperature, reaction time, and NaCl concentration. After the hydrolysis is, the mixture is filtered into two parts, including: (i) a solid part containing mainly fish bones, used as raw materials for HA preparation; (ii) a liquid fraction consisting of soluble proteins, insoluble proteins (viscous), and crude lipids.

- *Reduction of the lipid content in FPH using lipase:* To continue hydrolysis of liquid fraction obtained from the last fraction by using lipase. FPH with low lipid content will be used as a supplement in feed for white-leg shrimp in the period of 20 - 55 days old. At this stage, the study parameters include weight enzyme and reaction time.

- *Preparation of nano HA from catfish bones:* Raw fish bones, which were collected after the hydrolysis, were washed three times with distillation water and dried to constant weight. Then, samples were heated at different times, temperatures, and heating rates to obtain nano HA.

- *Application of FPH and HA mixture:* To investigate FPH and nano HA mixture's potential application for white-leg shrimp feed at 20 - 55 days old.

This approach aims to maximize the recovery of valuable compounds from the catfish by-products. Also, it used enzymatic agents that have safe and environmentally friendly properties. However, the thesis's scope does not focus on the purification of lipid and insoluble protein products, but only at the step of collecting these two products in raw form.

2.3. Characterization

2.3.1. Proximate analysis of the fish by-products

The crude protein content is determined by the Kjeldahl method. The ash and moisture content were determined by the standard AOAC method. The crude lipid content was analyzed by the Folch method.

2.3.2. Proximate analysis of obtained products

FPH and crude lipid: DH is determined by the DNFB method. The following formula determines NR: NR (%) = (Total nitrogen in FPH obtained from 100g of the by-products (g)) / (Total nitrogen 100 g of the by-product (g)) × 100. The amino acid profile is determined by high-

performance liquid chromatography (HPLC). Molecular weight is measured by gel permeable chromatography (GPC).

Hydroxyapatite: Metal content is determined by ICP-MS. Metal oxide content is measured by XRF spectroscopy. The elemental composition of HA is analyzed by XPS. The surface area, the crystallinity of HA is measured by BET and XRD, respectively. Chemical structure information was determined by FTIR spectroscopy. The morphology of HA was examined by SEM and TEM.

2.3.3. The investigated criteria during the experiment of the shrimp culturing

The environmental conditions: In detail, the temperature and pH were measured twice a day at 7:00 am and 3:00 pm, with a thermometer and a pH meter (HI98127 – Hanna, USA); alkalinity, nitrite, DO were measured every three days. Alkalinity was analyzed by acidic titration method, DO was analyzed by the Indophenol Blue method. Nitrite was determined by the SERA test kit.

Microbiological criteria: The density of total bacteria (TPC) and Vibrio bacteria was determined one time a week. The density of total bacteria was determined by the dilution and counting on Nutrient agar consisting of 1.5% NaCl (NA) [16]. Similarly, the density of total Vibrio bacteria was determined by dilution and counting on TCBS agar plates (Thiosulfate Citrate Bile Salt Sucrose). Typically, the initial water sample (concentration 100) was diluted with 0.85% brine to 3 different concentrations: 10^{-1}, 10^{-2}, 10^{-3}. Then, 100 µl from each dilution of the water sample was spread into the NA or TCBS medium dishes, repeating two times for each concentration. Samples were incubated at 24°C for 24 h, and bacterial density was calculated using the below equation: Bacterial density (CFU/mL) = Number of colonies × dilution × 10.

Quality monitor of postlarval shrimp: The quality monitor of postlarval shrimp was conducted by sampling 20 postlarval shrimps randomly and measuring total length in PL-12 using a microscope with an eyepiece microscope. Furthermore, the entire size and weight of postlarval shrimp in stages PL-20, PL-27, PL-34, PL-41, PL-48, and PL-55 were also measured using a ruler with an accuracy of 0.1 mm and a small balance with an accuracy of 1 mg. The survival rate was recorded at day 55 (PL-55). Survival rate (%) was calculated as the percentage of shrimp surviving to PL-55 in each tank divided by a number of shrimps at the start of the experiment. The yield was

calculated from the mean harvest weight of shrimp from each tank multiplied by the harvest density for that tank.

2.4. Statistical analysis

All statistical analyses were performed using Origin Version 8.5.2 statistical software (Origin Lab Corporation, Korea). Means were compared by one-way ANOVAs and Tukey's Multiple Comparisons. The statistical difference was defined at $p < 0.05$.

Chapter 3. RESULTS AND DISCUSSIONS

3.1. Chemical composition of catfish by-products

The chemical composition of catfish by-products was analyzed and shown in Table 3.1.

Table 3.1. Chemical composition of catfish by-products

Parameters	Content (%)	
Weight composition		
Head	45.25 ± 0.45	
Organs	21.30 ± 0.23	
Bones	28.95 ± 0.28	
Fins, tail	4.5 ± 0.15	
Chemical composition	**Wet basis**	**Dried basis**
Protein	14.66 ± 0.75	42.62± 0.75
Lipit	14.60 ± 0.63	41.85± 0.63
Moisture	65.11 ± 0.68	-
Ash	5.63 ± 0.15	15.53 ± 0.15

As can be seen in Table 3.1, the weight composition of the by-products contains mainly the head (45.25%) and bones (28.95%), organs (21.30%), and fins, tail (4.5%). The catfish by-products contain three main parts, including protein (42.62%), lipid (41.85%), and minerals (15.53%). These ingredients can be used to produce value-added products. However, the high lipid content (41.85%) can affect the selection of hydrolysis methods, reaction parameters, and agents.

3.2. Separation of lipid

The lipid extraction efficiency before hydrolysis is presented in Table 3.2. Accordingly, when the ingredients are cooked and pressed, a relative amount of lipid is separated (61.92%). In general, by using the above method, the remaining lipid content in the raw material is still relatively high (5.56%). The remaining protein content in the material is 10.48%.

