COMPARISON OF EXTRACTION METHODS AND CHARACTERISATION OF CHITIN
AND CHITOSAN WITH ANTIMICROBIAL AND ANTIOXIDANT PROPERTIES FROM
BLACK SOLDIER FLY *(HERMETIA ILLUCENS)* MEAL

by

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This thesis is dedicated to God the Almighty source of divine wisdom and to all in pursuit of academic excellence.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Chitin/chitosan is a natural biopolymer that has many applications in agriculture, medicine and biotechnology. Although synthesized by different organisms, crustacean shells dominate the production. The present study focused on the extraction of chitin from an insect source using conventional and alternative extraction methods; enzyme-, microwave- and ultrasound-assisted extraction, and compared the physicochemical characteristics of the end products. The biological activities of chitosan from three sources was also investigated. The conventional extraction method from BSFM resulted in 9.7% chitin yield, while the enzyme-, microwave- and ultrasound-assisted extractions gave 42.3%, 11.4% and 13.7% chitin on dry weight basis, respectively. FTIR patterns displayed bands corresponding to the stretching and vibration of OH, NH and CO bonds, confirming the presence of α-chitin/chitosan. Chitosan samples from three different sources displayed both antioxidant and antimicrobial activity. Chitosan MW and DD both had effects on biological activities; high MW chitosan showed better antimicrobial activity lower MW chitosan.

LIST OF ABBREVIATIONS USED

-NH$_2$	amino
-OH	hydroxyl
%	percentage(s)
°C	degree Celsius
BSFM	Black soldier fly meal
CCH	Chitosan extract obtained from conventional extraction
CE	Conventional extraction method
DA	Degree of acetylation
DD	Degree of deacetylation
DPPH	1,1-diphenyl-2-picrylhydrazyl
EA	Elemental Analysis
EAE	Enzyme – assisted extraction method
ECHa	Chitosan extract obtained from enzyme – assisted extraction, acetone wash
ECHb	Chitosan extract obtained from enzyme – assisted extraction, no acetone wash
EDTA	Ethylenediaminetetraacetic acid, a chelating agent (used as positive control)
FCHa	Fungal chitosan sample a
FCHb	Fungal chitosan sample b
FerroZine^{TM}	3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate
FTIR	Fourier transform infrared spectroscopy
g	gram(s)
GlcN	D-glucosamine
GlcNAc	*N*-acetyl-D-glucosamine
h	hour(s)
HMW	High molecular weight
kDa	kiloDalton(s)
LDC	Lobster shell-derived chitin
LDCH	Lobster shell-derived chitosan
LMW	Low molecular weight

MAE	Microwave – assisted extraction method
MCH	Chitosan extract obtained from microwave – assisted extraction
MW	Molecular weight
NMR	Nuclear magnetic resonance
rpm	revolutions per minute
SCH	Commercial chitosan
STC	Commercial chitin
TGA/DTG	Thermogravimetric and derivative thermogravimetric analysis
UAE	Ultrasound – assisted extraction method
UCH	Chitosan extract obtained from ultrasound – assisted extraction
w/v	weight/volume
w/w	weight/weight
μL	microliter(s)
α	alpha
β	beta
γ	gamma

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CHAPTER 1

INTRODUCTION

1.1. Thesis Overview

The term biopolymer is used to identify polymers which can be synthesized from living organisms. Chitin and chitosan are examples of biopolymers that have received considerable research interests due to their potential applications in agriculture and food, biomedicine and pharmaceutical, papermaking and textile industries, cosmetics and wastewater treatment (Kumar, 2000). Structurally, chitin is a linear polysaccharide, made of N-acetyl-D-glucosamine units connected by β (1→4) linkages. Every year, approximately 100 billion tons of chitin are produced by crustaceans, mollusks, insects, fungi and related organisms every year (Muxika et al., 2017). When the acetyl-D-glucosamine units in chitin lose its acetyl groups in a process of deacetylation, the molecule is called chitosan.

Although chitin and chitosan can be extracted from various terrestrial and aquatic organisms, commercial chitin and chitosan are mostly extracted and obtained from crustacean wastes (i.e. crabs, shrimps and krill). Hence, most of the studies are based on the α-chitin/chitosan extracted from crustacean shells or animal source. Currently, chitin is commercially extracted using chemical methods which involve the removal of minerals and proteins using strong acids and bases at high temperatures. These processes not only require high energy consumption but create environmental issues as the effluents generated must be neutralized by adequate treatments (Pachapur et al., 2016). Besides, there is a high cost associated with the purification of chitin extracts from crustacean shells as well as the allergen they may possess to individuals who are at high risk to shell and seafood exposure. Furthermore, an examination of the extracts from this method has shown inconsistencies that have led to the production of chitin/chitosan with variable

physicochemical properties. (No et al., 2000b). As a result, alternate sources of raw materials and extraction methods may mitigate or reduce the drawbacks of the conventional source and process which need to be explored and investigated for their potential to extract chitin/chitosan.

Emerging research has examined sources such as insects and fungi as potential raw materials for chitin/chitosan production (Kaya et al., 2015a; Teng et al., 2001; Wu et al., 2005; Yen & Mau, 2007). Depending on the origin of a sample, chitin/chitosan can exhibit a variety of chemical, physical and biological properties (Usman et al., 2016). A combination of factors is observed to affect these activities and are being investigated and studied by researchers. Some of these factors or properties such as MW and DDA, depends on the source of the chitin/chitosan as well as their extraction methodology.

Novel technology such as microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE) and enzyme-assisted extraction (EAE) has been employed for the extraction of polysaccharides from different sources. A few studies have investigated to use of these methods for chitin production. However, they were utilized in the deproteinization i.e. removal of proteins, phase. In these studies, a significant reduction of time was observed, and chitin/chitosan of similar physical characteristics was obtained. At the time of this report, no studies have been found of these alternative methods in the full process of chitin recovery from either shrimp, insects or fungi. On the other hand, extraction methodologies have been shown not only have an impact on polysaccharide yield but also have shown an influence on the structural characteristics and biological activities of the chitosan (Ale et al., 2011; Dong et al., 2016; Wang et al., 2016).

Therefore, this project was focused on finding out an alternate source for its potential as a raw material for chitin production. In current study, MAE, UAE and EAE methods were used for chitin extraction from BSFM source. The physicochemical characteristics of these extracts obtained from

three different sources compared with the extract obtained from the conventional extraction method. Furthermore, the biological activity of chitosan obtained from three different sources (insect, fungi and crustacean) were investigated to determine if the origin of the sample as well as the extraction methods (for insect source) influences or affects the biological activity of chitosan.

1.2. Thesis Objectives

Specific objectives of this research work are;

- To investigate the extraction yield of chitin from black soldier fly meal (BSFM) using the conventional extraction method;

- To compare and evaluate the chitin yield based on alternative extraction methods such as microwave-, ultrasound- and enzyme-assisted extraction;

- To assess the impact of extraction methods on the physicochemical characteristics of chitin extracts with commercial chitin and lobster derived chitin (crustacean);

- To evaluate the physicochemical and functional properties of chitosan obtained from three different sources; insect (BSFM), crustacean and fungi.

1.3. Outline of Thesis and Organization

The thesis is divided into five chapters, including the present chapter. Chapter 2 provides a review of relevant works of literature done in the field of the study. It has a discussion on the different characteristics of chitin/chitosan regarding sources i.e. raw materials, extraction methods and procedures, structural, physicochemical and biological properties. Chapter 3 examines the yield of chitin from an insect source and investigates the potential of other extraction methods to obtain chitin. Physicochemical characteristics of the chitin from insect were examined with a comparison to available commercial chitin (crustacean source). Chapter 4 examines the physicochemical and

biological activities of chitosan obtained from insect, fungi and crustacean to determine the effect, if any, of raw material sources and other factors on chitosan biological activity. Chapter 5 summarizes the study and offers insights into future research, followed by references and appendices.

CHAPTER 2

LITERATURE REVIEW

2.1. Chitin

Chitin, a structural polysaccharide, is the primary constituent of the outer skeleton of crustaceans and insects. It is referred to as the second most abundant polymer on earth after cellulose. Chitin is a linear polymer mainly consisting of β-(1,4) linked 2-acetamido-2-deoxy-β-D-glucopyranose units and partially of β-(1,4)-linked 2-amino-2-deoxy-β-D-glucopyranose (N-acetylglucosamine) (Kaur and Dhillon, 2014). It was first discovered in mushrooms by Professor Henri Braconnot in 1811, and the name "chitin" came about in the 1830s when it was isolated in an insect (Odier, 1823).

Chitin occurs in nature as ordered crystalline microfibrils (Merzendorfer, 2006). Structurally, it is found in three polymorphic forms: α-chitin, β-chitin, and γ-chitin. α-Chitin is the most stable and abundant form of the three crystalline variations and is arranged in anti-parallel strands (Hamed et al., 2016). It serves as the resistance structure in insect cuticles, shells of crabs, lobsters and shrimp, and in fungal and yeast cell walls. It is also found in marine sponges. β-Chitin, on the other hand, is less stable than the α form. It is arranged in parallel chains and has been found in the extracellular fibers of diatoms, the pens of squid, and the spines and chaetae of certain annelids (Lavall et al., 2007). The least common form is γ-chitin, which can be found in the stomachs of squid and the cocoons of two genera of beetles (Rinaudo, 2006; Souza et al., 2011). γ-chitin has two chains in one direction and another chain running in the opposite direction, which has been considered to be a combination of the α and β structures rather than a different crystalline form (Roberts, 1992).

2.1.1. Sources of Chitin

Chitin is synthesized from many different organisms and is typically isolated from the cell walls of fungi and algae, the exoskeleton of insects, endoskeleton of cephalopods and shells of molluscs and crustaceans (Dhillon et al., 2013). However, the primary commercial sources of chitin are crab and shrimp shells.

2.1.1.1. Crustaceans

Crustaceans include crabs, lobsters, crayfish, shrimp, krill, woodlice and barnacles. Traditionally, crustacean shells as by-products of the seafood processing industry constitute the primary and commercial source of chitin. For instance, shrimp wastes contain high concentrations of protein, which stem primarily from the skeletal tissues. This skeletal tissue is comprised of a calcified protein-chitin matrix, which is responsible for the hard shells of crustaceans (Kjartansson et al., 2006). The crustacean shells are assembled from three fundamental components namely: (a) chitin, (b) minerals and (c) proteins. Chitin serves as the skeleton which is enriched with minerals, mainly inorganic carbonate salts that strengthen the shells while proteins render the shells as living tissues (Kaur & Dhillon, 2013). On a dry weight basis, crustacean shell waste consists of approximately 40% protein, 35% minerals, 20% chitin and 5% lipids. However, the actual chitin content will vary depending on species, the health of the animals, harvesting season and geographical location. For example, the chitin content in crab shells may be as high as 32% as compared to less than 20% in shrimp shells (Abdou et al., 2008; Synowiecki & Al-Khateeb, 2003).

Extraction of chitin requires the removal of proteins and a tiny amount of pigments and lipids by deproteinization and inorganic calcium carbonate by demineralization. In some cases, an

additional step of decolourization is applied to remove the excess residual pigments (Younes & Rinaudo, 2015). Chemical extraction procedures of chitin from shrimp wastes have shown inconsistent yields of chitin and chitosan (linear polysaccharide obtained by alkaline deacetylation of chitin), which may be attributed to protein contamination, inconsistent levels of deacetylation (DD), and high molecular weight (MW), leading to the production of chitin with variable physicochemical characteristics (No et al., 2000a). There are also additional problems such as environmental issues which arise due to the production of significant amount of concentrated alkaline waste, consumption of large volumes of fresh water, seasonal limitation of the supply of seafood shell and a high cost associated with chitin purification (Wu et al., 2005).

2.1.1.2. Insects

Insects are promising sources of new biomass because of their composition (i.e. rich in proteins, fats, and biopolymers), their cultivation possibilities (ability to grow on biological waste streams) and the high percentage of dry matter content (Dossey, 2010). There have been few studies on the chitin contents of insect species (Table 2.1), which varied from 10 to 36% of their dry weights (Gonil & Sajomsang, 2012; Liu et al., 2012; Majtán et al., 2007; Nemtsev et al., 2004; Paulino, et al., 2006; Sajomsang & Gonil, 2010). Badawy & Mohamed (2015) reported that chitin and chitosan obtained from six different insect species exhibited similar chemical structure and physicochemical properties to those of chitin and chitosan obtained from crustaceans. Similarly, Kaya et al. (2015a) demonstrated that the chitin extracted from two grasshopper species was similar in terms of characteristics to commercially available chitin derived from crabs and shrimps. These findings suggested that insects could be used as alternative sources for chitin and chitosan production. Insects are known to reproduce fast when environmental conditions are appropriate. This can potentially lead to an excessive increase in population, thus making them available for

chitin production (Zhang et al., 2000). Also, insect cuticles have been shown to have lower levels of inorganic material than crustacean shells, which make the demineralization treatment rapid and easier (Badawy & Mohamed, 2015).

Many insects naturally feed on organic wastes by incorporating the biomass nutrients into their biomass and in the process to reduce the amount of waste material. *Hermetia illucens*, better known as the black soldier fly (BSF), is one of the most important species that has been proposed as a converter of organic waste (Čičková et al., 2015). It is a beneficial insect that can be found all over North America and around the world. The adult flies do not eat, sting or bite. Thus, the risk of disease transmission is avoided. BSF larvae thrive on a wide range of decaying organic matter and are used in composting piles as they have the potential of converting organic waste into rich fertilizer. BSF is also reported to be a good source of proteins, lipids, and chitin (Waśko et al., 2016). According to the study by Waśko et al. (2016), physicochemical properties of chitin isolated from both adult and larvae of BSF was not significantly different other insects or crustaceans' shell . Thus, processes similar to methods employed for extraction of chitin from crustaceans have been adopted to isolate chitin from insect species. However, due to the presence of little or no inorganic materials, very low concentrations of acid and alkali are required.

Table 2.1. Chitin values obtained from dry weight of insects

Samples	% chitin content	Reference
Holotrichia parallela	15	Liu et al., 2012
Bombyx mori	15 - 20	Zhang et al., 2000
Palomena prasina	10.8	Kaya et al., 2015b
Melolantha melolantha	13 - 14	Kaya et al., 2014b
Geolycosa vultuosa	8 - 8.5	Kaya et al., 2014d
Hogna radiata	6.5 - 7	Kaya et al., 2014d
Cicada sloughs	36	Gonil & Sajomsang, 2012

2.1.1.3. Fungi

Chitin is widely distributed amongst members of the kingdom Mycota such as *Ascomycetes, Zygomycetes, Basidiomycetes*, and *Phycomycetes*. In these fungi, chitin is an integral part of the cell walls and structural membranes of mycelia, stalks, and spores. The chitin is responsible for maintaining the shape, strength, and integrity of the cell structure (Ruiz-Herrera, 2016), which comprises about 22 – 44% of the cell walls. Studies have shown that those belonging to the *Zygomycetes* class of fungi have higher amounts of chitin and chitosan in their cell walls as compared to their counterparts in the other classes (Table 2.2).

Fungal biomass is a rich and inexpensive source of chitin and can be exploited for chitin/chitosan extraction through environmentally safe methods such as mild alkaline and acidic treatments (Kaur & Dhillon, 2014). Thus, the production of chitin from the cell walls of fungi grown under controlled conditions offer greater potential for a consistent chitin product. Unlike the seasonal supply of crustaceans, fungal mycelia can be cultivated throughout the year by fermentation under submerged culture, which is rapid and better synchronized. For instance, the molecular weight and degree of deacetylation can be controlled by varying the fermentation conditions (Arcidiacono & Kaplan, 1992). Also, the fermentation process can be conducted in bioreactors with automated and controlled conditions to ensure homogeneous production of mycelium biomass from each batch (Ospina Álvarez et al., 2014). In addition, fungal mycelium has lower levels of inorganic materials compared to crustacean shells, and as a result, no demineralization treatment is required (Teng et al., 2001). Fungi are usually harvested at their late exponential growth phase to obtain the maximum yield for chitin and chitosan. Pochanavanich & Suntornsuk (2002) investigated four

different filamentous fungi that represent four different species, namely; *Aspergillus niger*, *Rhizopus oryzae, Lentinus edodes* and *Pleurotus sajo-caju* and two yeast strains, namely; *Zygosaccharomyces rouxii* and *Candida albicans* for their potential to produce chitosan on synthetic media. A higher chitosan yield of 138 mg/g dry weight (DW) representing 14% chitosan was achieved by *R. oryzae* followed by 107 mg/g DW representing 11% chitosan by *A. niger*.

Table 2.2. Chitin content on a dry weight basis of mycelium from different fungi

Fungi species	Chitin content (%)
Aspergillus niger	42.0
Aspergillus phoenicis	23.7
Mucor rouxii	9.4
Neurospora crassa	8.0 – 11.9
Penivillium chrysogenum	19.5 – 42.0
Trichoderma viridis	12.0 – 22.0
Saccharomyces gutulata	2.3
Blastimyces dermatidis	13.0

Modified from Synowiecki & Al-Khateeb, 2003

Biotech industries utilize fungi in different processes such as brewing and baking, antibiotics, pharmaceuticals, organic acid and enzyme production. *A. niger* strains are widely used for the production of citric acid (CA) and other biotechnological and pharmaceutical products on an industrial scale (Dhillon et al., 2013). CA is the most widely used organic acid in the beverage and other food and pharmaceutical industries as an acidifying or flavour-enhancing agent. Submerged fermentation has been employed for the industrial production of CA. The annual worldwide production of CA is estimated to be 1.7 million tons, which results in 0.34 million tons of *A. niger* mycelium waste (Dhillon et al., 2011; Wu et al., 2005). Chitin and subsequently chitosan can, therefore, be isolated from such a large amount of *A. niger* mycelium waste, which will serve as an inexpensive and rich alternative source.

Nonetheless, chitin and chitosan extraction from fungal source is similar to industrially utilized extraction processes except for the demineralization step due to low mineral content in fungal mycelia. Extraction procedure consists of three steps: (1) alkaline treatment to remove protein and alkali-soluble polysaccharides; (2) acid reflux to separate chitin and chitosan; and (3) precipitation of chitosan under alkaline conditions.

2.2. Chitosan

Despite chitin being abundant and having a wide range of beneficial properties and activities such as biodegradability, biocompatibility, non-antigenicity, and non-toxicity, it, however, has limited utility due to its poor solubility in water and most organic solvents. Solubility in dilute acids is improved by the conversion of chitin to chitosan through chemical or enzymatic processes, where N-acetyl-D-glucosamine units (GlcNAc) are transformed into co-polymers of GlcNAc and D-glucosamine units (GlcN) with free amino groups (–NH2; Fig. 2.1) (Aranaz et al., 2009; Hamed et al., 2016). Moreover, this deacetylation makes chitosan more positively charged with the ability to interact with diverse molecules (Muxika et al., 2017).
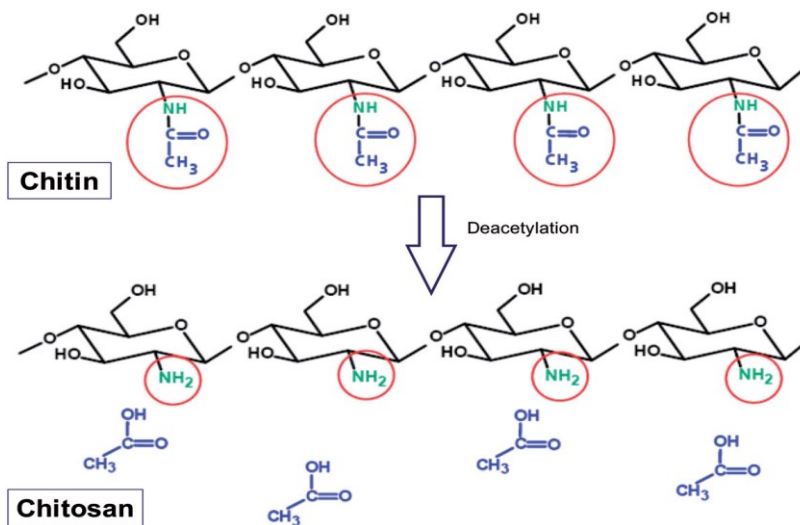
Figure 2.1. Chemical structure of chitin and deacetylated derivative chitosan, modified from Kaur & Dhillon, 2013

Chitosan is therefore a water soluble cationic polymer (at acidic pH) due to the positive charge on its amino groups (Muxika et al., 2017). This unique property makes it appropriate for its wide range of applications in foods, cosmetics, and pharmaceuticals. The cationic biopolymer can interact with anionic molecules such as glycosaminoglycans (GAG) and proteoglycans (Hamed et al., 2016).

The chemical conversion of chitin to chitosan is preferred due to its lower cost and scalability. Chemical deacetylation involves treatment with sodium hydroxides (40-50% concentrations) at high temperatures of $\geq 80°C$ (Hamed et al., 2016). Chitosan with different degrees of deacetylation is generated depending on the reaction time, the temperature and the concentration of the alkali solution (Teng, 2012). On the other hand, enzyme-assisted deacetylation of chitin is achieved using the enzyme chitin deacetylase, which catalyses the hydrolysis of N-acetamido bonds resulting in the conversion of chitin to chitosan (Zhao et al., 2010). This enzyme was first discovered in 1974 in *Mucor rouxii* extracts and was later found in several fungal strains, marine bacteria and insects (Zhao et al., 2010). In contrast to the chemical deacetylation procedure where large volumes of fresh water are consumed to neutralize the acids/alkalis used and in turn, generate large volumes of waste, enzymatic deacetylation is considered to be more environment friendly and generate homogenous, reproducible chitosan (Younes & Rinaudo, 2015). However, lack of adequate studies on chitin deacetylase isolation limits its application on a commercial scale (Zhao et al., 2010). This is most likely due to the cost associated with isolation since isolating pure forms of an enzyme from a natural source, especially at a commercial scale is highly expensive.

2.2.1. Attributes required for chitosan as a high value-added product

Three key features are considered for chitosan to be regarded as a high value-added product as follows: (a) molecular weight (MW); (b) degree of deacetylation (DD); and (c) purity, i.e., the absence of contaminants, such as inorganic materials. Physicochemical characteristics of chitosan are significantly affected by these key features, which in turn govern almost all of its applications. By controlling these features, chitosan can be tailor-made for specific use in industrial, pharmaceutical and agricultural applications.

The MW depends on the chain length and has been confirmed to influence the rate of biodegradation of chitin (Huang et al., 2005; Zhang & Neau 2001). Similar to its composition, the MW of chitosan varies with the source and the method of preparation (Samar et al., 2013). Natural chitin usually has molecular weight larger than 1,000 kDa while commercial chitosan products have molecular weight ranges of 100 – 1,200 kDa, depending on the process and grades of the product (Li et al., 1992). For some application purposes, such as protein recovery, low MW chitosan is usually desired to form complexes with polyanions, as high MW chitosan may result in poor solubility at neutral pH and high viscosity aqueous solutions (Wibowo et al., 2007). These can also limit its potential use in food, pharmaceutical, and agricultural industry. Studies by Park et al. (2003) and Hammond & Skonberg (2012) demonstrated that lower MW chitosan had higher divalent metal chelation, which could be due to : (i) low viscosity (ii) solvent accessibility and (iii) less steric hindrance. Together, these could make the lower MW chitosan have enhanced bio functionalities. The MW of chitosan can be determined by several methods such as light scattering spectrophotometry, gel permeation chromatography (GPC) and viscometry (Kumar, 2000).

Similarly, DD has been shown to influence the performance of chitosan. The DD, which refers to the ratio of GlcNAc to GlcN structural units is influenced by the procedures employed in the preparation of chitosan. DD of chitosan ranges from 56% to 99% with an average of 80%, depending on the crustacean species and method of preparation (No et al, 2000b). Chitosan solubility, charge distribution, moisture absorption and intrinsic viscosity are impacted by the DD (Aranaz et al., 2009). Infrared spectroscopy, pyrolysis gas chromatography, GPC, proton nuclear magnetic resonance ($^+$H-NMR) spectroscopy are some of the methods that have been reported for the determination of the degree of deacetylation of chitosan.

Product purity is equally essential for high-value product applications, particularly in the field of biomedicine, pharmaceuticals or cosmetics. Purity is quantified in terms of the remaining ash, proteins, insoluble fraction as well as bio-burden, i.e., microbes, yeasts, moulds and various endotoxins produced by these microorganisms. The purity of chitosan has been reported to influence its toxicological profile (Raafat & Sahl, 2009). Residual proteins in chitosan can cause allergic reactions such as hypersensitivity (Aranaz et al., 2009), which can potentially limit the use of chitosan in the biomedicine sector. Pure chitosan has been used in some specialty applications such as internal haemostatic dressing, drug delivery agent, tissue scaffolding, and in other healthcare-related products (Baker & Wiesmann, 2008; Baldrick, 2010).

2.2.2. Chitosan bioactivity and applications

Chitosan has attracted much attention because of its unique biological, chemical and physical properties. Chitosan has exhibited antioxidant, antifungal, antitumor and antimicrobial activities, immuno-enhancing effect and increased protective effect against infection with some pathogens (Chien et al., 2007; Qin et al., 2002; Tomida et al., 2009). Chitosan serves as a promising biopolymer with a variety of potential applications in several industries. Their biological activities

14

including antioxidant and antimicrobial, are being investigated for their potential use in the food industry to improve food safety, food quality and shelf-life (Hamed et al., 2016).

2.2.2.1. Antioxidant activity

Oxidative stress induced by oxygen radicals is believed to be a primary factor in various degenerative diseases as well as in the normal aging process (Halliwell et al., 1992). It reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Reactive oxygen species (ROS) in the forms of superoxide anion, hydroxyl radical and hydrogen peroxide are generated by the normal metabolic process and they can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides that are capable of damaging a wide range of essential biomolecules (Halliwell et al., 1992). Antioxidants are therefore substances that delay or prevent the oxidation of cellular oxidizable substrates. They exert their effects by scavenging ROSs, activating a battery of detoxifying proteins or preventing the generation of ROSs (Xing et al., 2004). Synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole, and propyl gallate may be used to retard lipid peroxidation (Wanita & Lorenz, 1996). However, their use is under strict regulations due to the potential health hazards associated with usage (Kim et al., 2001; Park et al., 2001). Therefore, the search for natural antioxidants as alternatives to synthetic ones is on the rise and of great interest among researchers.

The antioxidant activity of chitosan and its derivatives have been assessed in many studies. However, the mechanism of antioxidant activity is still disputable. While some studies indicate considerable *in vitro* antioxidant properties of chitosan (Park et al., 2003; Yen et al., 2007; 2008), other studies have shown low or no antioxidant activity of native chitosan. In contrast, activity was observed to significantly increase with modified chitosan extracts such as (i) chitosan grafted with

gallic acid; (ii) graft copolymerization of maleic acid sodium onto hydroxypropyl chitosan; and (iii) carboxymethyl chitosan sodium (Casettari, et al., 2012; Xie et al., 2001; Xing et al., 2005). Chitosan acts as an antioxidant by scavenging oxygen radicals such as hydroxyl, superoxide, alkyl and the highly stable DPPH radicals (Park et al., 2003). The ability to abstract hydrogen atoms easily from free radicals is directly correlated to the presence of amino and hydroxyl groups attached to C-2, C-3 and C-6 positions of the pyranose ring of the chitosan molecule (Xie et al., 2001). In the food industry, chitosan has been used as a food additive and its antioxidative activity is attributed to its chelation efficiency since by binding metal ions, it prevents the initiation of lipid oxidation (Guibal, 2004; Rhazi et al., 2002). Also, by simply preventing the contact of oxygen with packaged food, chitosan films and coatings were observed to reduce the rate of oxidation of packaged foods (Georgantelis et al., 2007).

2.2.2.2. Antimicrobial activity

The increasing consumer demand for foods without chemical preservatives is getting interest on the discovery of new natural antimicrobials (Wang, 1992). As a result, the antimicrobial activity of chitin, chitosan and their derivatives against different groups of microorganisms such as bacteria, yeast, and fungi has received a considerable attention (Yalpani et al., 1992). Due to the presence of the positive charge on the C-2 of the glucosamine monomer at below pH 6, chitosan is more soluble and has a better antimicrobial activity than chitin (Chen et al., 1998). The exact mechanism of the antimicrobial action of chitin, chitosan, and their derivatives has not been fully elucidated, but different mechanisms and hypotheses have been proposed. The most feasible hypothesis is a change in cell permeability of microorganisms due to the interactions between the polycationic chitosan and the electronegative charges on the cell surfaces. This interaction therefore leads to the leakage of intracellular electrolytes and proteinaceous constituents (Chien et

16

al., 2016; Severino et al., 2015; Xing et al., 2009). Other mechanisms mentioned in literature are (i) the interaction of diffused hydrolysis products with microbial DNA, which leads to the inhibition of the mRNA and protein synthesis (Devlieghere et al., 2004; Sudarshan et al., 1992; Yuan et al., 2016); (ii) inhibition of microbial growth by the chelation of nutrients and essential metals (Chien et al., 2016; Li et al., 2010; Yuan et al., 2016); (iii) formation of a polymer membrane on the surface of cells which prevents nutrients from entering the cell (Helander et al., 2001); and (vi) acting as an oxygen barrier which inhibits growth of aerobic bacteria (Yuan et al., 2016).

Chitosan is used in food preservation and packaging to reduce the use of chemical preservatives and to produce edible antimicrobial films as reviewed by Friedman & Juneja (2010). Their antimicrobial activity allows them to protect foods from microbial contamination. Hence, it is widely used for seed-coating, controlled release of product into the soil, frost protection, plant protection from microorganisms and as a protective coating for fruits and vegetables (Bautista-Baños et al., 2006; Devlieghere et al., 2004; Hadwiger et al., 2002). The antimicrobial activity of chitosan has been shown to be influenced by several factors such as the characteristics of chitosan (DD, MW) used, the pH of the medium, temperature, source of chitosan and the presence of several food components (Hosseinnejad & Jafari, 2016). Antimicrobial activity of chitosan was higher at low pH, which is due to the fact that the amino groups of chitosan become ionized at acidic pH whilst at higher pH (>6), chitosan tends to lose its charge and may precipitate from solution due to deprotonation of the amino groups (Claesson & Ninham, 1992). On the other hand, the effect of chitosan MW on its antimicrobial activity has generated equivocal results. Some studies reported that increase in MW leads to a decrease in activity, while in other studies, higher MW (HMW) chitosan displayed greater activity than lower MW (LMW) chitosan (Tikhonov et al.,

2006; Tokura et al., 1996). The DD, which determines the content of free amino groups is also an important chemical characteristic. The higher the DD, the higher the positive charge of the chitosan molecule and consequently, the stronger its antimicrobial activity (Takahashi et al., 2008). Likewise, the source of chitosan has been shown to influence its activity. In the study by Jeihanipour et al. (2007), fungal chitosan displayed lower antimicrobial activity than chitosan from crustacean shells. The results showed that less than 100 ppm of crustacean shell chitosan reduced more than 99% of the initial bacteria whereas more than 200 ppm was required for the fungal chitosan. However, both exhibited better inhibitory effects against gram-positive bacteria compared with gram-negative ones. Moreover, chitosan from shiitake stripes displayed more effective antimicrobial activity than chitosan from crab shells (Chien et al., 2016).

2.2.2.3. Applications

In recent years, the development of applications for chitin and its derivatives such as chitosan and chitooligosaccharides have significantly expanded due to the following major characteristics: (a) definite chemical structure; (b) polycationic, innocuous, biodegradable and biocompatible with many organs, tissues, and cells; (c) physically and biologically active; (d) can be chemically or enzymatically modified; and (e) can be processed into several forms such as flakes, beads, powders, membranes, gels, sponges, cottons, and fibres (Dhillon et al., 2013). As a result, they have been used in a wide variety of industrial and medical applications. Some of these are the biomedical, food, environmental, biotechnological and pharmaceutical industries (Kardas et al., 2013) as listed in Table 2.3.

