UNIVERSITI
MALAYSIA
KELANTAN

ANTIMICROBIAL PROPERTIES OF CHITOSAN EXTRACT FROM ETOK SHELL (*CORBICULA FLUMINEA*)

Nursyasya Athirah Binti Mohd Suhaimin

J20A0730

A reported submitted in fulfilment of the requirements for the degree of Bachelor of Applied Science (Bioindustrial Technology) With Honours

FACULTY OF BIOENGINEERING AND TECHNOLOGY

UMK

2024

DECLARATION

I declare that this thesis entitled "ANTIMICROBIAL PROPERTIES OF CHITOSAN EXTRACT FROM ETOK SHELL (*CORBICULA FLUMINEA*)" is the results of my own research except as cited in the references.

Signature : 

Student's Name : NURSYASYA ATHIRAH BINTI MOHD SUHAIMIN

Date : 29/2/2024

Verified by:

Signature :

Supervisor's Name : DR. ZUBAIDAH AIMI BINTI ABDUL HAMID

Stamp :

Date :

ACKNOWLEDGEMENT

Firstly, I would like to express my sincere gratitude to my thesis advisor, Dr.Zubaidah Aimi Binti Abdul Hamid, for her invaluable guidance and support throughout this project. I am especially grateful for her willingness to meet with me regularly, provide insightful feedback, and challenge me to think critically about my research. I would also like to thank my committee members, Syahakimi Bin Hasbi, Nur Zulaikal Binti Ramlee, Azisyamimi Binti Mohd, Nur Munirah Nadjwa Binti Nazri and Nur Qurratuain Binti Baharuddin for their helpful suggestions and feedback on my thesis.

Additionally, I would like to extend my sincere appreciation to Universiti Malaysia Kelantan because has provide all the facilities to use using laboratory work in thesis project. Because of this I enabled to successful to complete my research.

Finally, I would like to thank my family and friends for their love and support throughout this journey. Their encouragement and understanding helped me to stay motivated and persevere through challenging times in laboratory work.

SIFAT ANTIMIKROB BAGI EKSTRAK KITOSAN DARIPADA CENGKERANG ETOK (*CORBICULA FLUMINEA*)

ABSTRAK

Kemampanan, biodegradasi, biokompatibiliti, tidak toksik dan penjerapan kitosan membolehkan pelbagai kegunaan dalam makanan, rawatan air, pertanian, kosmetik dan ubat-ubatan. Matlamat penyelidikan ini adalah untuk mengekstrak kitosan daripada cengkerang etok dan menyiasat sifat antimikrobnya. Negeri Kelantan menawarkan banyak sumber etok yang boleh digunakan untuk menghasilkan pelbagai produk biojisim seperti kitosan. Prosedur pengekstrakan terdiri daripada empat fasa: penyahmineralan, penyahproteinan, penyahwarnaan, dan penyahetilasi yang menghasilkan kitin putih. Ekstrak etok diukur tahap penyahtilasi daripada hasil yang menggunakan analisis unsur, analisis spektroskopi inframerah transformasi Fourier (FTIR), dan aktiviti antimikrob telah disiasat dalam kajian ini. Hasil kitosan etok adalah peratusan hasil sebanyak 4%. Peratusan analisis unsur menunjukkan bahawa kandungan karbon dan nitrogen sampel adalah masing-masing 13.14% dan 3.38%. Selain itu, pengiktirafan khusus bagi kitosan jelas dipaparkan dalam spektrum FTIR. Puncak luas sekitar 3300-3600 cm^{-1} menunjukkan getaran regangan kedua-dua kumpulan O-H (hidroksil) dan N-H (amina) pada spektrum. Akhirnya, hampir kesemua sampel bertindak balas kepada mikrob *Escherichia coli* dalam ujian antimikrob berbanding mikrob lain.

ANTIMICROBIAL PROPERTIES OF CHITOSAN EXTRACT FROM ETOK SHELL (*CORBICULA FLUMINEA*)

ABSTRACT

Chitosan's sustainability, biodegradability, biocompatibility, nontoxicity, and adsorption enable a diverse variety of uses in food, water treatment, agriculture, cosmetics, and medicines. The aim of this research is to extract chitosan from etok shells and investigate its antimicrobial properties. The Kelantan region offers an abundance of etok sources that may be used to produce various biomass products such as chitosan. The extraction procedure consists of four phases: demineralization, deproteinazation, decolorization, and deacetylation which is produce a white chitin. The chitosan obtained from etok shell was measured by its degree of deactylation using elemental analysis, functional group by fourier transform infrared spectroscopy (FTIR), and antimicrobial activity were performed. The chitosan yield from etok is 4% by weight. The elemental analysis shows that the sample's carbon and nitrogen values are 13.14% and 3.38%, respectively. The FTIR spectrum also clearly demonstrates the particular identification of chitosan at a large peak at 3300-3600 cm^{-1} suggests stretching vibrations of both O-H (hydroxyl) and N-H (amine) groups on the spectrum. Finally, nearly all of the sample responds to the *Escherichia coli* germ in the antimicrobial test rather than another bacteria.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

NaOH	Sodium hydroxide	1
KOH	Potassium hydroxide	1
NH_2	Nitrenium ion	1
LMWS	Low molecular weight water-soluble	2
kDa	Kilodalton	2
pH	Power of hydrogen	2
CH3COOH	Acetic acid	2
HCl	Hydrochloric acid	2
$CaCO_3$	Calcium Carbonate	3
CaO	Calcium oxide	3
X-RD	X-ray diffraction	8
EtOH	Ethyl alcohol	11
MeOH	Methyl alcohol	11
FTIR	Fourier transform infrared	15
TGA	Thermogravimetry Analysis	16
NaOCL	Sodium Hypochlorite	16
CMC	Carboxymethyl chitosan	21
$-COCH_3$	Acetyl groups	21

LIST OF SYMBOLS

^{o}C	Celsius	18
%	Percentage	20
(=)	Double bond	24

CHAPTER 1

INTRODUCTION

1.1 Background of study

Corbiculacea fluminea or etok is a family of freshwater bivalve mollusks commonly known as freshwater clams or Asian clams. Etok have shells that are typically oval or triangular in shape, with distinct ridges or concentric lines (Crespo et al, 2015). The shells are frequently coloured in brown or green tones and are predominantly constituted of calcium carbonate. Chitosan is derived from chitin, a natural polysaccharide obtained from deacetylating chitin through a chemical process (Aranaz et al., 2021).

