ISOLATING CHITIN AND DERIVATIZING CHITOSAN FROM DEAD ADULT BLACK SOLDIER FLY

By

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ABSTRACT

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Ng Khai Xi

Insect, particularly black soldier fly can be a potential source of chitin and chitosan. In this study, dead adult black soldier fly bio-wastes was evaluated for its production of chitin and chitosan to add value to the waste. The sample was dried, sieved, grinded and defatted prior to the chitin extraction. The chitin which was extracted through chemical demineralization followed by deproteination, obtained the yield in dry weight basis of 10.44 ± 1.39%. The yield dropped to 5.70 ± 0.11% after decolorization, and a color change from dark to white was observed. The obtained chitin yield suggested milder condition was better for chitin extraction from BSF, coupled with high yield, high demineralization and deproteinization efficiency and shorter experimental time. The chitin content of dead adult BSF and extracted chitin were determined as 10.88 ± 0.18 and 36.52 ± 1.08 mg/g respectively. The low determined chitin content suggests a harsher acid hydrolysis treatment should be used to completely digest the chitin to glucosamine prior to spectrophotometric measurement. The extracted chitin was converted to chitosan I and II using two levels of deacetylation with the same NaOH concentration (12.5 M), 80ºC for 15 h for chitosan I and 50ºC for 6 h for chitosan II, with the occurrence of different extent of browning. The yield of chitosan I and II were 3.78 ± 0.16% and 3.79 ± 0.25% respectively. Commercial, pharmaceutical and chitosan I and II were compared using FTIR. As a result,

chitosan I and II exhibited a similar structure with commercial chitosan, with the presence of characteristic functional groups including amide I, amide III, C-O stretching and C-O-C asymmetric stretching that indicated as primary amino group at C2 position, as well as the presence of C-H bending and CH_3 symmetric deformation that was not observed in pharmaceutical chitosan. Both chitosan I and II were showed a high degree of deacetylation of 80.90 and 80.70%, suggested milder deacetylation temperature (<90°C) and short reaction time of 6 h is sufficient to obtain a high DDA (~80%) to indicate promising deacetylation on the chitin of BSF. This study reported that significant amount of chitin and chitosan could be extracted from dead adult BSF bio-wastes even with mild extraction condition, and the chitosan had similar characteristic with commercial chitosan from crab shell which suggested its used as alternative chitin and chitosan source.

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DECLARATION

I hereby declare that this final year project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

NG KHAI XI

APPROVAL SHEET

This final year project report entitled "**ISOLATING CHITIN AND DERIVATIZING CHITOSAN FROM DEAD ADULT BLACK SOLDIER FLY**" was prepared by NG KHAI XI and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Food Science at Universiti Tunku Abdul Rahman.

Approved by:

ChangYP

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(Dr. Chang Ying Ping) Date: 10 April 2023

Supervisor

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Date: 10 April 2023

PERMISSION SHEET

It is hereby certified that **NG KHAI XI** (ID No: **20ADB02454**) has completed this final year project report entitled "ISOLATING CHITIN AND DERIVATIZING CHITOSAN FROM DEAD ADULT BLACK SOLDIER FLY" under the supervision of Dr. Chang Ying Ping from the Department of Agricultural and Food Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project report in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

(NG KHAI XI)

UNIVERSITI TUNKU ABDUL RAHMAN

FACULTY OF SCIENCE

Date: 15 May 2023

SUBMISSION OF FINAL YEAR PROJECT

I, **NG KHAI XI** (ID No: **20ADB02454)** hereby certify that I have completed the final year project titled "**Isolating chitin and derivatizing chitosan from dead adult black soldier fly**" under the supervision of Dr Chang Ying Ping (Supervisor) from the Department of Agricultural and Food Science, Faculty of Science.

I understand that the University may upload the softcopy of my final year project in PDF to the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

(NG KHAI XI)

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LIST OF ABBREVIATIONS

ºC	Degree Celsius
BSF	Black soldier fly
cm^{-1}	Wavenumber
DA	Degree of acetylation
DDA	Degree of deacetylation
FTIR	Fourier transform infrared spectroscopy
h	Hour
M	Molar
mg/g	Milligram/gram
mg/mL	Milligram/milliliter
min	Minute
rpm	Revolutions per minute
SEM	Scanning electron microscope
v/v	Volume/volume
w/v	Weight/volume
XRD	X-ray diffractometry

CHAPTER 1

INTRODUCTION

1.1 Background of study

Nowadays, the continued rise in organic waste production around the world has been an emerging concern not only to human health, but also to the ecosystem (Siddiqui et al., 2022). This highlights the importance of waste treatment to solve the waste accumulation problem. Black soldier fly (*Hermetia illucens*) has been researched as a sustainable method for organic waste management. The hatched larvae black soldier fly can consume organic waste and convert it into valuable biomasses (Kim et al., 2021). And larvae BSF is known as a rich source of protein and fat, make it to be used as nutrient-rich animal feed (Dortmans et al., 2017). This means that larvae BSF can eliminate the organic wastes and converting themselves into high quality nutrients. Therefore, the facilities to breed black soldier fly has shown a markable increase prior to producing larvae BSF that is able to consume biological wastes.

However, black soldier fly actually consists of five development stages, which are eggs, larvae, prepupa, pupae and adult fly. And among the life cycles, only the larvae stage requires feeding, which means larvae is the only stage to process biowastes, because after growing into prepupa stage, their mouthpart is replaced with a hook-shaped structure. This means that the rest of the residues, particularly the dead adult of black soldier fly that will die after laying eggs, as well as those exoskeleton and the husks will be considered as waste product and

be discarded, which causing an increasing concern of farming waste (Dortmans et al., 2017).

In fact, although these waste residues to be discarded are lacking ability of bioconversion of waste, they can be a product of value as well. Besides of their high protein and fat residue content, Soetomans et al. (2020) stated that different parts of black soldier fly including prepupa, pupae, shedding, cocoons and adult flies are potential source of chitin. Chitin besides cellulose, belongs to the second most abundant types of polysaccharide in nature, which formed solely by N-acetylglucosamine monomers linked by β-1,4 covalent bonds, and is generally found in the cuticle of a variety sources including shrimps, crustaceans, fungi and insects to provide structural protection (Tan, 2021). And chitin can be derived to chitosan via deacetylation process, which is a copolymer polysaccharide consist of D-glucosamine and N-acetylglucosamine. Both chitin and chitosan offer useful properties such as biocompatibility, biodegradability, immunostimulant activity and non-toxicity, which make them a versatile material (Triunfo et al., 2022). And Pakizeh, Moradi and Ghassemi (2021) reported chitin and chitosan are widely applied in the fields of cosmetics, agriculture, food industry and wastewater treatments, with chitosan possess an extra application in pharmaceutical area than chitin. Therefore, these discardable BSF biowastes can be utilized as a potential source of chitin , as well as chitosan to make them comparative more valuable. And this project emphasized on the extraction of chitin and its deacetylation to chitosan from dead adult black soldier fly biowastes.

Currently, the solid wastes such as shrimp shell and crab shell from fishing industries are the major source of chitin, which contains an average chitin content of 15–40% (Kurita, 2006). However, this source is limited by seasonal availability and higher cost for production (Triunfo et al., 2022). Instead, another source of chitin, insects especially black soldier fly, offers high adaptability to the environment, and is not limited by seasonality. Thus it is having higher availability than the fishery waste, making it a potential source of chitin and chitosan (Triunfo et al., 2022). Recent studies have reported chitin could be extracted from all stages of black soldier fly, however there is very limited information regarding the suitability of chitosan produced from black soldier fly to be used for commercial purpose. Since the application of chitosan can vary with different properties, it is important to determine whether the final chitosan extracted from black soldier fly could possess similar properties as those commercial available chitosan mainly produced from crustacean sources. Therefore, extraction of chitin and chitosan from the dead adult black soldier fly bio-wastes, and characterization of the extracted chitosan was performed in this project to determine whether the BSF discardable wastes could obtain a significant amount of high quality chitin and chitosan that shows promising potential similar to the commercial ones.

1.2 Objectives

The purpose of conducting this project including:

1. To isolate chitin and determine chitin content of the dead adult black soldier fly bio-wastes.

2. To convert isolated chitin into chitosan using different levels of deacetylation processes.
3. To compare the structure and degree of deacetylation of the produced chitosan with a commercial available chitosan and a pharmaceutical grade chitosan.

CHAPTER 2

LITERATURE REVIEW

2.1 Black soldier fly (*Hermetia illucens*)

Black soldier fly (BSF), scientifically named as *Hermetia illucens*, is a true fly (Diptera) in Stratiomyidae family. Despite being originated in Americas. It can now be found in tropical and temperate regions all over the world (Wang and Shelomi, 2017). BSF is known as a holometabolous type of insect which undergoes a complete metamorphosis throughout its life cycle (Zimri, 2018). It consists of five development stages, including eggs, larvae, prepupa, pupa and adult flies. Black soldier fly has been reported to be used in organic waste management, especially the larvae stage which is the only stage involves feeding activity, due to their ability to decompose a wide variety of organic waste including food waste, kitchen waste, dried distillers' grains, rice straw, fish offal and many others (Wang and Shelomi, 2017; Shumo et al., 2019) during feeding, and can convert them into high quality nutrients for animal feeding, thus reducing the waste and pollution to the environment. The variety types of organic waste the larvae BSF can be fed on suggest them as polyphagous, and the high efficiency having by them to decompose those waste making black soldier fly superior in any other known species of insects (Kim et al., 2011).

The adult black soldier fly does not feed on since it loses its mouthpart as the digestive organ during the growth transformation after the larvae stage (Dortmans et al., 2017). It rarely encounters humans, does not consider as a

vector to transmit any specific diseases, thus BSF is considered as a non-pestilent fly (Wang and Shelomi, 2017). The adult BSF is transformed from pupa stage after undergoing the pupation process. It only lives for about one week after emerging. In adult stage, BSF does not feed, it only requires water, humid environment and warm temperature of 25–32ºC to ensure a successful reproduction before dying. Since only the larvae BSF is fed on, protein and fat content are accumulated from their diet as the main nutrient resource in this stage to provide essential energy for the pupation process as well as the reproduction needs (Dortmans et al., 2017), which allowed the subsequent stages including prepupa, pupa and the adult flies to have a great amount of nutrients as well. And as compare with other insects such as carrion and house flies with shorter development times, the larvae BSF has longer development times of over three weeks, which means a larger amount of nutrients will be accumulated in the larvae BSF, to produce larger pupae, as well as a larger adult fly (Wang and Shelomi, 2017). The consumption of larger amount of substrate suggested a higher nutrient in terms of protein and fat content that a black soldier fly contains, making it an alternative source of animal feed or human food source. The production of black soldier fly requires a humid environment with a relative humidity of 60–90% to prevent dehydration (Zimri, 2018). And the feeding source to the larvae BSF should contain a moisture content of 60–90% to ease the ingestion (Dortmans et al., 2017). These lead the BSF itself to have high moisture content as well. In terms of composition, all stages of BSF have relatively high fat and protein content, in a wide range of 30–58% and 4.8–32% respectively (Liu et al., 2017; Triunfo et al., 2022), in which the variation of

protein and fat content are largely dependent on the organic source that the larvae feeds on.

2.2 Chitin

Chitin is a homopolymer consist of N-acetyl-D-glucosamine monomers bounded by β-1,4 covalent bonds, and is the second abundant forms of polysaccharide exist in the nature besides cellulose. It is a structural component that is mainly presented in the exoskeleton and the shells of crustaceans and shrimps, the cuticle of insects and the cell walls of microorganisms including fungi, yeasts and algae (Komi and Hamblin, 2016). Chitin possesses a lot of biological properties including biorenewability, biocompatibility, biodegradability, environmental friendly and non-toxicity, these beneficial properties allow chitin to be used as chelating agent, wound-healing agents and also applied widely in the fields of medicine, agriculture, cosmetics, textile industries and wastewater treatment (Younes et al., 2014; Zargar, Asghari and Dashti, 2015). Chitin is highly hydrophobic and is practically insoluble in water and inorganic solvents such as diluted acids which is due to the high intra and intermolecular hydrogen bonding that leads to the rigid crystalline structure of chitin. As reported by Pakizeh, Moradi and Ghassemi (2021), chitin is only dissolved in inorganic acids that is highly concentrated, such as hydrochloric acid, phosphoric acid, sulfuric acid, dimethylformamide and lithium chloride. Hence, the solubility limitation causes it to be rarely applied in pharmaceutical industry, instead, it is often further derived into chitosan, which has even more versatile properties than chitin (Zargar, Asghari and Dashti, 2015).

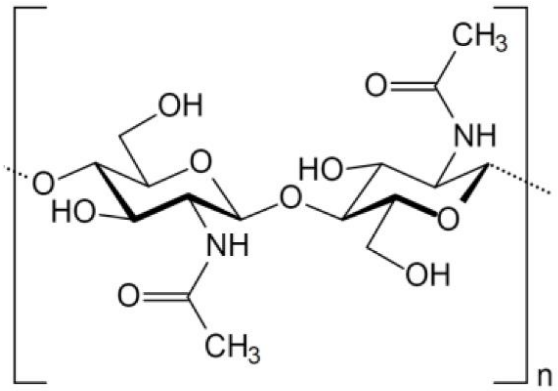

Figure 2.1: Chemical structure of chitin (Younes and Rinaudo, 2015)

Chitin can be classified to three polymorphic forms: α-form, β-form, and γ-form. These forms are differentiated according to their structural arrangement which the α, β and γ-forms of chitin are corresponding to anti-parallel, parallel and alternated alignments which is a combination of α and β arrangement structure, respectively (Knidri et al., 2018) as shown in Figure 2.2.2. α-chitin is the most common and stable form that is usually obtained from the crustacean and insect exoskeleton including cuticles of black soldier fly which requires structural rigidity to protect them. The antiparallel arrangement in α-chitin results in high density and strongly packing of hydrogen bonds, thus generate the high rigid structure. Instead, β-chitin which the polymer chains are arranged in parallel orientations, gives a lower packing of inter-sheet hydrogen bonds and thus a reactive and crystalline structure with lower rigidity that is easier to be destructed (Pakizeh, Moradi and Ghassemi, 2021). β-chitin is less common in nature and can be extracted from squid pens. And γ-chitin which is much rarer than both α and β forms can be isolated from fungi and yeast.

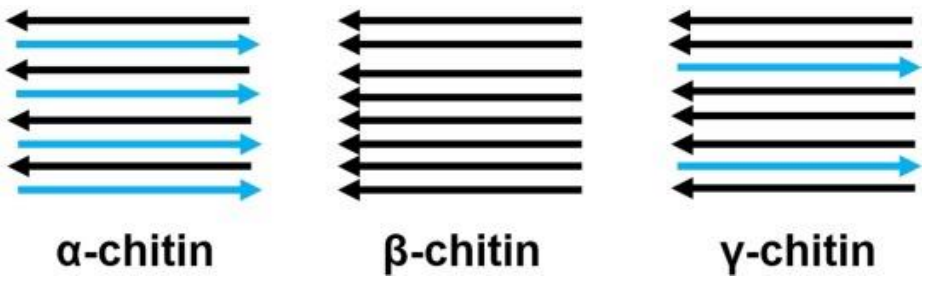

Figure 2.2: Polymorphic forms of α, β and γ-chitin (Knidri et al., 2018)

Nowadays, the major sources commercially chitin production are the shells, cuticles and the exoskeleton of crustaceans, mainly crabs and shrimps (Kamal et al., 2020). And recent studies have reported a numbers of species of insect to be used as chitin source as well. The chitin content of insects is mainly located in their cuticle. Since the body structure including the cuticle will change along with the development of insect stages, the chitin content contained in insects will be different with different stages of development (Zimri, 2018).

