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# CHITIN AND CHITOSAN RECOVERED FROM SHRIMP SHELL
## Structure, Characteristics and Applications

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## ABSTRACT


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Environmental pollution is the most noticeable problem over the world, recycling is recommended. Modern seafood production produces a large volume of waste. Chitin and chitosan which are produced through the food process are high-value natural compounds. Thus, chitin and chitosan have attracted many researchers who reported many experiments about extracting chitin and chitosan from seafood waste.

The chitin and chitosan's definition, structure, allomorphs, and properties were written in this thesis. Relationship of physical properties and biological activities, which affect directly to chitin and chitosan properties, was introduced. Chitin and chitosan are the second common compounds in nature just after cellulose. Crustaceans, microorganisms, and insects are the main sources of chitin and chitosan, in which three different types of chitin were found in various species.

The lab-scale and commercial-scale extracting chitin and chitosan were introduced, turning crustacean waste to chitin and chitosan were performed through deproteinization, demineralization, and deacetylation. Decolorization and preserving process of chitin and chitosan were optional process to produce the high quality chitinous product. Chemical and biological extraction was compared as well as the conditions of each step. Although chemical extraction had many drawbacks, but it was the most economical method to produce chitin products. Chitin and chitosan have great potential biological activities such as antioxidant, antihypertensive, anticoagulant, anti-inflammatory, antitumor, antidiabetic effects, antimicrobial, and hypocholesterolemic. Thus, chitin and chitosan were applied in medical, agriculture, and industrial domains. Some applications were discussed as drug delivery, wound healing, engineering tissue, water treatment or food preservation. In conclusion, challenges were written although chitin and chitosan are promising compounds in the future.



**Key words**
Chemical and Enzymatic Deproteinization, Chitin, Chitosan, Crustacean, Deacetylation, Demineralization.

**CONCEPT DEFINITIONS**

| | |
|---|---|
| $CaCl_2$ | Calcium Chloride |
| $CaCO_3$ | Calcium Carbonate |
| $Ca_3(PO_4)_2$ | Calcium Phosphate |
| Cd | Cadmium |
| Ch-Q | Chitosan-Quartzite |
| Ch-V | Chitosan-Vermiculite |
| $CH_3CH(OH)COOH$ | Lactic Acid |
| $CH_3COOH$ | Acetic Acid |
| Co | Cobalt |
| $CO_2$ | Carbon Dioxide |
| Cu | Copper |
| DD | Degree of Deacetylation |
| DM | Degree of Demineralization |
| DMAc | Dimethylacetamide |
| DP | Degree of Deproteinization |
| GlcN | D-glucosamine |
| GlcNAc | N-acetylglucosamine |
| HCOOH | Formic Acid |
| HCl | Hydrochloric Acid |
| Hg | Mercury |
| $H_2SO_4$ | Sulfuric Acid |
| KOH | Potassium Hydroxide |
| LiCl | Lithium Chloride |
| LP47 | Lactobacillus plantarum sp. 47 |
| $M_w$ | Molecular Weight |
| NaOH | Sodium Hydroxide |
| $NaBH_4$ | Sodium borohydride |
| $-NH_2$ | Amine group |
| -OH | Hydroxyl group |
| Pb | Lead |
| YPG medium | Yeast Extract–Peptone–Glycerol medium |

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# 1 INTRODUCTION

In recent decades, the demand for food has increased every day. Since products of seafood have grown in quantity and processing scale which leads to a large amount of waste have discharged in environment. Processing of seafood industry contributes to many types of waste from feeding to cooking (Perä 2019, 5). About 6 to 8 million tons of seafood waste are produced annually around the world, in which 1.5 million tons in only Southeast Asia. In developing countries, waste have thrown in landfills or the sea, whereas, in developed countries, it is very expensive to dispose of those wastes. For example, the cost to dispose seafood waste is US$150 per ton in Australia. Crustacean shell contains useful chemicals – protein, calcium carbonate ($CaCO_3$), and chitin. The potential value of these wastes for chemistry purposes has been ignored for a long time. However, scientists have found solutions for this plentiful and not expensive renewable resource. (Yan & Chen 2015.)

Chitin and chitosan are polysaccharides, which are abundant in nature, just after cellulose. Chitin is copolymers of structural units N – acetyl – Glucosamine, bonded together by β – 1,4 glycoside bonds. Chitin is present in the shell structure, the skeleton of crustaceans like shrimp, crab, squid pen, and insect. In addition, chitin is also present in the fungal cell wall. Chitosan can be obtained from chitin through deacetylation with alkalis and can also be naturally present in some fungi. With flexible biological properties, both of chitin and chitosan have potential in economic value. Commercial applications of chitinous products can be demoted by its crystallinity and insolubility. Many processes to extract chitin have been researched and improved to reach the highest efficiency. There are two main ways to extract chitin from raw materials: chemical extraction and biological extraction. In industry, chitin has been extracted by using chemical methods because of economic benefits, whereas biological methods have been used in laboratory scale. Both methods have advantages and disadvantages, the suitable method can be chosen depends on the purposes of final products. Chitin, chitosan, and their derivatives have diverse biological activities like biocompatible, biodegradable, resistant bacteria, fungi, antioxidant, cholesterol-lowering activity, increase strengthen of the immune system, and anticancer activity. So, chitin, chitosan, and their derivatives are applied widely in medicine and pharmacy, in industry and biotechnology, in agriculture and in environmental protection. Research and applications of chitin and chitosan have been noticed to scientists for many years. (Rinaudo 2006, Rinaudo & Younes 2015, Hamblin & Elieh-Ali-Komi 2016.)

The purpose of this thesis is to introduce valuable substances in seafood waste and how to extract them from raw sources. Besides that, applications of chitin and chitosan are covered to prove the value of substances then give a positive insight about this topic. This thesis will end with a conclusion that provides some challenges and achievements of scientists in extracting and producing chitin and chitosan.

## 2 STRUCTURE AND PROPERTIES OF CHITIN, CHITOSAN

The second most plenteous polysaccharide is chitin, just after cellulose. Chitin, chitosan, and cellulose have similarities in structure. In addition, properties of chitin and chitosan also affect its quality and applications such as solubility, molecular weight ($M_w$), or deacetylation results. Chitin is insoluble in common solvents whereas chitosan is easily dissolved in mild acids. (Rinaudo 2006, Younes & Rinaudo 2015, Hamblin & Elieh-Ali-Komi 2016.)

### 2.1 Chitin

Identified since 1811, Chitin has gradually become a promising material in future. Chitin's molecular formula is $(C_8H_{13}O_5N)_n$. Depending on sources of chitin, there are 3 forms of chitin, known as α–chitin, β–chitin, and γ–chitin. Each type of chitin has different properties which affect directly to quality and the purpose of chitinous products. Furthermore, the solubility of chitin is an important factor to evaluate other properties of chitin. (Rinaudo 2006, Hamblin & Elieh-Ali-Komi 2016.)

#### 2.1.1 Chemical Structure

Chitin $(C_8H_{13}O_5N)_n$ is a polysaccharide (β-(1-4)-N-acetyl-D-glucosamine), which is the second most popular nature polymer just after cellulose, identified first time in 1811 by Henrry Braconnot. Figure 1 below shows the chemical structure of chitin. Chitin has the same structure as cellulose; however, N-acetyl-D-glucosamines are units that build up to chitin, and these units bond together by β - (1-4) – glycosidic linkage (Hamblin & Elieh-Ali-Komi 2016.)

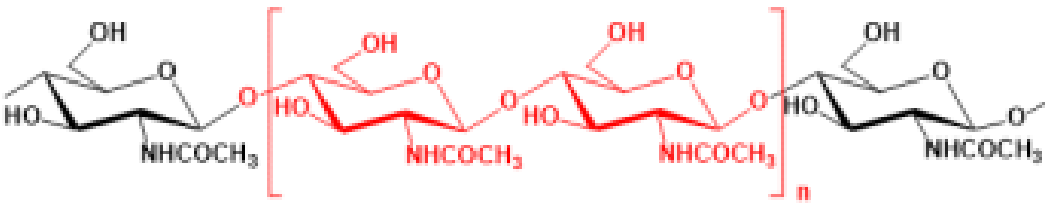



FIGURE 1. Chemical structure of chitin (adapted from Mohammed, Hussain, Haj 2017, 1)

### 2.1.2 Allomorphs of Chitin

Chitin has 3 forms of allomorphs, known as α–chitin, β–chitin, and γ–chitin (FIGURE 2). These forms have differences in aquation and dimension or a number of chitin lines in each component. α–chitin is the most stable and in solid form, the chains are arranged as parallel and in opposite direction type in chitin. β–chitin which includes chains that are parallel and in the same direction, has low hardness and high in aquation. In contrast, the form of γ–chitin arranges as 2 parallel and same direction chains then 1 in opposite direction. In nature, α–chitin is the most popular and usually very hard, whereas β–chitin and γ–chitin are elastic and flexible. (Rinaudo 2006.)

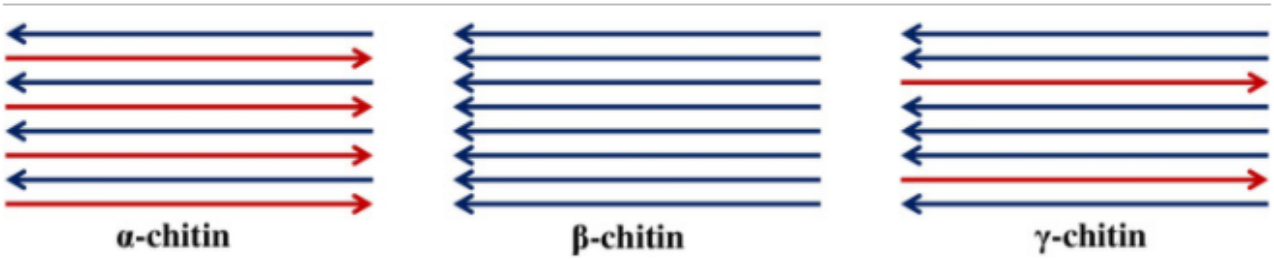



FIGURE 2. Arrangement of polymer chains in α–chitin, β–chitin and γ–chitin (adapted from Knidri, Belaabed, Addaou, Laajeb, Lahsini 2018)

In addition, to understand the structure of chitin arrangement and hydrogen bonds, the crystal structure of chitin should be researched. At solid-state, chitin has crystal structures are α and β. α–chitin is most popular in nature, present in fungal microflora, yeast cell walls, shrimp shells, lobsters, crab shells, and insect shells as well. α and β form of chitin crystal structure are shown in Figure 3. In both α and β form, the arrangement of chitin chains is firmly held by hydrogen bonds in sheet. Along *a* direction, hydrogen bonds help to maintain chains in range of 0.474 nm, they also contribute to hydroxymethyl groups along the *b* direction of α–Chitin chains. These features were not found in β–chitin which is more budging than α–chitin in crystal structure. In addition, the *a*c* projection is similar in both allomorphs of chitin. (Rinaudo 2006.)