Chitosan is extremely flexible and provides accurate results of chemical changes. Its sustainability, biodegradability, biocompatibility, nontoxicity, and adsorption enable a broad range of uses in food, water treatment, textiles, farming, cosmetics, and medicines (Aranaz et al., 2021). Through the process of deacetylation, chitin's acetamide groups are hydrolyzed to yield chitosan by the addition of concentrated NaOH or KOH. Long chitosan chains may surround four crystalline polymorphs: three hydrated and one anhydrous. Chitosan reactions are more flexible than cellulose reactions because of the presence of group NH_2 (Bradić, 2020a). It has been demonstrated that the presence of free amino groups and primary and auxiliary hydroxyl bunches is what causes the chitosan reactivity. It has been established that chitin and chitosan have numerous uses in the biological, pharmaceutical, and industrial domains (Aranaz et al., 2021). The previous research investigated the antibacterial activity of chitosan at low molecular weight water-soluble (LMWS) and chitosan with molecular weights of 1, 5, and 10 kDa at pH 5.4 and 7.4 in relation to a diverse range of drug-susceptible bacteria. (Park et al., 2015).

The antimicrobial of chitosan can vary depending on factors such as its molecular weight, degree of deacetylation which is the extent to which chitin has been converted into chitosan, and the specific microorganism targeted (Sawai, 2011). Chitosan is an effective

antibacterial substance against various microbial infections due to its wide range of actions and high rate of eradication of specific bacterial strains. Chitosan has been shown to have broad-spectrum antibacterial action against a variety of bacteria, fungi, and certain enveloped viruses. Chitosan is also used in food packaging to keep perishable items fresher longer by suppressing the growth of spoiling bacteria (Ebana et al., 2016). Chitosan extraction processes might vary depending on parameters such as chitin supply, desired chitosan characteristics, and intended applications (Shahidi et al., 1999). Chemical techniques, enzymatic hydrolysis, and physical treatments are frequently used for extraction. Chitosan extraction involves three step which is demineralization, deproteinization, and deacetylation (Dutta et al., 2004). These procedures help turn chitin into chitosan and eliminate contaminants like minerals and proteins. By treating the chitin source with an acid, such as acetic acid (CH3COOH) or hydrochloric acid (HCl), which solubilizes the minerals, demineralization is often achieved (Dutta et al., 2004). Thus, the antibacterial properties of etok chitosan extract was investigated in this work. The usefulness of these chitosan extracts was demonstrated by testing and optimisation to assure their performance in specific applications.

1.2 Problem statement

Chitosan is a useful biopolymer having numerous uses in a variety of industries, including food, medicine, agriculture, and wastewater treatment. Chitosan, which is widely available and reasonably priced, may be found in etok shell. However, due to the complexity of the extraction method, and the poor yield of chitosan, the extraction of chitosan from shrimp shell is a difficult undertaking (Taser et al., 2021). As a result, the issue for chitosan extraction from etok shell may be hard to extract because of high yield produce of pure chitosan with the least amount. By identifying particular research questions like these, this problem statement might be improved further which is what are the ideal circumstances (temperature, pH, length of time, and extracting agent concentration) for extracting chitosan from etok shell. It also to identifying how much may the extraction process be made to produce chitosan with greater purity and with less impurities and how can the yield of the chitosan derived from etok shell be increased. With this research can obtained the possibility that could etok shell chitosan have, and how does its quality and cost-effectiveness compare to that of other chitosan sources.

Clams of the *corbiculacea* family are common around the world and can be found in ponds, lakes, and rivers. Ions are well known for their capacity for growth in a range of aquatic settings (Sabapathy Allen, 2022). There are many compounds that have in the clam shells such as *corbiculacea* species. Calcium oxide, or CaO, can be produced from calcium carbonate (CaCO3), which is present in *corbiculacea* shells (Roslan et al., 2019). The calcium oxide from etok shell can be a biomass product. The use of the etok shell for this research also in Kelantan. The use of hydroxyapatite (HA), a group of calcium phosphate (CaP) that is similar to the composition of real bone that also have in the clam shells due to its outstanding biocompatibility and bioactive qualities (Varadavenkatesan et al., 2021). The shell is typically discarded, with the filling inside being utilised as a sustenance source. The shell will be numerous, but the excellent component will not be used. The fact that the resources in the etok can be used to produce biomass products like chitosan is a significant drawback.

1.3 Objectives

There are 3 objectives to achieved which is:

1. To extract chitosan from etok shell
2. To characterize the chitosan extract from etok shell
3. To investigate the antimicrobial properties of extracted chitosan from etok shell

1.4 Scope of study

Enhancing the extraction method to get the largest yield of chitosan from etok shells is the main goal of the study. Investigating how various extraction factors, such as temperature, pH, duration, and concentration of the extracting agent, affect the yield and purity of chitosan may be necessary (Fabre et al., 2022). First scope of study is chitosan characterization. The study may also contain a description of the extracted chitosan's molecular composition, level of deacetylation, and other physicochemical characteristics for example molecular weight for chitosan extraction may be led from 300 until 360 KDa (Mohammadi et al., 2023). Second

scope of study is impurity analysis. The study can look into if there are impurities in the extracted chitosan and come up with ways to lessen or get rid of them.

Third scope of study is applications of chitosan. The study can investigate how chitosan, which is derived from the etok shell, might be used in a range of industries, including food, medicine, agriculture, and wastewater treatment (Aranaz et al., 2021). Fourth scope of study is cost-effectiveness and environmental impact. When compared to alternative sources of chitosan, the study can assess the cost-effectiveness and environmental impact of chitosan extraction utilizing etok shell. Overall, depending on the research questions and aims, the study's scope may be broad or small. Additionally, the study may combine experimental work, analytical methods, and theoretical modelling.

1.5 Significance of study

The significance of study includes the sustainable chitosan the source. Etok shell is a naturally occurring by product of the processing of seafood, and using it as a source of chitosan is sustainable and environmentally benign. By minimizing waste and utilizing renewable resources, the study can help to build a circular economy. Second, the cost effectiveness of the study is significant. Etok shell is plentiful and easily accessible, and using it as a source of chitosan can lower production costs as compared to using other sources, including the shells of crustaceans.

The third significance of study is applications in a variety of fields. Chitosan, which is produced from the etok shell, has a wide range of potential uses in a variety of industries, including food, agriculture, wastewater treatment, and medicine. The research may aid in the creation of fresh, cutting-edge goods and technologies. The fourth significance of study is local economic development. By giving small-business owners new options and advancing the seafood processing sector, the study can support regional economic growth. The fifth significance of study is knowledge contribution. The work can add to our understanding of the extraction, characterization, and process optimization of chitosan. Other natural polymers and their potential applications may be affected by this. Overall, there are considerable societal, environmental, and economic advantages to researching chitosan extraction utilizing etok shell.