2.3 Chitin extraction

In crustaceans sources, the chitin is not found solely on their rigid shell, instead, it generally formed a complex network with proteins, and deposited with minerals, mainly the calcium carbonate as a mixed constituents contribute to the whole shells (Younes and Rinaudo, 2015). On the other hand, the chitin found in cuticles of insects is associated with large amount of proteins, lipids, minerals as well as some deep color pigment lead to their body color (Zimri, 2018). Therefore, chitin extraction from crustaceans requires two necessary steps, demineralization to remove inorganic minerals, deproteinization to remove proteins, and additional step of decolorization to remove those residue impurities and pigments. Whereas the insect chitin extraction especially black soldier fly used in this project requires an extra defatting steps to remove the high lipid

content, followed by the general demineralization and deproteinization as well as decolorization to produce pure chitin. Several studies have outlined the use of chemical and biological methods to isolate chitin (Zlotko et al., 2021). Biological methods often include the use of microorganisms to produce proteolytic enzyme-protease for deproteinization, and fermentation of bacteria to produce organic acid for demineralization (Arbia et al., 2013), which is environmental friendly, low cost and able to preserve the structure of chitin to a better extend. However, it is time-consuming and produces low chitin yields with low purity (Hahn et al., 2020). This indicates the efficiency of biological methods is lower as compare with chemical methods, with 5–10% of relatively higher protein residues remain in the isolated chitin after deproteinization (Younes and Rinaudo, 2015). Thus chemical methods which involves the use of reagents that offers higher efficiency as well as higher purity of chitin production (Kim and Park, 2015) is currently most applied on the chitin extraction from crustacean shells as well as insect such as BSF.

2.3.1 Sequence of chemical extraction

The sequence of demineralization and deproteinization can affect the yield of extracted chitin when using chemical extraction. According to Lertsutthiwong et al. (2002), the yield of chitin from shrimp shells was 20–27% when demineralization was carried out first before deproteinization. While the yield dropped to 15–20% when extraction of chitin started with deproteinization. The lower yield in cases where deproteinization was carried out before demineralization, was due to the removal of protein that serve as a protective layer to the chitin during deproteinization, causing the chitin to be exposed to

HCl in unprotected condition during demineralization later on. As a result, the inorganic material and solid fraction of chitin was subjected to unwanted, excessive hydrolysis, which experienced degradation as well as larger loss of solid material, leading to lower yield of chitin (Lertsutthiwong et al., 2002). Thus, when considering the extraction of chitin with higher yield, demineralization was done before deproteinization, which was the most frequently used sequence for recent studies.

2.3.2 Demineralization

Demineralization is the process of removing minerals from the cuticle of BSF. This can be achieved with the use of wide variety of acid reagents, including hydrochloric acid, nitric acid and sulfuric acid that belong to strong acid, and formic acid and acetic acid that belong to mild organic acid reagents, of which dilute hydrochloric acid has been researched as the most preferred reagent for both crustacean shells and insect cuticles (Abidin et al., 2020). Demineralization of BSF involves the decomposition of minerals by acid treatment into water-soluble salt and carbon dioxide, which the salts can be easily separated from chitin by washing and filtration (Hahn et al., 2020). The demineralization can be affected by the minerals content of shell, acid concentration, treatment duration and temperature, and the solute-to-solvent ratio (Younes and Rinaudo, 2015). In fact, the solute-to-solvent ratio depends on the acid concentration, this is because the minerals content in insect exoskeleton is mainly consist of calcium carbonate, and one molecule of calcium carbonate requires two molecules of HCl to be converted to calcium chloride, water and carbon dioxide according to the following equation:

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

Thus, the addition of acid should be at least two times greater than the amount of minerals in order to achieve a complete reaction.

Abidin et al. (2020) reported a HCl concentration of 0.25–4 M and the process temperature and time of 21–100ºC and 0.25–48 h respectively, are commonly used parameters for demineralization. Insects usually contain lower mineral content in the range of 2–10% as compare with crustacean shells that contain up to 50% (Kurita, 2006). Therefore, normally a milder condition is enough to complete the demineralization of insect. Recent studies have completed the demineralization of varies insects with varies methods. When focusing on the black soldier fly insect, only Smets et al. (2020) reported the use of high temperature (100ºC) along with 0.5 h of 1 M HCl treatment to carry out demineralization of larvae, prepupa and pupa BSF. On the other hand, other studies perform this step at room temperature, of which Soetemans, Uyttebroek and Bastiaens (2020) applied 1 M HCl at a solute-to-solvent ratio of 1:10 for 1 h under room temperature condition, Khayrova, Lopatin and Varlamov (2019) used 1% HCl at a ratio of 1:10 at 20ºC for 2 h. And Antonov et al. (2019) suggested the used of 5% HCl at room temperature for 2 h to achieve demineralization of adult BSF.

Besides the frequently used of HCl, there are only a few research that have conducted the demineralization using organic acid that is milder and more environmental friendly than HCl. Triunfo et al. (2022) demineralize the BSF samples by using 0.5 M formic acid for 1 h at room temperature. And Hahn et al

(2020) applied the same parameters using 0.5 M formic acid for the demineralization of BSF larvae exoskeleton. There is only reported one case for the use of formic acid was observed a similar demineralization efficiency as compare with HCl (Hahn et al., 2020). Since no adequate information for the overall efficiency achieved by organic acid demineralization, HCl is still the preferred reagent for most of the sources.

2.3.3 Deproteinization

Deproteinization is the process of removing protein content, thus purify the extracted chitin. Alkaline solutions such as sodium hydroxide, calcium hydroxide and potassium hydroxide were recently reported as effective deproteinization reagents (Younes and Rinaudo, 2015), in which sodium hydroxide (NaOH) is the most preferred reagent that is widely used for both crustacean and insect sources, often applied with concentration of 0.025–4 M at 25–150ºC, and duration ranging from 20 min to 96 h to complete the deproteinization (Abidin et al., 2020). In case of black soldier fly sample, normally a high temperature with low concentration of NaOH in the range of 0.5–2 M is applied. Truenfo et al. (2022) deproteinized the BSF biomasses using 2 h of 2 M NaOH treatment at 80ºC. Zlotko et al. (2021) and Smets et al. (2020) used 1 M NaOH at 80ºC with a duration of 24 h to achieve deproteinization of pupal exuviae BSF. Whereas Antonov et al. (2019) suggested the use of high temperature that reached 100ºC, together with 1.25 M of NaOH for 3 h to deproteinize the dead adult BSF. However, there are a few studies conducted deproteinization of insect under low temperature. Caligiani et al. (2018) treated the prepupae BSF with 4% NaOH at 40ºC for 1 h. Besides that, 2 M NaOH at

50ºC, and a reaction time of 18 h was applied for deproteinization of adult grasshoppers (Erdogan and Kaya, 2016). Although the use of high temperature for deproteinization is important for the solvent to penetrate and remove the protein, thus giving high deproteinization efficiency, it can possibly result in an undesirable chitin deacetylation and biopolymer hydrolysis which cause a lower molecular weight (Younes and Rinaudo, 2015). Instead, recent studies have suggested the use of lower temperature that is able to achieve the deproteinization of insect, including black soldier fly to a great extend as well, which consider low temperature as a possibly better processing to higher yield and better quality of chitin.

2.3.4 De-coloration

Despite being an additional step for chitin extracted from crustacean shells, decolorization is necessary for insect chitin to remove their common brown to dark color. The coloration of insect cuticle can be related to the pigments originated during the tanning process and cuticular sclerotization such as melanins and dopamine (Sugumaran, 2009). After demineralization and deproteinization of black soldier fly, a small amount of pigment and impurities could be removed, since the appearance of extracted chitin showed a brownish colour, which differs greatly with the white color as observed in commercial chitin. Thus, a de-coloration is required to remove the brown color of chitin. Besides that, According to Triunfo et al. (2022), the obtained chitosan from decolorized chitin had a more desire appearance which was whiter as compare with the chitosan from unbleached chitin. Nemtsev et al. (2004) reported the chitosan yield from bleached chitin of honeybee corpses was 16–25% which was

lower than the yield from unbleached chitin (20–30%). The difference in yield of chitosan from unbleached and bleached chitin might be explained by the presence of catechol compounds in unbleached chitin that cross-linked to the α-chitin, and hide the acetyl groups of chitin, thus hinder the access of NaOH from deacetylating the chitin (Truenfo et al. 2022). This observation suggests that de-coloration has significant effect on the appearance and yield of chitosan as well.

Recent studies have reported the use of sodium hypochlorite, acetone and hydrogen peroxide as decolorizing reagents. Ibitoye et al. (2018) applied 1% sodium hypochlorite solution in a solute-to-solvent ratio of 1:200 at room temperature for 3 h and repeated twice to decolorize the house cricket. On the other hand, Zimri (2018) applied a onetime treatment with high concentration of sodium hypochlorite (3%) for 3 h at room temperature for the decolorization of adult and pupal shells BSF. Furthermore, Triunfo et al. applied the use of 5% hydrogen peroxide at 90ºC for 2 h for adult, larvae and pupal exuviae BSF. Several studies reported the use of methanol-chloroform mixture to perform insect decolorization. However, organic solvents (methanol and chloroform) have little or no bleaching efficiency which is not able to break the complex bonds of chitin with tannins, melanins or catecholamines (Hahn et al., 2020). Thus, sodium hypochlorite and hydrogen peroxide are the preferred decolorizing reagents for insect, especially black soldier fly. There is no standard method for decolorization, and currently no study has compared the decolorization methods (Pakizeh, Moradi and Ghassemi, 2021). It depends on the raw materials used as well as the examination of extend of removing the brown color.

2.4 Chitosan

Chitosan is produced from the deacetylation process of chitin which involves the removal of acetyl groups from chitin monomers. Through the removal of acetyl groups, chitosan formed as a heteropolymer that consist of N-acetyl-glucosamine and glucosamine monomers arranged in a linear polymer with semi-crystals structure (Ibrahim and Zairy, 2014). The difference between chitin and chitosan is mainly indicated by the presence of amino group. Chitosan has a high degree of free amino groups (NH_2) which served as an active site for the chemical reactions to take place (Hahn et al., 2020). Besides that, the presence of amino groups at C-2 positions allows chitosan to have higher solubility as compare with chitin. The protonated NH_2 groups gives a pKa value of approximately 6.5 which allows the chitosan to dissolve in diluted acidic aqueous solutions (pH <6) (Aranaz et al., 2021). And the protonated amino groups make chitosan as the only positively charge polysaccharide present in nature, which differ it from other polysaccharides including chitin. After deacetylation, the primary amino group (NH_2) in C2 position as well as the primary and secondary OH groups at C6 and C3 positions, allow chitosan to be subjected to different modifications to have different properties and behaviors (Aranaz et al., 2021), thus implies chitosan to have more widely application as compare with chitin.

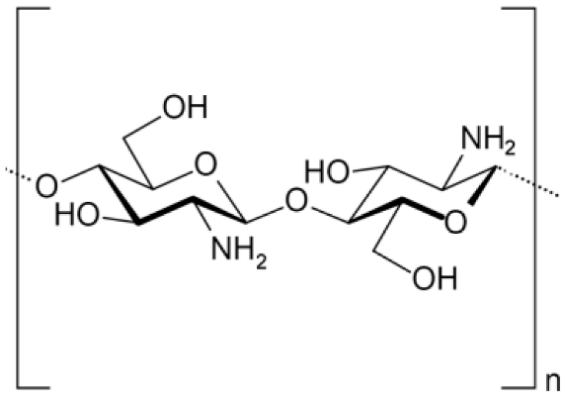

Figure 2.3: Chemical structure of chitosan (Younes and Rinaudo, 2015).

Pakizeh, Moradi and Ghassemi (2021) reported a wide application of chitosan in pharmaceutical and biomedical fields including antitumeric, hemostatic, immune assistance, anti-cholesteremic, analgesic effect and central nervous system depressant. Besides that, chitosan has a superior antimicrobial and antifungal activity, which is explained by its positive charges of amino groups that could bind with the negative charges carried by the surface of bacterial cell, to prevent cell permeability, as well as its ability to inhibit microbial RNA synthesis due to the binding of amino groups with the DNA cell (Croisier and Jerome, 2013). Several studies reported the antioxidant properties of chitosan credited to its scavenging ability via the reaction with free radicals. All of these applications rely on the positive charge amino groups present in chitosan, this highlights the importance of further conversion of chitin to chitosan.

2.5 Deacetylation

Deacetylation involves removing the acetyl groups from chitin to produce chitosan (Vicente et al., 2021). Recent studies had reported the use of chemical and enzymatic deacetylation (Ilyina, Tatarinova and Varlamov, 1999) to convert

chitin to chitosan. Enzymatic deacetylation involves the use of enzyme chitin deacetylases to catabolize the hydrolysis of N-acetylglucosamine bonds in chitin to obtain chitosan, although this method offers lesser degradable process due to its milder hydrolysis treatment (Tsigos et al., 2000), similar to the limitation for biological chitin extraction, it was not able to achieve an efficient deacetylation due to the high crystallinity of chitin (Wattjes et al., 2019). In this case, enzymatic deacetylation was difficult to achieve a high level of deacetylation degree. Therefore, chemical deacetylation was applied extensively for the current industrial scale of commercial chitosan production, not only due to its high efficiency, but also its lower cost requirement and the suitability to mass production (Hahn et al., 2020). Chemical deacetylation can be performed with the use of acids or alkaline solution, but typically strong acid solution was not commonly used, because of the high susceptibility of glycosidic bonds between N-acetylglucosamine towards the strong acid solution (Younes and Rinaudo, 2015). Thus, concentrated sodium hydroxide was the major and most commonly used deacetylation reagent.

The alkaline deacetylation consists of heterogeneous and homogeneous processes, which result in chitosan with different characteristics (Hahn et al., 2020). For instance, homogeneous deacetylation involves treating chitin with concentrated sodium hydroxide at room temperature for few hours, then it was allowed to dissolve in 0ºC of crushed ice. The chitosan produced from homogeneous deacetylation often shows a lower degree of deacetylation of 45-52% with acetyl groups distributed uniformly along the chitosan chains (Younes and Rinaudo, 2015). In contrast, the heterogeneous deacetylation which applies

the use of concentrated NaOH in hot temperature treatment for few hours, is able to produce a highly deacetylated chitosan with degree of deacetylation of around 85-99%, possessed irregularly distributed N-acetylglucosamine and glucosamine units, as well as acetyl groups with blockwise distribution along with the main polymer chain of chitosan molecular arrangement (Aiba, 1991). Recent studies reported the similar deacetylation parameters for both crustacean and insect sources. This was related to the same chitin form present in these two sources which was α-chitin (Zimri,2018). Badawy and Mohamed (2015) had completed the extraction from six different insect species and the study stated that these insect chitins showed structural arrangement and properties, similar to those of the commercial shrimp chitin, indicated the possibility of insect to be an alternative chitin and chitosan sources. The similar structure and the α-form suggested the deacetylation parameter require to break the chitin bonds for insect and crustacean could be similar as well.

Most of the studies reported the application of chemical heterogeneous deacetylation for the production of chitosan from insect as well as crustacean sources (Pakizeh, Moradi and Ghassemi, 2021; Hahn et al., 2020), because heterogeneous deacetylation could produce chitosan with degree of deacetylation up to 80–85%, which consider a characteristic for high solubility of chitosan, and this cannot be reached with homogeneous deacetylation. The deacetylation degree of chitosan from crustacean and insect obtained through heterogeneous deacetylation were similar, which are in the range of 56–98% and 62–98% respectively. Based on the study of Hahn et al. (2020), the heterogeneous deacetylation parameters for insect often apply the use of

concentrated NaOH in the range of 40–60%, temperature of 90–150ºC and duration of 1–9 h. And recent studies regarding the chitosan production from crustacean sources applied the similar range of parameters as well. Table 2.1 shows some heterogeneous deacetylation process for the production of chitosan from crustacean and insect which had been done by other studies.

Table 2.1: Heterogeneous deacetylation parameters for chitosan production from shrimp shells and insect reported in previous studies.