Table 2.3. Applications of chitin and chitosan

Application area	Specific use	Reference
Food	Preservation of food	Hamed et al., 2016
	Food wrapping and packaging	Friedman & Juneja, 2010
	Coating materials	Devlieghere et al., 2004
	Filtration and clarification of fruit juices	Chatterjee et al., 2004
	Food additives	Chen et al., 2005
Wastewater treatment	Removal/recovery of metal ions from aqueous waste water	Nechita, 2017
	Coagulation/flocculating agents for polluted waste wasters	Kumar, 2000; Nechita, 2017
Agriculture	Antimicrobial agents and biopesticides	Bautista-Baños et al., 2006
	Plant seed coating	Devlieghere et al., 2004
	Fertilizer	Devlieghere et al., 2004
Biomedical and pharmaceutical	Tissue engineering	Yang, 2011
	Wound dressing	Azuma et al., 2015
	Encapsulating agent	Alishahi et al., 2011
	Drug delivery, gene delivery	Wang et al., 2011; Jayakumar et al., 2010
Cosmetic	Ingredients for hair and skin care (conditioners)	Hamed et al., 2016; Rinaudo, 2006
Textile and Paper	Fibers for textile and woven fabrics	Dutta et al., 2004
	Paper and film	Dutta et al., 2004

2.3. Extraction Processes

Conventional heated reflux extraction is the most common method for extracting polysaccharides. The yield of this method depends on extraction time and temperature. The use of high temperatures and long extraction times may lead to the degradation of the polysaccharides and a decrease in their pharmacological activity (Jia et al., 2013). In recent times, several novel techniques are being employed for the extraction of polysaccharides such as microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE) and enzyme-assisted extraction (EAE). Each extraction method, however, has its advantages and disadvantages. It has been shown that the extraction procedures have a significant impact on the yield and to some extent, the structural characteristics of polysaccharides as well as their biological activities (Ale et al., 2011; Dong et al., 2016). In the study by Wang et al. (2016), MAE extracts displayed the best antioxidant activity while UAE extracts exhibited the least antioxidant activity. Moreover, MAE was observed to have a higher yield (Dong et al., 2016). The extraction method is therefore, a vital factor that may determine the characteristic attributes such as the MW, DD, purity, crystallinity and biological activity of the final purified chitin.

2.3.1. Conventional chemical extraction (CE)

Chitin extraction is conventionally carried out using the chemical extraction (CE) process as described earlier. However, the process is slightly modified depending on chitin source. The process begins with the removal of minerals usually by submersion in hydrochloric acid and then treated with an alkaline solution for the removal of proteins (Fig. 2.2). The order of the two steps may be reversed with no adverse effect on the yield and quality of the final product as shown in the study by Kjartansson et al. (2006). The conditions utilized in the industry not only vary widely but they differ in the type and concentrations of acid and base, time and temperature of the

treatments. Demineralization is generally achieved in 0.25 – 2 M Hydrochloric acid (HCl) at 0 -

100 °C for 1 – 48 h, whilst deproteinization is carried out with 0.125 – 2.5 M NaOH solution at 65

– 100 °C for 1 – 72 h (Percot et al., 2003; Tolaimate et al., 2003). The major concerns with this

process are that harsh acid treatments reduce the MW of chitin polymer while high concentrations

of NaOH and high temperature for deproteinization cause undesirable depolymerization and

deacetylation of the chitin polymer.
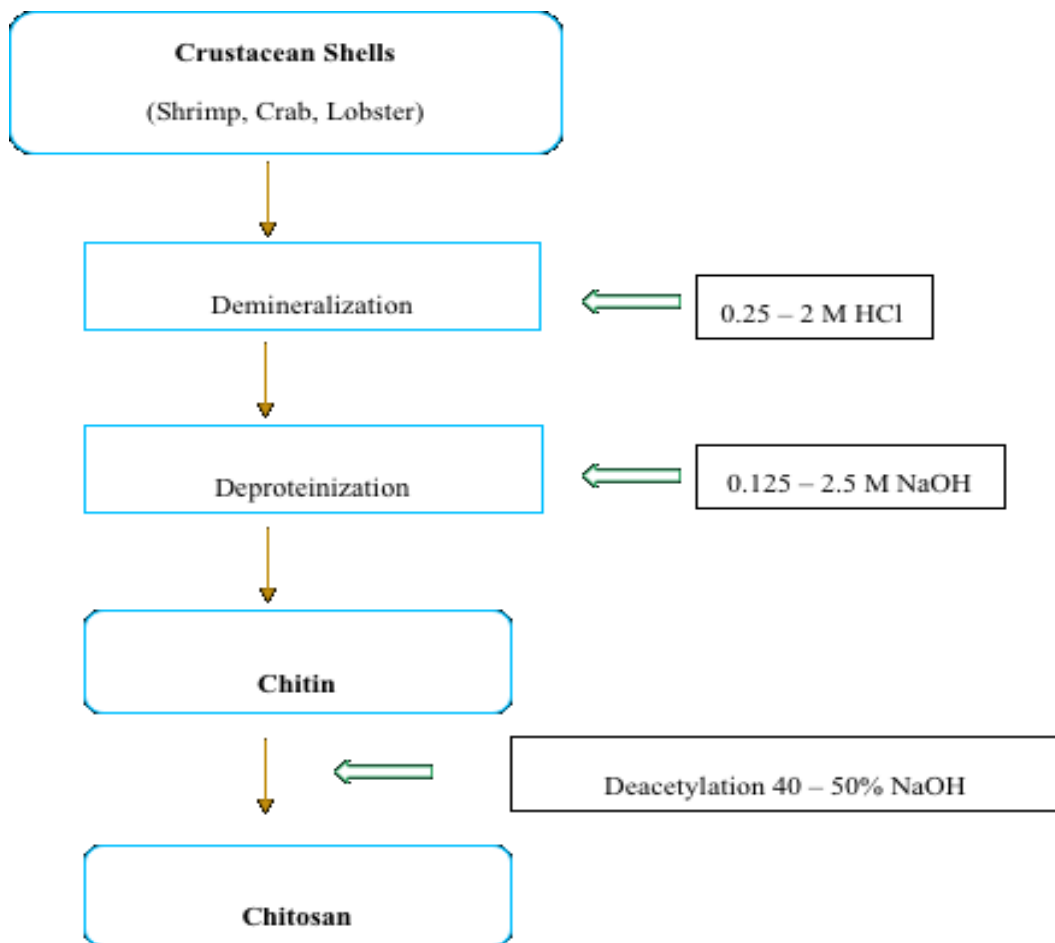
Figure 2.2. Flow diagram of the conventional extraction process for chitin and chitosan production

2.3.2. Microwave-assisted extraction (MAE)

In the last decade, MAE has been successfully applied for extraction of numerous biologically active compounds from a wide variety of natural resources (Cheong et al., 2016; Périno-Issartier et al., 2011; Rodriguez-Jasso et al., 2011; Thirugnanasambandham et al., 2015). MAE is based on the direct application of electromagnetic radiation to a material that can absorb electromagnetic energy (microwaves) and transform it into heat. Unlike conventional heating, microwave irradiation uses the three-dimensional heating of the reaction mass thereby performing chemical transformations in minutes rather than hours (Safari et al., 2014). Unlike the traditional methods, MAE has many advantages as it is faster and requires less solvent. MAE may provide better extraction rate and ultimately, produce a higher yield of substance without significantly changing or altering its nature (Samar et al., 2013). Al Sagheer et al. (2009) and Samar et al. (2013) adopted microwave irradiation technology for the conversion of chitin to chitosan. In this case, the process of deacetylation of chitin and the reaction time was significantly reduced compared to the conventional method. MAE was utilized for the first time in a recent study in the demineralization and deproteinization phases of chitin extraction from shrimp waste (El Knidri et al., 2016). It was observed that chitin and chitosan with similar characteristics were successfully extracted and also microwave technology significantly reduced total extraction time.

2.3.3. Ultrasound-assisted extraction (UAE)

UAE is another advanced method that consumes less energy, solvents and time. It can be defined as the application of high-intensity ultrasound to accelerate the extraction of solid material in a liquid solvent. The UAE method uses sound waves migrating through a medium, inducing pressure

variation and creating small vacuum bubbles or voids which collapse violently (cavitation). This results in localized pressure and heat, which help to solubilize polysaccharides (Kadam et al., 2015). It also has a higher extraction efficiency (Ying et al., 2011). UAE was applied for extraction of polysaccharides from plants (Ebringerová & Hromádková, 2010; Xia et al., 2011) and medicinal fungi (Chen et al., 2010). These suggested a significant reduction in extraction time and solvent requirements and thereby, lead to cost savings. Kjartansson et al. (2006) in their study, investigated the effect of ultrasound on the demineralization and deproteinization phases of chitin extraction from shrimp shells. The results showed that sonication was effective in removal of proteins, but no significant improvement was observed for the removal of minerals. However, the high energy input and pressure required for UAE could be disadvantageous since some of the chitin material could be solubilized and subsequently washed out with the reagents due to depolymerization. Consequently, optimization and moderation of extract conditions are advised to avoid structural alterations and other damages.

2.3.4. Enzyme-assisted extraction (EAE)

Due to the challenges of chemical extraction, biological extraction of chitin and chitosan from crustaceans have been explored (Arbia et al., 2013; Khorrami et al., 2011). In this process, proteolytic enzymes (enzymatic extracts) are used for deproteinization of the crustacean shells. Demineralisation may be performed either by lactic acid bacteria (microbial fermentation) or with HCl. Comparative study on the extraction of chitin from shrimp waste by chemical and microbial methods indicated that the microbial method was advantageous with regards to the biological extraction process (Khanafari et al., 2008). This is because it offered a homogenous and high purity final product and also reduces solvent consumption and energy input. Although various enzymatic treatments have been utilized for recovery of components from crustacean wastes, this has not

been commercially adapted for extraction of chitin. Single step extraction through enzymatic treatment is not feasible as these treatments are incapable of removing minerals completely from the protein-chitin matrix (Gortari & Hours, 2013). A combination of enzymatic deproteinization step and microwave-assisted demineralization step for chitin recovery was reported by Valdez-Peña et al. (2010).

2.4. Summary

Chitin and its derivative, chitosan, have attracted much attention owing to their beneficial properties, which are currently being applied in the agriculture, pharmaceutical, biotechnology and cosmetic industries. The main sources of raw materials for commercial chitin production are the cuticles of various crustaceans, mainly shrimps and crabs, generated as by-products from the seafood industry. Whilst the utilization of these seafood industry by-products contributes towards the management of environmental problems, drawbacks from the use of these raw materials make it necessary for the search of alternate raw materials that can be used for chitin production. The conventional method for the extraction and preparation of chitin and chitosan have been described in this review in addition to its disadvantages. Consequently, alternate sources to serve as raw material for chitin production, as well as extraction methods that will result in speedy, environmentally friendly and efficient chitin and chitosan production, need to be investigated.

Currently, insects and fungi have been explored for their potential as sources for chitin and chitosan production. While there have been successful chitin and chitosan production with the use of the conventional extraction method recorded for both insects and fungi, the identified challenges with this method of preparation remains. MAE, UAE and EAE have been successfully used for the extraction of polysaccharides from a variety of sources. Similarly, a few studies have adopted MAE, UAE and EAE in the deproteinization phase of the chitin production process. However,

studies utilizing these techniques for chitin production from crustacean are limited. Also, there is limited literature information on the application of these techniques for the production of chitin from insects and fungi. Active research on the application of these methods for chitin production from other sources will not only enhance the commercialization prospects of chitin and chitosan, but it will lead to the development of novel processing techniques that are not only eco-friendly but also time-saving leading to cost effectiveness.

CHAPTER 3

EXTRACTION AND PHYSICOCHEMICAL CHARACTERIZATION OF CHITIN FROM BLACK SOLDIER FLY (*HERMETIA ILLUCENS)* MEAL: COMPARISON OF DIFFERENT EXTRACTION TECHNIQUES

3.1. Introduction

Chitin exists abundantly in nature and is synthesized by many organisms including fungi (i.e. in the cell wall) and a broad variety of arthropods (i.e. crustacea, insecta and arachnida) where it forms part of the skeletal structure. Interest in chitin and chitosan has increased over the years because of their beneficial biological properties, which have been employed in agriculture, medicine, biotechnology and bioinspired material science (Philibert et al., 2016). Commercial chitin can be obtained from a few organisms including shrimps, lobsters, crab and generally waste products from the seafood industry (Kaya et al., 2015a). Hence, studies and research conducted on isolation and characterization of chitin and chitin-derivatives have focused generally on crustaceans such as crab, shrimp and krill (Zhao et al., 2010).

Insects, just like crustaceans, possess a chitinous exoskeleton and are found in nearly all environments with a total number of known species estimated to be over two million (Kaya et al., 2014b). In conducive environments, insects are known to reproduce quickly leading to population explosion that can be exploited for chitin production. A few of the studies on chitin and chitosan content of some insects include the beetle larva cuticle and silkworm (*Bombyx mori)* (Zhang et al., 2000), the bumblebee (*Bombus terrestris)* (Majtán et al., 2007) and the housefly (*Musca domestica*) (Ai et al., 2008). Liu et al. (2012) determined the chitin content of beetle (*Holotrichia parallela*) to be 15%, which is indicative of the potential of insects as viable sources of chitin production.

Black soldier fly (BSF; *Hermetia illucens*), is a recognized resource insect. Though native to the Neotropical ecozone i.e. tropical ecoregions of the Americas and South Americas, BSF in recent decades, has spread across all continents. By naturally feeding on organic wastes, BSF transforms biomass nutrients into its biomass and thereby reduces environmental waste. BSF larvae can thrive on a wide range of decaying organic matter and are used in composting piles as they have the potential of converting organic waste into rich fertilizer (Caligiani et al., 2018). BSF is also reported to be a good source of nutrients such as proteins, lipids and minerals (Spranghers et al., 2017). Based on their high protein content, BSF has been proposed to be used as a component of feed for poultry (Veldkamp et al., 2012), fish (St-Hilaire et al., 2007) and pigs (Newton et al., 2005). BSF biomass is also being exploited for the production of biodiesel due to the high-fat content in the prepupae (Li et al., 2015). Furthermore, BSF has been identified as a potential source of chitin by Waśko et al. (2016). In the study, it was observed that the chitin extracted from BSF had similar characteristics to chitin from other insects. As a result, BSF offers not only the advantage of being easily reared and utilized to degrade organic waste; it can also be used to produce chitin, proteins and fat that would be beneficial to the economy.

Chitin is currently extracted using chemical demineralization and deproteinization with strong acids and bases, respectively (Kjartansson et al., 2006). The use of these chemicals has been noted to be harsh as they may cause partial deacetylation of chitin and hydrolysis of the polymer, which results in inconsistent physicochemical properties. Moreover, disposal issues are created by the release of harmful waste products of residual acids and bases. Based on these reasons, it is imperative that alternate extraction methods that will result in rapid, environment friendly and efficient chitin production be investigated. While there has been successful chitin isolation from insects with the use of conventional extraction method, the identified challenges such as significant

amount of alkaline wastes generation resulting in environmental issues, with this method of preparation remains.

Microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE) and enzyme-assisted extraction (EAE) have been successfully utilized for the extraction of polysaccharides from a variety of sources such as seaweeds, plants, fungi and shrimp waste (Cheong et al., 2016; Gortari & Hours, 2013; Kjartansson et al., 2006; Samar et al., 2013). Likewise, a few studies adopted the use of MAE, UAE and EAE in one phase, i.e. mainly the deproteinization phase, of the chitin production process (Gortari & Hours, 2013). In these previous studies, the duration for chitin extraction was significantly reduced when compared to the conventional method. These methods can therefore be used for the extraction of chitin in addition to cost savings and offering sustainable alternatives for chitin production. An investigation into these extraction processes will provide to the industry at large, other potential cost-effective extraction methods to obtain chitin.

Therefore, this present study aimed to examine the potential of BSF as a source of chitin and the use of MAE, UAE and EAE as extraction methods were also evaluated. Additionally, the chitin extracts were examined and compared with lobster shell-derived chitin and commercial chitin obtained via conventional techniques to determine if the physicochemical characteristics of chitin extracts were influenced by the extraction methods employed.

3.2. Materials and Methods

3.2.1. Materials

Black soldier fly in the meal form (BSFM) was purchased from Enterra Feed Corporation (BC, Canada). Hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from Fisher Chemicals (ON, Canada). Protease from *Bacillus lincheniformis* (Alcalase® ≥2.4 U/g), sodium

phosphate monobasic, sodium phosphate dibasic, acetone, potassium bromide (KBr), commercial chitin (from shrimp shells) were purchased from Sigma-Aldrich (ON, Canada) and acetic acid from BDH VWR Analytical (USA). Lobster shell-derived chitin was provided by the Verschuren centre (Cape Breton, NS, Canada).

3.2.2. Extraction of Chitin

3.2.2.1. Proximate composition of BSFM

Proximate composition of BSFM was determined using the methods as described by AOAC, 1990. BSFM samples were weighed before and after oven-drying at 105°C for 24 h to determine the moisture content. The initial and final weight of samples were obtained before and after burning in a muffle furnace (Thermo Fisher Scientific, US) at 600°C for 3 h to measure the ash content. Lipid content in BSFM was analyzed using a Soxhlet apparatus (C. Gerhardt UK Ltd, UK) with petroleum ether for 3 h. The proximate composition was expressed as a percentage (%). Percentage carbon, nitrogen and hydrogen contents were determined using an elemental analyzer (PerkinElmer Inc., US). The proximate analysis was performed in triplicates.

3.2.2.2. Conventional chemical extraction (CE)

The chemical extraction was performed according to the method reported by Kaya et al. (2015b) with minor modifications. Demineralization was carried out by adding 1 M HCl to BSFM powder (1:10 w/v) with constant stirring at 70°C for 2 h. The sample was recovered by centrifugation, washed with distilled water to neutralize and dried for 20 h in an oven at 60°C. The demineralized sample was subjected to alkaline treatment to remove proteins. A 1 M NaOH solution was added to the sample (1:20 w/v) and was stirred at 80°C for 22 h. The residue was subsequently separated

by centrifugation, repeatedly washed with distilled water to neutralize the pH and dried in an oven at 60°C for 30 h. The extracted samples were stored in the freezer at -4°C for later use.

3.2.2.3. Microwave-assisted extraction (MAE)

The microwave-assisted extraction of chitin as described by El Knidri et al. (2016) was adopted with modifications. Samples of BSFM were placed into Teflon extraction vessels followed by the addition of 1 M HCl (1:10 w/v). Microwave irradiation of the mixtures was done at 70°C, 650 W microwave power for 15 mins for mineral removal using CEM Discover and Explorer microwave (MARS 6 230/60 910900, USA). The irradiated mixtures were centrifuged, washed thoroughly with distilled water and dried in an oven at 60°C for 20 h. The dried demineralized samples were placed in Teflon extraction tubes and 1 M NaOH at a ratio of 1:20 w/v was added for the removal of proteins. Extraction occurred at 70°C, 700 Watts microwave power for 40 mins microwave irradiation time. The residue from deproteinization step was recovered through centrifugation, washed with distilled water to neutralize the pH and dried in an oven at 60°C for 30 h. The extracted samples were stored in the freezer at -4°C for later use.

3.2.2.4. Ultrasound-assisted extraction (UAE)

Chitin was extracted from BSFM following the method of Kjartansson et al. (2006) with modifications. The samples were suspended in 1 M HCl (w/v) and sonicated at 20 kHz for 10 mins using a half inch (13 mm in diameter) probe with a removable tip. Sonication was performed at an amplitude of 70%, using a Sonics and Materials Inc. Ultrasonicator (USA VCX750). The beaker containing the sample was placed in cooling liquid (ice) to maintain an average temperature of 40°C – 45°C. The sonicated mixture was centrifuged, vacuum filtered, and the filtrate repeatedly washed with distilled water until neutral pH was attained. The demineralized residue was dried in

the oven for 20 h at 60°C. Removal of proteins was performed by adding 1 M NaOH at 1:20 w/v ratio to demineralized samples and sonicated at 20 kHz for 30 min at an amplitude of 70%. The residue was subsequently separated by centrifugation, repeatedly washed with distilled water to neutral pH and dried in the oven at 60°C for 30 h. The extracted samples were stored in the freezer at -4°C for later use.

3.2.2.5. Enzyme-assisted extraction (EAE)

Enzymatic extraction was performed using the enzyme Alcalase® at optimal conditions of pH and temperature according to the methods described by Ramakrishnan et al. (2013) and De Holanda & Netto, (2006) with modifications. The samples were suspended in 0.2 M sodium phosphate buffer (pH 7, 1:10 w/v) and heated to a temperature of 55°C. The Alcalase®enzyme in ratio 1:50 (w/w) was added to the mixture and enzymatic hydrolysis went on for 3 h. At the end of the hydrolysis, the temperature was increased to 90°C for 10 min to inactivate the enzyme and cooled immediately in an ice bath. The mixtures were centrifuged, residues were washed repeatedly with distilled water and dried in the oven at 60°C for 30 h. The extracted samples were stored in the freezer at -4°C for later use.

Chitin extraction was performed twice for each extraction method.

3.2.2.6. Decolourization and removal of pigments of extracts

The demineralized and deproteinized BSFM powder was treated with acetone (100 mg/mL) for 24 h at room temperature to remove oil and pigments. The samples were thereafter washed with distilled water and oven-dried at 55°C for 6 h.

The % yield of chitin before the removal of pigments was calculated as:

% yield of chitin = [weight of extracted chitin/weight of starting material] ×100

The total % yield of chitin after pigment removal was calculated as:

% yield of chitin = [weight of chitin (acetone washed)/weight of starting material] ×100

3.2.3. Characterization of Chitin

3.2.3.1. Elemental Analysis (EA)

Perkin Elmer series II CHNS/O Analyser 2400 (Perkin Elmer, USA) elemental analyzer was used to determine the percentage carbon (C), nitrogen (N) and hydrogen (H) contents of the chitin extracts. Chitin extracts were weighed using the Perkin Elmer Autobalance AD6000 (USA) with sample weights maintained between 1.5 and 2.5 mg. The weighed samples were inserted into the CHNS/O analyzer for sample combustion at 975°C with adequate oxygen supply and 500°C for reduction and detection via a thermal conductivity detector. The data were collected using the Perkin Elmer 2400 Data Manager.

3.2.3.2. Fourier transform infrared spectroscopy (FTIR)

The structural analysis of all chitin extracts was carried out using FTIR. Extracts were ground to a fine powder with KBr using an agate mortar and formed into a pellet under vacuum. FTIR spectra were recorded using a Nicolet 6700 Spectrometer (Thermo Instruments, Canada). 64 scans were taken with a resolution of 4 cm^{-1}. Transmittance was measured as a function of wavelength between 450 – 4000 cm^{-1}. The absorption peaks of FTIR spectra were used to examine the presence of or change in functional groups. Additionally, FTIR spectra of lobster shell-derived chitin and commercial chitin (shrimp shells) were performed for comparison.

The FTIR spectra were used to estimate the degree of acetylation (DA) of chitin in the extracts. The DA was calculated via a formula using absorbance values at 1658 cm^{-1} and 3450 cm^{-1} (Kaya et al., 2014a):

$$DA\ (\%) = [(A_{1658}/A_{3450}) \times 100] / 1.33.$$

3.2.3.3. Thermogravimetric and derivative thermogravimetric analysis (TGA/DTG)

The chitin extracts were studied concerning the kinetics of thermal decomposition using the Perkin Elmer STA 8000 system, with a temperature rate of change of 10°C min^{-1} heating gradually from 25°C to 650°C. The sample mass ranged between 10.35 mg – 15 mg. The thermogravimetric analysis was performed in a nitrogen atmosphere and data collected using Perkin Elmer's proprietary thermal software, i.e. Pyris Software.

3.3. Results and Discussion

3.3.1. Extract Yield

The percentage (%) yield of chitin from BSFM for four extraction processes; namely CE, MAE, UAE and EAE, were compared in this present study and is given in Table 3.1. The chitin yields ranged between 9.2 – 33.9 % with EAE having the highest yield. The dry weight of the general body of insects from previous studies have been determined to have chitin in the range of 10 – 36%, and these organisms have been suggested to be used as alternative sources for chitin production (Kaya et al., 2014b,c, 2015b; Liu et al., 2012; Zhang et al., 2000). Therefore, the similarity of chitin yield from BSFM to those from other studies suggests that BSF may be used as an alternative source for chitin production.

Table 3.1. Initial and final chitin extract yield (%w/w) from various extraction processes

Extraction Methods	Initial Yield (%)	Final yield (%)

CE	9.7	9.2
MAE	11.4	9.9
UAE	13.7	12.8
EAE	42.3	33.9

When the yields from the four extraction processes were compared, EAE gave the highest yield of 42.3% (Table 3.1). Though EAE had highest yield, it is likely that other excipients such as inorganic materials, protein and fibers were extracted alongside chitin. This was further confirmed by the reduction in the extract yield (approximately 20%) after acetone wash i.e. removal of pigments and oil as shown in Table 3.1 and agrees with the report by Gortari & Hours (2013). They reported that EAE processes alone were incapable of entirely removing minerals from the protein-chitin matrix in a single step. Thus, a demineralization step with acid solution may need to be applied to achieve complete mineral removal. On the other hand, UAE gave the higher yield (12.8%) when compared to MAE (9.9%), resulting in a 29% increase in yield between both processes. Also, UAE and MAE had higher yields than the conventional extraction method at approximately 39% and 7%, respectively. While extraction processes that result in high yields are beneficial and will be of good use for industries aiming to maximize production, it is imperative to note that high extract yield is not synonymous with an increased amount of active ingredients as seen with the EAE process. There is a high probability that the extract may contain high amounts of impurities and minimal compounds of interests.

3.3.2. Characterization of chitin

3.3.2.1. Elemental Analysis

In this study, the nitrogen (N) contents of the extracts ranged from 5.8 – 7.9 % (Table 3.2). The N content from BSFM decreased from 9.52% for all the extraction methods, which is an indication of deproteinization of chitin extracts. However, these values were lower than the theoretical value

of pure chitin i.e. completely acetylated chitin which has been speculated to be about 6.89%, and was considered to be an indicator of the purity of chitin (Liu et al., 2012; Majtán et al., 2007; Sajomsang & Gonil, 2010). Nitrogen levels higher than 6.89% imply that residual protein may be present in chitin sample, while levels lower than 6.89% suggest that inorganic materials may not have been entirely removed from the sample (Sajomsang & Gonil, 2010). Thus, the lower levels of N in the chitin extracts asides of the UAE extract, indicated the presence of inorganic materials. In comparison, commercial chitin (STC) also had N content of 6.57%, which is lower than pure chitin. The UAE extract and lobster shell-derived chitin (LDC) had higher N content of 7.94% and 6.92% respectively, and may be due to the presence of protein residues in chitin extracts. Similar results were obtained by Kjartansson et al. (2006) when ultrasonication was applied to the deproteinization stage of extraction of chitin from shrimp. It was observed that though the application of ultrasound yielded better results regarding the reduction of protein content, the process was still insufficient to remove all the proteins. It is imperative to note however, that extraction processes will not yield extracts void of impurities as clearly seen in the results of this study and previous studies. However, should optimal conditions for each extraction process be adapted, there may be a probability of obtaining extracts with minimal impurities.

Table 3.2. Results of elemental analysis (EA) of chitin extracts from various extraction processes, commercial chitin (STC), lobster-shell derived chitin (LDC) and BSFM

Samples	N (%)	C (%)	H (%)
CE	5.8	45.2	6.6
MAE	5.9	46.7	6.7
UAE	7.9	47.1	6.6
EAE	5.9	43.9	6.2
STC	6.6	44.9	6.6
LDC	6.9	46.6	6.7
BSFM	9.5	40.4	5.7

CE: conventional extraction method chitin extract
MAE: microwave-assisted extraction method chitin extract
UAE: ultrasound-assisted extraction method chitin extract
EAE: enzyme-assisted extraction method chitin extract

3.3.2.2. FTIR analysis

FTIR is an important technique for structural analysis of biomolecules and is especially useful in determining functional groups present in a given sample. When the FTIR spectra (Appendix A) of the chitin extracted from BSFM using various extraction methods were examined (Table 3.3), characteristic bands for α-chitin were observed. Amide I bands were observed as follows: CE, 1659 and 1626 cm^{-1}; MAE, 1658 and 1627 cm^{-1}; UAE, 1660 and 1626 cm^{-1}; EAE, 1655 and 1632 cm^{-1}; commercial chitin, 1663 and 1635 cm^{-1}; and lobster shell-derived chitin (LDC), 1654 and 1628 cm^{-1}. Likewise, amide II bands were observed at 1559 cm^{-1} for CE, MAE and commercial chitin; and 1558 cm^{-1} for both EAE and lobster shell-derived chitin (LDC). The existence of similar bands was noted in chitin extracted from insects in other studies (Kaya et al., 2015b; Liu et al., 2012; Waśko et al., 2016). For α-chitin, the amide I band is observed at two characteristic bands; 1660 and 1620 cm^{-1}, while it is only observed at 1660 cm^{-1} for β-chitin (Kaya et al., 2014a). Other characteristic bands for α-chitin are the N-H bend, C-N stretch (also known as amide II), O-H stretching, N-H stretching and asymmetric CH$_3$ stretching. These bands are respectively found within the following ranges in the FTIR spectra: 1600 – 1550 cm^{-1}, 3550 – 3200 cm^{-1}, 3310 – 2800 cm^{-1} and 3000 – 3000 cm^{-1} respectively. Thus, it can be inferred that the chitin structures are in α crystal form. However, the amide I band did not show a well-defined peak at 1630 cm^{-1} for EAE chitin extract (Appendix A; Fig A4). Another FTIR spectral characteristic for distinguishing α- and β-chitin is the CH ring stretching band, which is around 895 cm^{-1} in α-chitin and 890 cm^{-1} in β-chitin (Kumirska et al., 2010). Table 3.3 showed CH ring stretching bands of lobster shell-

derived chitin, commercial chitin and all the extracts were at 896 cm^{-1} apart from the EAE extract, which further confirmed that extracts from BSFM as α-chitin.

In summary, the FTIR spectra (Appendix A) of the extracts, lobster shell-derived and commercial chitins were similar to each other with minor differences noted in the intensities and wave numbers of the absorption spectra, especially in the EAE spectra which could be a result of the presence of impurities. Thus, it can be inferred that the extraction processes yielded chitin that was comparable or similar to the commercial chitin product.

Table 3.3. FTIR bands and interpretations of chitin extracts from various extraction processes, commercial chitin and lobster shell-derived chitin

Vibration modes (Kaya et al., 2014a; Kaya et al., 2015a)	Wavenumber (cm^{-1}) frequency					
	CE chitin	MAE chitin	UAE chitin	EAE chitin	Commercial chitin	Lobster shell-derived chitin
OH stretching	3443	3441	3443	3428	3488	3443
NH stretching	3269-3107	3265-3108	3265-3108	3270-3107	3266-3105	3271-3207
CH$_3$ sym. and CH$_2$ asym. stretching	2926	2924	2924	2925	2933	2934
CH$_3$ asym. stretching	2891	2891	2865	2854	2891	2890
C=O secondary amide stretching (Amide I)	1659	1658	1660	1655	1663	1654
C=O secondary amide stretching (Amide I)	1626	1627	1626	1632	1635	1628
NH bending and CN stretching (Amide II)	1559	1559	1558	1549	1559	1558
CH$_2$ bending and CH$_3$ deformation	1430	1431	1433	1454	1418	1419
CH bending and sym. CH$_3$ deformation	1378	1378	1378	1376	1379	1378
CH$_2$ wagging (Amide III)	1315	1315	1315	1315	1314	1314
Asym. bridge oxygen stretching	1158	1158	1155	1155	1159	1159
Asym. in-plane ring stretching	1116	1116	1116	1113	1117	1119
C-O-C asym. stretching in phase ring	1073	1073	1073	1073	1072	1074
C-O asym. in phase ring	1028	1028	1027	1031	1027	1026
CH$_3$ wagging	953	953	953	953	952	953
CH ring stretching	896	896	896	875	896	896

3.3.2.3. DA

The DA values of chitin extracts from the various extraction processes, lobster shell-derived chitin and commercial chitin were calculated using the formula (in section 3.2.3.2). DA values of chitin extracts were in the range of 80 – 110% (Table 3.4) with MAE extract having the lowest DA value at 80.6% and EAE extract having the highest at 110.3%. The DA is one of the essential characteristics of chitin and is dependent on the source material and extraction methods. The FTIR absorption peak at 1658 cm^{-1} denotes the absorbance of the amide I band as a measure of the N-acetyl group content while the peak at 3450 cm^{-1} denotes the absorbance of the hydroxyl band.

Table 3.4. Degree of acetylation (DA) values (%) of chitin from various extraction methods, commercial chitin (STC) and lobster shell-derived chitin (LDC).