Table 3.2. Effects of lipid extraction methods

Method	Recovered lipid (%)	Lipid remain (%)	Protein remain (%)
Cooking – Pressing	61.92 ± 0.85	5.56 ± 0.48	10.48 ± 0.53
Non-separation	0	14.60 ± 0.63	14.66 ± 0.75

3.3. The hydrolysis of catfish by-products by alcalase

3.3.1. Effects of the fish size

At the same hydrolysis conditions: the enzyme–substrate ratio 0.6%, temperature 50°C, natural pH, the ratio of water/material 1:1, NaCl concentration 1.5%, reaction time 4 h. From Table 3.3, the by-product from small fish (500 - 900 g/head) has a negligible effect on DH and NR. For the bigger fish (900-2000 g/cow), both DH and NR were decreased. This is explained that the by-products from the bigger size of fish contain a high lipid content, which will inhibit the hydrolysis process.

Table 3.3. Effect of fish size on the DH and NR of the FPH

Size of catfish (g/fish)	Moisture (%)	Protein (%)	DH (%)	NR (%)
500 – 700	68.30 ± 0.85	13.60 ± 0.60	37.85^{a} ± 0.98	81.55^{a} ± 1.12
700 – 900	68.10 ± 0.75	13.68 ± 0.55	35.63^{b} ± 1.05	80.78^{ab} ± 0.85
900 – 1100	67.40 ± 0.70	14.25 ± 0.72	33.75^{c} ± 0.95	$80,42^{ab}$ ± 1.02
1100 – 1500	66.50 ± 0.75	14.70 ± 0.80	31.92^{d} ± 1.08	79.38^{cb} ± 1.05
1500 – 2000	65.30 ± 0.58	14.20 ± 0.65	28.98^{e} ± 1.13	78.45^{db} ± 1.25
Mix	65.50 ± 0.65	14.50 ± 0.85	31.33^{d} ± 0.87	79.22^{cb} ± 1.05

3.3.2. Effect of the enzyme–substrate ratio on the hydrolysis

The effect of the enzyme–substrate ratio on the hydrolysis process is shown in Figure 3.1a.

According to Figure 3.1a, when no external enzymes were added, DH reached 6.48% after 3 h. The DH increased significantly up to 20.58% when alcalase was added at enzyme–substrate ratio of 0.1% (v/w). The DH was slowly increased from 23.98 to 30.69% in the ratio range of 0.2–0.4% (v/w) and reached the highest value of 30.69% at a ratio of 0.4%. Afterwards, it was nearly unchanged or slightly reduced with the enzyme–substrate ratio over 0.4% (v/w). Meanwhile, NR was increased significantly when the enzyme–substrate ratio increased from 0 to 0.6% (v/w). Notably, the

NR value was increased rapidly from 37.22% to 78.98% in the range of 0.1 to 0.2% (v/w). As the enzyme–substrate ratio increased from 0.3 to 0.4% (v/w), the NR increased slightly from 81.16% to the highest value of 82.24%. After that, there was a slight decrease in the range of 0.5–0.6% (v/w).

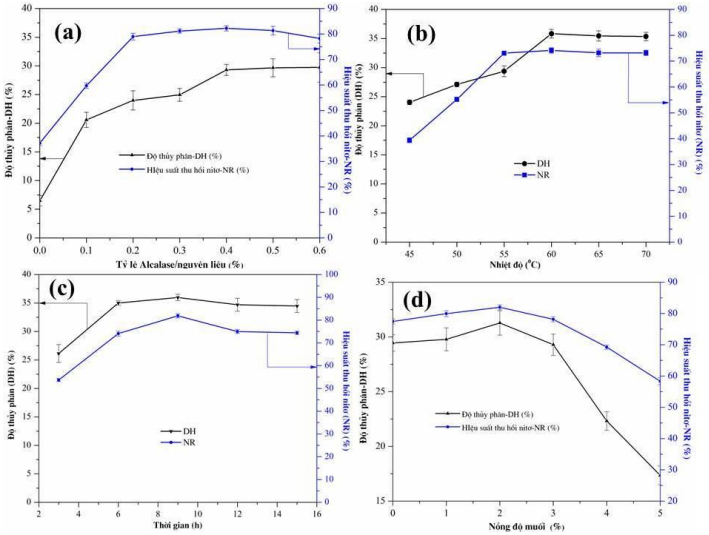

Figure 3.1. Effect of (a) enzyme rate, (b) temperature, (c) time, (d) NaCl concentration on DH and NR of the FPH.

Thus, the enzyme–substrate ratio of 0.4% is the most suitable for hydrolysis to obtain the FPH with the highest DH and NR values.

3.3.3. Effect of the reaction temperature on the hydrolysis

Figure 3.1b shows that the hydrolysis temperature was increased from 45 - 60^{o}C; the DH value was increased from 24.04% to 35.83%. Then, the reaction occurred at 70^{o}C; the DH was decreased slightly to 34.67%. Similarly, when the hydrolysis temperature increases from 45 - 70^{o}C, the NR value increases rapidly from 39.39% to 74.15% and decreases slightly to 73.19%.

Thus, the temperature of 60^{o}C is the most suitable for the hydrolysis of the by-products to obtain the FPH with the highest DH and NR values.

3.3.4. Effect of the reaction time on the hydrolysis

Figure 3.1c shows that both DH and NR values were increased from 1 to 9 h and kept stable or decreased slightly after nine hours.

The DH was increased quickly from 3 h to 6 h, and reached the highest value of 35.97% at 9 h. The NR was increased from 53.71% (3 h) to the maximum value of 81.88% (9 h). However, the NR was decreased slightly to 74.98% (12 h) and 74.41% (15 h).

Thus, the reaction time of 9 h is most appropriate to reach the highest values of DH and NR.