CHAPTER 2

LITERATURE REVIEW

2.1 Etok (*corbicula fluminea*)

Etok or its scientific name *Corbicula fluminea* is a shellfish that settles in a water area bordering freshwater and saltwater areas, but rather a lower river area than the estuary near the sea. It is a rat mollusk shellfish (Bodon et al., 2020). It includes *Corbicula fluminea* and *Cyrenacea*, *Sphaeriacea*. *Corbiculacea fluminea* is a little clam with an inflated shell that ranges in shape from slightly rounded to triangular. The shell, which has multiple thick concentric ridges, is the most obvious characteristic (Sabapathy, 2022). Typically, the shell is olivaceous to black and pale brownish or yellowish brown (Figure 2.1). Each valve has three cardinal teeth inside, and the lateral teeth have a lot of serration. The colour of the nacre can range from white to salmon or dark purple (Sabapathy, 2022). Its lifespan is one to seven years, and although its shell is typically less than 25 mm long, it can grow to a length of 50 to 65 mm. Freshwater species of *Corbiculacea fluminea* are found naturally in southern and eastern Asia, including Russia, Thailand, the Philippines, China, Hong Kong, Taiwan, Korea, and Japan as well as Africa (Sabapathy, 2022).

Figure 2.1: *Corbiculacea fluminea* species

(Source: Sabapathy, 2022)

2.1.1 Morphological studies of etok

Examining and characterising the surface and internal structures of etok (*Corbiculacea fluminea*) shells is a necessary step in morphological searches. Etok shells frequently have an oval or long form. They have two valves or halves that are hinged together, resulting in bivalve shells. The species, age, and environmental conditions are just a few examples of the variables that might affect the size of the shells (Freer, Greenwood, Chung, Pannell, & Cusack, 2010). The outside of etok shells has distinctive characteristics that help with species identification. These characteristics could include radial ribs, concentric growth lines, and delicate sculpture patterns. These traits can vary in order and density between species and even within an individual. Etok shells have layers made up of both organic and inorganic materials. The organic periostracum, which is the topmost layer, offers defence. Calcium carbonate crystals are arranged in a prismatic or columnar pattern in the middle layer, which is referred to as the prismatic layer. The innermost layer, also referred to as the nacreous or shiny layer, is made up of brick-like calcium carbonate crystals called aragonite crystals. The chemical makeup of etok shells can be determined by using analytical methods including Fourier-transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD).

2.1.2 Phytochemical studies of etok shell

Phytochemical studies of etok shells involve the identification and analysis of bioactive compounds present in the shells. While the term "phytochemical" is commonly associated with plant-based compounds, it can be extended to include non-plant sources such as mollusk shells. Chitosan is one of the major bioactive compounds found in etok shells. It is a polysaccharide derived from the deacetylation of chitin, the primary structural component of mollusk shells. Chitosan has attracted significant attention due to its various biological activities, including antimicrobial, antioxidant, anti-inflammatory, and wound-healing properties. Phytochemical studies focus on quantifying the chitosan content and characterizing its properties in etok shells. Etok shells, like other mollusk shells, contain minerals such as calcium carbonate, which constitutes the majority of the shell's composition. Phytochemical analyses may involve the determination of mineral content, including calcium, magnesium, and trace elements. These minerals contribute to the structural integrity of the shell and may also have potential health benefits. Some etok shells exhibit pigmentation, which can be attributed to the presence of pigmented compounds. Phytochemical studies aim to identify and characterize these pigments, which may have potential applications in various industries, such as food, cosmetics, and dyes. Phytochemical studies of etok shells may also explore the presence of phenolic compounds. Phenolics are known for their antioxidant and potentially health-promoting properties. The identification and quantification of phenolic compounds contribute to understanding the nutritional and functional value of etok shells. Phytochemical studies of etok shells help in understanding the chemical composition, nutritional value, and potential applications of these shells. The findings contribute to the development of value-added products, such as nutraceuticals, functional food ingredients, or natural additives, from etok shells.

2.1.3 Pharmalogical studies of etok

Pharmacological studies of etok shells involve investigating their potential therapeutic and pharmacological properties. Etok shells, particularly their chitosan content, have shown potential in promoting wound healing. Chitosan possesses antimicrobial properties, accelerates tissue regeneration, and exhibits hemostatic activity. Pharmacological studies can explore the use of etok shell extracts or chitosan derived from the shells in the development of wound dressings, gels, or creams for improved wound management. Chitosan derived from etok shells has been studied for its anti-inflammatory properties. It can inhibit the release of inflammatory

mediators, reduce edema, and modulate immune responses. Pharmacological investigations can further explore the mechanisms of action and potential applications of etok shell extracts or chitosan in the management of inflammatory conditions. Some studies suggest that etok shells exhibit antioxidant activity. Antioxidants help protect cells from oxidative stress and can have potential health benefits. Pharmacological studies can focus on identifying and characterizing the antioxidant compounds present in etok shells and assessing their potential therapeutic applications.

Etok shells, particularly chitosan derived from them, possess antimicrobial properties. Chitosan can inhibit the growth of various bacteria and fungi, making it a potential natural antimicrobial agent. Pharmacological studies can investigate the efficacy of etok shell extracts or chitosan against specific pathogens and explore their application in the development of antimicrobial agents or coatings.

2.2 Chitosan

Economically sustainable is the manufacture of chitosan from food waste collected from shells. Direct extraction of chitosan from fungi using an alkaline and acidic process. chitosan extracted from shell sources, including its composition, characteristics, and functions (Bradić, 2020b). They provide useful knowledge on how chitosan derived from crab shells is used in many fields. Chitin is N-deacetylated to produce chitosan, a cationic biopolymer. Some fungi, including the Mucorales types, have this partly acetylated glucosamine biopolymer in their cell walls. However, it is mostly caused by N-deacetylation of chitin (Bradić, 2020b).

2.3 Extraction method of chitosan

The first step in separating the desired natural products from the raw materials is extraction. According to the extraction principle, there are several extraction procedures, including solvent extraction, distillation, pressing, and sublimation. The technique with the highest usage is solvent extraction. Following are the steps that the extraction of natural compounds moves through as the solvent absorbs into the solid matrix. The solute is then diffused out of the solid matrix after first dissolving in the solvents. The extracted solutes are gathered for results. The extraction will be made easier by any element that increases the above

steps' diffusivity and solubility. The extraction efficiency is influenced by the characteristics of the extraction solvent, the size of the raw materials' particles, the solvent to solids ratio, the extraction temperature, and the extraction time (Zhang, Lin, & Ye, 2018).

For solvent extraction, the choice of the solvent is important. The selection of solvents should take into consideration selectivity, solubility, cost, and safety. According to the principle of similarity and impermissibility (like dissolves like), solvents whose polarity values are close to those of the solute's are likely to perform better, and vice versa. Alcohols (EtOH and MeOH) are all-purpose solvents used in solvent extraction for research on phytochemicals (Zhang et al., 2018). The traditional chemical extraction process was used to remove chitin. Demineralization, deproteination, and deacetylation were the three main processes that were taken in the extraction of chitosan. 10 g of the sample were treated with 2N hydrochloric acid for 2 hours at a solid-to-solvent ratio of 1:15 while being continuously stirred at 150 rpm in an incubator shaker at room temperature (Varun et al., 2017).

