UNIVERSITÀ POLITECNICA DELLE MARCHE

FACOLTÀ DI INGEGNERIA

Corso di Laurea Magistrale in Environmental Engineering

CHARACTERIZATION AND POTENTIAL USE OF MOLLUSC BY-PRODUCTS AS BIOFERTILIZER

Relatore:

Prof. Francesco Fatone

Correlatora:

Prof. Anna Laura Eusebi

Tesi di laurea di**:**

Santoshi Gedela

Anno Accademico

(2021-2022)

Abstract

Seafood is one of the essential sources of nutrients and bioactive compounds for human consumption throughout the world. The literature review reveals that the processing of the seafood industry is leading to the generation of a tremendous quantity of by-products and discards annually. The production of seafood by-product/discards waste volume by 30-70% of the whole seafood after industrial processing. The crucial problems associated with the seafood processing industry are waste disposal, and improper waste management of seafood waste can cause negative impacts on the environment and human health. So far, the utilization of seafood wastes was confining at a relatively lower level due to the lack of inadequate knowledge.

The non-edible residues coming from the seafood processing industry contain an appreciable number of biomolecules such as minerals, proteins, polysaccharides (chitin), carotenoids, vitamins, polyunsaturated fatty acids, peptides, collagen, glycosaminoglycans, and lipids. The acknowledged high-value compounds in the seafood waste are still untapped because of the lack of appropriate management. Recovering those biomolecules can be a great way to improve global food security and mitigate environmental problems associated with seafood by-products/discards. However, the concepts of circular economy, sustainability, and green chemistry encourages the vaporization of seafood by-product waste into value-added chemicals and materials as a sustainable alternative compared to the exploitation of more conventional resources.

The work carried out in this master thesis is a part of an international research activity funded under Horizon 2020 (Project "**SEA2LAND** - Producing advanced bio-based fertilizers from fisheries wastes") and focused on the characterization and pre-treatment of waste from the fishing industry to produce bio-fertilizers.

The sub-objective of the **SEA2LAND** project mainly focused on the Process leftover that comes from **CO.PE.MO.** (Seafood processing industry) is in the port of Ancona, Italy. **CO.PE.MO** produces 1-3 tons/day of seafood wastes/by-products and is majorly composed of mollusc shell waste, such as mussel, clam, and murex. They have three major separate lines of seafood processing waste for mollusc shells, such as mussel, clam, and murex. Apart from those three lines, they mix all the three-line waste in one container. In the original seafood processing, they have separated the organic fraction from each kind of shell. In addition to the mainline, they also separated waste shells that are not useable for seafood

processing. In my research, I collected the available samples from three lines processed by the **CO.PE.MO**.

This research aimed to study the Sampling and characterization of raw waste, settling tests, bio methanation potential, Pre-treatments for inorganic and organic fractions from raw waste, and chemical extraction of chitin on the mollusc (mussel, clam, and murex) by-products that come from the **CO.PE.MO** (Seafood processing industry). In the stage of sampling and characterization, the raw samples of mussel, clam, and murex were carried out on the shredding pump test to crush the samples and separate the solid(shell) and organic parts from each waste. The shredding test was conducted based on the different dilution factors [total waste (water + sample) (kg) /sample (kg)] of each sample, which includes mussel (5, 3, and 2), clam (3, 2.47, 2.22, and 2), and murex (4.14, 2.57, and 2). After the shredding test, we have the solid part (shell) at the bottom and the liquid portion, which included a little organic (meat part) at the top, to be measured. The Imhoff cone or settling test was conducted on the liquid waste (from the shredding pump) to observe the settleable characteristic for 45 minutes of a period. The settling fractions from each sample from the Imhoff cone test and sloid part (shell) were carried out for laboratory analysis to determine the moisture, dry matter, ashes content. The carried-out mass balances for each sample obtained from the shredding pump test with different dilution factors I acknowledged before to check the best operating condition. The bio methanation potential (BMP) test conducted on the liquid portion includes a small contribution of the organic part (meat part) from the shredding pump test to check the biogas production.

The initial step of pre-treatments was to separate the organic (meat) and inorganic(shell) parts from the mussel, clam, and murex waste by cooking the waste sample. In addition to the mussel, clam, and murex waste, mixed waste was considered raw waste (without separating the organic and inorganic fractions). The waste characterization was carried out for both mussel, clam, and murex waste on the shell and organic (meat) parts and mixed waste (considered only raw waste) by both proximate and elemental analysis in the laboratory. The analysed waste characterization determines the waste compositions in terms of the moisture, dry matter, ashes, nutrients, cations, and heavy metals contents in percentage on both shell and organic parts.

FTIR analysis was carried out on shell waste (manually separated the shell and meat part from the raw waste) of mussel, clam, and murex to understand the compounds of chitin, protein, and CaCO3. In addition, FTIR analysis was carried out on the commercial chitin to make a

spectrum as a reference to understand the removal of impurities, such as protein and minerals from our shell waste.

The chitin extraction process was conducted only on the shell part (manually separated the shell and organic (meat) part from the raw sample) of mussel, clam, and murex by using chemical methods. In this project, chitin extraction was followed mainly by the three-step process, which includes pre-treatments, deproteinization (DM), and demineralization (DM). In the pre-treatment stage, the manually separated shells of the mussel, clam, and murex were washed with tap water and then dried in the oven at a temperature of 35℃ for 24 hours. The deproteinization process aims to eliminate the protein content was processed out by the 1M NaOH solution [40 grams NaOH (solid) dissolved in one litter of water]. In this project, the deproteinization step was carried out based on the different operating conditions, which are different chemical dosages of NaOH (10, 15, and 20 ml) per one gram of sample and durations of (1, 2, and 3 hours) at temperature 70℃. To observe the maximum deproteinization yield for different chemical dosages in terms of weight loss percentage. In the deproteinization step, the completion of the process controlled with the solution reaches the steady-state ph. The demineralization process was carried out on the deproteinized sample to remove the mineral content (CaCO3). The demineralization step was proceeded by 1M HCl (83 ml concentration of HCL added to one litter of water) solution. The demineralization step was conducted based on the different chemical dosages of (10 and 20 ml) per one gram of sample and durations of (1, 2, 2.33, and 4 hours) at ambient temperature (approximately at 30℃). To observe the maximum demineralization yield in terms of weight loss percentage. In the demineralization step, the completion of the process controlled with the solution reaches the steady-state ph.

In addition, the deproteinization and demineralization processes were carried out separately on the fresh samples. The end processes of both DP and DM controlled with the solutions reach until the pH constant. The samples obtained from the deproteinization, and demineralization process were carried out by FTIR analysis. And then correlate the spectra with reference spectra of commercial chitin we declared before to observe the protein and CaCO3 removal.

Acknowledgments

I would like to thank the **Università Politecnica Delle Marche** for affording me the valuable opportunity to pursue my master's course here.

Besides, I would like to express my sincere gratitude to my supervisors **prof. Francesco Fatone and prof. Anna Laura Eusebi** for the continuous support of my thesis, for their patience, motivation, and immense knowledge.

My appreciation also extends to my secondary supervisors **……** for their valuable comments, mentoring, and encouragement.

Last but not the least, I want to thank all the professors and tutors that kindly support me during this master course.

Dedication

I dedicate my dissertation work to my Family and specially to my supportive mother and father. A special feeling of gratitude to my love, Venky whose words of encouragement and push for tenacity ring in my ears.

Table of contents

Contents

List of figures

List of tables

Chapter 1: Introduction

1.1. Statement of problem:

Demand for good quality protein is increasing throughout the world for food and proper health benefit applications. Proteins from seafood products are well recognized to possess good nutrition characteristics. The demand for seafood is rising everywhere, driven by an increase in population and awareness of the health benefits associated with its consumption (Sasidharan & Venugopal, 2020).

Seafood is one of the major primary sources of nutrients and bioactive compounds for human consumption. The growing demand for seafood consumption had led to an increase in seafood production. The global seafood production in the year between 2004 to 2018 was 134.3 MT (2004) to 178.5 MT (2018). At the same period, the global aquaculture capture production has increased from 41.9 to 96.4 MT (FAO, 2018, 2020). In general, seafood includes finfish species, mollusc shells (mussels, oysters, clams, squid, etc.), and crustacean shells (crab, prawns, lobster, and shrimp, etc.), which can be harvested from marine, freshwater, and estuaries habitat. Based on literature, the processing of the seafood industry leads to the generation of a tremendous quantity of by-products and discards. The growth of seafood production has led to an increase in the by-products volume, which can count for 30–70% of whole seafood after industrial processing (Bruno et al., 2019).

The different seafood by-products produced by shellfish industries in Italy are referred to in 2016 based on the EUMOFA database as shown in Table 1.1 and Table 1.2 (Nisticò, 2017).

Table 1-1. Volumes (expressed in tons) of the shellfish industry products in the EU for the year 2016 obtained from the European Market Observatory for Fisheries and Aquaculture products (EUMOFA) database: mollusc (Cephalopods and bivalves)

Cephalopods					Bivalves				
Italy	Cuttlefish (Sepia)	Octopus	Squid	other Cephalopods	Clam (Chamelea)	Scallop (Pecten)	Mussel (Mytilus)	Oyster (Ostrea)	other Mussels
volume in (tons)	24,058.39	63,884.13	101342.40	4723.3	19,315.10	8248.27	46,364.11	6368.3	11,612.40

Table 1-2. Volumes (expressed in tons) of the shellfish industry products in the EU for the year 2016 obtained from the European Market Observatory for Fisheries and Aquaculture products (EUMOFA) database: crustacean

crustacean									
Italy	European Lobster (Homarus)	Norway Lobster (Nephrops)	Red Lobster (Palinurus	European Crayfish (Astacus)	King/Striped Prawn (Aristaeomorpha/Penaeus)	Brown Crab (Cancer)	Common Shrimp (Cragon)	other Shrimps	other Crustaceans
volume in (tons)	4481.49	10,223.54	1242.9	6154.3	33,482.08	4161.79	392.46	41,823.06	2610

The crucial problems associated with the seafood processing industry are waste disposal. So far, the utilization of seafood wastes was confining at a relatively lower level due to the lack of inadequate knowledge (Hamed et al., 2016; Kocaman et al., 2016). The non-edible residues that come from the seafood processing industry contain an appreciable amount of biomolecules or valuable compounds such as minerals, proteins, polysaccharides (chitin), carotenoids, vitamins, glycosaminoglycans, peptides, collagen, polyunsaturated fatty acids, and lipids. The acknowledged high-value compounds in the seafood waste are still untapped because of the lack of appropriate management. In the past decades to now, seafood processing waste (by-products and discards) was treated as unwanted material and used as animal feeds, fertilizers, or discarded about (30-70%) in the environment can cause negative impacts on the environment and human health (Bruno et al., 2019; Sasidharan & Venugopal, 2020).

The work carried out in this thesis mainly focused on mollusc waste, which includes mussel, clam, and murex. The mollusc waste was analyzed by the pre-treatments, characterization, settling test, bio methanation potential, and chitin extraction on the three samples. Based on the literature, a few studies have investigated the waste characterization of mollusc waste. Moreover, they carried out waste characterization majorly on the proximate analysis. There is no proper investigation on the heavy metals, cations, and nutrients compositions of waste. However, to the best knowledge of me no study investigates the murex waste characterization and chitin extraction phenomena. There is a lack of information on the use of different chemical dosages, temperatures, and particle sizes that can influence the chitin yield. There is no appropriate information on the process control during the processes of deproteinization and demineralization (Abdulkarim et al., 2013; Alabaraoye et al., 2018; Danarto & Distantina, 2016; Harmami et al., 2019; Idacahyati et al., 2020; Ni'mah et al., 2019).

Therefore, to fill this research gap, this thesis aims to carry out the complete waste characterization of mussel, clam, and murex waste to analyse the heavy metals, cations,

nutrients on both fractions of organic (meat) and inorganic (shell). In addition, to control the deproteinization and demineralization with different chemical dosages and durations.

1.2. Importance and necessity of study

Global fish production (from capture fisheries and aquaculture) is projected to increase from 178.5 MT in the base period of (2017-2019) to 200 MT by 2029. By 2029, 58% of the fish available for human consumption projected to originate from aquaculture, up from 53% in (2017-19) (OECD/FAO, 2020). Based on the above acknowledgment, the seafood by-products will increase soon, and it is necessary to treat the seafood waste into valuable material.
Modern seafood processing industries result in the amassment of a large volume of by-products. The non-edible residues contain a considerable amount of biomolecules such as proteins, polysaccharides, lipids, carotenoids, vitamins, minerals, etc. The acknowledged high-value compounds in the seafood waste are still untapped because of the lack of appropriate management. Recovering those biomolecules can be a great way to improve global food security and mitigate environmental problems associated with seafood by-products/discards. Moreover, inadequate disposal of waste also has negative impacts on both environment and human health (Bruno et al., 2019; Yadav et al., 2019).

In the field of study, renewable energy resources have been increased in the last decades due to the high economic growth of interests in the valorization of sustainable feedstocks that are useful for obtaining valuable chemicals and materials. In the past studies, mainly focused on the valorization of both agricultural and municipal wastes are used as a starting material for the production of new material and chemicals (Nisticò, 2017). So far, the utilization of seafood wastes was confining at a relatively lower level due to the lack of inadequate knowledge. The conversion of seafood by-products into high-value components can pave the way for the complete valorization of seafood discards and by-products, increase the limited resources and provide solutions to the associated environmental problems (Bruno et al., 2019).

The process leftovers from seafood industries fractionate into solid waste and few contributions of the organic fraction. Solid fractions consist of shell waste (mollusc and crustacean shell), and the organic fraction contains (remaining shell meat and seaweed). The present study only focused on mollusc processing waste, which includes the main chemical components of **chitin**, **protein**, and **minerals**. Most industries use these chemicals as a primary chemical. The recovery of chitin from shell waste is important instead of dumping it or disposal of the way back into the ocean (Hülsey, 2018). Seafood waste utilization will create benefits for the

environmental and economic points of view (Yang et al., 2019; Nisticò, 2017; Morris et al., 2019; COM, 2015).

The sources of chitin come from exoskeleton waste of molluscs (mussels and oyster shells), crustacean shells (prawn, crab, shrimps, and lobsters), fish scales (silver and pang), insects, the walls of fungi, and microorganisms. Chitin is commonly mixed with protein and minerals (Alabaraoye et al., 2018; Abdulkarim et al., 2013)**.** The crustacean shells include prawns, crab, shrimp, and lobster (Yadav et al., 2019). Both insects and crustacean shells contain a relatively high amount of pure chitin mixed with proteins and minerals ($CaCO_3$) (Hülsey, 2018)**.**

Based on the sources, chitin in nature is presents mainly in three different types of crystalline forms: α, β, and γ. α chitin occurs in the shrimp shells, lobsters, cell walls of fungi, crab, prawn, and shellfish. In α chitin, the polysaccharide (biopolymer) chains have been structured in an anti-parallel orientation which allows maximum bonds. The α chitin is the most stable form of chitin in nature results in chitin fibrils with high crystallinity index of 80%. The β chitin has been found in the squid pens, tubes synthesized by vestimentiferan worm and pogonophoran. β chitin is rarely associated with protein content in the tubes synthesized by the pogonophoran, vestimentiferan worms, and squid pens. The β chitin arrangement of the polymer chain is in the parallel orientation, with the crystallinity index of chitin fibrils is about 70%. γ chitin is the combination of α- and β chitin-type arrangement in which two similar polymer chains are arranged alternatively with one anti-parallel chain (Rinaudo, 2006); (Yadav et al., 2019)**.**

Proteins are nutrient-rich and mainly suitable for fertilizer production and feedstock for animals. The application of calcium carbonate ($CaCO_3$) is utilizing in various industries as pigments and fillers material. Chitin is a natural biopolymer material that is associated with many excellent properties such as biodegradability, ability to form film, biocompatibility, and non-toxicity. The main applications of chitin can find in different industries such as food, pharmaceutical, cosmetics, biochemicals, and agriculture (Hülsey, 2018; Yang et al., 2019; Alabaraoye et al., 2018).

1.3. Objective and Scope of the work

The concepts of circular economy, sustainability, and green chemistry encourages the valorization of seafood by-product waste into value-added chemicals and materials as a sustainable alternative compared to the exploitation of more conventional resources (Nisticò, 2017).

The main objectives and scope of the thesis are to provide a clear and comprehensive study on the utilization of mollusc biowaste into value-added chemicals and materials based on the concept of environment, land management, health, circular economy, green chemistry, and sustainability.

The work carried out in this master thesis is a part of an international research activity funded under Horizon 2020 (Project "**SEA2LAND** - Producing advanced bio-based fertilizers from fisheries wastes") and focused on the characterization and pre-treatment of waste from the fishing industry for the production of bio-fertilizers.

The sub-objective of the **SEA2LAND** project mainly focused on the mollusc shells that come from CO.PE.MO, Ancona, Italy. They have three major separate lines of seafood processing waste for mollusc shells, such as mussel, clam, and murex. Apart from those three lines, they mix all the three-line waste in one container. In the original seafood processing, they have separated the organic fraction from each kind of shell. In addition to the mainline, they also separated waste shells that are not useable for seafood processing. In my research, I collect the available samples from different lines processed by the CO.PE.MO.

In my thesis work, I mainly focused on the characterization and pre-treatments for different lines of mollusc waste. Below are the tasks I performed on the lab.

a) Sampling and characterization of raw waste
b) Pre-treatment for inorganic and organic separation from raw waste
c) Chemical extraction of chitin from mollusc shell
d) Settling tests
e) BMP tests

1.4. Preface

This study is structured as follows: Chapter 2 shows the overview of Mollusc by-products characterization, Chitin extraction methods from seafood processing, FTIR analysis, bio methanation potential (BMP), and Protein hydrolysates recovery from seafood processing. Chapter 3 represents the material and methodology followed in this study, which includes the

characterization of biowaste by both elemental and chemical analysis based on percentages of dry matter and raw waste. In the shell waste, the chitin extraction process can be carried out by using chemical methods with the following stages of deproteinization and demineralization. In addition to chitin extraction, the process optimization is conducting on various operating conditions(such as different chemical dosages and temperatures) that can influence the yield of the DP, DM, and chitin. The FTIR analysis spectra was analyzed on the raw samples and commercial chitin as reference spectra. In the chitin extraction process, samples that came from the deproteinization and demineralization stages were analyzed based on the FTIR analysis to make individual spectra related to DP and DM. And then, to make a correlation between both deproteinized and demineralized FTIR spectra with the original spectra of the raw sample. To check the best efficient removal of both protein and CaCO3 obtained in which chemical dosage and temperature. Finally, FTIR analysis was conducted on the final product of chitin and then compared the chitin spectra with commercial chitin spectra. The results and discussions are given in Chapter 4, and Chapter 5 contains the summary of this study and the conclusions.

Chapter 2: State of Art

2.1. Introduction

In this chapter, we tried to review the available literature that deals with mollusc by-products. This chapter provides a detailed study on the following topics, which mainly include:

1. Mollusc by-products characterization
2. Chitin extraction methods from mollusc shells
3. FTIR analysis
4. Bio methanation potential (BMP)

2.2. Mollusc by-products characterization

In this topic, the literature review was carried out on the characterization of molluscs (such as mussel and clam) by-products. The waste characterization of mussel shells was summarized on the different fractions (shell and meat). Moreover, those fractions were carried out based on a dry and wet basis. The clam characterization was specified in the shell part on a dry basis only. However, no proper study investigates the murex waste characterization.

Several studies mentioned that mussel shells contain 95 to 99% percent of the weight as CaCO3 and organic matrix, making this material an excellent potential alkalinity source for treatment systems (Chakraborty et al., 2020; Hamester et al., 2012; Lertwattanaruk et al., 2012; Uster et al., 2014). Based on the richest source of biogenic CaCO3, shell wastes are suitable to prepare high purity CaCO3 powders, which have been extensively applied in various industrial products such as paper, rubber, paints, and pharmaceuticals. Moreover, some authors explained that the composition of mollusc shells is 95% to 99% calcium carbonate and (0.1-5%) organic matrix (Barros et al., 2009; Krutof et al., 2020; Naik & Hayes, 2019; Zhang & Zhang, 2006). Therefore, mussel shells are a low-cost source of CaCO3 where mussel aquaculture is practised.