Source	Condition ; Obtained degree of deacetylation (DDA)	Reference
Shrimp shells	50% NaOH at 135ºC for 3.5 h; DDA = 90%	(Kurita, Kamiya & Ishimuraa, 1991)
Shrimp shells	50% NaOH at 100ºC for 5-6 h; DDA = 76%	(Ocloo et al., 2011)
Shrimp shells	50% NaOH at 90-100ºC for 3-5 h; DDA =80%	(Benhabiles et al., 2012)
BSF larvae	50% NaOH at 100ºC for 2 h; DDA = 90%	(Khayrova, Lopatin and Varlamov, 2019)
Mayfly adult	60% NaOH at 150ºC for 6 h; DDA = 84.3%	(Tan et al., 2018)
BSF adult	48% NaOH at 100ºC for 4 h; DDA = 93%	(Triunfo et al., 2022)

While there were some exception studies showed that lower temperature than 90ºC could achieve the degree deacetylation range as well. For instance, Trung et al. (2020) produced chitosan from shrimp shell chitin using 50% NaOH at

65ºC for 12 h and obtained the DDA of 85%. Besides that, another study reported the chitosan from horsefly larvae produced using 60% NaOH at 70ºC for 8 h obtained DDA of 90.3% (Ai et al., 2008). These suggested the use of lower temperature heterogeneous deacetylation could possibly result in high deacetylation degree of chitosan from insect as well, which could be a better treatment when applied on insect such as black soldier fly that consist of melanin pigment capable of binding to the deacetylated chitosan through high temperature to give an undesirable color (Khayrova, Loapatin and Varlamov, 2020; Islam, Khan and Alam, 2016).

2.6 Characterization of chitosan

Characterization of chitosan is an important section prior to justify the identity of a substance as a chitosan, as well as to determine its properties related to different physical and biological application (Yuan et al., 2011). Typically, the quality of produced chitosan in terms of its deacetylation degree as well as purity could be analyzed with the use of Fourier Transform Infrared Spectroscopy (FTIR). The spectrum of FTIR obtained from the analysis will observe peak at different frequency based on the analyzed range, which is often between 4000 to 400 cm^{-1} for the chitin and chitosan samples (Varma and Vasudevan, 2020). These peak represented as different functional groups, which can be used to access the purity and the identity of chitosan. Chitosan consists of protonated amino groups (NH_2) after the deacetylation of chitin, which is responsible for its extensive application (Aranaz et al., 2021), this could be indicated as some specify characteristic peak for the FTIR analysis of chitosan. Besides that, degree of deacetylation could be used as chitosan indicator. Degree of deacetylation

(DDA) is a measurement of the molar fraction of glucosamine in the chitosan that consist of N-acetylglucosamine and glucosamine monomers (Jiang et al., 2017). High DDA indicates higher chitosan content. Recent studies had reported the relationship between DDA to the solubility of chitosan, which is the major properties for its suitability in pharmaceutical used that chitin could not be directly applied. Younes and Rinaudo (2015) reported a product with DDA of higher than 50% could be identified as chitosan, which is due to its increasing protonated amino groups that allow it to become soluble (Aranaz et al., 2021). Thus, the solubility in aqueous solution such as water and the diluted acid solution which chitin could not dissolve in (Pakizeh, Moradi and Ghassemi, 2021), serve as another characteristic for chitosan.

CHAPTER 3

MATERIALS & METHODS

3.1 Materials

3.1.1 Raw sample

The raw material used in this project, the bio-wastes of black soldier fly (*Hermetia illucens*) were collected from BioLoop Sdn Bhd (Sendirian Berhad), a biotechnology company located at Teluk Intan, Perak. The dead adult and exoskeleton of black soldier fly were separated from the bio-wastes, and the packed dead adult bio-wastes were stored at a freezer at -4ºC prior to be analyzed in this research.

3.1.2 Chemical reagents

The chemical reagents used in this project and their brand were listed in Table 3.1.

Table 3.1: List of chemicals used and the brand.

Chemicals	**Brand**
Acetic Acid Glacial	Bendosen
Acetone	Chemiz
Acetylacetone	R&M Chemicals
Boric Acid	ChemSoln
Bromocresol Green	Bendosen
Copper Sulfate	QReC™

D-Glucosamine Hydrochloride	Alfa Aesar
Di-methylaminobenzaldehyde (DMAB)	Merck
Ethanol (95%)	Rank Synergy
Hydrochloric Acid (37%)	Fisher Scientific
Methanol (99.8%)	ChemSoln
Methyl Red	Bendosen
Petroleum Ether (40-60°C)	Chemiz
Phenolphthalein	R & M Chemicals
Phosphoric Acid	QReC™
Potassium Bromide	Fisher Scientific
Potassium di-Hydrogen Phosphate	Bendosen
Potassium Sulfate	SYNERLAB
Sodium Carbonate	Chemiz
Sodium Hydroxide	SYNERLAB
Sodium Hypochlorite	ChemSoln
Sodium Nitrite	Nacalai Tesque
Sulfuric Acid (98%)	Fisher Scientific
2-Thiobarbituric Acid	Tokyo Chemical Industry

3.1.3 Instruments

The instruments and equipment used in this research were listed in Table 3.2.

Table 3.2: List of instruments/equipment used and their model and brand.

Instruments	**Brand / Model**

Centrifuge Machine	MIKRO 22 R
Distillation Unit	BUCHI / K-355
Drying Oven	Binder
Grinder	NIPPON / NBL-C501SS
Hot Plate	Thermo Scientific
Moisture Analyzer	A&D / MX-50
Scrubber	BUCHI / K-415
Shaking Incubator	INFORS / 230 VAC
Sieve Shaker	Retsch / AS200 basic
Soxtherm	Gerhardt / SE416
Spectrophotometer	Thermo Scientific / G10S UV-Vis
Speed Digester	BUCHI
Vacuum Pump	GAST / DOA-P504-BN
Water Bath	Gerhardt / SE-416
Weighing Balance	METTLER TOLEDO / ME1002
FTIR Instrument	Perkin Elmer / Spectrum RX 1

3.2 Overview of the design of experiments

This project consists of 9 experiments. First, the BSF samples were prepared properly, then was determined for moisture content. The BSF samples were then defatted, meanwhile determined for their fat content, and proceeded to protein determination. After composition determination was done, the sample was subjected to chitin isolation, then the obtained chitin yield was determined. The chitin was then undergone chemical deacetylation to produce chitosan I and II. After determined the yield, chitosan I and II was determined for their chitosan

content, then was subjected to FTIR analysis to identify their chitosan structure and determine degree of deacetylation, meanwhile to compare with the commercial and pharmaceutical chitosan.

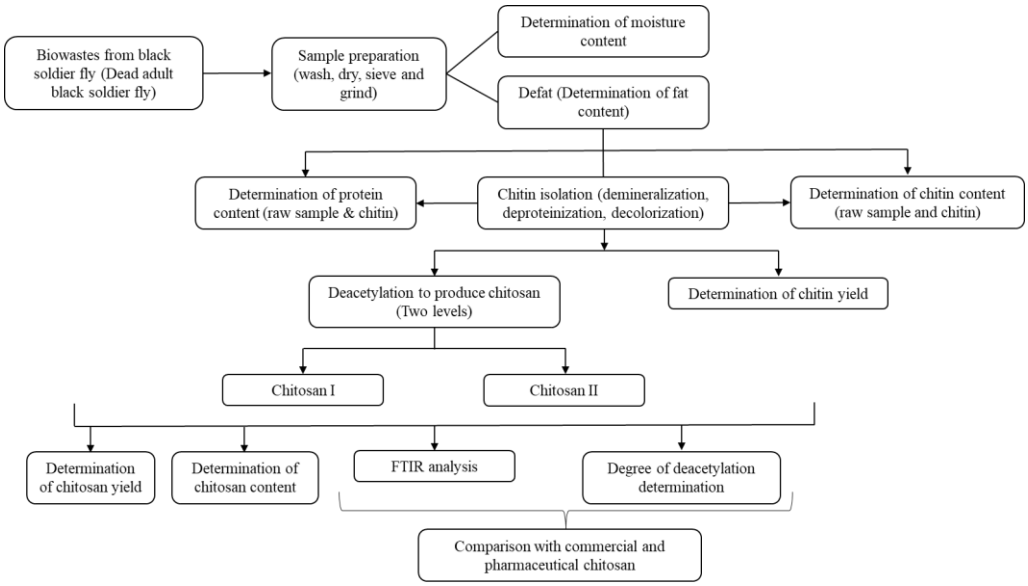

Figure 3.1 Overview of the experimental design in this project.

3.3 Sample preparation

Approximately 100 g of dead adult black soldier fly bio-wastes were weighed in an aluminum tray using a weighing balance. The weighed samples were transferred to a kitchen sieve and washed several times using tap water until clean. The washed samples were then transferred back to the aluminum tray and were allowed to dry at 70°C for 2 days using a drying oven. After drying, the samples were sieved using a sieve shaker, then was grinded into small particle powder form using a grinder, and the prepared samples were stored in a Scott bottle prior to further analysis.

3.4 Determination of moisture content

The moisture content of the sample was measured in triplicate using a moisture analyzer. The moisture analyzer was pre-set to Mid accuracy which target for 5 g of samples, drying temperature of 105°C and the moisture content was set to display as moist/d (dry) in percentage. After this, about 5 g of sample was placed evenly on the sample pan in the moisture analyzer, the lid of the moisture analyzer was closed, and "START" button was pressed to start the analyzer. Then the moisture content (%) displayed on the screen of moisture analyzer after the end of analysis was recorded.

3.5 Determination of fat content

3.5.1 Preparation of extraction beaker & sample

The extraction beakers were added with 3 pieces of boiling stones, and was dried in a drying oven for 1 h at 105°C, then were cooled down in a desiccator for 1 h. After this, the extraction beakers were weighed using an analytical balance. For the preparation of sample, approximately 5 g of sample was weighed on a piece of 150 mm filter paper. The weighed sample in the filter paper was wrapped, and was then inserted to the bottom of a thimble. A piece of cotton wool was inserted to cover on top of the sample, then the thimble with sample was put into a thimble holder and was inserted into the extraction beaker. After this, 90 mL of petroleum ether (boiling point of 40–60°C) was poured into the extraction beaker in a fume hood.

3.5.2 Sample extraction

Before extraction using the fat analyzer, the system solvent storage tank was checked on to make sure it was not full. Then the fat analyzer was switched on based on these steps:

Turn on main power > pump > tap water > computer

After this, the extraction beakers were placed into the fat analyzer, and Soxtherm Manager on desktop was selected. “PETROLEUMETHER” parameter was selected and checked as correct. Then “Run” tab was clicked to start the fat extraction.

3.5.3 After extraction

After the extraction has completed, the extraction beakers were taken out and put into the insert rack with the aid of a clamp, and the remaining and recovered solvent was collected from the solvent storage tank. The thimble holder together with thimble was removed in a fume hood. And the sample was removed from the thimble and collected as the defatted sample for subsequent experiments. The extraction beakers with oil were dried at 105°C for 1 h using a drying oven, then cooled in a desiccator for 1 h. After this, the extraction beakers with oil were weighed, and the percentage of fat content of sample was determined using the following equation:

$$\left[\frac{(\text{Extraction beaker} + \text{boiling stone} + \text{oil weight}) - (\text{Extraction beaker} + \text{boiling stone weight})}{\text{Original sample weight}}\right] \times 100\%$$

3.6 Determination of protein content

3.6.1 Digestion

The scrubber and the Speed Digester were switched on, the preheating temperature was set to level 8, and the Speed Digester was heated up for 10–15 min. Approximately 2 g of the BSF sample was weighed in a sample tube, and the sample weight and position of the tube in the rack were recorded. A catalyst (7 g potassium sulfate + 0.8 g copper sulfate) was added to the sample, then 20 mL of 98% sulfuric acid was added to sample in a fume hood. A "Blank" that contain only sulfuric acid without sample was prepared. The sample tubes were inserted into the rack, then installed into the instrument, then the digestion process was started. The digestion was ended when the sample turned from black to blue or green colour clear solution, then the rack was removed from the instrument and cooled down for 1 h. After that, the Speed Digester and the scrubber were switched off.

3.6.2 Distillation

The Distillation Unit was switched on and preheated. A conical flask with a volume of 250 mL was prepared as the receiving vessel for each sample tube. Each conical flask was filled with 25 mL of 4% boric acid as the receiving solution and 5 drops of Bromocresol green indicator was added into the boric acid. After a preheating step of a distillation unit, the sample tube and the conical flask were inserted into the distillation unit and receiving vessel compartment respectively. Forty mililiters of water followed by 60 mL of 32% sodium hydroxide were added to the sample tube, and the distillation was started. The

sample was distilled until the solution colour in the receiving vessel changed from slightly pink to blue. After that, the solution inside the sample tube was thrown inside the sink with running tap water, and the distillation unit was cleaned prior to next distillation.

3.6.3 Titration

The boric acid solution of each distilled sample was titrated with 0.25 M sulfuric acid (or 0.1 M hydrochloric acid). In this project, the distillate from the digesta of the prepared black soldier fly was titrated using 0.25 M sulfuric acid, while the distillate from the digesta of the extracted chitin was titrated using 0.1 M hydrochloric acid. The titration was stopped when the blue colour of boric acid mixture turned back to pink colour. After this, the volume of acid required to reach the end point was recorded, and the percentage of protein of sample was calculated using the following equation, which the nitrogen to protein conversion factor of 4.67 was used for black soldier fly sample (Janssen et al., 2017):

$$\frac{\text{mL acid (sample} - \text{blank)} \times \text{Normality of acid } \times 14.0 \times 4.67 \times 100}{\text{sample weight } \times 1000}$$

The protein content of dead adult black soldier fly and chitin were measured in triplicate samples.

3.7 Chitin isolation

The extraction of chitin from dead adult black soldier fly in this project was done by using chemical methods which offered high efficiency, high purity as well as high yield as described by Kim and Park (2015). Isolation of chitin consists of

demineralization, deproteinization and decolorization processes. The experiment was done in triplicate samples.

3.7.1 Demineralization

Demineralization of dead adult black soldier fly was performed using the method provided by Antonov et al. (2019). About 3 g of sample was weighed into a conical flask with a volume of 50 mL, then 5% of hydrochloric acid was added to the sample-containing flask at a solid to liquid ratio of 1:4 (w/v). The reaction was carried out at constant stirring with a magnetic stirrer, under room temperature for 2 h. After this, the solution containing the precipitate was filled with distilled water and washed for several times until the water turned to clear. Then the solution was removed, and the precipitate inside the conical flask was dried at 60°C for 24 h prior to the deproteinization process.

3.7.2 Deproteinization

Chemical deproteinization of demineralized sample was carried out using the parameters provided by Pakizeh, Moradi and Ghassemi (2021). The hydrochloric acid-treated samples were treated with 2 M sodium hydroxide at a solid to liquid ratio of 1:4 (w/v). The deproteinization process was carried out at 45°C and 100 rpm constant stirring for 4 h using a shaking incubator. After 4 h of alkaline treatment, the suspension was washed several times with distilled water until a clear solution was obtained. Then the precipitates were filtered through a 1 µM filter paper (70 mm) using a vacuum pump. Following this, the obtained samples were dried at 60°C for 24 h prior to the decolorization process.

3.7.3 Decolorization

The decolorization of extracted chitin was performed based on the procedure provided by Ibitoye et al. (2018) with slight modifications. The samples were treated with 3% of sodium hypochlorite solution at a solid to liquid ratio of 1:200 (w/v), and the reaction was done at room temperature and constant stirring for 3 h. After this, the samples were filtered with 1 µM filter paper (70 mm) using a vacuum pump. The whole decolorization process was carried out once more until the chitin samples were fully decolorized. Then, the decolorized samples were dried at 60°C for 24 h, and the chitin weight was recorded.

