MINISTRY OF EDUCATION AND TRAINING
NHA TRANG UNIVERSITY

PHAM THI DAN PHUONG

PREPARATION OF CHITOSAN LACTATE FROM MOULTED SHRIMP SHELLS (*LITOPENAEUS VANNAMEI*) AND ITS APPLICATION IN FLOCCULATING MICROALGAE *NANNOCHLOROPSIS* SP.

SUMMARY OF PH.D. THESIS ON SEAFOOD PROCESSING TECHNOLOGY

KHANH HOA - 2023

NOVELTIES OF THE THESIS

Title: Preparation of chitosan lactate from moulted shrimp shells (*Litopenaeus vannamei*) and its application in flocculating microalgae *Nannochloropsis* sp.
Major: Seafood Processing Technology.
Code: 9540105.
Student: Pham Thi Dan Phuong
Course: 2018 – 2022
Supervisors: 1. Assoc. Dr. Nguyen Van Hoa
2. Assoc. Dr. Huynh Nguyen Duy Bao
Institution: Nha Trang University

The novelties of the thesis:

1. The thesis has successfully developed a process for producing chitin and chitosan from the moulted shrimp shells, which are solid wastes generated from intensive shrimp farms. The mass of shells is estimated to be about 5% of the total shrimp production. The obtained chitin/chitosan product is of commercial quality (remaining mineral and protein content < 1%). Noted, the mineral and protein contents in the moulted shells are significantly different from those of the shrimp shells generated from shrimp processing factories. Therefore, the demineralization and deproteinization processes were carried out using diluted acidic and alkaline solutions (3-4%) in a short reaction time (3-6 h). As a result, the production yield was high (~23%). The prepared chitosan has a high DD (about 90%), high solubility (~ 99%), and M_w of 500 kDa.
2. The thesis has proposed a process for producing chitosan lactate with high solubility (> 99%) from prepared chitosan.
3. The prepared chitosan lactate was used for flocculating microalgae *Nannochloropsis* sp. The recovery efficiency was of ~ 90% after 120 min at the chitosan lactate concentration of 250 ppm and pH 10.0.

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PREFACE

1. The necessary

Fishery sector is one of the key economic sectors of Vietnam, bringing a large source of foreign currency revenue for the country. In 2022, total exported value from fishery products turnover reached 11 billion USD, of which the exported value from shrimp products was the highest, reaching 4.1 - 4.2 billion USD. Currently, the model of intensive shrimp farming has been widely implemented and brought about high economic efficiency in many localities throughout the country. In addition, the excellent control of the environmental conditions helps to reduce shrimp's risk of diseases. However, due to the high density of shrimp farming, a large amount of wastes is generated during the farming process, including leftovers, feces, suspended matters, and moulted shells of shrimp etc. These wastes have a potential to pollute the farming environment, causing diseases for shrimp. In particular, the moulted shells of shrimp can be recovered by siphoning method and used as a source of raw materials for producing chitin and chitosan. Utilization of the moulted shells as raw materials for chitin and chitosan production not only increases the value for shrimp farming, but also reduces environmental pollution, improves value for the shrimp supply chain as well as contributes to the sustainable development of the seafood industry.

On the other hand, the demand for using microalgae in aquaculture feeds has been increasing. Microalgae are essential nutrients for bivalve molluscs, fish and crustaceans at the larval stage. In which, the microalgae *Nannochloropsis* sp. has been commonly used as feed for aquatic larvae due to its small size, high bioactive compounds, and nutritional value. However, one of the major problems that need to be solved is to find out an effective method to harvest the microalgae *Nannochloropsis* sp. with low cost and high product quality being used for aquaculture feeds.

Chitosan is a biopolymer used for many applications, such as in agriculture, wastewater treatment, food, cosmetics, and medicine. However, its applications are still limited because chitosan is only soluble in some dilute acid solutions. To increase the solubility, chitosan is derived into oligochitosan, chitosan salts, nanochitosan by attaching hydrophilic groups into chitosan structure. Chitosan lactate is a derivative of chitosan, which is well-soluble in water, non-toxic, and non-acidic odour. Therefore, chitosan lactate can be dissolved in water for harvesting marine microalgae. The flocculation mechanism is said to be due to electrical neutralization between chitosan lactate with positive charge and microalgae with negative charge… In addition, chitosan lactate retains its coating-forming properties to protect microalgae cells, avoid swelling and rupture that affect biological activities of pigments.

From the above considerations, the thesis "Preparation of chitosan lactate from moulted shrimp shells (*Litopenaeus vannamei*) and its application in flocculating microalgae *Nannochloropsis* sp." was carried out.

2. The objectives

The main objectives of the project are:

- To use moulted shells from intensive shrimp farms to produce high purity chitin and

chitosan.

- To develop a process for preparing chitosan lactate with a high solubility from the obtained chitosan.
- To identify the suitable conditions for using chitosan lactate to harvest marine microalgae *Nannochloropsis* sp.

3. Research scope and contents

In order to achieve the above objectives, three research contents need to be carried out:

- Preparation of chitin and chitosan with high purity from moulted shrimp shells.
- Preparation of chitosan lactate from moulted shrimp chitosan with a high solubility.
- Investigation of using chitosan lactate for flocculating microalgae *Nannochloropsis* sp.

4. The scientific and practical significance of the thesis

- This is the first time, moulted shrimp shells generated from intensive shrimp farms have been evaluated the yield, chemical composition and physicochemical characteristics. Moulted shells were also used to produce chitin and chitosan.
- Successfully developed a process for producing chitin and chitosan from moulted shrimp shells with high purity, less chemicals used, short processing time, and without decolorization step.
- Successfully developed a process for preparing chitosan lactate with high solubility (> 99%) by solid-liquid method, which can be easily applied at a large scale.
- Successfully identified the suitable conditions for using chitosan lactate in combination with pH adjustment to flocculate marine microalgae *Nannochloropsis* sp. with high flocculation efficiency (~90% after 2 h).
- Based on the results of the thesis, a new approach has been opened up on utilization of moulted shells from intensive shrimp farms to produce value-added products (chitin, chitosan, chitosan lactate), leading to reduce adverse effects on the environment during intensive shrimp farming.
- The utilization of chitosan lactate for harvesting microalgae being used in aquafeeds enhances a circular economy and sustainable development of the aquaculture industry.

CHAPTER 1. INTRODUCTION

1.1. The moulted shrimp shells

The intensive and super-intensive aquaculture models can reduce environmental pollution and diseases. It also increases the stocking density and crop of the year. In addition, the daily siphoning of wastes to clean the ponds makes it possible to collect a large amount of moulted shrimp shells. In Vietnam, only approximately 10% of farms (mainly in the Mekong Delta) are applied the intensive farming model, but they produce about 80% of the total shrimp output. Among them, exported value of *vannamei* shrimp accounted for 72% of total exported value of shrimp in 2020. According to the Directorate of Fisheries (February 2022), the production of white-leg shrimp was estimated at 25,500 tons (up 6.3% over the same period last year).

The moulted shrimp shells generated during the farming process can be collected by siphoning method. The mass of moulted shells increases proportionally with the production of shrimp. The main components of the moulted shell include chitin and minerals, which should be used to produce value-added products. The use of moulted shells leads to reduce environmental pollution.

Before moulting, shrimp shell has gone through the calcification stage to create a certain hardness. Therefore, the moulted shells contain a high mineral content. Minerals of moulted shrimp shells exist in a form of amorphous crystals of calcium and calcium carbonate and are surrounded by a lipid-protein substrate. Thus, the protein and lipid contents are low. Although the mineral content is high, they are interspersed with chitin-protein fibers that form thin, porous plates. Therefore, chitin can be recovered by demineralization and deproteinization without decolorization. At the same time, the production process is proposed to create high-quality products (mineral and protein content < 1%) with less chemicals used and short reaction time.

1.2. Production of chitosan and chitosan lactate

Chitosan is widely used in biomedical, food, agricultural, biotechnology, and pharmaceutical industries due to its unique natural polycationic and safe biopolymer. In addition, chitosan has antifungal, antibacterial and antioxidant activities, and film-forming, gel-forming, colour-adsorbing, and solution-cleaning abilities. However, its applications are limited because it is only soluble in some dilute acids. To overcome this problem, converting chitosan into water-soluble chitosan salt is of a great interest to scientists today. Chitosan salt (water-soluble chitosan) is formed when amine groups at position 2 of chitosan are protonated ($-NH_3^+OCOR^-$) in an acidic medium (inorganic and organic).

So far, chitosan is produced from chitin through deacetylation process, which removes acetyl groups from the chitin molecules. Chitosan salt is prepared by reacting chitosan with organic or inorganic acids under suitable conditions. The salt products are obtained after filtration and washing to remove excess acid. The factors affecting the formation of chitosan salts include the chitosan particle size, the ratio of chitosan to solvent, the concentration and type of acids, and the reaction temperature and time. There are three methods to prepare chitosan salts: (1) chitosan reacts with acids in a solid state in an alcohol medium, (2) chitosan reacts with vapor acids in a solid state, (3) chitosan is completely dissolved in an acidic medium to form a gel, then spray-dried/freeze-dried to form a powder or dried to form a film.