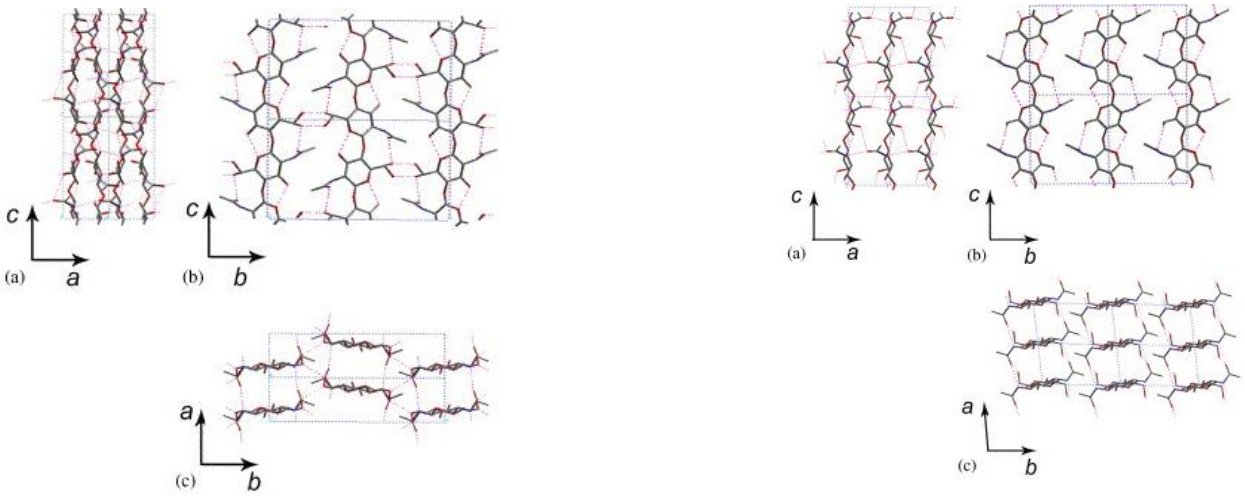



FIGURE 3. Crystal structure of α–chitin (A) and β–chitin (B): (a) *a*c* projection; (b) *b*c* projection; (c) *a*b* projection (adapted from Rinaudo 2006)

Dimensions of chitin's structure are shown in Table 1. There are 3 parameters that correspond to the *a*, *b*, and *c* direction in figure 3. In *a* and *c* direction, α and β–chitin have similar dimensions. Other way, in *b* direction, α–chitin dimension is longer 2 times than β–chitin. These parameters and symmetry elements of α–chitin and anhydrous β–chitin which are given in table 1 are currently accepted. (Rinaudo 2006.)

TABLE 1. Dimension of α and β–chitin's crystal structure (adapted from Rinaudo 2006)

| | a(nm) | b(nm) | c(nm) |
|---|---|---|---|
| α–chitin | 0.474 | 1.886 | 1.032 |
| β–chitin (anhydrous) | 0.485 | 0.926 | 1.038 |

### 2.1.3 Solubility of Chitin

Chitin is a natural polymer, but it is a hydrophobic polymer. So, both α and β–chitin are insoluble in water and almost common solvents. It can be soluble in some special solvents such as formic acid (HCOOH), dichloroacetic acid ($C_2H_2Cl_2O_2$), or trichloroacetic acid ($C_2HCl_3O_2$) (Hamblin & Elieh-Ali-Komi 2016). A research was succeeded by obtaining a complex solvent of chitin and lithium chloride (LiCl) in dimethylacetamide (DMAc) and N-methyl-2-pyrrolidone which were also used for solving

cellulose (Rinaudo 2006). Besides that, calcium chloride dihydrate ($CaCl_2.2H_2O$) saturated methanol, hexafluoro isopropyl alcohol, and hexafluoroacetone sesquihydrate were applied as well, but DMAc/LiCl mixture was the most common method used to soluble chitin over the world (Rinaudo 2006). According to Vicendon's experiment (Vicendon 1996, 233-237), chitin was dissolved in concentrated phosphoric acid (minimum 75%) at room temperature. In this experiment with an unchanged level of acetylation, the viscosity of solvents decreased quickly in first 12 hours, and during first hours in phosphoric solution, despite $M_w$ were decreased, regenerated chitin was not modified chemically (Vicendon 1996, 233-237). Vicendon's method is usually used to create colloid chitin that uses for chitinase's research.

Alkali chitin was obtained by dissolving chitin in high concentrated sodium hydroxide (NaOH) solution at low temperature. Initially, chitin is put in high concentrated NaOH at 25˚C for 3 hours or more; alkali chitin is collected at around 0˚C. This method is used to create film chitin which has good mechanical properties and transparent (Rinaudo 2006). In addition, β–chitin at solid-state can be transformed to α–chitin by treatment with strong hydrochloric acid (HCl) (>7M) then washing with water. An important characteristic which regards enzymatic and chemical transformations of chitin is the potential reactivity of β–chitin (Rinaudo 2006). According to Mark–Houwink equation, molecular mass of chitin can be determined following viscosity and some solvents properties.

$$[\eta] = \mathbf{K^*M^{\mathit{a}}} \quad (1)$$

With [η] is chitin's intrinsic viscosity, which is measured the contribution of solute to the viscosity of a solution; M is average molecular mass; K and *a* are constants the values of which depend on the nature of the polymer, given in Table 2. (Rinaudo 2006.)

TABLE 2. Mark–Houwink parameters of chitin (adapted from Rinaudo 2006)

| Solvents | K(ml/g) | *a* | T (˚C) |
|---|---|---|---|
| 2.77M NaOH | 0.1 | 0.68 | 20 |
| DMAc/LiCl 5% | $7.6*10^{-3}$ | 0.95 | 30 |
| DMAc/LiCl 10% | $2.4*10^{-1}$ | 0.69 | 25 |

## 2.2 Chitosan

Chitosan is a derivative of chitin; it was created by deacetylation in which acetyl group was replaced. Chitosan easily dissolves in mild acids. The solubility of chitosan is the main factor which decides properties of chitosan and affects to its application. Furthermore, Degree of Deacetylation (DD) and $M_w$, which are factors that evaluate the quality of chitosan, are concerned. (Younes & Rinaudo 2015.)

### 2.2.1 Chemical Structure

Chitosan is a polymer, in which glucosamine and N-acetyl-glucosamine bond together by β - (1-4) – glycosidic linkage. Chitosan, which is also a product of deacetylation of chitin, can be dissolved in aqueous acid media when DD reaches 50 %. From chitin, chitosan can be converted by enzymatic method or chemical hydrolysis. Figure 5 illustrates the chemical structure of chitosan. (Hamblin & Elieh-Ali-Komi 2016.)

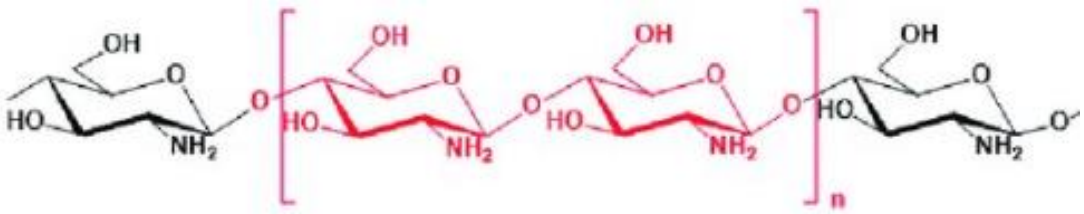



FIGURE 5. Chemical structure of chitosan (adapted from Mohammed et al 2017, 1)

### 2.2.2 Solubility of Chitosan

Chitosan can be dissolved easily in HCl, lactic acid ($CH_3CH(OH)COOH$), mild acetic acid ($CH_3COOH$) (1% or 0.1 M), and some organic acids. Not only does DD affect the solubility of chitosan, but also the location of acetyl groups in the main chain is an agent. pH and pK of acid were investigated as the main role in the protonation of chitosan and its solubility. With low DD chitosan, solubilization occurs when the degree of ionization of chitosan around 0.5, the corresponding pH in HCl is 4.5-5. Chitosan chlorhydrate salt was formed when dissolved chitosan in HCl 1M. A pK=6±0.1 acidic solution is created when chitosan chlorhydrate is directly soluble in water. As a result, with a pH below 6, chitosan can be dissolved. (Rinaudo 2006.)

The solubility of chitosan depends on deacetylation, the distribution of acetyl groups, and its $M_w$. The solubility of chitosan is usually tested in $CH_3COOH$ 1% or 0.1M. This parameter is very difficult to control because it is depended on deacetylation, the concentration of ion, pH value, the distribution of acetyl group along the chain, extraction and drying conditions, $M_w$ as well. In addition, the solubility of chitosan is also affected by the intra-chain hydrogen bonds between hydroxyl (-OH) in the Chitosan structure. Because the free amine ($-NH_2$) groups at the C-2 position of monomer D-glucosamine (GlcN) are protonated to $NH_3^+$, homogeneous reactions are allowed to perform. (Rinaudo 2006, Younes & Rinaudo 2015.)

### 2.2.3 Degree of Deacetylation and Molecular Weight of Chitosan

Chitosan is a product of partial deacetylation of chitin, it is a copolymer of N-acetylglucosamine (GlcNAc) units and GlcN units. Biological properties and activities of chitin and chitosan strongly depend on $M_w$ and DD. The DD is defined as the percentage of GlcN monomers present in the chitosan structure.

$$\text{DD (\%)} = \frac{N(GlcN)}{N(GlcN)+N(GlcNAc)} * 100\% \quad (2)$$

Where, N(GlcN) is average number of GlcN, N(GlcNAc) is average number of GlcNAc (Czechowska-Biskup, Jarosińska, Rokita, Ulański, Rosiak 2012, 1).

The DD is proportional to the $NH_3^+$ group in molecule, therefore, polyelectrolyte properties and solubility of chitosan are affected by the DD. Besides that, the distribution of acetyl groups along the chain is an important reason which changes some physical properties of chitosan (Younes & Rinaudo 2015). Many techniques have used to determine the DD of Chitosan such as infrared spectroscopy, elementary analysis, and potentiometric titration, but $^1H$ liquid-state and solid-state $^{13}C$-NMR are recommended (Czechowska-Biskup et al. 2012, 1-2; Younes & Rinaudo 2015).

Furthermore, the $M_w$ of chitosan and its distribution are also important characteristics to evaluate quality of chitosan. The $M_w$ of chitosan can be calculated from Mark–Houwink relation, equation (1) above, in which *a* and K values are known. Besides that, the solvent is important in $M_w$ determination. In this

method, intrinsic viscosity can be determined by automatic viscometer then $M_w$ also can be calculated. Parameters for chitosan solvents are given in table 3.

TABLE 3. Mark–Houwink parameters of chitosan solutions (adapted from Rinaudo 2006)

| **Solvent** | ***K* (mL/g)** | ***a*** | ***T* (°C)** |
|---|---|---|---|
| 0.1 M AcOH/0.2 M NaCl | $1.81\times10^{-3}$ | 0.93 | 25 |
| 0.1 M AcOH/0.02 M NaCl | $3.04\times10^{-3}$ | 1.26 | 25 |
| 0.2 M AcOH/0.1 M AcONa/4 M urea | $8.93\times10^{-2}$ | 0.71 | 25 |
| 0.3 M AcOH/0.2 M AcONa (DA=0.02) | $8.2\times10^{-2}$ | 0.76 | 25 |
| 0.3 M AcOH/0.2 M AcONa (0<DA<0.03) | $7.9\times10^{-2}$ | 0.796 | 25 |
| 0.02 M acetate buffer/0.1 M NaCl | $8.43\times10^{-2}$ | 0.92 | 25 |

# 3 SOURCES OF CHITIN

In nature, chitin exists in composition of shrimp, crab' shells or cuttlefish pens (seafood processing's wastes), insects' cuticles, fungal cell wall, or mushrooms. However, for industrial purposes, chitin is mainly provided by seafood waste (crustaceans). (Rinaudo 2006; Knezevic-Jugovic, Petronijevic, Smelcerovic 2010, 25-33; Younes & Rinaudo 2015; Seenuvasan, Sarojini, Dineshkumar 2019, 115-130.)

## 3.1 Sources from Crustaceans

About 20 million tons of seafood waste, which proportion around 25% total of catching and producing seafood over the world, are produced through processes. This source is rich in protein, so environment will be affected, thus chitin production is one of solutions to reduce pollution (Brück, Slater, Carney 2010, 11-19). Seafood waste sources contain 16 - 49% Chitin (according to pieces and seasons), 20 – 40% protein, 20 – 60% $CaCO_3$. Chitin's allomorphs and content are shown in Table 4. In addition, lipids, materials, and other constituents also are contained in these waste sources. In chitin manufacturing, the value of process will be improved if all products are used totally and environment also avoids pollutants. Currently, waste sources from seafood processing are the main source to produce chitin and chitosan (Brück et al. 2010, 11-19).