3.7.4 Chitin yield

The yield of chitin extracted from dead adult black soldier fly in percentage was calculated according to the following equation:

$$\text{Chitin yield (\%)} = m_2/m_1 \times 100$$

where m_1 is the weight of dead adult black soldier fly sample and m_2 is the weight of extracted chitin.

3.8 Determination of chitin content

The chitin content determination was carried out according to the method provided by Wai (2018).

3.8.1 Preparation of reagents and chemicals

The reagents and chemicals required in this experiment including D-glucosamine hydrochloride, 2 M hydrochloric acid, 1 M sodium hydroxide, 1% potassium di-hydrogen phosphate, 0.5% phenolphthalein, 4% acetyl acetone

solution and Ehrlich reagent. The D-glucosamine hydrochloride, 2 M hydrochloric acid, 1 M sodium hydroxide, 1% potassium di-hydrogen phosphate were prepared by using distilled water as the diluent. Phenolphthalein (0.5%, v/v) was prepared using 95% ethanol, acetyl acetone solution (4%, v/v) was prepared by dissolving in 1.25N of sodium carbonate solution. For the preparation of Ehrlich reagent, 2.5 g of di-methylaminobenzaldehyde (DMAB) was dissolved in 50 mL of methanol, then 50 mL of concentrated phosphoric acid was added to the mixture and placed on an ice bath.

3.8.2 Conversion of chitin to glucosamine

Approximately 0.1 g of dried sample was weighed in a test tube and was digested with 5 mL of hydrochloric acid (2 M) by boiling the mixture on a hot plate for 2 h. A control without sample was prepared without boiling as well. After that, the tubes were briefly cooled by placing at room temperature, and 1 mL of the supernatant was transferred into a new tube using a micropipette, then mixed with 2 drops of 0.5% phenolphthalein. 1 M sodium hydroxide was added gradually until the mixture turn to pink colour. Next, 1% potassium di-hydrogen phosphate was added to the mixture until the pink colour disappear. The mixture was topped up to 5 mL using distilled water, and 3 mL of supernatant was transferred to a new tube.

3.8.3 Preparation of standard curve using D-glucosamine hydrochloride

Ten miligrams of D-glucosamine hydrochloride was dissolved in 10 mL of distilled water for the preparation of 1 mg/mL stock solution. Then 0.02, 0.04, 0.06, 0.08 and 0.1 mL of the stock solution were pipetted to the tubes

respectively to construct a calibration curve with different concentration of glucosamine solution in a range of 20, 40, 60, 80, and 100 µg. After that, the solutions were topped up to 3 mL by using distilled water.

3.8.4 Determination of glucosamine content

An aliquote of 1 mL acetyl acetone solution (4%) was added to the supernatant (sample) or D-glucosamine solution, the mixture was boiled for 20 min for glucosamine condensation. After briefly cooled the tubes at room temperature, 6 mL of 95% ethanol, followed by 1 mL of Ehrlich reagent were added to the mixture. Then the tubes were incubated in a water bath at 65°C for 15 min. The solution was cooled to room temperature, and the absorbance was measured at 530 nm against a reagent blank (control) using a spectrophotometer. After this, the glucosamine concentration of the sample was determined from the equation obtained from the standard curve.

3.8.5 Calculation of chitin content

According to Kasongo et al. (2020), chitin content was calculated from glucosamine content by substituting the obtained glucosamine content to the following equation: $M = m \times 203/179$, where M is the chitin content, m is the determined mass of glucosamine, 203 is the molecular weight of acetylglucosamine anhydride, and 179 is the molecular weight of glucosamine.

3.9 Deacetylation

Deacetylation is the process of converting chitin into chitosan. In this project, alkaline treatment was performed for deacetylation of chitin. Two types of

deacetylation conditions were used to produce two levels of chitosan. Triplicate of the assay was done for each level of chitosan production.

3.9.1 Production of chitosan I and chitosan II

The deacetylation of chitin to chitosan was conducted based on the procedure of Marei et al. (2016) with different parameters to obtain two products, namely chitosan I and chitosan II. For the production of chitosan I, the extracted chitin was added with 12.5 M of sodium hydroxide with a solid to liquid ratio of 1:15 (w/v), at 80°C and 150 rpm of constant stirring, using a shaking incubator for 15 h. For chitosan II, chitin was treated with 12.5 M of sodium hydroxide with a ratio of 1:15 (w/v), at 50°C and 150 rpm of constant stirring, using a shaking incubator for 6 h.

3.9.2 Chitosan collection

At the end of the deacetylation process, both chitosan products were recovered using the same method as described by Yean (2016) with slight modifications. First, the pH of chitosan-contained solution was adjusted to around 7.0 with 5 M of hydrochloric acid solution. After this, the chitosan was precipitated by adding 95% of ethanol with a sample to reagent ratio of 1:1.5 (v/v). The precipitate was then separated through centrifugation at 4°C and 5000 rpm for 15 min. Following this, the solution was removed, and the chitosan pellet was washed with 10 mL of 70% ethanol for three times, then subsequently with 10 mL of 95% ethanol. The collected chitosan was dried in an oven at 55°C for 24 h. Then the weight of chitosan was recorded.

3.9.3 Determination of chitosan yield

The yield of chitosan deacetylated from isolated chitin of adult black soldier fly wastes in percentage was calculated in chitin-based and raw sample-based according to the equation as followed:

Chitosan yield (%, based on chitin) = $m_4/m_3 \times 100$

Chitosan yield (%, based on sample) = $m_4/m_5 \times 100$

where m_4 is the weight of obtained chitosan, m_3 is the weight of extracted chitin, and m_5 is the weight of dead adult black soldier fly sample.

3.10 FTIR analysis & degree of deacetylation determination

Chitosan I and II, a commercial chitosan from crab shell and pharmaceutical used chitosan were characterized using Fourier Transform Infrared Spectroscopy. Using FTIR, the degree of deacetylation was determined based on the equation provided by William and Wid (2019). First, the obtained chitosan was grinded into powder form, then was added with dried potassium bromide (KBr) and mixed well by using a mortar pestle. Following this, the sample-potassium bromide mixture was pressed into a disc by applying hydraulic pressure for few seconds. After that, the disc was inserted into the FTIR instrument, and the FTIR spectra of the chitosan was analysed from 400 to 4000 cm^{-1}. The obtained FTIR spectra was recorded, and the degree of deacetylation was determined based on the equations as followed:

$$\mathrm{DA}(\%) = \left[\left(\frac{A_{1320}}{A_{1420}}\right) - 0.3822\right]/0.03133$$

$$\mathrm{DDA}(\%) = 100 - \mathrm{DA}(\%)$$

Where DA is the degree of acetylation (%), A_{1320} is the absorbance at peak area for band 1320 cm^{-1}, A_{1420} is the absorbance at peak area for band 1420 cm^{-1} and DDA is the degree of deacetylation (%).

3.11 Determination of chitosan content

The chitosan content of obtained chitosan in this project was determined using the spectrophotometric method done by Badawy (2012) with slight modifications. The experiment was done in triplicate samples.

3.11.1 Preparation of reagents

The reagents and chemicals used in this experiment were 1% aqueous acetic acid, 0.5 M of sodium nitrite, 0.1 M sodium hydroxide and 0.04 M of 2-Thiobarbituric acid solution, in which all the reagents were prepared by dissolving in distilled water. The prepared 0.5 M sodium nitrite should be kept between 0 to 4°C. For the preparation of 2-Thiobarbituric acid (0.04 M), 0.576 g of 2-Thiobarbituric acid was dissolved in 100 mL of distilled under warming conditions for 15 min.

3.11.2 Preparation of standard curve

First, 1 mg/mL of chitosan stock solution was prepared by dissolving 0.1 g of commercial chitosan sample in 100 mL of acetic acid solution (1%, v/v). Then 0.01, 0.02, 0.03, 0.04, 0.05 and 0.06 mL of stock solution was pipetted into tubes respectively to make chitosan standard solutions with different amount of chitosan in a range of 10 to 60 µg. Each tube was then topped up to 1 mL using distilled water.

3.11.3 Procedure

First, the sample solution was prepared by dissolving 0.1 g of BSF – derived chitosan sample in 100 mL of acetic acid solution (1%, v/v). An aliquot of 0.05 mL was pipetted into a tube, and was topped up to 1 mL using distilled water. Then 0.2 mL of 0.5 M sodium nitrite was added for deamination of chitosan, and the mixture was mixed manually. The depolymerization-deamination reaction was carried out by incubating the tubes at 80°C for 30 min in a water bath. After that, 0.2 mL of sodium hydroxide was added to the tubes and shaken properly to raise the pH to 8, followed by the addition of 0.5 mL of 0.04 M thiobarbituric acid with a briefly shaking. The tubes were then incubated at 80°C for 10 min. Consequently, the solution was briefly cooled, and the absorbance at 555 nm was measured against a blank (control with distilled water and other reagents without sample) by using a spectrophotometer. The chitosan content of the chitosan sample was then determined using equation obtained from the standard curve.

3.12 Statistical analysis

All results were reported as mean ± standard deviation. Except the determination of degree of deacetylation from FTIR, all the experiments were done in triplicate. T-test was performed for the assays of chitosan yield and chitosan content determination to find out whether significant difference between chitosan I and chitosan II exist. The significant difference was indicated by *p* value less than 0.05 at ($p < 0.05$) level.

CHAPTER 4

RESULTS

4.1. Determination of moisture content

Table 4.1 shows the moisture content of raw sample (dead adult black soldier fly) and prepared sample (after washed, dried, sieved and grinded). The moisture content of prepared sample was applied in calculating the assays in dry weight basis.

Table 4.1 Moisture content of raw and prepared dead adult black solider fly.

Sample	Moisture content (%)
Raw sample	58.69 ± 1.38
Prepared sample	2.49 ± 0.09

Values represent mean ± SD (n=3).

4.2. Determination of fat content

Table 4.2 shows the crude fat content of dead adult black soldier fly bio-wastes in dry weight basis. The fat content was calculated as percentage.

Table 4.2 Crude fat content (dry weight basis) of dead adult black soldier fly.

Sample	Fat content (%)
Dead adult black soldier fly	12.24 ± 0.20

Values represent mean ± SD (n=3).

4.3 Determination of protein content

Table 4.3 shows the crude protein content of dead adult black soldier fly bio-wastes and extracted chitin in dry weight basis, which the low protein content of extracted chitin showed a high efficiency of deproteinization process during chitin extraction. The protein content was calculated as percentage.

Table 4.3 Crude protein content (dry weight basis) of dead adult black soldier fly and extracted chitin.

Sample	Protein content (%)
Dead adult black soldier fly	64.64 ± 0.26
Extracted chitin	1.91 ± 0.04

Values represent mean ± SD (n=3).

4.4 Chitin isolation

4.4.1 Demineralization

Table 4.4 shows the yield of precipitate in dry weight basis after chemical demineralization using HCl and dried overnight. The yield was calculated as percentage.

Table 4.4: The % yield of precipitate (dry weight basis) after demineralization with HCl.

Sample	Yield (%)
Residue after demineralization	30.80 ± 1.35

Values represent mean ± SD (n=3).

4.4.2 Deproteinization

Table 4.5 shows the yield of chitin in dry weight basis after deproteinization using NaOH. The yield was calculated as percentage based on the mass of dead adult black soldier fly without demineralization.

Table 4.5: The % yield of extracted chitin (dry weight basis) after deproteinization with NaOH.

Sample	Yield (%)
Extracted chitin	10.44 ± 1.39

Values represent mean ± SD (n=3).

4.4.3 Decolorization

Table 4.6 shows the yield of chitin in dry weight basis after decolorization. The yield was calculated as percentage based on the mass of dead adult black soldier fly. Figure 4.1 shows the extracted chitin from dead adult black soldier fly before and after decolorization. Chitin appears black color before decolorization, while change to white color after bleaching.

Table 4.6: The % yield (dry weight basis) of extracted chitin after decolorization process.

Sample	Yield (%)
Extracted chitin	5.70 ± 0.11

Values represent mean ± SD (n=3).

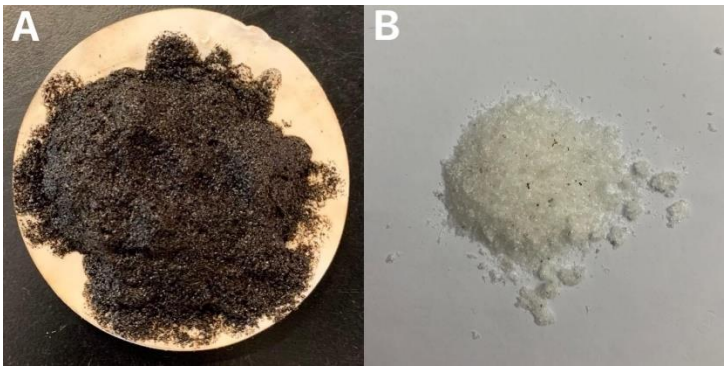

Figure 4.1: Extracted chitin from dead adult black soldier fly (A) before decolorization and (B) after decolorization.

4.5 Determination of chitin content

Figure 4.2 shows the standard curve of glucosamine at different concentrations (0 – 100 µg). Glucosamine is the hydrolysed form obtained after the acid hydrolysis of N-acetylglucosamine which is the chitin monomer. The obtained R-squared value was 0.9894 which is higher than 0.95 and close to 1, indicated that the obtained data was fit to a straight line and the generated equation of standard curve was highly reliable to be used for the determination of glucosamine content, as well as to further estimate the chitin content.

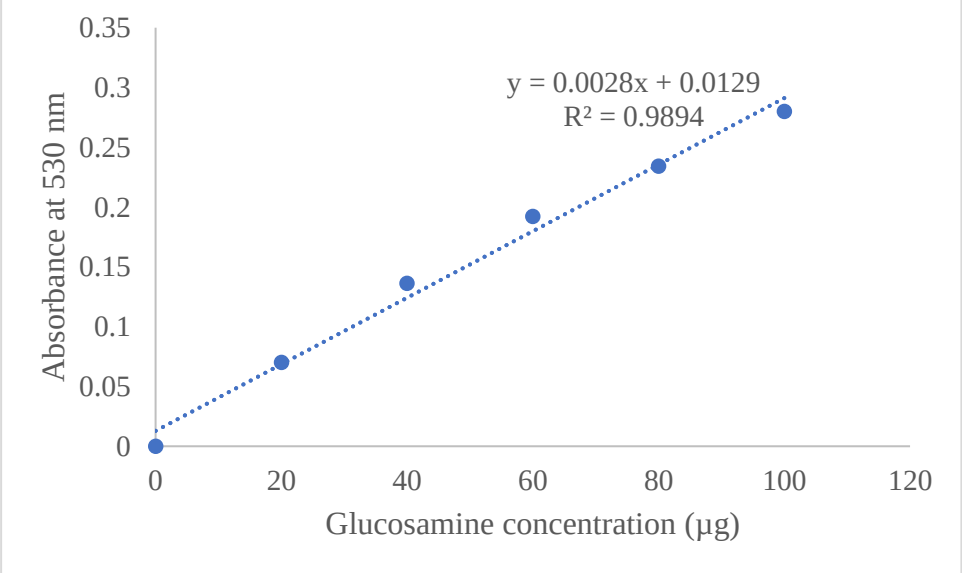

Figure 4.2: Standard curve for glucosamine content determination.

Table 4.7 shows the chitin content of dead adult black soldier fly bio-wastes and extracted chitin in dry weight basis, which was determined from the obtained glucosamine content. The chitin content was calculated as mg/g which was mg of chitin content in 1 g of sample.

Table 4.7: Chitin content (dry weight basis) of dead adult black soldier fly and extracted chitin.

Sample	Chitin content (mg/g)
Dead adult black soldier fly	10.88 ± 0.18
Isolated chitin	36.52 ± 1.08

Values represent mean ± SD (n=3).