Table 2-1. represents the waste composition of mussel shell

Reference		**(Naik & Hayes, 2019)**	**(Martínez-García et al., 2017)**	**(Iriani et al., 2020)**	**(Buasri et al., 2013)**
Waste source		blue mussel shell (Mytilus edulis	mussel shell pre-heated at 135°C for 30 min. (dry matter)	freshwater mussel shells were dried at 60°C for 6 hours. (Dry matter)	The shells were dried in an oven. The dried waste shells were calcined at 700–1,000◦ C in an air atmosphere with a heating rate of 10 C/min for 4 hours.
Test method	-		XRF	AOAC	
parameters	value				
Dry matter	wt%				
moisture	wt%	5.09		0.41	
volatile matter	wt%	95-99	2.15	93.01	
ash	wt%	0.1-5		3.44	
protein	wt%			0.18	
fat	wt%			2.97	
carbohydrate	wt%			1.80	
crude fiber	wt%			61.39	
Ca	wt%				
Cao	wt%				98.37
CaCO3	wt%		94.66		
SiO2	wt%		2.58		
Al2O3	wt%		0.01		
Fe2O3	wt%		0.01		
K2O	wt%		0.01		
MgO	wt%		0.28		
Na2O	wt%		0.51		0.94
SO3	wt%		0.31		0.29
SO4	wt%				
SrO	wt%		0.19		0.16
K	mg/kg				
Mg	mg/kg				
Na	mg/kg				
P	mg/kg				
Al	mg/kg				
Cd	mg/kg				
Cl	wt%		0.01		0.04
Cr	mg/kg				
Cu	mg/kg				
Fe	mg/kg				
Pb	mg/kg				
Zn	mg/kg				

Table 2-2. represents the waste composition of mussel shell and mussel meat

Table 2-3. represents the waste composition of clam shell

Reference		**(Barbachi et al., 2019)**	**(Barros et al., 2009)**	**(Tavares et al., 1998)**	(Bernárdez & Pastoriza, 2011)
Waste source		Mussel shell (dry matter)	Mussel shells were dried at 190℃ for 18 min and then calcining at 500℃ takes 15 min. (dry matter)	Mussel meat (dry matter)	Live Mussel meat (dry matter)
Test method	-				AOAC
parameters	value				
Dry matter	wt%				
moisture	wt%	0.674	0.79	72.12	
volatile matter	wt%	5.7			
ash	wt%			2.42	8.9
protein	wt%			20.50	59.8
fat	wt%			3.24	
carbohydrate	wt%			1.70	24.9
crude fiber	wt%				
Ca	wt%	77.029			
Cao	wt%				
CaCO3	wt%	94.42	>95.00		
SiO2	wt%	1.22			
Al2O3	wt%		<0.1		
Fe2O3	wt%		<0.1		
K2O	wt%		<0.1		
MgO	wt%				
Na2O	wt%				
SO3	wt%				
SO4	wt%				
SrO	wt%				
K	mg/kg				
Mg	mg/kg	2080			
Na	mg/kg	16220			
P	mg/kg				
Al	mg/kg	310			
Cd	mg/kg	670			
Cl	wt%	0.247			
Cr	mg/kg				
Cu	mg/kg				
Fe	mg/kg	200			
Pb	mg/kg				
Zn	mg/kg				

Reference		**(Lertwattanaruk et al., 2012)**	(Ademolu, Akintola, Olalonye, et al., 2015)	(Ademolu, Akintola, Olalonye, et al., 2015)	**(Finkelstein et al., 1993)**	**(Finkelstein et al., 1993)**
Waste source		Clam shell (dry matter)	mercenaria clam shell (dry matter)	meretrix lusoria clam shell	Ocean quahog sea clam shell (dry matter)	
Test method	-	XRF	AOAC	AOAC		surf sea clam shell (dry matter)
parameters	value					
Dry matter	wt%					
moisture	wt%	0.26	2.22	0.00	4.82	
volatile matter	wt%					10.14
ash	wt%		8.47	9.32		
protein	wt%		0.22	0.30		
fat	wt%		0.47	0.56		
carbohydrate	wt%		84.60	85.61		
crude fiber	wt%		4.02	4.21		
Ca	wt%				38.50	
Cao	wt%	53.99				36.35
CaCO3	wt%	96.80			95.73	
SiO2	wt%	0.84				91.65
Al2O3	wt%	0.14				
Fe2O3	wt%	0.06				
K2O	wt%	0.03				
MgO	wt%	0.08				
Na2O	wt%	0.39				
SO3	wt%	0.16	0.12			
SO4	wt%	0.06	0.04			
SrO	wt%					
K	mg/kg		700.00	800.00	700.00	
Mg	mg/kg		59240.00	56710.00	200.00	800.00
Na	mg/kg		1100.00	1300.00	5300.00	300.00
P	mg/kg		630.00	710.00		6900.00
Al	mg/kg					
Cd	mg/kg				<1	
Cl	wt%	0.02			0.06	<1
Cr	mg/kg				13.00	0.12
Cu	mg/kg				18.00	9.00
Fe	mg/kg		48110.00	42610.00	20.00	9.00
Pb	mg/kg				1.20	195.00
Zn	mg/kg		1310.00	1220.00	<5	

2.3. Chitin extraction methods from mollusc shells

Chitin is the most abundant polysaccharide in the marine ecosystem and firstly was identified in 1884 (Abdulkarim et al., 2013; Yadav et al., 2019). Chitin or poly β-(1–4) N-acetyl-D-glucosamine is the second most important natural polymer on earth after cellulose. The chitin is a nitrogenous polysaccharide which pure state is yellowish or white, and it is also tasteless and odorless. The major primary source of chitin is from crustacean shells obtain from the seafood process industry. The crustacean shells include prawns, crab, shrimp, and lobster (Yadav et al., 2019). Both insects and crustacean shells contain a relatively high amount of pure chitin mixed with protein and minerals (CaCO3) (Hülsey, 2018). Based on the sources, chitin in nature presents mainly three different types of crystalline forms α, β, and γ. α chitin occurs in the shrimp shells, lobsters, cell walls of fungi, crab, prawn, and shellfish. In α chitin, the polysaccharide (biopolymer) chains have been structured in an anti-parallel orientation which allows maximum bonds. The α chitin is the most stable form of chitin in nature results in chitin fibrils with high crystallinity index of 80%. The β chitin has been found in the squid pens, tubes synthesized by vestimentiferan worm and pogonophoran. β chitin is rarely associated with protein content in the tubes synthesized by the pogonophoran, vestimentiferan worms, and squid pens. The β chitin arrangement of the polymer chain is in the parallel orientation, with the crystallinity index of chitin fibrils is about 70%. γ chitin is the combination of α- and β chitin-type arrangement in which two similar polymer chains are arranged alternatively with one anti-parallel chain (Rinaudo, 2006; Yadav et al., 2019).

The chitin has excellent properties such as biocompatibility, biodegradability, and non-toxic. It is not soluble in water and most of the organic solvents because of its hydrophobic nature. It is only soluble in few solvents such as hexafluoroacetone and hexafluoro-isopropanol. In general, due to the lack of solubility, low porosity, and highly ordered crystalline structures to resistant to physical and chemical agents. Based on all the parameters, the application of chitin is limited in many cases. For the above-mentioned reasons, chitin can be deriving into soluble forms especially, the most important derivative of chitin is chitosan, which is soluble in both organic and inorganic acids. The chitosan can obtain through the partial deacetylation of chitin. (Hamed et al., 2016; Kaczmarek et al., 2019; Rameshthangam et al., 2020; Yadav et al., 2019).

Chitosan (β- (1-4) -2-amino-2-deoxy-D-glucopyranose) is a linear polymer obtained from partial deacetylation of chitin by both chemical and biological methods. In general, chitosan has main pieces of functional groups such as hydroxyl and amino, and the existence of these groups allows the modification of chitosan to obtain valuable compounds. The amino group modified compounds that contain superior properties such as anti-fungal, anti-bacterial, non-toxic, biodegradability, and biocompatibility. The hydroxyl group allows the acetylation reaction and grafting. The chemical method of chitin deacetylation can proceed through the hydroxide compounds such as (KOH or NaOH)

or acids. The different degrees of deacetylation of chitosan are generated based on the operating temperature, time, and alkali solution concentration. The biological process of chitosan extraction from chitin can be utilization by enzymes or microbes. The types of enzymes used in the enzymatic process contain: 1) specific enzymes such as chitosanases and 2) non-specific enzymes such as cellulases, lipases, papain, lysozyme, hemicelluloses, pectinases, and pepsin. Chitosanases are presented widely in nature, as well as they have found in plants, bacteria, viruses, and fungi (Danarto and Distantina, 2016; Bruno et al., 2019). Nowadays, the most used method for the deacetylation of chitin with the utilization of a strong alkaline solution to produce chitosan (Kaczmarek et al., 2019; Abdou et al., 2008). Figure 2-1 represents the chitosan extraction by the chemical and biological methods by deacetylation of chitin (Hamed et al., 2016).

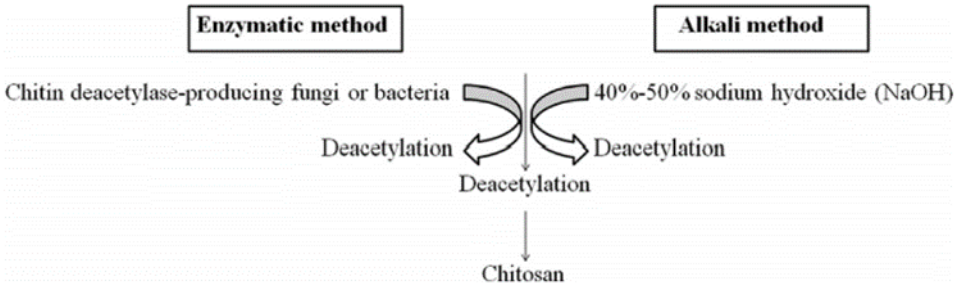

Figure 2-1-chemical and biological method of chitosan extraction (Hamed et al., 2016)

The main difference between chitin and chitosan is their solubility and degree of deacetylation. The chitosan can be soluble in many aqueous acid solutions, which are mainly citric, acetic, formic, and lactic acid below its pKa value (pH value is <6.5). the quality of chitosan can depend on two main factors such as molecular weight and degree of deacetylation (Danarto and Distantina, 2016; Kaczmarek et al., 2019).

Chitin is closely associated with components such as protein, inorganic materials, which are mainly calcium carbonate and lipids. The extraction of chitin from the shell needs to remove the impurities from the chitin shell waste (Alabaraoye et al., 2018).

They are two main extraction methods available for the recovery of chitin from shell waste. The first one is chemical extraction while the second one is the biological methods. The process of chemical extraction mainly involves three steps: deproteinization, demineralization, and decolorization. The two most common biological methods used for chitin extraction include: 1) fermentation using microorganisms and 2) Enzymatic deproteination (Hamed et al., 2016; Kaczmarek et al., 2019; Rameshthangam et al., 2020; Yadav et al., 2019).

Currently, chemical methods of chitin extraction are the most widely used in laboratory and industrial-scale production. The chemical process mainly involves the use of strong acids and bases with high temperatures. The chemical extraction methods of chitin need high energy requirements and is associated with several negative impacts on the environment. Moreover, the liquid obtained from the

chemical extraction process needs proper treatment before disposing and increases the purification cost of chitin.

Generally, the removal of both proteins and minerals (calcium carbonate, calcium phosphate, and calcium chloride) from seafood waste is conducted by the deproteinization and demineralization processes. The first step of the chemical method is deproteinization, which is processed by alkaline solutions. Among all the other alkali solutions, sodium hydroxide (NaOH) is the preferential reagent with concentrations ranging from 0.125 to 5.0 M NaOH. The sodium hydroxide also incorporates the additional benefits for hydrolysis of biopolymer, dropping of molecular weight, and partial deacetylation of chitin.

The second step process, demineralization, involves the decomposition of calcium carbonate (CaCO3) into calcium chloride (CaCl2) including the discharge of carbon dioxide. The demineralization process treating with acidic solutions such as HCl, H2SO4, CH3COOH, HNO3, and HCOOH. In the demineralization process, hydrochloric acid is the superior reagent among all other acids due to its high removal efficiency of the minerals

The reaction shown in Equation 1 represents the demineralization process of seafood waste into calcium chloride and the release of carbon dioxide (Yadav et al., 2019); (Nisticò, 2017).

$2HCl + CaCO3 \rightarrow CaCl2 + H2O + CO2 \uparrow$

The final step is the depigmentation process to remove the pigments such as carotenoids. The depigmentation process is performed by acetone or sodium hypochlorite (NaClO3) and is mostly the use of acetone (Arbia et al., 2013; Dhillon et al., 2013; Kaczmarek et al., 2019; Rameshthangam et al., 2020; Yadav et al., 2019).

The chemical extraction process creates a lot of negative impacts on both environment and health. The consideration of negative contributions, increasing the use of biological extraction methods since it is a cheaper and safer treatment for chitin extraction with desired properties. However, the biological process is time-consuming. The most used biological methods are Enzymatic deproteination and fermentation using microorganisms. The use of chemical methods is growing very fast because of the less processing time for chitin and its derivative recovery. In the enzyme deproteinization method, Proteases and proteolytic enzymes are required for the removal of protein from seafood waste. The large-scale sources of proteolytic enzymes are animals, plants, and microbes. There are some examples of proteases such as pepsin, pan-creatin, papain, and trypsin. This enzymatic process can perform the deproteinization either before or after the step of demineralization. This deproteinization process using both crude and purified extracted enzymes with purified enzymes is more expansive than crude enzymes. The sources of crude enzymes mainly come from bacteria and fish viscera (Yadav et al., 2019); (Arbia et al., 2013); (Dhillon et al., 2013). The enzymatic process of deproteinization has lower

removal efficiency of protein than the chemical methods. After the enzymatic deproteinization, 5-10% of the protein remained also associated with the chitin. The remaining percentage of protein needs further treatment by the alkali solution (NaOH) to increase the chitin purification (Kaur and Dhillon, 2015). Figure 2-2 represents the chemical and biological methods for the extraction of chitin (Yadav et al., 2019).

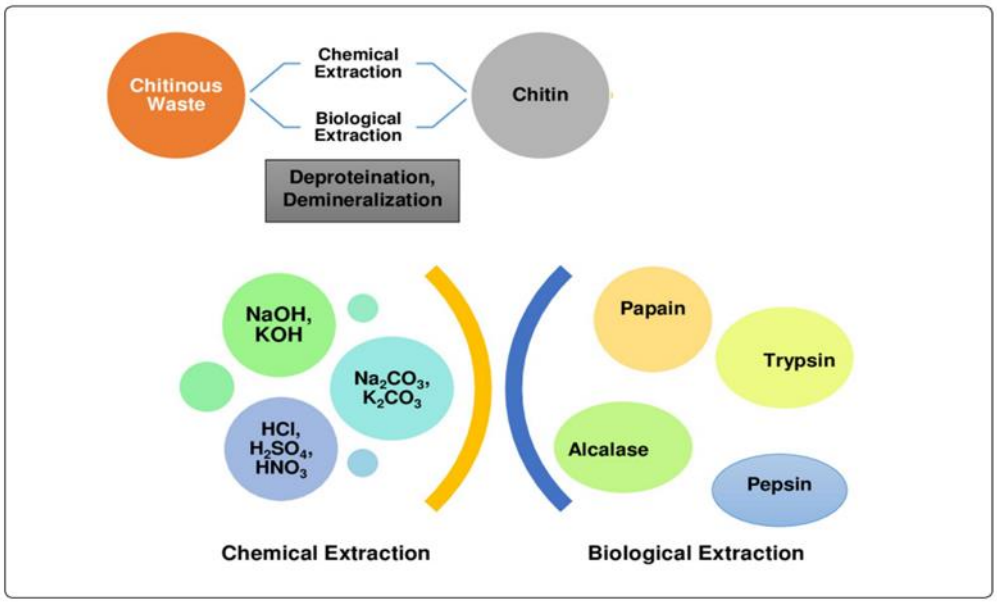

Figure 2-2- chemical and biological extraction of chitin

In the biological process, Lactic acid is applied for the removal of minerals from seafood waste that include calcium carbonate and calcium chloride instead of the chemical method with acidic solutions (HCl) (Rameshthangam et al., 2020; Yadav et al., 2019). Figure 2-3 represents both chemical and biological methods of chitin extraction from seafood waste (Arbia et al., 2013; Dhillon et al., 2013; Hamed et al., 2016).

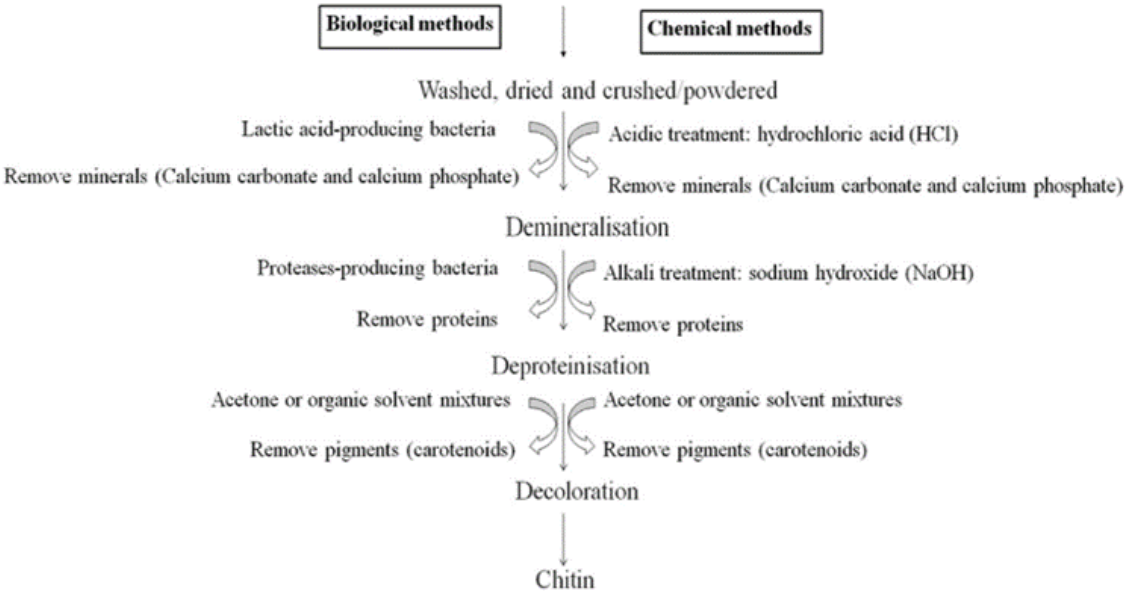

Figure 2-3- chemical and biological extraction methods of chitin (Hamed et al., 2016)

The removal or purification quality of impurities such as protein and calcium carbonate from the shell waste is estimated based on many studies. Physicochemical parameters such as the degree of acetylation, intrinsic viscosity, solubility, and molecular weight, are considered in the estimation of chitin purification. Some research showed that the chitin is soluble in phosphoric, sulfuric, and hydrochloric acid, but it is not soluble in nitric acid (Alabaraoye et al., 2018).

The chitin can be utilized in many industries such as agricultural, biochemical, food industries, and fertilizer. An essential quality of chitin fertilizer is its carbon to nitrogen ratio, which is the ratio of the mass of carbon to nitrogen. This ratio, 6–7 for chitin and chitosan, determines how fast the fertilizer decomposes and becomes available for the plant (USDA, 2011). Another important fertilizer characteristic is the 'N–P–K ratio,' the percentage of nitrogen (N), phosphorus (P), and potassium (K) that the fertilizer contains; chitin has no phosphorus or potassium (Kaplan et al., 2016).

Based on the drawbacks of the chemical methods of chitin and chitosan extraction from seafood waste, it is necessary to optimize and control the process. To do this, focusing more on the different particle sizes, temperature, duration, and chemical dosages can be essential for the extraction process of chitin and chitosan. The following papers I mentioned in summary Table 1 represent the chemical extraction procedures for chitin and chitosan.

Table 2-4. Summary Table

Biowaste	Target compounds	Extraction method	Results	Reference
Mussel shell	**Chitin and chitosan**	**Chemical method:** Pre-treatment: The collected mussel shells were washed with distilled water and then dried in the oven at 35℃ temperatures to have constant weight. A sample of 100 grams was taken for the extraction process. Demineralization: The dried shells were a reduced size and soaked into 0.68M HCL solution (1/10 w/v) at room temperature (30℃) for 6 hours. Deproteinization: demineralized shells were soaked into 0.62M NaOH solution (1/10 w/v) at room temperature (30℃) for 16 hours. The chitin was grounded and sieved through 150µm. Deacetylation: The obtained chitin was soaked with 25M NaOH solution (1/10 w/v) at 70℃ for 20 hours	**The results obtained by the deproteinization, demineralization, and deacetylation are 9.99, 51.62, 23.25, and 15.14%.**	**(Abdulkarim et al., 2013)**
a) Mollusc waste (oyster and mussel shell) **b)** crustaceans waste (crab and prawn) **c) fish scales waste (silver and pang)**	**Chitin**	**Chemical method:** **Pre-treatment:** The collected samples were washed with running warm water, and then the cleaned shell was dried in the oven at 35℃ for 12-24 hours. **Deproteinization:** The deproteinization was carried out by10% NaOH (1:10v/w) at room temperature (30 °C) for 18–24 hours. The resulting solid product was dried to have a constant weight at 35°C to 60 °C for 24 hours. **Demineralization:** The deproteinized sample was done by 10% HCl solution (1:10 w/v) at room temperature (30°C) for 18–24 h. The resulting solid product was dried to have a constant weight at 35 °C to 60 °C for 24 hours. **Depigmentation:** Decoloration step in the formation of chitin was omitted in this research work.	**The results obtained that protein and chitin percentages of oyster, mussels, crab, prawn shell, pang scales, and sliver scales are 98.85, 86.42, 63.73, 58.80, 44.36, 40.22%, and 69.65, 35.03, 60, 40.89, 35.07, 31.11%.**	**(Alabaraoye et al., 2018)**

Green mussel shell	**Chitin and chitosan**	**Pre-treatment:** The collected cleaned shells were crushed and then sieved into 200mm. In the extraction process, they have taken 50 grams of powder. **Deproteinization:** The deproteinization was done by 1M NaOH (1:10v/w) at 70°C temperature for 1 hour. **Demineralization:** The deproteinized sample was treated with 1M HCl solution (1:10 w/v) at 70°C temperature for 1hour. Depigmentation: The Decoloration process was done with hot acetone solution (1:10 w/v) for 1hour. **Deacetylation:** The process was done by NaOH (1:10v/w) at two different temperatures as treatment1 at high temperature (90-100°C for 2 hours) and treatment2 at room temperature for 24 hours.	**The results obtained for deproteinization, demineralization, and Depigmentation yields were 92.58, 43.84, and 41.60. the chitin deacetylation yields, degree of treatment1, and treatment 2 are 28.0, 39.5, 79.80, and 61.40 %.**	**(Danarto & Distantina, 2016)**
Green mussel shell	**Chitin and chitosan**	**Chemical method:** **Deproteinization:** The deproteinization was done by 3% NaOH (3:1 v/w) and then stirred with the magnetic stirrer for 1 hour. The solution was heated on the hot plate at 80°C for 30 minutes. **Demineralization:** The demineralized sample was treated by 1.25M HCl solution (3:1 w/v) heated on the hot plate at 75°C temperature for 1hour. **Deacetylation:** The deproteinization processed by 50% of NaOH (1:20) at high-temperature 90-100°C for 1hour. The sample was dried in the oven at 60°C.	The deacetylation degree of chitin is 43.05 %.	**(Idacahyati et al., 2020)**
shrimp, mussel, squid pen, and crab shells	**Chitin and chitosan**	**Chemical method:** **Pre-treatment:** Each sample was ground in the mill and sieved through the 40-mesh sieve. A powdered sample of 50 grams of each was taken for the extraction process of chitin and chitosan. **Demineralization:** The demineralized sample was treated with 2M HCl solution at room temperature between 4-24 hours	**The results obtained by the recovery of both chitin and water-soluble chitosan shrimp, mussel, squid pens, crab shells are 28.88, 16.35, 47.07, 19.33%, and 16.79, 4.19, 37.87, 5.48%. The**	**(Ni'mah et al., 2019)**

		for static and stirred at 50 rpm conditions. **Deproteinization:** The residue of the filtered sample was treated with 2.5M NaOH at 60°C. The remaining residue from the demineralization was washed with distilled water and dried in the oven at 50°C. **Deacetylation:** The deproteinized sample was treated with 50% NaOH at 60°C. The dry residue was added to 2% (w/w) acetic acid. Finally, 30% H2O2 is added to the solution and lasts for 4hours. The chitosan sample was kept in the oven at 60°C.	**deacetylation degree of shrimp, mussel, squid pens and crab shells are 64.18, 35.03, 58.04, and 53.91%.**	
Shrimp and mussel shells	**Chitin and chitosan**	**Chemical method:** **Pre-treatment:** Each sample was ground in the mill and sieved through the 40-mesh sieve. A powdered sample of 50 grams of each was taken for the extraction process of chitin and chitosan. **Demineralization:** The demineralization process is carried out with 50 g was soaked in 500 mL of HCl 7% and then filtered. **Deproteinization:** The residual powder from demineralization was soaked in 500 mL of NaOH 10% and then filtered. Finally, the chitin was washed with 125 ml of ethanol 96%. **Deacetylation:** The chitin sample of 10 grams was soaked in 20 ml of 50% NaOH and washed with distilled water, then dried in the oven. The residual of 1 gram chitosan was soaked in 10 ml of acetic acid with 2% acetic acid and 4 ml of 30% H2O2. The mixture was left to react using a magnetic stirrer hotplate before being filtered to obtain the filtrate.	**The results obtained by the recovery of both chitin and water-soluble chitosan shrimp, mussel shells are 39.04, 18.68 % and 27.03, %.**	**(Harmami et al., 2019)**

2.4. FTIR analysis

The work carried out in this thesis is focused on the extraction of chitin from the shell of mussel, clam, and murex. The shell waste contains valuable compounds such as protein, CaCO3, and chitin. So, here we need to identify those compounds before going to chitin extraction by using the Fourier transform infrared spectrometry (FTIR). The extracted chitin is often evaluated by FTIR analysis to observe the functional groups of chitins**.**

(Brugnerotto et al., 2001) explained that the comparison of different IR spectra based on different modes such as drift mode with the powder form of KBr, transmission spectra with KBr pellets or pads, and ATR (Attenuated total reflectance) spectra using a standard ZnSe crystal (angle of incidence 45°C). Figure 2-4 represents the comparison of different IR spectra.