3.3.5. Effect of the NaCl concentration on the hydrolysis

From Figure 3.1d, when the salt concentration was increased from 0 - 5%, both DH and NR values were increased slightly from 29.45% to 31.28% and from 77.45 to 81.98%, respectively. After that, both DH and NR were gradually decreased to 17.35% and 58.34%, respectively. At the salt concentration of 0 - 2%, both DH and NR values increase rapidly and reach the highest value of 31.28% and 81.98%. However, at the salt concentration of 3%, both DH and NR values were decreased slightly. The difference was not statistically significant ($p > 0.05$). At the concentration of 4-5%, the DH and NR were failed very quickly.

Therefore, the optimum salt concentration in hydrolysis is 2%.

3.4. The hydrolysis of the FPH by lipase

3.4.1. Effect of the weight enzyme on the hydrolysis

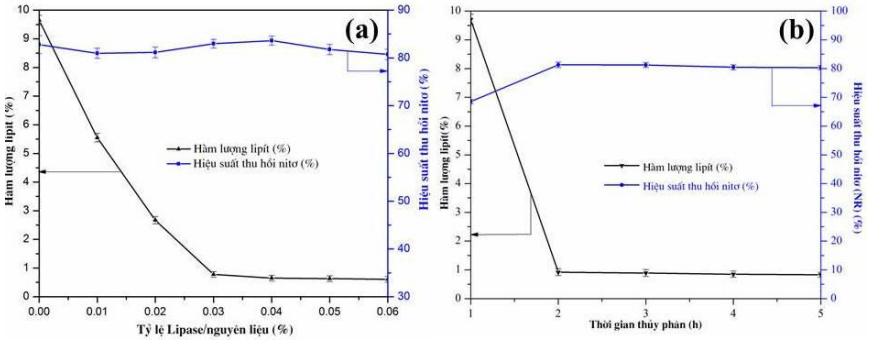

Figure 3.2. Effect of (a) weight enzyme, (b) hydrolysis time on lipid and NR content.

The effect of weight enzyme on the hydrolysis of the FPH obtained from stage 1 is shown in Figure 3.2a. Accordingly, when the of weight enzyme was 0 – 0.06%, the lipolysis in protein solution occurs strongly; lipids were hydrolyzed to form free fatty acids, the lipid content in the final solution was dropped quickly from 9.65% to

0.78%. When the of weight enzyme was 0.03 – 0.06%, the lipid content in the solution is not reduced significantly. There is no statistically significant difference (p> 0.05). Meanwhile, the NR value was increased slightly from 80.96% to 82.97%, corresponding to the enzyme rate from 0.02% to 0.03%, respectively. At the of weight enzyme was 0.03 - 0.06%, the NR was 83.62% and 80.73%, respectively. Therefore, the optimal of weight enzyme loading is 0.03%. The lipid content of final FPH is low (0.38% wt.), which is suitable for supplementary feed for postlarvae.

3.4.2. Effect of the reaction time on the hydrolysis

The effect of the hydrolysis time using lipase on the protein hydrolysis from stage 1 is shown in Figure 3.2b. The optimal hydrolysis time for pangasius hydrolysis phase 2 is 2 hours. After hydrolysis at stage 2 by lipase enzyme, protein hydrolysate had a low lipid content (0.83%), which is suitable for supplementary feed for postlarvae.

3.5. Chemical composition of the obtained hydrolysate (FPH)

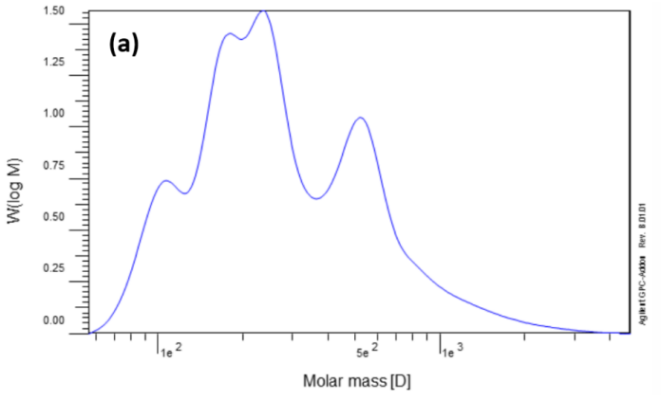

Figure 3.3. The molecular weights distribution of the FPH

Figure 3.3 presents the molecular mass distribution (M_w) of the FPH peptides. The results show that the percentage of peptides is 10% (<71.6 Da), 20% (71.6 – 171 Da), 20% (171 – 247 Da), 20% (246 – 403 Da), 20% (403 – 676 Da), 10% (> 676 Da).

Most peptides have an Mw of less than 1000 Da. The Amino acid compositions of FPH prepared are shown in Table 3.5. The FPH contained eight essential amino acids as well as 12 non-essential amino acids. The total amino acid content was 420.16 mg/g of

protein, in which the total essential amino acids (TEAA) was 19.37% w/w, and the total hydrophobic amino acids were 33.56% w/w. Some primary amino acids of the FPH include ornithine (46.11 mg/g of protein), aspartic (42.85 mg/g of protein), glycine (40.92 mg/g of protein), alanine (39.88 mg/g of protein), and glutamic (38.40 mg/g of protein). This FPH with rich in amino acids can be used for the production of consciousness shrimp feeding.