TABLE 4. Allomorph and contents of chitin in some different sources (adapted from Jones, Kujundzic, John, Bismarck 2020)

| Allomorph | Sources | Chitin content (%) | Other materials |
|---|---|---|---|
| α | Crustacean shells | Chitin makes up to 50% of sources' dry weight. | |
| | Lobster | 16-23 | 20-60% Calcium or Magnesium Carbonate, 20-40% of protein |
| | Crab | 25-30 | |
| | Krill | 34-49 | |
| β | Cuttlefish pens | 31-49 | Protein and materials |

### 3.2 Sources from Microorganism

Chitin exists popularly in microbial world, it is contained in fungi, yeasts, molds, algae, and in some bacteria as well. Chitin is found as main element in fungal cell walls and septa of Ascomycetes, Zygomycetes, Basidiomycetes, and Deuteromycetes (Knezevic-Jugovic et al. 2010, 25-33). Chitin occurs in 3 types of allomorphs, and the most common form can be found in fungal is α–chitin. In Knezevic-Jugovic et al.'s research (2010, 25-33), chitin contained in fungal cell walls with different valuables from 2% up to 60% in several species of fungi. Species of Zygomycetes contain higher chitin and especially chitosan, particularly in Mucor rouxii, Mucor mucedo, Rhizomuco Rhizopus oryzae. On another hand, chitin and acidic polysaccharides are contained with significant quantities (26%-65%) in Ascomycetes and Basidiomycetes (Synowiecki and Al-Khateeb 2003). However, some species do not contain or contain chitin as a minor component. Table 5 shows the contents of chitin in some microorganism and their conditions to produce chitin.

TABLE 5. Chitin in microorganism (adapted from Knezevic-Jugovic et al. 2010, 27-29)

| Species | Cultivation media | Cultivation conditions | Efficiency % (based on dry mycelia weight) |
|---|---|---|---|
| Mucor rouxii | Yeast Extract–Peptone–Glycerol (YPG) medium | SmF, Erlenmeyer flask, 28˚C, 400 rpm.<br>SmF, Erlenmeyer flask, 28˚C, 170 rpm, 48h. | 4%-8% chitin<br>8.9% chitin and 7.3% chitosan |
| Mucor sp<br>Rhizopus sp | YPG medium | SmF, Erlenmeyer flask, 30˚C, 150 rpm. | 25% chitosan |
| Rhizopus oryzae | Yeast Mannitol Broth (YMB) medium | SmF, Erlenmeyer flask, 30˚C, 180 rpm, 15-21 days. | 14% chitosan |
| Cunninghamella elegans | Yam bean media and 4 traditional culture medias. | SmF, Erlenmeyer flask, 28˚C, 150 rpm, 72h. | 44% chitin and 6.6% chitosan |

According to Knezevic-Jugovic et al. (2010, 25-33) research, content, properties, and weight of chitin and chitosan which collected from microorganisms are affected by cultivation media, cultivation conditions, or fermentation techniques. More detailed information is addressed in Table 5. These factors decide cost of chitin products, from that, chitinous compounds can be evaluated their suitability for industrial purposes. Microorganism is an abundant source, but economic issues are barriers to produce chitinous products. As a result, the research needs to be focused on improving recovery efficiency by choosing and using new microbial sources which contains higher chitin/chitosan, optimizing the fermentation conditions and extraction processes. In addition, the structure/function relationships of compounds and applications should be made clear, thus their industrial synthesis can be easier to control and got more effective. Moreover, the advances in molecular biology lead to appearance of products with higher capability of chitin by using genetic transformation of fungi. (Knezevic-Jugovic et al. 2010, 25-33.)

### 3.3 Sources from Insects

Chitin, melanin, and protein are major elements of insect's cuticles. Mosquitoes, cockroaches, honeybees, silkworms, and nematodes are insects in which chitin's composition and biosynthesis are found. For instance, in Nwe, Furuike, Tamura (2010, 3-9) research, 23%-32% of Chitin, 35%-45% proteins, 30%-40% melanin, and 3% minerals build up to organic matrices of honeybees. Otherwise, in organic matrices of silkworms, chitin makes up about 20% and other components, proteins, minerals, and fat, are contained. Table 6 shows chitin's composition in insect cuticles from different sources.

TABLE 6. Chitin content in different insect sources (adapted from Seenuvasan et al. 2019, 117)

| Organism | Chitin content (%) |
|---|---|
| Blattella (cockroach) | 18.4 |
| Coleoptera (ladybird) | 27-35 |
| Diptera (fly) | 54.8 |
| Pieris (butterfly) | 64 |
| Bombyx (silkworm) | 44.2 |
| Galleria (wax worm) | 33.7 |

# 4 PRODUCTION OF CHITIN AND CHITOSAN

Generally, in extraction, the most common method to separate a substance from materials or matrix is dissolving that substance in one or a mixture of solvents. After that, the substance-solution will be collected then the substance able to be removed from solution by partial methods such as concentration, precipitation, crystallization. Based on differences in physical and chemical properties of solutes and solvents. The extraction of most biological polymer has been applied to this method, for instance, alginate, agar, gelatin. However, chitin extraction takes place in the opposite direction. Because of its insolubility, all other components in raw materials are dissolved instead of dissolving and collecting chitin directly. For this reason, many difficulties occur during production of chitin. Firstly, unnecessary components need to remove by many steps and processes. Secondly, due to the complex structure of crustacean shells as mentioned in section 3.1, unnecessary components cannot be removed totally from raw materials. These explain why chitin production technology requires many different methods and there are still have many issues that need to be investigated. In addition, chitin has many properties base on structure, raw material's properties, and methods of extraction also. Therefore, in chitin and chitosan production technology, its purposes need to be considered to choose the extraction technology with right parameters. (Rinaudo 2006, Younes & Rinaudo 2015.)

## 4.1 Chemical Extraction

Chitin is contained in cuticles of various crustaceans, and this also the main source to produce chitin. In crustaceans' exoskeleton, chitin, proteins, and minerals bond together to build an external form. Therefore, proteins and minerals will be removed in the procedure of chitin production by deproteinization and demineralization process. Non-chitin compounds such as proteins, minerals, lipids, pigments, and other compounds are not same in different sources. For instance, minerals in squid pens are lower than in spider-crab's shell. To remove proteins and minerals from crustacean sources, chemical, biological, or bio-chemical extraction technology can be applied. However, chemical extraction is the most commonly applied method on commercial-scale; its advantages are short processing time, simple and easy to apply on large scale, besides that, eco-unfriendly, uneconomical, and negative effects on properties of products are drawbacks of chemical extraction. (Rinaudo 2006, Younes & Rinaudo 2015, Seenuvasan et al. 2019.)

Chitin can be extracted by several techniques; it is up to the raw materials. These techniques, in general, have same three main processes such as deproteinization, demineralization, and decolorization (Rinaudo 2006, Younes & Rinaudo 2015). With raw materials, initially, it will be washed, dried, crushed, and ground to a powder before transferring to the main process (Rinaudo 2006, Younes & Rinaudo 2015). Figure 6 illustrates shortly chitin production process.

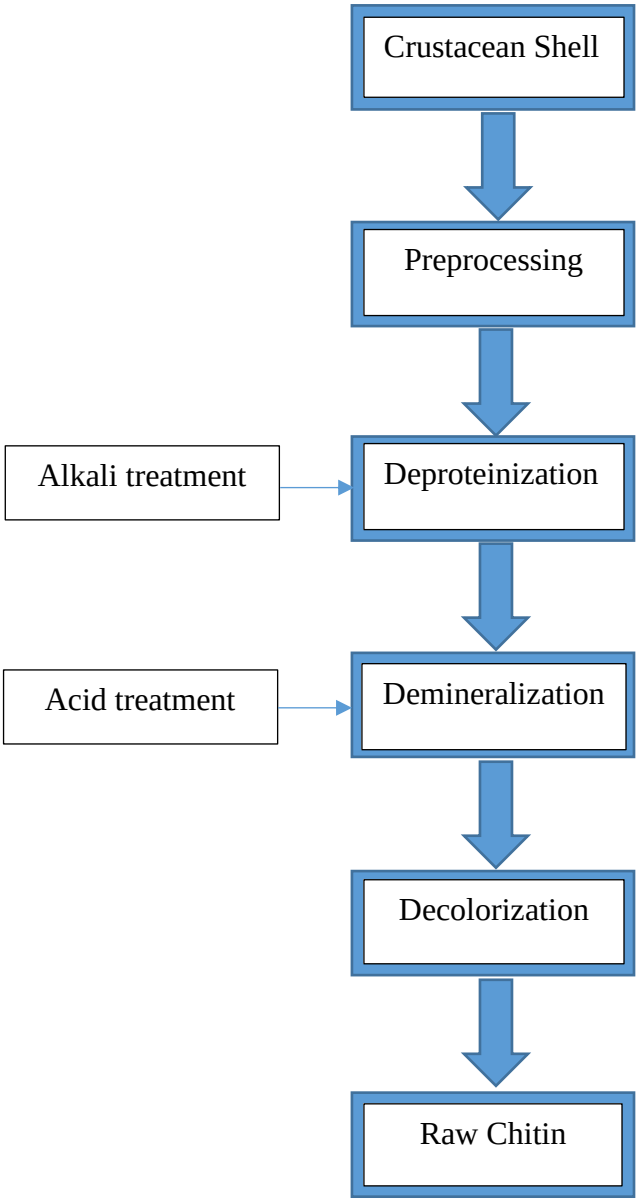



FIGURE 6. Chitin production process by chemical extraction

### 4.1.1 Chemical Deproteinization

Using chemicals to depolymerize the biopolymer is deproteinization process, in which chemical bonds between proteins and chitin is disrupted. The contained proteins are the main cause of allergy in biomedical, so deproteinization is important. Chemical method is the most common method to deproteinize. Crustacean's waste deproteinization is carried out by chemicals such as NaOH, sodium carbonate ($Na_2CO_3$), sodium bicarbonate ($NaHCO_3$), potassium hydroxide (KOH), potassium carbonate ($K_2CO_3$), calcium hydroxide ($Ca(OH)_2$), etc. However, NaOH is a preferential reagent with concentrations ranging from 0.125M to 0.5M, operates at room temperature or up to 160˚C during hours or days. NaOH either leads to deproteinization or results in hydrolysis of biopolymer, affecting its $M_w$, and deacetylation of chitin. Deproteinization's conditions are shown in Table 7 below. (Younes & Rinaudo 2015; Synowiecki & Al-Khateeb 2003.)

### 4.1.2 Chemical Demineralization

Crustacean waste (crab or shrimp' shell) usually consists a large amount of inorganic minerals mainly $CaCO_3$ and Calcium Phosphate ($Ca_3(PO_4)_2$) which need to be removed. So, demineralization is one of the most important steps in producing chitin. It is generally accomplished by acid treatment. In Younes & Rinaudo's report (2015), dilute HCl is recommended for the demineralization step. This step is easily completed because demineralization involves decomposition of $CaCO_3$ into calcium chloride ($CaCl_2$) and carbon dioxide ($CO_2$) as shown:

$$2\ HCl + CaCO_3 \rightarrow CaCl_2 + H_2O + CO_2 \uparrow$$

Other minerals consisted in shellfish cuticles perform similarly with $CaCO_3$. After the above step, salt solutions can be removed by filtration, chitin is collected by washing with diluted water. Same as deproteinization, according to research, various demineralization's conditions are used. It depends on the mineralization degree of each shell, extraction time, temperature, particle size, acid concentration, and solute/solvent ratio. Among these factors, the acid concentration is the most important condition, since this process needs 2 molecules of HCl to transfer 1 molecule of $CaCO_3$ to $CaCl_2$. The process will be finished when pH towards to neutral or reaction's environment turns to acidity at medium value at the end of the reaction. (Younes & Rinaudo 2015.)