Extraction Methods	CE	MAE	UAE	EAE	STC	LDC
DA (%)	96.4	80.6	97.5	110.3	97.5	98.2

According to Samjonsang and Gonil (2012), DA values of chitin higher than 100% implies the presence of mineral residues while DA value much less than 100%, implies that protein residues may be present. In earlier studies, the DA values for chitin isolated from different organisms were determined to be 102% for cicada sloughs, 104% for rice-field crab shells, 87% for bumblebees, 93% for the beetle and 99% for shrimp (Kaya et al., 2014b; Matjan et al., 2007). From the DA values reported in other studies, a 100% value was not obtained. This implied that some mineral or protein residues were still present in the chitin samples, which should be expected as the possibility of obtaining a pure sample from extraction may not be possible. Consequently, just as noted in previous studies, the results in this study also suggest the presence of some protein and mineral residues in the chitin extracts as seen in the MAE extract and EAE extract. On the other hand, CE and UAE extracts had high DA values of 96.4% and 97.5% respectively which were also

similar in range to DA value obtained for commercial chitin (97.5%) and lobster shell-derived chitin (98.2%). This suggests that the extraction methods and source of chitin utilized may have an overall effect on the DA values and affected chitin composition.

3.3.2.4. TGA

The thermograms of the chitin extracts (Appendix B) in this study are similar to previous studies i.e. the weight of the sample decreasing with temperature and mass loss was observed at two stages. Details of the TG-DTG results are presented in Table 3.5., and is similar to decomposition temperatures of chitin from other studies which have been determined to be between 300° and 400 °C (Juárez-de la Rosa et al., 2012; Kittur et al., 2002; Paulino et al., 2006). From the DTG curves, two peaks are recognized, and they can be associated with the important thermal transformations of the chitin structures. The first peak which is related to the first mass loss is observed between 0° and 150°C and is attributed to the evaporation of water already within the polymer structure. The second mass loss occurs between 150° and 650°C with DTG_{max} peaks between 360° and 390°C for all the extracts. This loss can be attributed to several processes such as degradation, depolymerization and denaturation. In this phase, the following occurs: aliphatic compounds (CH_2, CH_3 functional groups) are separated from the chitin structural ring. After DTG_{max} peak, the amide groups (C=O, N-H), saccharide structure (C-O-C, C-O-P and P-O-P), and the phosphodiester groups (CO, P=O and PO_2) are degraded (Juárez-de la Rosa et al., 2012). The DTG_{max} disintegration temperatures of α-chitin isolated from other organisms such as crab, krill, shrimp and insects ranged between 350° and 390°C (Juárez-de la Rosa et al., 2012; Kaya et al., 2014b; d). Thus. the TGA results of chitin extracts agree with results from previous studies.

The thermal stability of the extracted chitin from BSFM fell in the order CE > MAE > EAE > UAE. Though it would have been expected that the DTG_{max} of the chitin extracts be similar with little or no disparity, this was not the case. It can therefore be inferred that the extraction processes utilized to extract chitin may have affected the overall composition of chitin extract. The high energy input and pressure required for UAE and MAE could be detrimental to the integrity of the extracts, as these technologies have been associated with depolymerization of the linear chain molecules (Grönroos et al., 2004). On the other hand, when compared with chitin from crustacean, insect chitin is noted to be more stable i.e. CE > LDC > STC, which was also observed in the study by Kaya et al. (2015b).

Table 3.5. Results of TG-DTG analyses of chitin from various extraction methods, commercial chitin (STC) and lobster shell-derived chitin (LDC)

Sample	Temperature range (°C)	Mass loss (wt %)	Total mass loss (wt %)	DTG_{max} peak (°C)
CE	0-150	2.9	80.8	386.3
	150-650	77.9		
MAE	0-150	3.45	80.5	380.9
	150-650	77.1		
UAE	0-150	3.5	82.1	365.2
	150-650	78.6		
EAE	0-150	2.2	66.2	369.0
	150-650	64.1		
STC	0-150	3.3	85.2	379.9
	150-650	81.9		
LDC	0-150	3.8	83.9	384.5
	150-650	80.1		

3.4. Conclusion

The potential of using BSFM as an alternative source for chitin production was investigated in this study. The microwave-, ultrasound- and enzyme- assisted extraction processes were evaluated for their potential to be used as alternative extraction methods to obtain chitin. All four extraction methods were successful in isolating chitin. The physicochemical properties of the chitin extracts in this study were compared to those of commercially available chitin and lobster shell-derived chitin. The comparisons indicated similarity between the chitin extracts from the insect source (BSFM) regarding crystal structure (IR), N content and DA values with commercially available chitin. Also, the thermal stability of insect chitin was higher than chitin sourced from lobster shell. Thus, the findings from this study suggest that the black soldier fly can be used as an alternative source of chitin and be used for chitin production.

The disparities observed in the physicochemical properties were noted in the extracts from the other extraction processes which may be attributed to the presence of impurities in the extracts. There is a high probability that the extracts obtained from EAE had inorganic materials present, while the extracts from MAE and UAE may have undergone further depolymerization. Besides, it is possible that the parameters and conditions set for MAE and UAE may have contributed to further breakdown of the polysaccharide molecule. Though higher yields of chitin were obtained from the other extraction processes, the actual amount of active ingredient may be minimal when compared to amount obtained from CE. However, the application of these technologies (MAE and UAE) gave the advantage of lesser extraction time when compared to CE and are likely to be eco-friendly. While extraction conditions may influence and affect the final outcome of extraction products both quantitatively and qualitatively, there are other factors such as raw materials, location of raw materials and species, that should be considered. Findings from this study suggest

that MAE and UAE were more effective and beneficial extraction processes than EAE for chitin extraction. However, further research will need to be conducted to obtain optimal extraction conditions for MAE and UAE to determine the best fit for efficient chitin extraction.

CHAPTER 4

A COMPARISON OF PHYSICOCHEMICAL, ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES OF CHITOSAN FROM CRUSTACEAN, INSECT AND FUNGI

4.1. Introduction

Chitosan is a biopolymer derived from partial deacetylation of chitin, a major component of crustacean shells, fungal cell wall and insect cuticles. The deacetylation of chitin to produce chitosan is accomplished using three different methods, namely; chemical (hot alkaline), microbial, and enzyme-assisted methods. Chitosan is commercially obtained through alkaline deacetylation of chitin from crustaceans using hydroxides, in most cases, sodium hydroxide (NaOH) and at high temperatures (130 °– 140 °C). When the degree of deacetylation is 50% or higher, chitosan starts to solubilize in aqueous acidic medium due to the protonation of its amino groups and thereby, allow the polymer to interact with diverse types of molecules (Younes et al., 2014).

Interest in chitosan resides in the fact that it exhibits desirable biological and functional traits including antimicrobial and antioxidative properties. Its antimicrobial activity against bacteria has been reported in the literature (No et al., 2007; Rabea et al., 2003; Shahidi & Abuzaytoun, 2005). Chitosan has subsequently been studied for use in food packaging and preservation (Friedman & Juneja, 2010). Similarly, the antioxidant activity of chitosan has been investigated and as a result it has been explored for use in different applications in the food industry (Chien et al., 2007; Georgantelis et al., 2007; Martín-Diana et al., 2009; Raybaudi-Massilia et al., 2009). The molecular weight, degree of deacetylation and chitosan source (i.e. raw material and extraction method) are some of the important properties that have been considered to influence its biological

activities. A majority of the studies on the antimicrobial and the antioxidant activities of chitosan have focused on crustacean shell chitosan with limited research on chitosan from insect and fungi.

In this study, the physicochemical characteristics, antioxidant and antimicrobial activity of chitosan obtained from insect chitin extracted by different extraction methods were examined and compared with chitosan sourced from fungi, lobster shell and commercial chitosan obtained by conventional method.

4.2. Materials and Methods

4.2.1. Materials

Sodium hydroxide (NaOH) and 95% ethanol were purchased from Fisher Chemicals (Belgium). Sodium acetate (CH_3COONa) was purchased from OmniPur® (Germany) and acetic acid (CH_3COOH) from BDH VWR Analytical (USA). FerroZine^{TM}, DPPH, iron (II) chloride ($FeCl_2$), deuterium oxide (D_2O), acetic acid-d4 (CD_3COOD), ninhydrin reagent, potassium bromide (KBr), glucosamine hydrochloride, commercial chitosan (from shrimp shells), EDTA, Mueller-Hinton agar 2 (MHA), Mueller-Hinton Broth (MHB) and Tryptic soy agar (TSA) were purchased from Sigma-Aldrich (ON, Canada).

Five strains of bacteria were used in this study, namely; *Klebsiella pneumoniae* (Kleb), *Staphylococcus aureus* ATCC 6538 (SA 6538), *Staphylococcus aureus* ATCC 25928 (SA 25928), *Streptococcus mutans* SA38 (SA 38), and *Escherichia coli* ATCC 25922 (E. coli), which were provided by the Department of Biology, Cape Breton University (NS, Canada). Lobster shell-derived chitosan was provided by the Verschuren center (Cape Breton, NS, Canada) and fungal chitosan samples provided from a pre-commercial source.

4.2.2. Extraction of Chitosan

Chitosan was obtained by removal of acetyl groups from chitin using NaOH according to a previously described procedure (Trung et al., 2006). The chitin extracts obtained from the four extraction processes in section 3.2.2 were treated with 50% (w/w) NaOH (100 mg/mL) for 20 h at 65°C. Thereafter, the extracted chitosan was washed using distilled water until the pH was neutralized, followed by overnight oven-drying at 55°C. The % yield of chitosan was calculated as:

Initial yield of chitosan (%) = (weight of extracted chitosan/weight of chitin) $\times$ 100

Purification of chitosan

Purification of chitosan extracts was carried out by treating the extracts with 0.1 M acetic acid (1mg/ml) for 18 h at 60°C with constant stirring. The filtrates were recovered by vacuum filtration and the dissolved chitosan samples were reprecipitated by the addition of 1 M NaOH to increase the pH of the solution to 8.5 – 9.0. The samples were subsequently recovered by centrifugation for 20 min at 4,000 rpm, washed repeatedly with distilled water to neutralize the pH and the precipitate was freeze dried. The % yield after purification was calculated as:

Final yield (%) = (weight of chitosan precipitate/weight of starting material) $\times$ 100

4.2.3. Characterization of chitosan

4.2.3.1. Elemental Analysis (EA)

The percentage carbon (C), nitrogen (N) and hydrogen (H) contents of the chitosan samples were determined as described in section 3.2.3.1.

4.2.3.2. Fourier transform infrared spectroscopy (FTIR)

The structural analysis of all chitosan samples was studied using FTIR as described in section 3.2.3.2.

4.2.3.3. Thermogravimetric and derivative thermogravimetric analysis (TGA/DTG)

Thermal decomposition of chitosan samples was determined as described in section 3.2.3.3.

4.2.3.4. Quantitative determination of chitosan

The quantitative determination of chitosan in the samples was performed according to the method reported by Prochazkova et al. (1999) with minor modifications. The assay principle was based on the reaction of ninhydrin with a primary amino group, i.e. GlcN (2-amino-2-deoxy-β-D-glucopyranose) to form a coloured reaction product called Ruthermann's purple. Chitosan solution was prepared by dissolving chitosan samples in 1% acetic acid solution (1mg/ml). To 2 ml of chitosan solution, 1 ml of ninhydrin reagent was added, mixed thoroughly and incubated in boiling water bath for 10 min. Once the sample cooled to room temperature, 5 ml of 95% ethanol was added to each tube and vortexed. The mixture (200 μL) was then transferred to a 96-well plate and absorbance was measured at 570 nm. Glucosamine Hydrochloride (G-HCl) was used to obtain a standard curve and chitosan content of the extracts was expressed as % GlcN equivalent.

4.2.3.5. Molecular weight (MW) determination of chitosan

The molecular weight distribution of chitosan samples was measured by gel-permeation chromatography (GPC) with a refractive index (RI) detector (Agilent, 1260 Infinity II Multi-Detector GPC/SEC System, CA, USA). The samples were dissolved in 0.1 M sodium acetate/0.2 M acetic acid buffer (pH 4.4) at a concentration of 2 mg/mL and were filtered through 0.45 μm

pore size syringe filter (Basix^{TM}, Fisher Scientific, NH, USA) before injection. The GPC/SEC system calibration and performance were verified using Agilent GPC/SEC Calibration kits containing pullulan polysaccharides with molecular weights ranging from 0.18 – 642 kDa. The GPC measurements of chitosan was carried out by eluting samples through 2 × PL aquagel-OH MIXED-M columns (300 × 7.5 mm, 8 μm, Agilent, CA, USA) with 0.1 M sodium acetate/0.2 M acetic acid solution used as mobile phase at a flow rate of 1 mL/min. A differential refractive index increment (dn/dc) value of 0.142 for chitosan was used and the data obtained were analyzed using the Agilent GPC/SEC software manager.

4.2.3.6. Proton nuclear magnetic resonance analysis (^{1}H NMR)

Chitosan samples were analyzed by proton nuclear magnetic resonance spectroscopy (^{1}H NMR) on a Bruker Avance II 400 MHz spectrometer. The solutions of chitosan were prepared by dissolving 10 mg of samples in 1 ml of CD$_3$COOD/D$_2$O (2% v/v) solution, and the mixture was stirred at 70°C until complete dissolution of the polymer. Approximately 1 ml of the chitosan sample solution was transferred to 5 mm NMR tubes. The sample tube was inserted in the magnet and allowed to reach thermal equilibrium at 70°C for 10 min before performing the experiment.

The degree of deacetylation (DD) was calculated using integrals of the peak of proton H1 of deacetylated monomer (H1-D) and of the peak of the three protons of acetyl group (H-Ac) according to Lavertu et al. (2003);

$$DD\% = \left(\frac{H1D}{H1D + \frac{HAc}{3}} \right) \times 100$$

4.2.4. Antioxidant assays

4.2.4.1. Chelating ability on ferrous ions

Chelating ability was determined according to the method described by Dinis et al. (1994) with modifications. Each chitosan sample (5 mg/mL, 1.35mL) in 0.2% acetic acid solution was mixed with 50 μL of $FeCl_2$ (2 mM) for 10 min (RT). The reaction was initiated by the addition of 100 μL of FerroZine^{TM}. The mixture was shaken vigorously and incubated for another 10 min (RT). The mixture (200 μL) was then transferred to a 96-well plate and absorbance was measured at 562 nm against a blank. All assay components except the sample, which was substituted with an equal volume of deionized water was used as a blank. Ethylenediaminetetraacetic acid (EDTA) was used as a positive control. The inhibition percentage of ferrozine-Fe^{2+} complex formation was calculated using the formula given below:

$$\text{Metal chelating effect (\%)} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

4.2.4.2. Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals

The DPPH radical scavenging activity of chitosan samples was determined according to the method described by Kaya et al. (2014c). Each chitosan sample (5 mg/mL, 4 mL) in 0.2% acetic acid solution was mixed with 1 mL of methanolic solution containing DPPH radicals. The mixture was shaken vigorously and left to stand for 30 min in the dark and the absorbance was then measured at 517 nm against a blank. The blank experiment contained all assay components except the sample, which was substituted with an equal volume of deionized water. Ascorbic acid was used as a positive control. The scavenging ability was calculated as follows:

$$\text{Scavenging ability (\%)} = [(\Delta A_{517} \text{ of control} - \Delta A_{517} \text{ of sample}) / \Delta A_{517} \text{ of control}] \times 100$$

4.2.5. Antimicrobial assay

4.2.5.1. Inoculum and sample preparation

Each bacterial strain was sub cultured overnight at 37°C on tryptic soy agar plates. Bacterial colonies from the plates were thereafter suspended in 9% sterile saline solution and suspension adjusted to achieve a turbidity equivalent to a 0.5 McFarland standard, which contains about 1 - 2 x 10^8 colony forming units (CFU)/ml.

Chitosan extracts were dissolved in 1% (v/v) acetic acid solution at a concentration of 5mg/ml. After stirring and dissolution of chitosan, the solutions were sterilized by autoclaving at 121°C for 20 min and stored at 4°C for later use.

4.2.5.2. Kirby-Bauer disc diffusion assay

A 100 µL aliquots of each bacterial suspension were spread on the surface of the solidified agar medium using a L-form glass spreader. Chitosan sample discs, prepared by impregnating 50 µL (250 µg/disc) of chitosan solution on sterile filter paper discs (6 mm) followed by air-drying, were placed on the top of the agar plates. The plates were then incubated at 37°C for 24 h. Gentamycin discs (10 µg, antibiotic) were used as positive control. Filter paper discs loaded with 50 µL of acetic acid excluding chitosan was used as negative control. The presence of inhibition zones was measured in millimeters (mm) and considered as evidence of antimicrobial activity.

4.2.5.3. Broth dilution assay (bacterial colony count)

E. coli was chosen as a model bacterium for this assay. Chitosan polymer suspension were prepared in 1% (v/v) acetic acid at a concentration of 1% (w/v) before being added to MHB to

give a final chitosan concentration of 0.1% (w/v). Bacterial inoculum suspension (0.5 McFarland standard) was diluted with MHB to obtain a final inoculum concentration of about 5×10^5 CFU/ml. A 50 µL of diluted inoculum were inoculated into 5 ml of chitosan/MHB solution and mixed thoroughly. The solutions were thereafter incubated at 37°C for 24 h with samples being withdrawn at different time points: 0, 2, 4, 8, 12, and 24 h, during the incubation period. A 100 µL of serially diluted (1×10^{-5}) samples were spread on the surface of the solidified MHA medium using a L-form glass spreader and incubated at 37°C for 48 h. MHB with 1% acetic acid was prepared as negative control while MHB with only the bacterial inoculum was prepared as blank. The bacterial colonies were counted, and the results were expressed as log CFU/ml. The antimicrobial activity of the chitosan samples was investigated depending on the time-course change of the number of viable colonies in comparison with the number of colonies present in MHA plates containing the blank sample.

4.3. Statistical analysis

Quantification of chitosan, antioxidant and antimicrobial assays were performed in triplicate. The results were expressed as mean ± standard deviation and analysed for statistical significance at $p < 0.05$, using the GLM procedure in Minitab 17 Statistical Software (Minitab Inc., PA, USA). One-way analysis of variance (ANOVA) was carried out using the Tukey's multiple comparison test.

4.4. Results and Discussion

Chitosan obtained from insect chitin from the four processes in chapter 3 are noted, CCH for conventional extraction method, MCH for microwave-assisted extraction method, UCH for ultrasound-assisted extraction method and ECH for enzyme-assisted extraction method. Two

samples were prepared for the EAE chitosan extracts; one from EAE chitin that was subjected to acetone wash (ECHa) as a decolourization step, and the second from EAE chitin with no acetone wash (ECHb) in order to examine the effect (if any) of acetone wash on the chitin extract from EAE method. Fungal chitosan samples received from a pre-commercial source are noted FCHa and FCHb. Lobster shell-derived chitosan is noted LDCH and commercial chitosan is noted SCH.

4.4.1. Chitosan yield

The percentage (%) yield of chitosan obtained from chitin extracted from BSFM for four extraction processes, namely; CCH, MCH, UCH and ECH was compared in this present study. Table 4.1 shows the initial chitosan yield from chitin extracted from BSFM *via* the different extraction techniques. From the results, conventional chemical extraction method had the highest chitosan yield of 88%, from its chitin extract, with ultrasound-assisted chitin extract having the least chitosan yield. The chitosan yield from BSFM in this study is relatively higher than previous reports on insect derived chitosan yield of 74 - 76% (Kaya et al., 2015a) and ornate rock lobster (*Panulirus ornatus*) derived chitosan yield of 74.3% (Oduor-Odeto et al., 2007). The high yield indicates the potential of BSFM as a sustainable and potential alternative source for chitosan production.

Since similar conversion methods were employed, it was expected that the chitosan % yields from chitin extracts be same, however, the results show otherwise. This further buttress the fact that higher yields of an extract is not necessarily synonymous with higher yields of the active ingredients as can be seen in this case. When the final chitosan yields were compared, the % yield of CE, MAE and UAE were in the range 6 - 8% with a 1 - 2 % increase yield observed i.e. CE yield was 2% > than UCH. EAE on the other hand, had the highest chitosan yield (23%) which as noted from the previous chapter may contain other inorganic materials and impurities.

The yield of chitosan from EAE chitin with no acetone wash (ECHb) was lower than the yield from EAE chitin with acetone wash. It is possible that the acetone wash removed some of the impurities in the chitin extract to give a higher yield of chitosan.

Table 4.1. Chitosan yield (%w/w) from insect chitin obtained from various extraction processes

BSFM chitosan	Initial Yield (%)	Final yield (%)	% Change
CCH	87.5	8.5	90.3
MCH	62.5	7.1	88.6
UCH	45.0	6.2	86.2
ECHa	55.0	23.3	57.6
ECHb	41.3	n/a	n/a

4.4.2. Characterization of chitosan

4.4.2.1. Elemental analysis

The results of the elemental analysis of the chitosan polymers employed in this study are given in Table 4.2. The nitrogen content of CCH, MCH, FCHa, LDCH and SCH were comparable with N content noted for chitosan from a previous study (Blanco et al., 2013), which ranged between 6 – 8%. On the other hand, UCH, ECHa, ECHb and FCHb had N values lower than 5% with ECHb having the least N value of 3.52%. Also, both EAE chitosan extracts had lower N content than its chitin extract (Section 3.3.2.1) Likewise, UCH had a lower N content. This may be due to the polymer being further hydrolyzed down to dimers and monomeric units resulting in solubilization in the aqueous fraction, which could have been lost in the conversion process.

Interestingly, while FCHa had N value similar to commercial chitosan product (SCH), its counterpart, FCHb had a lower N value. While we are unaware of the particular extraction conditions utilized for the production of each fungal chitosan, it can be assumed that different extraction protocols or fungal sources may have been employed, thus yielding products with varied

N values. From the present results, it is possible that the chitosan source may have an influence on the N value content of the extract.

Table 4.2. Results of elemental analysis (EA) of chitosan samples from three sources; insect chitin obtained from various extraction processes, fungi and crustacea: lobster and shrimp

Samples	N (%)	C (%)	H (%)
CCH	6.85	43.65	7.16
MCH	6.21	44.87	7.25
UCH	4.44	42.48	6.14
ECHa	4.64	39.87	6.25
ECHb	3.52	44.35	7.05
FCHa	7.06	40.72	7.15
FCHb	3.71	33.70	6.26
LDCH	7.67	41.45	6.82
SCH	7.51	41.03	6.81

ECHa: chitosan from EAE insect chitin with acetone wash
ECHb: chitosan from EAE insect chitin with no acetone wash

4.4.2.2. FTIR analysis

FTIR spectra of chitosans were examined and characteristic absorption peaks for the amide I band (carbonyl $v(C=O)$) and amide II (amine $v(NH_2)$ at 1650 cm^{-1} and 1590 cm^{-1}, respectively, were noticed (Peng et al., 1994; Qu et al., 2000). According to Table 4.3 and Figures in Appendix C, the FTIR spectra of insect, fungal and crustacean chitosan showed the presence of these bands. This is an indication of chitosan formation, which is also in agreement with literature reports. The absorption band at 3000 – 3500 cm^{-1} was due to symmetric stretching vibration of NH_2 and OH groups while the peak at around 2885 cm^{-1} was due to C-H stretching in the pyranose ring. The detailed spectral bands of the chitosan extracts are presented in Table 4.3.

When the spectra of the insect chitosan from the different extraction processes were compared, similar absorption peaks were observed in the chitosan extracts with minor differences noted in

the intensities and wave numbers of the absorptions especially, with the EAE chitosan extracts (ECHa and ECHb). These minor differences were also observed in the FTIR spectra of the chitin extracts, as noted in Chapter 3. In the chitosan FTIR spectra (Appendix C), the intensity of the band peaks at the amide I and C -H stretching region of the EAE chitosan spectra (Fig. C4 & C5) were larger than in the spectra of the other chitosan extracts. In addition, the EAE spectra do not show a defined and well detailed structure like the other spectra, which could be attributed to different packaging of the macromolecules or weaker inter- and intra-molecular hydrogen bonding in the chitosan extract (Kurita et al., 2005). Furthermore, unlike the other spectra that seem to show a doublet peak at the amide I region (1620 -1650 cm^{-1}), the fungal spectra only display one distinct peak (Fig. C6 & C7). These differences when observed in the amide I region of chitin are usually used to differentiate between the α- and β- configurations. Though there is limited information on the FTIR structural differences of α- and β- chitosan, one may assume that these differences in the chitin structure may be applicable and, in this case, may infer that the fungal chitosan may be of β- configuration. However, most of the studies on a β- chitosan show that they are primarily obtained from squids (He et al., 2016; Jung & Zhao, 2013). The study by Jung & Zhao (2013) further revealed that the inter- and intramolecular hydrogen bonding and CH stretching of chitosan structure changed with changes in the DD and MW. The NH bands in higher DD α-chitosan samples were not distinguished as in the lower DD samples while there were no significant differences in the OH bands. FCHa and FCHb have DD values of 94 and 92% respectively and thus, could possibly explain the differences noted in their spectra when compared to the others.

Overall, the FTIR spectra of the chitosans from the three different sources can be said to be comparable or similar to each other and in the α- configuration.

Table 4.3. FTIR bands and interpretations of chitosan from insect chitin obtained from various extraction processes, fungal chitosan, lobster shell-derived chitosan and commercial chitosan

Vibration modes (Kaya et al., 2014a; Kaya et al., 2015a)	Wavenumber (cm⁻¹) frequency							
	CCH	MCH	UCH	ECHa / ECHb	FCHa	FCHb	LDCH	SCH
ν(NH2) assoc. in primary amines and ν(OH) assoc. in pyranose ring	3441	3441	3437	3428 / 3429	3440	3429	3442	3422
ν(C-H) in pyranose ring	2852	2852	2852	2852 / 2852	2875	2871	2881	2878
ν(C=O) in $NHCOCH_3$ group (Amide I)	1658	1652	1658	1652 / 1653	1658	1652	1652	1653
ν(NH_2) in $NHCOCH_3$ group (Amide II)	1597	1579	1579	1577 / 1574	1599	1598	1597	1595
δ(CH_2) in CH_2OH group	1422	1421	1422	1426 / 1423	1421	1420	1420	1420
δ_s(CH_3) in $NHCOCH_3$ group	1380	1379	1379	1379 / 1376	1385	1385	1376	1378
δ(C-H) in pyranose ring	1323	1322	1321	1321 / 1321	1320	1316	1322	1323
ν_s (C-O-C) glycosidic linkage	1155	1155	1157	1153 / 1157	1155	1155	1154	1156
ν_{as} (C-O-C) glycosidic linkage	1090	1081	1073	1070 / 1074	1076	1083	1085	1077
Pyranose skeletal ring vibrations	895	896	895	878 / 875	890	895	896	895

4.4.2.3. Quantification of Chitosan

The reaction of ninhydrin (triketohydrindene hydrate) with a primary amino group to form a coloured product, diketohydrindylidene-diketohydrindamine (Ruhemann's Purple) has been known and studied for years and have been used for amino acid analysis and chitosan quantification in drug formulations (Leane et al., 2004). When the assay was first used to quantify chitosan (Curotto & Aros, 1993), it was demonstrated that the assay could be used reliably in the quantification of the chitosan samples studied. Thus, it is expected that GlcN units in the chitosan extracts would give a positive ninhydrin reaction since the reaction is specific to primary amino groups. The ninhydrin reaction assay was therefore used as a quick and crude test to confirm and determine the quantity of active ingredient (chitosan) in the sample extracts obtained. The differences in reactivity of chitosan samples are showed in Fig. 4.1.

Assuming that each GlcN unit reacts with ninhydrin (i.e. stoichiometric reaction), then the reaction of chitosan with ninhydrin is expected to be directly proportional to its GlcN content. The present results were however contrasting. Assuming that the monosaccharide GlcN gives the maximum obtainable yield assigned as 100%, the reactivity of all samples was therefore low i.e. < 50%. This was similar to the observations noted in the study by Prochazkova et al. (1999), where chitosan with different degrees of deacetylation and molecular weight gave rise to different results. In their study, chitosan with DD of 99% gave a 41% colour yield, also < 50%. The low colour yield per mol of the amino groups is related to the availability of amino groups, which is based on the hydrolysis of the chitosan molecule. Though this method was found to be rapid, sensitive and reproducible it is very dependent on the properties of the chitosan used. Thus, the low yields observed in the chitosan extracts could be as a result of (i) type of chitosan; in this case, probably the source, (ii) lack of complete hydrolysis of the chitosan extracts which is dependent on the MW,

(iii) DD, and (iv) the possibility due to occurrence of side reactions between some amino groups and ninhydrin or ninhydrin intermediates (Prochazkova et al., 1999). While there has been no direct information of the effect of chitosan source (i.e. crustacea, fungi and insect) on chitosan quantification, studies have shown that the MW and DD can influence its degree of hydrolysis and colour yield (Leane et al., 2004; Prochazkova et al., 1999). Ultimately, these characteristics can be tied to the chitosan source. From the results, LDCH with highest MW (section 4.4.2.4) and DD (section 4.4.2.5) was observed to have low chitosan yield i.e. based on the ninhydrin quantification. It could thus be inferred that the MW and DD, in this case, may have resulted in partial hydrolysis of LDCH resulting in low chitosan quantity in the sample.
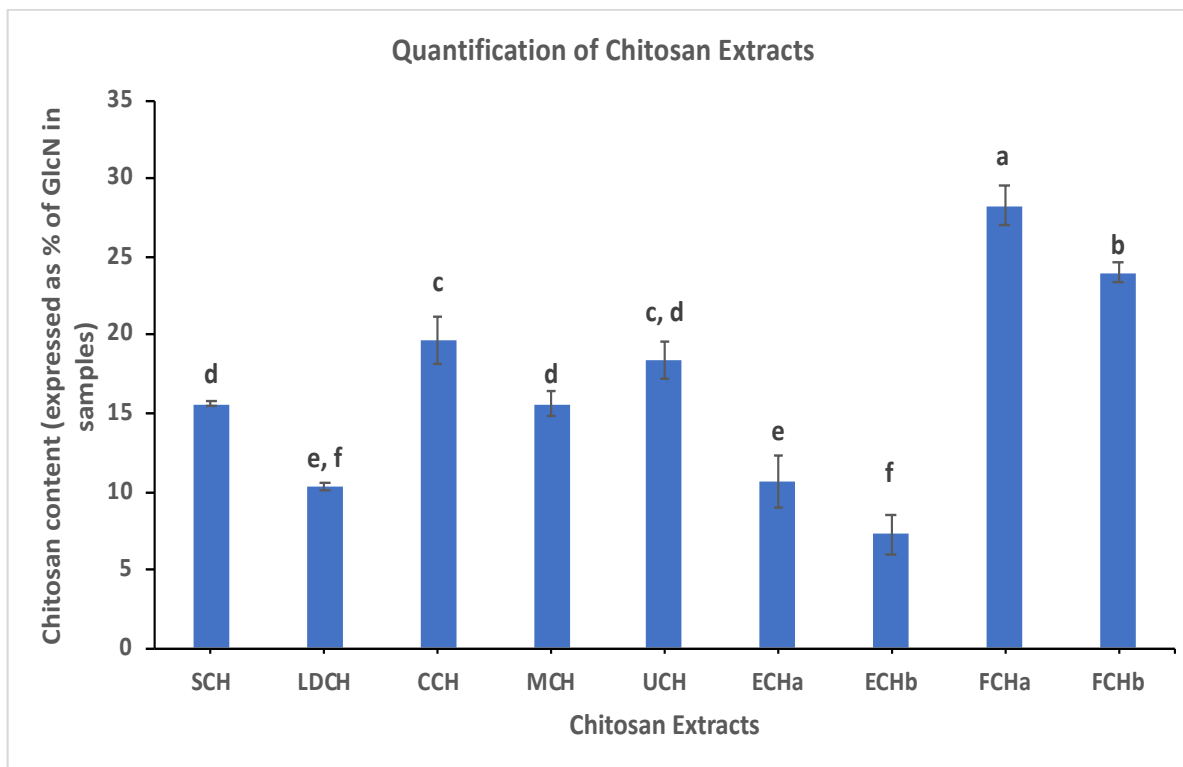

Figure 4.1. Quantity of chitosan (%) in extracts expressed as % GlcN; bars with different letters represent significant different mean values P < 0.05

4.4.2.4. Molecular Weight (MW)

Chitosan is a very attractive polymer because of the diversity linked to its chemical structure which is expressed by its molecular weight (MW) ranging from oligo-chitosan to low, medium or high molecular weight. The MW as stated earlier is an important feature of chitosan as it significantly affects the physicochemical properties. A common issue that is related to the MW is the definition of the terms oligo-chitosan, low, medium and high molecular weight chitosan (Verlee et al., 2017). The boundaries between these terms are usually vague and it is quite important to utilize similar terminology for chitosan MW description. Therefore, in this study, the boundaries and ranges utilized are: low molecular weight (LMW) chitosan > 16 kDa up to 190 kDa; medium molecular weight (MMW) chitosan > 190 – 300kDa; and MW > 300 kDa, is referred to as high molecular weight (HMW) chitosan.