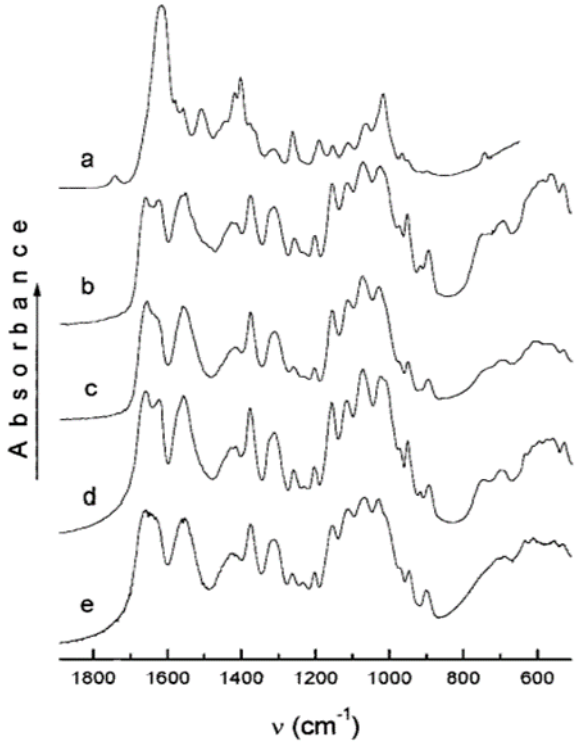

Figure 2-4- Comparison of IR spectra (shown in absorbance) of α-and β-chitin (shrimp and squid, respectively) recorded under different sampling techniques. For α -chitin: (a) ATR on film, (b) DRIFTS on powder, (c) Standard transmission on film, (d) Standard transm

The following summary table 6 represents the functional groups of chitins by FTIR analysis based on the different sampling techniques.

Table 2-5. Summary table

Reference	**(Silverstein et al., 2005)**	**(Idacahyati et al., 2020; Silverstein et al., 2005)**	**(Brugnerotto et al., 2001)**	**(Brugnerotto et al., 2001)**	**(Ramasamy et al., 2014)**	**(Brugnerotto et al., 2001; Ni'mah et al., 2019)**
Sample	Common functional groups	Chitin	Chitin	Chitin	Standard chitin	Mussel chitin
Condition	FTIR spectra were analyzed in the radiation band between 4000-400 cm^{-1} with the KBr powder.	FTIR was used to identify the functional groups in chitin and radiation band between 4000-400 cm-1 with the KBr powder.	FTIR spectra were analyzed in the radiation band between 4000-400 cm^{-1} with the KBr pellets (1 mg in 100 mg of KBr).	FTIR spectra were analyzed in the radiation band between 4000-400 cm-1 with the KBr pellets (1 mg in 100 mg of KBr) by transmission mode.	FTIR spectra were analyzed in the radiation band between 4000-400 cm^{-1} with the KBr powder.	FTIR spectra were analyzed in the radiation band between 4000-400 cm^{-1} with the KBr pellets
Functional group	cm^{-1}	cm^{-1}	cm^{-1}	cm^{-1}	cm^{-1}	cm^{-1}
OH Stretching	3550-3200	3429.3	3461	3426	3431	3394
NH Stretching	3500-3400	3429.3				
CH Stretching	3000-2840	2922.16	2907	2925	2925	2982
NH Stretching	2800-2000	2522.89	2551			2519
NH Stretching	2800-2000	2372.44				
NH bending	1655-1250	-	1553 1322	1558 1315	1583 1316	1464
C=O Stretching	1870-1540	1784.15	1626	1661	1624	1788
CH bending	1468-1442	1467.83				
CO Stretching	1260-1000	1082.07			1068	
C-C Stretching	1200-800	864.11			893	
CN Stretching	800-666	707.88				711
C-C-O or C-O-C	1260-1000		1072 1023	1074 1029		1082 868
C-C bending	below 500				464	
OH bending	1420-1330					

2.5. Bio methanation potential

The separation of solid (shell) and organic (meat) fractions from the raw waste samples, such as mussel, clam, and murex, were conducted out by a shredding pump. After the shredding test, we had the solid part (shell) at the bottom and the liquid portion, which included a little organic (meat part). The bio methanation potential (BMP) test was carried out on the liquid portion with a small contribution of the organic fraction (meat part) obtained from the shredding pump is treated by anaerobic digestion to observe the biogas production.

The $BDCH_4$ (Biodegradability based on Methane Yield) and methane potential of substrate are commonly determined using the Biochemical Methane Potential (BMP) test (Fannin et al., 1986, 1987; Owen et al., 1979). In the BMP test, t, the investigated material is mixed with active anaerobic inoculum collected from a full-scale digester plant. The mixture is incubated under mesophilic or thermophilic conditions for 30-60 days or even longer. The mixing is important for optimal mass and heat transfer. (Labatut et al., 2011; Owen et al., 1979). The test runs until the material is considered fully degraded or the daily gas production is less than 1% of the accumulated gas production as recommended by German standard (VDI, 2006), which depends on the physical and chemical properties of the material and the activity of the inoculum. During the test, the volume of produced gas is measured using either manometric or volumetric methods. The BMP is expressed as the volume of methane per gram of organic material added, which is often based on volatile solids (VS) or chemical oxygen demand (COD) (Strömberg et al., 2014). Several factors, which are mainly temperature, pH, inoculum preparation, inoculum to substrate ratio (ISR), substrate concentration, and mixing, can affect the BMP test results.

Inoculum plays a vital role in the BMP test and is the most complex factor that affects the results of the test and is also the most difficult for standardization due to the diversities of microorganisms included and their metabolic activities. Different approaches exist for inoculum preparation and storage prior to the BMP test, e.g., pre-incubated the inoculum at 35 ± 2°C for up to 7 days to reduce background gas production and decrease the influence of the blanks (ISO11734, 1995). filter the inoculum with a size of 2 mm sieve to remove grit or large particles (Browne & Murphy, 2013), and store the inoculum at 4°C (Cabbai et al., 2013), etc. The inoculum storage conditions, and preparation (includes pre-incubation and filtration) influence the metabolic activities of the microorganisms, secretion of the extracellular

enzymes, and consequently, hydrolysis of the substrate (Sambusiti et al., 2014). However, to date, no qualified recommendation exists for inoculum preparation prior to a BMP test.

Substrate concentration has been considered as an important parameter that can influence the efficiency of the AD process. At low substrate concentration, the microorganisms might exhibit low metabolic activity due to the low availability of substrate. The substrate concentration could not be too high also, as the overload situation might lead to inhibition caused by the accumulation of intermediate products (Taherzadeh & Karimi, 2008; Zhang et al., 2014).The German standard VDI 4630 (2006) suggests using an inoculum with a VS of 1.5-2.0% and an ISR $\geq$ 2.In a batch BMP test, different mixing types are applied, e.g., mixing by manually shaking (Kafle & Kim, 2013) or with the aid of a magnetic bar (Raposo et al., 2011), shakers (Guendouz et al., 2010), and stirrers driven by geared motors (Raposo et al., 2011). In addition to mixing types, the mixing mode (i.e., continuous, or intermittent) and intensity at different frequencies and speeds can further influence the test. However, the results related to mixing are conflicting.

Chapter3: Material and methods

3.1. Introduction

In this chapter, we discussed the various materials and methodologies involved to carry out the Mollusc waste (mussel, clam, and murex), such as sampling and characterization of raw waste, settling tests, pretreatments for organic (meat), and inorganic (shell), chemical extraction of chitin, FTIR analysis, and Bio methanation potential.

3.2. Materials

The sub-objective of the **SEA2LAND** project mainly focused on the Process leftover that comes from **CO.PE.MO (s**eafood processing industry), which is in the port of Ancona, Italy. **CO.PE.MO** produces 1-3 tons/day of seafood wastes/by-products and is majorly composed of mollusc shell waste. The production activity of Co.Pe.Mo. is articulated through different steps: purification by washing (Figure 3-1), selection and sorting on special machines controlled by qualified personnel (Figure 3-2) and packaging (Figure 3-3).

Figure 3-1- Washing and purification of fished products

Figure 3-2- Selection and sorting of market-value product. Clam (top) and murex line (bottom).

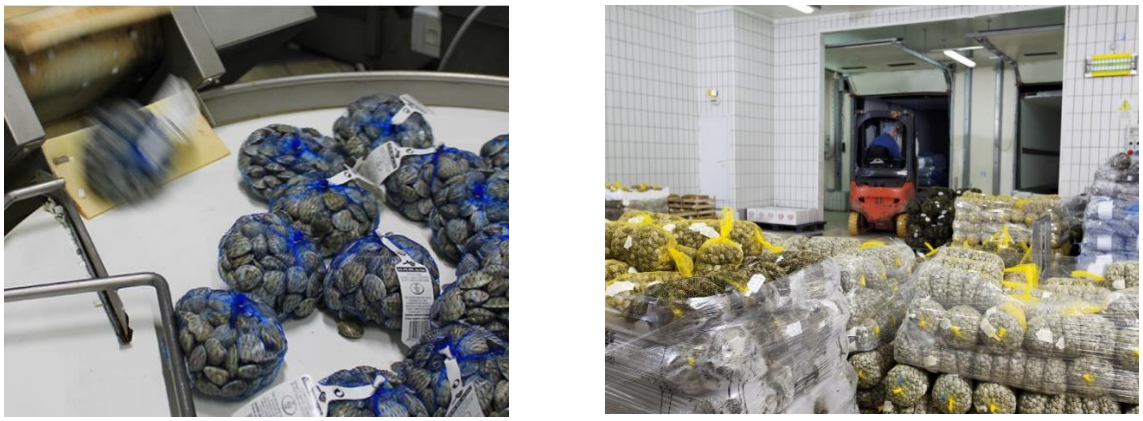

Figure 3-3- Packaging and distribution of final product

During the selection and sorting phase, there is a generation of by-products mainly composed of mollusc not suitable for the market since they are undersized, fouled with barnacles, broken shells or unwanted species.

There are three major separate lines of seafood processing: one for mussels, one for clams, and one for murex. In each line, workers remove the not-market value product and throw it in 100l bins close to them. Figure 3-4 shows an example of the production line dedicated to clam. Then they mix all the three-line waste in one container stored in a cool room before being disposed.

Figure 3-4- Production line for clam packaging

In my research, I collected the available samples from three lines processed by the CO.PE.MO. Figure 3-5 represents the three main lines of seafood processing in CO.PE.MO, which includes mussel, clam, and murex.

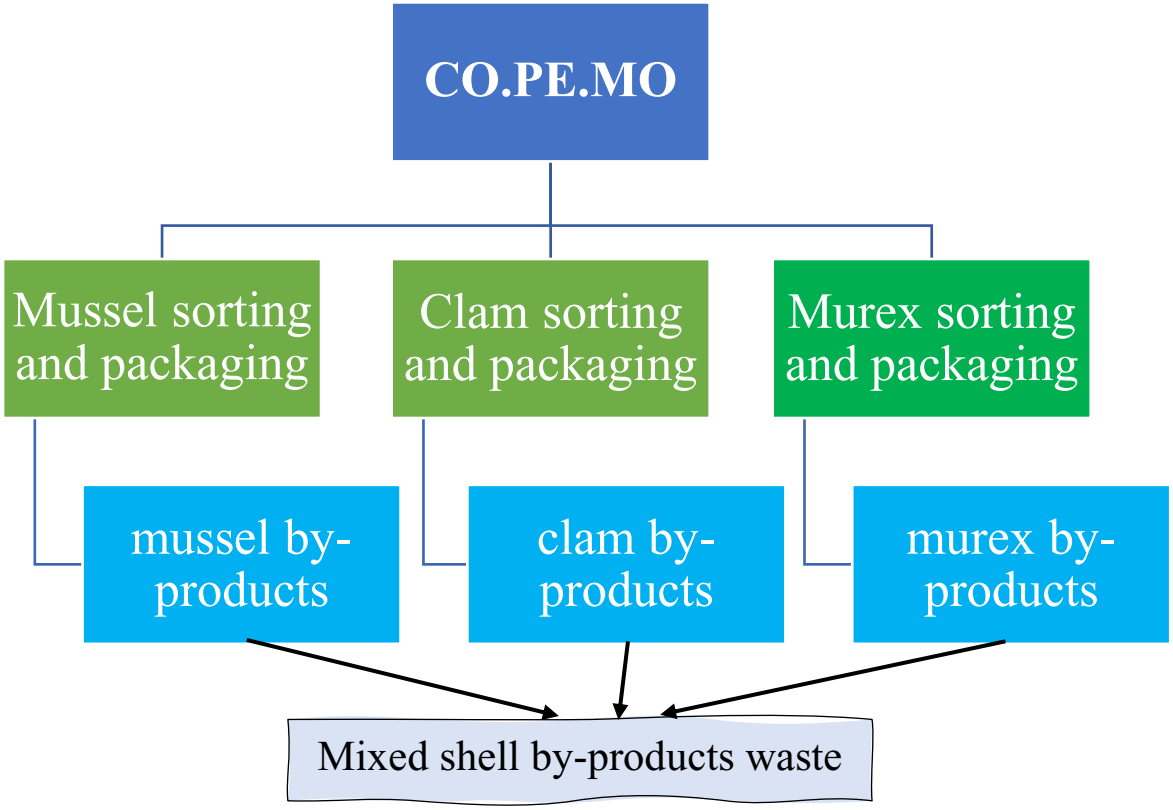

Figure 3-5- main lines of seafood processing waste

Figure 3-6 shows the raw waste samples of mussel, clam, murex, and mixed waste samples.

a) Mussel waste

b) Clam waste

c) Murex waste

d) Mixed waste

Figure 3-6- Raw waste samples

3.3. Methodology of the study

In this thesis work, I mainly focused on the characterization and pre-treatments for three lines of mollusc waste. The following tasks I performed in the laboratory are mentioned below:

f) Sampling and characterization of waste
g) Separation of organic and inorganic fraction
h) Settling tests
i) BMP tests
j) Chemical extraction of chitin from mollusc shell

3.3.1. Sampling and characterization of sample

Four different side-streams were identified:

1. Clam (*chamelea gallina*) by-products
2. Mussel (*Mytilus galloprovincialis*) by-products
3. Murex (*Bolinus brandaris*) by-products
4. Mixed shellfish by-products.

The first three samples were collected directly from each production line.

Specifically, 2 kg of SFB were collected from the 100l bin and stored separately to the other coming from distinct packaging lines. Another 2 kg were also collected from one 500l bin in the cool room as a sample of mixed waste.

Following the same procedure, three sampling campaigns were conducted. The first in March, the second in April and the last one in May to see if any variability would occur.

For the characterization of the shellfish by-products, *ad hoc* methods for numerous parameters do not exist. Therefore, methods already used for the characterization of other biomasses were adopted and/or minimally modified to describe shellfish by-products properties, useful to predict their recovery as fertilizers. All analyses were performed in duplicate, and the average values are obtained from three different sets of sample.

A representative quantity of each sample was manually separated and weighed (more or less 1 kg for shells and 200 gr for meat) to initially assess the yield of meat compared to the shell in terms of fresh weight (FW). The analyzed waste characterization determines the waste compositions in terms of the moisture, dry matter, ashes, nutrients, cations (Na, Mg, K, and Ca) and heavy metals (Zn, Cu, Cd, Fe, Mn, and Cr, etc.) contents in percentage on both shell and organic parts. The chemical composition of the raw waste (without separating the organic and inorganic fractions) was calculated backwards.

3.3.1.1. Characterization procedures

The dry matter test was conducted at a temperature of 105ºC until we achieved the constant weight **(UNI EN 13040:2002)** on the different fractions of inorganic and organic from the mussel, clam, murex, and mixed waste (raw waste). Based on the dry matter, calculated the total solids (TS) % for each fraction of the waste.

The ashes content test was conducted on each fraction of samples by weighing sample after calcination in a furnace overnight at 550 °C (APHA, 1998).

The protein content of each fraction of waste on the dry matter was carried out by the Kjeldahl method and total protein using a conversion factor of 6.25 **(AOAC 2000.11)**. The total fat content of each fraction of waste on the dry matter was carried out by the gravimetric method based on Soxhlet extraction by hexane and ethanol **(Adani et al., 1995).**

The cations such as Na, Mg, K, and Ca content of each fraction of waste on the dry matter determined by ICP-MS after acid digestion **(EPA, 1998).** The obtained results in terms of g/kg-

1 DM. The content of heavy metals in each fraction of waste on the dry matter, determined by ICP-MS after acid digestion **(EPA, 1998).** The obtained results in terms of mg/kg-1 DM.

3.3.2. Separation of organic and inorganic fraction

Due to the different chemical compositions of the shell (inorganic fraction of waste) and the residual meat (organic fraction of waste), specific valuable products are expected to be recovered from each fraction. On the other hand, it was hard and time-consuming to separate the inorganic and organic parts from the raw waste manually. Therefore, some trials were carried out to check the feasibility of using a shredding pump for the separation of inorganic fraction (shell) from the organic fraction (residual meat). As a consequence, also a size reduction of the sample will be achieved.

3.3.2.1. Shredding pump test

3.3.2.1.1. Instrumental setup

Initially, the shredding pump was submerged in a tank to carry out the test. The dimension of the tank used for the shredding test was a depth of 58 cm, a height of 95 cm, and a total volume of 34320 cm^3. The shredding pump used in this project works at a minimum water level of 13 cm. Based on the minimum water level and tank area, we calculated the water volume of 22 L. Figure 3-7 represents the shredding pump test set-up.

Figure 3-7- Shredding pump set up

3.3.2.1.2. Shredding test process

The shredding test was conducted individually on the different weights of the mussel, clam, and murex waste diluted with a fixed water volume of 22 L to understand the best operating conditions.

Mussel

In the first attempt, we considered the 5.5 kg weight of mussel waste with a water volume of 22 L added to the tank. And then, we turned on the switch to carry out the shredding during the time that was needed for observing the proper crushing in the tank. After the shredding test, we observed the solid part (shell) at the bottom and the liquid portion, which included a little organic(meat part) at the top. The liquid portion was separated from the bottom part manually, and then the weight of both solid and liquid waste was measured. Figure 3-8 represents the crushed shell in the bottom after the removal of the liquid portion.

Figure 3-8- crushed shell in the bottom after removal of liquid portion from shredding tank

In the second and third attempts, we considered the 11kg and 22kg weight of the mussel shell with a fixed volume of 22 L of water to carry out the shredding test. The procedure was the same as I mentioned before. And then, we calculated the dilutions factors based on the total waste [shell waste (kg)+water(kg)] divided by the shell waste. The dilution factor for the tests with mussel shells were 5, 3, and 2.

Clam

In the shredding pump test, we considered the different weights of clam waste, such as 11, 15, 18, and 22 kg diluted with a water volume of 22 L added to the tank individually. And then, we turned on the switch to carry out the shredding during the time that was needed for observing the proper crushing in the tank. And then, we calculated the dilutions factors based on the total waste [shell waste (kg)+water(kg)] divide by the shell waste. The dilution factors of the clam refer to three different conditions were 3, 2.5, 2.2, and 2. Finally, we performed the

moisture content and ashes on the solid part that comes from the shredding test for each dilution factor as same as for mussel. Figure 3-9 represents the complete process of the shredding test.

a) Raw mussel b) set up(loading)

c) once extracted the pump d) crushed shell in the bottom

Figure 3-9- shredding test process of clam

Murex

In the shredding pump test, we considered the different weights of murex waste, such as 7, 14, and 22 kg diluted with a water volume of 22 L added to the tank individually. And then, we turned on the switch to carry out the shredding without considering the duration of shredding until the proper crushing was observing in the tank. And then, we calculated the dilutions factors based on the total waste [shell waste (kg)+water(kg)] divide by the shell waste. The dilution factors of the murex refer to three different conditions were 4, 2.6, and 2. Figure 3-10 represents the process of the shredding test.

a) Raw sample

b) crushed shell in the bottom

Figure 3-10- shredding test process for murex

Finally, we performed laboratory analysis to calculate the moisture content and volatile solids content on the raw waste and solid (crushed shell) part (from shredding test) for each dilution factor of mussel, clam, and murex waste.

Moisture test

The moisture test was carried out at a temperature of 105ºC until all the water contained in the sample was evaporated. The procedure of Moisture test is as follows:

a) To take the weight of empty crucible (w1)
b) Take the weight of (sample + crucible) (w2).
c) Set the oven at the temperature of 105°C
d) Take out the crucible from the oven and put it in the desiccator for 20 minutes to cool down.
e) And then note down the final weight of the sample (w3).
f) Finally, we can calculate the moisture content of the sample by using the following formula:

   Moisture content (%)=[(w3-w1)-(w2-w1))/(w2-w1)] *100

   Dry matter content (TS%)=[(w3-w1)/(w2-w1)]*100

Volatile solids content

The ashes content test was conducted at the temperature of 550°C in the furnace overnight on the dry matter that comes from the moisture test. The procedure of Volatile solids content is as follows:

a) To take the weight of (sample + crucible) that comes from moisture test (w4).

b) Set the furnace at the temperature of 550°C and stick a note on the furnace for safety conditions.
c) Process the test at overnight.
d) Take out the crucible from the oven and put it in the desiccator for 20 minutes to cool down.
e) And then note down the final weight of the sample (w5).
f) Finally, we can calculate the TVS/TS content of the sample by using the following formula:

   Volatile solids fraction (TVS/TS%) (%)=(w4-w5)/(w4-w1) *100

After, the obtained results represent the moisture content, dry matter (TS%), and ashes content(TVS/TS%) for each dilution factor of mussel, clam, and murex waste on the solid (crushed shell) fraction from shredding tank bottom. For each dilution factor on the different samples, we calculated the recovered amount of total solids (kg) from the bottom part(shell) from the shredding test was obtained by the multiplication of total solids(%) and shell weight (kg) after the shredding test.

3.4. Settling test

After shredding the waste, two fractions are easily detected in the tank: a liquid fraction composed of added water and the lighter solid (residual meat) and a solid fraction consisting in the heavier shredded shell that immediately settled on the bottom tank. Therefore, the residual meat should be separated from the shell but mixed with water.

A settling test using an Imhoff cone was conducted on the liquid fraction to observe how much solids can be recovered by settling after 45 minutes.

After each shredding test at different dilution factors, a homogenized sample (1L) from the liquid fraction was collected. The sample was put in the Imhoff cone (1L of capacity) and let settle (Figure 3-11). We monitored the behaviour of the Imhoff test for every 15 minutes (0, 15, 30, and 45 minutes) of duration for each dilutions factor of mussel, clam, and murex.