Demineralization's conditions are also diverse, concentration of HCl is changed from 0.125M up to 2M, reaction's temperatures are from very low to room temperature from 0.5 hour to 48 hours. Table 7 presents some conditions for chitin production according to Younes & Rinaudo report (2015).

TABLE 7. Conditions for chitin production (adapted from Younes & Rinaudo 2015)

| Source | Deproteinization | | | | Demineralization | | |
|---|---|---|---|---|---|---|---|
| | NaOH concentration (M) | Temperature (˚C) | Number of Baths | Time (h) | HCl concentration (M) | Temperature (˚C) | Time (h) |
| Shrimp | 0.125<br>0.75 | 100<br>100 | 1<br>1 | 0.5<br>-* | 1.25 | Room | 1 |
| Shrimp | 1.25 | 100 | 1 | 0,5 | 1.57 | 20-22 | 1-3 |
| Crab | 0.5 | 65 | 1 | 2 | 1.57 | Room | 5 |
| Crab | 1 | 80 | 1 | 3 | 1 | Room | 12 |
| Crab | 1 | 100 | 1 | 36 | 2 | Room | 48 |
| Crab | 1 | 100 | 3 | 72 | 1 | Room | -* |
| Crab | 1.25 | 85-90 | 3 | 24 | 1.37 | Room | 24 |
| Crab/Lobster | 2.5 | Room | 3 | 72 | 11 | -20 | 4 |
| Krill | 0.875 | 90-95 | 1 | 2 | 0.6 | Room | 2 |
| Lobster | 1 | Room | 5 | 12 | 2 | Room | 5 |
| Squid | 2 | Room | 2 | A night | 1 | Room | A night |
| Lobster | 10%** | 100 | 1 | 2.5 | 10%**HCl, 90%** HCOOH | Room | 18 |
| Krill | 3.5%** | 25 | 1 | 2 | 3.5%** | 20 | 1.5 |
| Lobster | 5%** | 80-85 | 2 | 0.5 | 5%** | 70 | 4 |
| Crawfish | 3.5%** | 65 | 1 | 2 | 1 | Room | 0.5 |
| Crab | 1 | 50 | 1 | 6 | 1 | 20 | 3 |
| Shrimp | 1%** | 65 | 1 | 1 | 0.5 | Room | -* |
| Shrimp | 3%** | 100 | 1 | 1 | 1 | Room | 0.5 |
| Shrimp | 4%** | 100 | 1 | 1 | 5%** | Room | -* |

* Some values were not mentioned in experiment/ **the concentration was mentioned as (w/w)

During demineralization, chitin chains may be degraded under some conditions. To avoid this problem, demineralization accomplished at room or normal temperature (20-25˚C). In addition, mild acids such as ethylenediaminetetraacetic acid (EDTA), $CH_3COOH$, $H_2SO_4$ are also applied to minimize chitin degradation; chitins which are rich in remaining ash content will give better results in this case.

Demineralization using HCl is also usually accomplished after 2 to 3 hours under stirring, but time is flexible among treatment methods as can be seen in Table 7. Demineralization can be performed in several days; ash content can be slightly reduced but polymer degradation can occur. In general, demineralization is carried out at high temperature in order to accelerate the reaction, because at high temperature, solvent gets into the chitin matrix easily. Besides that, acceleration of reaction strongly depends on interfacial area between chitin matrix and solvent. Though high acid concentration, high temperature, long time of reaction, and interfacial area control the final properties of chitin. Finally, to have high a quality of chitin, demineralization should be performed by using mild acids under room temperature with the presence of the stoichiometric contained amount of 0.25M HCl. (Younes & Rinaudo 2015.)

### 4.1.3 Decolorization and Processes Preserving Chitin Structure

The final step in recovery of chitin is decolorization, which means colored pigments will be removed in this stage. In crustacean shell, such as shrimp, crab contain pigments in which carotenoid group is major. Acetone and organic solvent mixture are recommended in this process. For instance, acetone is used to treat the samples in ten minutes after that, samples will be dried at ambient temperature for 2 hours, and the samples will be removed all pigments. The dewatering process is also an additional step to remove all residual matter, in which chitin samples will be washed by distilled water, then samples will be dried at 60˚C in 24 hours. (Seenuvasan et al. 2020, 116.)

Chitin collected from crustacean waste has 2 main types α and β–chitin; chitin content in each type of sources is also different. Besides that, deproteinization and demineralization conditions are depended on the type of sources and required quality. As can be seen from Table 4, Chitin produced from shrimp's waste is α–Chitin, and from cuttlefish's pen is β–Chitin; with 2 sources, production condition is different because of differences in structure and content. Moreover, deproteinization and demineralization can be performed several times by using HCl and NaOH at low concentration in order to reach purity requirements. Table 8 compares chitin production from different sources (Younes and Rinaudo's research 2015.)

TABLE 8. Chitin production from different sources (adapted from Younes & Rinaudo 2015)

| Source | | Number of Deproteinization baths | Number of Demineralization baths | Degree of acetylation |
|---|---|---|---|---|
| | | 0.3M NaOH, 80˚C, 1h | 0.55M HCl, 25˚C, 2h | |
| Cirripedia | Anatife | 4 | 2 | 100 |
| Reptantia Brachyura | Red crab | 3 | 5 | 97 |
| | Marbled crab | 3 | 3 | 99 |
| | Spider crab | 3 | 3 | 96 |
| Reptantia Macrura | Lobster | 3 | 3 | -* |
| | Crayfish | 7 | 3 | 100 |
| | Slipper lobster | 3 | 2 | -* |
| | Freshwater crayfish | 3 | 2 | -* |
| Natantia | Pink shrimp | 3 | 3 | 100 |
| | Grey shrimp | 2 | 2 | 100 |
| Stomatopoda | Squilla | 3 | 3 | 100 |
| Cephalopoda | Squid | 2 | 2 | 100 |

* Some values were not mentioned in experiment.

### 4.1.4 Chitosan Production by Chemical Deacetylation

Chemical deacetylation is the process in which the acetyl group on C2 glucosamine will be replaced by -$NH_2$ group; and chitosan is the product of this process (Hajji, Younes, Ghorbel-Bellaaj, Hajji (R), Rinaudo, Nasri, Jellouli 2014). The deacetylation process can be performed by either acids or alkalis. When using acid treatments, glycosidic bonds are affected (polymer chains are cut) so alkali deacetylation is preferred. In alkali methods, acetyl groups are separated by treating with hot concentrated alkalis (NaOH is more efficient than KOH) in few hours, then insoluble residue chitosan is created as the final product with DD up to 85-99% (Hajji et al. 2014). Deacetylation is shown in figure 7 below.

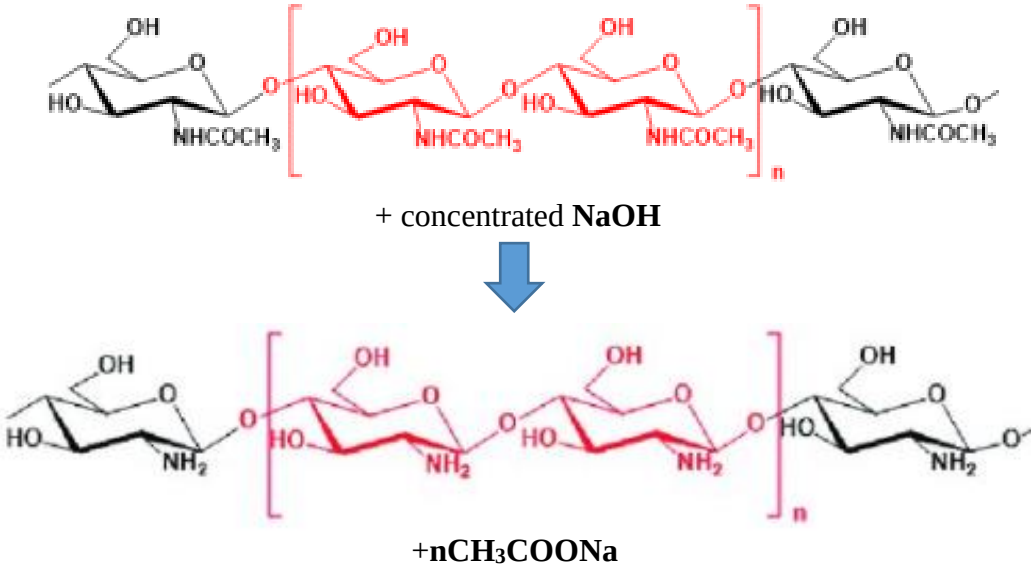


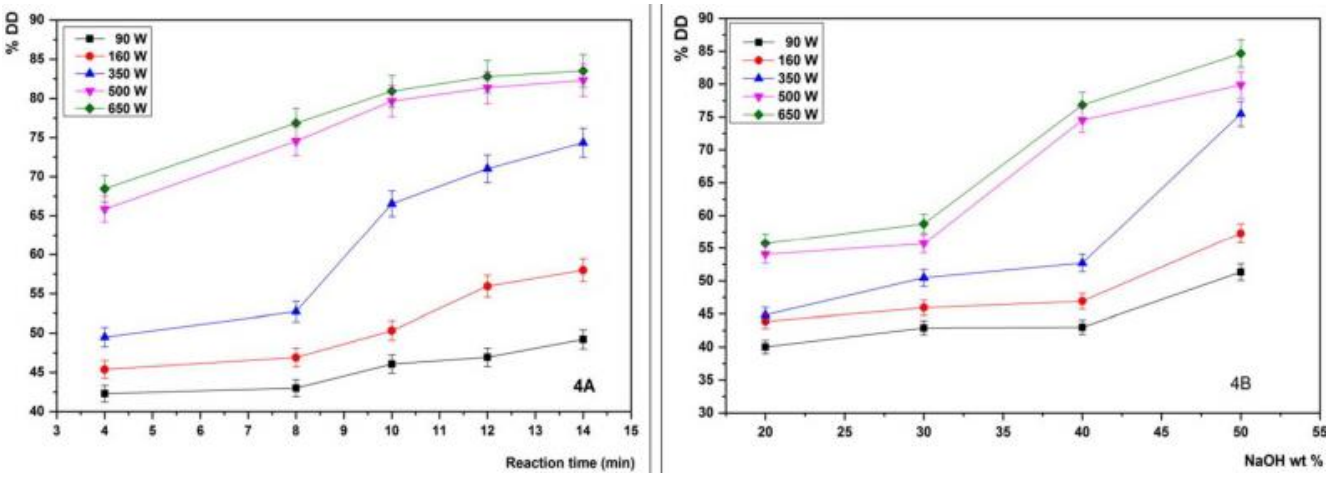

FIGURE 7. Reaction of deacetylation (adapted from Mohammed et al 2017)

The efficiency of deacetylation and characteristics of chitosan base on many parameters which depend on sources of chitin and chitosan's properties. According to Pires, Vilela, and Airoldi (2013, 221-224), effects of temperature, deacetyl time, and concentration of NaOH on deacetylation were proved. Reaction time is the most influential factor to chitosan properties; when the time of deacetylation is 24 hours, DD is higher than in case 3 hours deacetylation with the same conditions. The temperatures of reaction were also noticed, at room temperature (24˚C) the time of reaction is quickest. Additionally, concentrations of NaOH slightly affected this experiment. However, the prepared sample set up at 393K for 24 hours, the higher NaOH concentration leads to different results. Produced chitosan, which had higher DD and weak charges, contained nanoparticles (smaller than 50nm). Conditions of this experiment are illustrated in Table 9 below.