The molecular weight range for chitosan samples (Table 4.4) was analysed using GPC, which provided information on the weight average molecular weight (MW, represents the molecular size of the sample), and the number average molecular weight (Mn, provides information on the statistical average of all polymer chains within a sample) in the region of 1-500 kDa. The dispersity index (Đ) measures the broadness or heterogeneity of molecular weight distributions within a polymer sample. It is represented as MW/Mn, thus a higher difference between MW and Mn indicates a more heterogenous or wider molecular weight distribution. From the table, insect chitosan was seen to be LMW while fungal and lobster-derived chitosan extracts were HMW. As expected, commercial chitosan (SCH) was LMW. On a general note, the differences observed in the MW of the chitosan extracts may be due to the differences in DD and the chitosan source (El Knidri et al., 2016). In addition, the MW differences noted in the insect chitosan extracts could be as a result of several factors in the extraction procedure such as the temperature of the treatments,

concentration of alkali, reaction time and more importantly, the treatment of chitin extract. In this study, the application of microwave and ultrasound irradiation may have caused additional degradation and splitting of the chitin polymer chain resulting in polysaccharides with lower MW. Consequently, it can be inferred that the insect chitosan extracts are similar in terms of MW to commercially available LMW chitosan and may exhibit similar bioactivity while, FCHa/b and LDCH, on the other hand, may exhibit bioactivity characteristics of HMW chitosan extracts.

Table 4.4. Molecular weight distribution of chitosan samples from three sources; insect chitin obtained from various extraction processes, fungi and crustacea: lobster and shrimp

Samples	Weight average molecular weight, Mw (kDa)	Number average molecular weight, Mn (kDa)	Dispersity index (Đ)
CCH	113.22	98.33	1.15
MCH	71.58	50.75	1.41
UCH	97.16	62.83	1.55
ECHa	80.05	46.22	1.73
ECHb	86.73	62.51	1.39
SCH	167.88	144.12	1.16
FCHa	380.94	n/a	n/a
FCHb	312.64	n/a	n/a
LDCH	> 500	n/a	n/a

n/a: not determined

4.4.2.5. H^1 NMR analysis of chitosan extracts

The H^1 NMR spectra of the chitosan extracts are given in Appendix D. The DD% was calculated using the equation given in section 4.2.3.6 and values given in Table 4.5. Chitosan is highly hygroscopic and must be carefully dried to eliminate moisture that could contribute to hydroxyl band intensities and incorrect determination of degree of deacetylation (DD). Thus, the H^1 NMR analysis of chitosan extracts was done in this study with the aim of determining the DD% of the extracts, as this technique allows for a direct determination of DD% (Hirai et al., 1991).

Table 4.5. Degree of deacetylation (DD) values (%) of chitosan from three sources; insect chitin obtained from various extraction processes, fungi and crustacean: lobster and shrimp

Chitosan source	CCH	MCH	UCH	ECHa	ECHb	FCHa	FCHb	LDCH	SCH
DD (%)	82.05	86.18	84.02	81.55	73.33	94.32	91.90	97.11	83.28

4.4.2.6. TGA

The thermograms of the chitosan extracts (Appendix E) in this study are similar to previous studies i.e., the weight of sample decreasing with temperature and mass loss was observed at two stages. Details of the TG-DTG results are presented in Table 4.6. and is similar to decomposition temperatures of chitosan from other studies, which have been determined to be between 280°C and 308°C (Abdou et al., 2008; Kaya et al., 2014a; Paulino et al., 2006). From the DTG curves, two peaks are recognized, which can be associated to the important thermal transformations of the chitosan structures as noted in the DTG of chitin samples. The first peak, which appears between 0°C and 150 °C can be attributed to evaporation of residual water in the polymer structure. The second decomposition peak occurs between 200°C and 650°C and can be related to the decomposition of glucosamine and residual acetyl glucosamine in the chitosan chain.

Unlike the chitin extracts that had varying DTG_{max} disintegration temperatures (Section 3.3.2.4), chitosan structures from the BSFM had similar DTG_{max} temperatures, with differences noted in the EAE chitosan extracts. The DTG_{max} temperatures of fungal chitosan extracts was lower than insect and crustacea chitosan. Thermal decomposition temperatures of fungal chitosan was observed to be lower than shrimp chitosan in the study by Yen & Mau (2007). It can thus be inferred that chitosan from crustacea and insect are more thermally stable than fungal chitosan. Chitosan extract from BSFM and lobster could be more thermally stable than commercial chitosan, which was also observed in the study by Abdel-Rahman et al. (2015), where isolated chitosan from

shrimp shells were more thermal stable than commercial chitosan. Initial water loss from chitosan extracts were in the range of 3 – 8%, comparable to other studies, but was highest for FCHb which had an initial water mass loss of 11%. In addition, FCHb had the lowest DTG_{max} temperature of 263°C. While the total mass loss of FCHb was in similar ranges with other extracts, the higher initial mass loss and lower DTG_{max} temperature allude to the fact that the source of chitosan and extraction methodology may have an impact on the overall chitosan structure. On the other hand, EAE chitosan extracts, ECHa and ECHb exhibited the lower total mass loss i.e. 65% and 61% respectively, which may be attributed to the presence of inorganic materials in the extract.

Table 4.6. Results of TG-DTG analyses of chitosan from three sources; insect chitin obtained from various extraction processes, fungi and crustacea: lobster and shrimp

Sample	Temperature range (°C)	Mass loss (wt %)	Total mass loss (wt %)	DTG_{max} peak (°C)
CCH	0-150	4.57	73.97	302.56
	150-650	69.40		
MCH	0-150	4.21	72.99	304.25
	150-650	68.68		
UCH	0-150	4.49	71.53	303.81
	150-650	67.04		
ECHa	0-150	3.10	65.14	298.66
	150-650	62.04		
ECHb	0-150	3.51	60.65	298.56
	150-650	57.14		
FCHa	0-150	4.39	74.89	280.67
	150-650	70.50		
FCHb	0-150	11.03	71.13	262.77
	150-650	60.10		
LDCH	0-150	4.91	73.99	308.79
	150-650	69.08		
SCH	0-150	6.61	77.92	296.15
	150-650	71.31		

Comparing the main degradation temperatures (DTG$_{max}$) of chitin molecules extracted in Chapter 3 (section 3.3.2.4) and chitosan molecules, it was observed that chitin molecules are degraded in higher temperature than chitosan molecules i.e. chitin molecules are more thermal stable than those of chitosan which is consistent with literature reports (Abdou et al., 2008; Kaya et al., 2014a). It is presumed that the reason for this difference is the presence of the *N*-acetylated polymer units in chitin, which are more stable than the amine polymer units of the chitosan molecule (Paulino et al., 2006).

On a different note, a correlation analysis using the coefficient of determination value (R^2) was used to test the relationship, if any, between the MW and DD on the total mass loss of the chitosan samples. For MW, an R^2 value of 0.5828 indicated a linear relationship with the total mass loss. Similarly, an R^2 value of 0.6485 demonstrated the linear relationship between the DD and total mass loss. These trends suggest that as the DD and MW increased, the total mass loss of chitosan samples also increased. Thus, a higher DD or MW of chitosan may result in a higher mass loss. This is however not a definite/conclusive assumption, since the data simply suggests these relationships and not a significant impact.

4.4.3. Antioxidant activity

4.4.3.1. Chelating effect on ferrous ions

The highest Fe (II) chelating activity of chitosan extracts was observed in LDCH extracts (Fig. 4.2). FCH extract had higher chelating ability than the insect and commercial chitosan extracts, which agrees with previous report (Yen et al., 2007). As expected, EDTA which was used as the standard had higher chelating ability than all the chitosan extracts, also at par with previous report (Lin & Chou, 2004). According to Inoue et al. (1988), the chelation of metal (Cu^{2+}) ions involved

the binding of Cu^{2+} with the hydroxyl group on C6 and the amino group on C2 of the chitosan molecule. Likewise, it may be inferred that ferrous ion chelation also involves the binding of Fe^{2+} to the same C atoms as well as NH_2 atoms. As stated earlier and in the study by Qin (1993), the ion-chelating ability of chitosan is significantly affected by its DD, which was similarly observed in this study. From the results in Table 4.6 (Section 4.4.2.6), the chelating abilities of the chitosan extracts correlate with their DD. LDCH with highest DD of 97%, displayed highest chelating activity while ECHb with DD of 73% had lowest chelating ability which agrees with previous studies (Yen et al., 2007; 2008). Also, apart from ECHb, insect chitosan extracts had similar chelating abilities to commercial chitosan (SCH).

Interestingly, from Fig. 4.2, it seemed that there was no significant difference in the chelating ability of the insect chitosan samples (i.e. CCH, MCH, UCH and ECH) and the extraction processes employed may not have an influence on this ability. However, this was not the case. When the chelating abilities of the insect chitosan were compared to each other, significant differences were observed as shown in Fig. 4.3. The chelating metal abilities were as follows: UCH > ECHa > CCH > MCH > ECHb. Thus, UCH showed significantly higher ability on ferrous ion chelating activity than the other extracts.
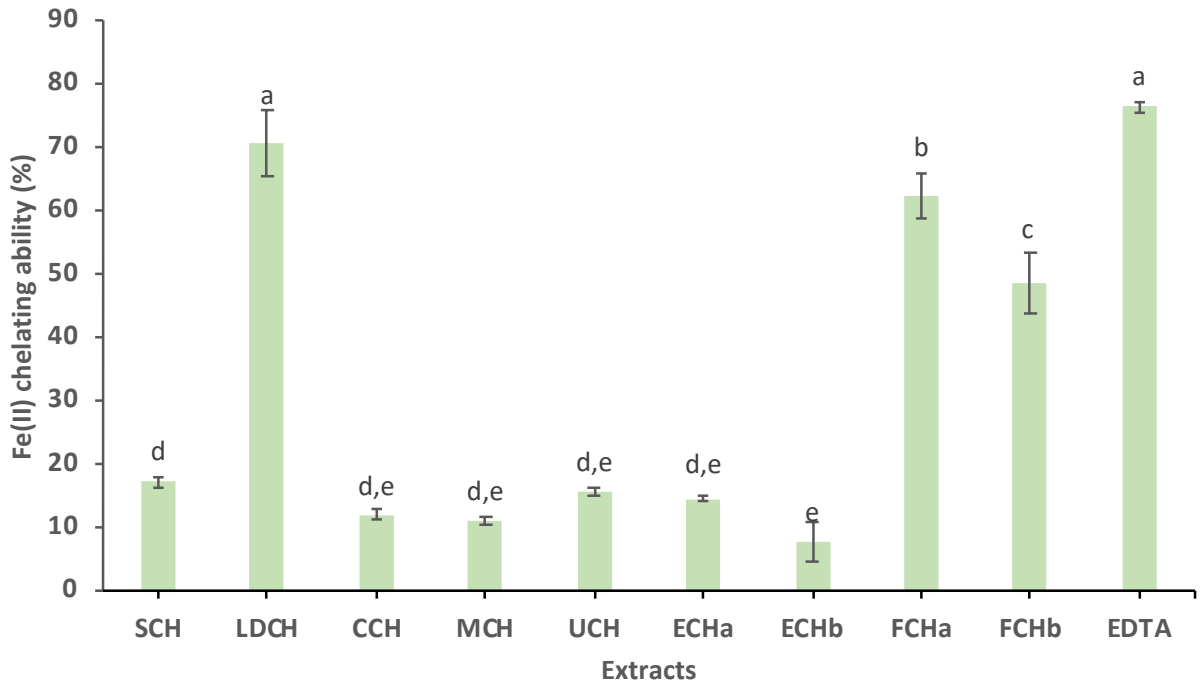
Figure 4.2 Fe (II) – chelating ability of chitosan extracts and EDTA; bars with different letters represent significant different mean values $P < 0.05$
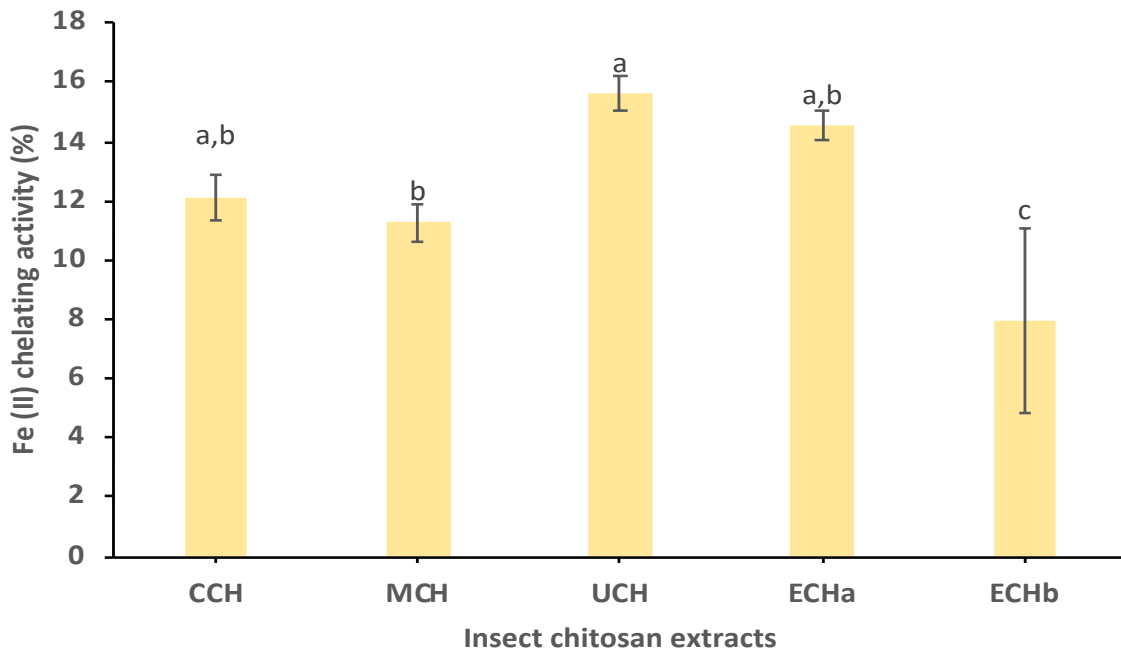

Figure 4.3. Fe (II) – chelating ability of insect chitosan extracts; bars with different letters represent significant different mean values $P < 0.05$

4.4.3.2. Scavenging activity of DPPH radical

All chitosan samples showed scavenging ability for DPPH radicals. However, their activity was lower than that of Ascorbic acid, which was used as a positive control (Fig. 4.4). FCHa and LDCH displayed greater ability to scavenge DPPH radicals than insect chitosan extracts. Apart from ECHb, the insect chitosan extracts had higher scavenging abilities than commercial chitosan, which is in agreement in part to the results obtained in the studies by Song et al. (2013) and Yen et al. (2007). Comparatively, chitosan samples from crab and fungi were more effective scavengers for DPPH than insect chitosan. However, the present study suggested that only FCHa was significantly different, had higher scavenging ability when compared to insect and shrimp chitosan and was similar in capacity to chitosan from lobster. Similar to the trend observed with Fe (II) chelating ability, the scavenging ability was almost synonymous with the DD. Though LDCH, in this case did not display the highest ability to scavenge DPPH radicals, its ability was not significantly different from FCHa (DD values of 97% and 92%, respectively). The effect of antioxidants on DPPH scavenging was considered to be due to their abilities to donate their hydrogen atoms (Zhang et al., 2013). Consequently, DPPH scavenging ability demonstrated by the chitosan extracts may be attributable to a strong hydrogen-donating ability.
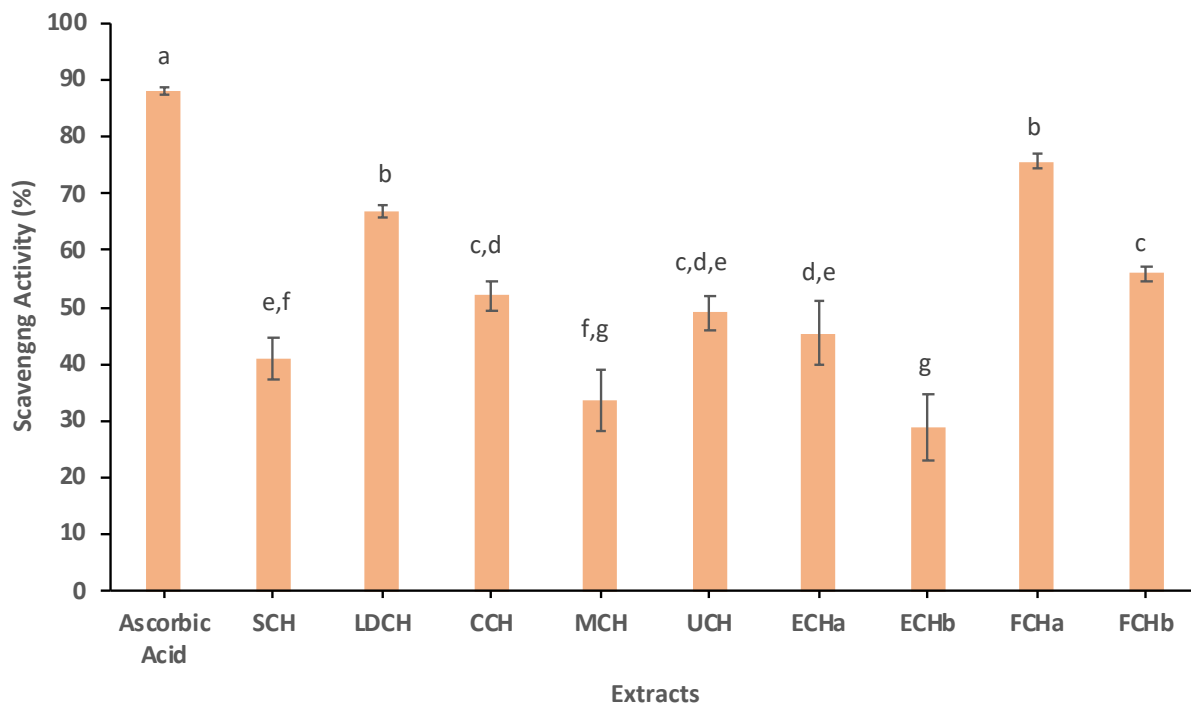
Figure 4.4. Scavenging effect of chitosan extracts and ascorbic acid on DPPH; bars with different letters represent significant different mean values $P < 0.05$

Previous studies show that the antioxidant activity of chitosan was mainly related to its low molecular weight (Vinsova & Vavrikova, 2011) and also to the content of active hydroxyl and amino groups present in the polymer chains (Feng et al., 2008). Chitosan with high degree of deacetylation have more amino groups to enhance their antioxidant activity as observed in the present study. Likewise, LMW chitosan were reported to have higher antioxidant activity than HMW chitosan. Contrastingly, the HMW chitosans were noted to display higher antioxidant activity in the present study. While these results may seem to differ, the definitions and boundaries given for MW vary from study to study. For example, in the study by Sun et al. (2007), LMW chitosans were 2.30, 3.27 and 6.12 kDa and HMW was 15.25 kDa. Also, Feng et al. (2008) showed that LMW chitosan (2.1 kDa) had much higher antioxidant properties than 210 kDa MW chitosan.

It is proposed that polysaccharides with smaller MW would have more reductive hydroxyl groups to scavenge free radicals on per-unit-mass basis (Liu et., al 2010). In this study, the higher DPPH scavenging ability exhibited by insect chitosan could therefore be as a result of its MW, which is lower than the commercial chitosan.

While there is limited information on the effect of extraction methodologies on the antioxidant abilities of chitosan, studies have reported varying antioxidant activities of polysaccharides extracted using different extraction methods. For example, in the study by Wang et al. (2009), polysaccharides extracted *via* microwave-assisted extraction showed greater antioxidant activity compared to hot-water extracted polysaccharides. Similarly, Zhang et al. (2013), showed that extracts obtained *via* ultrasound and microwave displayed significantly higher DPPH scavenging ability than extracts obtained *via* chemical and enzyme-assisted methods. It is presumed that the microwave and ultrasonic treatments may have induced the depolymerization of the polysaccharide molecule, thereby increasing the solvent accessibility and reduced stearic hindrances (Yang et al., 2008). On the other hand, the study by Zhang et al. (2013), showed that enzyme-assisted polysaccharide extracts demonstrated better metal chelating ability than extracts obtained *via* other extraction methods.

The antioxidant activities of natural polysaccharides have been suggested to be related to their molecular weight, structure and conformation as well as monosaccharide composition (Wang et al., 2009; 2011; 2016), suggesting that the antioxidant activities of polysaccharides are not a function of a single factor but a combination of several factors. It is therefore probable that different factors utilized in the extraction protocol such as temperature, solvents concentration and pH may have influenced the bio-activity of the final polysaccharide extract.

4.4.4. Antimicrobial activity

The results of the disc diffusion antimicrobial assay of the chitosan samples are shown in Table 4.7. The negative control treatment (1% acetic acid) had no inhibitory effect on any of the tested microorganisms. The inhibition zone diameters of the antibiotic (positive control treatment) are also presented in Table 4.7. The results obtained for chitosan samples indicated that chitosan showed little or no inhibition on all the tested microorganisms. Only LDCH showed antimicrobial activity against all tested microorganisms. MCH, UCH, ECHa, FCHa and LDCH showed activity against *S. aureus 6358*. None of the chitosan samples exhibited any inhibition against *S. aureus 25928*, with the exception of LDCH. The SCH sample displayed some antimicrobial activity against *E. coli,* which is evidenced by the observed inhibition zone observed. Although not properly defined, inhibition zones (noted as NDI, Fig 4.5) were observed for the samples against *Kleb.* and *S. aureus 38,* which was an indication that the samples may have antimicrobial activity against these organisms. It would seem therefore that the chitosan samples may have displayed higher antimicrobial activity against gram-positive than gram-negative bacteria. Though, this was not consistent, and thus may only be a probability, higher antimicrobial activity against gram positive bacteria have been observed in other studies (Dutta et al., 2009; No et al., 2002). Although the effect of chitosan MW on its antimicrobial activity is yet to be fully determined as earlier mentioned in Chapter 2, the results in this assay seem to agree with the inference that HMW chitosan displayed greater activity than LMW. LDCH, having the highest MW, displayed antimicrobial activity against all the tested microorganisms. Similarly, the antimicrobial activity can be attributed to the high DD of LDCH samples, as chitosan samples with higher DD has been reported to possess stronger antimicrobial activity (Takahashi et al., 2008). The antimicrobial

activity of chitosan has been noted to be dependent on the presence of a positive charge. Thus, it is expected that chitosan with higher DD would have more effective activity because it would contain a higher concentration of positive charges. However, the initial expectation of clear and defined inhibition zones as observed in other studies was not obtained in this study. The results indicate that the chitosan samples may have minimal antimicrobial activity. It is probable that the chitosan samples in this assay were incapable of fully diffusing through the paper disks, as it was observed that the chitosan droplets formed a thin layer of film on the surface of the paper disc. Consequently, the chitosan samples were unable to interact with the microbial cell walls and inhibit microbial growth. This assumption is not unlikely as the study by Foster & Butt (2011) revealed that films made from chitosan displayed no antibacterial activity but chitosan solutions and gels inhibited the growth of microbial organisms.

The results of the bacterial colony counting assay (Fig. 4.6) on the other hand, indicated that all chitosan samples displayed antimicrobial activity against *E. coli*. After 48 h incubation period, the number of colonies observed in chitosan solutions were lower than the numbers obtained in the blank and negative control, indicating antimicrobial activity, which is consistent with results from other studies (Raafat & Sahl, 2009). From Fig. 4.6, samples taken between 0 - 2h displayed no growth, inclusive of the blank and negative samples. It could be that the initial concentration of microbial cells present in the solutions was too low i.e. very dilute, and thus needed additional time to develop and multiply. However, after 4 h, a significant growth of colonies was measured in the blank and the negative control but none in the chitosan samples. After 8 h, colony growth was observed in chitosan samples except FCHb, SCH and LDCH. Though these numbers were lower than the numbers in the blank and negative controls. The antimicrobial activity of these samples at 8 h time point was in the order MCH > UCH > CCH > ECHa > FCHa > ECHb. At 12

h and 24 h time points, FCHb, SCH and LDCH chitosan samples exhibited growth of microbial cells in addition to the other samples but no cells were observed in MCH, UCH and ECHa. Thus, it is probable that chitosan samples may have delayed the rate of growth of the microbial cells between 0 – 8 h and after 8 h may cause the death of some cells reflecting the reduction in number of colonies counted for 24 h time point. Unlike the results obtained from the disc diffusion assay, where HMW chitosan displayed greater activity, the LMW chitosan samples were observed to display greater antimicrobial activity when compared with the HMW chitosan samples, apart from LDCH. Unlike the results of the disc diffusion assay, these results seem to agree with the alternate inference that LMW display greater antimicrobial activity than HMW chitosan. Previous studies carried out on *E. coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae* proved that greater reduction of microorganism growth was observed for LMW chitosan (Jing et al., 2007; Tikhonov et al., 2006; Tsai et al., 2004). It was therefore postulated that attraction, ionic interaction and mobility of smaller chains facilitated the adoption of an extended conformation, which led to effective binding to cell membrane surfaces than for bigger chains (Kumar et al., 2005). While the specific mechanism(s) of antimicrobial activity of chitosan is yet to be fully elucidated, several hypotheses, as mentioned in Chapter 2, have been proposed. The speculation therefore supports the hypothesis that the antimicrobial mechanism is as a result of electrostatic reaction between the positively charged chitosan molecules and the negatively charged microbial cell membranes. These interactions would thereby lead to the leakage of proteinaceous and other intracellular constituents of bacterial cells (Helander et al., 2001; Liu et al., 2004). Thus, chitosan with higher DD, which has higher positive charge, would be expected to have stronger antibacterial activity. However, this was not the case and a possible explanation is that the longer chains i.e. HMW chitosan, would have increased number of charged sites and will tend to form clusters by

molecule segregation while in solution, hence the reduction of antimicrobial activity when compared to LMW chitosan (Assis et al., 2002). Furthermore, Younes et al. (2014) demonstrated that antibacterial activity of chitosan against gram-negative increased with decreasing DD and MW, as was noted in the results of the assay in this present study. The chitosan samples with lower DD, i.e. insect chitosan with DD in the range 82 – 86%, exhibited higher antimicrobial activity.

Since SCH was LMW, it was expected that similar antimicrobial activity to insect chitosan would be observed. However, the results indicated that insect chitosan displayed greater activity than the commercial chitosan. For the FCHa, the 8 h incubation had some microbial growth but not the 12 – 24 h incubation, while FCHb displayed some growth at both 12 and 24 h incubation. Chitosan from the different sources, i.e. crab, fungi and insects, have been studied for their effect on antimicrobial properties. In the study by Jeihanipour et al. (2007), the antimicrobial activity of fungal chitosan when compared to chitosan from crustacean, was lower. The presence of some impurities or the physicochemical properties of the fungal chitosan was speculated to be the cause of its lower antimicrobial activity. Kaya et al. (2015a), on the other hand, demonstrated that chitosan from grasshoppers exhibited stronger antimicrobial effectiveness against gram-negative bacteria than gram-positive bacteria. Furthermore, chitosan prepared from ground and entire crab leg shell exhibited drastic differences in their functional characteristics as noted in the study by Byun et al. (2013). Ground shell chitosan, with higher DD, showed higher antimicrobial activity than chitosan produced from entire crab leg.

Therefore, while it has been established that chitosan exhibits antimicrobial activity against microorganisms, the activity is dependent on many factors such as its MW, DD, source (raw material), pH of the medium, purity of the chitosan extract and the type of microorganisms. These

need to be considered when measuring and determining antimicrobial activity of chitosan from different sources.

Table 4.7. The diameters of inhibition zones (expressed in mm) against Gram-negative and positive bacteria after 24 h incubation at 37 °C

Samples	Kleb.	SA 6358	SA 25928	SA 38	E. coli
Gentamycin (+)	21.67 ± 2.08	21.67 ± 1.53	23.67 ± 0.58	19.67 ± 0.58	18.33 ± 0.58
CCH	NDI	NI	NI	NDI	NI
MCH	NDI	5.33 ± 4.62	NI	NDI	NI
UCH	NDI	5.50 ± 4.91	NI	NI	NI
ECHa	NDI	6.67 ± 5.77	NI	NDI	NI
ECHb	NI	NI	NI	NI	NI
SCH	NI	NI	NI	NI	7.67 ± 6.66
FCHa	NDI	5.33 ± 4.62	NI	NDI	NI
FCHb	NI	NI	NI	NI	NI
LDCH	9.67 ± 0.58	9.00 ± 1.00	5.33 ± 4.62	9.67 ± 0.58	6.67 ± 5.77
Acetic Acid (-)	NI	NI	NI	NI	NI

NDI: No definite inhibition zone
NI: No inhibition
Diameter of the inhibition zone includes disc diameter. Values are reported as means ± SD of three separate experiments.
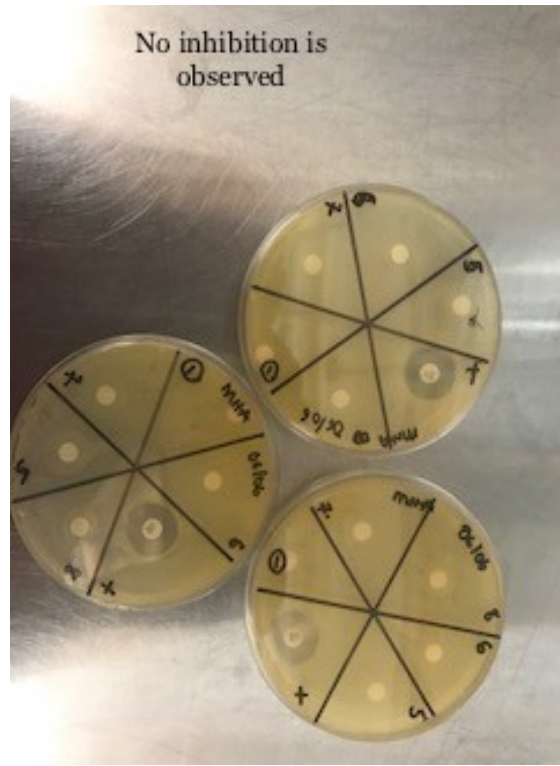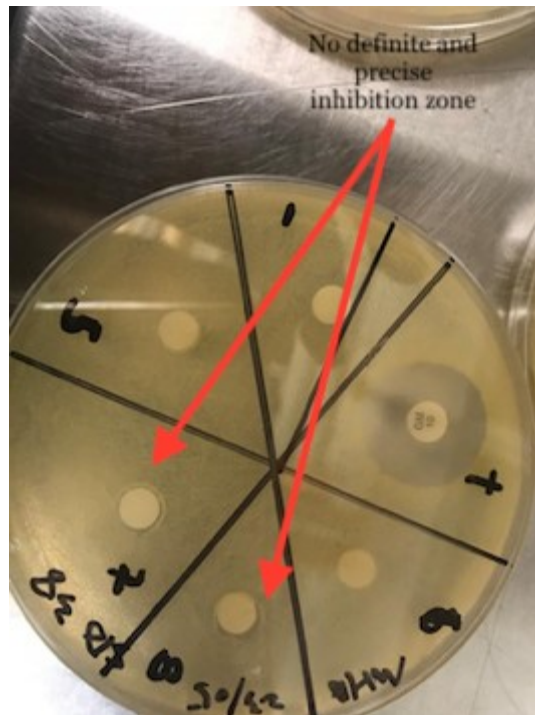
Figure 4.5. Disc diffusion assay plates showing (a) inhibition zones (b) no inhibition zones (NI) and (c) no definite and precise inhibition zones (NDI)
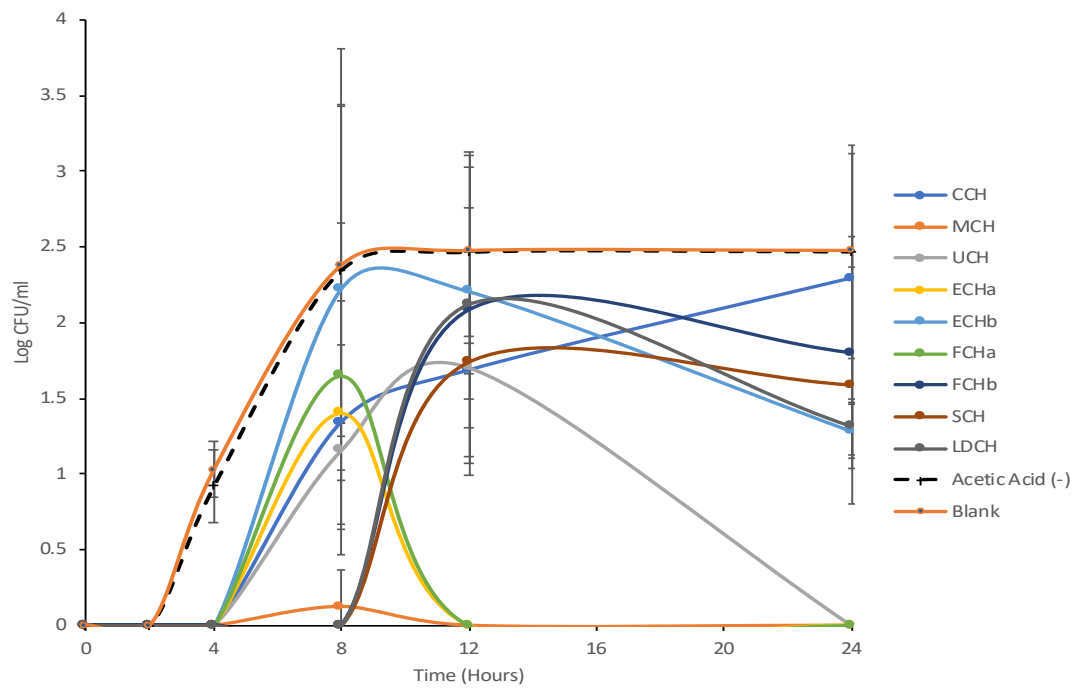
Figure 4.6. Growth curve showing the antimicrobial effect of chitosan samples on *E. coli* at different time points after 48 h incubation estimated through viable colony counts

4.5. Conclusion

The results of this study indicate that the source of raw materials for chitosan does play an important role in its physicochemical characteristics. Though the differences may not be quite significant, it is important and crucial to factor in this when comparisons of chitosan are done. For the first time, the effect of DD and MW on TGA was examined. In particular, a linear relationship was associated with these, a higher DD or MW resulted in higher mass loss, although additional studies will be required in order to substantiate the assumption. Furthermore, the antioxidant and antimicrobial results indicated that the chitosan samples although exhibiting these abilities, the physicochemical properties associated with each chitosan sample influences its activities. Similar to other studies, our results indicated that MW as well as DD of chitosan are important factors to be considered when assessing the biological activities of chitosan.