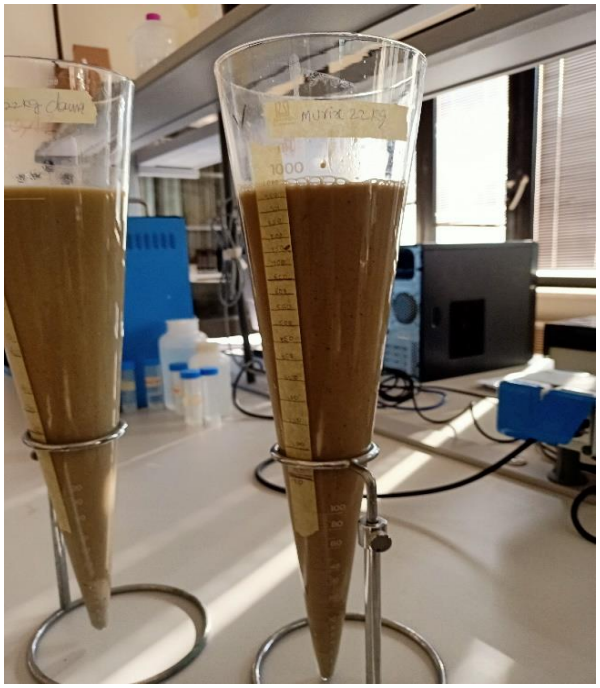

Figure 3-11- Settling test on the liquid part comes from shredding test

After 45 minutes duration, we observed and noted down the different fractions that can be detected:

1. settleable fraction
2. supernatant fraction
3. floating fraction (it was detected only in some tests)

Each fraction was separately collected and laboratory analysis (TS%, TVS/TS%, TKN) were performed on them.

3.5. Bio methanation test (BMP)

The bio methanation potential (BMP) test was conducted to check the biogas production of the liquid fraction recovered after shredding the waste that, as above mentioned it consists of the added water plus the lighter solids like residual meat.

The collected samples from shredding tests performed at a dilution factor equal to 2 were used as substrates. While the anaerobic sludge from the anaerobic digestor of Falconara Marittima wastewater treatment plant was used as inoculum. In the BMP test, there was no addition of nutrients because the liquid part already contained them.

3.5.1. Test procedure

Before going to the BMP test, we performed a laboratory analysis to characterize the sludge and substrates (mussel, clam, and murex):

- Total solids (TS%)

- Total volatile solids fraction (TVS/TS)

The total solids and volatile solids were conducted on the based on the same procedure I declared in the 3.3.2.1.2 section.

In this test, the considered ratio of VSS inoculum/VSS substrate was set equal to 2 and serum bottles of 250 ml volume were used. Mesophilic conditions (35°C) were set by putting the bottles in a temperature-controlled fridge. Biogas production was measured by the volumetric method reading the decrease of water in the graduated cylinder connected to the bottle. Gas analysis was carried out to know the amount of CH4 and CO2 in the biogas.

The BMP test was carried out on:

1. mussel liquid waste (+inoculum)
2. clam liquid waste (+inoculum),
3. murex liquid waste (+inoculum)
4. blank (inoculum without the addition of substrate).

The characterization of both substate and inoculum was mentioned as shown in Table 3-1 and Table 3-2.

Table 3-1- substrate characterization

Sample	**tare (g)**	**Lordo (g)**	**P105(g)**	**TS%**	**P550(g)**	**TVS/TS%**
clam	57.931	94.397	59.915	**5.44**	58.446	**74.06**
murex	65.937	115.116	68.870	**5.96**	67.4366	**70.00**
mussel	62.757	107.840	65.136	**5.28**	63.4547	**70.66**

Table 3-2- sludge characterization

tara	**peso 105**	**ml**	**MLSS mg/l**	**TARA**	**lordo 550**	**MLVSS mg/l**	**MLVSS/MLSS**
0.8063	1.6933	**50**	17740	48.4252	48.75	11336	0.64

The operation conditions of the BMP test were mentioned as shown in below Table 3-3 to Table 3-5.

Table 3-3- operating conditions of the BMP

sample	inoculum ml	inoculum g	VSS inoculum g	TVS substrate to dose g	substrate g
clam	180	180	2.04	1.02	**25.3**
murex	180	180	2.04	1.02	**24.4**
mussel	180	180	2.04	1.02	**27.4**
blanket	180	180	2.04	0	25

Table 3-4- indicates the total volume (substrate + inoculum)

sample	**total volume**
	ml
clam	205
murex	204
mussel	207
blanket	205

Table 3-5- represents the VSS load

sample	VSS load
	kgVSS/m3/d
clam	5.0
murex	5.0
mussel	4.9
blanket	0.0

The background methane production from the inoculum (blank test) is subtracted from the methane production obtained with the substrates (mussel, clam, and murex) treated with anaerobic sludge.

3.6. Chemical extraction of chitin from mollusc shell

3.6.1. Chemical's solutions

Deproteinization:1M NaOH solution (40 grams NaOH (solid) dissolved in one liter of water)
Demenerization:1M HCl solution (83 ml of HCl 37% added to one liter of water).

3.6.2. Chitin extraction

The chitin extraction process was conducted only on the shell part (manually separated the shell and organic (meat) part from the raw sample) of mussel, clam, and murex by using chemical methods. In this project, chitin extraction followed mainly three steps:

1. pre-treatments,
2. deproteinization (DP)
3. demineralization (DM).

In the pre-treatment stage, the manually separated shells of the mussel, clam, and murex were washed with tap water and then dried in the oven at a temperature of 35℃ for 24 hours.
The deproteinization process aims to eliminate the protein content and was processed out by the 1M NaOH solution at temperature 70℃. In this project, the deproteinization step was carried out based on different operating conditions:

- different chemical dosages of NaOH (10, 15, and 20 ml) per one gram of sample
- different durations (1, 2, and 3 hours)

At the end of the deproteinization process, the sample was filtered by a Whatman filter paper (2 microns porosity). After the filtration step, the samples were washed with distilled water for 5 to 7 baths. And then, the obtained sample was kept in the oven at 60ºC for 24 hours. After 24 hours, the sample was removed from the oven and kept in the desiccator for 20 minutes to cool down. Finally, the deproteinization yield was estimated in terms of weight loss. To observe the maximum deproteinization yield that can obtain by which chemical dosage and duration used can be mentioned in the results section.
The demineralization process was carried out to remove the mineral content (CaCO3). The demineralization step was proceeded by 1M HCl solution at ambient temperature (approximately at 30°C). The demineralization step was conducted based on:

- the different chemical dosages of (10 and 20 ml) per one gram of sample
- different durations of 1, 2, 2.33, and 4 hours.

Similarly, to deproteinization, at the end of the demineralization process, the sample was filtered, washed with distilled water dried in the oven at 60ºC for 24 hours. Then, the demineralization yield was measured in terms of weight loss percentage.
Additionally, we measured the pH and temperature every 15 minutes in both steps (DP and DM).

3.6.3. Chitin quality investigation through FTIR

To assess if the chitin extraction was successful, FTIR analysis was carried out. A first investigation on the mollusc shell was also performed to identify the functional groups of chitin, protein, and CaCO3.
The Fourier transform infrared spectrometry (FTIR) has been extensively developed over past decades and provides several advantages. Radiation containing all IR wavelengths (4000-400cm^-1) is split into two beams. One beam is of fixed length, the other of variable length (movable mirror). The varying distance between the two path lengths results in a sequence of constructive and destructive interferences and hence variations in intensities: an interferogram.

Fourier transformation converts this interferogram from the time domain into one spectral point on the more familiar form of the frequency domain. Smooth and continuous vibrations of the length of the piston adjust the positions of mirror B; Fourier transformation at successive points throughout this variation gives rise to the complete IR spectrum. Passage of this radiation through a sample subject the compound to broadband of energies. In principle, the analysis of one broadband pass of radiation through the sample will give rise to a complete spectrum. The following Figure 3-12 shows the schematic representation of an FTIR spectrometer.

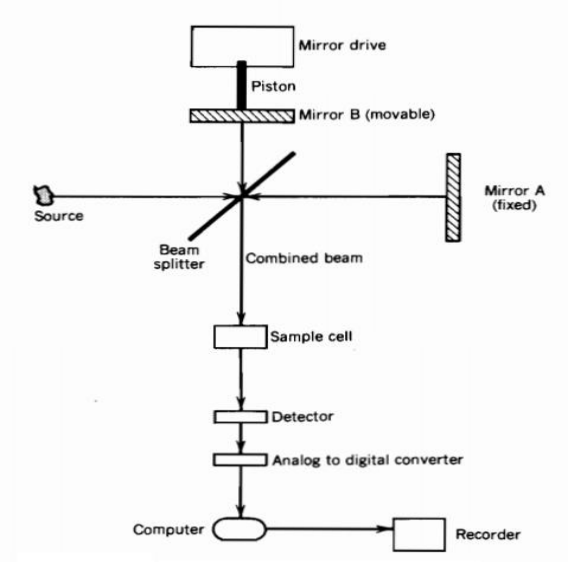

Figure 3-12- Schematic representation of FTIR spectrometer

There are several modes for acquiring FTIR spectra such as Attenuated Total Reflectance (ATR), Transmission mode, Diffuse Reflectance (DRIFT) and Specular Reflectance. Both powdered samples and pressed disks can be used. In particular, a mixture of the sample and an IR transparent matrix (such as KBr) is often used when dealing with chitin identification. The pellet (pressed disk) technique depends on the fact that dry, powdered potassium bromide can be compacted under pressure to form transparent disks. The sample (0.5-1 mg) is intimately mixed with approximately 100 mg of dry, powdered KBr.

The transmission mode with KBr pads, DRIFT (with KBr powder reflectance mode), and UATR (Universal Attenuated Total Reflection) were first tested to understand the influence of FTIR acquisition modes. The best performing was used for the next analysis.

In addition, FTIR analysis of transmission mode was carried out on the commercial chitin to make a spectrum as a reference to understand the removal of impurities, such as protein and minerals from our shell waste. Then deproteinization and demineralization efficiency was also evaluated by FTIR analysis comparing the obtained spectra with the reference spectrum of commercial chitin if the case.

Chapter 4 Results and discussions

4.1. Introduction

This chapter mainly focused on the results obtained from the tests explained in chapter 3, which includes Sampling and characterization of waste, Separation of organic and inorganic fractions, Settling tests, BMP tests, and chemical extraction of chitin from Mollusc shells.

4.2. Sampling and characterization of waste

In this step, manual separation was conducted on the fresh samples of mussel, clam, and murex to remove the meat fraction from the shell. And then, compared the meat fraction with the shell fraction. The physicochemical composition of seafood byproducts such as mussel, clam, and murex, was conducted on both shell and meat parts. In addition, the mixed waste was characterized as raw waste (without separation of the meat and shell). The waste characterization was carried out three times (March, April, and May) on the mussel, clam, murex, and mixed waste to observe any changes in the sampling phase.

The analyzed waste characterization determines the waste compositions in terms of moisture, ashes, dry matter, nutrients, cations (Na, Mg, K, and Ca), and heavy metals (Cu, Zn, Cd, Cr, Fe, etc.). Finally, the waste compositions were considered by the average of the three trails. The chemical composition of the raw waste (without separating the organic and inorganic fractions) was calculated backwards.

Table 4-1 represents the three average samples of waste characterization, the meat fraction of the mussel was (32.54), clam (18.17), and murex (14.65). here we observed that the highest meat fraction was thus found in the mussel waste.

Table 4-1- Fractions of organic and shell from fresh sample

separation of shell and organic part from fresh sample	
sample	average (%w/w FM)
Murex shell	85.35
Murex organic	14.65
mussel shell	67.46
mussel organic	32.54
clam shell	81.83
clam organic	18.17

Table 4-2 summarizes the average values of moisture content, dry matter, and ashes content of the shell, and organic fractions of mussel, clam, murex, and mixed waste were determined. There are no significant differences in terms of dry matter (DM) and ash content on the three

main shell samples of mussel, clam, and murex. The murex waste as considered as raw sample (there is no separation between organic and shell fraction).

Table 4-2- Average values of %DM, VS% and %ashes

sample	DM%	%VS (DM)	% ASHES (DM)
Murex shell	92.48	5.93	94.07
Murex organic	28.16	89.89	10.11
mussel shell	93.65	3.95	96.05
mussel organic	19.11	74.14	25.86
clam shell	93.00	3.94	96.06
Clam organic	24.60	76.09	23.91
mix	65.13	8.56	91.44

Table 4-3 shows the average values of crude protein, phosphorus, fats, and CaCO3 of the shell, and organic fractions of mussel, clam, murex, and mixed waste were determined. The phosphorus content is a little higher in the organic waste than in shells, while CaCO3 content is found high in the shell waste, which is approximately 87.50%. The mussel shells contain (91-95%) CaCO3 **(**C. A. Papadimitriou, 2014; Hyldig et al., 2020; Naik & Hayes, 2019). The clam shell contains (91.65-96.80%) of CaCO3 (Finkelstein et al., 1993; Lertwattanaruk et al., 2012). The obtained results of the mussel and clam were within the range of literature values. The protein content of the mussel shell (0.1-7%) (Hyldig et al., 2020; Iriani et al., 2020; Naik & Hayes, 2019). The obtained values of protein content in the mussel shell were within the range of literature. The protein content of the clam shell (0.22-0.30%) (Ademolu, Akintola, Olalonye, et al., 2015). The protein content of the calm shell was within the range of literature.

Table 4-3- Represents the three average values CaCO3, P, crude protein, and fats on the dry matter

	%DM			
sample	**CaCO3**	**P**	**crude protein**	**fats**
Murex shell	81.52	0.07	0.26	
Murex organic		0.84	64.33	17.38
mussel shell	87.50	0.05	0.41	
mussel organic		0.71	49.65	23.93
clam shell	87.35	0.04	0.12	
Clam organic		0.92	51.79	22.73
mix	79.50	0.10	0.74	

Table 4-4 shows the average values of cations such as Na, Mg, K, and Ca. Other authors presented 250-800 mg K/kg, 200-663 mg Mg/kg, 1100-6900 mg Na/kg and 600-700 mg P/kg for clamshell (Ademolu, Akintola, Adelabu, et al., 2015; Finkelstein et al., 1993; Lertwattanaruk et al., 2012; Nguyen et al., 2020). While for mussel shell, 50-166 mg K/kg, 181-1670 mg Mg/kg, 3264-6952 mg Na/kg are stated in literature (Hamed et al., 2016; Lertwattanaruk et al., 2012; Martínez-García et al., 2017; Papadimitriou et al., 2020).

Table 4-4- The average values of cations

Shellfish	**By-product**	**Na^+**	**Mg^{2+}**	**K^+**	**Ca^{2+}**
		mg kg^{-1} DM			
clam	organic	16'887 ± 3'035	2'924 ± 538	9'399 ± 3'341	47'780 ± 35'172
murex	organic	11'195 ± 6'336	5'430 ± 1'908	9'715 ± 815	19'763 ± 12'718
mussel	organic	13'699 ± 4'506	3'498 ± 1'598	9'324 ± 419	27'510 ± 17'664
clam	shell	6'406 ± 1'290	323 ± 132	426 ± 179	349'409 ± 15'518
murex	shell	5'812 ± 178	1'002 ± 281	662 ± 55	326'053 ± 11'122
mussel	shell	4'016 ± 113	1'223 ± 83	197 ± 51	349'981 ± 25'787
mix	all	5'997 ± 347	1'816 ± 598	970 ± 62	318'018 ± 13'978

The measured concentration of heavy metals, which is shown in Table 4-5 is validated by literature values. It is worth mentioning that high variability is detected compared to previously reported results since it strongly depends on the quality of the water where mollusc grows. For instance, published values for iron concentration in clamshell varies from 140 to 48,000 mg/kg on a dry basis (Ademolu, Akintola, Adelabu, et al., 2015; Nguyen et al., 2020) and our clamshell contains 1517 mg/kg of iron similarly to Li et al., 2020 (1119 mg Fe/kg). In general, the concentration of heavy metals is comparable to that of the organic fraction of municipal solid waste (Fisgativa et al., 2016), commonly used as by-product in aerobic and/or anaerobic treatments to produce fertilizers.

Table 4-5- The average values heavy metal

	Clam	Murex	Mussel	Clam	Murex	Mussel
	organic	**organic**	**organic**	**Shell**	**Shell**	**shell**
Al	808 ± 307	792 ± 454	723 ± 548	76.28 ± 46.04	712 ± 350	58.22 ± 32.80
As	14.53 ± 3.31	43.51 ± 24.60	28.95 ± 12.51	0.64 ± 0.29	2.78 ± 0.85	0.59 ± 0.30
Cd	0.40 ± 0.12	4.76 ± 5.62	4.04 ± 4.70	nd	0.74 ± 0.39	nd
Co	1.02 ± 0.24	1.05 ± 0.43	1.37 ± 0.58	0.93 ± 0.30	1.08 ± 0.09	0.98 ± 0.13
Cr	8.32 ± 4.41	8.30 ± 3.68	12.81 ± 17.07	8.14 ± 5.12	15.05 ± 4.06	2.54 ± 2.45
Fe	1'455 ± 668	845 ± 260	610 ± 246	1'517 ± 581	2'005 ± 70	1'247 ± 434
Mn	49.11 ± 12.94	35.17 ± 14.79	19.58 ± 4.05	13.48 ± 6.22	39.13 ± 15.00	8.04 ± 3.07
Hg	0.14 ± 0.04	0.38 ± 0.25	0.44 ± 0.23	0.72 ± 0.60	nd	nd
Mo	2.20 ± 0.67	1.30 ± 0.49	14.40 ± 19.54	0.91 ± 0.48	0.58 ± 0.09	2.60 ± 0.94
Ni	6.16 ± 1.14	5.99 ± 2.18	42.89 ± 67.16	10.19 ± 2.77	7.45 ± 1.43	8.85 ± 3.71
Pb	1.50 ± 1.07	0.84 ± 0.09	1.01 ± 0.69	nd	0.66 ± 0.06	0.72 ± 0.29
Cu	16.34 ± 3.69	117 ± 95	25.43 ± 18.13	6.50 ± 2.03	35.56 ± 25.69	6.41 ± 5.13
Se	2.25 ± .54	2.91 ± 1.24	3.67 ± .14	nd	nd	nd
Tl	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Zn	74.25 ± 38.52	164 ± 107	157 ± 46	2.88 ± .79	21.92 ± 3.91	6.77 ± 4.85

The mixed waste was characterized without separating the shell from the organic fraction. To compare the results with the separately collected sample, a mass balance was carried out to obtain the overall characterization of clam, mussel, and murex by-products before pre-treatment. Figure 4-1 illustrates the VS, ashes and Cations in raw waste; and Figure 4-2 presents the Heavy metals concentration in raw waste.

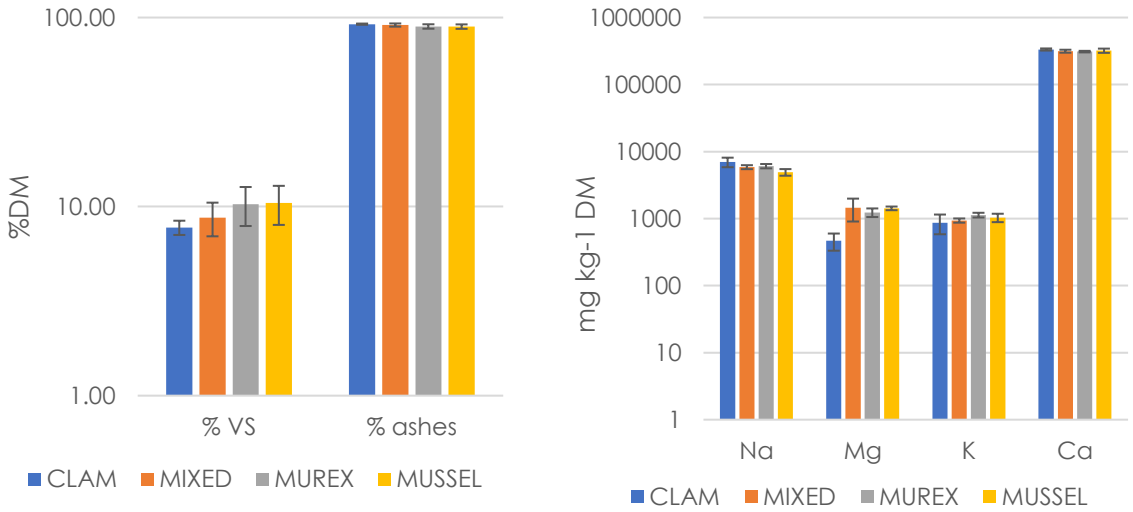

Figure 4-1- VS and ashes (left panel); Cations in raw waste (right panel)

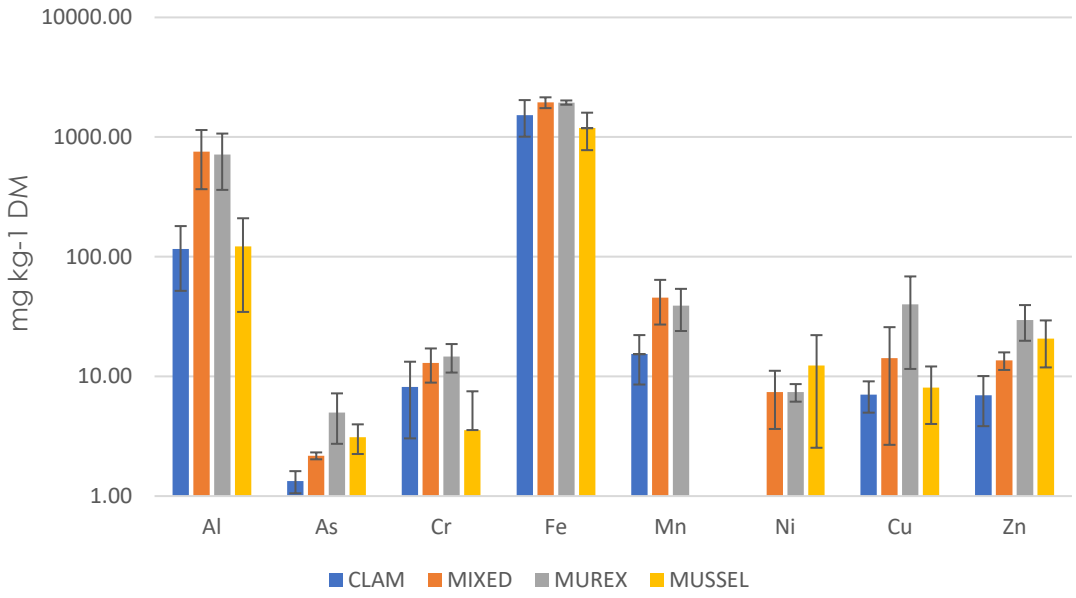

Figure 4-2- Heavy metals concentration in raw waste

There are no great differences between the three mollusc species confirming the homogeneity of the side-stream although the variability could occur daily and seasonally depending on the production activity of Co.Pe.Mo. It means the mixed side-streams is quite a homogenized by-product that ensures replicability and stability of the future valorisation chain. Figure 4-3 shows a comparison of the composition of different fraction and overall by-products, on a dry basis.