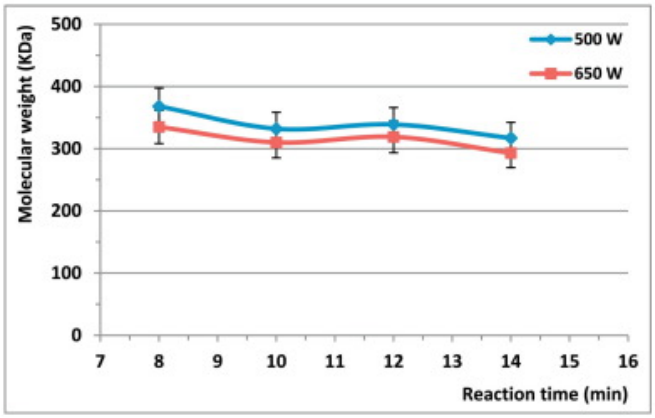
TABLE 9. Conditions of deacetylation applied for chitin from shrimp source (Pires et al. 2013, 222)

| Chitin sources | Deacetyl conditions | | |
|---|---|---|---|
| | NaOH Concentration | Temperature (˚C) | Time (hours) |
| Shrimp | 2M | 24 | 3 |
| | 10M | 24 | 3 |
| | 2M | 90 | 24 |
| | 10M | 90 | 24 |

According to Knidri, Dahmani, Addaou, Laajeb, and Lahsini (2019), chitin and chitosan were extracted in the microwave; time, power, and NaOH concentration affected directly on DD and $M_w$ of chitosan. From Figure 8A, when increasing the reaction time, the DD increases. In addition, the deacetylation reaction accelerated when higher powers (500-650W) were applied. With the NaOH concentration, DD grows when the concentration of NaOH increases (Figure 8B). It was found that with the highest concentration of NaOH (50%) and a power range from 500 to 650W the DD has the greatest values. With reaction time between 8 minutes to 12 minutes under microwave power 500W and 650W, the chitosan's $M_w$ changes between 300 and 360 kDa (Figure 8C). By using microwave extraction, produced chitosan has medium and high $M_w$. Figure 8 illustrates parameters that affected DD and $M_w$ of chitosan in the research of Knidri et al. (2019).




A B




C

FIGURE 8. Effect of reaction time (A), NaOH concentration (B) on DD and reaction time on $M_w$ (C) (Knidri et al. 2019)

The repeated alkali treatment is also an important parameter in deacetylation, it strongly affected DD and $M_w$ (Tolaimate, Desbrières, Rhazi, Alagui, Vincendon, and Vottero 2000). All of researches proved that NaOH concentration, reaction time, temperature, and repeat of alkali treatment affects directly DD and $M_w$ of chitosan. In addition, the environment conditions were reported that affected DD and solubility of chitosan by Ngo and Ngo (D) (2017). The DD was improved, and the solubility was better under ultrasound with a low concentration of NaOH. In order to decrease depolymerization of chitosan during deacetylation, the nitrogen environment was applied instead of an air environment (Younes & Rinaudo 2015). To study influences of parameters in deacetylation, 7 factors were applied: temperature, alkali reagent and its concentration, number of successive baths, performance time, addition of sodium borohydride ($NaBH_4$), and reducing reagent (Younes, Ghorbel-Bellaaj, Chaabouni, Rinaudo, Souard, Vanhaverbeke, Jellouli, and Nasri 2014). The results proved that temperature and alkali reagent are major factors, the DD was also significantly greater by using more alkali baths, alkali's concentration, and reaction time. Whereas atmospheric conditions and reducing reagent ($NaBH_4$) only affected $M_w$ and reducing depolymerization.

### 4.2 Biological Extraction

Chitin production by chemical extraction has many advantages such as simple, effective, easy to perform at large scales. However, this process also has many drawbacks that affect directly to the environment, generally, chitin extraction is a process where demineralization, deproteination, and deacetylation are applied as a chemical extraction technique. These steps occur in the environment of concentrated acids and alkali under high- temperature setting. This type of process has pros and cons at the same time; however the disadvantages site accounts for the majority. The process always asks for high energy, increasing in chitin purification cost, and impaired physiochemical properties of extracted products. The most common biological methods used to extract chitin were reported. The first method is using proteolytic enzymes in deproteinization and the second is using microorganisms to digest both protein and minerals. These methods which responses the requirements of "green chemistry" (cleaner, eco-friendly, and economic), are attracting many researchers. (Younes & Rinaudo 2015; Seenuvasan et al. 2020.)

### 4.2.1 Enzymatic Deproteinization

In enzymatic deproteinization, proteolytic enzymes such as proteases are used to remove protein content during chitin production from seafood waste. The main source of proteolytic enzymes are plants, microbes, and animals. Many proteases such as papain, trypsin, pepsin, devolvase, alcalase, and pancreatin could be used to deproteinization and reduces steps in next processes like deacetylation and depolymerization. Accomplishing the deproteinization either before or after demineralization can modify the accessibility of the enzymes. Commercially, both crude and purified proteases are used in the deproteinization step. However, purified enzymes are more expensive than crude enzymes; not only cheaper than purified enzymes but also crude enzymes are more effective because of coexisting proteases. Fish viscera and bacteria are the main sources of crude enzymes, in which bacteria proteases are used popularly. Furthermore, protease enzymes are also found in marine animals and can be recovered in active and stable forms. This is a valuable and economical source, since in several countries the by-product of the seafood industry is about 50% of the harvest. Hence, recycling and using seafood waste to produce protease enzymes should be concerned in chitin extraction. (Younes & Rinaudo 2015; Seenuvasan et al. 2020.)

In the past 10 years, many reports have performed the application of bacterial proteases in chitin extraction (Synowiecki & Al-Khateeb 2000; Hamdi, Hammami, Hajji, Jridi, Nasri, and Nasri (R) 2017; Doan, Tran, Nguyen, Vo, Nguyen (D), and Wang 2019). Synowiecki & Al-Khateeb (2000) applied Alcalase 2.4L in order to extract chitin and produce valuable proteins hydrolysate. The given flowsheet in Figure 9 below shows the process of chitin and protein hydrolysate production from shrimp shell waste, which was performed by Synowiecki & Al-Khateeb (2000). 10% (w/v) HCl solution was used in demineralization for 100g of the shells at 20˚C for 30 minutes with a ratio 1: 20(w/v) of shells to acid. After that, demineralized shells were prepared as a suspension in water then the mixture was exposed to protein hydrolysis with pH 8.5 and at 55˚C by 20 Anson Units/kg (AU/kg) of concentration of enzyme. Furthermore, 4M NaOH solution was used to maintain pH during the hydrolysis. To stop the hydrolysis, 4M HCl was used to decrease pH to 4.0 for 30 minutes. The product was centrifuged at 4000xg (where xg stands for times gravity) in 15 minutes. The final steps were washing with water, ethanol (10:1, v/w), and acetone (10:1, v/w) respectively to collect crude chitin. In this experiment, the achieved hydrolyzate is a potential source for amino acid in the food industry, but this process also had difficulties because of residual peptides and amino acids in the material after hydrolyzing. This method obtained good results in purity of chitin, in the last product content, only 4 % of proteins existed, that purity responded for many no-medical purposes. (Synowiecki & Al-Khateeb 2000.)

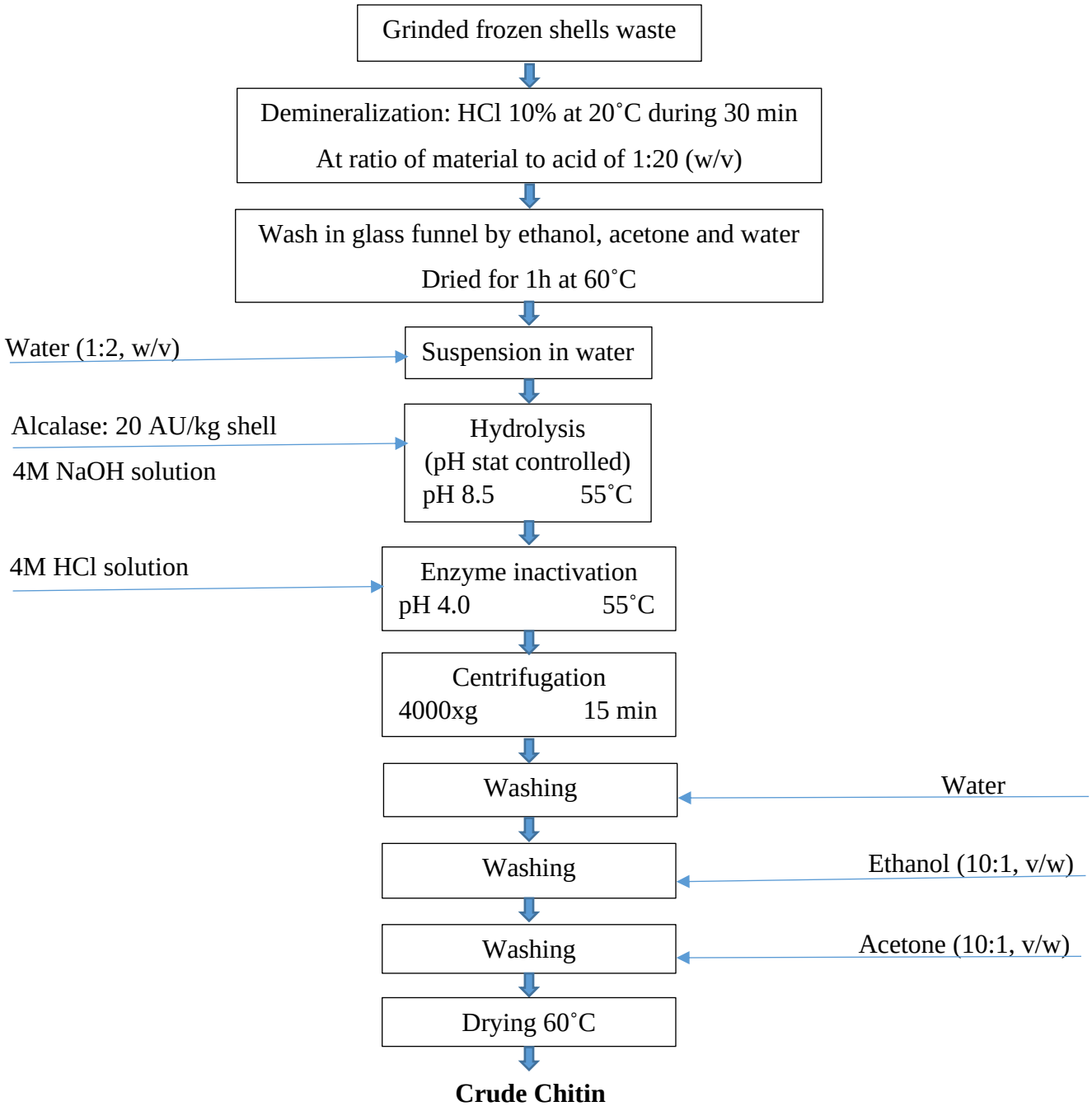



FIGURE 9. Chitin production from shrimp waste by using enzymes

In Hamdi et al.'s experiment (2017), digestive alkaline proteases from blue crab (Portunus segnis) viscera were used in deproteinization in extracting chitin from shrimp and crab shells. In addition, Purafect (R 2000E) and Neutrase P1236 are used to control the experiment. Firstly, shell powders mixed with distilled water. Conditions of reactions were adjusted to pH 8.0, 50˚C; pH 10.0, 50˚C; pH 7.0, 50˚C for Portunus segnis proteases, Purafect, and Neutrase respectively. Then the deproteinization occurred in 3 hours. After that, mixtures were heated at 90˚C for 20 minutes to inactivate enzymes. The

deproteinized products were washed till reach pH neutral and dried at 50˚C for a night. The demineralization of deproteinized shell powders was completed by using HCl 0.55M (1:10 w/v). High deproteinization degree (around 85% and 91% for blue crab and shrimp shells, respectively) were reached in this experiment by using Enzyme/Substrate ratio = 5 units/mg (U/mg) proteins, at 50˚C and pH=8.0, for 3 hours. Figure 10 illustrates the steps of this experiment.