4.6 Deacetylation to produce chitosan

4.6.1 Appearance of produced chitosan I and II

Figure 4.3 shows the appearance in terms of color of chitosan I (deacetylated with 12.5 M NaOH at 80°C for 15 h) and chitosan II (deacetylated with 12.5 M NaOH at 50°C for 6 h). Different deacetylation resulted in different colors of chitosan which chitosan I gives a dark brown color while chitosan II displays a bright yellow brown color.

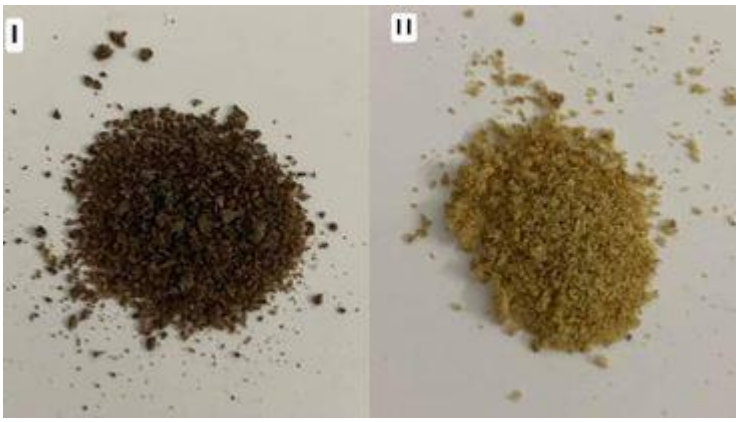

Figure 4.3: (I) Chitosan I and (II) chitosan II produced from isolated chitin of dead adult black soldier fly using different deacetylation processes.

4.6.2 Yield of produced chitosan I and II

Table 4.8 shows the yield of chitosan I and chitosan II produced by the deacetylation of chitin. The yield was calculated as percentage based on the mass of extracted chitin and the mass of dead adult black soldier fly respectively. There was no significant difference ($p > 0.05$) for the yield of chitosan I and II.

Table 4.8: The % yield of extracted chitosan I and chitosan II after different levels of chitin deacetylation.

Sample	**Yield (%)**	
	Chitin based	**Dead adult black soldier fly based**
Chitosan I	66.39 ± 2.75	3.78 ± 0.16
Chitosan II	66.42 ± 4.37	3.79 ± 0.25

Values represent mean ± SD (n=3).

4.7 FTIR analysis

Figure 4.4 shows the FTIR spectra of chitosan I (deacetylated with 12.5 M NaOH at 80°C for 15 h) and chitosan II (deacetylated with 12.5 M NaOH at 50°C for 6 h) as compare with the pharmaceutical chitosan. Figure 4.5 illustrates the FTIR

spectra of commercial chitosan. Table 4.9 shows the functional group identified from the peak from each frequency detected in chitosan I and II, pharmaceutical and commercial chitosan. The band at 3436 to 3460 cm^{-1} represents the O-H stretching (Varma and Vasudevan, 2020), the bands at 2917–2928 and 2850 cm^{-1} were corresponding to the asymmetric and symmetric CH_2 stretching, respectively (Murillo et al., 2017). C=O stretching of amide I is indicated by the band at 1637 cm^{-1} (Queiroz et al., 2015). 1420 cm^{-1} shows the band of CH_2 stretching bending (Ardila et al., 2017). The peaks at 1381–1383 cm^{-1} that close to ~1375 cm^{-1} indicates the presence of CH bending CH_3 symmetric deformation (Ibitoye et al., 2018). The bands at 1260–1319 cm^{-1} represent the amide III band that showed in a range of 1250 to 1350 cm^{-1} (Ji et al., 2020). The bands at ~1153 cm^{-1} (1153–1161 cm^{-1}) correspond to C-O-C asymmetric stretching (Queiroz et al., 2015). The bands at 1073–1119 and 1025–1031 cm^{-1} which were near to the range of 1066–1100 and 1028–1040 cm^{-1} confirms the presence of C-O stretching (Queiroz et al., 2015, Chai and Isa, 2013). And the characteristic band formed at 876–900 cm^{-1} represents the C-H bending (Queiroz et al., 2015). Based on Table 4.7, only pharmaceutical chitosan showed the peak at 2850 and 1420 cm^{-1} correspond to CH_2 symmetric stretching and CH_2 stretching bending respectively, whereas chitosan I and II and commercial chitosan observed the peak at ~1381 cm^{-1} (C-H bending and CH_3 symmetric deformation) which was absence in pharmaceutical chitosan.

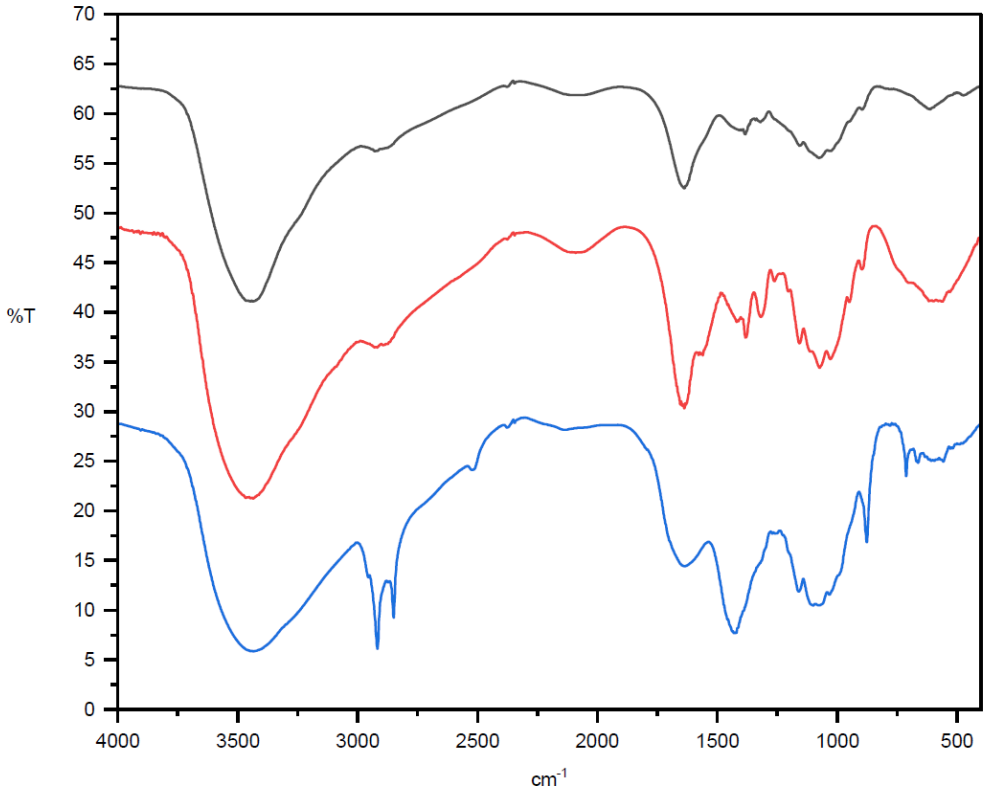

Figure 4.4: FTIR spectra of chitosan I (black), chitosan II (red) compared to the pharmaceutical chitosan (blue).

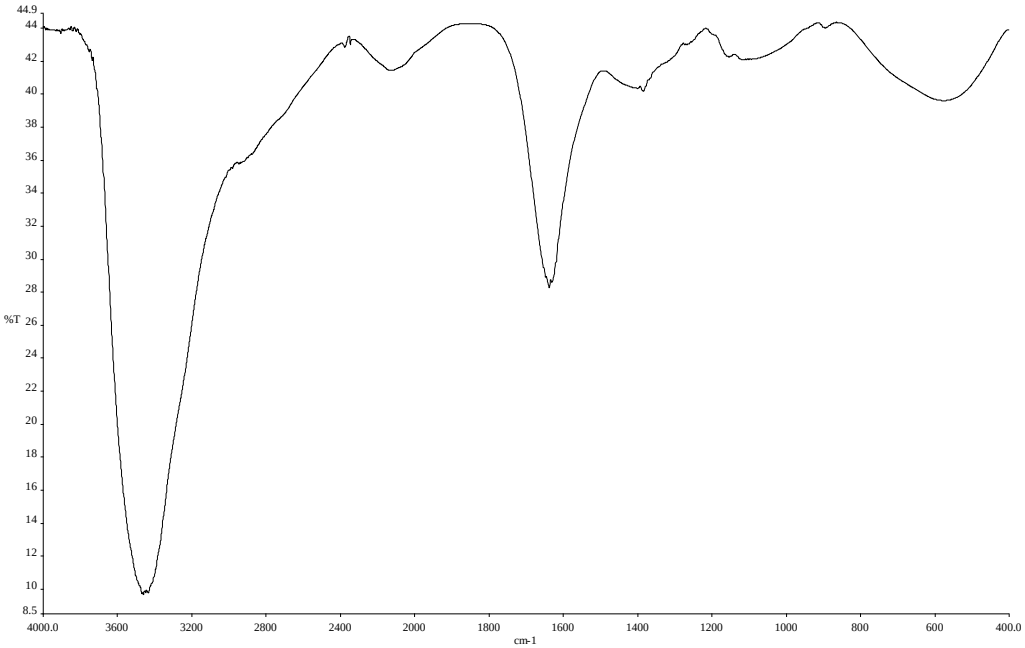

Figure 4.5: FTIR spectra of commercial chitosan.

Table 4.9: Represented functional groups for the peaks and their corresponding frequencies in chitosan I and II, pharmaceutical and commercial chitosan.

Represented functional group	Frequency (cm^{-1})			
	Chitosan I	**Chitosan II**	**Pharmaceutical chitosan**	**Commercial chitosan**
O-H stretching	3436	3436	3435	3460
CH_2 asymmetric stretching	2928	2924	2917	2928
CH_2 symmetric stretching	-	-	2850	-
Amide I (C=O stretching)	1637	1637	1637	1637
CH_2 stretching bending	-	-	1420	-
C-H bending CH_3 symmetric deformation	1383	1381	-	1383
Amide III	1315	1319	1260	1260
C-O-C asymmetric stretching	1155	1157	1161	1153
C-O stretching	1075,1025	1073, 1025	1100, 1031	1119
C-H bending	894	900	876	892

4.8 Degree of deacetylation determination

Table 4.10 shows the degree of deacetylation of chitosan I and chitosan II, pharmaceutical and commercial chitosan, in which the degree of deacetylation of pharmaceutical chitosan was the highest (86.60%), followed by commercial chitosan (81.76%), chitosan I and II were both lower than the former chitosan, which had the similar degree of deacetylation (80.90% and 80.70% respectively).

Table 4.10: Degree of deacetylation of chitosan I, chitosan II, pharmaceutical and commercial chitosan determined from FTIR.

Sample	Degree of deacetylation (%)
Chitosan I	80.90
Chitosan II	80.70
Pharmaceutical chitosan	86.60
Commercial chitosan	81.76

4.9 Determination of chitosan content

Figure 4.6 shows the standard curve of chitosan at different concentrations (20–60 µg). The obtained R-squared value was 0.9811 which was higher than 0.95 and close to 1, indicated that the obtained data was accurate and consistent to fit to a straight line and the generated equation of standard curve could be used for the determination of chitosan content.

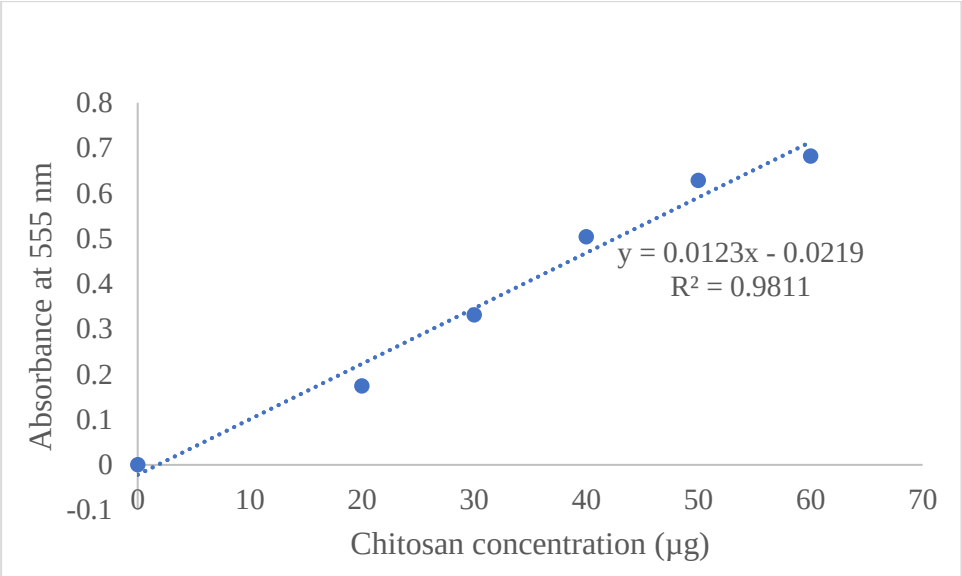

Figure 4.6: Standard curve for chitosan content determination.

Table 4.11 shows the chitosan content of chitosan I and chitosan II. The chitosan content was calculated as mg/g which was mg of chitosan in 1 g of sample. There was no significant difference ($p > 0.05$) between the chitosan content of chitosan I and II.

Table 4.11: Chitosan content of chitosan I and chitosan II determined by spectrophotometry method.

Sample	Chitosan content (mg/g)
Chitosan I	981.41 ± 85.57
Chitosan II	871.92 ± 67.68

Values represent mean ± SD (n=3).

CHAPTER 5

DISCUSSION

5.1 Moisture content of dead adult black soldier fly

The moisture content of raw and prepared dead adult black soldier fly bio-wastes were determined and shown in Table 4.1. The moisture content of dried sample was important to express the results obtained in the subsequent assays in dry weight basis, for comparison with most other recent studies. The moisture content of adult BSF was 58.69 ± 1.38%, which was similar as compared to the data provided by Holeh et al. (2022) who stated that prepupa of BSF had 44% of dry matter (56% moisture content), which followed the trend discovered by Liu et al. (2017) who mentioned that the prepupa stage of BSF had similar amount of dry mass than the adult BSF, which means that adult BSF would have higher moisture content compared with the prepupa.

One of the requirements for chitin extraction is that the moisture content of prepared sample must be lower than 5%. This is because high moisture content may alter the concentration of reagents used during the extraction assay. During deacetylation process of chitin, moisture content higher than 5% would reduce the concentration of NaOH, resulting in lower rate of removing the acetyl group from chitin, thus affect the quality of chitosan in terms of degree of deacetylation (Ahing and Wid, 2016). Since chemical method was applied to extract chitin in this project, the adult BSF should be processed properly to have moisture content less than 5% as well, to avoid changing the concentration of acid and alkaline

used which may affect the demineralization and deproteinization efficiency respectively, resulting in the production of low quality chitin. After drying at 70ºC for 2 days, the moisture content in the residue sample was 2.49 ± 0.09%. This achieved the moisture content requirement which was below 5%. Tema Process (2022) stated that low temperature and slow dehydration process using oven drying method was suitable for drying BSF to avoid losing valuable nutrients and prevent unwanted sample loss and burning of BSF. This explained the use of lower temperature (70°C) instead of 105°C generally used for drying, to achieve slow dehydration of BSF (2 days) prior to preserve the original properties of the dead adult BSF to a great extend. Besides that, the obtained value was similar with the result reported by Zulkifli et al. (2022), in which the moisture content of larvae BSF processed using oven drying method at 50ºC for 36 h was 3.21 ± 0.03%. This finding supported the parameters used for oven drying in this project did not over-process the sample.