1.3. Harvesting marine microalgae *Nannochloropsis* sp.

Up to now, the recovery of microalgae biomass is still a problem in Vietnam. It requires a high investment cost in equipment and technology for commercial microalgae harvesting systems. Besides, effective harvesting methods are only applied for a few large and freshwater microalgae species. The current solutions commonly applied are: (i) microalgae are cultured at aquaculture farms or (ii) Microalgae are harvested by centrifugation and then refrigerated until supplied to hatcheries. In addition, many harvesting methods have been studied and applied, such as gravity

sedimentation, microfiltration, coagulation/flocculation, flotation, and a combination method. However, these methods still have some limitations, such as high investment energy costs, toxicity of chemicals used for flocculation, and less feasible to scale up to large production.

In recent years, microalgae flocculation using biopolymers has been applied to replace toxic chemicals. It has a low investment cost and simple technique, making it easy to apply in practice. However, the recovery ability for marine microalgae is not high due to a high salt content, which affects the flocculation process.

Chitosan salt has a high solubility in a wide pH range, which extends its applications. Chitosan lactate is used for harvesting microalgae *Nannochloropsis* sp., which reduces transportation costs from microalgae farms to aquaculture farms and maintains the required quality (bioactive substances and growth resilience). Besides, the researchers indicated that the flocculation method with organic agents is suitable for large-scale applications because of its high efficiency, low operating costs, suitability for many algae species, fast, minimal maintenance requirements, and environmentally friendly.

CHAPTER 2. MATERIALS AND METHODS

2.1. Materials

Moulted shrimp shells, microalgae *Nannochloropsis* sp.

2.2. Methods

The overall experimental schematic diagram is presented in Figure 2.4.

Explain the diagram:

(1) Evaluation of the potential use of moulted shrimp shells as raw materials for chitin and chitosan production

This section aimed to evaluate the potential for obtaining moulted shrimp shells and the changes in chemical composition with age during intensive culture. Besides, it provides the ability to use moulted shells as raw materials for chitin and chitosan production.

(2) Production of chitin and chitosan from moulted shrimp shells

This section aimed to find out the suitable conditions for recovering chitin and chitosan from moulted shrimp shells via demineralization, deproteinization, and deacetylation.

(3) Production of chitosan lactate from moulted shrimp shell chitosan

This section aimed to find out the suitable conditions for preparing chitosan lactate from chitosan, including swelling time, pH, acid concentration, reaction time, and temperature.

(4) Applications of chitosan lactate for harvesting *Nannochloropsis* sp.

This section aimed to find out the suitable conditions for harvesting *Nannochloropsis* sp. by using chitosan lactate.

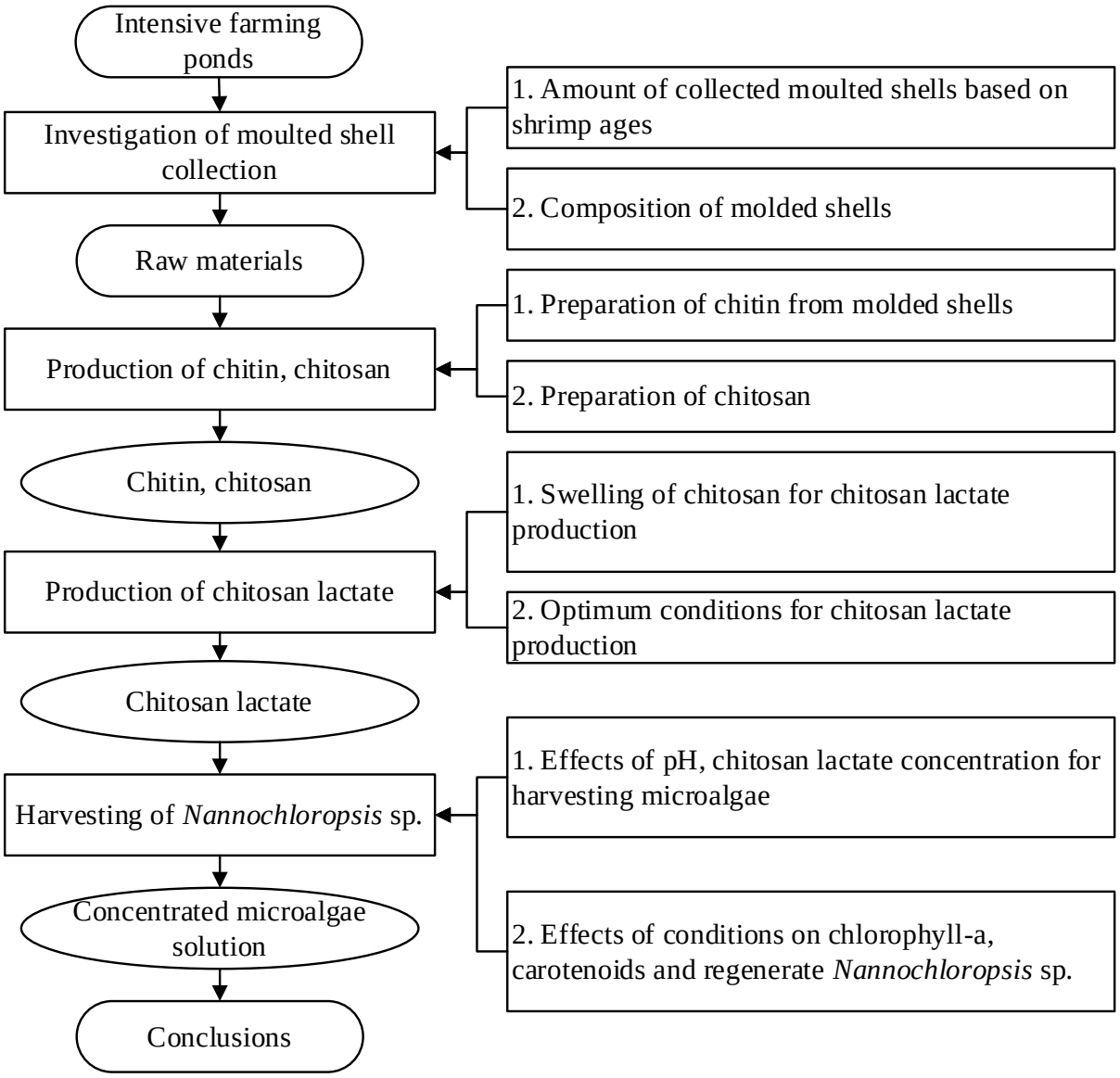


Figure 2.4. The overall experimental schematic diagram**.**

2.3. Characterization

The mineral and moisture content were determined according to the AOAC method (1990). The protein content in moulting shell was determined by Biuret method, protein content in chitin and chitosan was determined by Micro-Biuret method; the protein content in microalgae was determined by the Biuret method, according to Dorsey et al. (1978). Chitin content was determined according to the method of Black and Schwartz (1950). The deacetylation of chitosan was determined according to Wu and Zivanovic (2008). The swelling of chitosan in the solvent was determined according to the method of Minh et al. (2017). The lipid content was determined according to Bligh and Dyer (1959). Carbohydrate content was determined by a phenol-sulfuric acid method (Nielsen, 2010). The content of chlorophyll-a and carotenoids was determined based on Sumanta et al. (2014). Cell density was determined using a Neubauer-improved erythrocyte counting chamber. Control the salinity of the seawater environment with microalgae culture with the S-10E refractometer. Microalgae microscopic image was taken on microscope Olympus BX41.

The M_W of chitosan was determined by internal viscosity (Mark-Houwink, 2001). The solubility of chitosan and chitosan lactate was determined by the method of Minh et al. (2019). The morphology of moulted shell, chitin, and chitosan was measured by SEM. The chemical structure of shrimp shells, chitin, chitosan, and chitosan lactate was measured by FT-IR in the range 500 – 4000 cm^{-1} at a resolution of 16 cm^{-1} within 32 scans. The relative crystallinity (CrI) of the samples was determined by the XRD spectrum. Nuclear magnetic resonance spectroscopy (^{1}H NMR) of chitosan, chitosan lactate was measured on a spectrometer AV300 MHz, Bruker, USA.

2.4. Calculation

The yield of moulted shells recovered from intensive culture was estimated by the following formula:

$$G = \frac{M_S}{M_O} \qquad (1.1)$$

In which: G (kg/kg) is the yield of moulted shell recovered from intensive farming. M_S (kg) is the amount of moulted shells, and M_0 (kg) is the amount of commercial shrimp harvested from the same intensive culture crop.