Table 3.4. The properties of the protein hydrolysate from the catfish by-product

Parameters	Content/characteristic
Protein content (g/l)	33.20 ± 2.15
Lipid content (%)	1.93 ± 0.05
Dried material content (%)	40.6 ± 2.32
Acid amine content (mg/g)	420.16
Color	Dark brown
Odor	Natural aroma

Table 3.5. Amino acid composition of the protein hydrolysate from the catfish by-product

Amino acids	Content (mg/g)	Amino acids	Content (mg/g)
Arginine	30.24	Methionine	14.38
Serine	5.04	Valine	12.45
Aspartic	42.85	Phenylalanine	3.56
Glutamic	38.40	Cysteine/Cystine	10.97
Hydroxylproline	26.54	Isoleucine	3.56
Glycine	40.92	Tyrosine	36.17
Threonine	12.45	Leucine	7.41
Alanine	39.88	Ornithine	46.11
Aminobutyric acid	2.82	Lysine	15.57
Proline	18.83	Histidine	12.01
Total essential amino acids			**81.39**
Total non-essential amino acids			**338.77**
Total amino acids			**420.16**

Table 3.6. Fatty acid composition of crude fish oil from the catfish by-products

Fatty acid	Content (mg/g)	Fatty acid	Content (mg/g)

C14:0 (Myristic)	13.28 ± 0.10	C18:3 (Linolenic)	18.32 ± 0.03
C16:1 (Palmitoleic)	5.42 ± 0.10	C20:4 (Arachidonic)	1.44 ± 0.07
C18:0 (Stearic)	41.51 ± 0.35	C20:5n3 (Eicosapentaenoic)	2.21 ± 0.06
C18:1n9c (Oleic)	7.99 ± 0.15	C22:6 (docosahexaenoic)	0.57 ± 0.05
C18:2n6c (Linoleic)	1.53 ± 0.04		
Total saturated fatty acids		**54.79 ± 0.25**	
Total unsaturated fatty acids		**36.91 ± 0.18**	

3.6. Preparation of HA from catfish bones

3.6.1. The chemical composition of raw fishbones and HA

The parameters of the chemical composition of raw fishbone and HA obtained by different pretreatment methods are shown in Table 3.7 & 3.8.

Table 3.7. The chemical composition of raw fishbones obtained from different pretreatments

Hydrolysis using alcalase			Boiling method		
Protein (%)	Lipid (%)	Ash (%)	Protein (%)	Lipid (%)	Ash (%)
25.70 ± 0.25	9.54 ± 0.12	51.06 ± 0.35	27.85 ± 0.25	8.73 ± 0.12	50.06 ± 0.35

Table 3.8. The chemical composition of HAs from different pretreatment methods

Hydrolysis using alcalase				Boiling method			
Protein (%)	Lipid (%)	Moisture (%)	Ash (%)	Protein (%)	Lipid (%)	Moiture (%)	Ash (%)
ND	ND	0.40 ± 0.04	99.2 ± 0.75	0.82 ± 0.04	ND	0.45 ± 0.05	98.73 ± 0.63

3.6.2. Preparation of raw fishbones

After treating catfish by-products with alcalase, the raw catfish bones have lower organic content (protein, lipid) of 35.24%; ash content was higher (51.06%) compared to that of pretreated by the boiling method (protein, lipid) was 36.58% with an ash content of 50.06%. HA had a higher purity by treating with enzyme than that of the sample pretreated by boiling method.

Specifically, the HA obtained from waste treatment with alcalse, no protein was detected. Meanwhile, the HA obtained from boiled by-products still contains 0.82% protein.

Therefore, the thesis selected raw fishbones obtained after the hydrolysis using alcalase to be the raw material to investigate to find the best conditions to get HA.

3.6.3. Effect of heating temperature on HA properties

Figure 3.4 shows TEM images of HA obtained at different heating temperatures. Accordingly, HA particles have a nanometer size, about 30 - 300 nm. In particular, the obtained HA particles have uniform sizes (about 50-70 nm) at a temperature of 700^{o}C. Meanwhile, the other samples had larger and different particle sizes. This can be explained that organic compounds have not been completely removed at 600^{o}C. At the temperature of 900 - 1000^{o}C the HA was destroyed partially to tricalcium phosphate ($Ca_3(PO_4)_2$) and tetracalcium phosphate ($Ca_4P_2O_9$) by following reactions:

Step 1 (dehydrate): $Ca_{10}(PO_4)_6(OH)_2 \rightarrow Ca_{10}(PO_4)_6(OH)_{2-2x}O_x + xH_2O$

Step 2 (Distroy): $Ca_{10}(PO_4)_6(OH)_2 \rightarrow 2Ca_3(PO_4)_2 + Ca_4P_2O_9 + H_2O$

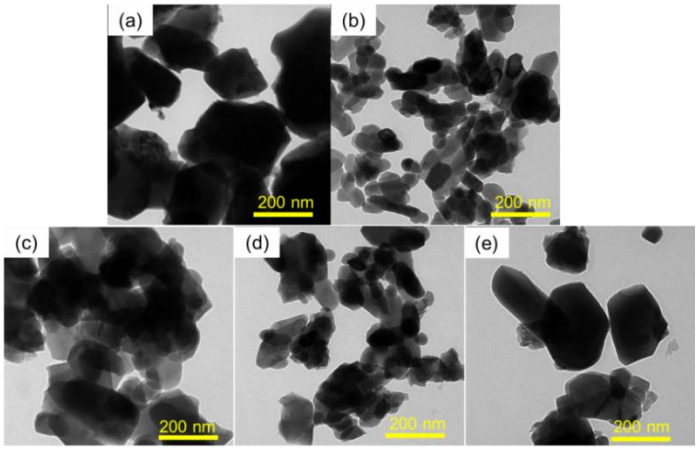

Figure 3.4. TEM image of HA obtained from fishbones calcined at (a) 600^{o}C, (b) 700^{o}C, (c) 800^{o}C, (d) 900^{o}C và (e) 1000^{o}C.