5.2 Fat and protein content of dead adult black soldier fly

The nutritional composition of dead adult black soldier fly bio-wastes, including fat and protein content, were 12.24 ± 0.20% and 64.64 ± 0.26% respectively. As compared with the result of postmortem adult BSF provided by Liu et al. (2017) which reported 21.6 ± 0.36% of crude fat content and 57.6 ± 0.26% of crude protein content, the results in this project showed a lower fat content and higher protein content. The fluctuation of nutritional composition might be due to different feed types and feeding rate during the development stage of BSF. Liu et al. (2017) stated that depending on the manure fed on, BSF would exhibit a variety range of crude fat and protein content. For example, the crude protein

content of BSF in the larvae stage was within the range of 34 to 42% when chicken feed was used. In contrast, it raised to 38–46.3% when the feed was changed to byproduct derived from food. Besides that, the research done by Chia et al. (2020) indicated that the fat content of larvae BSF varied in a range of 17.1 to 49.05% when it was reared with different feeds and supplement. Therefore, different protein and fat content from different feeding types may contribute to varied composition of BSF. Besides feeding source, nutritional composition of BSF also affected by processing method, in which the nutrients in BSF might experience loss during the drying process. Monisha and Loganathan (2021) reported that significant difference existed in crude protein (44.63% and 39.74%) and fat content (23.44% and 20.68%) of prepupa BSF when different processing methods (freeze drying and sun drying) were used. This supported that the severity of drying condition used might also be one of the reasons for the fluctuation of nutritional composition in this project as compare with other studies.

When compared with the larvae and prepupa stage of BSF, dead adult BSF showed higher protein content (64.64%) and lower fat content (12.24%), which larvae BSF recorded fat and protein content of 18–33% and 32–53% respectively (Lu et al., 2022), and prepupa BSF contained 20–37% and 32–44% of fat and protein content (Caligiani et al., 2018). These shows that the nutritional composition of black soldier fly varies with different stage of development as well. The high protein content of larvae and dead adult might be speculated as larvae stage demonstrates high protein and fat catabolism prior to the completeness of growth while the adult stage requires protein and fat for

reproduction purpose. A higher protein content in adult indicates the energy requirement for reproduction might be higher than metamorphosis (Triunfo et al., 2022). While the highest fat content of the prepupa BSF might be due to energy requirement to support high level of metabolism to undergo metamorphosis into the next development stage (Zulkifli et al., 2022). Overall, the results obtained in this project could support dead adult BSF as the best protein source among all the development stages based on its highest protein content.

5.3 Chitin isolation

The isolation of chitin from dead adult black soldier fly biowastes was performed using chemical demineralization and deproteinization. Instead of using biological extraction which requires strict condition in terms of pH and temperature for protease enzyme to induce the deproteinization reaction, chemical extraction offers higher efficiency as well as higher purity of chitin production which was important for the subsequent chitosan production (Kim and Park, 2015). And the sequence of chitin extraction step used in this project was demineralization followed by deproteinization which had proved to result in higher yield as compare with deproteinization before demineralization (Lertsutthiwong et al., 2002).

5.3.1 Recovery from demineralization

The biomass recovery of dead adult black soldier fly after demineralization, as shown in Table 4.4 was 30.80 ± 1.35%, and was greatly lower than the result of approximately 68% obtained from Zimri (2018) and Triunfo et al. (2022). Most

studies including Zimri (2018) filtered the demineralized residue using a vacuum pump filtration, which almost all of the residues could be collected on the filter paper. In current work, due to machine and time limitation, instead of applying the vacuum pump, the residues were simply washed by adding distilled water, then allowed to precipitate before pouring the supernatant into sink. This step was repeated several times until the residue was cleaned. A significant amount of sample was loss through pouring, resulting in such low mass recovery.

5.3.2 Decolorization

The yield of extracted chitin from dead adult black soldier fly in dry weight basis after chemical demineralization and deproteinization was 10.44 ± 1.39%, which was similar to the result reported by Triunfo et al. (2022) that showed a chitin yield of 9 ± 0.4% from dead adults BSF (7% moisture content). After decolorization, the yield further decreased from 10.44 to 5.70%, which was now similar with the chitin yield results of adult BSF reported by Zimri (2018) in a range of 4.5 to 5.4%. Decolorization, also known as bleaching, is the process of removing impurities, excess minerals which were not remove during the demineralization step, and pigment such as melanin that adhered naturally to the chitin via complex bonds, resulting in a higher purity of extracted chitin with bright white color. The decrease in yield before and after bleaching process indicated decolorization as an indispensable stage besides demineralization and deproteinization prior to complete the chitin extraction, since it avoids those non-chitin substances to be accounted as chitin. Therefore, the yield of bleached chitin was more reflective of the true yield. Triunfo et al. (2022) reported a drop of yield from 9 to 6% after decolorization as well. Figure 4.1 shows the color

change of extracted chitin from black to white after decolorization, which the white color was more related to the appearance of commercial chitin. These results stresses the importance of determining chitin yield after decolorization.

5.3.3 Yield of extracted chitin

As compare with the research done by Zimri (2018) which reported the yield of extracted chitin from adult BSF in a range of 4.5 to 5.4% using different parameters of chemical extraction. the chitin yield in this project (5.70%) was similar. Zimri (2018) discovered a trend that decreased duration, temperature and concentration of chemical would result in a higher chitin yield. With similar condition being used for demineralization, the duration and temperature used during deproteinization in this project was 4 h and 45ºC, which were both milder than the condition used by Zimri (2018) in a great extent (10–12 h, 85–100ºC). And according to Table 4.3, the protein content of unbleached extracted chitin was 1.91 ± 0.04%, showing a high deproteinization efficiency. This suggested milder condition was better for chitin extraction from black soldier fly sample, in fact offering the same high efficiency of demineralization and deproteinization processes coupled with high yield and shorter experimental period.

While the yield exhibited a large difference when comparing between different biomasses of black soldier fly, Triunfo et al. (2022) reported the chitin yield of dead adult stage (6%) was the lowest, compared with 10% from larvae and 23% from pupal exuviae. Besides that, according to the results provided by Soetemans et al. (2020), among five life stages of black soldier fly, the extracted

chitin yield of adult flies was the lowest (5.6%), followed by prepupa (9.1%), larvae (9.5%), pupae (10.3%), pupae cocoon (23.8%) and larvae shedding (31.1%). Therefore, besides the chemical extraction parameter and the types of chemicals used, the yield of chitin of black soldier fly varies significantly depending on the stages of development as well as the analyzed body part. And the data obtained in the mentioned studies and in this project reflected the dead adult of black soldier fly was not the best recommended chitin source when comparing with other body parts, instead, the larvae shedding and the exuviae of prepupa of BSF deliver the most promising chitin source.

5.4 Determination of chitin content

The chitin content of dead adult black soldier fly was determined based on glucosamine content using colorimetric method. Chitin is an abundant polysaccharide that consist of N-acetyl-D-glucosamine monomer units arranged as a linear polymer (Komi and Hamblin, 2016). Generally, it is difficult to determine the content of polymer due to its complex structure, thus, acid hydrolysis is the first step for determining the chitin content. The addition of acid can hydrolyse the covalent bonds between each N-acetylglucosamine units, cause the cleavage of glycoside bonds, and then remove acetyl group from the N-acetylglucosamine monomers (Wu, 2004). As a result, the chitin present in the sample was depolymerized and deacetylated into the end product, glucosamine. And the glucosamine content in acid hydrolysate can be quantified by using chromatography or colorimetric method then calculated back to chitin content. Katano et al. (2016) reported chromatography method was lack of sensitivity due to the effect of hydrolysate components on the retention

characteristic, thus colorimetric method is more suitable in glucosamine determination assay. The colorimetric method used in this project was applied with acetylacetone and Ehrlich reagent. After acid hydrolysis and neutralized using NaOH solution, the glucosamine residue was acetylated with acetylacetone to produce a pyrrol derivative, then react with Ehrlich reagent in ethanol in proper condition to give the end solution a pink (red violet) colour, then the absorbance of solution was read at 530 nm to quantify the glucosamine (Rosenlund, 1956).

The determined chitin content from dead adult black soldier fly was 10.88 ± 0.18 mg/g, which was 1.09%, and was extremely low as compared with the result reported by Soetemans, Uyttebroek and Bastiaens (2020) who obtained a chitin content of 8.4% from adult black soldier fly using glucosamine measurement. The possible reason for obtaining such low chitin content might be due to improper conditions of acid hydrolysis. Hondt et al. (2020) stated that acid hydrolysis using 6 M of HCl at 110ºC was the best condition for black soldier fly sample to convert chitin into glucosamine, and incomplete conversion would occur when milder conditions was used. Besides that, the research done by Katano et al. (2016) reported that the extend of chitin conversion increased with increased acid hydrolysis duration, where a significant amount of glucosamine was produced after 2 h and continued to increase until a constant level after 6 h. Hondt et al. (2020) mentioned that a duration of close to 10 h was optimum for the black soldier fly sample to complete the chitin conversion to glucosamine since degradation of monomers would be observed due to over hydrolysis treatment. This was supported by the result of Wu (2004) which showed an

increased glucosamine content from 5.36 to 7.00% as the hydrolysis time increase from 0.5 to 3 h and experienced a drop to 5.71% after 18 h of hydrolysis. Therefore, these studies indicated that the parameters for acid hydrolysis must be optimum to avoid either incomplete conversion or excessive hydrolysis. As compare with the mentioned studies, 2 M HCl and 2 h of hydrolysis time used in this project was too mild which only a small fraction of chitin content was converted into glucosamine. Absorbance at 530 nm is mainly for detection of pink compounds generated due to the interaction of glucosamine with acetylacetone then with Ehrlich reagent. In other word, only the converted glucosamine can contribute to pink compound that is responsible for the absorbance reading, and the rest of unpolymerized chitin and unacetylated monomers in the form of N-acetyl-D-glucosamine would not be quantified, resulting in lower calculated chitin content than expectation.

Apart from N-acetyl-D-glucosamine and glucosamine, chitin also consist of acetate monomer (Hondt et al. 2020). As it is difficult to ensure a complete conversion of all monomer units to glucosamine via acid hydrolysis, instead of using only glucosamine content, N-acetyl-D-glucosamine and acetate content should be taken account when calculating the chitin content. The low chitin content in this project reflected the sole determination of glucosamine content was not reflecting the actual chitin content from dead adult black soldier fly.

After chitin isolation, the chitin content of extracted chitin was determined, which was 36.52 ± 1.08 mg/g (3.65%). Although the chitin content of extracted chitin was about three times higher than the dead adult black soldier fly, which

indicated a successful extraction of chitin from raw sample, it was not logic since the obtained result indicated an extracted chitin only contained 3.65% of chitin content. This was still related to the condition of acid hydrolysis. According to Hondt et al. (2020), a more pure chitin should be treated with longer hydrolysis period since high purity will cause a delay in releasing of N-acetylglucosamine, resulting in slower conversion to glucosamine. Therefore, since the acid hydrolysis duration of extracted chitin in this project was same as the dead adult black soldier fly which was only 2 h, it would have even smaller fraction of glucosamine to be converted due to the delay of hydrolysis. Besides that, Wu (2004) reported that when reaching the best optimum hydrolysis time, the glucosamine content obtained from commercial chitin was only 68.66%, this meant that there was a significant amount of other monomers besides glucosamine that contribute to the chitin content. In contrast, the determined chitin content from commercial chitin using High Performance Liquid Chromatography (HPLC) obtained from Hondt et al (2020) was 92% when acid hydrolyzed for 6 to 10 h, and the calculation of chitin content included the determined mass of N-acetylglucosamine, glucosamine and free acetate. Thus, the obtained chitin content in this project would be lesser due to improper calculation which relied only on the glucosamine content. Apart from that, Khayrova, Loapatin and Varlamov (2020) stated that pure chitin cannot be obtained from the ontogenesis stages of black soldier fly including prepupa, pupa and adult, which was due to the naturally formation of melanin that bound strongly to the chitin and cannot be removed completely by decolorization. Therefore, the decolorized chitin might still contain with some chitin-melanin complex, serve as non-chitin substances present in chitin. Another possible

reason for the impractical chitin content of extracted chitin might be due to the concentration of solution that was too high. Katano et al. (2016) suggested 0.01 g of pure chitin sample to be used when applying colorimetric determination method, while 0.1 g of sample was used in this project. Since the extracted chitin sample was known to have very high glucosamine content, it would give an extreme intense of pink color, which might be too concentrated to be read by the spectrophotometer. As a result, the absorbance of extracted chitin at 530 nm in this project was more than 1.0 which shown the concentration might exceed the detectable range of glucosamine content, resulting in such low chitin content.

5.5 Deacetylation of chitin to chitosan I and II

The extracted chitin was undergone two levels of deacetylation processes with 12.5 M, 15 h, 80 ºC and 12.5M, 6 h, 50 ºC for production of chitosan I and II respectively.

5.5.1 Appearance of chitosan I and II

As shown in Figure 4.3, both chitosan I and II appeared as brown color which was different from the respective decolorized chitin with white color. This was similar with the result reported by Triunfo et al. (2022). This study related the browning from chitin to chitosan to the application of high temperature during the deacetylation reaction, in which the high temperature could cause saccharide dehydration, leading to the formation of double bonds, that meant the occur of chemical binding reaction. This was supported by the study of Islam, Khan and Alam (2016) which stated there might be chemical binding of pigments during heat treatment which led to a color changes. In this case, there might be some

pigment impurities which were not removed by the decolorization process. Khayrova, Lopatin and Varlamov (2021) reported the adult BSF contained melanin which was the pigment essential for its dark brown color. Melanin would bound to chitin to form a strong covalent bond, and this complex cannot be completely removed during the decolorization. And during deacetylation processes, the high temperature might cause the chemical binding of melanin with chitosan, and possibly some extra melanin formation which led to the regeneration of dark brown color. And the different intensity of brown color shown by chitosan I and II which gave dark brown and bright yellow brown color respectively, might because of the different temperature used which possibly led to different chitosan-melanin complex formation.

5.5.2 Yields of chitosan I and II

The yield of chitosan I and II determined from chitin was 66.39 ± 2.75 and 66.42 ± 4.37%, whereas the chitosan yield related to dead adult black soldier fly was 3.78 ± 0.16 and 3.79 ± 0.25, which there was no significant difference ($p > 0.05$) observed between both yield calculated based on dead adult BSF sample or based on chitin. This shows that 80 ˚C and 50 ˚C have similar deacetylation effect. When concentration and temperature were similar, long duration would not result in large different yield. This was supported by the experiment done by Tokatli and Demirdoven (2017) which obtained similar chitosan yield from shrimp waste of 76.88% and 76.38% when a large different of deacetylation duration was applied (1 h and 12 h) while obtained a different chitosan yield of 77.15 and 83.91% when only small change on the temperature (120 to 100ºC) was made. This shows that temperature had greater effect than the duration

during deacetylation process. The similar yield obtained from chitosan I and II might reflected the deacetylation levels used (15 h and 6 h, 80 and 50ºC) have the same extend of removing the acetyl groups from chitin, thereby showing no significant difference. Besides the deacetylation process, the yield of chitosan was affected by the sequence and parameters of chemical extraction of chitin before deacetylation. William and Wid (2019) reported that even using the same deacetylation condition, different chitosan yield of 19.01 and 22.22% was obtained when different sequence of chitin extraction was performed. When demineralization was carried out first, the yield of chitosan would be higher since it experienced lesser loss of solid material due to the protection of native protein chain that prevent chitosan from being over hydrolyzed. This suggested the sequence of chitin isolation used has more significant effect than deacetylation condition on the yield of chitosan. And the parameters of chitin extraction were also important to the contribution of chitosan yield because different levels of extraction could result in different demineralization and deproteinization efficiency, which indicated as the residue protein and mineral content present in the extracted chitin that may influenced the yield of chitosan. Therefore, the chitin used to deacetylate into chitosan I and II could be extracted using different sequence and parameters of demineralization and deproteinization to produce chitosan with different yield.