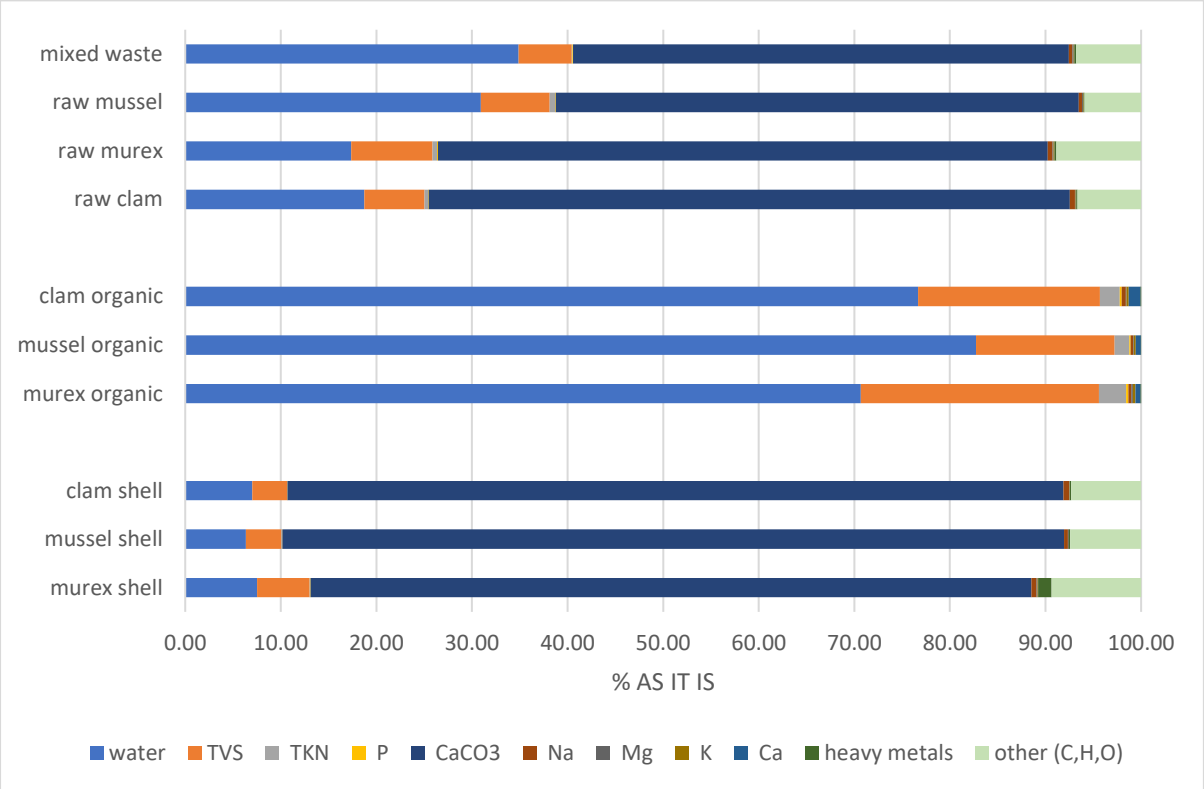

Figure 4-3- A comparison of the composition of different fraction and overall by-products, on a dry basis

4.3. Separation of organic and inorganic fractions

Table 4-6 mentioned that the measured weights of solid (in the bottom of the tank) and liquid (upper part of the tank) portions that come from the shredding pump test. Each dilution factors of mussel, clam, and murex. Here we observed that decreasing the dilution factors leads to an increase in both crushed shells in the bottom and the liquid portion volumes.

Table 4-6- waste type, dilution factor, water volume, waste weight crushed shell on the bottom, and liquid fraction

waste type	dilution factor	Water volume	Waste weight	Crushed shell on the bottom		Liquid fraction	
		l	kg	kg	%	Kg	%
CLAM	3.00	22	11	9	27.27	24	72.73
CLAM	2.47	22	15	11.5	31.08	25.5	68.92
CLAM	2.22	22	18	14	35.00	26	65.00
CLAM	2.00	22	22	17.8	40.45	26.2	59.55
MUREX	4.14	22	7	6.2	21.38	22.8	78.62
MUREX	2.57	22	14	11	30.56	25	69.44
MUREX	2.00	22	22	18.5	42.05	25.5	57.95
MUSSEL	5.00	22	5.5	3.5	12.73	24	87.27
MUSSEL	3.00	22	11	8	24.24	25	75.76
MUSSEL	2.00	22	22	16	36.36	28	63.64

Table 4-7 represents the total solids and volatile solid contents in percentages conducted on the crushed shell portion that comes from the shredding test for each dilution factor

Table 4-7- Total solids and volatile solid content on the crushed shell

waste type	dilution factor	TS%	TVS/TS%
clam	3.00	88.19	2.81
clam	2.47	91.35	2.58
clam	2.22	89.12	2.87
clam	2.00	84.61	2.82
murex	4.14	77.89	12.35
murex	2.57	82.15	4.56
murex	2.00	85.27	2.98
mussel	5.00	88.33	4.18
mussel	3.00	83.10	6.00
mussel	2.00	77.56	6.42

After shredding, two fractions were easily detected: the crushed shell immediately settled on the bottom tank and a liquid fraction composed of the added water and residual meat or lighter particles coming out from the shredding process. Both fractions were sampled to investigate TS% (Table 4-8). Figure 4-4 illustrates settling performance graph.

Table 4-8- Total solid in different fraction

waste type	**dilution factor**	TS on the solid fraction		TS on the liquid fraction	
		kg	%	Kg	%
CLAM	3.00	7.94	92.85	0.61	7.15
CLAM	2.47	10.51	92.19	0.89	7.81
CLAM	2.22	12.48	93.96	0.80	6.04
CLAM	2.00	15.06	91.51	1.40	8.49
MUREX	4.14	4.83	93.12	0.36	6.88
MUREX	2.57	9.04	89.16	1.10	10.84
MUREX	2.00	15.78	90.35	1.69	9.65
MUSSEL	5.00	3.09	93.03	0.23	6.97
MUSSEL	3.00	6.65	91.70	0.60	8.30
MUSSEL	2.00	12.41	91.66	1.13	8.34

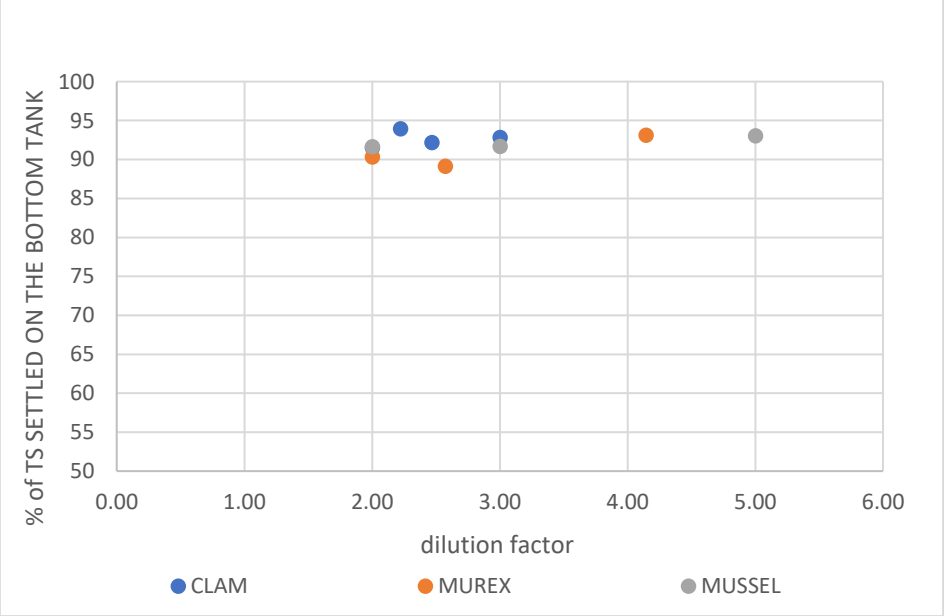

Figure 4-4- Settling performance graph

Here we can observe that for increasing or decreasing the dilution factor, there is no significant differences in the total solids settling on the bottom tank.

4.4. Settling tests

A settling test using an Imhoff cone was conducted on the liquid fraction to observe if other solids can be recovered by settling after 45 minutes.

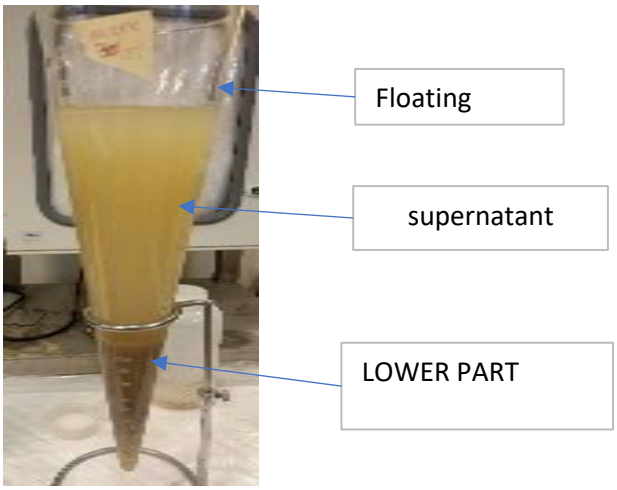

Figure 4-5- Settling performance of liquid waste

Good settleability was observed good settleability in different sample obtaining two volume fractions: denser on the bottom and poor in solids on the upper part.

Some samples of mussel and murex have an extra part called the floating part. TS% and TVS/TS were investigated on each fraction collected separately after 45 min of settling.

Table 4-9-TV and TVS/TS

		Lower part/settleable			supernatant			Floating part		
WASTE TYPE	dilution factor	Volume	TS %	TVS/TS %	Volume	TS %	TVS/TS %	Volume	TS %	TVS/TS %
CLAM	3.00	9.0%	2.87	79.62	91.0%	2.52	78.86			
CLAM	2.47	3.5%	8.14	47.72	96.5%	3.32	70.90			
CLAM	2.22	6.0%	6.12	62.96	94.0%	2.89	75.26			
CLAM	2.00	4.0%	7.62	62.39	96.0%	5.24	72.39			
MUREX	4.14	13.0%	5.33	44.24	83.0%	0.86	66.20	4.00%	3.86	81.83
MUREX	2.57	60.0%	5.65	64.83	35.8%	2.55	79.79	4.20%	2.09	67.94
MUREX	2.00	65.0%	8.78	39.69	35.0%	2.57	73.11			
MUSSEL	5.00	2.5%	2.93	84.78	97.5%	0.91	73.17			
MUSSEL	3.00	23.0%	3.29	83.19	67.0%	2.00	19.70	10%	3.10	78.82
MUSSEL	2.00	8.0%	4.32	88.80	92.0%	4.00	73.41			

We can observe (Figure 4-6) adding so much water to obtain a low amount of solids. Since the solids on the bottom tanks were almost the same in the different test conducted at different dilution factors, it means that this lower TS% is only caused by addition of more water.

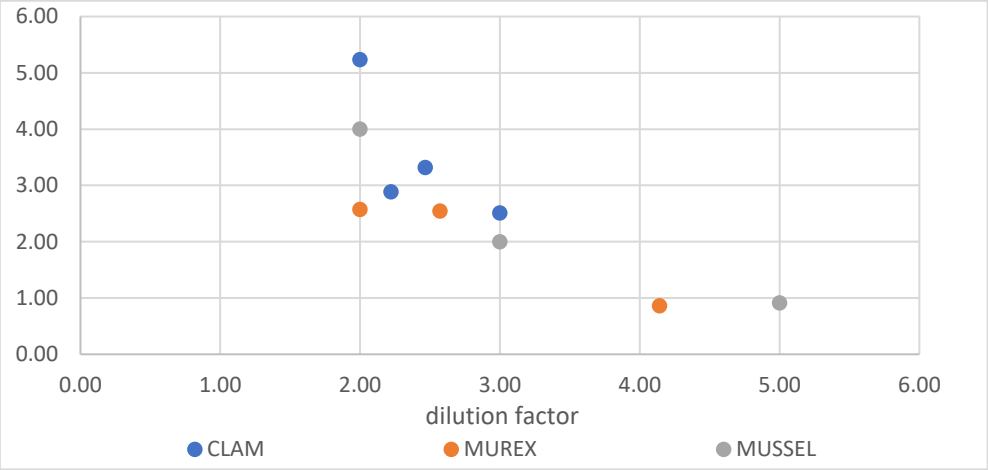

Figure 4-6- represents the calculation of % of liquid fraction and % total solids recovery

4.4.1. Mass balances

The mass balances were carried out on the solid (crushed shell part) and liquid part that comes from shredding pump test for each dilution's factors of mussel, clam, and murex. Table 4-10 to Table 4-12 represent the mass balances for each fraction of mussel, clam, and murex shell. Here we observed that increasing the source of waste leads increase the total solids in the bottom. Finally, we concluded that shredding test even go with low water volumes means dilution factor less than 2.

Table 4-10- The mass balances for each fraction of mussel

	5.5KG:22L			**11kg:22l**				**22kg:22l**		
	tank bottom	**settle Imhoff**	**supernatant Imhoff**	**tank bottom**	**settle Imhoff**	**supernatant Imhoff**	**floating Imhoff**	**tank bottom**	**settle Imhoff**	**supernatant Imhoff**
waste (KG)	3.5	0.6	23.4	8	5.75	16.75	2.5	16	2.24	25.76
TS(KG)	3.09	0.018	0.21	6.65	0.189	0.07	0.08	12.41	0.097	1.03
moisture (KG)	0.41	0.582	23.19	1.35	5.561	16.68	2.42	3.59	2.143	24.73
TVS(KG)	0.13	0.01	0.16	0.40	0.16	0.01	0.06	0.80	0.09	0.76

Table 4-11- The mass balances for each fraction of clam

	11KG:22L			**15kg:22l**			**18kg:22l**			**22kg:22l**		
	tank bottom	**settle Imhoff**	**supernatant Imhoff**	**tank bottom**	**settle Imhoff**	**supernatant Imhoff**	**tank bottom**	**settle Imhoff**	**supernatant Imhoff**	**bottom tank**	**settle Imhoff**	**supernatant Imhoff**
waste (KG)	9	2.16	21.84	11.5	0.8925	24.6075	14	1.56	24.44	17.8	1.048	25.152
TS(KG)	7.94	0.062	0.05	10.51	0.073	0.85	12.48	0.095	0.75	15.06	0.080	1.37
moisture (KG)	1.06	2.098	21.79	0.99	0.820	23.76	1.52	1.465	23.69	2.74	0.968	23.78
TVS(KG)	0.22	0.05	0.04	0.27	0.03	0.60	0.36	0.06	0.57	0.43	0.05	0.99

Table 4-12- The mass balances for each fraction of murex

	7KG:22L				14kg:22l				22kg:22l		
	tank bottom	**settle Imhoff**	**supernatant Imhoff**	**floating Imhoff**	**tank bottom**	**settle Imhoff**	**supernatant Imhoff**	**floating Imhoff**	**tank bottom**	**settle Imhoff**	**supernatant Imhoff**
waste (KG)	6.2	2.964	18.924	0.912	11	15	8.95	1.05	18.5	16.575	8.925
TS(KG)	4.83	0.158	0.16	0.04	9.04	0.848	0.23	0.02	15.78	1.456	0.23
moisture (KG)	1.37	2.806	18.76	0.88	1.96	14.152	8.72	1.03	2.72	15.119	8.70
TVS(KG)	0.60	0.07	0.11	0.03	0.41	0.55	0.18	0.01	0.47	0.58	0.17

4.5. Bio methanation test:

The collected samples from shredding tests performed at a dilution factor equal to 2 were used as substrates. While the anaerobic sludge from the anaerobic digestor of Falconara Marittima wastewater treatment plant was used as inoculum. In the BMP test, there was no addition of nutrients because the liquid part already contained them.

Once the completion of the substrate and sludge characterization, the bmp test was started. During this period, we always monitored the waster column levels to calculate cumulate gas in (ml). The cumulative is useful to calculate the (ml) of biogas production per day. During this test we conducted the gas analysis for 10 ml gas to check the production(%CH4%), CO_2, and water in ppm by using Brunel gas chromatography. once we know the % CH4 and ml biogas/day, then we calculated the volume of methane in (ml).

Table 4-13 represents the gas analysis for (10 ml of gas sample) during the BMP test to check the biogas production. We observe that blanket is still producing the methane, and other samples are of % CH4 is around 55%, and CO2 is around 28%.

Table 4-13- gas analysis on the blanket, mussel, clam, and murex

	MUSSEL			BLANKET			MUREX			CLAM		
DATE	CO2 %	CH4 %	extra%	CO2 %	CH4 %	extra%	CO2 %	CH4 %	extra%	CO2 %	CH4 %	extra%
4/28/2021	16.17	23.04	60.79	17.71	42.11	40.17	18.39	42.72	38.89	23.98	47.34	28.68
4/30/2021	22.55	56.44	21.02	15.68	35.47	48.84	17.04	28.95	54.01	19.29	57.67	23.05
5/7/2021	19.35	56.22	24.43	18.13	45.59	36.27	19.07	51.93	29.00	18.24	53.75	28.00
5/11/2021	18.18	51.26	30.57	19.63	53.47	26.90	17.47	49.32	33.22	21.95	55.37	22.69
5/18/2021	16.73	53.39	29.87	20.53	56.89	22.58	18.64	54.89	26.46	23.84	55.00	21.16
5/24/2021	16.91	54.49	28.60	20.41	57.26	22.34	16.57	55.32	28.11	23.84	55.90	20.27

The duration of the test lost for 27.7 days and then we calculated the volume of biogas in terms of gram of VSS added. At the end of test, we calculated the biogas for blanket, mussel, clam, and murex in in ml per day was calculated. After that we calculated the net banket biogas production by simply subtract the biogas production of substrate and the blanket production. Finally, we calculated the biogas production in terms of gram of VSS feed. The biogas production in terms of gram of VSS (biogas ml/kgVSS) for mussel, clam, and murex was 0, 76.41, and 4.25. Figure 4-7 represents the cumulative biogas production duration the test period.

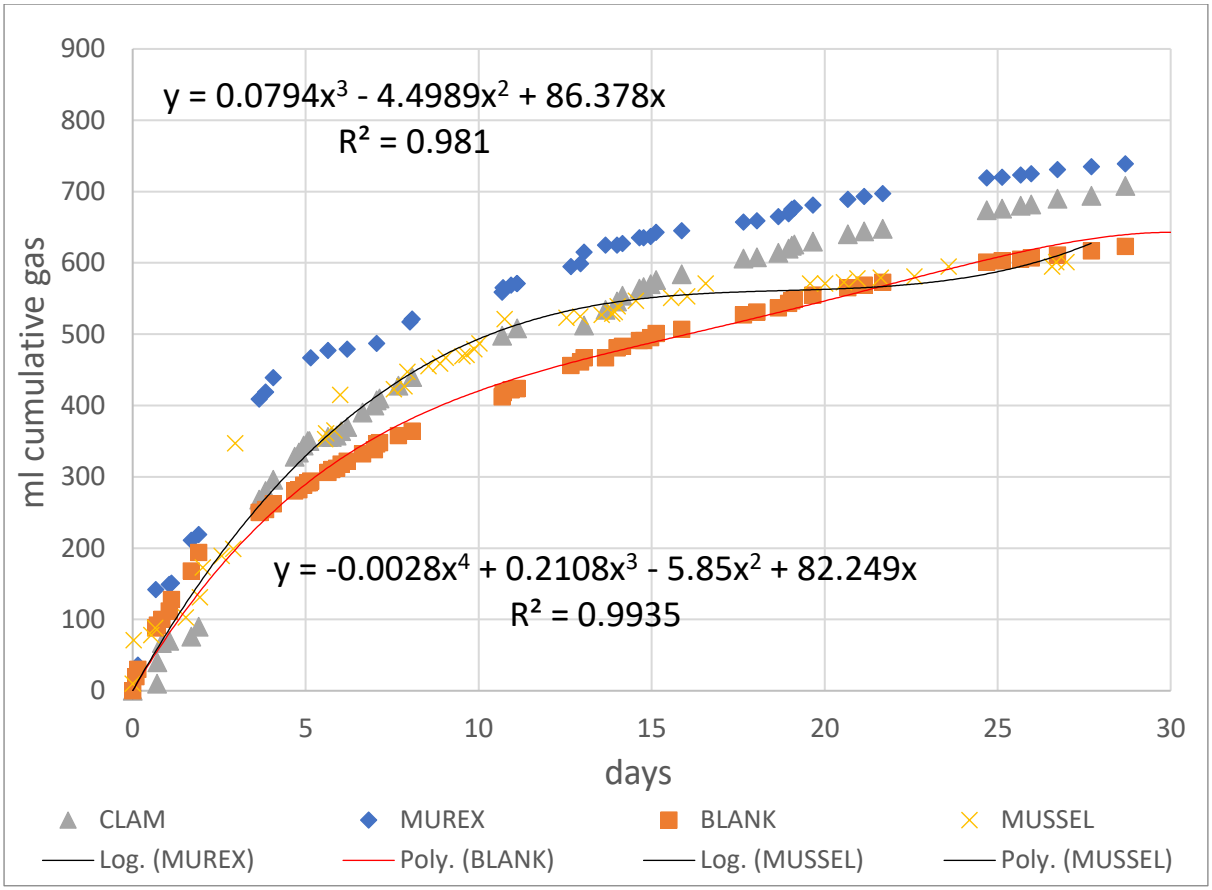

Figure 4-7- cumulate gas production from the BMP test

Figure 4-8 represents the biogas products after removing the blanket production from the substrate.

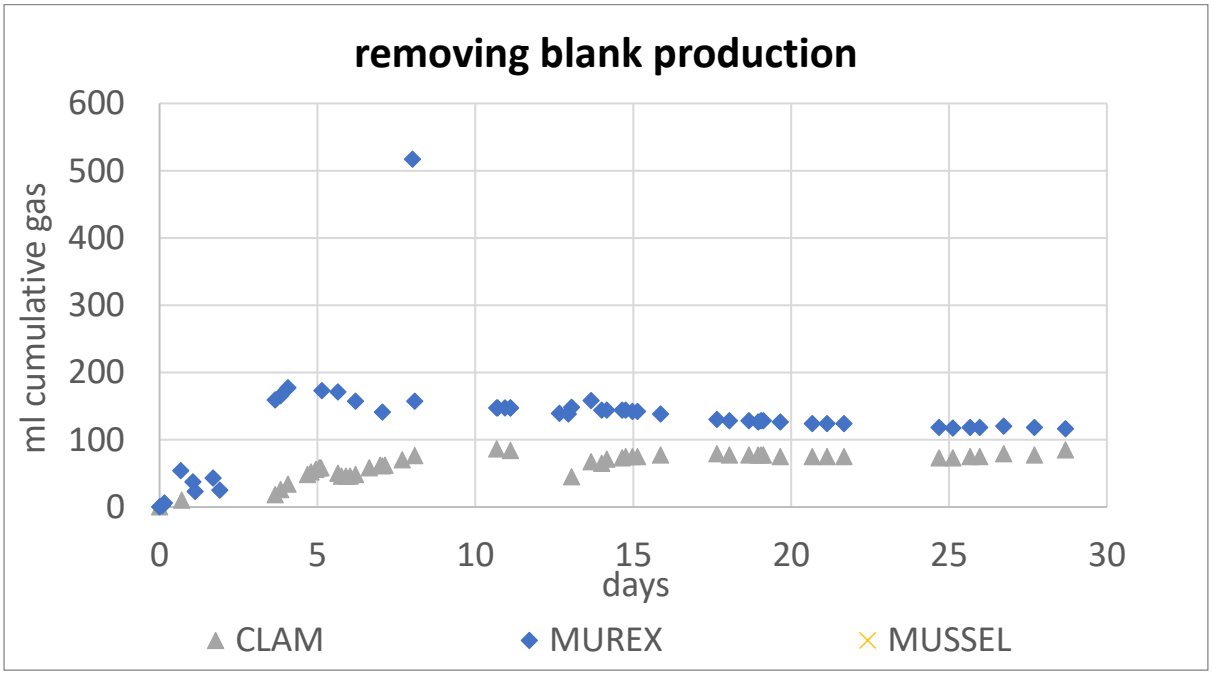

Figure 4-8- removing the bank production from the substrate of mussel, murex, and clam

We conducted the analysis on the final sample in the laboratory. Initially we filtered the sample by using 0.45-micron filter paper. The filtered fraction was carried out to analyze the pH, alkalinity, N-NH4, cations, anions. This analysis was done because we had the problems in biogas production to check any inhibitory action in the test. Here we observed that N-NH4 should be >200 is good for BMP but our case it is more 800. It was the one of the reason for test went wrong. Table 4-14 to Table 4-17 represent the pH, alkalinity, N-NH4, cations, and anions characterization on the final sample of BMP test.