The recovery efficiency (RE) was determined using the formula (1.2). Where, M and R (g) are the weight of the initial sample and the sample after treatment.

$$RE\ (\%) = \frac{R*100}{M} \qquad (1.2)$$

The flocculation efficiency of microalgal biomass was determined through the settling efficiency of microalgae biomass. Flocculation efficiency (FE) was determined by measuring the optical density of microalgae cells before and after coagulation of the solution in the culture medium. Optical density (OD) was measured on a UV-VIS spectrophotometer at 490 nm (the wavelength chosen after scanning the sample on the DR6000 spectrophotometer was the highest detected). The formula for calculating the settling efficiency (1.3) by optical densitometry (Şirin et al., 2012):

$$FE\ (\%) = \frac{OD_đ - OD_S}{OD_đ} \text{ x } 100 \qquad (1.3)$$

Which, $OD_đ$: optical density of algae solution before coagulation, OD_s: optical density of solution after coagulation.

Determination of content recovery efficiency of colorants (chorophyll-a: $RE_{Ch\text{-}a}$, carotenoids total: $RE_{Carotenoids}$) is the following (1.4) và (1.5):

$$RE_{Ch-a}(\%) = \frac{Ch_S*100}{Ch_T} \qquad (1.4)$$

$$RE_{Carotenoids}(\%) = \frac{C_S*100}{C_T} \qquad (1.5)$$

Which, Ch_T, C_T(g) is chlorophyll-a and carotenoid contents in the original sample; Ch_S, C_S is chlorophyll-a and carotenoid contents in the harvested sample.

2.5. Statistical analysis

The statistical difference was defined at $p < 0.05$. All statistical analyses were performed using IBM SPSS Statistics. Microsoft Excel, Visio Professional, and Origin software were used to draw the figures.

CHAPTER 3. RESULTS AND DISCUSSIONS

3.1. The moulted shrimp shells

3.1.1. Current status of moulted shrimp shells in intensive shrimp farms

The survey at some intensive shrimp farms found that the moulted shrimp shells were generated continuously during the farming process. Therefore, the mass of moulted shrimp shells increases significantly with a rapidly increase in the farming area. This may pollute the culture water or the pond area if the moulted shrimp shells are not collected and treated properly. Meanwhile, the moulted shrimp shells contain valuable components such as chitin and minerals and they can be easily collected by siphoning method. Therefore, assessment of the yield and utilization of moulted shrimp shells for producing value-added products are necessary.

3.1.2. Variations in the yield of moulted white-leg shrimp shells during the intensive farming process

The yield of moulted shrimp shells that can be recovered from the intensive farms at the day of culture of more than 40 days was about 280 (kg/pond/crop) for a 1,500 m^2 pond, and the stocking density of 200 shrimp/m^2. Therefore, the recovery ratio of moulted shrimp shells shrimp was 0.056 for the commercial shrimp production of 4,500 - 6,000 kg/pond/crop (average 5,000 kg/pond/crop). This means that for every 1 ton of commercial shrimp production, about 56 kg of the dry moulted shell was obtained from an intensive farms. Thus, the yield of moulted shrimp shells was equivalent to about 5% of commercial shrimp production.

3.1.3. Chemical composition of moulted shrimp shells

The chemical compositions of the moulted shells of shrimp *vannamei* at the day of culture of 40 - 90 days were of chitin (20 – 24% DM), minerals (52 – 61% DM), and protein (10 – 13% DM). The experimental production of chitin according to the process proposed by Toan et al. (2006): demineralization with 4% HCl (1:5, w/v) at 30^{o}C for 12 h and deproteinization with 4% NaOH (1:5, w/v) at 30^{o}C for 24 h. The results showed that the production yield of chitin was 23-24%, and the remaining mineral and protein content in chitin were low (≤1%).

3.2. Production of chitin from moulted shrimp shells

3.2.1. Demineralization

The moulted shells (dry and wet) were treated with different concentrations of HCl at a temperature of 30-70^{o}C for 1-12 h. The results presented in Figure 3.5 and Figure 3.6 indicated that, HCl concentration was an important factor affecting the demineralization efficiency, while temperature and time had negligible influences. To reduce production costs when applied in a large scale and limit the adverse effects on the quality of chitin, and at the same time satisfy the condition that the remaining mineral content in raw chitin is approximately 1%, the thesis proposed the suitable demineralization conditions for both wet and dry moulted shrimp shells were: HCl 4%, at 30^{o}C for 3 h. The residual mineral contents of the chitin obtained from wet and dry moulted shrimp shells were 1.3 ± 0.2%, and 1.3 ± 0.1%, respectively. As a result, the chitin recovery efficiencies were 41.6 ± 1.5% for wet moulted shrimp shells and 36.3 ±

2.1% for dry moulted shrimp shells.

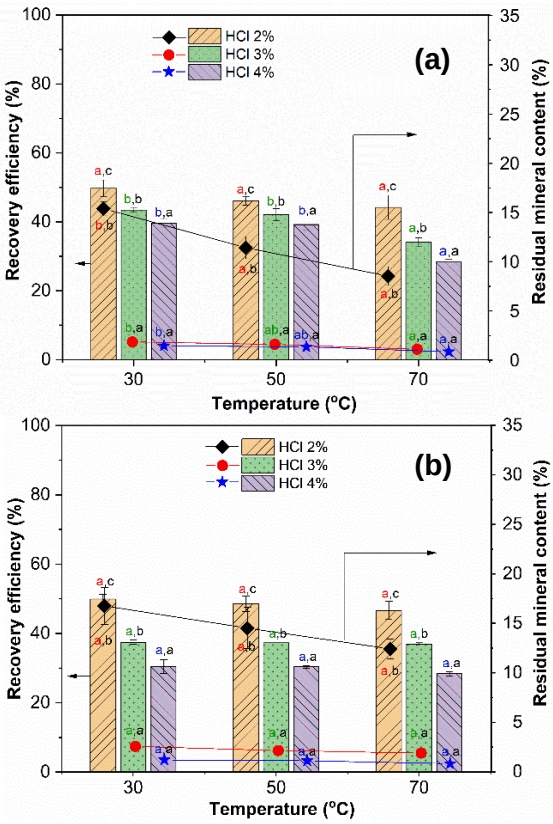
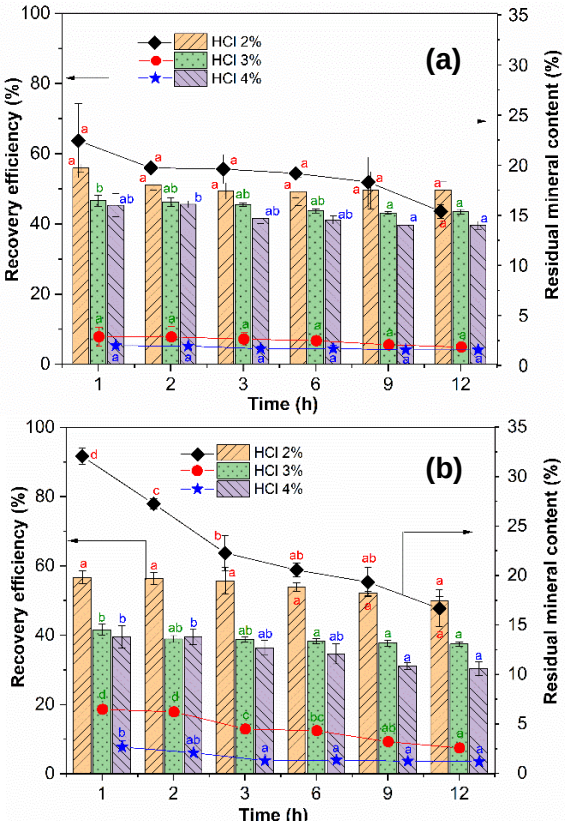


Figure 3.5. Effects of HCl concentration (%), temperature on recovery efficiency and residual mineral content in raw chitin.



Hình 3.6. Effects of HCl concentration (%), time on recovery efficiency and residual mineral content in raw chitin.

Note: The same conditions of reactions: raw material/HCl solution ratio: 1:5 (wet moulted shells: a), 1:15 (dry moulted shells: b).

Different letters (colour by concentration column (red: HCl2%, green: HCl3%, blue: HCl4%), black only by temperature) indicate significant differences ($p < 0.05$).

3.2.2. Deproteinization

Figure 3.2a and 3.2c show the effects of NaOH concentrations (2, 3, and 4%), temperatures (30, 50, and 70°C), and reaction times (3, 6, 9, 12, and 24 h) on chitin recovery efficiency and residual protein content in the chitin. Both NaOH concentration and temperature strongly affected the deproteinization efficiency. Deproteinization efficiency increased with increasing NaOH concentration and temperature (Figure 3.7 and Figure 3.8). To achieve the quality requirement of remaining protein content < 1%, the suitable deproteinization conditions were of 3% NaOH solution and at temperature of 50°C. The sample treated in 3% NaOH solution at 50°C for 6 h had a remaining protein content of < 1% and the chitin recovery efficiency 32.4% for wet moulted shrimp shells and 26.3% for dry moulted shrimp shells.