CHAPTER 5

CONCLUSION

The feasibility of employing the insect, black soldier fly, as a raw material for preparing chitin and chitosan was evaluated by comparing its structures, physicochemical properties, and biological activities with commercially available chitin and chitosan extracted from crustacea and fungi. Also, with the recent interest in green technology and eco-friendly extraction methodologies, novel extraction technologies were evaluated for their potential as alternative extraction methods for chitin production. Additionally, the impact of these extraction technologies on structural properties and biological activities were examined.

In this study, chitin was derived from an insect source; black soldier fly meal (BSFM), using the conventional extraction method as well as more recent extraction processes (microwave-, ultrasound- and enzyme-assisted extraction). BSFM was investigated for its potential as a source of raw material for chitin production. The alternate extraction methods were explored to determine if chitin extracts of similar physicochemical characteristics to conventional chitin will be produced. The four extraction methods were successful in isolating chitin. Comparison of chitin extracts with commercially available chitin and lobster shell-derived chitin, indicated similarity regarding crystal structure (IR), N content and DA values. Although more yield was obtained in the other extraction processes, a higher yield may not be representative of a high yield of the targeted compound, as extracts may contain higher amounts of excipients.

The application of these technologies, MAE and UAE, however gave the benefit of reduced extraction time when compared to CE and are likely to be more environmentally friendly. Although extraction conditions may influence and impact the final outcome of products both quantitatively and qualitatively, other factors such as raw materials, location of raw materials and

species, also need to be taken into consideration. The results of the study suggest that MAE and UAE were more effective and beneficial extraction processes than EAE for chitin extraction. In the future, optimal extraction conditions for MAE and UAE need to be evaluated in order to establish the best fit for efficient chitin extraction.

The physicochemical properties and biological activities of chitosan from three sources; insect, fungi and crustacean, were for the first time, evaluated and compared in this study. Though all chitosan extracts displayed characteristic bands synonymous to the α- configuration in the IR spectra, the intensities and wavelengths differed to some extent. The MW and DD of chitosan are key parameters that have been reported to influence and impact its characteristics and functions. In agreement with previous research and literature Insect chitosan are seen to be in the range of LMW chitosan, while chitosan from fungi and lobster were in HMW range. Also, when compared to fungi and lobster, the insect chitosan had lower DD. The impact of these 2 attributes on the total mass loss were therefore tested. The results seem to suggest that a linear relationship exists between the MW and DD on the total mass loss. As MW and DD increased, the total mass loss of chitosan also increased. To the best of our knowledge, this is the first study to test this relationship and thus the data may not be regarded as conclusive, it however suggests that these relationships may exist. The existence or non-existence of this relationship will therefore need to be evaluated and examined in future research.

The increasing interest in chitosan has been as a result of its biological functions which was also investigated in this study with focus on its antioxidant and antimicrobial properties. In line with previous studies, the DD and MW are observed to influence these properties. Though all the chitosan samples displayed antioxidant activity, there were significant differences observed. High DD and MW was synonymous with higher antioxidant activity. Thus, LDCH with highest DD has

highest antioxidant activity. The impact of the different extraction methods was also assessed. In this study, UCH displayed greater chelating ability on Fe (II) than the other extracts. Though the effect of extraction methodologies on chitosan antioxidant ability is limited, other studies have reported that the method of extraction influenced the antioxidant potential of polysaccharides. As observed in previous reports, the antimicrobial activity of the chitosan samples yielded varying results. While the observation from the disc diffusion assay seems to suggest that HMW chitosan had higher antimicrobial activity, the other broth dilution assay results suggest otherwise. A study of previous work from literature has not led to any conclusive data on the influence of DD, MW and source on chitosan antimicrobial activity, as well as whether chitosan has higher activity on gram-positive or on gram-negative bacteria. Chitosan samples were observed to act differently in both assays though it could be inferred that it displayed some amount of antimicrobial activity. Therefore, though the samples exhibited biological functions, the physicochemical properties associated with each chitosan sample, as well as other factors, such as temperature, pH, source of raw material need to be taken into consideration when measuring or determining these activities.

Taken together, our results demonstrated that BSFM could be a new source of chitin/chitosan. Compared with the commercial chitin/chitosan primarily obtained from shrimps and crabs, the chitosan obtained from BSFM has its own advantages, such as no seasonal limit on accessibility to raw materials, low inorganic salt content and no regional limit on industrial production. In addition, the data showed that chitosan from BSFM had antioxidant activity, suggesting that insect chitosan may be used as a possible food ingredient or in the pharmaceutical industry. These studies have important value in the utilization and exploitation of BSFM.

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APPENDIX A: FTIR SPECTRA OF CHITIN EXTRACTS
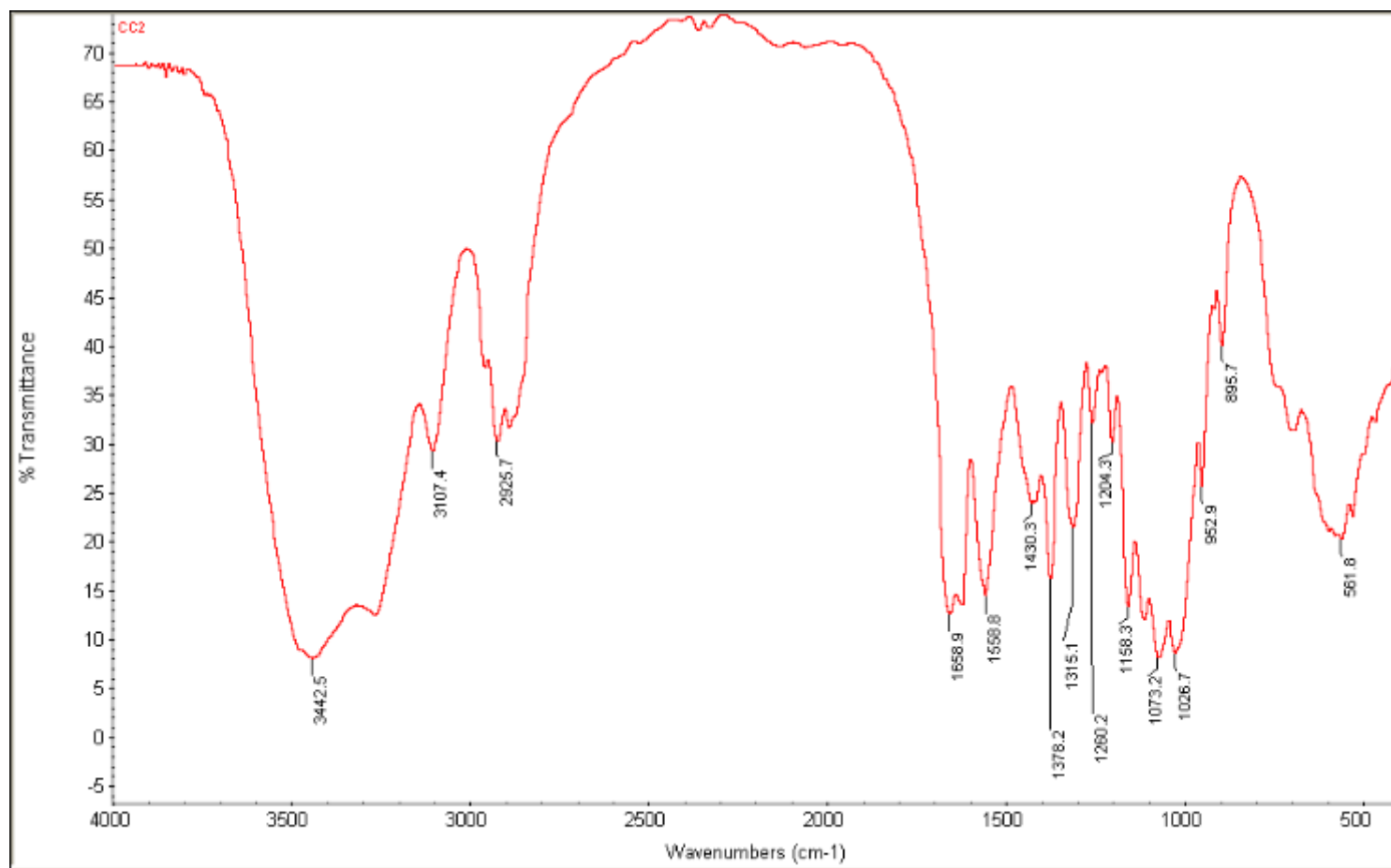
FIG A1. FTIR SPECTRUM OF BSFM CHITIN EXTRACT FROM CONVENTIONAL CHEMICAL EXTRACTION
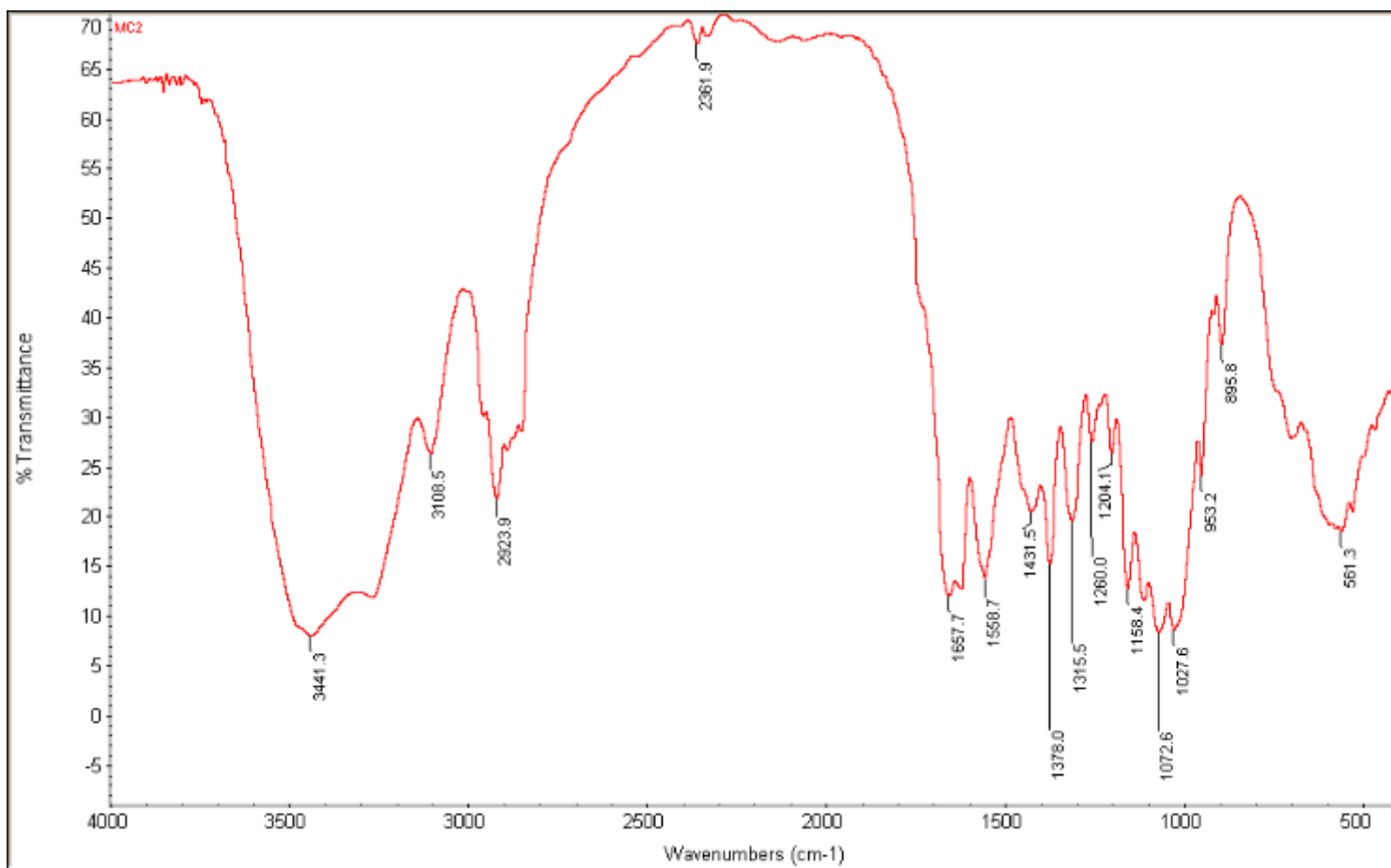
FIG A2. FTIR SPECTRUM OF BSFM CHITIN EXTRACT FROM MICROWAVE - ASSISTED EXTRACTION
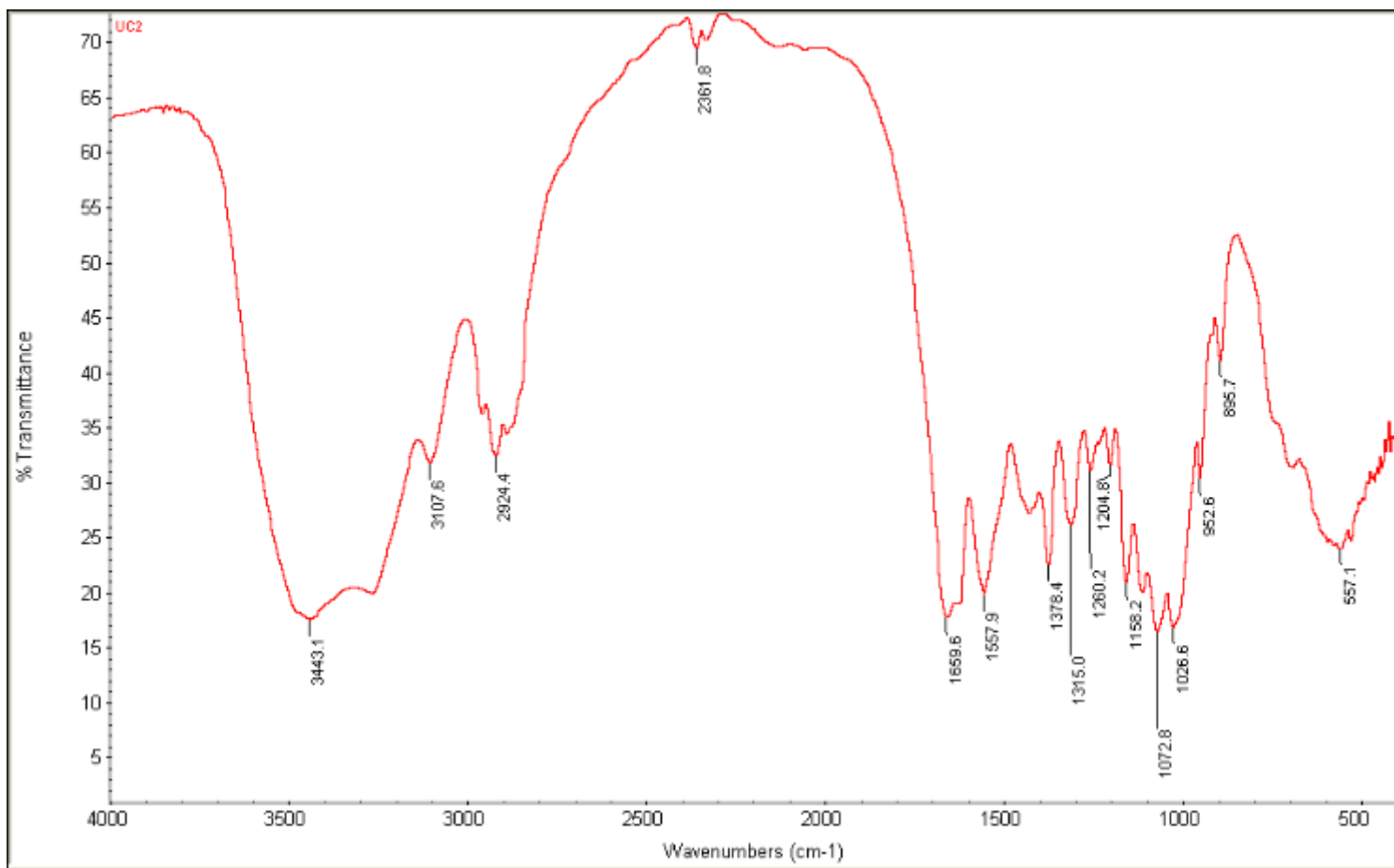
FIG A3. FTIR SPECTRUM OF BSFM CHITIN EXTRACT FROM ULTRASOUND - ASSISTED EXTRACTION
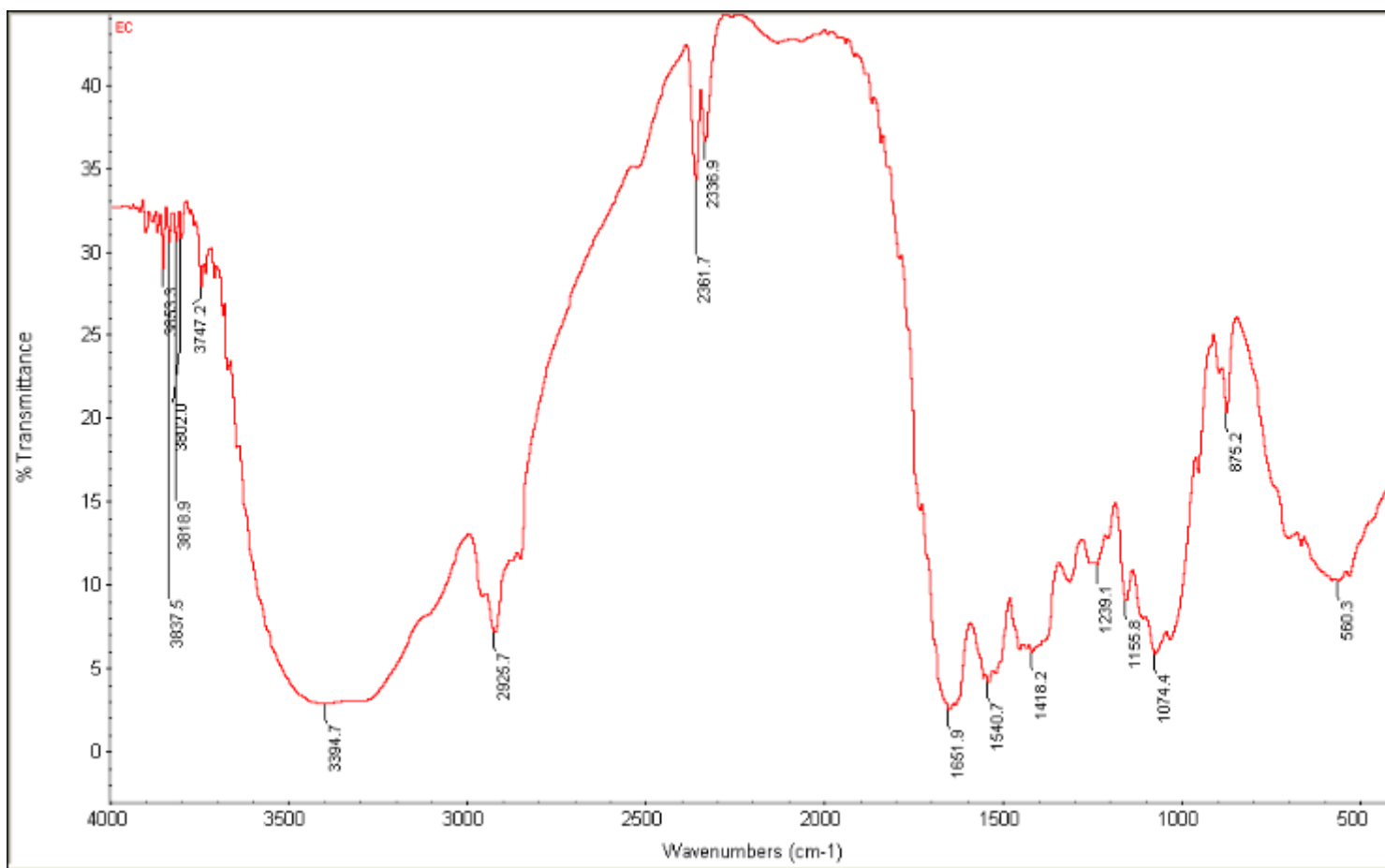
FIG A4. FTIR SPECTRUM OF BSFM CHITIN EXTRACT FROM ENZYME - ASSISTED EXTRACTION
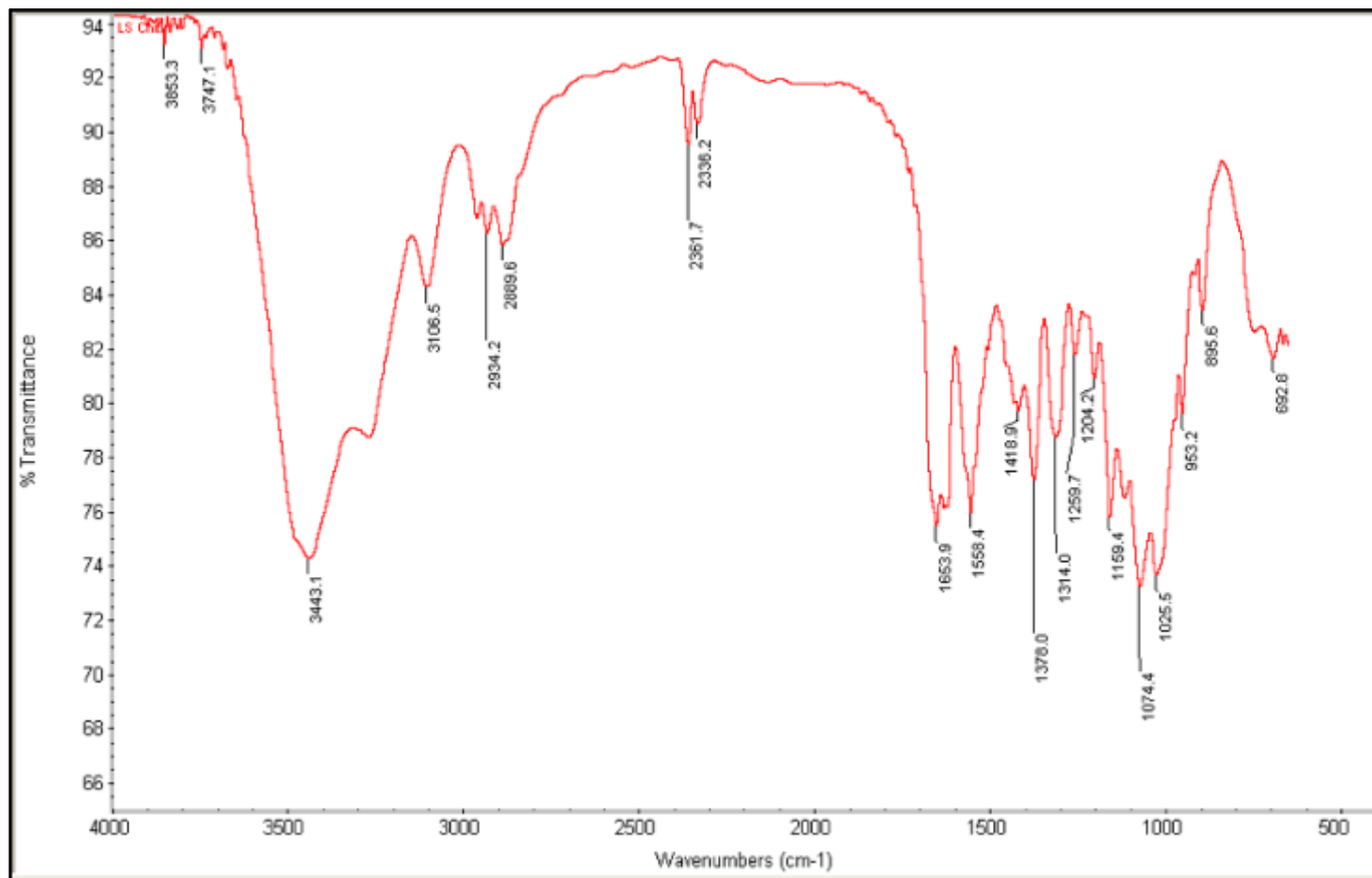
FIG A5. FTIR SPECTRUM OF LOBSTER SHELL-DERIVED CHITIN