The temperature affects the crystallinity of HA particles obtained by XRD spectrum (Figure 3.4). The results showed that raw fishbones were amorphous without any characteristic peaks of HA. This can be explained that the HA in the raw fishbone is covered

with organic layer accounts for more than 50%. When calcined at 600°C, the organic part was significantly decomposed with characteristic peaks appeared at 2θ 32°, 40°, and 50°. However, there were still existed a wide peak for amorphous form at 45°, showing that the organic part has not been entirely decomposed. By heated at 700 - 1000°C, all products showed the characteristic peaks of HA without any peaks of the organic component. However, the intensity of the peaks is different between the products heated 700 - 800oC compared to that of samples heated at 900 - 1000°C. This is due to the breakdown of HA to the β-HA form above 900°C.

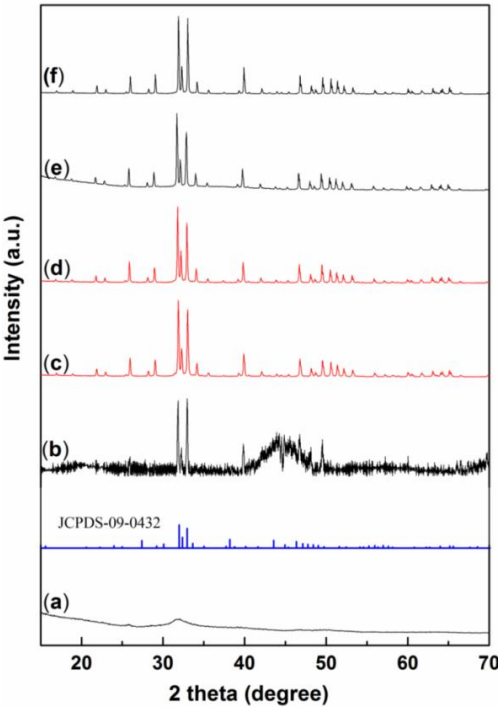

Figure 3.5. The XRD patterns of (a) raw fishbone, HA (b) 600 °C, (c) 700°C, (d) 800°C, (e) 900°C, (f) 1000°C for 2 h and HA standard JCPDS-09-0432.

When the temperature was increased to 700, 800, and 900°C, sharp peaks formed. This showed that the organic matter was removed, and the collected HA had a high crystal structure. All peaks of HA are arranged to correspond to standard HA JCPDS (see Figure

3.5). Furthermore, at 900°C, there were no new peaks, indicating a high purity HA. In previous studies, HA will convert to β - tricalcium phosphate (β - TCP) at high temperatures. Similarly, in this study, the peak of β-TCP was generated at 1000°C.

From the above results, the thesis chooses the most suitable temperature that is 700^oC.

3.6.4. Effect of heating time on HA properties

At the same heating rate 5^oC/min and 700^oC, different calcined time, HA product has a different color, recovery efficiency, size, shape, and crystallinity, as shown in Table 3.9, Figure 3.6, Figure 3.7.

Table 3.9 shows that the whole HA sample collected from catfish bone has gray-green color after 60 min. This is due to the presence of Cu^{2+} in HA. The color of HA turns milky white for 2 h, which is due to the removal of Cu^{2+} in the HA. After 5 hours, the obtained HA has appeared in ivory white. The HA samples are hard at 1 hour and become porous after 2 h. On the other hand, the recovery efficiency of HA was decreased gradually with the extended time. It may be due to the loss of more water in the HA. However, the difference in recovery efficiency was not statistically significant ($P < 0.05$).

Table 3.9. Effect of heating time on HA properties

Heating Time (h)	Properties of HA		Recovery efficiency (%)
	Color	Morphology	
1	Green gray	Hard	57.2
2	Milky white	Porous, fine	56.7
3	Milky white	Porous, fine	56.3
4	Ivory-white	Porous, fine	55.8
5	Ivory-white	Porous, fine	55.5

Figure 3.6 shows the morphology of HA at different heating times. All samples were in the range of 300 nm with high porosity. However, HA particle size is not uniform (30 - 300 nm) and agglomerated at 1 hour (Fig. 3.6a). After 2 h, HA particles were small and uniform (50 - 70 nm). This can be explained that organic compounds are eliminated. However, HA particles will be larger when heated at 4 to 5 hours (100-200 nm) due to the particles' adhesion.

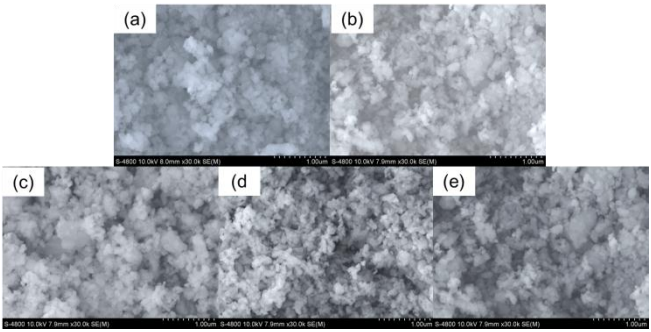

Figure 3.6. SEM images of HA from different temperatures (a) 1, (b) 2, (c) 3, (d) 4, and (e) 5 h.

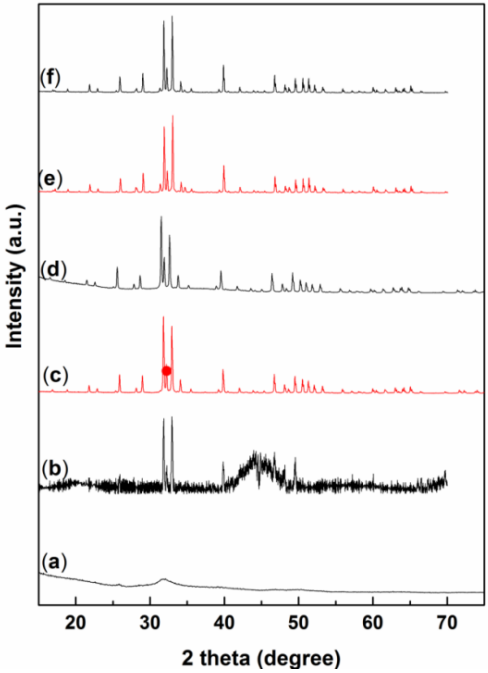

Figure 3.7. XRD patterns of (a) raw fishbone and HA from different heating temperatures (b) 1, (c) 2, (d) 3, (e) 4, and (f) 5 h.