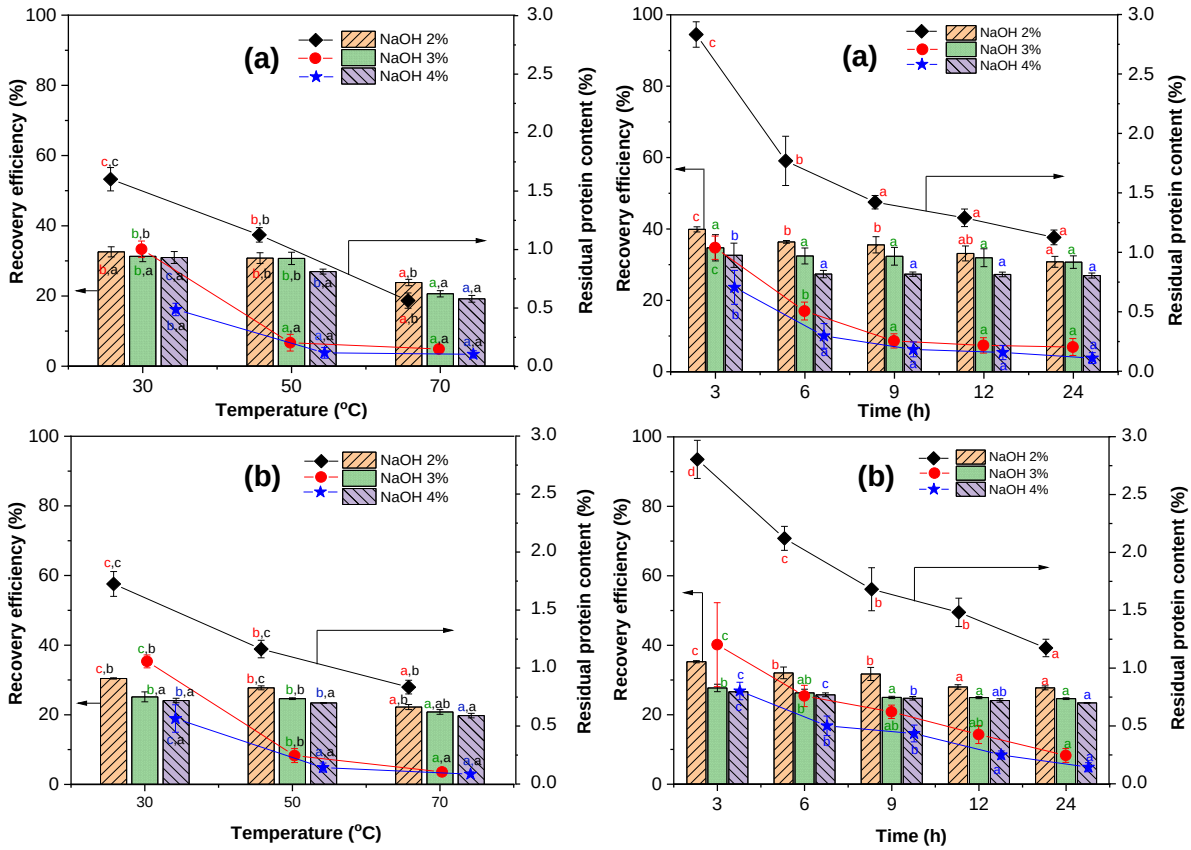


Figure 3.7. Effects of NaOH concentration (%), temperature on the recovery efficiency and residual protein content in chitin.

Figure 3.8. Effects of NaOH concentration (%), time on the recovery efficiency and residual protein content in chitin.

Note: General conditions of reactions: Ratio of chitin/NaOH solution 1:5 for both wet (a) and dry (b) moulted shrimp shells.

Different letters (colour by concentration column (red: NaOH 2%, green: NaOH 3%, blue: NaOH 4%), black only by temperature) indicate significant differences ($p < 0.05$).

3.2.3. Deacetylation

Table 3.7. Effects of temperature and reaction time on DD, viscosity, solubility, recovery efficiency, mineral and protein contents of chitosan

Temp. (°C)	Time (h)	DD (%)*	Viscosity (cPs)*	Solubility (%)*	Recovery efficiency (%)*	Ash (%)*	Protein (%)*
80°C	12	83.5 ±1.4^{a}	1.227 ±38^{c}	97.0 ±0.8^{a}	24.2 ± 2.5^{a}	0.5 ±0.1^{a}	0.11±0.0^{b}
	24	86.7 ±0.7^{a}	986 ±24^{b}	99.0 ±0.1^{b}	23.6 ± 2.9^{a}	0.5 ±0.1^{a}	0.07±0.0^{ab}
	36	90.2 ±1.7^{b}	639 ±32^{a}	99.0 ±0.2^{b}	23.3 ± 3.0^{a}	0.3 ±0.1^{a}	0.02 ±0.0^{a}
90°C	12	86.1 ±0.1^{a}	558 ±51^{b}	97.7 ±0.7^{a}	24.2± 2.6^{a}	0.3 ±0.2^{a}	0.15 ±0.1^{a}
	24	89.2 ±1.6^{b}	479 ±72^{b}	99.0 ±0.2^{b}	23.0 ± 2.4^{a}	0.2 ±0.1^{a}	0.10 ±0.1^{a}
	36	90.5 ±1.3^{b}	342 ±13^{a}	99.1 ±0.2^{b}	22.1 ± 3.3^{a}	0.1 ±0.0^{a}	0.09 ±0.0^{a}

**Calculated on absolute dry matter content. Different superscript letters indicate significant differences ($p < 0.05$).*

Chitin obtained from the moulted shrimp shells was deacetylated with 50% NaOH solution at different conditions: temperature (80, and 90°C) and time (12, 24, and 36 h) to obtain chitosan. The results are presented in Table 3.7. Accordingly, the

deacetylation efficiency and solubility of chitosan increased with increased processing temperature and processing time. However, at high temperature and long processing time resulted in lower viscosity and chitosan recovery efficiency.

To meet the requirements of chitosan products with DD ~ 90%, and solubility ~ 99%, the suitable deacetylation conditions were: 50% NaOH at 90°C for 24 h, the chitosan recovery efficiency 23%, viscosity of chitosan 479 cPs and high purity.

3.2.4. Proposing the process for chitin and chitosan production from moulted shrimp shells

The process of producing chitin and chitosan from the moulted shrimp shells was carried out through two steps of demineralization and deproteinization (without decolorization step) in a short time. Demineralization conditions were 4% HCl at 30°C for 3 h and deproteinization conditions were of 3% NaOH at 50°C for 6 h. The obtained chitin was of high quality (mineral and protein contents less than 1%). Chitin was deacetylated with 50% NaOH (w/v) at 90°C for 24 h (ratio 1:5, w/v). After filtering and washing to neutral, a purified chitosan product was obtained.

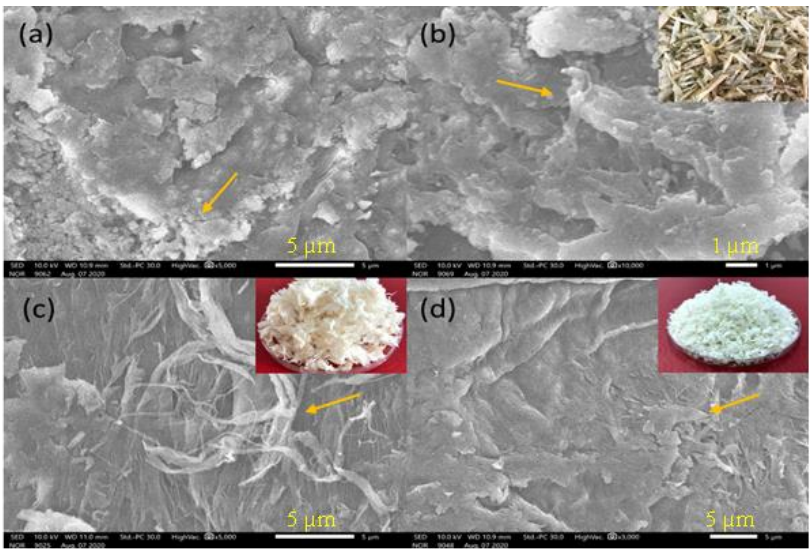


Figure 3.12. SEM images of moulted shrimp shells (a,b) at different magnifications, (c) chitin and (d) chitosan.

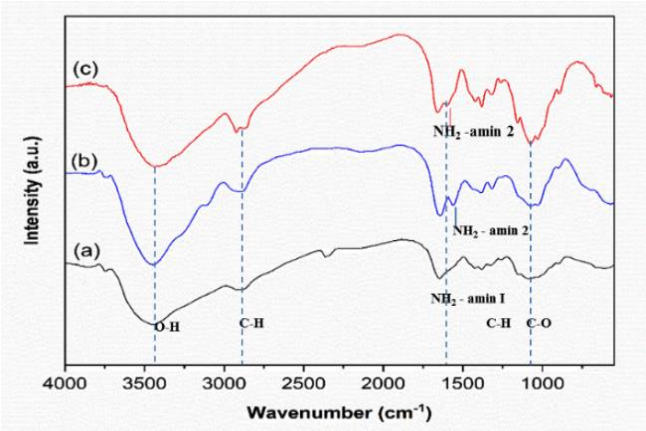


Figure 3.13. FTIR spectra of chitin (a), chitosan (b) from moulted shrimp shells, and chitosan (c) from processed shrimp shells.