FIG A6. FTIR SPECTRUM OF COMMERCIAL CHITIN

FIG B1. TGA/DTG OF BSFM CHITIN EXTRACT FROM CONVENTIONAL CHEMICAL EXTRACTION
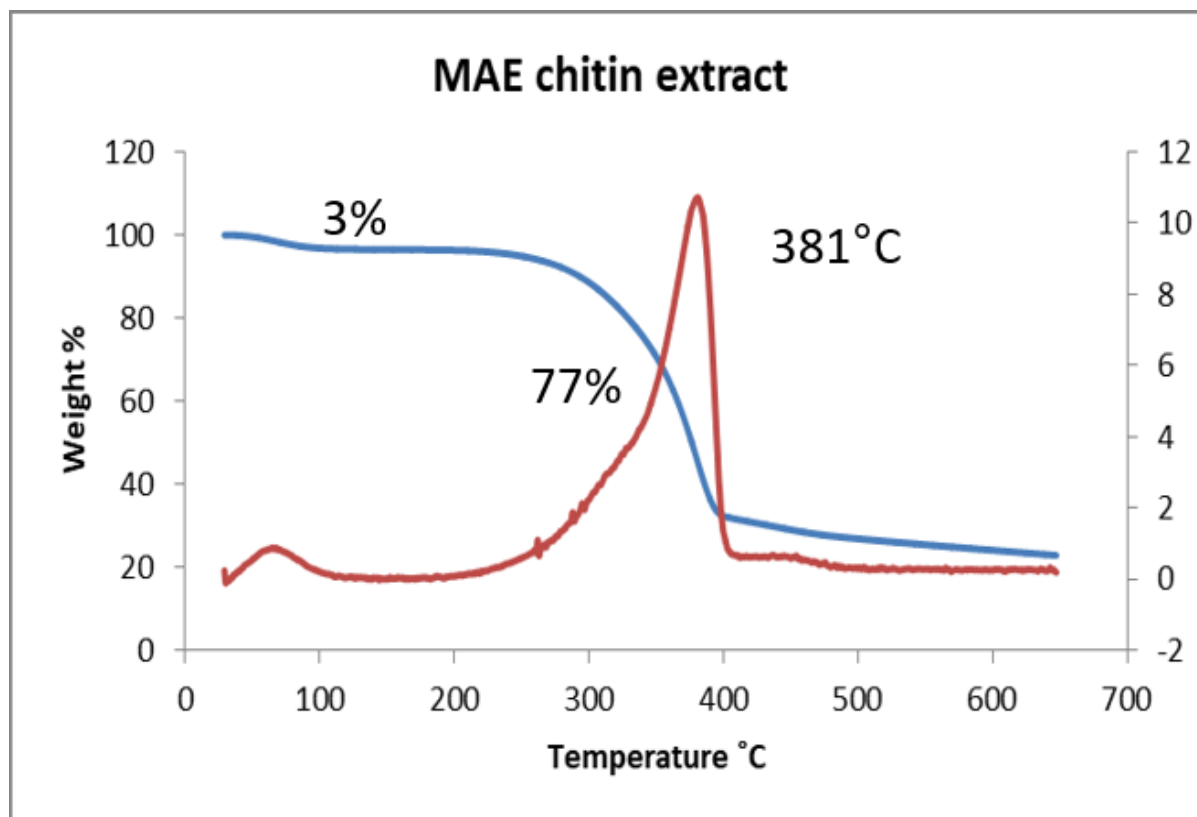
FIG B2. TGA/DTG OF BSFM CHITIN EXTRACT FROM MICROWAVE - ASSISTED EXTRACTION
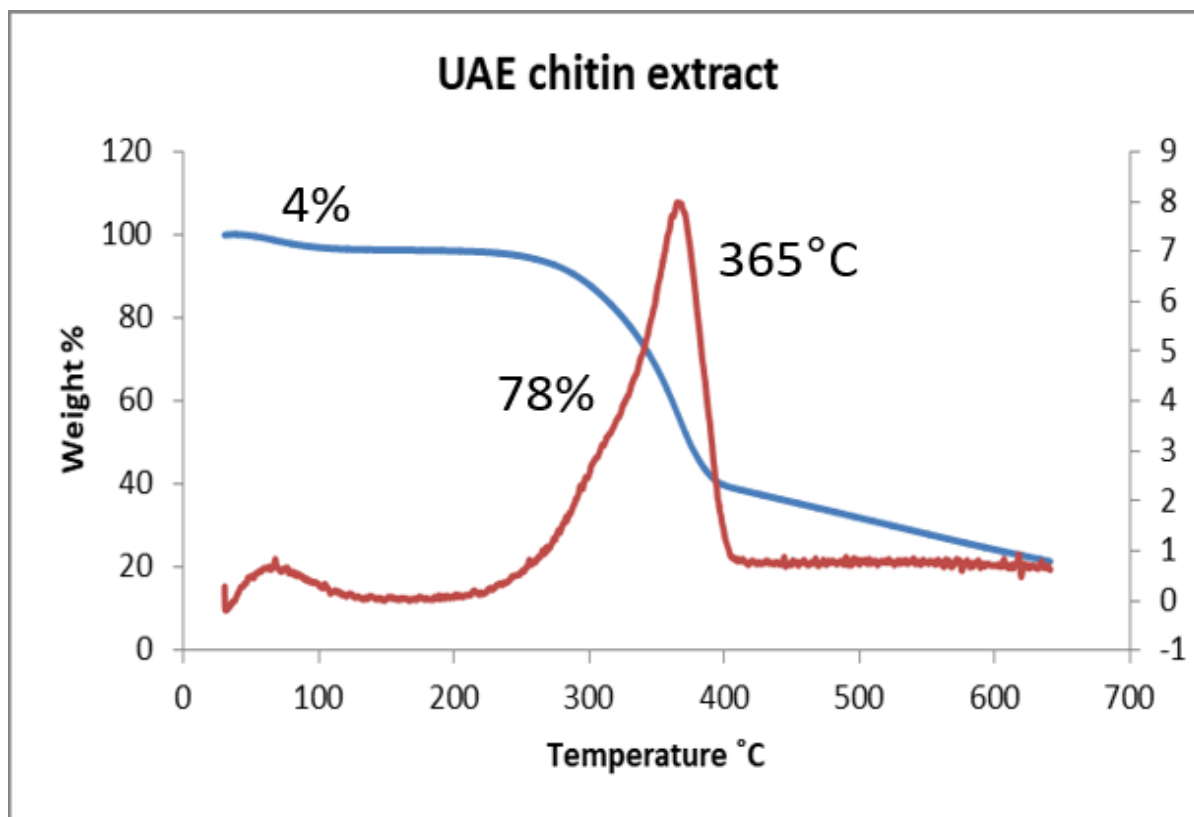
FIG B3. TGA/DTG OF BSFM CHITIN EXTRACT FROM ULTRASOUND - ASSISTED EXTRACTION
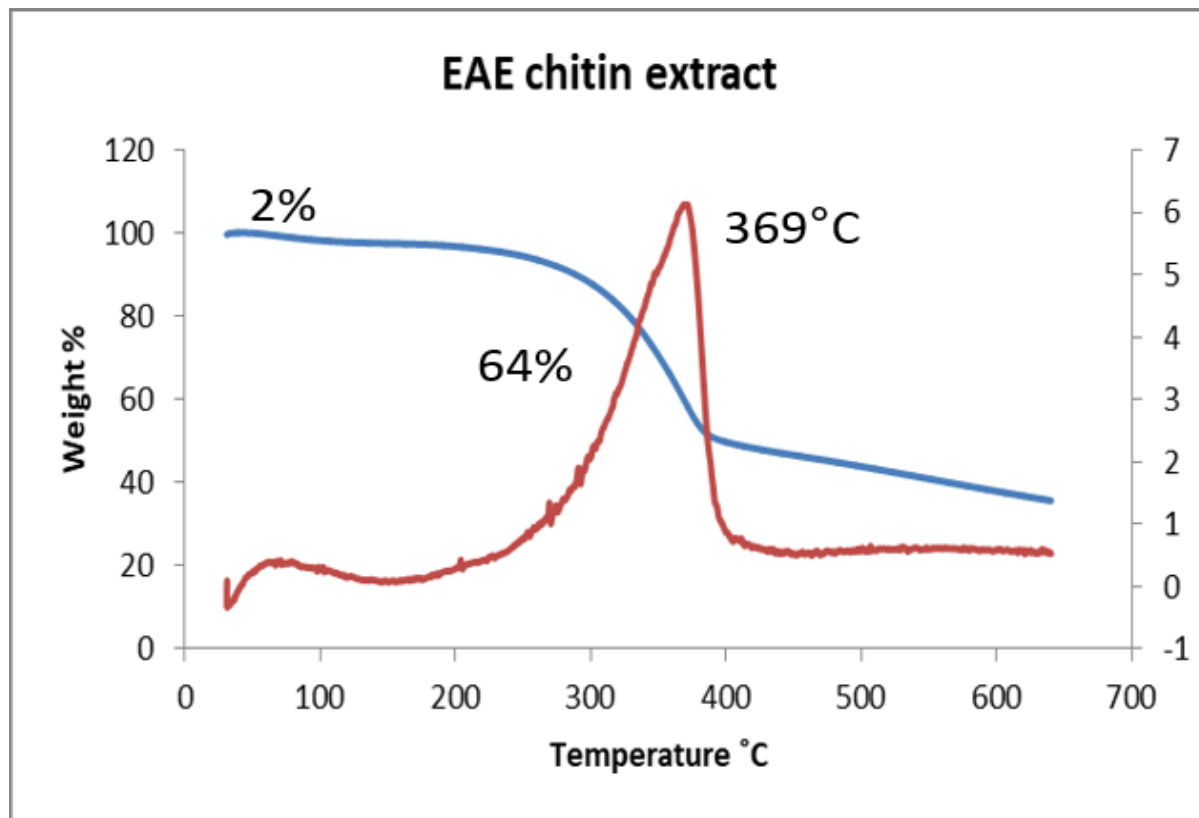
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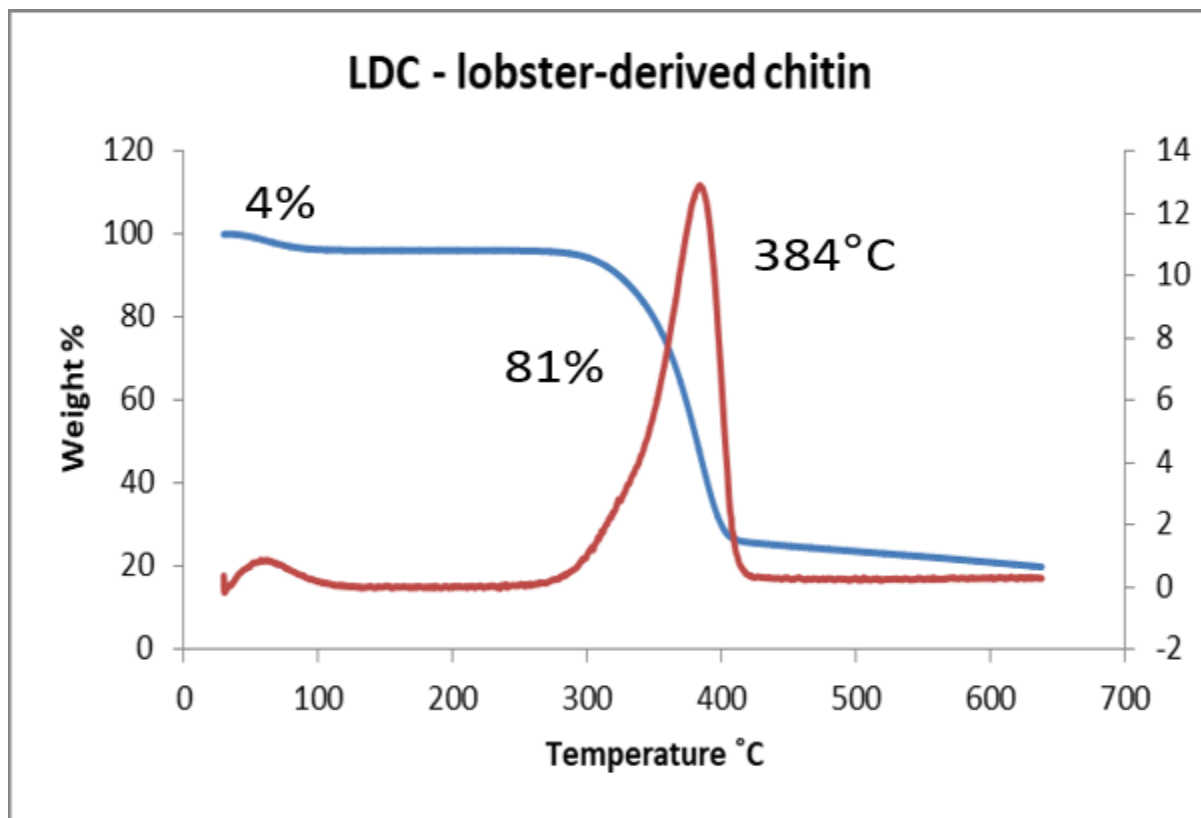
FIG B5. TGA/DTG OF LOBSTER SHELL-DERIVED CHITIN
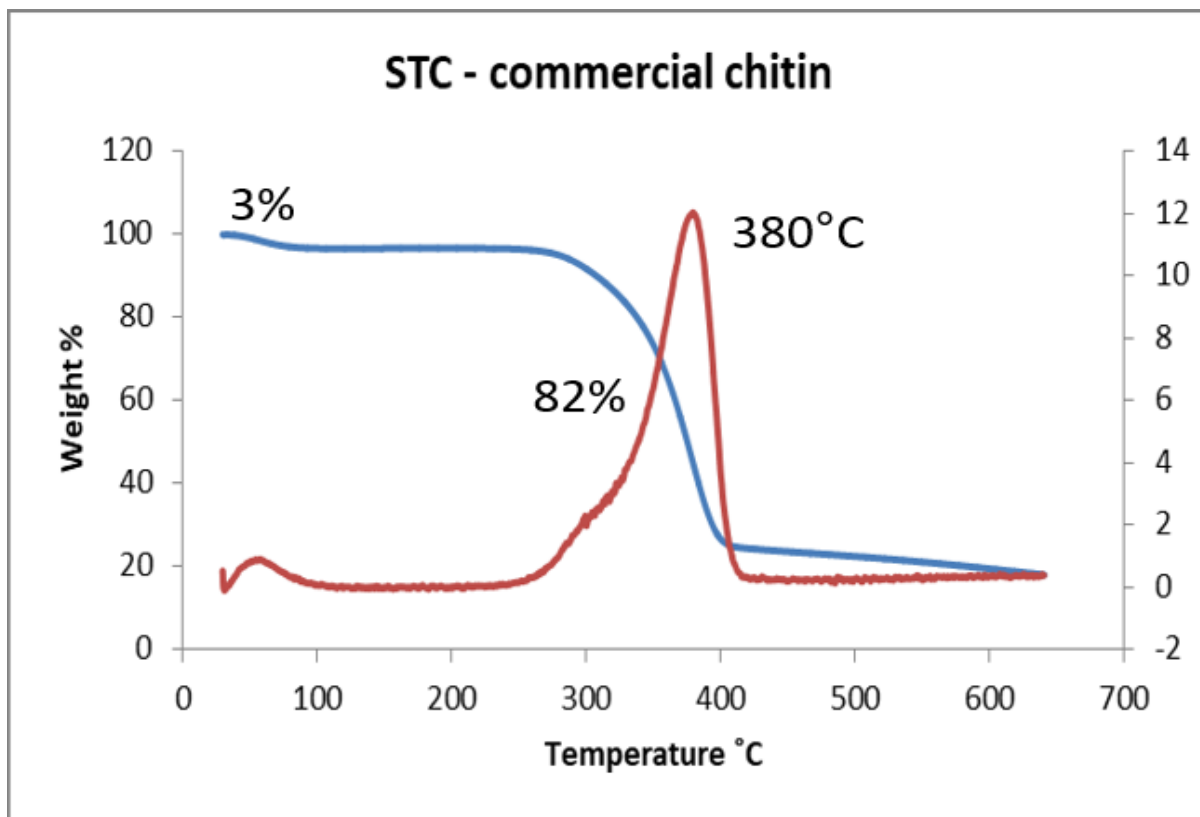
FIG B6. TGA/DTG OF COMMERCIAL CHITIN
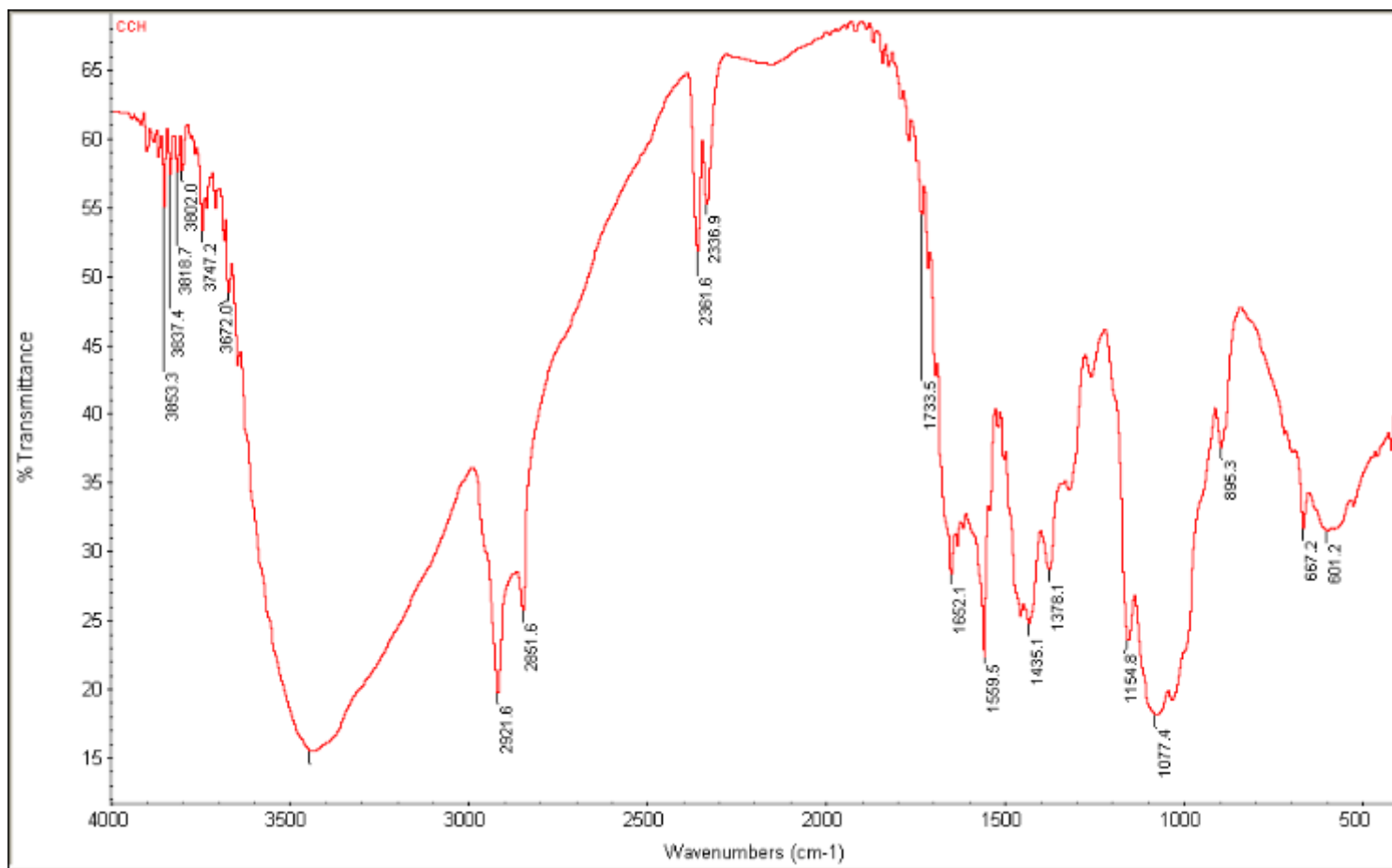
FIG C1. FTIR SPECTRUM OF INSECT CHITOSAN FROM CONVENTIONAL CHEMICAL CHITIN EXTRACT (CCH)
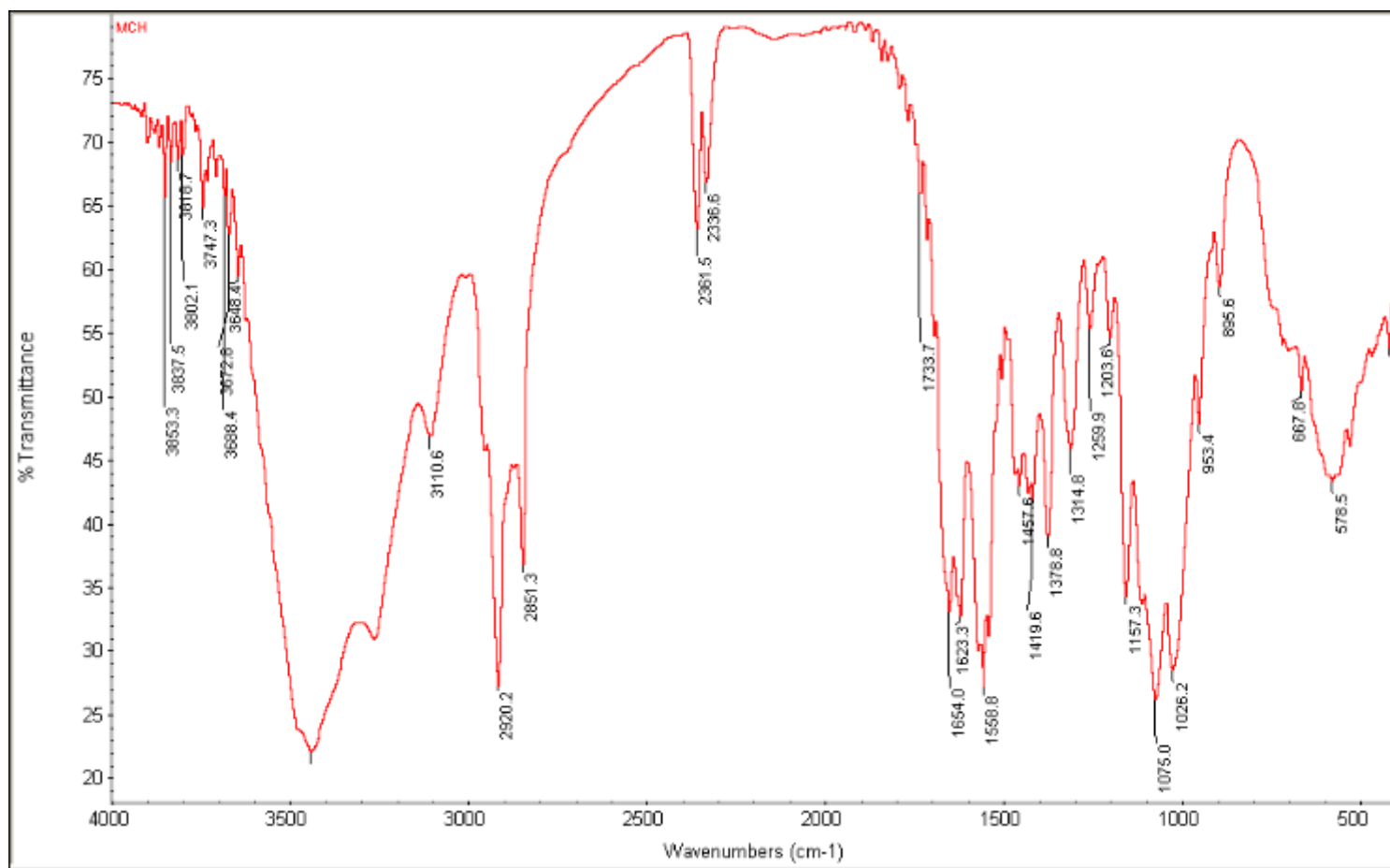
FIG C2. FTIR SPECTRUM OF INSECT CHITOSAN FROM MICROWAVE - ASSISTED CHITIN EXTRACT (MCH)
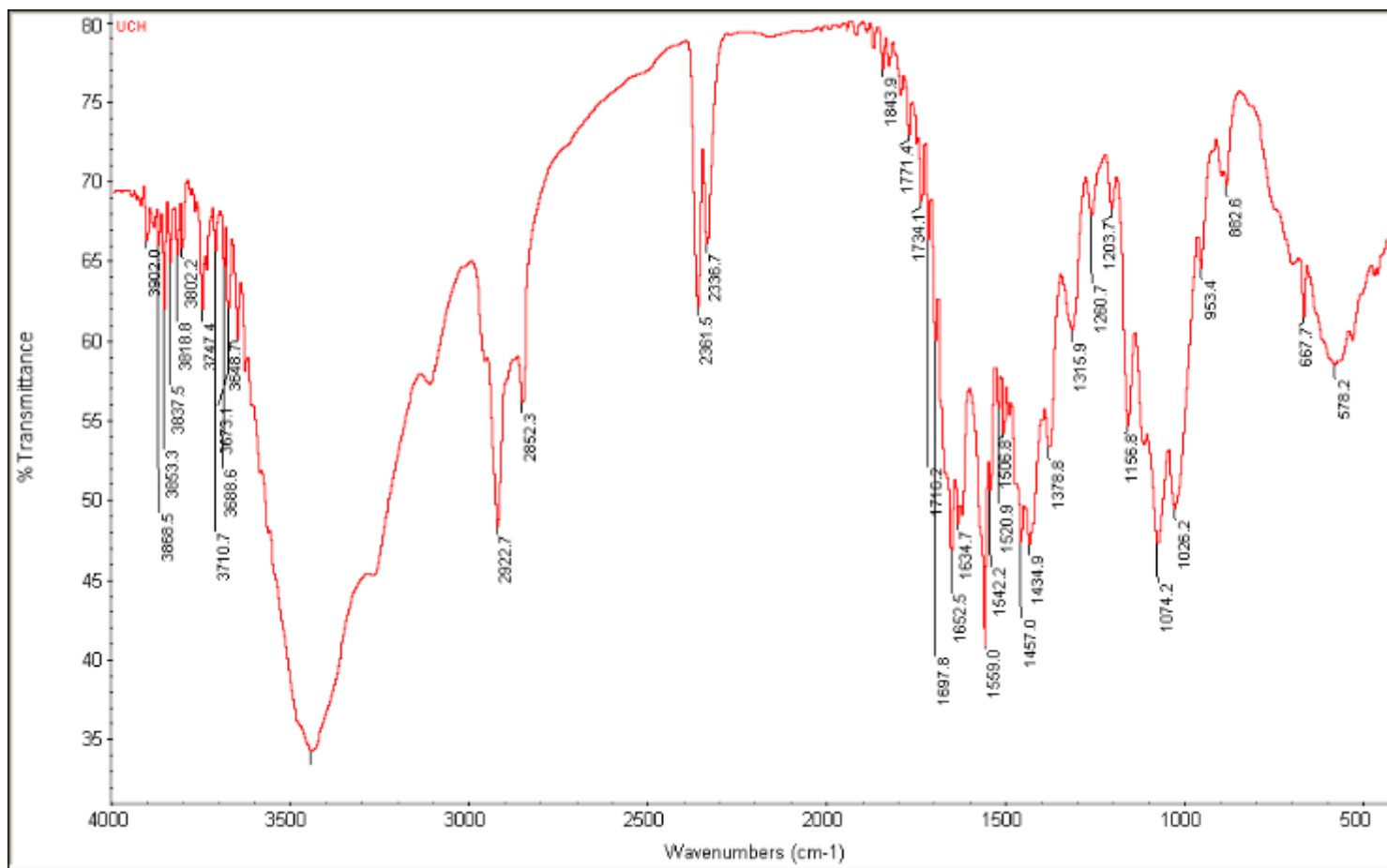
FIG C3. FTIR SPECTRUM OF INSECT CHITOSAN FROM ULTRASOUND - ASSISTED CHITIN EXTRACT (UCH)
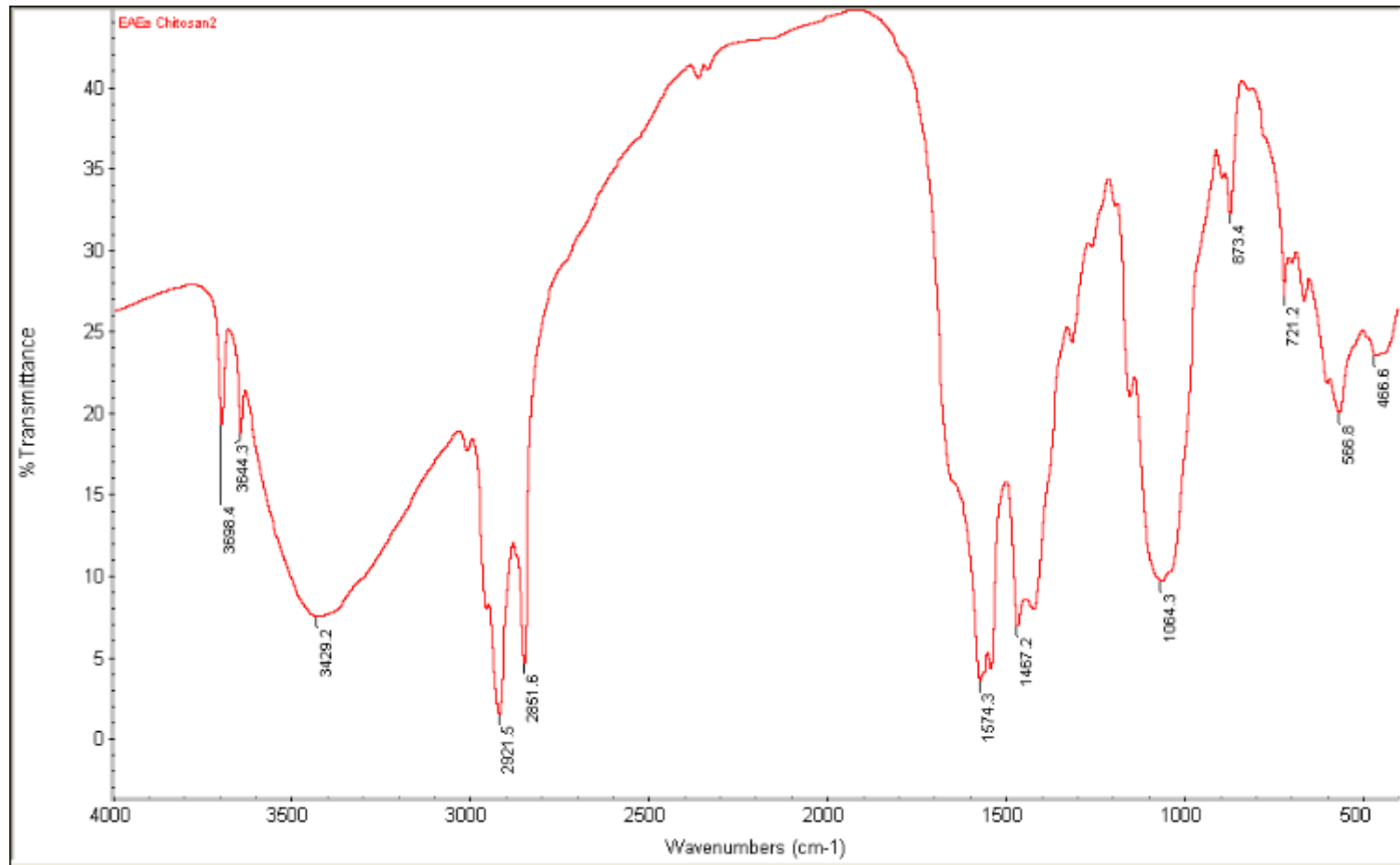
FIG C4. FTIR SPECTRUM OF INSECT CHITOSAN FROM ENZYME- ASSISTED CHITIN EXTRACT (ECHA)
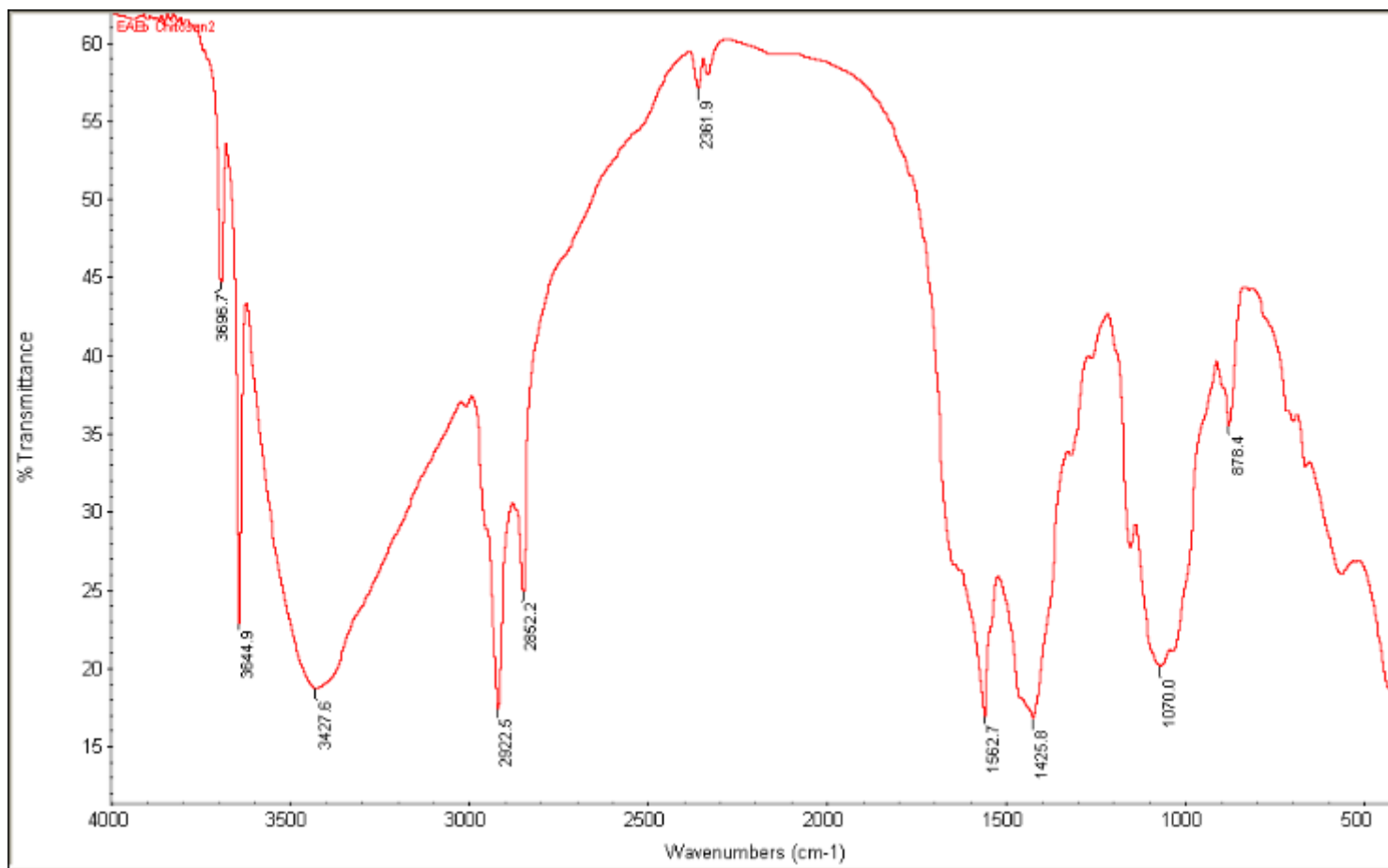
FIG C5. FTIR SPECTRUM OF INSECT CHITOSAN FROM ENZYME- ASSISTED CHITIN EXTRACT (ECHB)
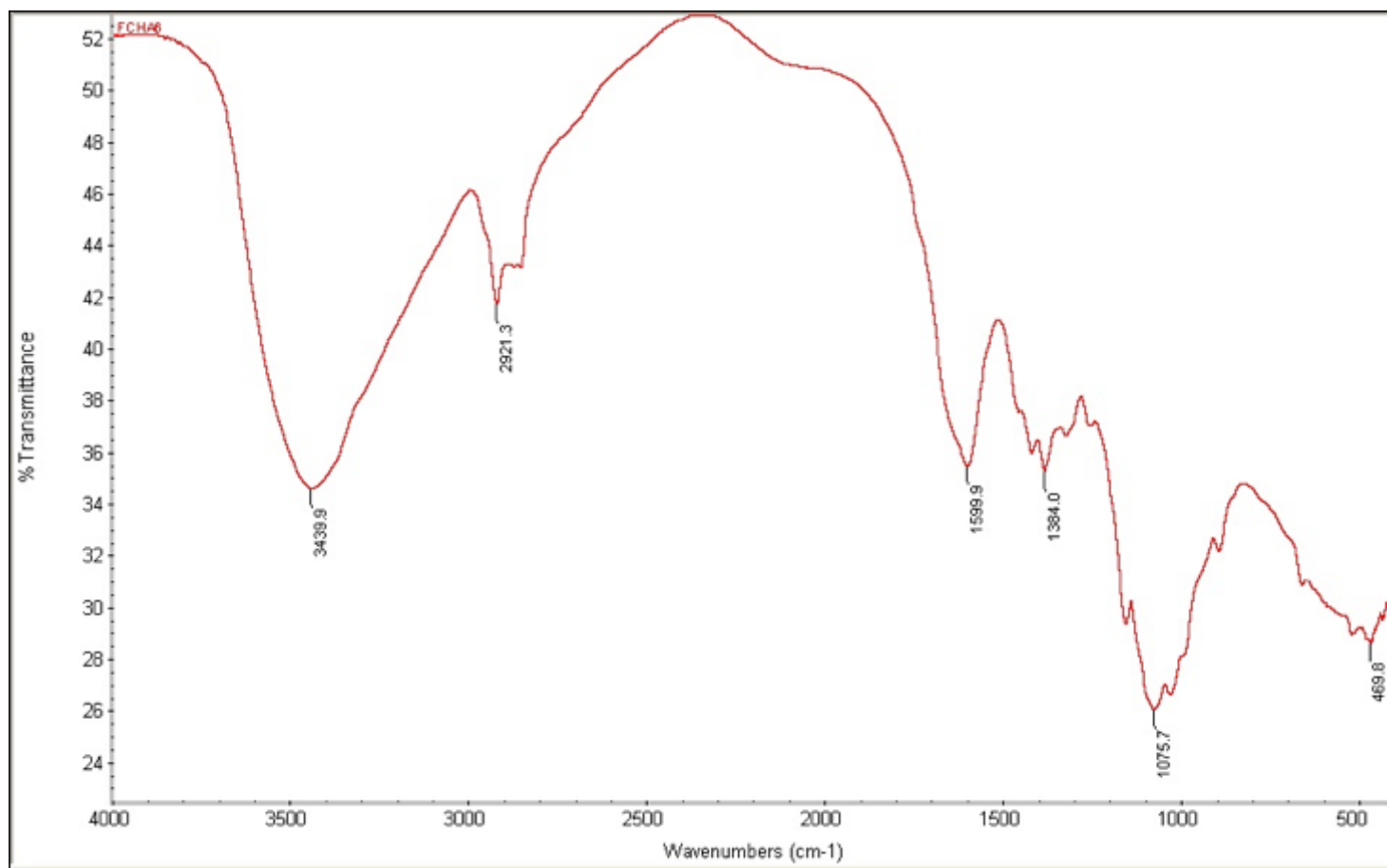
FIG C6. FTIR SPECTRUM OF FUNGAL CHITOSAN EXTRACT (FCHA)
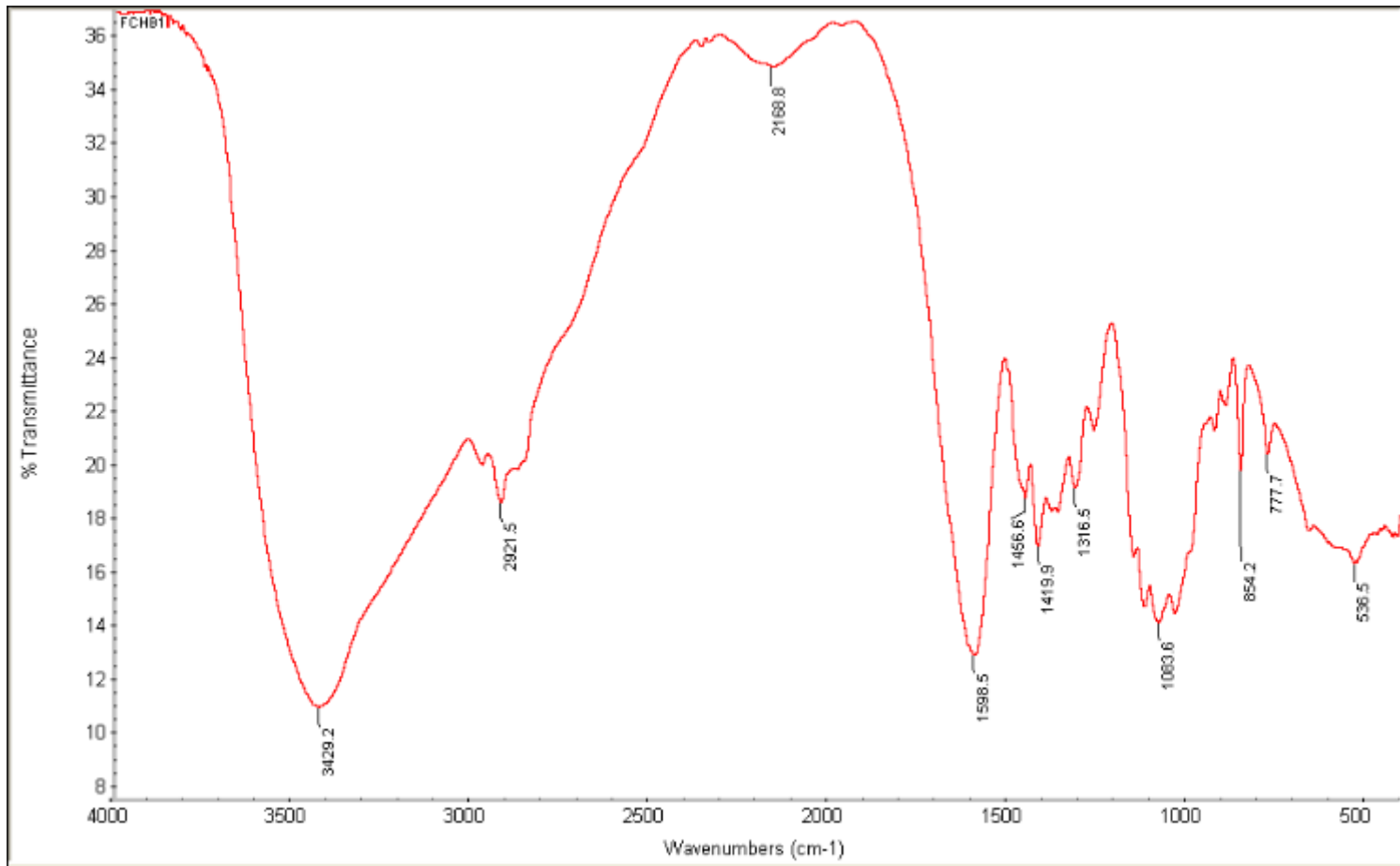