Figure 3.7 shows XRD patterns of raw fishbones and HA samples obtained at different times (1-5 h). The results showed that raw fishbone was amorphous without any characteristic peaks of HA. It can be explained by the presence of organic layers (about 50%) on

the surface of HA. After heating for 60 min, the above organic part was decomposed, and the characteristic peaks of HA appeared at angles of 2θ 32°, 40°, and 50°. However, there still existed a wide peak characteristic for amorphous form at 45^{o}, showing the organic part has not been entirely decomposed. After 2 to 5 h, all samples gave a similar standard spectrum with peaks of HA; there were no peaks of the organic part. However, the intensity of the peaks is different between the product heated at 2 h and 3-5 h. This is due to the breakdown of HA to the β-HA form above 2 hours.

Based on the above results, the most suitable heating time is 2 h.

3.6.5. Effect of heating rate on HA properties

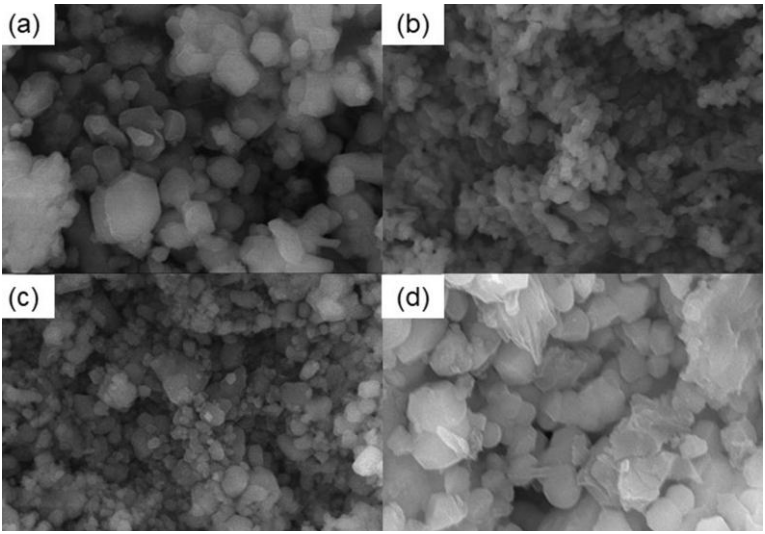

Figure 3.8. SEM images of HA from different heating rates (a) 3, (b) 5, (c) 7, and (d) 10^{o}C/min.

The heating rate has a great influence on the combustion process to remove the organic part in the raw fishbone and the rate of steam evaporation from the sample. Therefore, it affects the shape, size, and porosity of HA products. If the heating rate is high, the water evaporation on the surface of HA is rapid, creating a hard film on the surface to prevent water movement from the inside to the product's surface. Therefore, the product was dried unevenly and formed a different color of HA products. Besides, by quick heating, HA could be degraded and converted to β-HA,

resulting in a low recovery efficiency, large HA particles, and low porosity. The results are presented in Table 3.10, Figure 3.8, and Figure 3.9.

Table 3.10. Effect of heating rate on HA properties

Heating rate (^{0}C/min)	Properties of HA		Recovery efficiency (%)
	Color	Morphology	
3	Milky white	Porous, fine	57.1
5	Milky white	Porous, fine	55.6
7	Ivory-white	Porous, fine	55.3
10	Ivory-white	Porous, fine	55.1

Table 3.10 shows the effect of different heating rates on the properties of prepared HA samples at the same temperature of 700°C for 2 h. At the heating rate of 3^{o}C/min, the HA was milky white. When the heating rate increases from 5°C/min, the HA sample turns ivory-white. This is explained that the removal of water was completely at a high heating rate (> 3^{o}C / min). All HA samples are porous and fine. Besides, the recovery efficiency was decreased slightly with an increase in the heating rate. However, the difference in recovery was not statistically ($P < 0.05$).

Figure 3.8 shows the shape and size of HA at different heating rates. All samples are between 30 and 300 nm in size and high porosity with a rough surface. However, HA particles are not uniform and agglomerated at heating rates of 5, 7, and 10^{o}C/min. It is due to the removal of the water occurred on the surface at high heating rates.

Figure 3.9 shows the XRD spectra of raw fishbone and HA samples obtained at different heating rates at 3, 5, 7, and 10^{o}C/min. The raw fishbone was amorphous without any characteristic peaks of HA. It is because the raw fishbone is covered with a thick organic layer. After calcination, the above organic quantity was decomposed. There was no peak for the organic part.

From the above results, the most suitable heating rate is 3^{o}C/min.

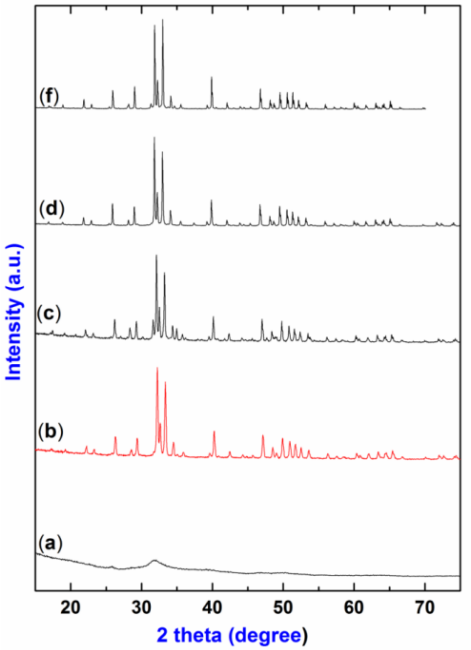

Figure 3.9. XRD patterns of (a) raw fishbone and HA from different heating rates (b) 3, (c) 5, (d) 7, and (e) 10^{o}C/min.