The obtained products are highly purity. The content of heavy metals (As, Hg, Cd, Pb) was below the allowable level. The morphology of chitin and chitosan was

compared with the moulted shrimp shell in Figure 3.12, showing the purity of the product. This is also more evident in XRD analysis (Figure 3.15), at 2θ in 30 - 50°, there were no peaks of $CaCO_3$ in the chitosan spectrum and no impurity peaks in the NMR spectrum (Figure 3.14). The obtained chitosan had a crystallinity of 65.72%. When compared with chitosan from processed shrimp shells, the chemical structure and crystal structure of chitosan from moulted shrimp shells were not different, as shown in Figures 3.13 and 3.15.

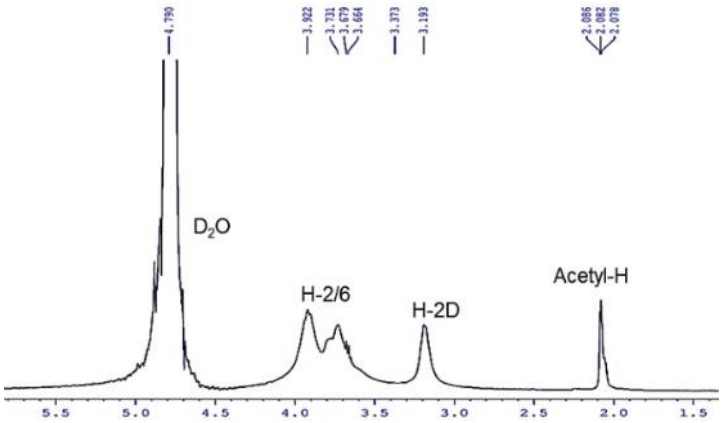


Figure 3.14. ^{1}H NMR spectrum of chitosan from moulted shrimp shells.

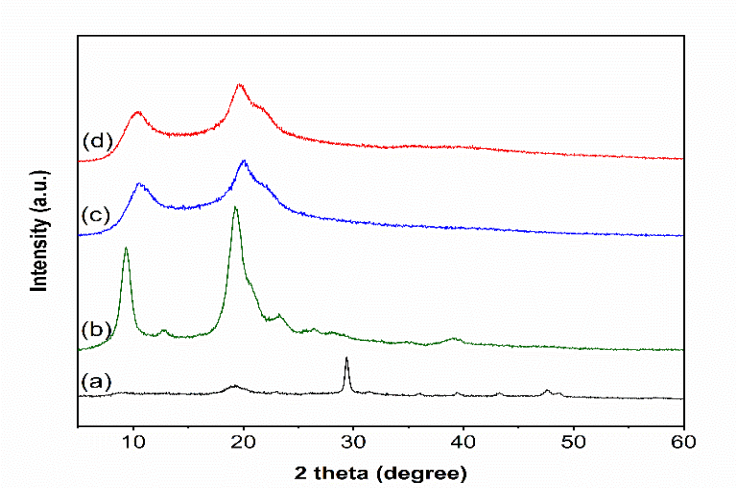


Figure 3.15. XRD patterns of moulted shrimp shells (a), chitin (b), chitosan (c) from moulted shrimp shells, and chitosan (d) from processed shrimp shells.

3.3. Production of chitosan lactate

The chitosan obtained from the moulted shrimp shells was used for the preparation of chitosan lactate in the condition of chitosan swelling in a mixture of ethanol/water with different ratios and soaking times. Then, the swollen chitosan was reacted with lactic acid at different concentrations, temperatures, and times.

3.3.1. Effect of EtOH/H_2O ratio on swelling index

The influences of EtOH/H_2O ratio (%, v/v) on chitosan swelling, state, solubility and chitosan lactate recovery efficiency are presented in Table 3.11 and Figure 3.16. The solubility of chitosan salt samples treated in EtOH/H_2O was significantly higher than that of the control chitosan sample. However, there was no significant difference in swelling index between chitosan samples treated in EtOH/H_2O ratio from 10 to 50%.

In addition, after reacting with lactic acid, the solubility of chitosan lactate increased, resulting in a decreased recovery efficiency.

The chitosan lactate samples treated with EtOH/H_2O ratio of 70% and 80% had higher swelling index and higher recovery efficiency compared to other samples. Therefore, to save solvent, the suitable soaking conditions were of EtOH/H_2O 70% and soaking time 2 h. The obtained chitosan lactate had swelling index ~450%, recovery efficiency ~ 152% and solubility ~ 98%.

Table 3.11. Effects of EtOH/H_2O ratio (%. v/v) on swelling, solubility, and recovery efficiency

EtOH/H_2O ratio (%)	Swelling index (%)*	Solubility (%)*	Recovery efficiency (%)*
Control	-	83.7 ± 2.1^{a}	138.3 ± 3.3^{b}
0	506.0 ± 28.3^{d}	-	-
10	504.0 ± 27.9^{d}	-	-
20	501.2 ± 27.8^{d}	-	-
30	493.7 ± 26.9^{cd}	-	-
40	474.4 ± 23.3^{cd}	-	-
50	466.8 ± 14.4^{bcd}	98.2 ± 0.9^{b}	132.1 ± 1.2^{a}
60	452.3 ± 4.7^{bc}	98.3 ± 1.4^{b}	143.6 ± 2.7^{bc}
70	450.6 ± 6.5^{bc}	98.1 ± 0.3^{b}	151.0 ± 1.5^{d}
80	428.8 ± 2.0^{ab}	98.0 ± 0.8^{b}	151.6 ± 0.5^{d}
90	402.3 ± 3.4^{a}	96.3 ± 0.3^{b}	146.3 ± 5.0^{cd}

**Calculated on absolute dry matter content. Different superscript letters indicate significant differences ($p < 0.05$). Because chitosan lactate form gels and are dissolved in solvents after the reaction when chitosan is swollen in solvents with different EtOH/H_2O ratios in the range of 10 - 40%. Therefore. it is not possible to calculate the solubility and recovery efficiency.*

Figure 3.16. (a) Chitosan swells in EtOH/H_2O at different ratios, (b) Swollen chitosan reacts with lactic acid, and (c) chitosan lactate.

3.3.2. Effects of soaking time

The effects of soaking time on the swelling index, solubility and recovery efficiency are presented in Table 3.12. The results showed that the swelling index of chitosan was the highest after 0.5 h of soaking. To achieve chitosan lactate with the highest solubility (98.5%) and the highest recovery efficiency (151.1%), chitosan needs to be soaked in EtOH/H_2O 70% for 4 h (Figure 3.17).

Table 3.12. Effects of soaking time on the swelling index, solubility and recovery efficiency of chitosan lactate

Soaking time (h)	Swelling index (%)*	Solubility (%)*	Recovery efficiency (%)*
0.25	425.2 ± 3.2^{a}	90.7 ± 1.8^{a}	140.4 ± 3.4^{a}
0.5	445.6 ± 3.3^{b}	93.8 ± 1.7^{ab}	143.9 ± 2.5^{ab}
1	446.3 ± 2.7^{b}	94.4 ± 1.5^{abc}	144.6 ± 4.2^{ab}
2	447.8 ± 2.8^{b}	94.9 ± 1.1^{bcd}	146.2 ± 2.9^{ab}
3	448.3 ± 1.5^{b}	95.0 ± 1.1^{bcd}	147.7 ± 3.1^{ab}
4	451.2 ± 3.2^{b}	98.5 ± 0.3^{d}	151.1 ± 2.2^{b}
6	450.9 ± 6.4^{b}	98.1 ± 0.4^{cd}	151.0 ± 1.6^{ab}
8	445.7 ± 3.8^{b}	98.1 ± 0.3^{cd}	148.7 ± 2.7^{ab}
10	445.4 ± 2.2^{b}	97.8 ± 0.8^{cd}	148.2 ± 4.0^{ab}
12	445.8 ± 3.1^{b}	96.1 ± 0.4^{bcd}	147.5 ± 2.8^{ab}

** Calculated on absolute dry matter content. Different superscript letters indicate significant differences ($p < 0.05$).*

Figure 3.17. Chitosan was swelled in 70% EtOH/H_2O for 4 h. Chitosan before swelling (a) and chitosan after swelling (b) under the microscope (10x).