FIG C7. FTIR SPECTRUM OF FUNGAL CHITOSAN EXTRACT (FCHB)
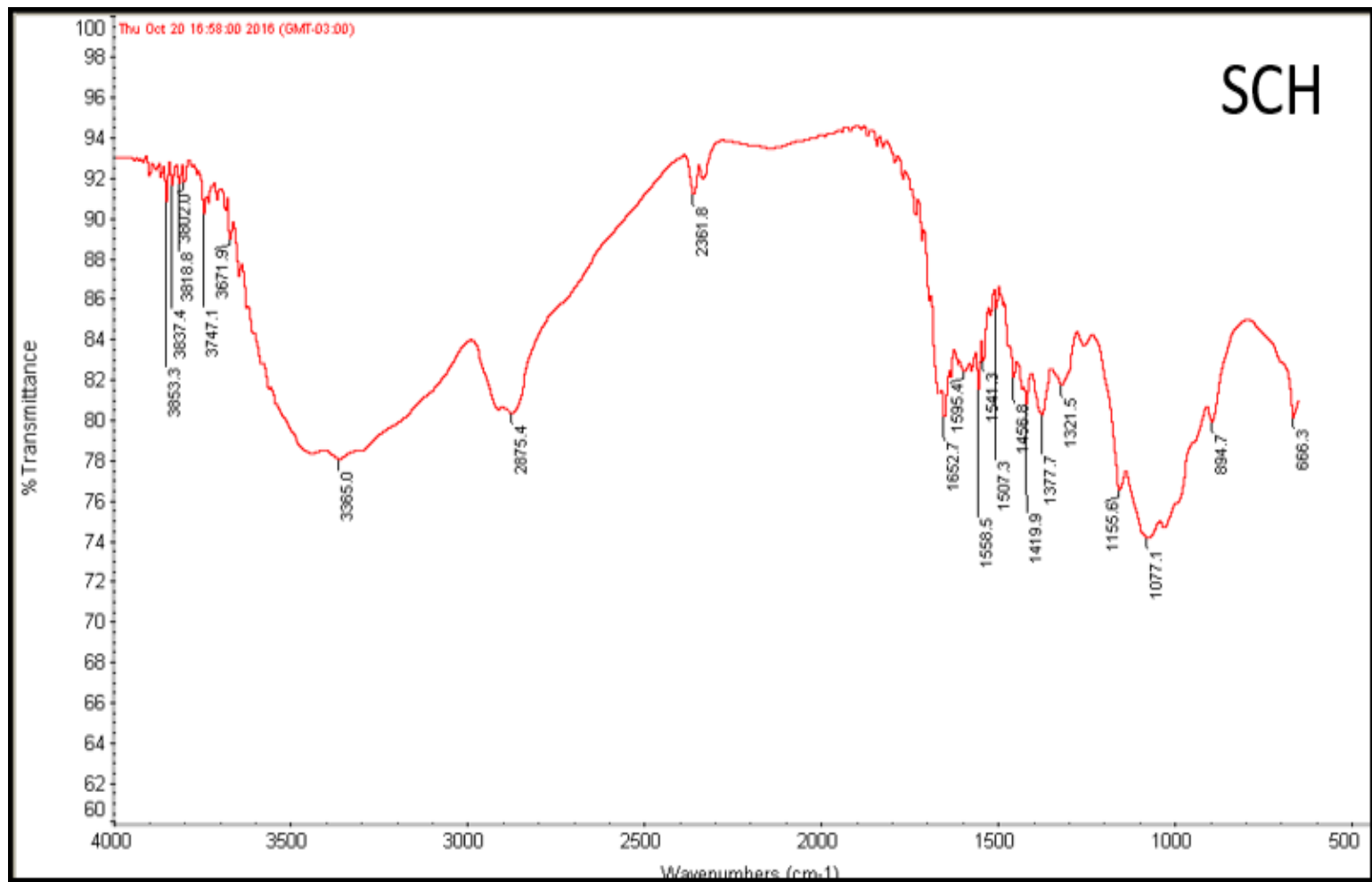
FIG C8. FTIR SPECTRUM OF COMMERCIAL CHITOSAN (SCH)
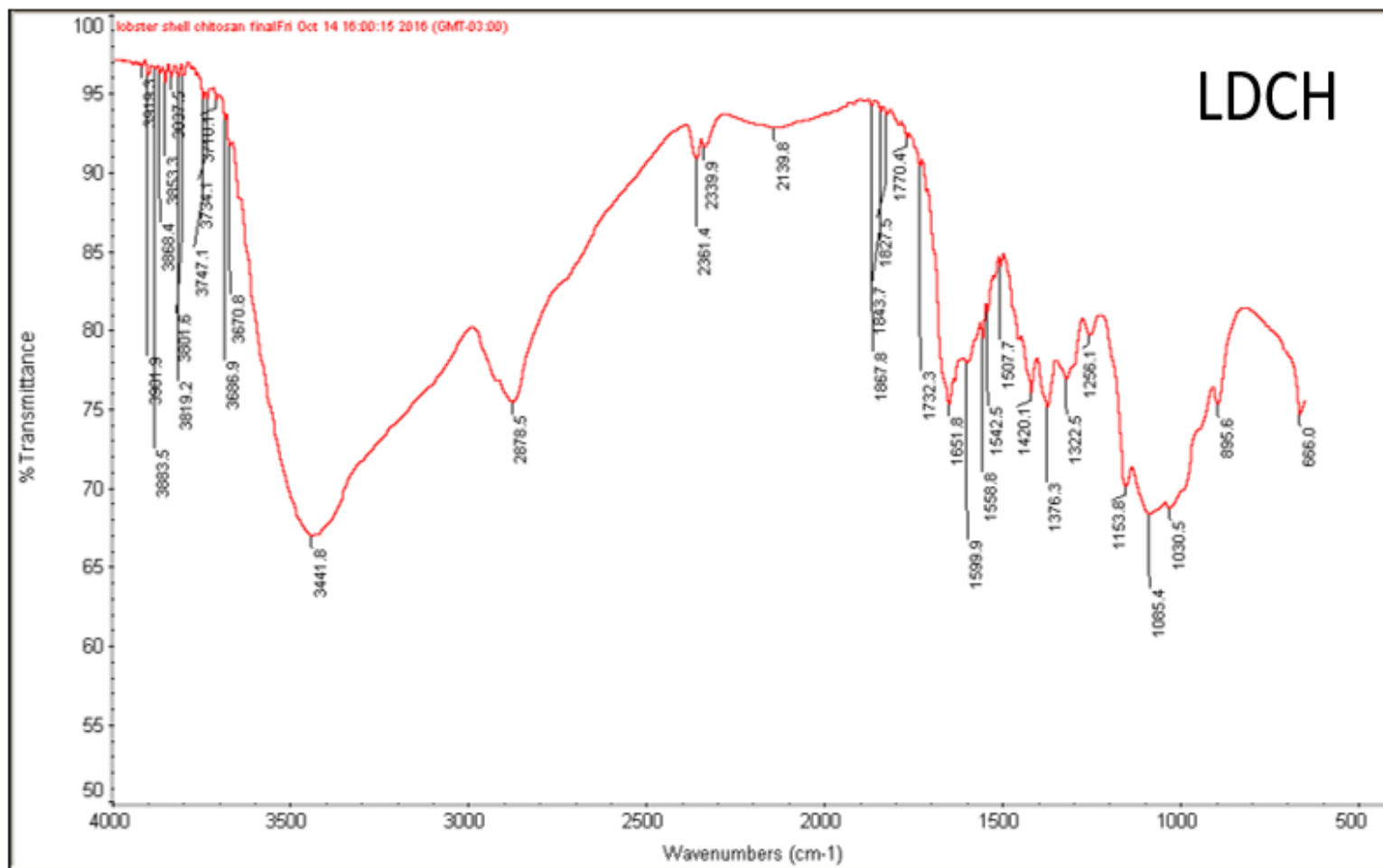
FIG C9. FTIR SPECTRUM OF LOBSTER SHELL DERIVED CHITOSAN (LDCH)
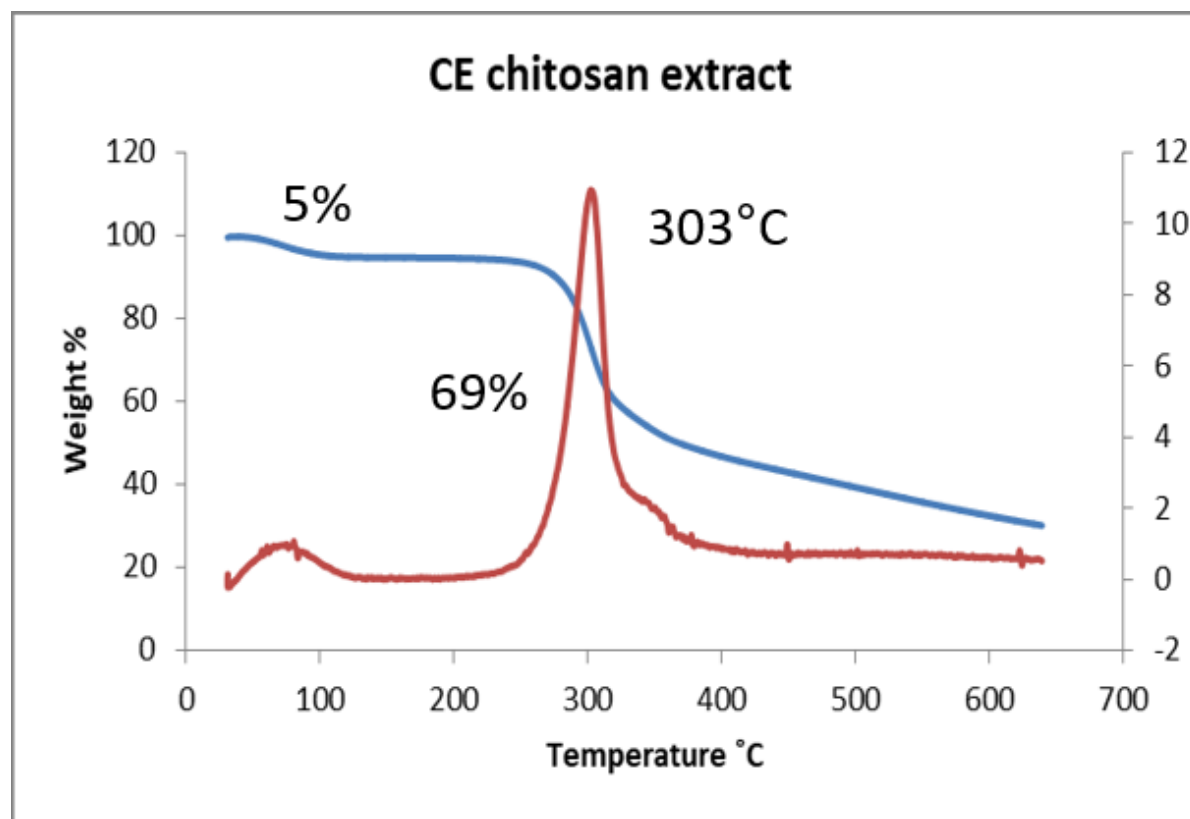
FIG D1. TGA/DTG OF INSECT CHITOSAN FROM CONVENTIONAL CHEMICAL CHITIN EXTRACT (CCH)
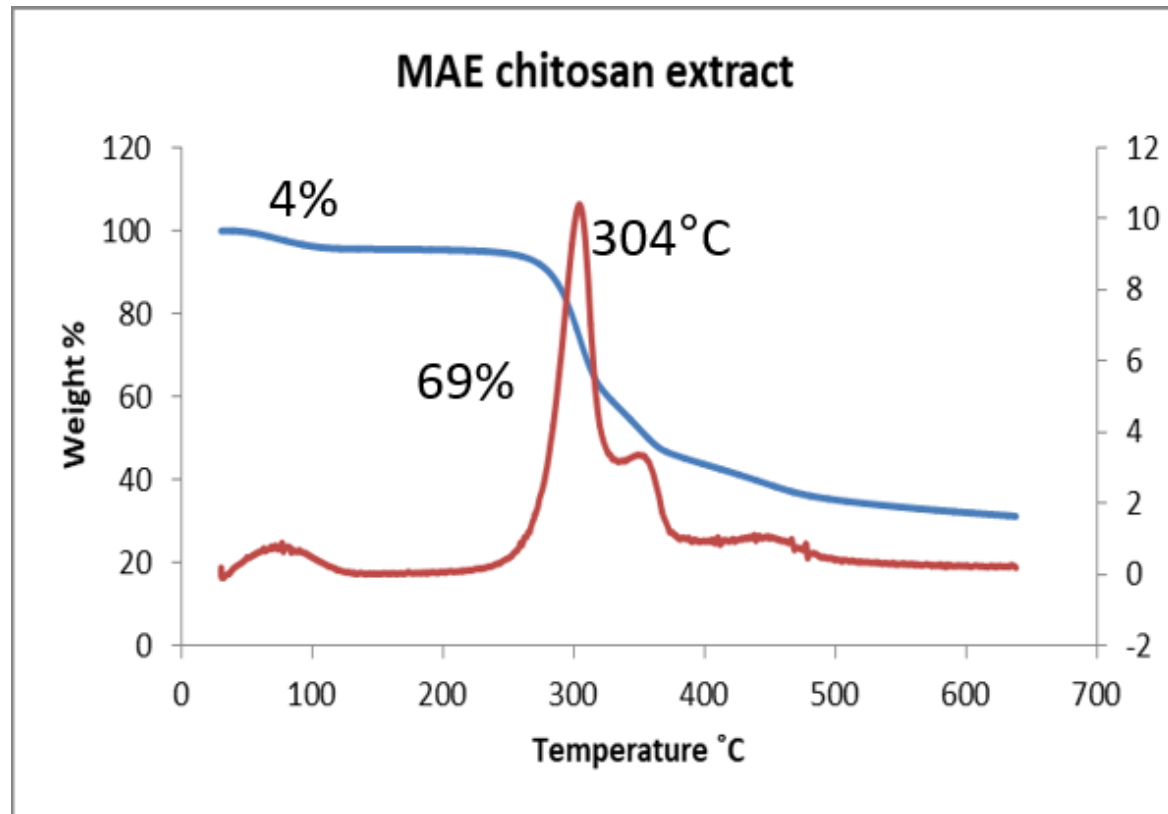
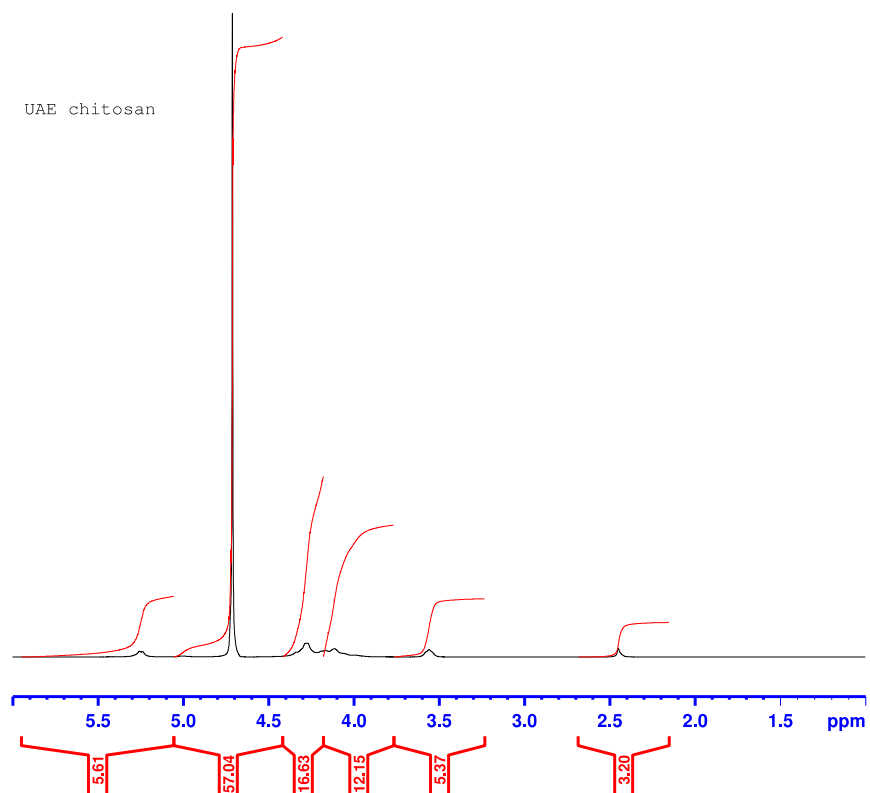**FIG D2.** TGA/DTG OF INSECT CHITOSAN FROM MICROWAVE - ASSISTED CHITIN EXTRACT (MCH)
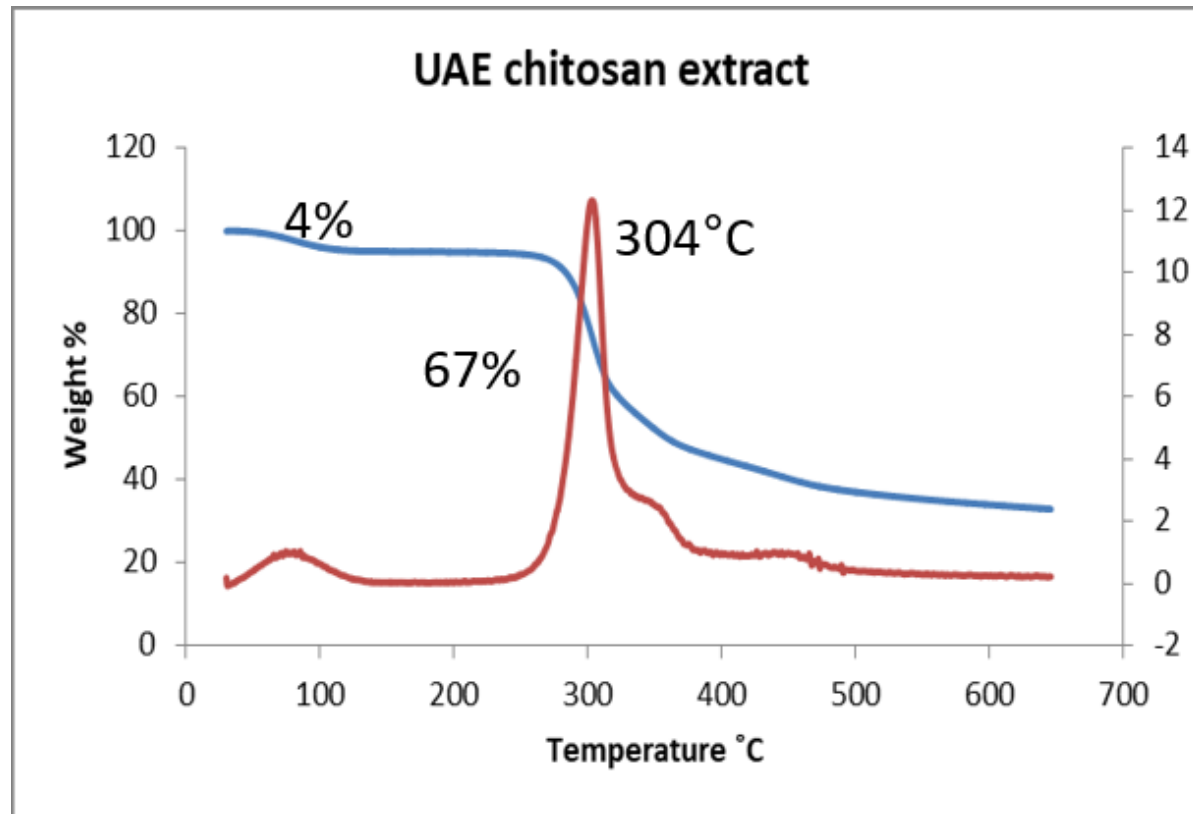
FIG D3. TGA/DTG OF INSECT CHITOSAN FROM ULTRASOUND - ASSISTED CHITIN EXTRACT (UCH)
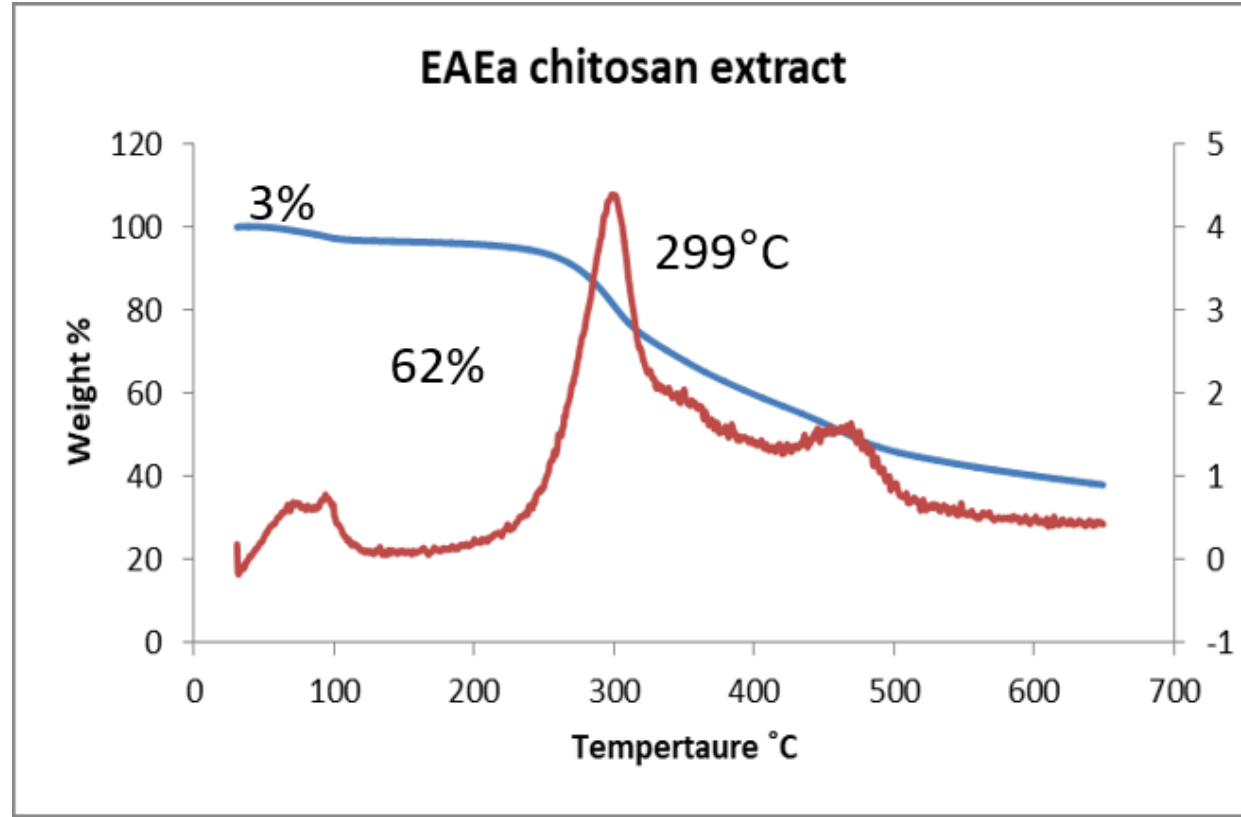
FIG D4. TGA/DTG OF INSECT CHITOSAN FROM ENZYME- ASSISTED CHITIN EXTRACT (ECHA)
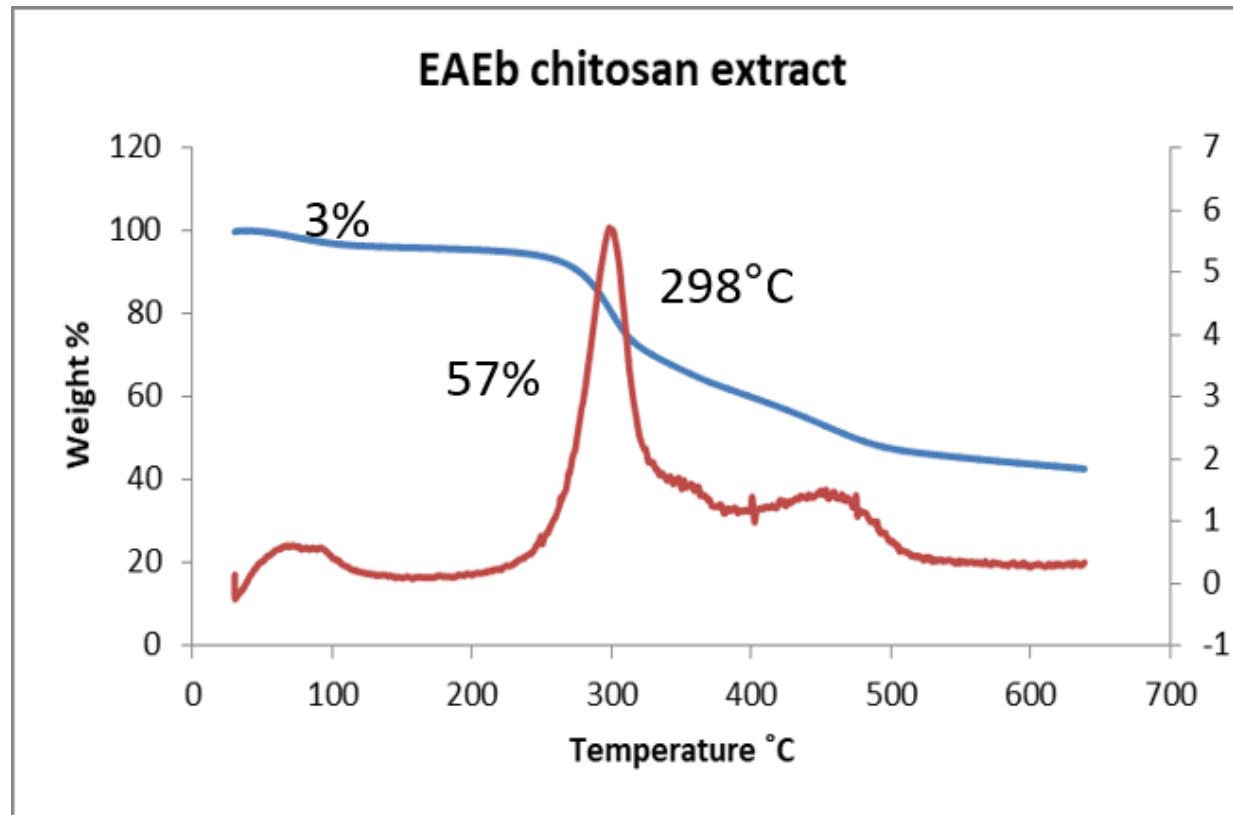
FIG D5. TGA/DTG OF INSECT CHITOSAN FROM ENZYME- ASSISTED CHITIN EXTRACT (ECHB)
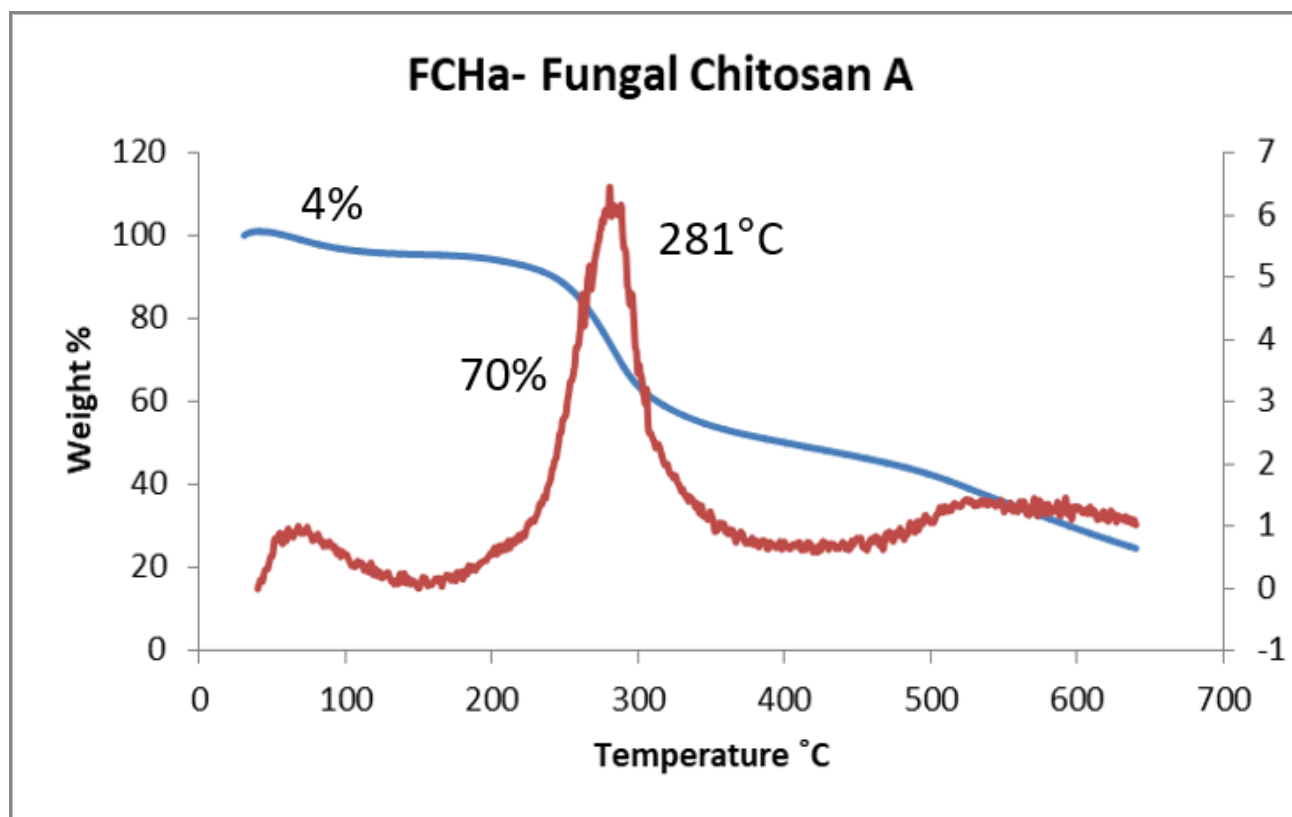
FIG D6. TGA/DTG OF FUNGAL CHITOSAN EXTRACT (FCHA)
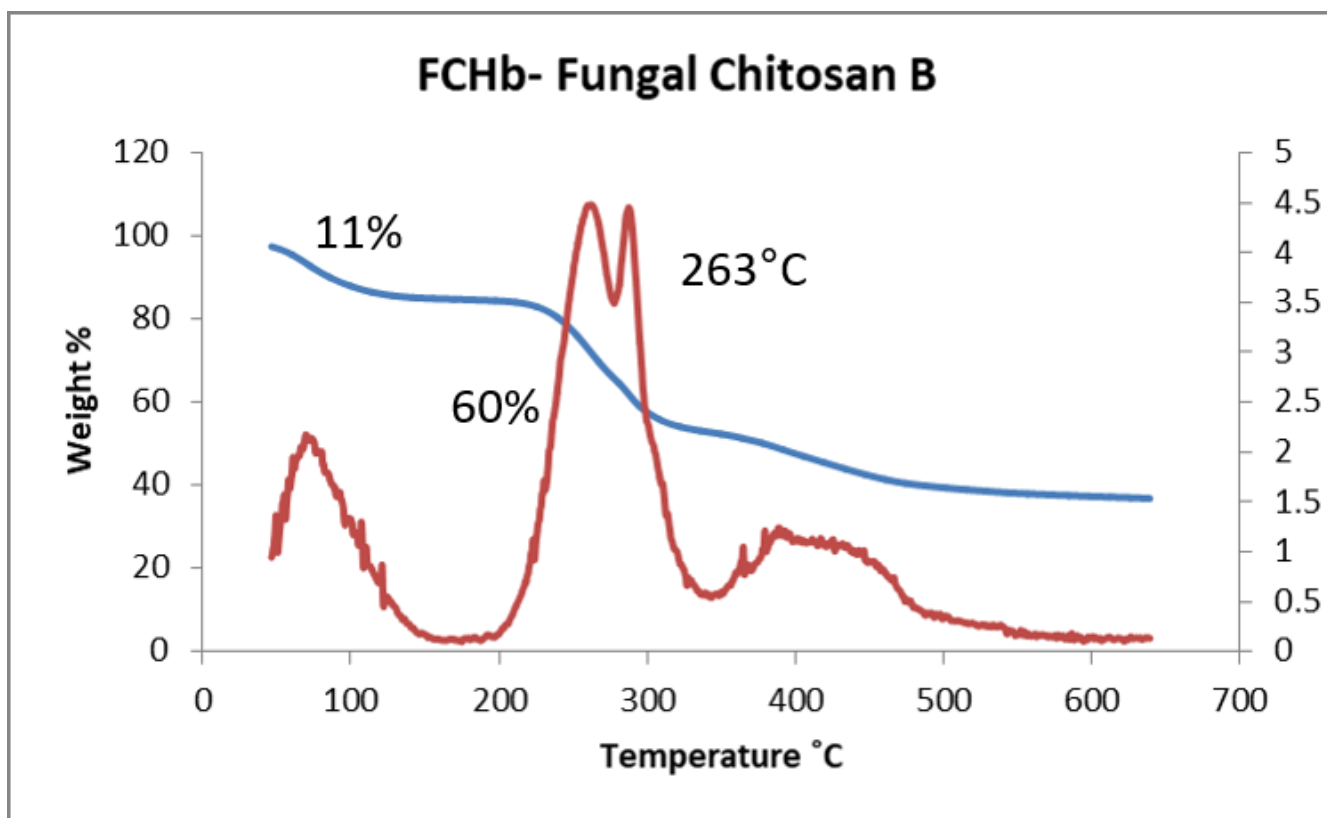
FIG D7. TGA/DTG OF FUNGAL CHITOSAN EXTRACT (FCHB)
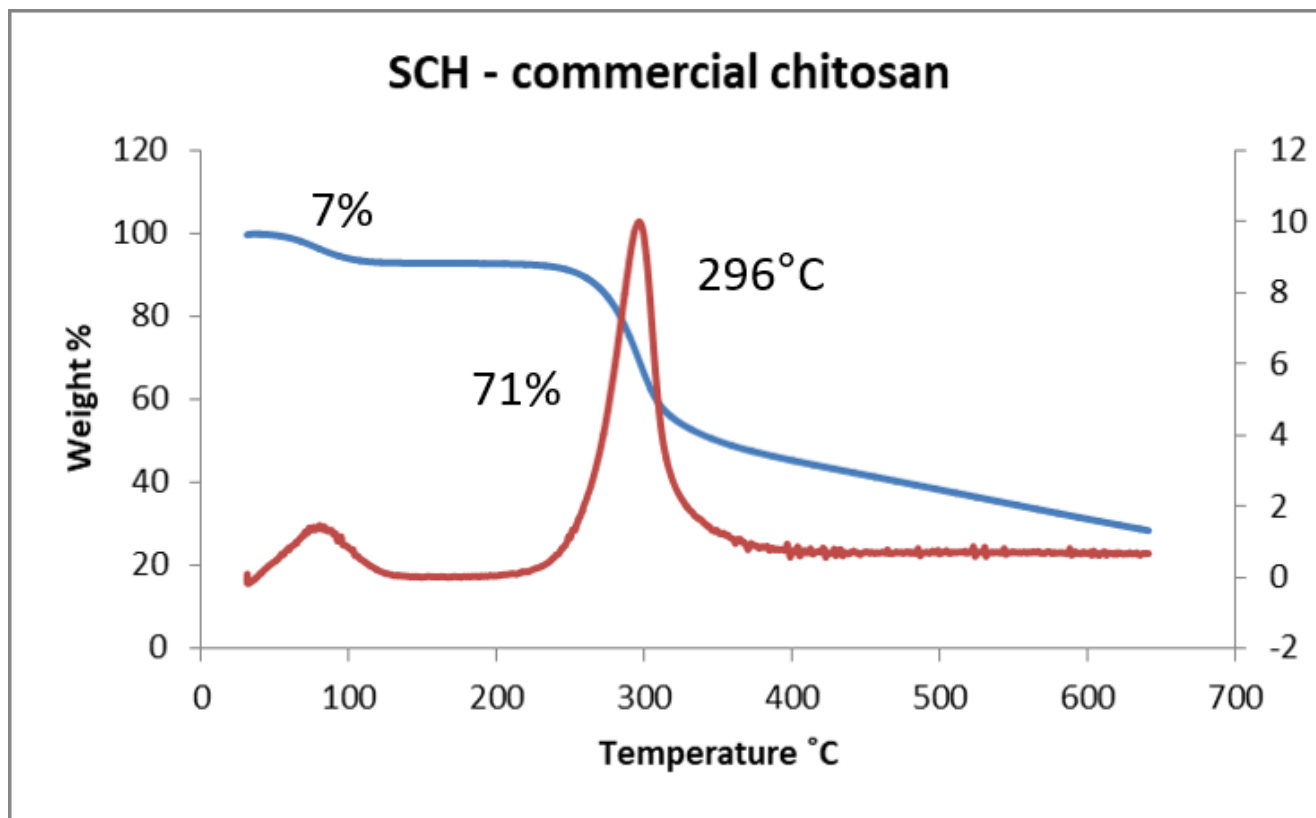
FIG D8. TGA/DTG OF COMMERCIAL CHITOSAN (SCH)
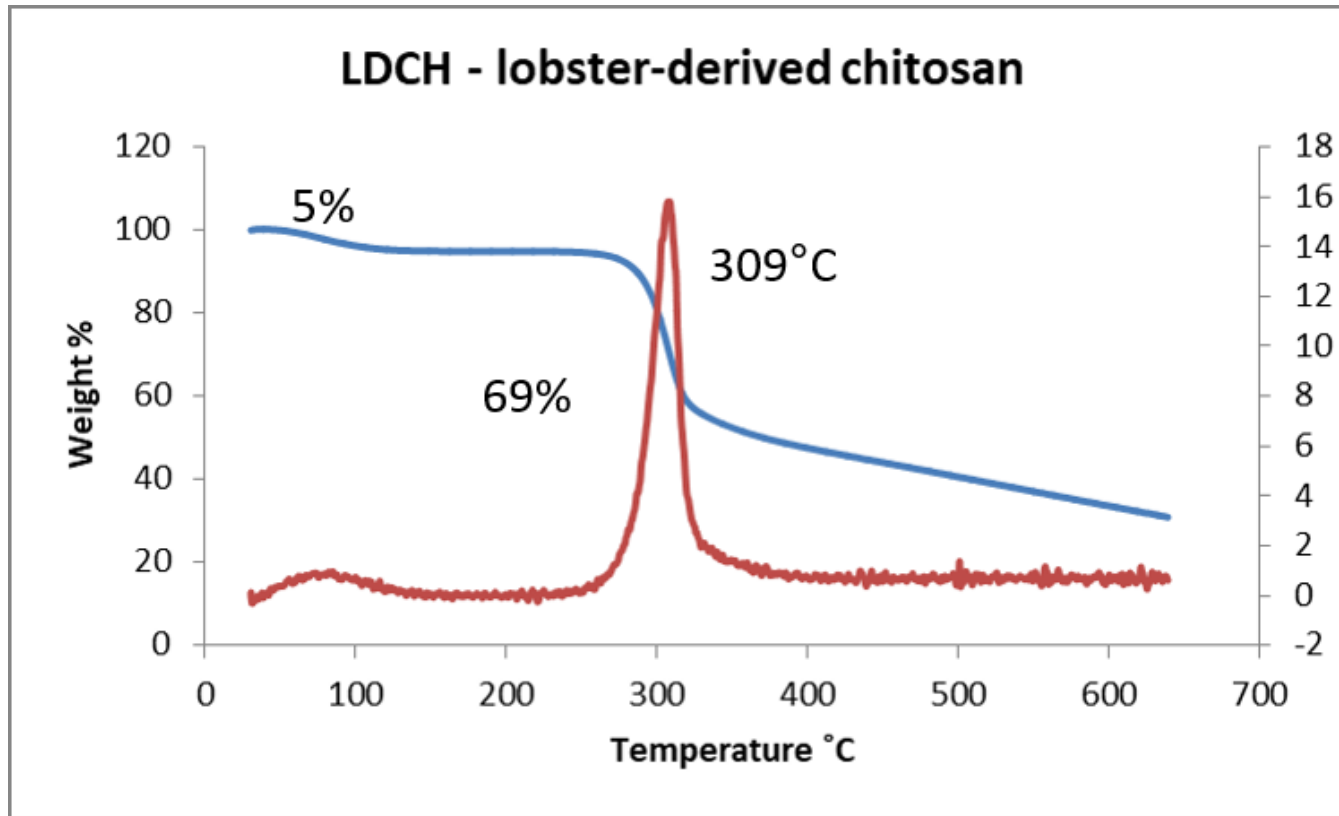
FIG D9. TGA/DTG OF LOBSTER SHELL DERIVED CHITOSAN (LDCH)
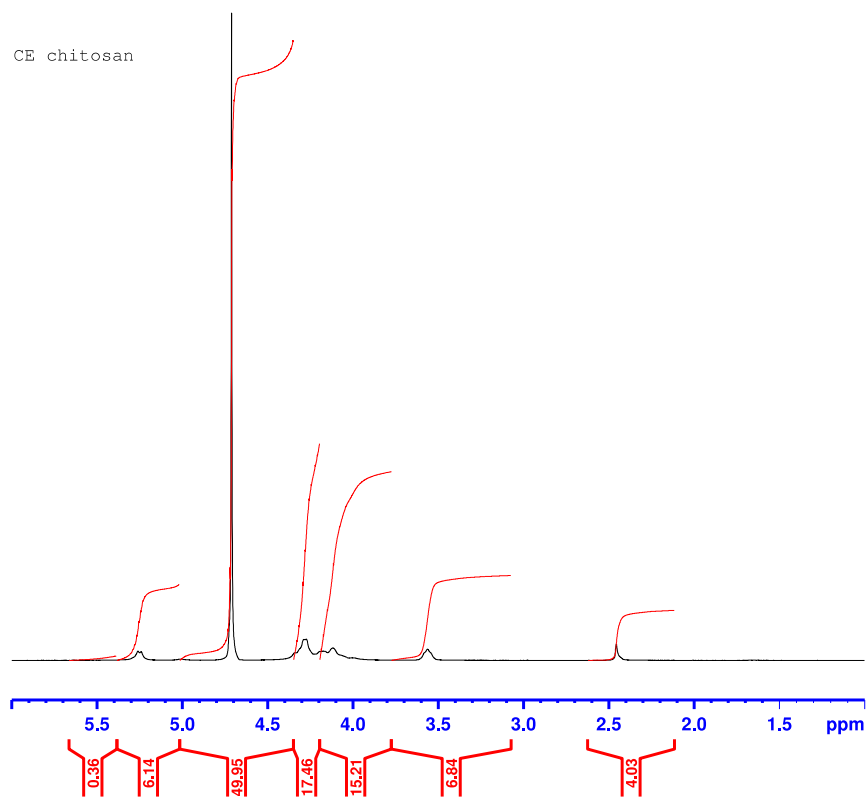
FIG E1. THE H¹ NMR SPECTRUM OF INSECT CHITOSAN FROM CONVENTIONAL CHEMICAL CHITIN EXTRACT (CCH)