Table 4-14- pH and alkalinity

	pH	**alkalinity**			partial alkalinity		total alkalinity
	pH	**NHCl**	**V Campione**	**VHCl**	**mg CaCO3/l**	**VHCl**	**mg CaCO3/l**
clam	8.92	0.0373	**2**	3.722	3471.70	0.718	4141.41
murex	8.94	0.0373	**2**	3.466	3232.91	0.796	3975.38
mussel	8.89	0.0373	**2**	3.606	3363.50	0.664	3982.84

Table 4-15- N-NH4

	N-NH4			
	NHCl	**V Campione**	**V HCl**	**mg N-NH4/l**
clam	0.0373	**2**	3.766	853.84
murex	0.0373	**2**	3.916	893.02
mussel	0.0373	**2**	4.338	1003.23

Table 4-16- anions

	anions							
	Cl	**NO2**	**N-NO2**	**NO3**	**N-NO3**	**PO4**	**P-PO4**	**SO4**
clam	1492.10	1.19	0.36	2.29	0.52	4.29	1.40	219.08
murex	1093.41	0.45	0.14	5.26	1.19	3.72	1.21	164.38
mussel	1099.72	0.43	0.13	2.95	0.67	5.95	1.94	49.90

Table 4-17- cations

	cations					
	Na	**NH4**	**N-NH4**	**K**	**Mg**	**Ca**
clam	701.06	469.24	364.96	275.06	107.13	88.87
murex	535.32	513.48	399.37	167.14	107.04	63.41
mussel	575.65	477.69	371.53	168.19	90.08	91.57

4.6. Chemical extraction of chitin from Mollusc shells

Before going to the chitin extraction method, here we considered the raw mussel, clam, and murex shell (manually separated shell from the meat) dried in the oven at 35ºC to remove the moisture content. The dried samples of mussel, clam, and murex shell was carried out for FTIR analysis to identify the chitin, protein, and CaCO3 compounds.

The FTIR analysis was carried out with three different modes, which are the transmission mode with KBr pads, DRIFT (with KBr powder reflectance mode), and UATR (Universal Attenuated Total Reflection) were first tested to understand the influence of FTIR acquisition modes. Moreover, to identify the spectra with best resolution.

Figure 4-9 represents the FTIR spectra, which was conducted on the calm shell by those modes I declared before. Here I observed the absorbance with 3417 cm^{-1} refers to OH stretching, 2975 cm^{-1} refers to CH stretching, 1788 cm^{-1} refers to C=O stretching, 1476 refers to NH bending, 1080 refers to CO stretching, and 856 refers to C-C stretching (Harmami et al., 2019; Idacahyati et al., 2020; Silverstein et al., 2005).

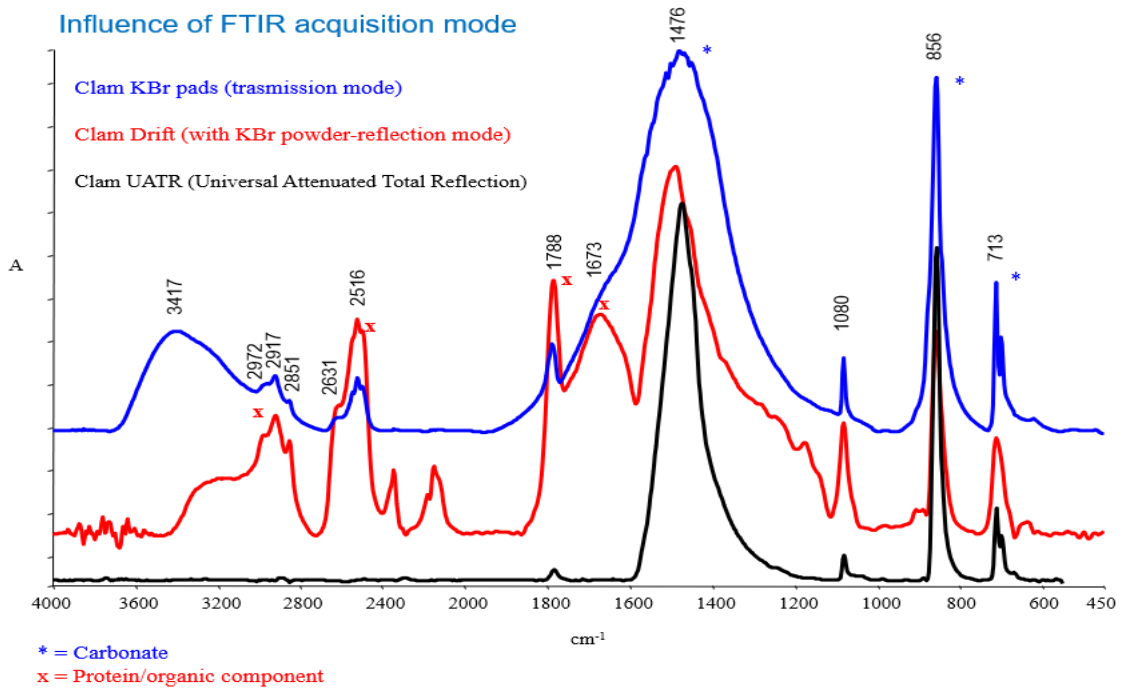

Figure 4-9- different modes of FTIR analysis on the calm shell

Based on the previous knowledge on the different modes of FTIR analysis, we obtained better spectra by transmission mode on the KBr pellets. Figure 4-10 represents the FTIR analysis on the raw sample of mussel, clam, and murex shell with transmission mode with KBr pellets.

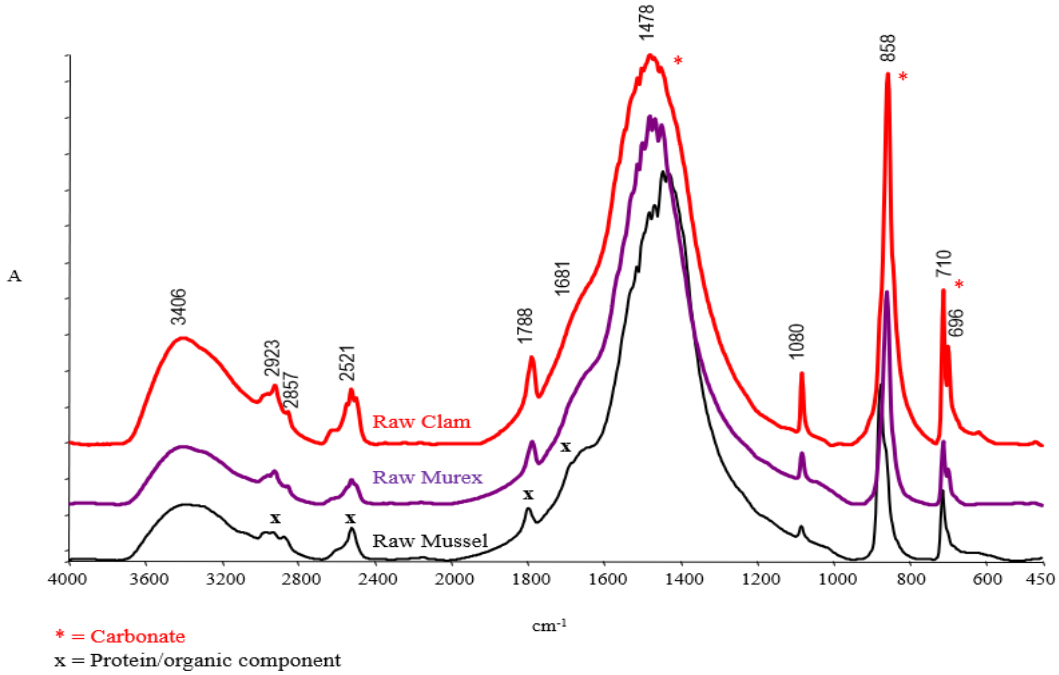

Figure 4-10- FTIR analysis on the raw shell samples of mussel, clam, and murex by transmission mode

In addition, FTIR analysis of transmission mode was carried out on the commercial chitin to make a spectrum as a reference to understand the removal of impurities, such as protein and minerals from our shell waste. The chemical extraction method of chitin was conducted by the step which includes pre-treatments, deproteinization, and demineralization.

The first trial of chitin extraction was carried out on the mussel and clam shell. The shell was dried in the oven at 35°C for 24 hours and then make them into powder form by manually by hammer. And then, sieve the powder with 355-micron sieve with particle sizes passing through a sieve was considered for chitin extraction.

In the chitin extraction process, the deproteinization step was carried out with 1M NaOH solution of 10 ml of NaOH added per one of gram of sample at 70°C for 3 hours. At the end of the deproteinization process, the sample was filtered by a Whatman filter paper (2 microns porosity). After the filtration step, the samples were washed with distilled water for 5 to 7 baths. And then, the obtained sample was kept in the oven at 60ºC for 24 hours. After 24 hours, the sample was removed from the oven and kept in the desiccator for 20 minutes to cool down. Finally, the deproteinization yield was estimated in terms of weight loss.

The sample obtained from the despotized sample carried for dimerization step with 1M HCl solution of 10 mg of HCl added per one gram of sample at ambient temperature of approximately 30°C for one hour. Similarly, to deproteinization, at the end of the demineralization process, the sample was filtered, washed with distilled water dried in the oven at 60ºC for 24 hours. Then, the demineralization yield was measured in terms of weight loss

percentage. Finally, we obtained the product of chitin and the processes of deproteinization, and demineralization was not controlled in terms of test lost at which time.

Table 4-18 represents the deproteinization, demineralization, and chitin yields. Here we conducted the FTIR analysis to quantify the chitin extraction quality. Here we observed lots of calcium carbonate peaks means that there is no complete removal of $CaCO_3$. Moreover, the shell contain (90-95%) of $CaCO_3$ (Naik & Hayes, 2019).

Table 4-18- deproteinization and chitin yield

sample for DP	DP yield (weight loss)	DM yield (weight loss)	chitin yield %
mussel	8.76	55.2	39.87
clam	12.38	54.5	40.88

We repeated demineralization step (bath one) on the same sample that comes from deproteinization step by increasing the duration from 1 hour to 3 hours at ambient temperature. This process was controlled with pH probe to observe the solution reaches the constant pH. Table 4-19 represents demineralization yield and chitin yield. Here we noticed that there is still calcium carbonate in the sample because it is not reliable to literature review on the $CaCO_3$.

Table 4-19- first bath of demineralization yield

DP sample for DM	DM yield (weight loss) %
mussel	52
clam	51.05

We again conducted the demineralization step (second bath) on the on the same sample that comes from deproteinization step with 3 hours of duration at ambient temperature. The obtained results of %DM yield of clam, and mussel was 52.35 and 57.33. here we noticed that the dosage of HCl dosage is not sufficient to remove the complete $CaCO_3$ form the sample.

In the second trail. We conducted the deproteinization process at ambient temperature for 2 hours with the NaOH dosage of 10 ml/g. Table 4-20 represent the deproteinization yields in terms of weight loss percentages of mussel, clam, and murex. Here we observed that this results are somehow related to the literature values between (0.1-7%) (Lertwattanaruk et al., 2012; Naik & Hayes, 2019).

Table 4-20- Deproteinization yield at ambient temperature for mussel, clam, and murex

sample	duration	DP yield %	dosage of NaOH (ml/g)	temperature
clam	2	5.04	10	30
murex	2	3.43	10	30
mussel	2	6.08	10	30

In the second trails we conducted the demineralization on the fresh samples of mussel, clam, and murex.

4.6.1. Demineralization on the fresh samples

Based on the previous trails we concluded that the dosage of HCl is not sufficient to remove the $CaCO_3$ form the sample. So here demineralization process was conducted at ambient temperature for 3 hours of duration with the chemical dosage of 20ml/g.

In this step, we controlled the process with pH and temperature. Table 4-21 represents the pH variations during demineralization on fresh sample. Here I observed that pH ranges from (0.4-1.43) when we treated demineralization with fresh samples.

Table 4-21- pH variations during demineralization on fresh sample

pH variations during demineralization on fresh sample								
mussel			clam			murex		
duration (min)	PH	temperature	duration (min)	PH	temperature	duration (min)	PH	temperature
0	0.49	22	0	1.06	22	0	0.44	22.30
14	0.85	22.8	23	1.28	23	17	0.8	23.20
31	0.95	23.4	40	1.36	23	31	0.81	23.50
49	0.98	24.4	58	1.39	24.4	51	0.83	24.50
65	0.99	25.1	77	1.4	24.9	72	0.84	25.40
85	1	25.5	96	1.4	25.1	90	0.84	25.90
107	1.01	25.8	116	1.43	25.6	113	0.85	26.10
132	1.01	25.9	140	1.43	25.7	136	0.85	26.40
153	1.01	26	162	1.43	25.9	159	0.85	26.20
176	1.01	25.7	185	1.43	25.7	184	0.85	26.00

Figure 4-11 represents the pH variations during demineralization on fresh sample. Here I observed that pH ranges from (0.4-1.43) when we treated demineralization with fresh samples. I concluded that the pH is a good identification for test lasting.

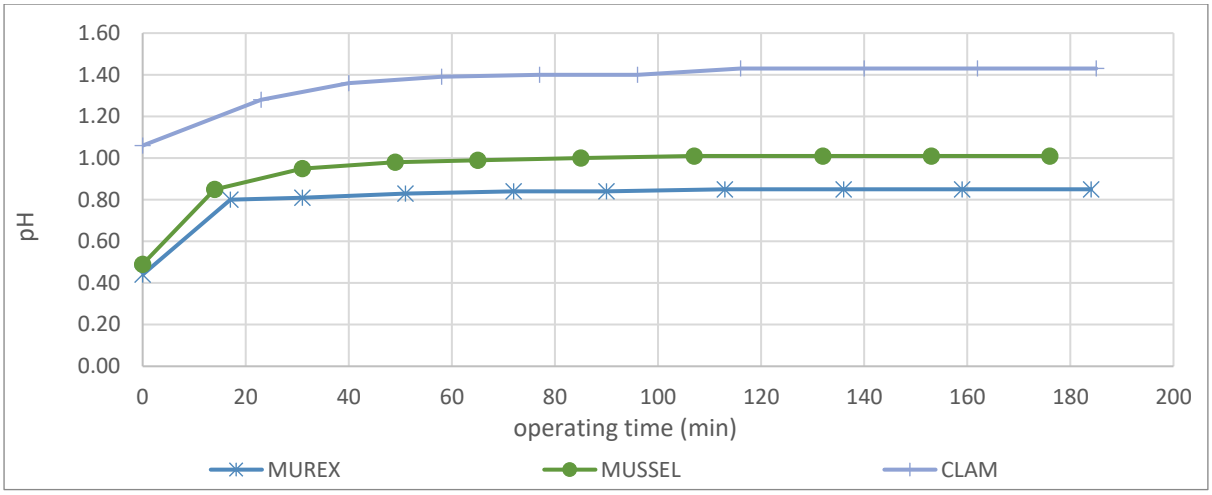

Figure 4-11- pH variations during demineralization on fresh sample

Table 4-22 represents the demineralization yields on the fresh samples of mussel, clam, and murex.

Table 4-22- demineralization yield on the fresh samples

demineralization yield on the fresh samples				
sample	duration	temperature	DM %	dosage (mg/l)
mussel	2 hours 56 minutes	24.66	92.86	20
clam	3 hour 5 minutes	24.53	96.87	20
murex	3 hour 4 minutes	24.95	92.52	20

Here we conducted the FTIR analysis on the demineralized samples to understand the $CaCO_3$ by the UATR (Universal Attenuated Total Reflection) mode. And then compared FTIR spectra of demineralized samples with the commercial machine spectra.

Figure 4-12 and Figure 4-13 represents FTIR analysis on the demineralized samples and comparison of demineralized sample with commercial chitin. Here we observed that there is no more $CaCO_3$ in the samples.

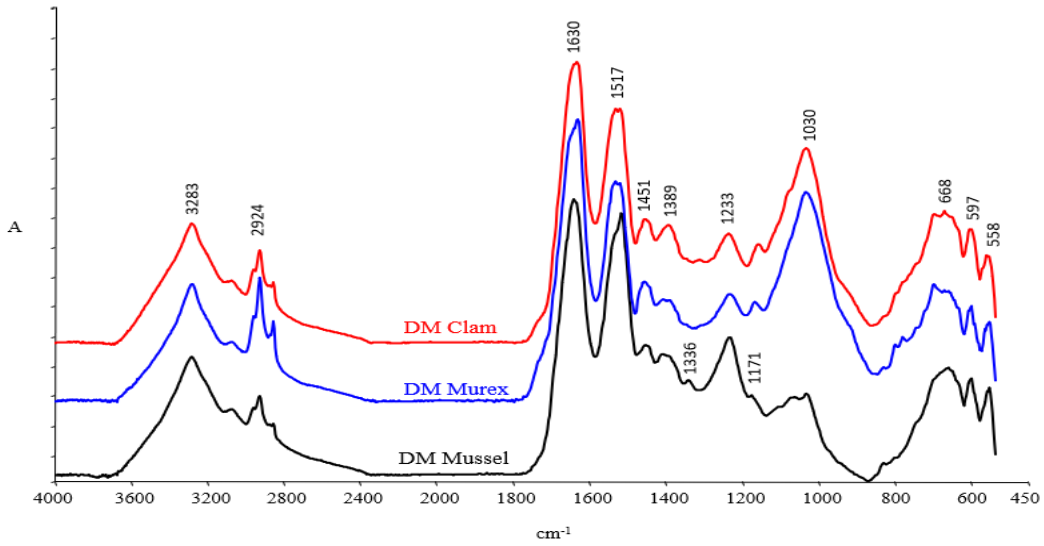

Figure 4-12- FTIR spectra on the demineralized sample

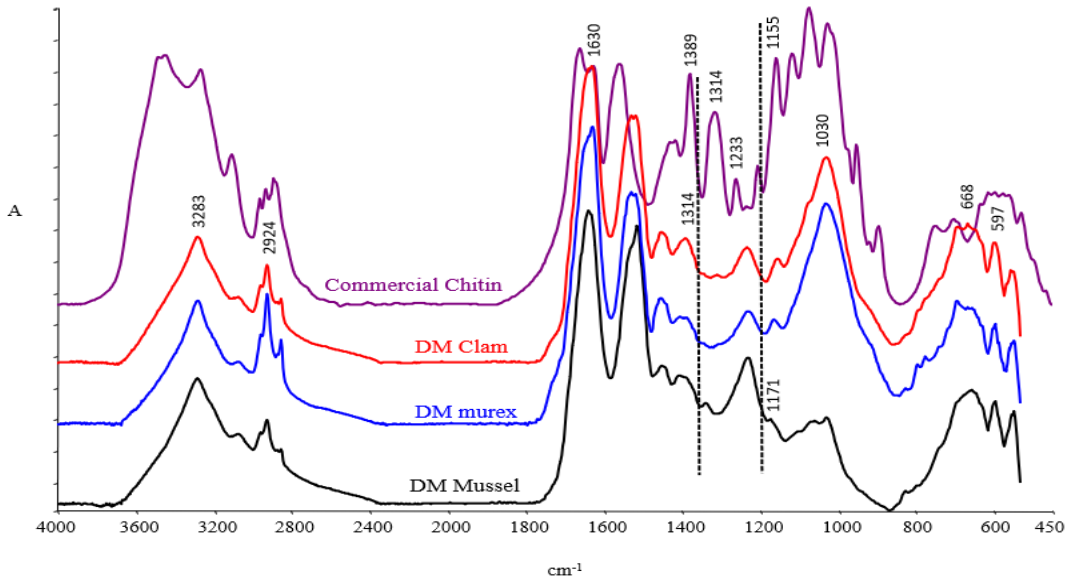

Figure 4-13- comparison between the demineralized sample with commercial chitin

In this third step we wanted to optimize the process of deproteinization with different chemical dosages, duration, and temperatures to understand the deproteinization yields. The maximum yield of deproteinized samples is going for demineralization step was discussed.

4.6.2. Deproteinization on the fresh samples

In the first step, we started with deproteinization with different chemical such as (10, 15, and 20 mg) added per one gram of sample at 70 °C for 2 hours on the mussel, clam, and murex shell. To understand the deproteinization yields with refers to different chemical dosages. This stage of deproteinization step we controlled the process with measured pH and temperatures.

Table 4-23 and Table 4-25 represents the deproteinization processes controlled with temperature and pH for mussel, clam, and murex. Here I conclude that pH is not a good indication for deproteinization test.

Table 4-23- Deproteinization process for mussel

deproteinization for mussel				
		Ph		
observation period	**duration (min)**	**mussel (10ml)**	**mussel (15ml)**	**mussel (20ml)**
15:15	0	11.13	11.25	11.72
15:30	15	10.83	11.23	11.83
15:45	30	10.51	11.18	11.48
16:00	45	10.5	11.36	11.17
16:15	60	10.49	11.27	11.26
16:30	75	10.62	11.17	11.32
16:45	90	10.48	11.16	11.14
17:00	105	10.53	11.23	11.27
17:15	120	10.47	11.33	11.31

Table 4-24- deproteinization process for clam

deproteinization for clam				
		Ph		
observation period	**duration (min)**	**clam(10ml)**	**clam (15ml)**	**clam (20ml)**
15:15	0	11.25	11.41	11.76
15:30	15	11.16	11.39	11.78
15:45	30	10.67	11.27	11.44
16:00	45	10.63	11.26	11.27
16:15	60	10.63	11.24	11.17
16:30	75	10.61	11.23	11.3
16:45	90	10.62	11.23	11.12
17:00	105	10.61	11.21	11.33
17:15	120	10.63	11.26	11.26

Table 4-25- deproteinization process for murex

deproteinization for murex				
		pH		
observation period	duration (min)	murex (10ml)	murex (15ml)	murex (20ml)
15:15	0	11.28	11.21	11.66
15:30	15	11.2	11.14	11.66
15:45	30	11.23	11.17	11.47
16:00	45	11.17	11.13	11.19
16:15	60	11.04	11.12	11.3
16:30	75	11.02	11.13	11.15
16:45	90	11.16	11.14	11.23
17:00	105	11.02	11.13	11.33
17:15	120	11.01	11.11	11.46

Figure 4-14 to Figure 4-16 represents the graphical representation in terms of variation in of pH and duration during the deproteinization test.