3.3.3. Effects of lactic acid concentration

Chitosan, after soaking in 70% EtOH/H_2O medium for 4 h, was reacted with lactic acid at different concentrations (0.5; 0.6; 0.7; 0.8; 0.9; 1.0, and 1.5 M). The results presented in Table 3.13 showed that the recovery efficiency and solubility of chitosan lactate increased with increasing lactic acid concentration. The chitosan lactate sample reacted with lactic acid concentration of 1M reached the highest solubility of 98.5% and recovery efficiency of 152%. There was no statistical difference when the lactic acid concentration increased to 1.5 M.

Table 3.13. Effects of lactic acid concentration on the solubility and recovery efficiency of chitosan lactate

Acid lactic concentration (M)	Solubility (%)*	Recovery efficiency (%)*
0.5	56.2 ± 2.6^{a}	114.4 ± 6.5^{a}
0.6	72.8 ± 2.7^{b}	132.0 ± 4.4^{b}
0.7	83.2 ± 2.7^{c}	140.5 ± 1.1^{c}
0.8	92.6 ± 1.6^{d}	141.7 ± 1.2^{c}
0.9	95.4 ± 0.8^{de}	144.0 ± 1.6^{c}
1.0	98.5 ± 0.2^{e}	151.8 ± 1.8^{d}
1.5	98.8 ± 0.1^{e}	151.5 ± 1.9^{d}

** Calculated on absolute dry matter content. Different superscript letters indicate significant differences ($p < 0.05$)*

3.3.4. Effects of reaction time

Table 3.14 presents the effects of reaction time on the solubility and recovery efficiency of chitosan lactate. The solubility and recovery efficiency of chitosan lactate increased during the first 2.5 h of reaction time, and the both remained unchanged. The solubility and recovery efficiency of chitosan lactate at 2.5 h reaction were of 99.5% and 152.0%, respectively.

Table 3.14. Effects of reaction time on the solubility and recovery efficiency of chitosan lactate

Reaction time (h)	Solubility (%)*	Recovery efficiency (%)*
0.5	97.7 ± 0.5^{a}	150.8 ± 0.4^{a}
1.0	98.2 ± 0.5^{ab}	151.1 ± 2.0^{a}
1.5	98.5 ± 0.2^{ab}	151.8 ± 1.8^{a}
2.0	99.4 ± 0.3^{b}	151.7 ± 2.8^{a}
2.5	99.5 ± 0.3^{b}	152.0 ± 1.4^{a}
3.0	99.5 ± 0.3^{b}	151.8 ± 0.2^{a}

** Calculated on absolute dry matter content. Different superscript letters indicate significant differences ($p < 0.05$).*

3.3.5. Proposing the process for chitosan lactate production

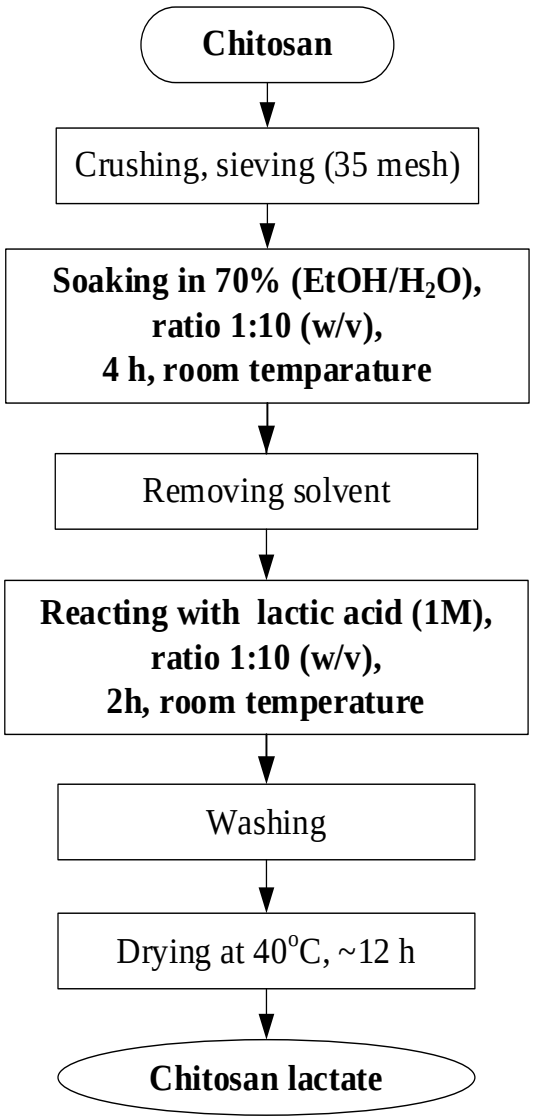


Figure 3.18. Proposed process for chitosan lactate production.

Chitosan lactate was produced from chitosan prepared from moulted shrimp shells, as shown in Figure 3.18. Chitosan was crushed, sieved with 35 mesh size, and soaked in an EtOH/H_2O 70% at the chitosan/solvent ratio of 1:10 (w/v) for 4 h at room temperature. After that, the swollen chitosan was reacted with 1.0 M lactic acid in EtOH (1:10. v/v) and stirred for 2 h at room temperature. Excess acid was removed by washing

twice with 85% EtOH (10 min/time) and soaking in EtOH 99.5% (30 min). The chitosan lactate was dried at 40°C until the moisture content reached 9 - 10%.

The physicochemical properties of obtained chitosan lactate were compared with the chitosan produced by Vietnam Food Company (Vietnam) and the commercial product of the MarkNature company (USA) (Table 3.16).

Table 3.16. Physicochemical properties of chitosan lactate from different sources

Parameters	Chitosan lactate from		
	Moulted shrimp shells	VNF company	MarkNature company
Viscosity (cPs)	105 ± 2[b]	172 ± 18[c]	36.3 ± 5.2[a]
Solubility (%)	99.4 ± 0.3[b]	96.0 ± 0.9[a]	99.6 ± 0.2[b]
Zeta potential (mV)	99.3 ± 1.6[b]	91.7 ± 2.9[a]	99.1 ± 1.9[b]
Recovery efficiency (%)	151.7 ± 2.8	143.6 ± 1.1	-
Appearance	Light yellow	Light yellow	Light yellow
Color of 1% chitosan lactate	White, clear	White, clear	Light yellow, clear
Moisture (%)	9.5 ± 0.6[a]	9.2 ± 0.7[a]	8.8 ± 0.2[a]
pH of 1% chitosan lactate	4.7 ± 0.2[a]	4.7 ± 0.3[a]	4.7 ± 0.2[a]

** Results are calculated on absolute dry weight; (-) No analysis. Different superscript letters indicate significant differences ($p < 0.05$).*

The results presented in Table 3.16, Figure 3.20 and Figure 3.21 show that the physicochemical properties of chitosan lactate obtained from moulted shrimp shells were comparable to the chitosan lactate from VNF company (prepared from processed shrimp shells) and commercial chitosan lactate salt (MarkNature company).

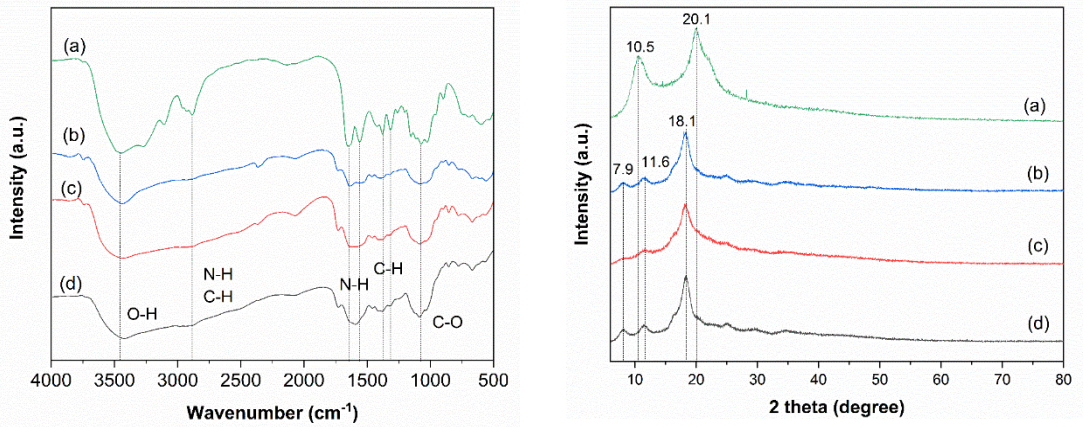


Figure 3.20 (A) FTIR spectra and **Figure 3.21** (B) XRD patterns of (a) chitosan and (b) chitosan lactate from moulted shrimp shells and (c) chitosan lactate from processed shrimp shells produced by Vietnam Food Company, (d) commercial chitosan lactate from Mark Natural Company.