2.4 Characterization of chitosan extract

2.4.1 Properties in etok shell

Studies have shown that extracts or preparations derived from *Corbiculacea* shells possess antibacterial activity against various bacterial strains. This antimicrobial effect may be attributed to the presence of bioactive compounds, such as chitosan, in the shells. Chitosan has known antimicrobial properties due to its ability to disrupt bacterial cell walls and inhibit microbial growth. *Corbiculacea* shell extracts have also exhibited antifungal activity against different fungal species. The antifungal effect may be attributed to the presence of chitosan or other bioactive components in the shells. Chitosan can inhibit fungal growth by disrupting the cell membranes of fungi.

The antimicrobial mechanism of *Corbiculacea* shells is likely related to the presence of chitosan, which possesses a cationic nature. Chitosan can interact with microbial cell membranes, leading to membrane disruption, leakage of intracellular components, and

ultimately cell death. Additionally, chitosan may interfere with microbial enzyme activity and DNA replication, further contributing to its antimicrobial effects.

2.4.2 Application in etok shell

Etok shells can be used in agriculture and horticulture as a soil amendment or fertilizer. The shells are rich in calcium carbonate, which can help neutralize acidic soils and improve soil fertility. They can also provide slow-release calcium, contributing to plant growth and development. Etok shells have been investigated for their potential use in water treatment processes. The shells contain calcium carbonate, which can help remove heavy metals and other contaminants from water through a process called adsorption. Crushed or powdered shells can be used as a natural and cost-effective filtration medium for water treatment applications.

Etok shells, specifically the chitosan derived from them, have shown promise in various biomedical applications. Chitosan possesses antibacterial properties and biocompatibility, making it suitable for wound healing and tissue engineering. It can be incorporated into dressings, scaffolds, and drug delivery systems for improved wound management and tissue regeneration. The high calcium carbonate content in etok shells makes them useful in environmental remediation efforts. The shells can be utilized in the treatment of acidic mine drainage or other contaminated environments to neutralize acidity and promote the precipitation of heavy metals. Crushed etok shells can be used as a component in the production of construction materials. The shells' calcium carbonate content provides strength and durability to the resulting products, such as concrete, mortar, or bricks. This application can help reduce the reliance on traditional aggregates and contribute to sustainable construction practices. Etok shells can be processed and incorporated as feed additives in animal husbandry. The calcium content of the shells can contribute to the mineral balance and bone health of livestock, particularly poultry and swine.

2.4.3 Benefit of properties in etok shell

The antimicrobial activity of Etok shell can help protect against bacterial and fungal infections. It also can be used to preserve food. The benefit of etok shell is can be used to develop natural pesticides and herbicides that can control the growth of plant pathogens.

2.5 Antimicrobial of chitosan extract

2.5.1 Antimicrobial properties in etok shell

Corbiculacea, commonly known as Asian clams or freshwater clams, are bivalve mollusks that inhabit freshwater environments. The shells of *Corbiculacea* have been studied for their potential antimicrobial properties. The antimicrobial properties of *Corbiculacea* shells make them potential candidates for various applications. They can be explored for the development of antimicrobial coatings, films, wound dressings, or drug delivery systems. Incorporating *Corbiculacea* shell extracts or chitosan derived from the shells can help impart antimicrobial properties to these materials.

2.5.2 Antimicrobial susceptibility with standard antibiotics

The standard procedure for determining antimicrobial susceptibility is the Kirby Bauer agar diffusion method, which has a strong collection of research. 6 mm-diameter discs of white filter paper have been absorbed with known concentrations of antibacterial substances. The agent's name and concentration are recorded on each disc. To quantitatively establish the minimum concentration (in mg/ml) of an antimicrobial agent needed to inhibit or kill the bacteria, dilution testing is utilized. This is accomplished by adding directly to an agar pour, a broth tube, or a micro-broth panel two-fold dilutions of the antimicrobial agent. The Minimum Inhibitory Concentration (MIC) is the lowest concentration at which the organism exhibits observable growth inhibition (Antimicrobial Susceptibility Testing - Microbiology Resource Center - Truckee Meadows Community College,).

2.6 Analysis chitosan extraction of etok shell

The well diffusion method was used to examine this characteristic of chitosan oligomers. Nutrient agar was employed to test the antibacterial property. 28 g/L of Nutrient agar was utilized as the media. For the autoclave broth, this broth was divided into flasks according to the quantity of bacteria to be examined. After being autoclaved, broth was allowed to cool to 40°C before being seeded with 1% of the mother culture's cultured broth and

thoroughly mixed. This research is preliminary; the future studies will also include positive and negative controls. Three duplicates of the test were run (Varun et al., 2017).

2.7 Identification organic compounds using FT-IR

Extracted chitosan was verified by a solubility test in diluted acetic acid, and its characterization was carried out using Fourier transform infrared spectroscopy (FT-IR), which produced the spectra of standard chitosan and extracted chitosan (Figure 2.2). The sample was then examined and characterized based on the standard chitosan's absorption spectrum (Varun et al., 2017). Table 2.1 shows the difference clam using FTIR analysis.

Table 2.1: Difference between clam, function group and bending spectrum. (Source: Varun, 2017)

Raw material	Function group	Bending	Source
Coelomactra antiquata 1	N-H group	2925 cm^{-1}	(Wu, 2019)
Clam shell 1	–NH2	3470 cm−1	(Li, 2020)
Clam shell 2	–NH2	3390 cm−1	(Li, 2020)
Clam shell 3	–OH	3332 cm−1	(Li, 2020)
Clam shell 4	CH2	2424 cm−1	(Li, 2020)
Coelomactra antiquata 2	N-H group	2921 cm^{-1}	(Wu, 2019)

Figure 2.2: FTIR machine at FSB, Campus Jeli, Kelantan

2.8 Characterization scanning by Thermogravimetric Analysis (TGA)

A thermal analysis method called thermogravimetric analysis (TGA) is used to examine how materials break down in relation to temperature. In a TGA experiment, the sample is continuously monitored for weight changes while being heated at a steady rate in a controlled atmosphere, usually nitrogen. The sample experiences thermal decomposition as the temperature rises, which causes weight loss. Important details regarding the material's composition, thermal stability, and decomposition kinetics can be learned from the rate and degree of weight loss. TGA is widely used to characterise a wide range of materials, including polymers, pharmaceuticals, and ceramics, in many different fields, including chemistry, materials science, and environmental science (Saadatkhah, 2020).