Chitosan I and II showed a similar yield (3.78 and 3.79%) with the study of Triunfo et al. (2022) which reported a chitosan yield of 3 ± 0.3% from dead adult BSF. This supported the conditions used in this project offered proper deacetylation effect, and meanwhile showed that when different deacetylation

parameters with similar or proper effect was applied, the yield of chitosan would not vary greatly among the same sources of sample. Instead, large different in the yields could be observed when comparing between the homogeneous and heterogeneous deacetylation. Hahn et al. (2020) reported when homogeneous deacetylation that refers to an application of room temperature treatment, the yield of chitosan from larvae exoskeleton might be 4 times lower than the heterogenous deacetylation (high temperature application). Besides that, the yield varies greatly as compared with other body parts and stages of black soldier fly as well. When considering the use of heterogeneous deacetylation and the yield based on the original BSF biomass, the chitosan yield from bleached larvae and pupal exuviae of BSF were 3% and 10% respectively (Triunfo et al., 2022), The study of Mirwandhono, Nasution and Yunilas (2022) showed the chitosan yield from prepupa of BSF was 10.85% whereas the larvae exoskeleton part of BSF showed a chitosan yield of 16% (Hahn et al., 2020). Apart from that, when comparing with the adult stage of different insect species, the chitosan yield from adult grasshopper was 5.7% (Hahn et al., 2020). Moreover, the study of Miranda et al. (2016) reported 8.1% from adult *Brachystola magna*, and Kim et al. (2017) reported 1.8% of chitosan yield from adult crickets, which were all quite differ from the chitosan yield of adult BSF. Furthermore, in terms of comparison among different sources, recent studies reported the chitosan yield obtained from insect samples was in the range of 2–8%, whereas ranged from 4–15% for the chitosan yields of crustaceans (Hahn et al., 2020). These reflected that the yield of chitosan was affected by the deacetylation method (heterogeneous or homogeneous), the development stages and body parts, the types of species and also the sources.

5.6 FTIR analysis

The FTIR spectra and the bands at their corresponding frequencies represented different functional groups of chitosan I and II, pharmaceutical and commercial chitosan were shown in Figure 4.7.1, Figure 4.7.2 and Table 4.7. The strong bands including O-H stretching (3435–3460 cm^{-1}), CH_2 asymmetric stretching (2917–2928 cm^{-1}), C-O stretching (1073–1119 cm^{-1} and 1025–1031 cm^{-1}) and C-H bending (876–900 cm^{-1}) representing the characteristic of polysaccharide structures (Queiroz et al., 2015, Cahu et al., 2012). These confirmed the identity of chitosan as an amino polysaccharide. As reported by Zimri (2018), the bands at ~1650 cm^{-1} and ~1590 cm^{-1} that correspond to C=O stretching (amide I) and NH_2 bending (amide II) were the characteristic of chitosan. All the four chitosan samples exhibited the C=O stretching (amide I) but were shown an absence in NH_2 bending (amide II) band, this might be due to the instrumental factor during detection which probably cause the characteristic NH_2 band to be overlapped by other bands. Instead, all the chitosan samples produced band between 1220 and 1020 cm^{-1}. Saraswathy et al. (2001) reported the characteristic bands between 1220 and 1020 cm^{-1} represented as free primary amino group (NH_2) that located at C_2 position of glucosamine, which glucosamine is the deacetylated monomers of chitosan, and this indicates successful deacetylation of chitin into chitosan in current work. Apart from the functional groups correspond to amide I and amide II , the band at ~1325 and ~1260 cm^{-1} which indicated as C-N stretching and NHCO group respectively represent amide III that can serve as another characteristic band for the chitosan (Queiroz et al., 2015, Ibitoye et al., 2018). Since chitosan I, II, pharmaceutical and commercial chitosan showed the amide III band (1315, 1319, 1260, 1260 cm^{-1} respectively), the FTIR analysis could

support the success conversion of chitin to chitosan I and II. When comparing among the chitosan samples, other than the peaks observed in all the chitosan samples, chitosan I and II and commercial chitosan were lack of the CH_2 symmetric stretching (2850 cm^{-1}) and CH_2 stretching bending (1420 cm^{-1}). Instead, they exhibited a peak at 1381–1383 cm^{-1} (C-H bending and CH_3 symmetric deformation) which was not observed in the pharmaceutical chitosan. This reflected that the production of chitosan I and II from dead adult black soldier fly was more similar with commercial chitosan than the pharmaceutical chitosan.

5.7 Degree of deacetylation determination

The degree of deacetylation of the chitosan I and chitosan II produced in this project, as well as the pharmaceutical and commercial chitosan that used as a comparison, were determined, which were 80.90, 80.70, 86.60 and 81.76%, representing high DDA value that resembled near-complete deacetylation. The DDA of chitosan I and II was almost the same, this reflected the use of deacetylation conditions for chitosan I and II which were 12.5 M NaOH, 80ºC, 15 h, and 12.5 M NaOH, 50ºC, 6 h respectively, offered the same effect of removing the acetyl groups. According to Hahn et al. (2020), in most cases, to produce chitosan with high degree of deacetylation, the required deacetylation condition including 40–60% of NaOH, 90–150ºC of temperature and 1–9 h of duration. As compare with that, only the concentration of NaOH was within the range, the temperature and duration used in this project were different from the range, which both temperature used were relatively milder. Among three parameters, time is having the least effect on deacetylation. When proper

concentration of NaOH and temperature was used, the deacetylation process could complete in a short time, the increase of reaction time could increase the extend of deacetylation, but only to a certain point and once reaching that end point, time will not have significant effect on the deacetylation process anymore. The same DDA obtained from both deacetylation process suggested 6 h is enough to reach the reaction end point. Further extending the deacetylation time beyond 6 h would not produce any significant increment of DDA. Besides that, the two levels of temperature used (80 and 50ºC) were both lower than the reported range (90–150ºC), however, the obtained DDA from chitosan I and II were within the range of 62–98% as reported by Hahn et al. (2020) as the DDA of insect chitosan produced with heterogeneous deacetylation. This suggested even the use of deacetylation temperature was lower than 90ºC which is typically the minimum temperature used for insect heterogeneous deacetylation, it was able to remove the acetyl groups from chitin of BSF to a great extent as well.

The similar degree of deacetylation obtained from chitosan I and II showed that 50ºC and 80ºC offered the same deacetylation efficiency. According to the study of Lertsutthiwong et al. (2002), when using low temperature which was 40ºC to carry out the deacetylation process, the DDA could only reach to about 80% when using a very long duration (more than 8 days), whereas the use of high temperature which was 90ºC, was able to obtain DDA of more than 85%. Current work shows that, when one-step deacetylation is performed, temperature and time parameters of 50ºC and 6 h is sufficient to obtain the maximum DDA which peaks around 80%. Further increment in temperature (80ºC) and time (15h) does not significantly improve the DDA. Lertsutthiwong et al. (2002) reported that

high DDA of more than 85% could be obtained with low temperature treatment when the process was carried out twice. Hence, in order to produce chitosan with higher DDA (>80 ºC), the deacetylation condition used for chitosan I could either change to a multi-step reaction or applied with higher temperature of more than 90ºC.

When comparing with commercial and pharmaceutical chitosan, DDA of commercial chitosan (81.76%) was similar with chitosan I and II, this shows that the chitosan from dead adult black soldier fly could potentially be used as commercial chitosan when produced using one-step low temperature deacetylation process. Whereas the DDA of pharmaceutical chitosan (86.60%) was higher than chitosan I and II, possibly indicated high temperature deacetylation of more than 90ºC was one of the requirement to produce pharmaceutical grade chitosan. This was supported by Lee et al. (2022) who reported a DDA of 85.71% of chitosan from larvae black soldier fly that was similar with 86.60% of pharmaceutical chitosan when using similar concentration of NaOH (55%) and time (12 h) but a higher temperature (95ºC) deacetylation condition as compare with this project.

The DDA of chitosan I and II were much lower than the data of dead adult black soldier reported by Triunfo et al. (2022), which was 93%. This was due to the higher liquid ratio and temperature used for the mentioned study. With similar NaOH concentration used, Triunfo et al. (2022) applied a chitin to NaOH ratio of 1:20 and temperature of 100 ºC which was both higher than the condition used in this project (solid: liquid ratio of 1:15 and 50 and 80ºC). According to Hahn

et al. (2020), chitosan exhibited 43% of DDA when using 120ºC and a solid: liquid ratio of 3:40, which increase to 77% when increasing temperature and the liquid ratio to 140ºC and 1:16 respectively, which support the use of 100ºC that achieve the temperature requirement for heterogeneous deacetylation and higher reagent volume could give a higher DDA than the result obtain in this project. Besides that, different determination method might result in large variety of DDA as well. According to Biskup et al. (2012), the DDA of chitosan determined using titration method as applied by Triunfo et al. (2022) would be higher as compare with the FTIR method used in this project. In this case, the obtained DDA of commercial chitosan and chitosan from dead adult black soldier fly in the study of Triunfor et al. (2022) and this project were 92 and 93%, and 81.76, 80.90 (chitosan I) and 80.70 (chitosan II) respectively, followed the trend as mentioned by Biskup et al. (2012), whereas the study of Zimri (2018) gave an even lower DDA of commercial and adult BSF chitosan, which were 70 and 69% with the elemental analysis-based determination. The study of Triunfo et al. (2022) showed that when using the same deacetylation process, similar DDA were obtained from larvae, pupal exuviae and dead adults of BSF, which were 94, 89 and 96%. Therefore, the degree of deacetylation of black soldier fly was depended solely on the deacetylation processes, and was not exhibited large variations among different development stages.

5.8 Determination of chitosan content

The chitosan content of chitosan I and II were determined using the sodium nitrite and thiobarbituric acid spectrophotometric method in this project. Instead of earlier chitosan determination method which relied on the glucosamine

determination using HCl hydrolysis, which there might be some of the content not completely hydrolysed by HCl and not included in measurement, causing an underestimated chitosan content, this method involves dissolving the chitosan samples in acetic acid solution, which the whole initial sample can be quantified, giving a higher accuracy of chitosan content determination (Badawy, 2012).

The determined chitosan content of chitosan I and II were 981.41 ± 85.57 and 871.92 ± 67.68 mg/g respectively, in which no significant difference ($p > 0.05$) was observed between them. The high chitosan content for both samples was expected because both chitosan samples were fully dissolved in 1% (v/v) acetic acid solution before starting the content determination. Hahn et al. (2020) reported the solubilization of chitosan in dilute acidic solutions indicated a non-degraded and high degree of deacetylation of chitosan. The study of Romanazzi et al. (2009) showed that 1% acetic acid solution was able to dissolve chitosan. These meant that the solubility in diluted acetic acid indicate the identity of chitosan. Besides that, based on the result in FTIR analysis and degree of deacetylation determination, both chitosan I and II exhibited the similar functional groups as well as the degree of deacetylation with the commercial chitosan that serve as the standard solution in this assay. Since the chitosan content of chitosan I and II was determined from the equation generated by commercial chitosan-based standard curve, ideally high chitosan content would be determined from them. The slightly lower of chitosan content detected in chitosan II might be due to low purity of chitosan II that lead to some impurities present in the chitosan solution and accidentally pipetted to the assay mixture,

which affected the concentration of solution, thus lower the absorbance reading, corresponded to lower chitosan content.

5.9 Limitation, future study and recommendation

5.9.1 Limitation

When conducting this project, benchwork duration was one of the limitations that restricted the expansion of this study. Since the experimental period during the first stage of final year project was conducted during short trimester, the restricted period could only allow a limited scope of experiment, including only one type of black soldier fly bio-wastes (dead adult black soldier fly) was able to be analyzed, and thus this study was lacking comparison with other collected bio-wastes which was the exoskeleton part. Besides that, during the sample preparation stage, the total mass of sample required to complete all the experimental analysis was underestimated, and after chitin had deacetylated to chitosan, there was a problem during the purification of chitosan, which the wrong purification method was selected, causing most of the chitosan sample dissolved in acetic acid solution failed to be recovered, hence there was no enough sample to perform the molecular weight determination of chitosan, which was one of the major properties related to the chitosan application, this result in an incomplete characterization of the produced chitosan in this study.

5.9.2 Future study

In terms of future study, the obtained high protein content of the dead adult black soldier fly bio-wastes suggested besides the chitin and chitosan, further economically analysis could be conducted to determine the potential of its

protein content for human use. Besides that, this study proved that chitosan from black soldier fly could be produced with low temperature deacetylation to obtain great extent of deacetylation and similar properties with commercial chitosan, which served as a better option as compare with the high temperature used for other studies. And the result obtained in this study showed that there was a color change from white to brown color during the conversion of bleached chitin to chitosan, with lower temperature treatment tend to have lighter brown, and thus better appearance. This suggested future study with the use of adult black soldier fly sample could apply the use of low temperature deacetylation to achieve a minor color change. Meanwhile, future study could focus on the solution to either prevent or decolorize the brown color formation of chitosan.

5.9.3 Recommendation

In terms of recommendation for the better performance of this study, preliminary test and the trial of experiment could be conducted before the actual analysis to prevent underestimation of require raw sample. Besides that, for the determination of chitin content assay, the acid hydrolysis time used could be increased to achieve a more complete conversion of glucosamine. And since measured glucosamine alone cause an underestimation of chitin content, further determination could be carried out to measure other monomers of chitin including N-acetylglucosamine and free acetate so that all glucosamine-related compounds could be taken into consideration during the calculation of chitin content, to avoid underestimation. Furthermore, higher temperature of more than 90ºC or a multi-step low temperature deacetylation could be used for chitosan I to determine whether chitosan from adult black soldier fly could reach an even

higher degree of deacetylation for wider application. Also, other analysis such as XRD, SEM and the molecular weight determination could be conducted to further characterize the produced chitosan.

CHAPTER 6

CONCLUSION

This study had determined the potentiality of chitin and chitosan extraction from the dead adult black soldier fly bio-wastes. After preparation including defatting, chitin was isolated from the dead adult black soldier fly, which obtained the yield of 10.44 ± 1.39% after demineralization and deproteinization. After decolorization, the yield was determined as 5.70 ± 0.11% which was more closed to other studies, together with a color change of chitin from deep black to bright white that was more related to the commercial available chitin. The obtained chitin with similar yield and high efficiency of demineralization and deproteinization as compare with other studies suggested milder condition was better for chitin extraction from black soldier fly sample. The chitin content of the dead adult black soldier fly and its extracted chitin were 10.88 ± 0.18 and 36.52 ± 1.08 mg/g respectively. The determined low chitin content from raw sample as compare with other study was due to the application of milder acid hydrolysis treatment, resulting in incomplete reaction which only small fraction of chitin converted to glucosamine, whereas the low and illogical chitin content from extracted chitin was due to higher purity that cause a further delay in glucosamine conversion and the presence of chitin-melanin complex that accounted as a part of the chitin.

The isolated chitin was deacetylated to chitosan I and II using different levels of deacetylation processes which were 12.5 M NaOH at 80°C for 15 h and 12.5 M

NaOH at 50°C for 6 h respectively. Their yield were determined as 3.78 ± 0.16 and 3.79 ± 0.25%, which no significant difference was observed, indicated the use of 80 and 50°C possessed similar deacetylation effect, and showed that temperature had greater effect than the duration during deacetylation process, thus further increasing the duration would not have significant effect when two levels of temperature offers the same deacetylation effect. The obtained result and the study of William and Wid (2019) suggested the sequence of chitin isolation has more significant effect than deacetylation condition on the yield of chitosan. The appearance of chitosan I and II changed to different extend of brown color after the deacetylation of white color chitin, with chitosan I observed a darker brown color and chitosan II displayed a bright yellow brown color. The browning was because of the chemical binding of melanin to chitosan during the heat treatment, leading to the regeneration of melanin brown color.