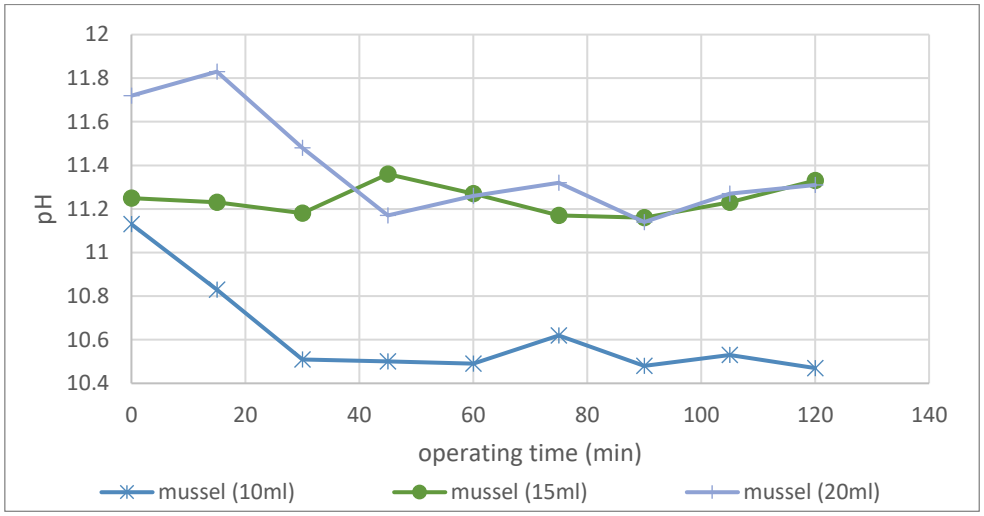

Figure 4-14- the variation pH deproteinization test for mussel

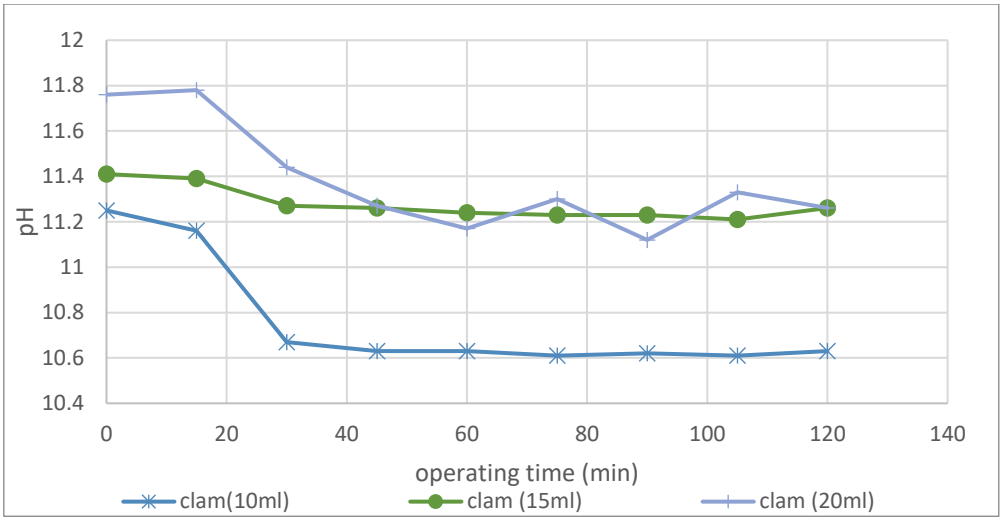

Figure 4-15- the variation pH deproteinization test for clam

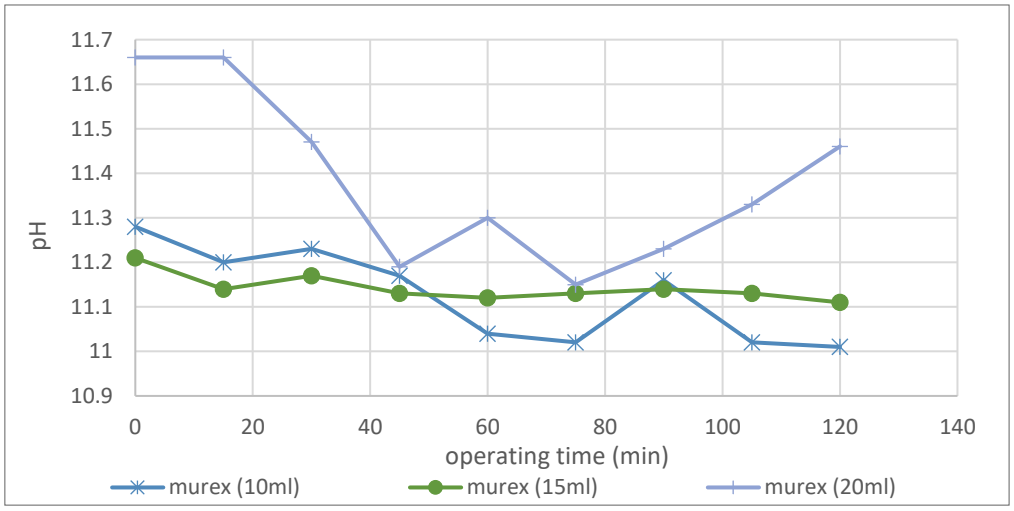

Figure 4-16- the variation pH deproteinization test for murex

Table 4-26 represents the different yields of deproteinization based on the chemical dosages and temperatures were observed.

Table 4-26- different yields of deproteinization processes of mussel, clam, and murex

sample (fresh) for DP	dosage of NaOH (ml/g)	DP yield (weight loss) %	Temperature	Duration (hours)
mussel 10 ml	10	17.66	73.56	2
mussel 15 ml	15	14.01	72.25	2
mussel 20 ml	20	9.60	70.92	2
clam 10 ml	10	7.78	74.56	2
clam 15 ml	15	5.07	71.56	2
clam 20 ml	20	7.68	71.17	2
murex 10 ml	10	17.01	74.78	2
murex 15 ml	15	11.56	74.38	2
murex 20 ml	20	11.84	71.33	2

Figure 4-17 represents the different yields of deproteinization based on the different chemical dosages. Here we noticed that the dosage of 10 ml/g gives the maximum yield of DP.

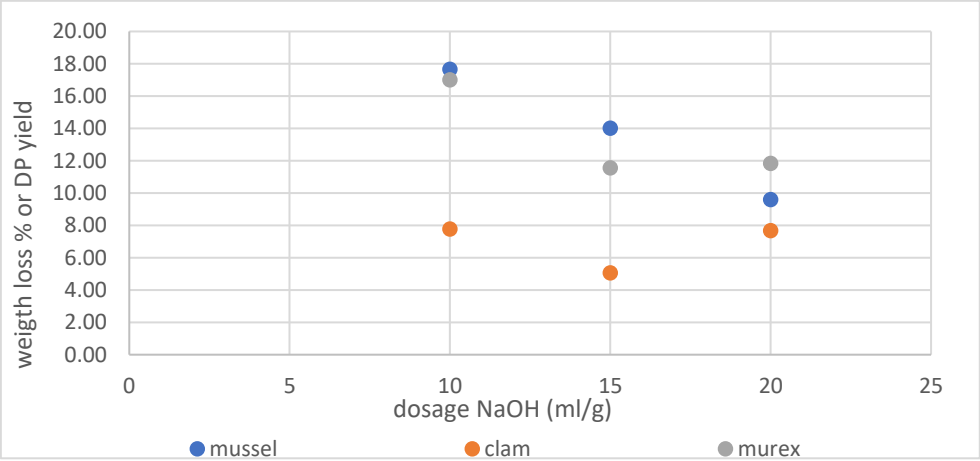

Figure 4-17- the yields of deproteinization based on the different chemical dosages

In this trail of deproteinization, the removal efficiencies of protein were conducted by the TKN analysis on the deproteinized samples. Before this analysis we conducted the TKN analysis on the fresh samples of mussel, clam, and murex to make comparison with deproteinized samples.

Table 4-27 represents the TKN analysis on the raw and deproteinized samples of mussel, clam, and murex was determine. Here we observed that compared with the fresh sample of

N%TS we almost removed the protein content (it is just like an indication for observing the protein removal).

Table 4-27- N%TS and N removal efficiencies based on the raw and deproteinized samples

sample	N%TS	N removal %
raw mussel shell	1.416	
Dp M10ml	0.048	96.619
DP M15ml	0.065	95.398
DP M20ml	0.103	92.752
clamshell	1.132	
DP C10ml	0.003	99.765
DP C15ml	0.047	95.868
DP C20ml	0.018	98.434
murex shell	1.509	
DP MX10ml	0.049	96.723
DP MX15ml	0.027	98.207
DP MX20ml	0.050	96.709

Based on the maximum yield we obtained from the dosage of 10 ml/g samples was considered for the demineralization step to remove the calcium carbonate. In this DM step, we considered the chemical dosage of HCl is 20ml/g at ambient temperature for 2 hours on the mussel, clam, and murex samples (Table 4-28). Here we observed that the complete removal CaCO3 and is comparable with the literature values.

Table 4-28- represents the demineralization yields of deproteinized samples of mussel, clam, and murex

DP samples for DM	**dosage of HCL (ml/g)**	**DM yield (weight loss) %**	**temperature**	**duration**
mussel	20	97.21	room (30)	2
clam	20	97.88	room (30)	2
murex	20	96.22	room (30)	2

In the fourth trail, we conducted the chitin extraction by the deproteinization with 10 ml/g NaOH dosage for one hour and demineralization with 20ml/g of HCl dosage. In this step we controlled the deproteinization and dimerization process with monitor of temperatures and pH (until the solution reaches steady state). Table 4-29 represents the deproteinized yield of the

samples of the mussel, clam, and murex. Table 4-30 represents the demineralization processes controlled with temperature and pH for mussel, clam, and murex.

Table 4-29- the deproteinization yields

sample (fresh) for DP	**dosage of NaOH (ml/g)**	**Dp yield (weight loss) %**	**temperature**	**duration (hours)**
mussel	10	9.90	79.80	1
clam	10	6.92	75.80	1
murex	10	11.36	74.20	1

Table 4-30- The demineralization processes controlled with temperature and pH for mussel, clam, and murex

demineralization on deproteinized sample							
		mussel		clam		murex	
time of observation	duration(min)	pH.	temperature	pH.	temperature	pH.	temperature
15:10	0						
15:30	20	4.7	25.8	4.42	26.1	0.7	25.7
15:50	40	4.83	26.3	4.71	26.8	0.69	26.6
16:10	60	5.1	27.1	5.05	27.3	0.68	27.6
16:30	80	5.23	27.6	5.23	27.8	0.68	28.3
16:50	100	5.44	28	5.48	28.2	0.68	29.1
17:10	120	5.62	28.3	5.7	28.4	0.68	29.7
17:30	140	5.82	28.7	5.98	28.9	0.68	29.9
17:50	160	6.01	29	6.14	29.2		
18:10	180	6.18	29.4	6.31	29.5		
18:30	200	6.31	29.7	6.44	29.9		
18:50	220	6.33	30.1	6.5	30.2		
19:10	240	6.52	30.2	6.61	30.5		

Figure 4-18 represents the pH variation during the demineralization processes. Here we observed that pH of the samples quite strange because the washing section in the deproteinization was not done properly for mussel, and calm and murex is good. Table 4-31 represents the demineralization yields of the samples.

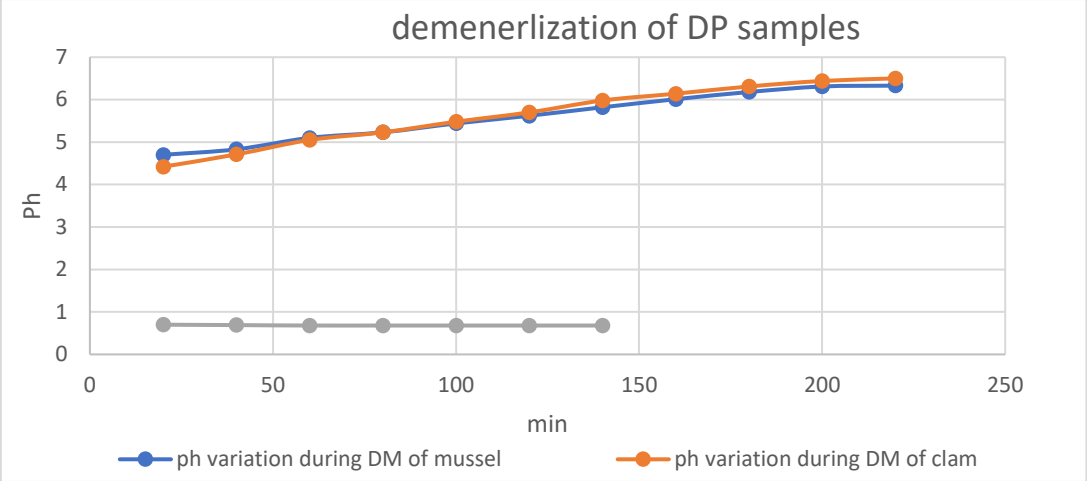

Figure 4-18- pH variation during the demineralization of DP samples

Table 4-31-The demineralization yields of the samples

DP sample for DM	dosage of HCL (ml/g)	DM yield (weight loss) %	temperature	duration (hours)
mussel	20	95.49	28.35	4.00
clam	20	97.23	28.57	4.00
murex	20	96.87	28.13	2.33

After the demoralization, FTIR analysis was conducted on the samples that come from demineralization step.

Figure 4-19 represents the chitin spectra of mussel, clam, and murex. Here we observed that the intermediate absorbances 2516 and 1797 cm^{-1} was occurred, which refer to carbonic acid because in the deproteination stage washing was not good. Based on the improper washing, when you add the acid (HCl) to the sample for demineralization process first the dosage is be consumed for making the solution neutral. And then, the demineralization process will start in that case we have problem in terms of the chemical dosage will not be sufficient for removing the demineralization process.

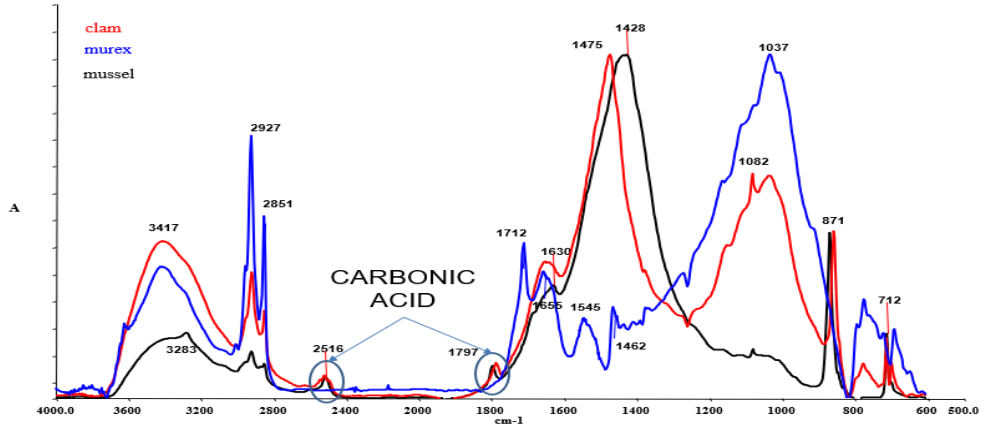

Figure 4-19- chitin spectra

4.6.3. Summary on the deproteinization test at lab scale:

Here I mentioned that the different trails were conducted on the deproteinization process in terms of different operational condition such as different dosages of chemicals, duration, and temperatures. To understand the how they can influence on the deproteinization yield (% of weight loses) were addressed.

Table 4-32 represents the deproteinization yield with different chemical dosages, temperature, and durations were observed. Here we observed that the maximum yield deproteinization obtained with the chemical dosage of NaOH at 10ml/g with the duration of 2-3 hours.

Table 4-32- the deproteinization yield with different chemical dosages, temperature, and durations

sample	duration	DP yield %	dosage of NaOH (ml/g)	temperature
mussel	3	8.76	10	70
mussel	2	17.66	10	73.56
mussel	1	9.90	10	79.80
mussel	2	14.01	15	72.25
mussel	2	9.60	20	70.92
mussel	2	6.08	10	30
clam	3	12.38	10	70
clam	2	7.78	10	74.56
clam	1	6.92	10	75.80
clam	2	5.04	10	30
clam	2	5.07	15	71.56
clam	2	7.68	20	71.17
murex	2	17.01	10	74.78
murex	1	11.36	10	74.20
murex	2	3.43	10	30
murex	2	11.56	15	74.38
murex	2	11.84	20	71.33

Figure 4-20 to Figure 4-22 represents the observation deproteination yields with different chemical dosages, durations, and temperatures.

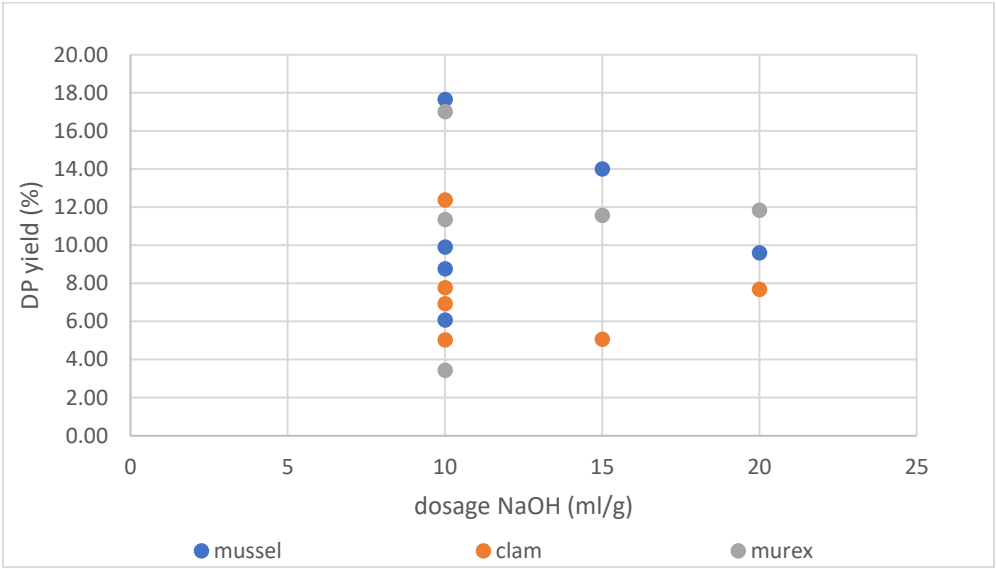

Figure 4-20- DP yield vs dosage (mg/l)

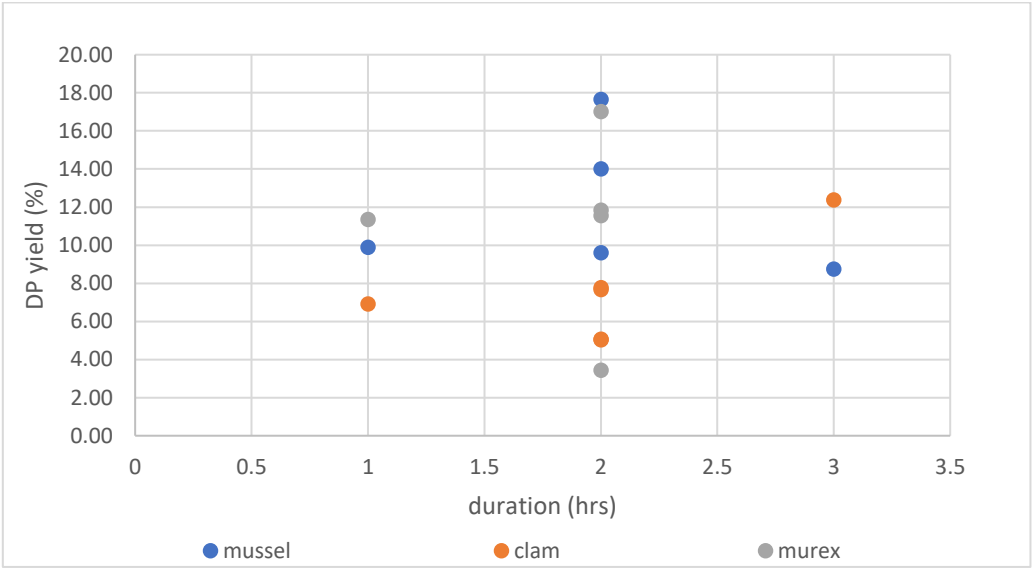

Figure 4-21- DP yield vs duration

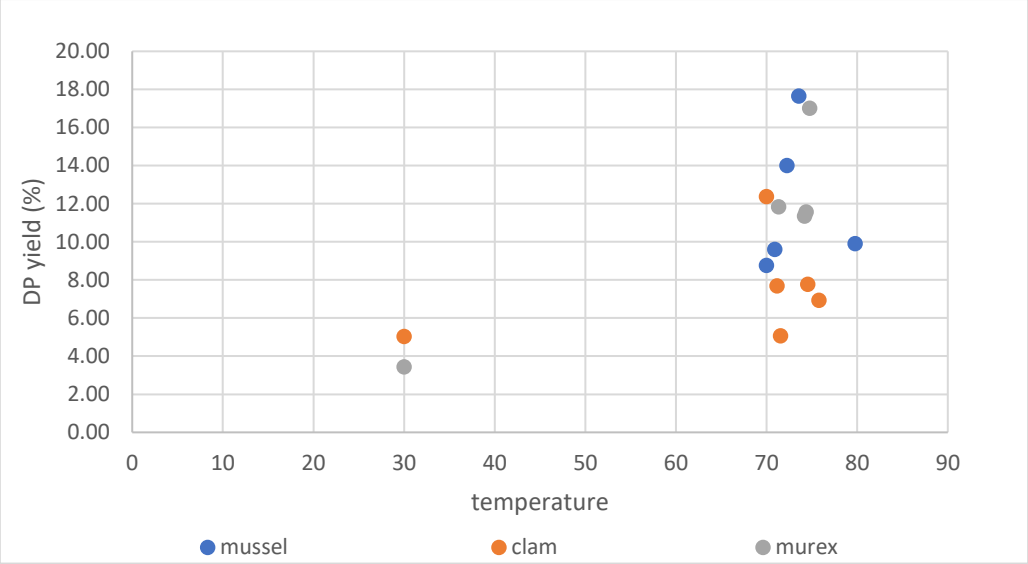

Figure 4-22- DP yield vs temperature

4.6.4. Summary on the demineralization test at lab scale

Here I mentioned that the different trails were conducted on the demineralization process in terms of different operational condition such as different dosages of chemicals, duration, and temperatures. To understand the how they can influence on the demineralization yield (% of weight loses) were addressed. Table 4-33 represents the demineralization yield with different chemical dosages, temperature, and durations were observed. Here we observed that the maximum yield demineralization obtained with the chemical dosage of HCl at 10ml/g with the duration of 2-3 hours.