3.7.The properties of nanohydroxyapatite

The HA sample was obtained from raw fish bones at the best conditions and further characterized for chemical composition, crystallinity, and surface properties.

Table 3.11. Elemental composition of HA from catfish bones

Content (mg/kg)							Ca/P
Ca	P	Na	K	Mg	Zn	Al	
302903	165539	4439.30	963.73	2.03	138.69	0.35	1.83

Table 3.11 shows the main elemental components in the HA sample, including calcium and phosphorus, with a Ca/P ratio of 1.83. This ratio is quite close to Ca/P ratio in animal and human bones (Ca/P ratio of 1.67). Besides, there are many trace elements, including Na, K, Mg, Zn, and Al. On the other hand, the content of calcium, phosphorus (over 90%) is a potential source of minerals for the feed white shrimps at 20 - 55 days old. The heavy elements such as Cd, Hg, and Pb was not found in the HA sample (lower than the detection threshold ICP-MS),

indicating that the HA produced is high purity and safe. The elemental composition was also analyzed by XPS, and the Ca /P ratio was 1.82 (Figure 3.10a).

Figure 3.10b shows the average surface area, pore-volume, and pore size are 2.87 m^2/g^{-1}, 0.020 m^3/g^{-1}, and 1.20 nm, respectively. The results indicate that HA has high porosity and excellent application potential of HA.

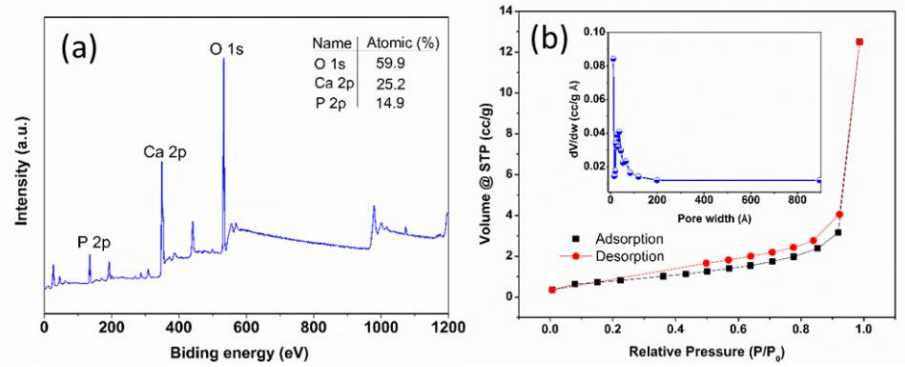

Figure 3.10. (a) XPS spectrum of HA, (b) surface property of HA calcined at 700°C, 3°C/min, 2h.

3.8. Application of the FPH and HA mixture

3.8.1. Culture environmental parameters

The pH varied between 7.55 to 8.05 in the morning and 7.75 to 8.15 in the afternoon Dissolved oxygen (DO) in all five treatments were maintained around 5.5 mg/L. The alkalinity of the experiment ranges from 103.3 mg $CaCO_3$/L to 105.8 mg $CaCO_3$/L, which is well suitable for shrimp to grow. The average NO_2^- contents in the five treatments ranged from 0.30 mg/L - 0.55 mg/L. Our results illustrate that the physical and chemical conditions of rearing water were within suitable ranges for the survival and development of L. *vannamei* (post 20 - post 55). Notably, the supplement of a mixture of FPH and HA from by-product Trafish in feed did not alter the water quality for shrimp culture.

3.8.2. Total bacteria and Vibrio bacteria

After 8 days of stocked, Treatments 1 and 2 had the highest Vibrio density, 0.14×10^3 CFU/mL, and was significantly different ($p < 0.05$) compared to treatments 3, 4, 5. Similarly, after the 15 days of stock, the density of Vibrio bacteria of treatments 1 and 2 was

highest with 0.58 × 10^3 CFU/mL and significantly different ($p < 0.05$) compared to treatments 3, 4, 5. On day 36, the density of total Vibrio bacteria of 5 treatments was significantly increased with 2.57-3.81 × 10^3 CFU/mL and accounted for 6.3 - 9.1% compared to the total number of bacteria in the shrimp culture environment.

The results of 55 days showed that treatments 4, 5 had the lowest Vibrio density, 0.09 × 10^3 CFU/mL, and it was significantly different ($p < 0.05$) compared to treatments 1 and 2. It has been shown that in the culture water, the density of Vibrio bacteria lower than 6.5 × 10^3 CFU/mL does not affect the health of farmed shrimps.

3.8.3. Factors on the growth of postlarval shrimps

The results of survival and yield of shrimps indicate that the supplement of a mixture of FPH and HA from Trafish by-products in the feed may improve the production of L. *vannamei* compared with the non-added treatments. The mix of FPH and HA in the feed is suitable for shrimp growth. By adding 5% mixture of FPH and HA (80: 20), shrimp grew by 0.97g, which is 123.5% in volume compared with without supplement. Also, the shrimp length was 7.37 mm, which is equivalent to 112% in size compared to the control sample. After the 55-day trial, the survival rate (%) and yield were 97.30 ± 2.85% and 3.52 ± 0.35 kg/m^2, respectively.