The produced chitosan I and II were compared with commercial available chitosan and pharmaceutical grade chitosan using the FTIR analysis. All the chitosan samples were observed with the presence of characteristic functional group for chitosan, including C=O stretching amide I, amide III and the characteristic bands between 1220 and 1020 cm^{-1} that represented the free NH_2 groups at C2 position of glucosamine. And both chitosan I and II was observed the more similar structure and functional groups with commercial chitosan than pharmaceutical chitosan, which there were lack of the CH_2 symmetric stretching that observed on pharmaceutical chitosan, while exhibited a peak represented as C-H bending and CH_3 symmetric deformation that did not observe in the pharmaceutical chitosan. Chitosan I and II were determined high degree of

deacetylation as 80.90 and 80.70% which was similar to the commercial chitosan (81.76%), indicated both of them possess similar properties with commercial chitosan, and suggested the use of milder deacetylation temperature (<90°C) and short reaction time (6 h) was able to remove the acetyl groups from chitin of BSF to a great extent. Overall, this study concluded that chitin could be extracted from the dead adult black soldier fly, and the converted chitosan with similar properties and structure to commercial chitosan suggested insect black soldier fly as an alternative source of chitin and chitosan besides the common crustacean source.

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APPENDICES

APPENDIX A

Moisture content of original sample (black soldier fly adult before preparation) and prepared sample (black soldier fly adult after dried, sieved and grinded) determined using moisture analyzer.

Sample	Replicate	Sample Weight (g)	Moisture Content (%)
Original sample	1	5.004	58.20
	2	5.003	57.63
	3	5.004	60.25
Prepared sample	1	5.002	2.58
	2	5.002	2.41
	3	5.001	2.48

APPENDIX B

Crude fat content of prepared sample (black solider fly adult after dried, sieved and grinded) in dry weight basis determined using fat analyzer.

Sample	Replicate	Extraction Beaker + Boiling stone weight (g), M1	Sample weight (g), M_0	Extraction Beaker + Boiling stone + oil weight (g), M2	Crude fat content (wb) (%) $(\frac{\textbf{Weight of oil}}{\textbf{Original sample weight}})$ ×100%
Black soldier fly adult	1	126.1446	4.8777	126.7374	12.15
	2	126.6078	4.8785	127.1987	12.11
	3	126.6817	4.8763	127.2898	12.47

APPENDIX C

Crude protein content of prepared sample (black solider fly adult after dried, sieved and grinded) in dry weight basis and the chitin extracted from defatted black soldier fly adult determined using Kjeldahl method.

Sample	Replicate	Sample weight	Titration volume (Final reading – initial reading) (mL)	Crude protein content (%)
Black soldier fly adult	1	2.0009	42.5 – 3.0 = 39.5	64.37
	2	2.0014	44.7 - 5 = 39.7	64.68
	3	2.0003	42.9 – 3.1 = 39.8	64.88
Extracted chitin	1	2.0008	6.0 – 0.0 = 6.0	1.93
	2	2.0005	11.8 – 6.0 = 5.8	1.86
	3	2.0002	17.8 – 11.8 = 6.0	1.93

Volume for blank = 0.1 mL

APPENDIX D

(%) yield of dried, sieved, grinded and defatted black soldier fly adult in dry weight basis after demineralization treatment using 5% HCl in sample to reagent ratio of 1:4, 2 h and room temperature.

Sample	Replication	Sample weight (g)	Sample +conical flask (g)	Sample + conical flask after demineralization (g)	Yield (%)
Black soldier fly adult	1	3.00	73.95	72.02	32.10
	2	3.00	64.66	62.64	29.40
	3	3.00	71.85	69.88	30.90

Sample used in dry weight basis = 3.333295

(%) yield of extracted chitin (demineralized) after deproteinization treatment using 2 M NaOH in sample to reagent ratio of 1:4, 4 hours, 45°C and 100 rpm.

Sample	Replication	Weight of filter paper (g)	Weight of filter paper + chitin (g)	Yield (%)
Black soldier fly adult (after demineralization)	1	0.6466	0.9484	9.05
	2	0.6580	1.0057	10.43
	3	0.6540	1.0488	11.84

Sample used in dry weight basis = 3.333295

(%) yield of extracted chitin after decolorization.

Sample	Replication	Sample weight	Yield (%) (chitin based)	Yield (%) (raw sample based)
Extracted chitin	1	1.0123	53.55	5.59
	2	1.0070	55.64	5.81
	3	1.0100	54.67	5.71

APPENDIX E

Absorbance at 530 nm, glucosamine content and chitin content of prepared sample (black solider fly adult after dried, sieved and grinded) and the chitin extracted from black soldier fly adult (after decolorization).

Sample	Replication	Sample weight (g)	Absorbance	Glucosamine content (µg)	Chitin content (µg)	µg/g	mg/g
Black soldier fly adult	1	0.1009	0.340	116.82	132.48	10941.91	10.94
	2	0.1004	0.341	117.18	132.89	11030.02	11.03
	3	0.1002	0.330	113.25	128.43	10681.5	10.68
Extracted chitin	1	0.1001	1.070	377.54	428.16	35643.94	35.64
	2	0.1004	1.135	400.75	454.48	37722.6	37.72
	3	0.1002	1.087	383.60	435.03	36181.01	36.18

APPENDIX F

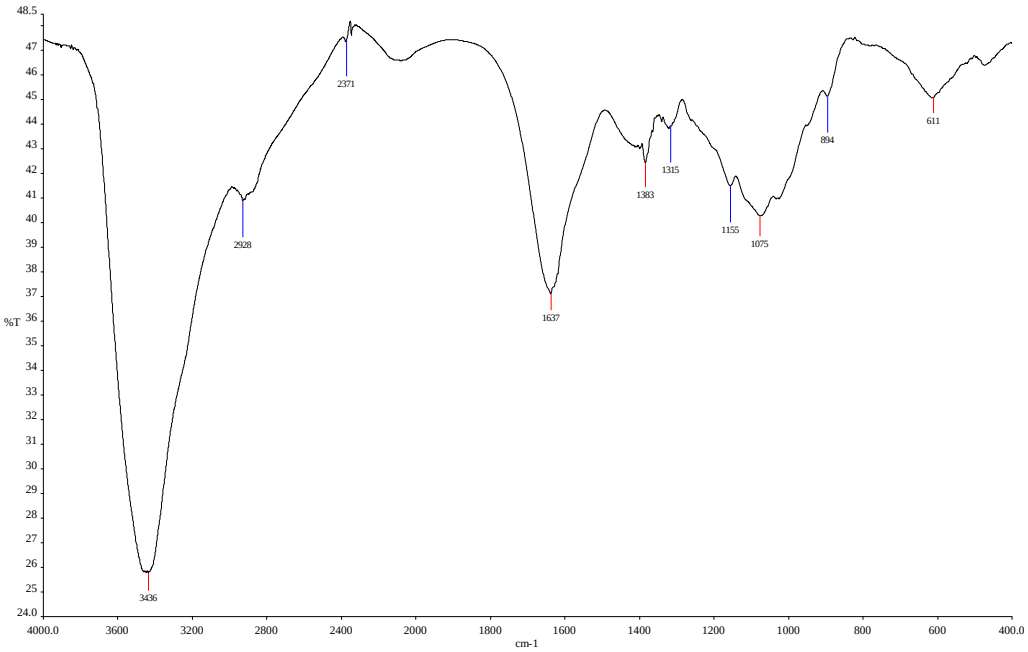
(%) yield of chitosan I (treated with 12.5 M NaOH, 80°C and 15 h) and chitosan II (12.5 M NaOH, 50°C and 6 h) (chitin-based) after deacetylation of chitin using NaOH treatment.

Sample	Replication	Sample weight (g) (before deacetylation)	Sample weight (g) (after deacetylation)	Yield (%)	p-value (at p<0.05 level)
Chitosan I (12.5M NaOH, 80°C and 15 hours)	1	0.6006	0.4175	69.51	
	2	0.6000	0.3858	64.30	
	3	0.6003	0.3924	65.37	
					0.9925
Chitosan II (12.5M NaOH, 50°C and 6 hours)	1	0.5501	0.3851	68.04	
	2	0.5505	0.3753	61.47	
	3	0.5521	0.3384	69.75	

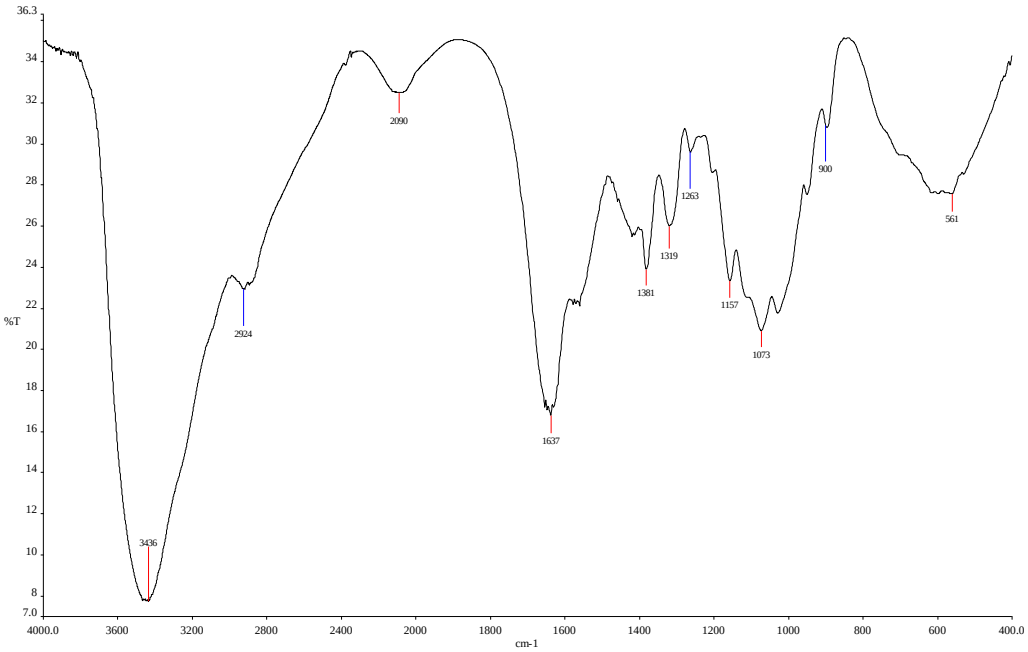
(%) yield of chitosan I (treated with 12.5 M NaOH, 80°C and 15 h) and chitosan II (12.5 M NaOH, 50°C and 6 h) (raw sample-based) after deacetylation of chitin using NaOH treatment.

Sample	Replication	Sample weight (g) (before deacetylation)	Sample weight (g) (after deacetylation)	Yield (%)	p-value (at p<0.05 level)
Chitosan I (12.5M NaOH, 80°C and 15 hours)	1	0.6006	0.4175	3.96	
	2	0.6000	0.3858	3.67	
	3	0.6003	0.3924	3.73	
					0.9550
Chitosan II (12.5M NaOH, 50°C and 6 hours)	1	0.5501	0.3851	3.88	
	2	0.5505	0.3753	3.50	
	3	0.5521	0.3384	3.98	

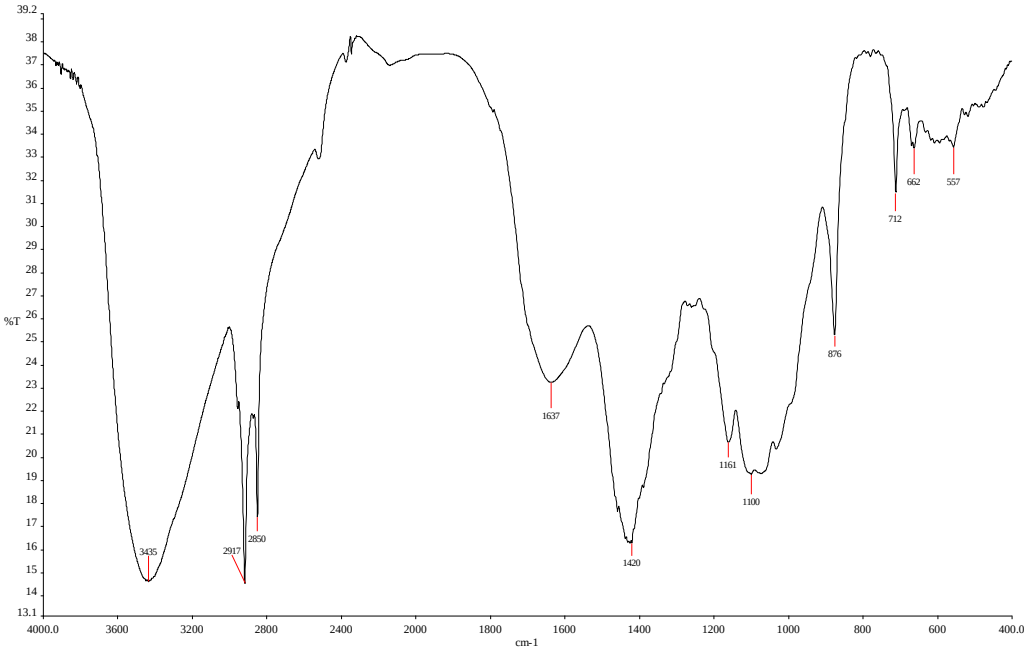
APPENDIX G



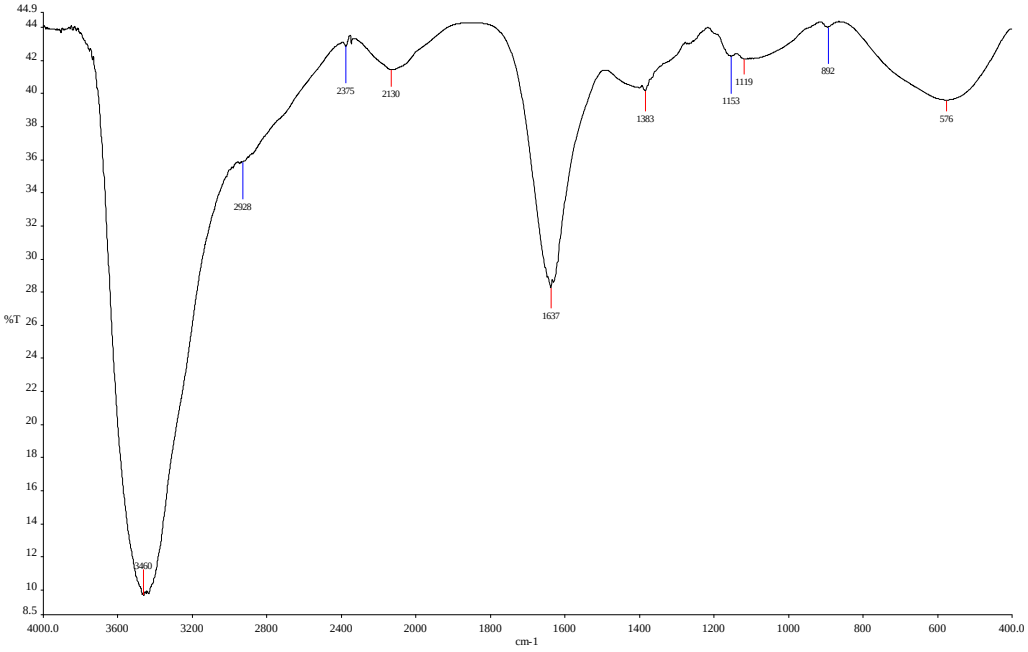
FTIR spectra of Chitosan I



FTIR spectra of Chitosan II



FTIR spectra of pharmaceutical chitosan



FTIR spectra of commercial chitosan (crab shell)

APPENDIX H

Absorbance at 555 nm and chitosan content of chitosan I and chitosan II determined using spectrophotometry method.

Sample	Replication	Absorbance	Chitosan content (µg)	mg/g	p-value (p<0.05 level)
Chitosan I (80°C, 15 hours)	1	0.591	49.83	966.6	0.1568
	2	0.525	44.46	889.27	
	3	0.629	52.91	1058.37	
Chitosan II (50°C, 6 hours)	1	0.518	43.89	877.89	
	2	0.554	46.82	936.42	
	3	0.471	40.07	801.46	

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