FIG E2. THE H[1] NMR SPECTRUM OF INSECT CHITOSAN FROM MICROWAVE – ASSISTED CHITIN EXTRACT (MCH)

UAE chitosan

5.61   57.04   16.63   12.15   5.37   3.20

5.5   5.0   4.5   4.0   3.5   3.0   2.5   2.0   1.5   ppm

FIG E3. THE H[1] NMR SPECTRUM OF INSECT CHITOSAN FROM ULTRASOUND – ASSISTED CHITIN EXTRACT (UCH)

EAEa Chitosan

5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 ppm

0.78 5.29 48.79 27.02 4.62 3.59 7.78 2.14

FIG E4. THE H[1] NMR SPECTRUM OF INSECT CHITOSAN FROM ENZYME – ASSISTED CHITIN EXTRACT (ECHA)

EAEb chitosan

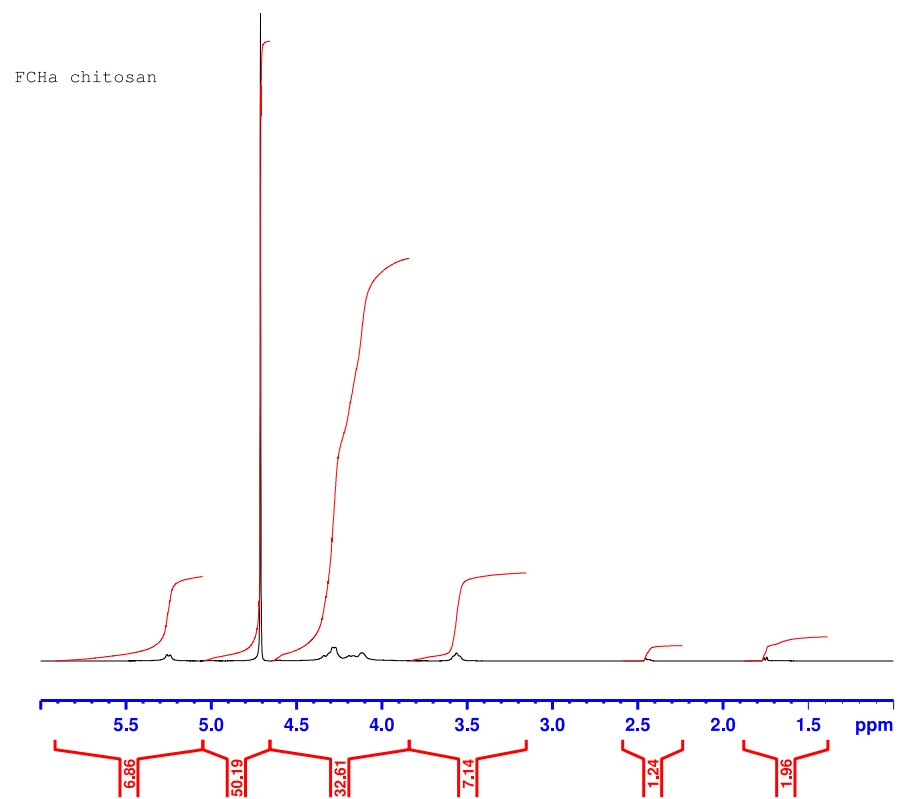**FIG E5.** THE H^1 NMR SPECTRUM OF INSECT CHITOSAN FROM ENZYME – ASSISTED CHITIN EXTRACT (ECHB)

FCHa chitosan

5.5   5.0   4.5   4.0   3.5   3.0   2.5   2.0   1.5   ppm

6.86   50.19   32.61   7.14   1.24   1.96
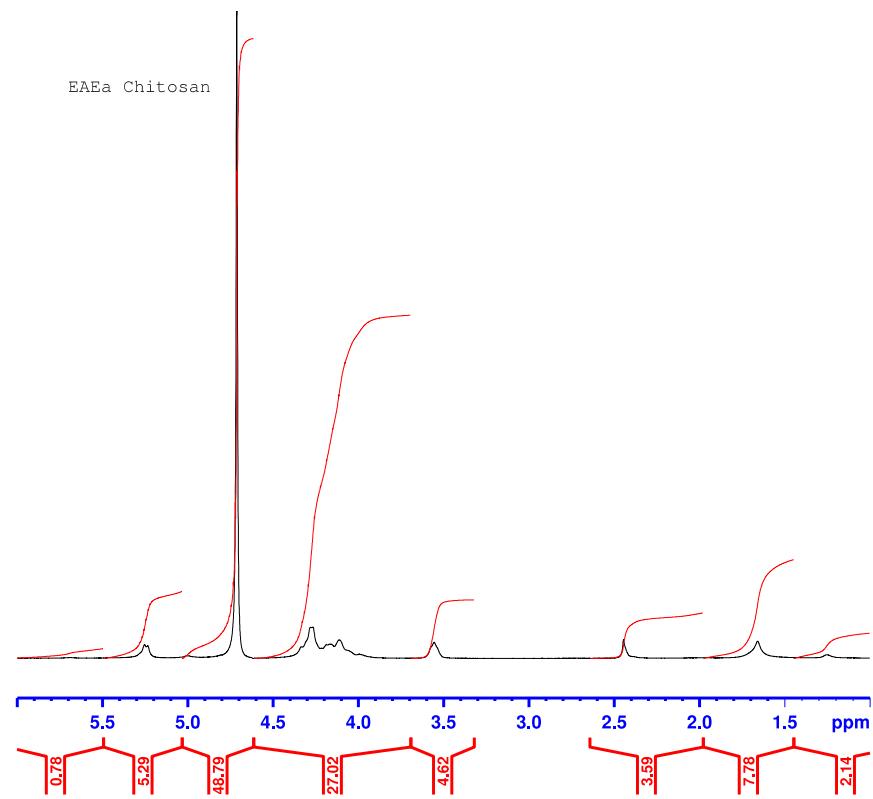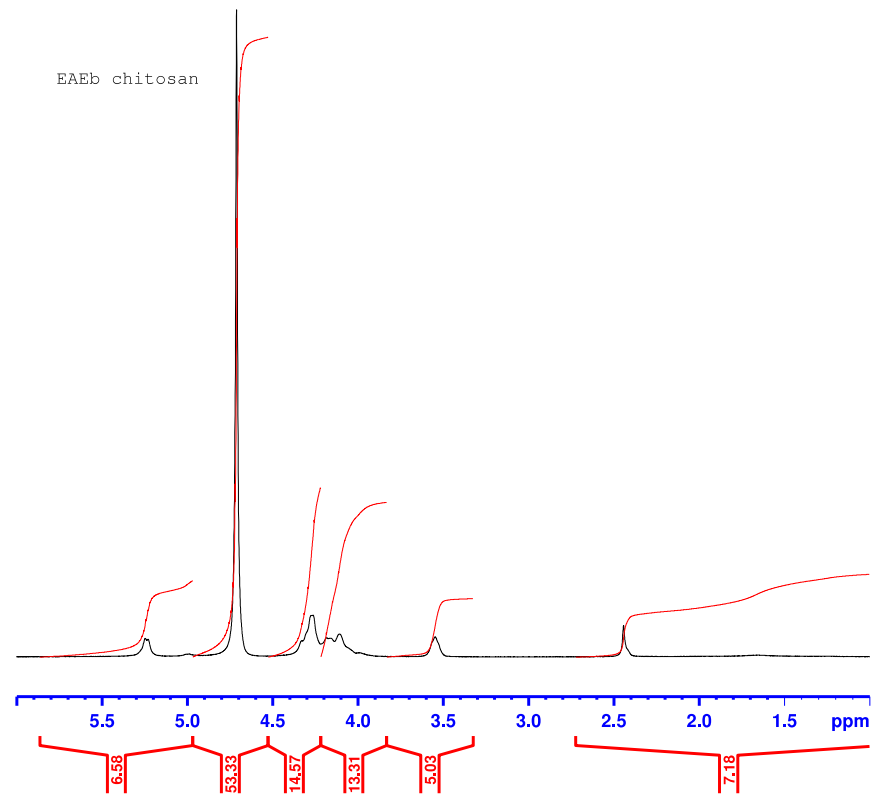
FIG E6. THE H^1 NMR SPECTRUM OF FUNGAL CHITOSAN EXTRACT (FCHA)

FCHb chitosan

FIG E7. THE H^1 NMR SPECTRUM OF FUNGAL CHITOSAN EXTRACT (FCHB)

Commercial chitosan (SCH)

5.28   48.66   15.84   13.72   5.90   7.42   3.18
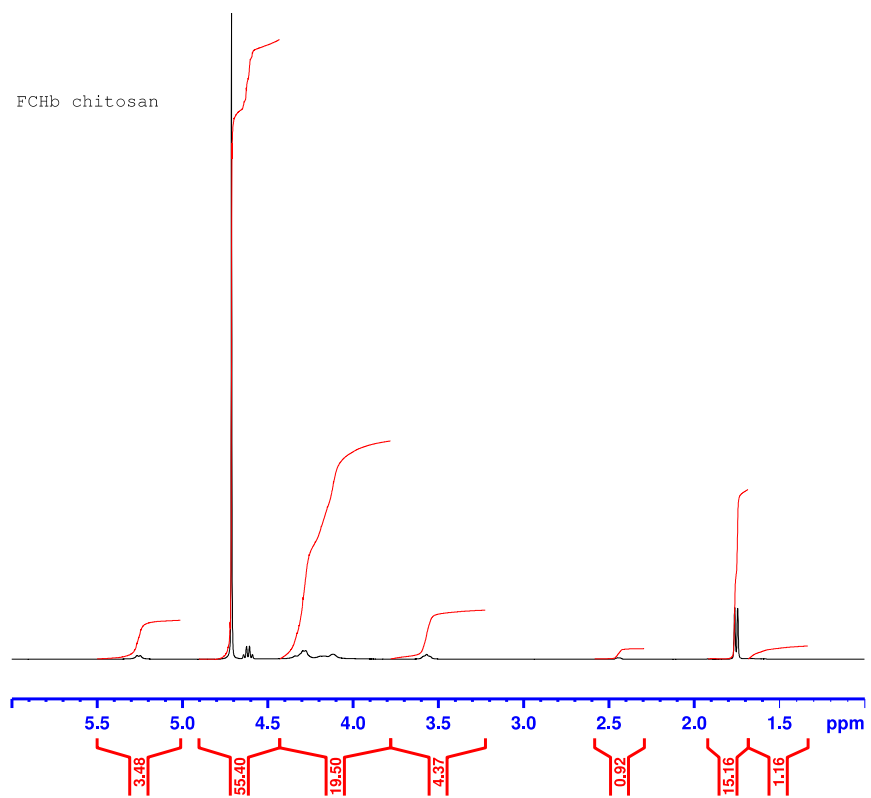
FIG E8. THE H[1] NMR SPECTRUM OF COMMERCIAL CHITOSAN (SCH)

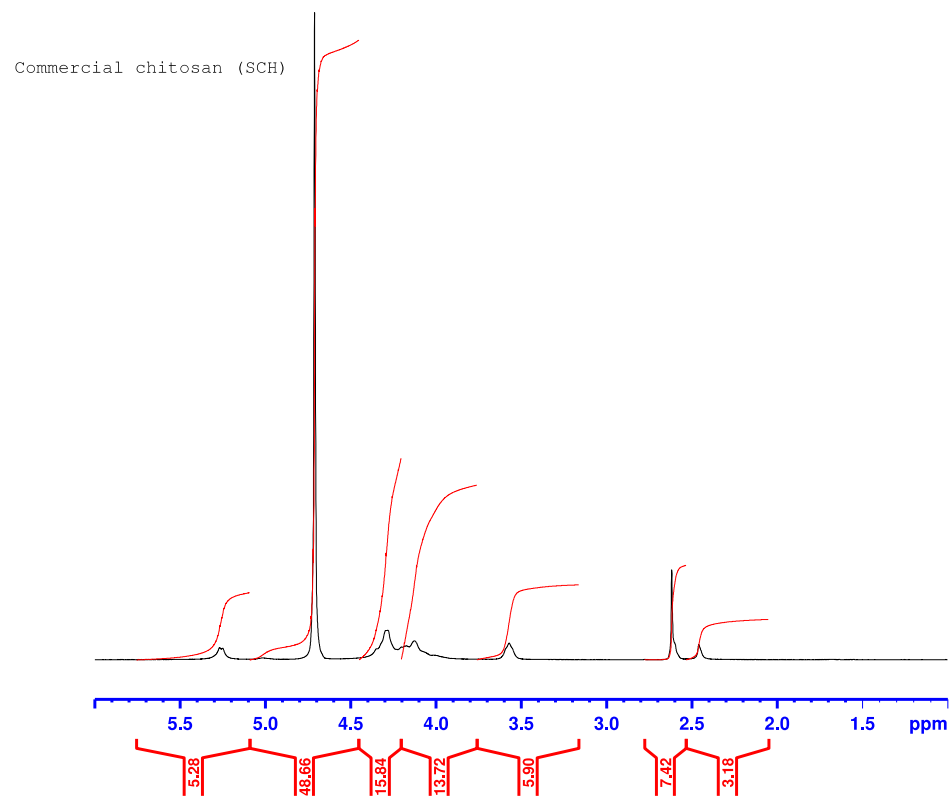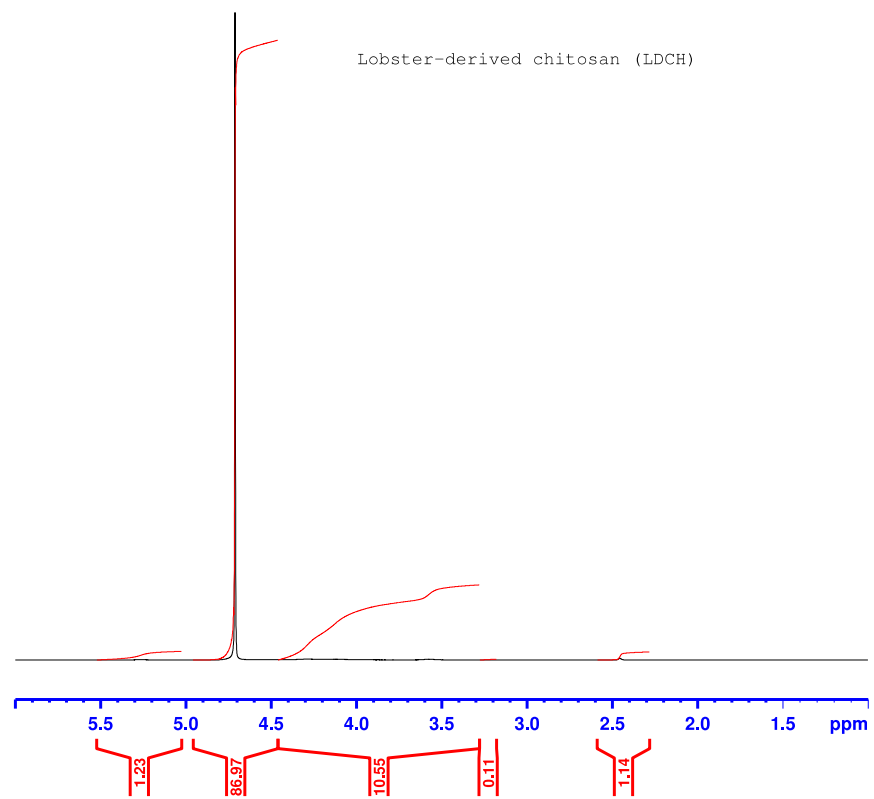Lobster-derived chitosan (LDCH)

FIG E9. THE H[1] NMR SPECTRUM OF LOBSTER SHELL DERIVED CHITOSAN (LDCH)