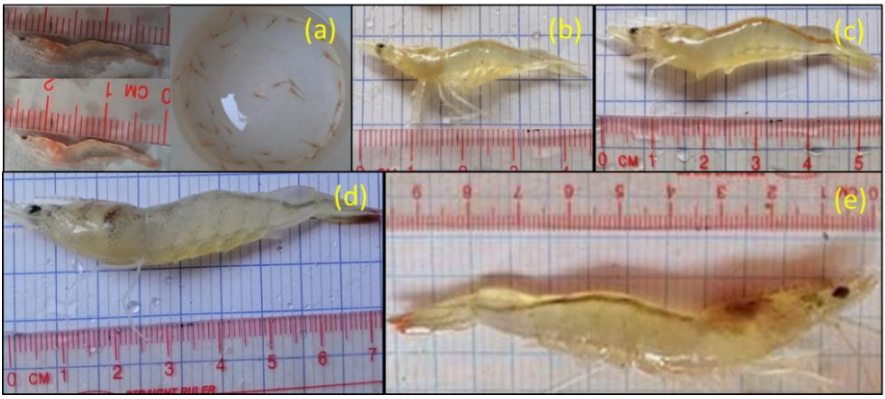

Figure 3.11. Photographs of *L. vannamei* at the ages of (a) 20 days, (b) 27 days, (c) 34 days, (d) 41 days, and (e) 55 days.

3.8.3.4. Checking of shrimp quality at 55 days old

Shrimps were shocked in formol solution for 30 minutes, resulting in 100% alive shrimp, shrimp are good quality. Morphology

and surface structure of the shrimp shell were determined by X-ray diffraction (XRD) and electron microscope (SEM):

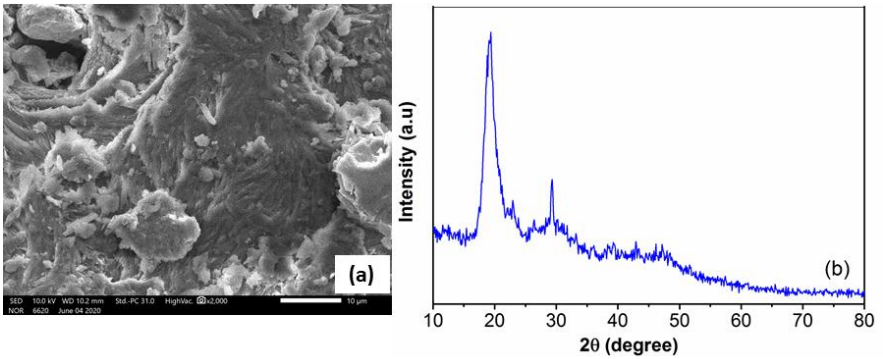

Figure 3.12. SEM image và XRD pattern of shrimp shells at 55 days old, which were feed by the mixture of FPH and HA.

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

1. Protein hydrolysate (DH 35%, M_W < 1000 Da, nitrogen total of 33.2%, lipid total of 1.93%, acid amine total of 420.16 mg/g protein) can be recovered from catfish by-products by an enzymatic hydrolysis method. Stage 1, the enzyme is alcalase and the best suitable conditions: ratio water/substrate 1:1, NaCl concentration 2%, the of enzyme–substrate ratio 0.4%, 60^oC, 9 h. Stage 2, the enzyme is lipase, and the best suitable conditions: the of weight enzyme 0.03%, 60^oC, 2 h.
2. Hydroxyapatite (50 – 70 nm, Ca/P ratio of 1.83, BET area 2.87 m^2/g, pore-volume of 0.02 m^3/g, the pore size 1.2 nm) can be prepared by calcination method. The most suitable conditions: temperature of 700^oC, the heating rate of 3^oC/min, 2 h.
3. The mixture of FPH and HA (FPH/HA 80:20) in the feed (5%) is suitable for shrimp growth at the age of 20 – 55 days old. Shrimp grew by 0.97g (123%) in weight and 7.37 mm (122%) in length compared to the control sample. The supplement of a mixture of FPH and HA from by-product Trafish in feed did not alter the water quality for shrimp culture.

Recommendations

1. It is necessary to do more experiments and evaluate the hydrolysis process and calculate the economic efficiency before applying it to large-scale production.
2. It is necessary to add the mixture of hydrolyzed protein solution and hydroxyapatite collected from pangasius waste to feed for other subjects.

PUBLICATIONS

Proceedings:

1. **Pham Viet Nam**, Nguyen Van Hoa,Trang Sĩ Trung. Biological hydroxyapatite nanomaterial extracted from fish bones. *Proceedings of First International Conference on Material, Machines and Methods for Sustainable Development,* Danang 5/2018.
2. **Pham Viết Nam**, Nguyễn Văn Hòa,Trang Sĩ Trung. Thu nhận hydroxyapatit có kích thước nano từ xương cá sử dụng phương pháp enzym và xử lý nhiệt. *Kỷ yếu Hội nghị Khoa học Công nghệ sinh học toàn quốc*, Hà Nội 10/2018.
3. **Pham Viet Nam**, Nguyen Van Hoa, Tran Thi Lan Anh, Trang Si Trung, Recovery of bioactive components from catfish (*Pangasius hypophthalmus*) by-products towards zero waste processing, *Proceedings of Vietnam – Japan Science and Technology Symposium,* Hanoi 2019.

Papers:

1. **Pham Viet Nam**, Nguyen Van Hoa, Tran Thi Lan Anh, Trang Si Trung (2020). Towards zero-waste recovery of bioactive compounds from Tra fish (*pangasius hypophthalmus*) by-products using an enzymatic method. *Waste and Biomass Valorization*, 11 (8) 4195-4206.
2. **Pham Viet Nam**, Nguyen Van Hoa, Trang Si Trung (2019). Properties of hydroxyapatite prepared from different fish bones: a comparative study. *Ceramics International*, 45 (16) 20141-20147.
3. Nguyễn Lê Bá Quảng, **Phạm Viết Nam**, Phạm Anh Đạt, Nguyễn Văn Hòa (2018). Thu nhận và xác định tính chất của hydroxyapatit kích thước nano từ xương cá: (1) phương pháp xử lý nhiệt. *Tạp chí Khoa học - Công nghệ Thủy sản*, Số 3, tr. 53 – 60.
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