Figure 2.3: TGA machine at Campus Jeli, Kelantan

2.9 Pathogenic bacteria

Bivalves, such as oysters, clams, scallops, and mussels, as well as other species like crustaceans like lobsters, crabs, and prawns, all fall under the category of shellfish. Numerous variables affect how well an antibiotic treatment works, but three key ones are the antibiotic itself, the target pathogen, and the patient's bodily system (Li et al., 2017). Vibrio organisms are widely distributed in marine waters. A few species, particularly V. parahaemolyticus and V. vulnificus, are capable of inflicting gastroenteritis in humans. Dinoflagellate algae toxins are responsible for some of the most severe and fatal episodes of shellfish food poisoning. Even the names of these illnesses which is paralytic shellfish poisoning, amnesic shellfish poisoning, and neurotoxic shellfish poisoning could prevent people from consuming shellfish (Hariharan & Amadi, 2016).

2.9.1 *Vibrio parahaemolyticus* and *vibrio vulnificus*

Cell adhesins, hemolysins, toxins, immunological modulators, proteases, and lipases are just a few of the virulence factors that the bacteria *Vibrio parahaemolyticus* and *Vibrio vulnificus* exploit to spread disease. According to recent reports, Vibrio species can use type VI secretion systems (T6SS) to kill the gut microflora, giving them an advantage over other pathogens. Vibrio species are successful at spreading disease because of their interactions with and impacts on host cells. The identification of compounds that suppress virulence could result in the creation of recent therapies for gastrointestinal illnesses. Due to the fact that these compounds do not actually kill bacteria, the development of antimicrobial resistance is not under as much pressure (Boyd, 2019).

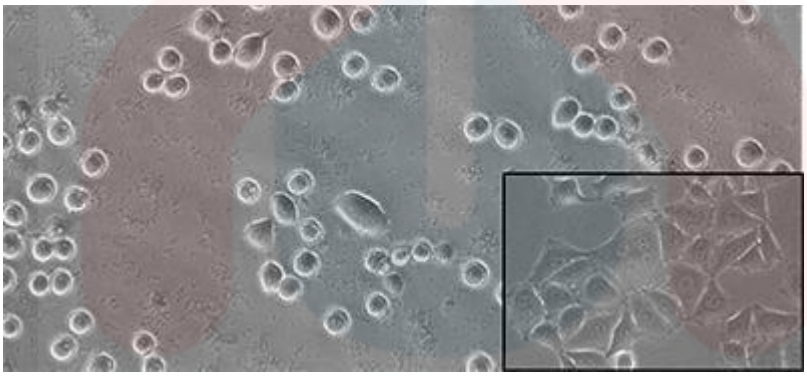


Figure 2.4: *Vibrio Parahaemolyticus* And *Vibrio Vulnificus*

Source: (Boyd, 2019)

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Ethanol (EtOH), Hydrocloric acid (HCl), Sodium hydroxide (NaOH), Sodium Hypochlorite (NaOCL), Distilled water, Ampicillin, Distilled water, Nutrient agar powder, Nutrient broth powder.

3.2 Methods

Methodology of preparation of chitosan extract and the analysis in this research proposal which is yield of extraction, disk diffusion method, FT-IR and element analysis.

3.2.1 Preparation of etok shell samples

The fresh sea water of *corbicula fluminea* or etok was obtained in market area Pasir Mas, Kelantan. The shell was washed until clean and dried in temperature 65℃.

3.2.2 Preparation of sample

The etok shell washed using tap water and rinsed with the distilled water. The sample was dry with dry tissue before dried in oven at temperature 60℃ for 6 hours. After that, ground the sample using blender to make it into powder form. The etok powder were kept in the room temperature for the future extraction process.

3.2.3 Chitosan extraction from etok shell

3.2.3.1 Demineralization

First step is demineralization process. 1 L of 1 M HCl was added to 100 g of etok shells. The reaction was proceeded at room temperature using magnetic stirrer and stirred with beaker and stirrer for 2 hours. The demineralize shells was filtrated and washed with distilled water until neutral pH. Shell was neutralized by immersing in distilled water for 10 min and dried in an oven at 70 ^{o}C.

3.2.3.2 Deproteinization

Next step is deproteinization process. 1 M NaOH was added to the dried demineralized shells at a solid/liquid ratio of 1:10 (g/mL) which is 10 g of sample to 100 mL of solvent. Reaction carried out under agitation at 80 ^{o}C for 3 hours.

3.2.3.3 Decolorization

After that decolorization,the dried deproteinized shell stirrer by magnetic stirrer using sodium hypochlorite (NaOCL) to ensure the chitosan become white colour. These processes have been done in 3 hours. The solid was filtrated and washed with distilled water until it achieved neutral pH. Resulting chitin will be dry in an oven at 70 ^{o}C.

3.2.3.4 Deacetylation

The third step is deacetylation process. In the result, Deacetylation of chitin was achieved by reacting chitin with 12.5 M NaOH at a solid/liquid ratio of 1:10 (g/mL). The reaction proceeded with stirrer, beaker and hot plate for 6 or 10 hours. Resulting chitosan was filtrated, washed with distilled water until neutral pH and dried in an oven at 70 ^{o}C.

3.2.4 Preparation of medium

Antimicrobial activity was performed by preparing the nutrient agar medium. 28 g of Nutrient agar powder was mixed with 1 litre of distilled water. The pH was checked before to be use next process. The medium was cooled before going to autoclave to destroy unwanted

bacteria at temperature 121 ^{o}C for 15 minutes. The agar was poured in the petri dish and sealed with parafilm also stored in refrigerator before use in 8 ^{o}C temperature.

3.2.5 Susceptibility testing with standard antibiotics

The shells extracts prepared with concentration 0.005 g dissolved in 5 mL of distilled water. Next, Ampicilin prepared with concentration 1 g of Ampicilin to 20 mL of distilled water and stored in 18 ^{o}C. Ampicilin reacted as positive control meanwhile distilled water react as negative control. The standarded broth inoculum swabbed on nutrient agar by used sterile cotton swab. The difference of concentration shell extract was tested. Then the nutrient agar inoculated one by one and then incubated at 37 ^{o}C for 24 hours. The inhibition zone measured by caliper.

3.2.6 Fourier transform infrared spectroscopy (FTIR) analysis

Functional group of Corbiculacea shells obtained using fourier transform infrared spectroscopy (FTIR) test. Sample chitosan extraction of etok shell used to find characteristic and organic compound in etok shell. This involved grinding a solid sample with an infrared-transparent material to form a pellet or mixing a liquid sample with an appropriate solvent. Infrared spectrum obtained through the infrared solution software which is give information in transmittance mode. Infrared spectrum was obtained through infrared solution software which are show information in transmittance mode. The result can be showed in range of 400 cm-1 to 4000 cm-1 for both extracted and not extracted samples.