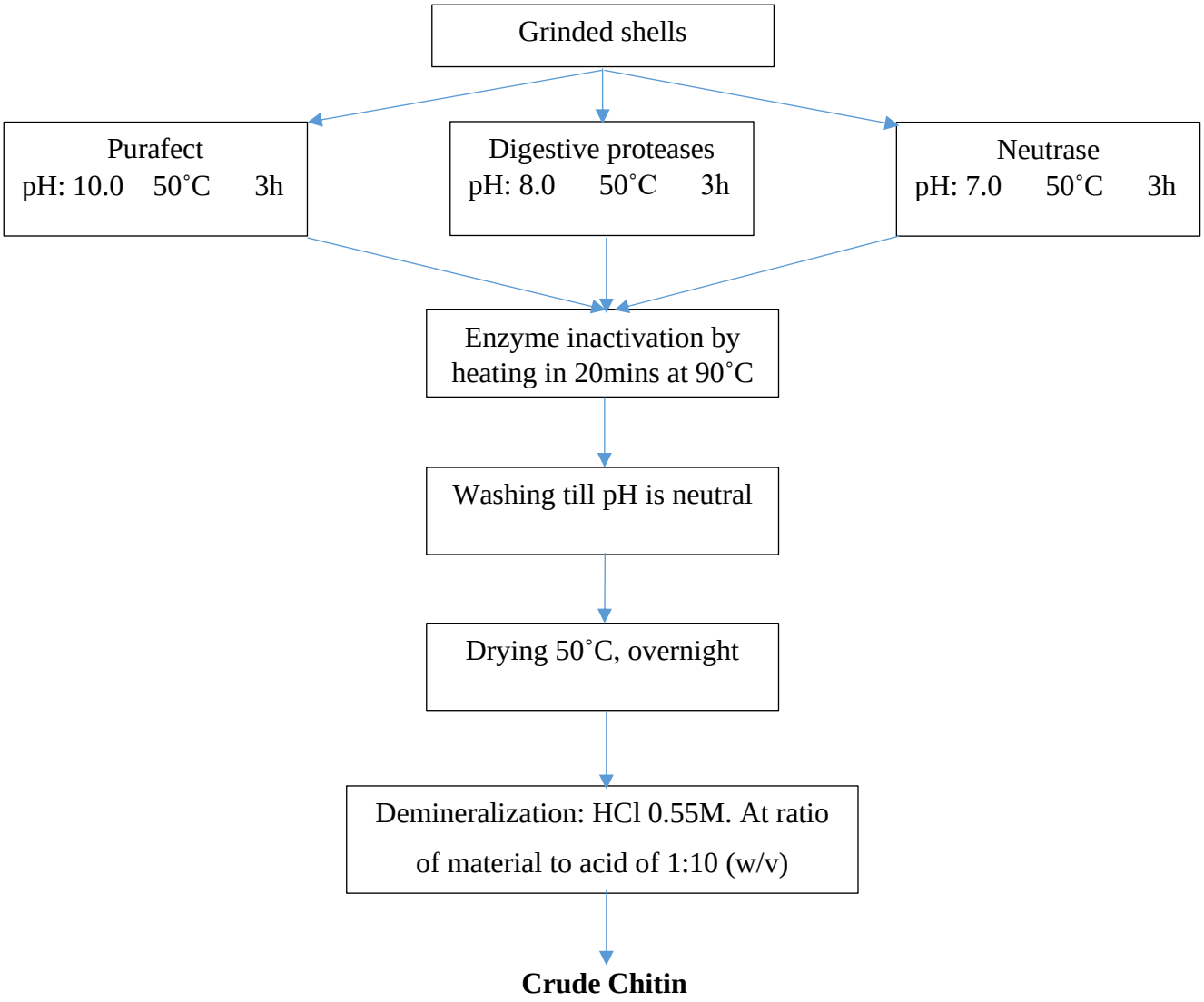



FIGURE 10. Chitin production from cab and shrimp shells by using digestive enzymes

The chemical process has much more efficiency compare to a clean process, knowns as enzymatic technique; 5%–10% remaining proteins existed in chitinous products. Additionally, to increase chitin's purity, NaOH solution can be applied to final chitin products. Moreover, in the chemical technique, the

efficiency and quantity of the last products are not significant affected by the order of demineralization and deproteinization. (Younes & Rinaudo 2015; Seenuvasan et al. 2020.)

### 4.2.2 Demineralization by Organic Acids

Demineralization is a major step in the chitin production process from crustacean waste. As mentioned above, demineralization, in general, accomplished by HCl. However, many disadvantages were found when using HCl in this step such as environmental effects, equipment corrosion, and chitin chain degradation. Therefore, some research has conducted on demineralization by lactic fermentation or using organic acids in the process. In Khong's report (2013, 9), 0.25M HCOOH and 0.25M citric acid was mentioned as a mixture of acid (ratio 1:2 respectively), which is one of the best ways to demineralize in chitin production. $CH_3CH(OH)COOH$ produced by sugar derived from fruit waste-stream was used to demineralize shrimp shells in Tan, Lee, and Chen' experiment (2020). Mahmoud, Ghaly, and Arab (2007, 1-9) extracted chitin from Pandalus borealis shrimp by organic acids ($CH_3CH(OH)COOH$ and $CH_3COOH$) from cheese whey fermentation. These experiments showed the effects of organic acid in demineralization, the results were positive although HCl had better effective.

Using organic acid to demineralize in chitin production is a potential method. The organic acid is "friendly" with the environment and can remain properties of chitin. Moreover, organic acid can be produced from low valuable sources or industrial process waste. Furthermore, salts collected from demineralization can be used as a food preservative. Especially when using organic acids to demineralize, chitin products have a higher purity that can respond to chitin purify requirements in medical and food industry. In addition, chitin also has good $M_w$ and viscosity. (Gortari & Hours 2013; Mahmoud et al. 2007, 1-9.)

In Mahmoud et al.' experiment (2007, 1-9), which extracted chitin from shrimp shells, organic acids were used as the main chemicals for demineralization. These acids were produced from cheese whey fermentation. The experiment was started by washing and crushing shrimp shells, followed that shells were deproteinized by Aspergillus niger (ATCC 16513) in 120 hours, then washed several times with distilled water. The deproteinized shells were dried in an oven at 60˚C. The demineralization was performed with 2 organic acids ($CH_3CH(OH)COOH$ and $CH_3COOH$) and HCl under two conditions. Acid concentrations and demineralization' conditions have followed the table 10.

TABLE 10. Demineralization conditions for each acid (adapted from Mahmoud et al 2007, 3)

| Acid | Concentration (g/l) | Temperature (˚C) | Time (hour) | Shells/Acid ratio (w/v) |
|---|---|---|---|---|
| HCl | 36.46 | 100 | 1 | 1:50 |
| | 61.98 | 24 | 6 | 1:10 |
| $CH_3CH(OH)COOH$ | 75.60 | 100 | 1 | 1:50 |
| | 75.60 | 24 | 6 | 1:10 |
| $CH_3COOH$ | 75.00 | 100 | 1 | 1:50 |
| | 75.00 | 24 | 6 | 1:10 |

The demineralized shells were continued to filtrate and washing with distilled water. The final step to acquire crude chitin is drying products in the oven at 105˚C for 24 hours. The last organic salts were used in food preservatives and they were also environmentally friendly de-icing/anti-icing agents (Mahmoud et al. 2007). The flowsheet in figure 11 illustrates the Mahmoud et al.' experiment.

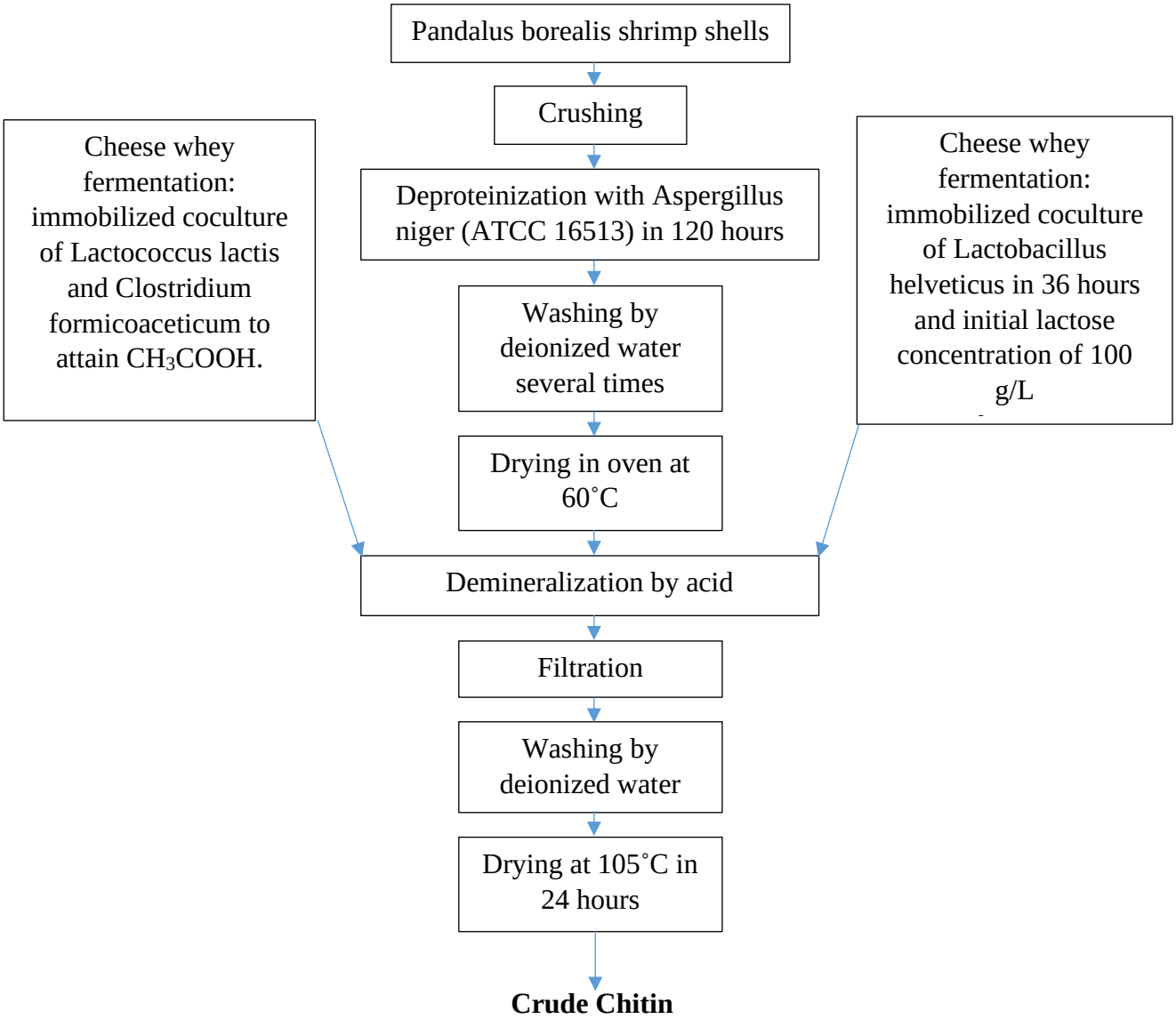



FIGURE 11. Chitin extracted from shrimp shells by organic acids

### 4.2.3 Fermentation

Deproteinization in the usage of fermentation technique is one of the efficient one. Due to this, the use of enzyme has a lower cost by using selected microbial strains and endogenous microorganisms. Fermentation which includes single-stage, two-stage, successive fermentation, or co-fermentation is the method in order to select microbial strains (Younes & Rinaudo 2015).