Table 4-33- summary table of demineralization test

sample	duration	DM yield %	dosage of HCL (ml/g)	temperature
mussel	1	55.2	10	30
mussel	3	52	10	30
mussel	3	57.33	10	30
mussel	3	92.86	20	30
mussel	2	97.21	20	30
mussel	4	95.49	20	28.35
clam	1	54.5	10	30
clam	3	51.05	10	30
clam	3	52.35	10	30
clam	3	96.87	20	30
clam	2	97.88	20	30
clam	4	97.23	20	28.57
murex	3	92.52	20	30
murex	2	96.22	20	30
murex	2.33	96.87	20	28.13

Figure 4-23 and Figure 4-24 represent the observation demineralization yields with different 10 ml/g and 20 ml/g of chemical dosages and durations. Here we observed that the maximum yield deproteinization obtained with the chemical dosage of HCl at 10ml/g with the duration of 2-3 hours.

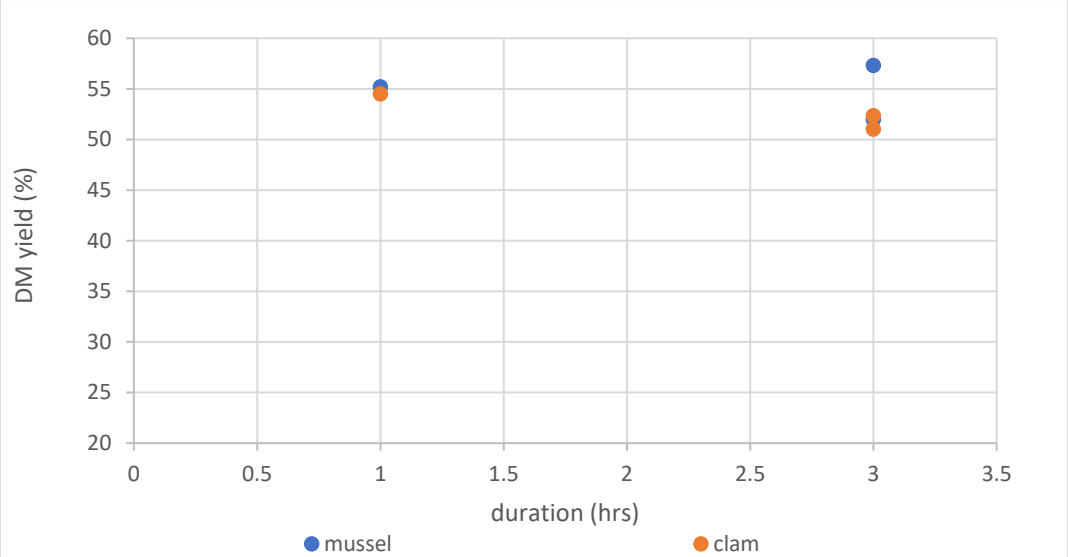

Figure 4-23- 10 ml/g of HCl dosage

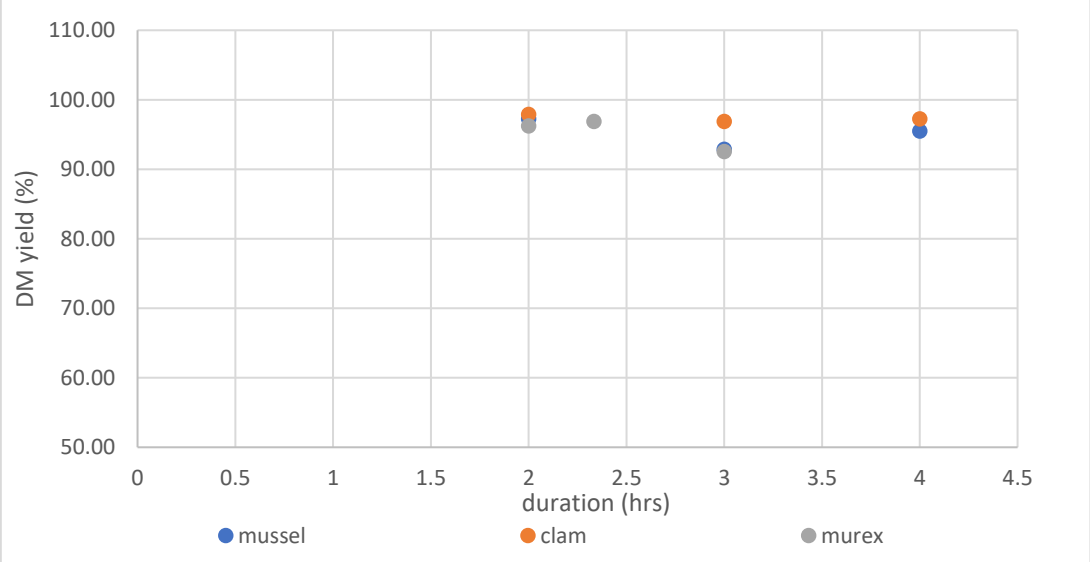

Figure 4-24- 20 ml/g of HCl dosage

Finally, the obtained chitin yields of mussel, clam, and murex was 4.06%, 2.57%, and 2.77% from the fourth trails. The conditions applied for this extraction was deproteinization of 10 ml/g for 1-2 hours all the samples and demineralization of 20 ml/g for 4, 4, and 2.33 hours.

Summery and conclusion

Seafood is one of the essential sources of nutrients and bioactive compounds for human consumption throughout the world. The literature review reveals that the processing of the seafood industry is leading to the generation of a tremendous quantity of by-products and discards annually. The production of seafood by-product/discards waste volume by 30-70% of the whole seafood after industrial processing. The crucial problems associated with the seafood processing industry are waste disposal, and improper waste management of seafood waste can cause negative impacts on the environment and human health. So far, the utilization of seafood wastes was confining at a relatively lower level due to the lack of inadequate knowledge

Global fish production (from capture fisheries and aquaculture) is projected to increase from 178.5 MT in the base period of (2017-2019) to 200 MT by 2029. By 2029, 58% of the fish available for human consumption projected to originate from aquaculture, up from 53% in (2017-19) (OECD/FAO, 2020)**.** Based on the above acknowledgment, the seafood by-products will increase soon, and it is necessary to treat the seafood waste into valuable material.

The concepts of circular economy, sustainability, and green chemistry encourages the valorization of seafood by-product waste into value-added chemicals and materials as a sustainable alternative compared to the exploitation of more conventional resources.

The work carried out in this thesis mainly focused on mollusc waste, which includes mussel, clam, and murex. The mollusc waste was analysed by the pre-treatments, characterization, settling test, bio methanation potential, and chitin extraction on the three samples. Based on the literature, a few studies have investigated the waste characterization of mollusc waste. Moreover, they carried out waste characterization majorly on the proximate analysis. There is no proper investigation on the heavy metals, cations, and nutrients compositions of waste. However, to the best knowledge of me no study investigates the murex waste characterization and chitin extraction phenomena. There is a lack of information on the use of different chemical dosages, temperatures, and particle sizes that can influence the chitin yield. There is no appropriate information on the process control during the processes of deproteinization and demineralization.

Therefore, to fill this research gap, this thesis aims to carry out the complete waste characterization of mussel, clam, and murex waste to analyse the heavy metals, cations,

nutrients on both fractions of organic (meat) and inorganic (shell). In addition, to control the deproteinization and demineralization with different chemical dosages and durations.

In the sampling and characterization of raw waste section, we analyzed the waste composition on both shell and organic fraction. The waste compositions in terms of moisture, ashes, nutrients, heavy metals. The results I obtained with this characterization was in lined with literature values. Finally, I concluded this section that there are no great differences between the three mollusc species confirming the homogeneity of the side-stream although the variability could occur daily and seasonally depending on the production activity of Co.Pe.Mo. It means the mixed side-streams is quite a homogenized by-product that ensures replicability and stability of the future valorisation chain.

In separation of organic and inorganic waste section, I carried out shredding pump test to separate the shell and organic fraction. Here we carried out shredding with different dilution factors to observe the good separation efficiency. Finally, I concluded this section that increasing the waste volume leads to lower the recovery of solids.

In the bmp test section, we observed that low production of biogas due to ammonia inhibition (more than 200 mg/l). The pH and alkalinity were within the range values. The chloride content is relatively very high maybe It could be a chance for test inhibition. Also, improper mixing can lead to accumulation of complex minerals etc,

In the chitin extraction section, we conducted a lot of trails to optimize and control the processes of both deproteinization and demineralization with different chemical dosages and durations to achieve the maximum yield. Here I concluded that the shell part contains more than 95% was the $CaCO_3$ and 0-5% is the protein content. The chitin content was very less (even negligible). For the demineralization process the dosage of chemical through stoichiometric analysis and processes was controlled with pH indication. Finally, the maximum yield of deproteinization was occurred with the dosage of 1M NaOH at 70°C for 2-3 hours period range. The maximum yield demineralization was occurred with the dosage of 1M HCl at 30°C for 2 hours period. The quality of chitin was quantified by the FTIR analysis.

References

Abdulkarim, A., Isa, M. T., Abdulsalam, S., Muhammad, A. J., & Ameh, A. O. (2013). Extraction and characterization of chitin and chitosan from mussel shell. *Civil and Environmental Research*, *3*(2), 108–114.

Adani, F., Genevini, P. L., & Tambone, F. (1995). A new index of organic matter stability. *Compost Science and Utilization*, *3*(2), 25–37. https://doi.org/10.1080/1065657X.1995.10701779

Ademolu, K. O., Akintola, M. Y., Adelabu, A. O. &, & Blessing A. (2015). Traditional utilization and biochemical composition of six mollusc shells in Nigeria. In *Rev. Biol. Trop. (Int. J. Trop. Biol. ISSN* (Vol. 63, Issue 2).

Ademolu, K. O., Akintola, M. Y., Olalonye, A. O., & Adelabu, B. A. (2015). Traditional utilization and biochemical composition of six mollusc shells in Nigeria. *Revista de Biologia Tropical*, *63*(2), 459–464. https://doi.org/10.15517/rbt.v63i2.15600

Alabaraoye, E., Achilonu, M., & Hester, R. (2018). Biopolymer (Chitin) from Various Marine Seashell Wastes: Isolation and Characterization. *Journal of Polymers and the Environment*, *26*(6), 2207–2218. https://doi.org/10.1007/s10924-017-1118-y

Barbachi, M., Biriane, M. El, Bouabaz, M., & Boudjellal, K. (2019). Physico-chemical analysis of mussel shells for use in civil engineering. *Applied Journal of Environmental Engineering Science*, *5*(2), 153–161.

Barros, M. C., Bello, P. M., Bao, M., & Torrado, J. J. (2009). From waste to commodity: transforming shells into high purity calcium carbonate. *Journal of Cleaner Production*, *17*(3), 400–407. https://doi.org/10.1016/j.jclepro.2008.08.013

Bernárdez, M., & Pastoriza, L. (2011). Quality of live packaged mussels during storage as a function of size and oxygen concentration. *Food Control*, *22*(2), 257–265. https://doi.org/10.1016/j.foodcont.2010.07.007

Brugnerotto, J., Lizardi, J., Goycoolea, F. M., Argüelles-Monal, W., Desbrières, J., & Rinaudo, M. (2001). An infrared investigation in relation with chitin and chitosan characterization. *Polymer*, *42*(8), 3569–3580. https://doi.org/10.1016/S0032-3861(00)00713-8

Bruno, S. F., Ekorong, F. J. A. A., Karkal, S. S., Cathrine, M. S. B., & Kudre, T. G. (2019). Green and innovative techniques for recovery of valuable compounds from seafood by-products and discards: A review. In *Trends in Food Science and Technology* (Vol. 85, pp. 10–22). https://doi.org/10.1016/j.tifs.2018.12.004

Buasri, A., Chaiyut, N., Loryuenyong, V., Worawanitchaphong, P., & Trongyong, S. (2013). Calcium oxide derived from waste shells of mussel, cockle, and scallop as the heterogeneous catalyst for biodiesel production. *The Scientific World Journal*, *2013*.

https://doi.org/10.1155/2013/460923

C. A. Papadimitriou. (2014). The use of crushed brick as an aggregate replacement in concrete. *International Masonry Conference 2014*, *September*, 1–12.

Cabbai, V., Ballico, M., Aneggi, E., & Goi, D. (2013). BMP tests of source selected OFMSW to evaluate anaerobic codigestion with sewage sludge. *Waste Management*, *33*(7), 1626–1632. https://doi.org/10.1016/j.wasman.2013.03.020

Danarto, Y. C., & Distantina, S. (2016). Optimizing deacetylation process for chitosan production from green mussel (perna viridis) shell. *AIP Conference Proceedings*, *1710*. https://doi.org/10.1063/1.4941494

Fannin, K. F., Conrad, J. R., Srivastava, V. J., Chynoweth, D. P., & Jerger, D. E. (1986). ANAEROBIC PROCESSES. *Journal of the Water Pollution Control Federation*, *58*(6), 504–510.

Fannin, K. F., Conrad, J. R., Srivastava, V. J., Chynoweth, D. P., & Jerger, D. E. (1987). ANAEROBIC PROCESSES. *Journal of the Water Pollution Control Federation*, *59*(6), 403–410. https://doi.org/10.2175/106143099x133695

FAO. (2018). The State of World Fisheries and Aquaculture 2018- Meeting the sustainable development goals. In *FAO*.

FAO. (2020). The State of World Fisheries and Aquaculture 2020. Sustainability in action. *FAO*, 224. https://doi.org/https://doi.org/10.4060/ca9229en

Finkelstein, A. D., Wohlt, J. E., Emanuele, S. M., & Tweed, S. M. (1993). Composition and Nutritive Value of Ground Sea Clam Shells as Calcium Supplements for Lactating Holstein Cows. *Journal of Dairy Science*, *76*(2), 582–589. https://doi.org/10.3168/jds.S0022-0302(93)77378-6

Fisgativa, H., Tremier, A., & Dabert, P. (2016). Characterizing the variability of food waste quality: A need for efficient valorisation through anaerobic digestion. *Waste Management*, *50*, 264–274. https://doi.org/10.1016/j.wasman.2016.01.041

Guendouz, J., Buffière, P., Cacho, J., Carrère, M., & Delgenes, J. P. (2010). Dry anaerobic digestion in batch mode: Design and operation of a laboratory-scale, completely mixed reactor. *Waste Management*, *30*(10), 1768–1771. https://doi.org/10.1016/j.wasman.2009.12.024

Hamed, I., Özogul, F., & Regenstein, J. M. (2016). Industrial applications of crustacean by-products (chitin, chitosan, and chitooligosaccharides): A review. In *Trends in Food Science and Technology* (Vol. 48, pp. 40–50). https://doi.org/10.1016/j.tifs.2015.11.007

Harmami, H., Ulfin, I., Sakinah, A. H., & Ni'mah, Y. L. (2019). Water-soluble chitosan from shrimp and mussel shells as corrosion inhibitor on tinplate in 2% NaCl. *Malaysian Journal of Fundamental and Applied Sciences*, *15*(2), 212–217. https://doi.org/10.11113/mjfas.v15n2.972

Hülsey, M. J. (2018). Shell biorefinery: A comprehensive introduction. In *Green Energy and Environment* (Vol. 3, Issue 4, pp. 318–327). https://doi.org/10.1016/j.gee.2018.07.007

Hyldig, G., Bald, C., Gutierrez, M., Martin, D. S., Simonsen, A., & Dam, L. (2020). *Hurdles and bottlenecks in maintaining and value adding of seafood side-streams*. *837726*.

Idacahyati, K., Amalia, Y., & Lestari, T. (2020). Decreased total cholesterol levels in rats administered with chitosan from Green mussel (Perna viridis L.) shells. *Pharmaciana*, *10*(2), 157. https://doi.org/10.12928/pharmaciana.v10i2.13976

Iriani, D., Hasan, B., & Sumarto. (2020). Physicochemical Characteristics of Freshwater Mussel (Pilsbryoconcha sp.) Shell from Sungai Paku Village Riau Province Indonesia. *IOP Conference Series: Earth and Environmental Science*, *430*(1). https://doi.org/10.1088/1755-1315/430/1/012003

Kafle, G. K., & Kim, S. H. (2013). Anaerobic treatment of apple waste with swine manure for biogas production: Batch and continuous operation. *Applied Energy*, *103*, 61–72. https://doi.org/10.1016/j.apenergy.2012.10.018

Kocaman, S., Ahmetli, G., Cerit, A., Yucel, A., & Gozukucuk, M. (2016). Characterization of Biocomposites Based on Mussel Shell Wastes. *International Journal of Materials and Metallurgical Engineering*, *10*(4), 438–441.

Labatut, R. A., Angenent, L. T., & Scott, N. R. (2011). Biochemical methane potential and biodegradability of complex organic substrates. *Bioresource Technology*, *102*(3), 2255–2264. https://doi.org/10.1016/j.biortech.2010.10.035

Lertwattanaruk, P., Makul, N., & Siripattarapravat, C. (2012). Utilization of ground waste seashells in cement mortars for masonry and plastering. *Journal of Environmental Management*, *111*, 133–141. https://doi.org/10.1016/j.jenvman.2012.06.032

Li, Y., Feng, Y., Xu, Z., Yan, L., Xie, X., & Wang, Z. (2020). Synergistic effect of clam shell bio-filler on the fire-resistance and char formation of intumescent fire-retardant coatings. *Journal of Materials Research and Technology*, *9*(6), 14718–14728. https://doi.org/10.1016/j.jmrt.2020.10.055

Martínez-García, C., González-Fonteboa, B., Martínez-Abella, F., & Carro- López, D. (2017). Performance of mussel shell as aggregate in plain concrete. *Construction and Building Materials*, *139*, 570–583. https://doi.org/10.1016/j.conbuildmat.2016.09.091

Naik, A. S., & Hayes, M. (2019). Bioprocessing of mussel by-products for value added ingredients. In *Trends in Food Science and Technology* (Vol. 92, pp. 111–121). https://doi.org/10.1016/j.tifs.2019.08.013

Nguyen, T. A. H., Ngo, H. H., Guo, W. S., Nguyen, T. T., Vu, N. D., Soda, S., Nguyen, T. H. H., Nguyen, M. K., Tran, T. V. H., Dang, T. T., Nguyen, V. H., & Cao, T. H. (2020). White hard clam (Meretrix lyrata) shells as novel filter media to augment the phosphorus removal from wastewater. *Science of the Total Environment*, *741*, 140483.

https://doi.org/10.1016/j.scitotenv.2020.140483

Ni'mah, Y. L., Harmami, H., Ulfin, I., Suprapto, S., & Welny Saleh, C. (2019). Water-soluble chitosan preparation from marine sources. *Malaysian Journal of Fundamental and Applied Sciences*, *15*(2), 159–163. https://doi.org/10.11113/mjfas.v15n2.971

Nisticò, R. (2017). Aquatic-derived biomaterials for a sustainable future: A European opportunity. In *Resources* (Vol. 6, Issue 4). https://doi.org/10.3390/resources6040065

OECD/FAO. (2020). OECD-FAO Agricultural Outlook 2020-2029. In *OECD Publishing Paris, /Food and Agriculture Organization of the United Nations, Rome*.

Owen, W. F., Stuckey, D. C., Healy, J. B., Young, L. Y., & McCarty, P. L. (1979). Bioassay for monitoring biochemical methane potential and anaerobic toxicity. *Water Research*, *13*(6), 485–492. https://doi.org/10.1016/0043-1354(79)90043-5

Papadimitriou, C., Anagnostopoulos, T., Anagnostopoulos, K., & Galinou-Mitsoudi, S. (2020). *The use of waste mussel shells as an aggregate replacement in concrete*.

Ramasamy, P., Subhapradha, N., Shanmugam, V., & Shanmugam, A. (2014). Extraction, characterization and antioxidant property of chitosan from cuttlebone Sepia kobiensis (Hoyle 1885). *International Journal of Biological Macromolecules*, *64*, 202–212. https://doi.org/10.1016/j.ijbiomac.2013.12.008

Raposo, F., Fernández-Cegrí, V., de la Rubia, M. A., Borja, R., Béline, F., Cavinato, C., Demirer, G., Fernández, B., Fernández-Polanco, M., Frigon, J. C., Ganesh, R., Kaparaju, P., Koubova, J., Méndez, R., Menin, G., Peene, A., Scherer, P., Torrijos, M., Uellendahl, H., … de Wilde, V. (2011). Biochemical methane potential (BMP) of solid organic substrates: Evaluation of anaerobic biodegradability using data from an international interlaboratory study. *Journal of Chemical Technology and Biotechnology*, *86*(8), 1088–1098. https://doi.org/10.1002/jctb.2622

Sambusiti, C., Rollini, M., Ficara, E., Musatti, A., Manzoni, M., & Malpei, F. (2014). Enzymatic and metabolic activities of four anaerobic sludges and their impact on methane production from ensiled sorghum forage. *Bioresource Technology*, *155*, 122–128. https://doi.org/10.1016/j.biortech.2013.12.055

Sasidharan, A., & Venugopal, V. (2020). Proteins and Co-products from Seafood Processing Discards: Their Recovery, Functional Properties and Applications. In *Waste and Biomass Valorization* (Vol. 11, Issue 11, pp. 5647–5663). https://doi.org/10.1007/s12649-019-00812-9

Silverstein, R. M., Webster, F. X., & Kiemle, D. J. (2005). Spectrometric identification of organic compounds 7ed 2005 - Silverstein, Webster & Kiemle.pdf. In *Microchemical Journal* (Vol. 21, p. 496).

Strömberg, S., Nistor, M., & Liu, J. (2014). Towards eliminating systematic errors caused by the experimental conditions in Biochemical Methane Potential (BMP) tests. *Waste*

Management, *34*(11), 1939–1948. https://doi.org/10.1016/j.wasman.2014.07.018

Taherzadeh, M. J., & Karimi, K. (2008). Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review. In *International Journal of Molecular Sciences* (Vol. 9, Issue 9, pp. 1621–1651). https://doi.org/10.3390/ijms9091621

Tavares, M., Do Amaral Mello, M. R. P., Campos, N. C., De Morais, C., & Ostini, S. (1998). Proximate composition and caloric value of the mussel Perna perna, cultivated in Ubatuba, Sao Paulo State, Brazil. *Food Chemistry*, *62*(4), 473–475. https://doi.org/10.1016/S0308-8146(97)00177-5

VDI. (2006). VDI 4630—Fermentation of organic materials, Characterisation of Substrate, Sampling, Collection of Material Data, Fermentation Tests [1872]VDI Gesellschaft Energietechnik (2006). In *VDI-Gesellschaft Energietechnik*.

Yadav, M., Goswami, P., Paritosh, K., Kumar, M., Pareek, N., & Vivekanand, V. (2019). Seafood waste: a source for preparation of commercially employable chitin/chitosan materials. *Bioresources and Bioprocessing*, *6*(1). https://doi.org/10.1186/s40643-019-0243-y

Zhang, W., Lang, Q., Wu, S., Li, W., Bah, H., & Dong, R. (2014). Anaerobic digestion characteristics of pig manures depending on various growth stages and initial substrate concentrations in a scaled pig farm in Southern China. *Bioresource Technology*, *156*, 63–69. https://doi.org/10.1016/j.biortech.2014.01.013