3.2.7 Characterization by Elemental analysis

Element analysis scanning was used to analyze the oxygen and nitrogen compound in the chitosan sample. For example, in chitosan have high of nitrogen and carbon also hydrogen compound. To learn more about the characteristics and purity of a modified form of chitosan with enhanced solubility and functionality, its elemental composition can be examined. Using

this method, the sample contents of carbon (C), hydrogen (H), nitrogen (N), and sulphur (S) are ascertained. Usually, the sample was heated up in an atmosphere with only oxygen and produced gases. After then analysed using methods like mass spectrometry or gas chromatography (Eddya, 2020). To determine the chemical composition of the etok chitosan sample, the collected data will be analysed. The degree of acetylation of the chitosan sample can be calculated from these data

$$DA = ((1)\left(\frac{C}{N}\right) - 5.145)/6.816 - 5.145 \quad X\ 100\ \%$$

3.2.8 Characterization scanning by Thermogravimetric Analysis (TGA)

A flexible analytical method for figuring out a material's composition and thermal stability is thermogravimetric analysis (TGA). The process involved determining the weight change as a function of temperature while heating a sample consistently at a controlled atmosphere which is frequently used nitrogen.

The sample is usually heated in a crucible from room temperature to the desired maximum temperature during a TGA experiment. Weight loss results from the sample of physical and chemical changes caused on by rising temperatures, including oxidation, decomposition, and evaporation. By using a balance that is attached with the TGA device to record the weight change, a thermogram plot of weight percentage against temperature or time. The thermogram used to calculate a number of parameters such as the rate of decomposition, the amount of residue left over after decomposition, and the temperature at which decomposition begins (Saadatkhah, 2020).

3.2.9 Analytical data

The measured of inhibition zones and absorbance readings were showed significance from disc diffusion method, FTIR, and elemental analysis.

The following formula was used to determine the dry weight chitin content of etok chitosan:

$$The\ percentage\ of\ chitin\ yield = 100 \times (produced\ chitin\ weight)/(initial\ weight)$$

CHAPTER 4

RESULT AND DISSCUSSION

4.1 Yield of Extraction

The most common source of chitosan, a biopolymer that is versatile and has potential uses in agriculture, environmental remediation, and medicine, is the shells of crustaceans like prawns and crab. Demineralization, deproteinization, decolorization, and deacetylation are the three primary steps in the extraction process (Kumari et al., 2015).

The goal of demineralization is to eliminate the calcium carbonate ($CaCO_3$) that is found in shells. The usual method for achieving this is to treat the shells for established amounts of time and temperature using a diluted acid solution, such as hydrochloric acid (HCl). A demineralized chitin-protein complex is left behind after the acid dissolves the $CaCO_3$. Proteins and pigments are then extracted from the complex by deproteinization, which frequently involves the use of sodium hydroxide (NaOH) at high temperatures. Peptide bonds are broken by the NaOH, which causes proteins to disintegrate and separate from the chitin. Lastly, by chemically eliminating the acetyl groups ($-COCH_3$) attached to its sugar units, deacetylation turns the extracted chitin into chitosan. Concentrated NaOH is often used for this, along with particular timing and temperature requirements. By modifying these variables, the degree of deacetylation which can affects the characteristics of chitosan can be regulated (Hossin et al., 2021). The produced chitin weight of chitosan sample was 20 grams and the percentage of chitin yield was 4 %.

4.2 Element Analysis Characterazation

. From elemental analysis, carbon, hydrogen, and nitrogen are the main composition of chitosan extract. Etok chitosan is created by deacetylating chitosan, a naturally occurring polysaccharide derived from chitin. Based on table 4.1 shown the elemental analysis of etok chitosan sample and its degree of deacetylation, carbon is the main component of organic compounds, and its presence in the chitosan structure of etok chitosan is indicated by the fact that carbon made up the majority of its composition which are 13.14 %. Only 0.03% of the molecular configuration is made up of hydrogen, which has a supporting role. Deacetylation and the transformation of acetyl groups into amino groups are represented by nitrogen, leading to up 3.38% of chitosan and is an essential component. By calculating the percentage of amino groups in the molecular structure, the degree of deacetylation which measured at 24.89%, implements additional data about the chitosan the main characters in the elemental analysis of chitosan are nitrogen, oxygen, hydrogen, and carbon. Their exact ratios, determined through advanced methods such as CHN analysis, provide a comprehensive depiction of the molecule's configuration. Carbon usually has the most prominence, making up about 45% of the cast. Next in line, with supporting roles of roughly 7% and 45%, respectively, are hydrogen and oxygen.

But the secret to chitosan's unique properties is nitrogen. The relative abundance of chitosan, which varies from 4% to 12%, sets it apart from chitin, its precursor. These free amine groups are what give chitosan its amazing reactivity and adaptability. They give it the capacity to bind with fats, metals, and even bacteria, which opens up a wide range of possible applications.

Understanding the chemical structure of chitosan requires an understanding of the degree of deacetylation (DD), which is the ratio of nitrogen to carbon. More free amine groups result from a higher DD, which can improve functionality. This important parameter can be precisely determined through elemental analysis, which helps researchers customise chitosan for particular uses.

Table 4.1: Elemental analysis of Etok chitosan sample and the degree of deacetylation

Name of sample		Chitosan	Degree of deacetylation (%)
Percent of elements (%)	Carbon	13.14	24.89
	Hydrogen	0.03	
	Nitrogen	3.38	

4.3 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The functional potential of chitosan, a naturally occurring biopolymer derived from crustacean shells, has captivated scientists. Its special qualities continue to stimulate research into a variety of fields, including food science and wound healing applications. To fully utilise its potential, however, one must comprehend its complex chemical structure. This is where the potent technique of Fourier-transform infrared spectroscopy (FTIR) comes into play, revealing the mysteries buried in the molecular structure of chitosan.