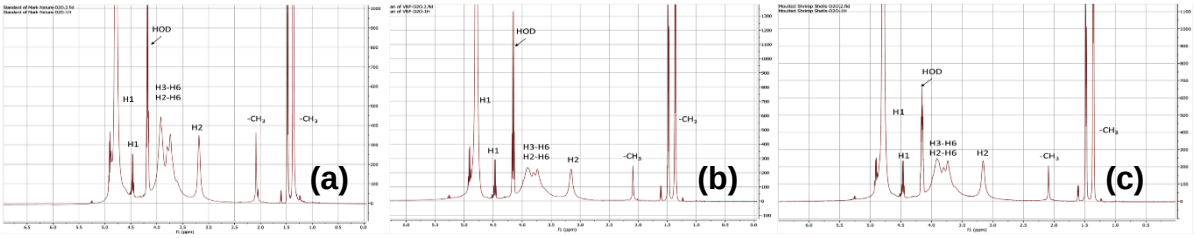


Figure 3.22. ^{1}H-NMR spectrum of commercial chitosan lactate from (a) Mark Natural Company, **Figure 3.23.** ^{1}H-NMR spectrum of chitosan lactate from (b) processed shrimp shells produced by Vietnam Food Company and **Figure 3.24.** ^{1}H-NMR spectrum of chitosan lactate from (c) moulted shrimp shells.

The results of FTIR spectrum analysis (Figure 3.20) confirmed the formation of chitosan lactate salt when using chitosan prepared from moulted shrimp shells. When compared with commercial chitosan lactate and chitosan lactate prepared from processed shrimp shells, no significant differences were observed in the number, location and intensity of peaks.

3.4. Application of chitosan lactate for flocculating *Nannochloropsis* sp.

3.4.1. Properties and composition of *Nannochloropsis* sp.

Nannochloropsis sp. with a size of 2 - 4 µm was propagated and stored at the Centre for Aquatic Animal Health and Breeding Studies, Nha Trang University. The microalgae sample had a cell density of 37.3 x 10^6 ± 2.6 (cell/mL), a green colour. The zeta potential was -6.1 ± 1.5 (mV), indicating that *Nannochloropsis* sp. had a negative surface charge. *Nannochloropsis* sp. contained a high content of carbohydrates (about 32.3%). The protein, lipids, and mineral contents were about 26.9%, 16.2% and 8.2%, respectively. The chlorophyll-a and carotenoids contents were 10369 ± 174 µg/g and 7368 ± 129 µg/g, accordingly.

3.4.2. Effects of chitosan lactate concentration

To date, no studies have been carried out on the application of chitosan lactate to harvest microalgae. Chitosan lactate is considered safe and can be dissolved directly in water. Chitosan lactate has been applied in in flocculation technology for fruit juice, wine, tea, etc. From that basis, chitosan lactate (CTSs) is proposed as a flocculant to harvest microalgae, particularly marine microalgae *Nannochloropsis* sp. The influences of chitosan lactate concentration on the flocculation efficiency and morphology of the obtained microalgae are presented in Figure 3.25.

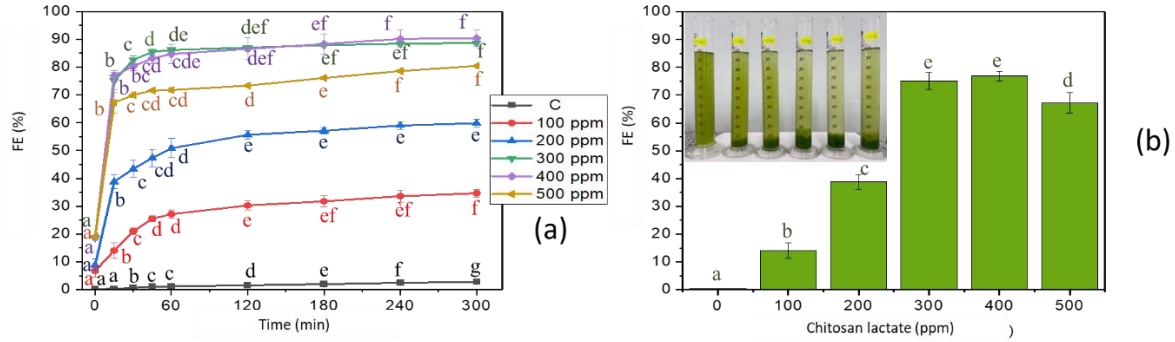


Figure 3.25. Effects of chitosan lactate concentration on flocculation efficiency (a) at settling time of 15 min (b). C sample: control.
Different letters indicate significant differences ($p < 0.05$).

Results presented in Figure 3.25a show that the samples treated with 300 and 400 ppm of CTSs had higher flocculation efficiencies (75 - 77%) compared to other samples throughout the settling process. At the settling time of 15 min, significant differences in flocculation efficiency (FE) between samples were observed (Figure 3.25b). The FE increased with increasing chitosan lactate concentration from 0 ppm to 400 ppm, but significantly decreased when CTSs concentration increased to 500 ppm. No significant difference in FE of the samples treated with 300 ppm and 400 ppm CTSs. Therefore, to save chitosan lactate, the concentration of 300 ppm was chosen for further investigation.

3.4.3. Effects of a combined method

The combination of chitosan lactate and pH adjustment aimed to achieve higher flocculation efficiency and to reduce disadvantages compared to using each method individually. Previous studies have demonstrated that the surface charge of microalgae and chitosan molecules varies with environmental pH and the optimal pH varies depending on the algae species. Results on the effects of environmental pH adjustment with different concentrations of CTSs are presented in Figure 3.26 and Figure 3.27.

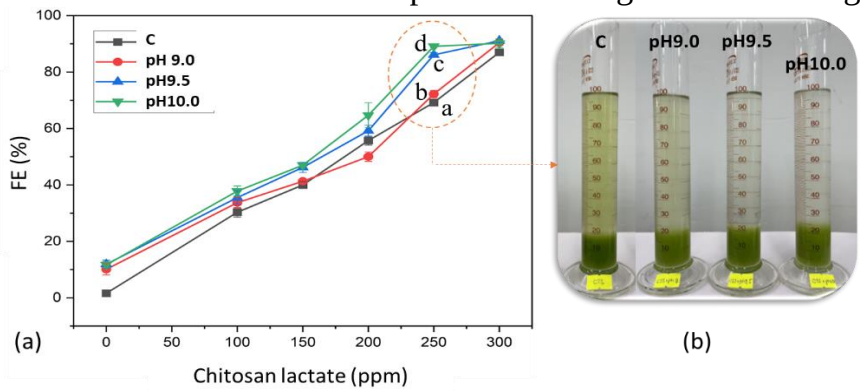


Figure 3.26. Effects of chitosan lactate concentration và pH on flocculation efficiency (a) *Nannochloropsis* sp. using CTSs 250 ppm and adjusted pH (b) after 120 min.
Different letters indicate significant differences ($p < 0.05$).

Figure 3.26 shows that at the settling time of 120 min, higher flocculation efficiency (FE) was observed in the samples treated with higher CTSs concentrations and higher pH values. The FE reached the highest value of 90.2% at the CTSs concentration of 250 ppm and pH 10. However, at CTSs concentration of 300 ppm, there was no significant difference in FE between the samples treated at different pHs. Thus, the suitable conditions for flocculating microalgae *Nannochloropsis* sp. were 250 ppm chitosan lactate used in combination with pH adjustment to 10.

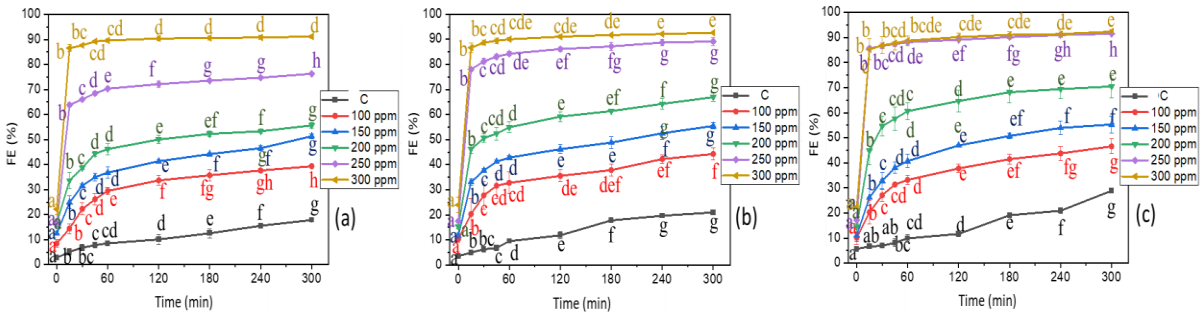


Figure 3.27. Effects of the combined method using chitosan lactate and pH adjustment (a, pH 9.0), (b, pH 9.5), (c, pH 10.0) on flocculation efficiency by harvest time. C sample: control.
Different letters indicate significant differences ($p < 0.05$).

Figure 3.27 shows that the chitosan lactate concentration and pH value affected the flocculation efficiency.. The higher the concentration of CTSs used resulted in the higher microalgae flocculation efficiency, reaching the highest value of over 90% after 5 h. When treated with CTSs concentrations lower than 300 ppm, the FE increased with an increased pH value. However, there was no significant difference in flocculation efficiency when treated with CTSs concentration of 300 ppm at different pH values ranging from 9.0 – 10.0.