Lactobacillus sp. strain is an ideal strain for simple single-stage fermentation for extracting chitin from seafood waste. In this process, demineralization and deproteinization occur at the same time in batch culture by microorganisms. $CH_3CH(OH)COOH$ and proteases are produced through the process. $CH_3CH(OH)COOH$ is obtained by alteration of glucose which brings about a decline in pH of silage thereby subduing the growth of spoilage microbes. Calcium lactate is precipitated by the reaction of $CH_3CH(OH)COOH$ and $CaCO_3$, it can be separated and recovered by washing with water. For Crab (Allopetrolisthes punctatus) shells, 99.6 and 95.3% degree of demineralization (DM) and degree of deproteinization (DP), respectively, were achieved with Lactobacillus plantarum sp. 47 (LP47) after 60 hours of fermentation (Castro, Guerrero-Legarreta, and Bórquez 2018). The fermentation of crab shells was accomplished at pH 6.0 ± 0.1, adjusted by $CH_3COOH$, using 10% LP47 inoculum, 15% sucrose, and 85% crab biomass. Besides, by using Pseudomonas aeruginosa A2, DM of 96% and DP of 89% were succeeded after 5-day fermentation of shrimp (Metapeneaus Monoceros) waste (Ghorbel-Bellaaj, Hmidet, Jelouli, Younes, Maâlej, Hachicha, and Nasri 2011).

Successive two-stage fermentation has been also accomplished to produce the final chitin crude from biomaterials. This method uses microorganisms that produce both organic acid and proteases or uses 2 types in which one produces organic acid, and one is a proteases producer. Lactobacillus paracasei KCTC-3074 and Serratia marcescens FS-3 were used to extract chitin from red crab shells in an experiment and resulted 94.3% of DM and 68.9% DP (Jung, Jo, Kuk, Kim, Oh, and Park 2007). Jung et al were the first team to succeed with successive fermentation, therefore, the DP was not satisfactory. In 2020, higher DP efficiency was obtained by using Bacillus amyloliquefaciens (Liu, Xing, Yang, Liu(S), Qin, Li, Yu, and Li (P) 2020). The raw material is shrimp (Litopenaeus vannamei) shells, by using Lactobacillus rhamnoides and Bacillus amyloliquefaciens, crude chitin was extracted with high quality. The result of DM was 97.5% and 96.8% for DP after 84 hours of fermentation, additionally, the final products were reached to quality of animal feed and food also.

Many factors affect the efficiency of fermentation such as species, the quantity of inoculum, carbon source concentration, pH during fermentation, temperature, and duration of fermentation. (Castro et al 2018; Ghorbel-Bellaaj et al 2011; Liu et al 2020; Gortari & Hours 2013.)

When compared to chemical processes of chitin extraction, biological methods are more productive and eco-friendly. However, many disadvantages can be seen through the above experiments such as long processing time (up to 5 days), poor accessibility of proteases (because of high residual proteins). As mentioned above, the crude chitin still contains minerals and proteins after extraction. Thus, mild acid could be used to remove residual proteins and minerals. (Younes & Rinaudo 2015.)

### 4.2.4 Chitosan Production by Enzymatic Deacetylation

As was mentioned in 4.1.4, chemical deacetylation uses concentrated alkali which has been a pollutant, besides that the energy needed to process is also high, with both causes above, the chemical deacetylation is non-eco-friendly. The chitosan products which have been obtained from the chemical method are large and diverse in the range of solubility. To overcome these disadvantages, same as chitin preparation, enzymes also were explored to apply in deacetylation. Then chitin deacetylase is a choice, it presents in several fungi (Mucor rouxii, Absidia coerulea, Aspergillus nidulans and two strains of Colletotrichum lindemuthianum, Penicillium oxalicum), insect (Lepidoptera, Hyphantria cunea) and bacteria (Nitratireductor aquimarinus) (Younes & Rinaudo 2015; Pareek, Vivekanand, Saroj, Sharma, Singh 2012; Yan, Zhao, Zhang, Guo, Wang, Zhao(K), Gao, and Wang(X) 2018; Chai, Hang, Zhang, Yang, Wang, Liu, and Fang 2020). Chitin deacetylase hydrolyses the N-acetamido group in the chitin structure to produce chitosan. However, the efficiency of chitin deacetylase reported is low for natural chitin which is insoluble. Thus, to enhance the deacetylation yield, pretreatment of chitin before enzymatic deacetylation is necessary with physical or chemical methods such as heating, sonication, grinding, derivatization, and interaction with saccharides (Zhao, Park, and Muzzarelli 2010).

# 5 APPLICATION OF CHITIN

Chitin and chitosan, which have potential in absorption capacity, nontoxicity, biodegradability, and biocompatibility, are natural polymers. Chitin and chitosan contain N-acetyl groups, amino groups, and -OH groups which able to possess biological activities. In addition, chitin, chitosan modifications by adding other functional groups such as carboxyl, glucan, peptide, phosphate, sulfate, saccharide diversified and increased biological activities of chitin, chitosan, and their derivatives (Knidri et al. 2018). Chitin and chitosan were confirmed to various biological activities and have potential in medical, agriculture, and industrial applications. Antioxidant, antihypertensive, anticoagulant, anti-inflammatory, antitumor, antidiabetic effects, antimicrobial, and hypocholesterolemic are chitin and chitosan's noticeable activities. (Knidri et al. 2018; Ahmad, Ahmad (R), Khan, Kant, Shahid, Gautam, Hasan, and Hassan 2020.)

## 5.1 Biomedical Applications

Each Chitin, Chitosan's properties and biological activities have applied for specific applications. In medical field, absorption promoters, hydrating agents, film production, and wound healing are significant applications of chitin and chitosan. They are formed to different shapes of structures such as fibers, powders, films, sponges, beads, solutions, gels, and capsules (Younes & Rinaudo 2015). Because of easy to apply, chitosan is used for drug delivery in implantable and injectable forms.

Form of fiber, filming chitin or chitosan are typically applied in tissue engineering and wound healing. Furthermore, chitosan can easily spread through transmucosal, so it has potential in vaccine delivery. Mucoadhesivity of chitosan and its cationic derivatives are admitted and proved to absorb drugs more efficient at neutral pH. N-lauryl-carboxymethyl-chitosan is a chitosan derivative without toxicity in membranes, therefore, it may be useful as a vehicle for hydrophobic cancer drugs. In addition, chitosan and its derivatives have applied for gene transfection. The efficiency of transfection improved when the alkyl side chain in N-alkylated chitosan, as well as Quaternized chitosan, were elongated to 8 carbons. Mixed $Ca_3(PO_4)_2$, citric acid, and chitosan or chitosan glycerophosphate is applied in bone repairing. In general, chitin and chitosan with different conformations have been researched and applied in pharmaceutical and biomedical domains. Table 11 shows selected chitin, chitosan applications. (Younes & Rinaudo 2015.)

TABLE 11. Pharmaceutical and biomedical applications of Chitin and Chitosan (adapted from Younes & Rinaudo 2015)

| **Conformation** | **Applications** |
|---|---|
| **Beads** | Drug delivery |
| **Microspheres** | Enzyme immobilization & Gene delivery vehicle |
| **Nanoparticles** | Encapsulation of sensitive drugs |
| **Coatings** | Surface modification & Textile finishes |
| **Fibers** | Medical textiles & Suture |
| **Nanofibers** | Guided bone regeneration & Scaffold for nerve tissue regeneration |
| **Nonwoven bioactive fibers** | Wound healing |
| **Films** | Wound care<br>Dialysis membrane<br>Antitumoral<br>Semi-permeable film for wound dressing |
| **Powder** | Adsorbent for pharmaceutical and medical devices<br>Surgical glove powder<br>Enzyme immobilization |
| **Sponge** | Mucosomal hemostatic dressing & Wound dressing<br>Drug delivery<br>Enzyme entrapment<br>Artificial skin |
| **Shaped objects** | Orthopedics & Contact lenses |
| **Solutions** | Cosmetics<br>Bacteriostatic agent<br>Hemostatic agent<br>Anticoagulants & Antitumor agent<br>Gene delivery<br>Spermicide |
| **Gels** | Delivery vehicle<br>Implants, coating & Tissue engineering<br>Wound dressing for wet treatment |
| **Tablets** | Compressed diluent, Disintegrating agent & Excipient |
| **Capsules** | Delivery vehicle |

### 5.2 Agriculture Applications

In recent decades, eco-friendly farming systems for sustainable agriculture has been developed surprisingly. Greater knowledge of using natural compounds, especially, chitin, chitosan, and their derivatives has revealed in order to minimize the risk of using chemical compounds. Chitin and chitosan are renowned by beneficial effects such as having an abundant supply of raw materials from nature, having natural origin, not containing any toxin, biodegradation, eco-friendly environmental, and having broad biological activities. As a result, chitin, chitosan, and their derivatives are greatly applied in agricultural fields. (Hadrami, Adam, Hadrami (I), and Daayf 2010; Knidri et al. 2018; Ahmad, Ahmad (R), Khan, Kant, Shahid, Gautam, Hasan, and Hassan 2020.)

According to their effects, chitin, chitosan, and their derivatives have biological activities which could be applied extensively in agricultural area. The control and management of plant's resistance to various pathogens have always been considered as one of the great examples for applications. For many years, chemical pesticides have been consumed because of their easy usage. Nevertheless, due to the high environmental risk, biological control of plant disease with bio-pesticide is highly recommended. Chitin, chitosan, and their derivatives are renowned biological control agents for their nontoxic, biodegradable, and biocompatible properties. They control pathogenic microorganisms by preventing growth, sporulation, spore viability, germination disrupting cell and inducement of different defense responses in host plants inducing or inhibiting different biochemical activities during the plant-pathogen interaction. Chitosan has been assayed for control numerous pre-and post-harvest diseases of many crops. For achieving the goal of sustainable agriculture, chitosan will become a popular plant protectant. (Hadrami et al. 2010.)

The use of bioactive compatible with the environment is one of the main challenges for modern agriculture. For this purpose, the use of chitin and its derivatives is a promising alternative, based on its biological activity and easy-to-obtain procedures. The ubiquity, biological and biocompatible properties of chitin and its derivatives settle them up as promising alternatives for agriculture. Its antiviral activity, together with the rest of recently-discovered properties are highly demanded in agriculture, while others, more established and still underestimated characteristics (e.g., antifungal and nematocidal activities) could result in great steps towards sustainable agricultural practices, by decreasing the use of chemical synthetic pesticides and bringing a new focus to modern phytopathology. The antimicrobial activity of chitosan against Gram-negative and Gram-positive bacteria as well as filamentous fungi and yeasts. The mechanism of antibacterial and antifungal action of chitosan on the cell wall of microorganisms and the

influence of physicochemical parameters of the polymer on its biological activity are an outstanding example of this application. Additionally, chitin, chitosan, and their derivatives also increase the quality of soil, water, and plants. Furthermore, nitrogen in nods of Leguminous plants can be improved by chitin products. (Hadrami et al. 2010; Dzung 2010, 619-629.)

Based on potential activities such as non-toxic, biodegradable, and environmentally friendly properties, chitin, chitosan, and their derivatives are very suitable for organic agricultural production models, and eco-sustainable agriculture. The effectiveness of these derivatives is usually slower than that of other agrochemical drugs, the effect is not stable because it is highly dependent on the $M_w$, the DD, environment, and different plants. (Hadrami et al. 2010.)

### 5.3 Industrial Applications

With special biological bioactivities, chitin and chitosan were applied in many sectors of industry. Chitin and chitosan were investigated wastewater treatment potentials, they can remove pigment, heavy or toxic metals. In addition, chitin and chitosan have applied to food and beverage, cosmeticss, biopharmaceutics, and tissue engineering. (Gamage & Shahidi 2007; Miretzki & Cirelli 2009; Sudha 2010, 561-576; Şenol, Gürsoy, Şimşek, Özer, and Karakuş 2020; Coura, Profeti, and Profeti (R) 2020.)