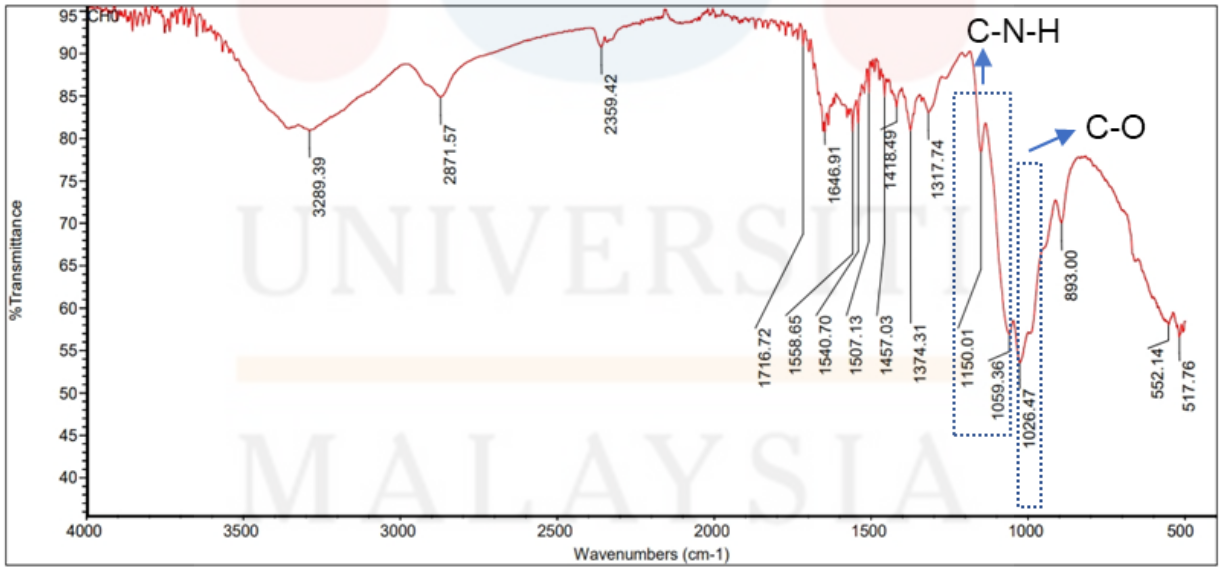


Figure 4.1: Result FTIR of commercial chitosan

Table 4.2: Peaks of chitosan commercial sample using FTIR and their compounds

Peaks	Assignment
1026.47	C-O
1059.36	C-N-H
1150.01	C-N-H
1317.74	R-C(=O)-R
1374.31	C-O
1418.49	C-O/C-H
1733.63	R-C(=O)-R
2359.42	C-N
2871.57	(−C(=O) −OH)
3648.57	(-OH)
3852.95	O-H

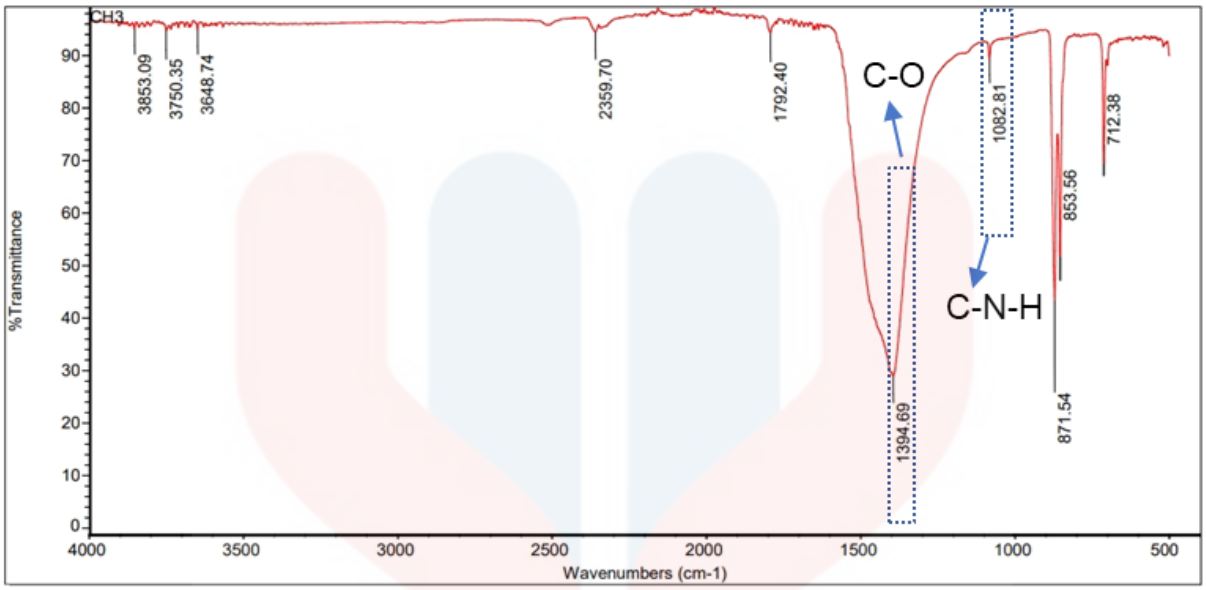


Figure 4.2: Result FTIR of sample chitosan of etok

Table 4.3: Peaks of chitosan sample using FTIR and their compounds

Peaks	Assignment
1082.81	C-N-H
1394.69	C-O
2359.70	C-N

Consider illuminating chitosan with an infrared kaleidoscope of light. Every molecule moves to a particular frequency, absorbing particular light wavelengths and exposing their functional groups that are hidden. Through the analysis of the FTIR spectrum, researchers are able to obtain valuable insights into the composition of chitosan.

Based on figure 4.1 of commercial chitosan result and figure 4.2 of sample etok chitosan, the FTIR spectrum clearly displays the distinct fingerprint of chitosan. Stretching vibrations of both O-H (hydroxyl) and N-H (amine) groups are indicated by a broad peak around 3300-3600 cm^{-1}. These functional groups are what give chitosan its distinctive cationic

nature and remarkable reactivity. The amine group, on the other conjunction is the star of the polysaccharide chain.

In figure 4.1 of commercial chitosan, was shown the wavelength for bond C-N-H was at 1150.01 cm^{-1} and 1059.36 cm^{-1} meanwhile for bond C-O was at wavelength 1026.47 cm^{-1}. For more peaks in figure 4.1 which was compound of bond had shown in table 4.2 with its assignment. Example, for assignment C-O have two peaks which is 1026.47 cm^{-1} and 1374.31 cm^{-1} meanwhile for assignment C-N-H was at wavelength 1059.36 cm^{-1} and 1150.01 cm^{-1}.

In figure 4.2 of sample etok chitosan, was shown the wavelength for bond C-N-H was at 1062.81 cm^{-1} meanwhile bond for C-O was at wavelength 1394.69 cm^{-1}. For more peaks in figure 4.2 which was compound of bond had shown in table 4.3 with its assignment. Example, for assignment C-O was at wavelength 1394.69 cm^{-1} and in addition wavelength for assignment C-N-H was at 1082.81 cm^{-1}

Based on table 4.2 of commercial chitosan and table 4.3 of sample etok chitosan, both of chitosan have the same functional group which is (C-O) and (C-N-H) which amine group in peak 1300cm^{-1} and 1059cm^{-1} - 1082cm^{-1}. The amine group indicates alkyl amine, the polysaccharide chain in chitosan.

4.4 Thermogravimetry Analysis (TGA)

Analysing the process of thermal decomposition in materials using thermogravimetric analysis, also known as TGA, is an effective technique. Using TGA to analyse chitosan, a biopolymer made from chitin, reveals information about its decomposition pattern and thermal stability (Rahman, 2022).