Comparison of the flocculation efficiency of microalgae *Nannochloropsis* sp.

using different types of CTSs is shown in Table 3.18 and Figure 3.29. The FE of the sample treated with chitosan lactate from the MarkNature company was significantly lower than that of other samples. There was no significant difference in FE when using chitosan lactate prepared from moulted shrimp shells and chitosan lactate from VNF company. Significantly higher FEs were observed in the samples treated with CTSs in combination with pH adjustment compared to the samples only treated with CTSs in all types of chitosan lactate used.

Table 3.18. Comparison of flocculation efficiency of microalgae *Nannochloropsis* sp. by different types of chitosan lactate (250 ppm) combined with pH adjustment to 10 after 15 min

Method	Flocculation efficiency (%)		
	CTSs from moulted shrimp shells	**CTSs from VNF**	**CTSs from MarkNature**
CTSs	58.8 ± 1.9^{b}	58.2 ± 2.4^{b}	52.5 ± 1.7^{a}
CTSs + pH 10.0	85.9 ± 0.5^{b}	87.5 ± 0.5^{c}	65.8 ± 0.7^{a}

** Values in the table with the same letter are not significantly different (p > 0.05).*

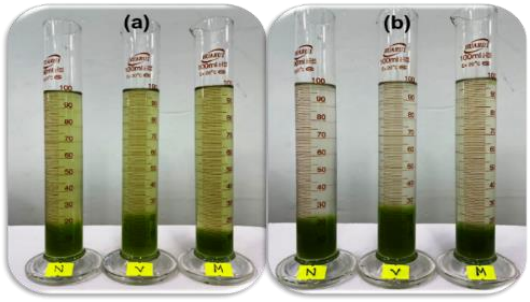


Figure 3.29. Effects of different types of chitosan lactate on the flocculation efficiency of *Nannochloropsis* sp. at the settling time of 15 min (a) only treated with chitosan lactate and (b) treated with chitosan lactate in combination with pH adjustment

Results presented in Figures 3.30a indicated that the flocculating method strongly affected the chlorophyll-a and carotenoids contents in microalgal cells, but did not affected the colour of mircoalgae cells (chloroplast pigment) (Figures 3.30b and 3.31).

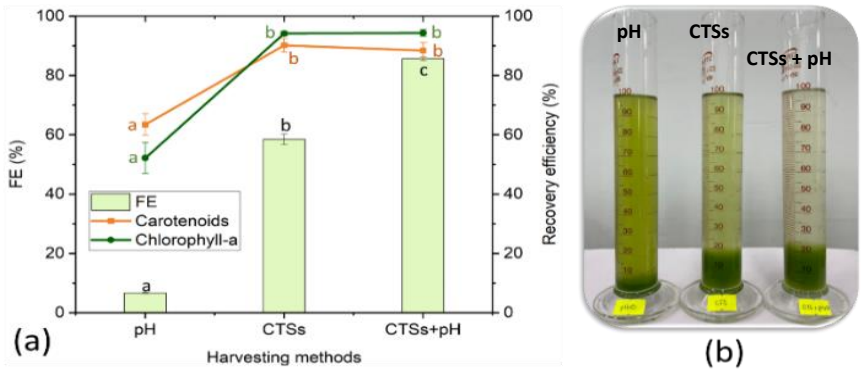


Figure 3.30. Effect of flocculating method on flocculation efficiency (FE). The relative recovery efficiency of pigments (chlorophyll-a and carotenoids) (a) and biomass of microalgae *Nannochloropsis* sp. collected by the difference (b) after 15 min of settling. *Different letters indicate significant differences (p < 0.05).*

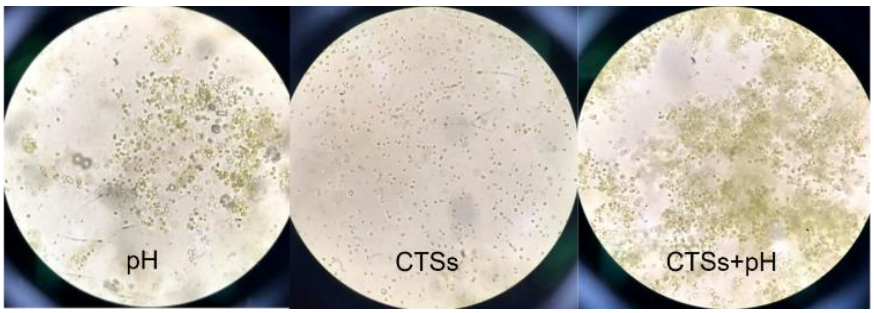


Figure 3.31. Cell morphology under a light microscope after 15 min of settling.

Table 3.19 and Figure 3.32 show the biomass of the microalgae *Nannochloropsis* sp. after harvest on the ability to grow and develop after 6 days of culture.

Table 3.19. The growth ability of harvested microalgae *Nannochloropsis* sp.

Culture time (day)	Cell density (cell/ml)	
	CTSs + pH 10	CTSs
1	$1.08 \times 10^6 \pm 0.04^a$	$1.29 \times 10^6 \pm 0.06^b$
6	$40.23 \times 10^6 \pm 1.8^a$	$41.63 \times 10^6 \pm 1.6^a$

Figure 3.32. The growth ability of microalgae flocculated by different methods after 1 day and 6 days of culture.

In conclusion, the chitosan lactate prepared from moulted shrimp shells was suitable for application in flocculation of microalgae *Nanochloropsis* sp. in combination with pH adjustment to 10.0. The flocculation efficiency was about 90% after 120 min, high recovery efficiencies of chlorophyll-a (~94%) and carotenoids (~88 – 90%).

CONCLUSIONS AND SUGGESTIONS

Conclusions

Based on the obtained results and analysis, some conclusions can be drawn out:

1. The moulted shells of *vannamei* shrimp (>60 days of culture) from intensive farming can be used to produce chitin and chitosan of commercial quality according to a process consisting of 3 main stages: (1) Demineralization with HCl 4% (1:5, w/v) at 30°C for 3 h, (2) Deproteinization with 3% NaOH (1:5, w/v) at 50°C for 6 h and (3) Deacetylation with 50% NaOH (1:5, w/v) at 90°C for 24 h. The proposed process has a high stability with chitin recovery efficiency of 32% and chitosan recovery efficiency of 23%.
2. Chitosan prepared from the moulted shrimp shells can be used to produce chitosan lactate by solid-liquid reaction method with a dilute lactic acid solution (1.0 M) in a process consisting of 2 main steps: (1) Soaking chitosan (35 mesh size) in 70% EtOH/H_2O for 4 h at room temperature, (2) The swollen chitosan (in solid state) reacted with 1.0 M lactic acid

(mixed in absolute ethanol) for 2 h at room temperature. The recovery efficiency was about 152% and the obtained chitosan lactate had a water solubility of over 99%, a viscosity of about 105 cPs.

3. Chitosan lactate prepared from moulted shrimp shells can be used as an effetive flocculant to harvest microalgae *Nannochloropsis* sp. The suitable flocculating conditions were 250 ppm chitosan lactate concentration in combination with pH adjustment to 10.0. The microalgae flocculation efficiency after 120 min was about 90% with high recovery efficiencies of chlorophyll-a (about 94%) and carotenoids (about 88-90%). The obtained microalgae cells were not broken.

Recommendations

Through the implementation of the thesis and based on the obtained results, the following recommendations are allowed:

- Continue to study the production of chitin, chitosan, and chitosan lactate from moulted shrimp shells at a pilot scale.
- Applying chitosan lactate to harvest microalgae in biomass ponds.

LISTS OF PUBLICATIONS

1. **Phạm Thị Đan Phượng**, Đỗ Quốc Dũng, Nguyễn Công Minh, Trang Sĩ Trung, Huỳnh Nguyễn Duy Bảo, Nguyễn Văn Hòa (2021). "Sản lượng vỏ tôm lột xác trong quá trình nuôi thâm canh và thử nghiệm sản xuất chitin". *Tạp chí Khoa học – Công nghệ Thủy sản*, 3, 50-56.
2. **Pham Thi Dan Phuong**, Trang Si Trung, Willem F. Stevens, Nguyen Cong Minh, Huynh Nguyen Duy Bao, Nguyen Van Hoa (2022). "Valorization of heavy waste of modern intensive shrimp farming as a potential source for chitin and chitosan production". *Waste and Biomass Valorization*, 13(2), 823-830.
3. Trang Si Trung, **Pham Thi Dan Phuong,** Nguyen Cong Minh, Nguyen Thi Nhu Thuong, Witoon Prinyawiwatkul, Huynh Nguyen Duy Bao, Nguyen Van Hoa (2022), "Swollen-state preparation of chitosan lactate from moulded shrimp shells and its application for harvesting marine microalgae *Nannochloropsis* sp.", *International Journal of Biological Macromolecules, x(xx), xxx.* (Under review).