#### 5.3.1 Industrial Wastewater Treatments

Environment pollution has been a global challenge since the end of the $20^{th}$ century. A healthy living environment is the most important mission for global in recent and future. Thefore, chitin, chitosan, and their derivatives have certain roles in environmental protection such as heavy metal, dye removal or metal, toxicities absorption (Sudha 2010, 561-576; Sarode, Upadhyay, Khosa, Mak, Shakir, Song, and Ullah 2019).

Industrial wastewater usually has high heavy metal concentrations, which affect to the environment and human health as well. Ion exchange and using safety biopolymer methods are feasibility solutions. Polymers have functional groups such as $-NH_2$, -OH, which increase metal ion absorption. Using chitosan to purify wastewater was accepted by the United States environmental protection agency (US EPA) with amounts of chitosan up to 10 mg/l. Not only does chitosan remove toxic metal Cadmium (Cd) and Lead (Pb), but the Cobalt-60 (Co) is also absorbed during treatment which prevents accidental

contamination. In Gamage and Shahidi (2007) experiment, Copper (Cu) (II) was removed with efficiency up to 97.5% from 50ppm (0.005%) metal solution and 56.5% for 100ppm (0.01%). It means that, Cu(II) was collected and recycled from metal solution. Gamage and Shahidi reported 5 metals in the experiment, the efficiency of each is shown in table 12.

TABLE 12. Chelation and Recovery for different metals (adapted from Gamage & Shahidi 2007)

| **Metal ion** | **Chelation (%)** | | **Recovery (%)** | |
|---|---|---|---|---|
| | **50 ppm** | **100 ppm** | **50 ppm** | **100 ppm** |
| Ni(II) | 99.6 ± 0.01 | 98.9 ± 0.00 | 87.0 ± 10.9 | 97.9 ± 1.69 |
| Co(II) | 76.6 ± 0.17 | 99.7 ± 0.00 | 52.2 ± 0.00 | 84.7 ± 3.18 |
| Cd(II) | 99.1 ± 0.01 | 99.1 ± 0.00 | 79.8 ± 0.20 | 74.3 ± 1.56 |
| Cu(II) | 99.9 ± 0.06 | 99.5 ± 0.00 | 97.5 ± 2.26 | 56.2 ± 0.51 |
| Ag(I) | 99.9 ± 0.00 | 99.9 ± 0.00 | 0.08 ± 0.00 | 0.00 ± 0.00 |

Mercury (Hg) (II) absorption by chitosan, its derivatives, and other component was reported in Miretzki and Cirelli's report (2009). As can be seen in graph 1, chitosan (Cs) absorbed about 450 mg/g Hg and graft polymerized cross-linked chitosan (GCSCS) had an outstanding result with 800mg/g.

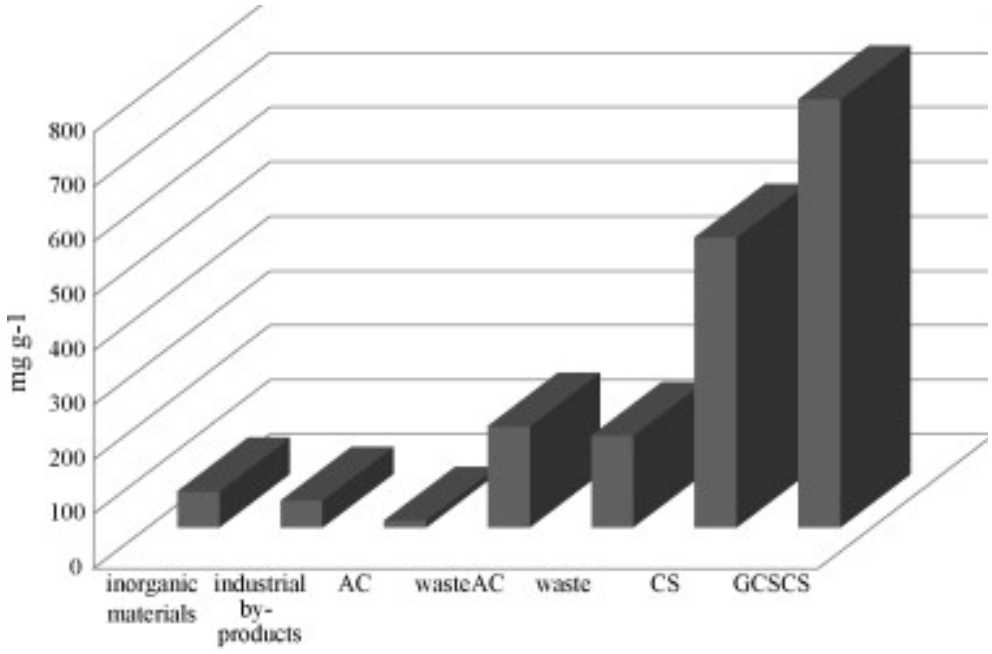



FIGURE 12. Hg(II) absorbed results by different absorbents. (Adapted from Miretzki & Cirelli 2009)

Besides metal in wastewater, organic pollutants are considered in industrial activities. Treatment and control of wastewater of food manufacture are significant challenges in recent years. Wastewater of food process contains high concentrated organic compounds, hence, chemical oxygen demand (COD),

biochemical oxygen demand (BOD), and solid are always high. Chitosan, is a natural polyelectrolyte polymer, has good ability in absorption, flocculation with organic compounds, especially, proteins in waste stream. Gamage and Shahidi (2009) reported the ability of chitosan in protein flocculation as well. Chitosan with higher DD carries more positive charges so it increases absorption, coagulation capacity. Proteins collected from the process will be used to produce animal feed.

Chitin, chitosan, and their derivatives are used to remove dyes in wastewater as well. Chitosan-Vermiculite (Ch-V) composite beads material removed Sunset Yellow FCF (Sy) and Brilliant Blue FCF (Bb) food dyes from aqueous solution (Şenol et al. 2020). The results showed that Ch-V removed 175.1 mg/g Sy and 181.6 mg/g Bb. Composite Chitosan-Quartzite (Ch-Q) was revealed about absorbing Reactive Black 5 (RB5) capacity. Ch-Q reached maximum the absorption capacity of 171.5 mg/g at 55˚C (Coura et al.2020).

### 5.3.2 Applications in Food Industry

Chitin, chitosan, and its derivatives are potential compounds used in the food industry. Chitin and chitosan carry special properties such as antimicrobial, antibacterial, antifungal, antioxidative. Thus, it is applied to food preservation, moreover, chitin and chitosan were used to produce function foods, additives in the beverage industry, and food packing. Application of chitin, chitosan, and their derivatives are illustrated in Table 13 below. In addition, chitosan is applied in cosmetic applications and drug delivery as well (Shelma & Sharma 2010, 507-515; Casadidio, Peregrina, Gigliobianco, Deng, Censi, Martino 2019). Not only delivers active ingredients (natural compounds), but also chitin and chitosan act as drug-like active ingredients. These compounds contain positive charges, which exploit the negative charges of skin under physiological conditions, thus an electrostatic interaction is established (Casadidio et al. 2019). With these biological properties, chitin and chitosan are applied in hair, skin, nails, and oral care.

TABLE 13. Application of Chitin and Chitosan in food industry

| Application Fields | Function | References |
|---|---|---|
| Antimicrobial Agent (food preservation) | Antimicrobial | Vidanarachchi, Kurukulasuriya, Kim 2010, 543-557. |
| | Antibacterial | |
| | Antifungal | |
| | Antioxidative | |
| Function Foods | Enhance Calcium absorption | |
| | Decrease Cholesterol and Lipid absorption | |
| | Used as Prebiotics | |
| Additives | Clarification, Acidity-adjustment agent of Wine and Beverage | Bornet & Teissedre 2010, 519-525. |
| | Increase product flavor | |
| | Remove heavy metal in wine | |
| | Suspension, stabilization agents | |
| | Water purification | |
| Food packing (films) | Control humidity of product and surroundings. | Priyadarshi, Rhim 2020 |
| | Water resistance, thermal stability | |
| | Food shelf-life extension | |
| | Control antioxidant and antimicrobial activities. | |
| | Control nutrients and flavor releasing | |

## 6 CONCLUSION

Chitin, which is the second most abundant compound in nature after cellulose, can be extracted from crustacean waste such as lobsters, shrimps, crabs, krill. With significant properties and biological activities, chitin has a great potential in various commercial fields. In chitin's extraction, many concentrated agents are used to obtain polymers with different properties ($M_w$, DD, and charge).

The chemical extraction of chitin is the most popular method to produce chitin from crustacean waste, this process used concentrated alkali and acid. In chitin, chitosan extraction, the most popular method is using NaOH (0.125M - 0.5M) for deproteinization and HCl (0.125M - 2M) for demineralization with a processing time from a day to 48 hours. Decolorization was also applied to remove pigments from crude chitin. Additionally, chitin extraction by using biochemical methods was also researched. Many proteases such as papain, trypsin, pepsin, devolvase, alcalase, and pancreatin were used in deproteinization and reduce steps in next processes like deacetylation and depolymerization. When proteases have been applied, deproteinization was completed with high effectiveness. Moreover, organic acids such as $CH_3COOH$, $CH_3CH(OH)COOH$, HCOOH, citric acid were applied to demineralization. Many experiments were performed to find out the conditions' effects on chitin, chitosan extraction. These factors are temperature, alkali reagent, and its concentration, number of successive baths, performance time. These conditions affect directly chitin, chitosan properties. In chitin, chitosan production, deacetylation plays an important role, this process is usually performed by using alkali treatments to remove the acetyl group in glucosamine chains. Chitosan properties base on DD and $M_w$ which are affected directly by deacetylation.

Chitin production by chemical extraction has many advantages such as simple, effective, easy to perform at large scales. However, this process also has many drawbacks that affect directly to the environment; conventionally, the chemical chitin extraction involves steps such as demineralization, deproteinization, deacetylation which are performed by using high concentrated acids and alkalis at hot temperature (up to 160˚C). With those conditions above, the chemical process is required high energy and faced to several negative effects. For example, the cost of chitin's purification will be gain and physiochemical properties of chitin products will be impaired. Moreover, when using chemical extraction, valuable proteins cannot be used to produce animal feed because these proteins possess many harmful chemicals. Many reports point out that the biological process is eco-friendly and its chitinous products still maintain the chitin structure. On the other hand, deproteinization of shrimp shells by various proteolytic microorganisms

makes chitin achieved have higher $M_w$ than the chemical process. In overview, the biological method of extraction chitin provides many advantages such as simple manipulation, lower energy, and higher regeneration in relatively less time and solvent consumption. However, long processing time (up to several days), limited in laboratory scale are drawbacks of this method. Whereas chemical extraction of chitin and chitosan occurs in a short time and can be used in industrial scale. In addition, the final products of the chemical method have high DD (%) and do not contain organic salts. Nevertheless, the chemical method is not environmentally friendly and other waste in the process such as minerals and proteins cannot be used for other purposes.

Chitin, chitosan's biological activities and properties which are applied for both plants and animals extend the applications of chitin, chitosan, and derivatives. Moreover, it was investigated in wastewater treatment with abilities as heavy metal, dye removal or metal, toxicities absorption. The result also showed that chitosan has good ability in absorption, flocculation with organic compounds, especially, proteins in waste stream. Finally, chitin, chitosan, and its derivatives are applied to the food and cosmetic industry. Interesting characteristics of chitin, chitosan, and its derivatives attract a great deal from global scientists to discover applications where chitin plays an important role in the improvement of materials and services. Various applications from biomedicals, food, pharmaceuticals to cosmetics made chitin a beneficial way to convert crustacean waste to valuable materials. They are also promising biomaterials for medical, tissue engineering applications in which the most important characteristics come from hydrophilic and antimicrobial properties. Being extracted from natural sources, chitin and chitosan are more advantages as compared to other polymer materials. Biodegradable and biocompatibility are properties that save cost and manpower in tissue engineering as scaffolds. However, controlling mechanical and physical properties of these materials are still challenges.

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