The sample is heated constantly in an atmosphere which, nitrogen during a thermogravimetric analysis of chitosan. Chitosan decomposes into various phases as the temperature rises. Chitosan loses moisture during the first stage, which usually noticed as a small weight loss at lower temperatures. Understanding the water content and moisture absorption properties of chitosan requires completing this step.

The next phases are where the majority of the chitosan degradation takes place. This is when the polymer chain breaks down, releasing evaporating substances of degradation and forming char residue. The temperature and rate of degradation could provide details about the kinetics of chitosan's degradation and thermal stability (Rahman, 2022).

Based on figure 4.3, was shown the chitosan sample graph. The weight of first stage was loss 22.07 % at temperature below 650℃ due to moisture content. The char residue was left in this process was 77.9242 % in total temperature for this process was 996.36 ℃. Meanwhile, figure 4.4 shows the commercial chitosan graph. In commercial chitosan it was two stage before chitosan degradation. During the first stage the weight loss of chitosan was 9.9773 % at temperature below 330℃ and the second stage the weight loss was 45.2021 % at temperature 350℃. The char residue was left in this commercial chitosan process are 44.7863 % in total temperature for this process was 995.78℃.

According to the data, the chitosan sample had more char residue remaining after degradation (77.9242%) than the commercial chitosan sample (44.7863%). The difference might result from variations in the chitosan samples' molecular weight or purity, and also from variations in the methods of extraction employed to produce them. Both samples have a similar total degradation temperature, which is approximately 996.36°C for chitosan and 995.78°C for commercial chitosan. Because the polymer chain breaks down at high temperatures, it is potentially determined that both samples have high thermal stability.

H. Moussout (2016) stated because polysaccharides have a strong affinity for water and are consequently easily hydrated, the first stage is intended for water loss. Meanwhile. correspond to the vaporisation and removal of volatile products, along with the thermal breakdown of the primary chitosan chain in the second stage (Moussout, 2016).

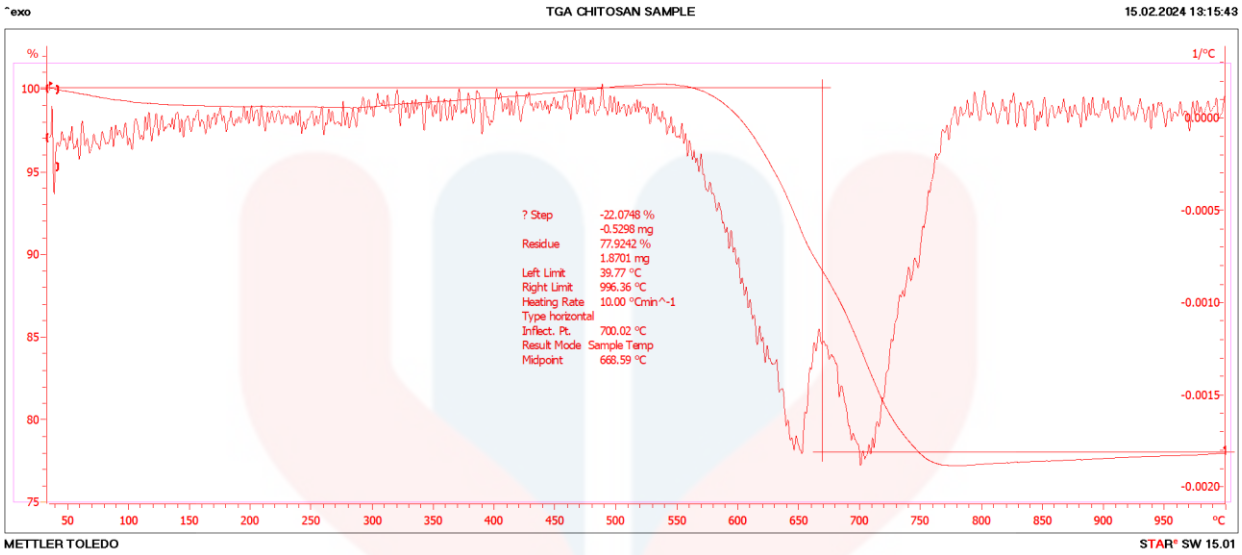


Figure 4.3: Result TGA of sample chitosan of etok

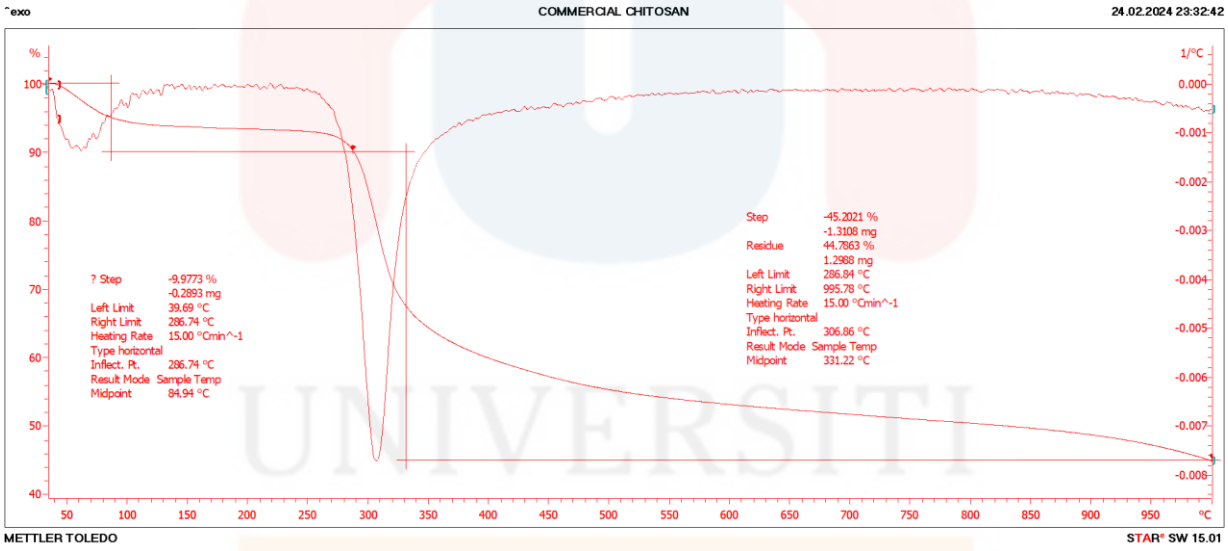


Figure 4.4: Result TGA of commercial chitosan of etok

4.5 Antimicrobial Properties

The results of the study, which tested chitosan's antimicrobial activity against *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus Aureus* are displayed in the table the diameter of inhibition zone. It has been demonstrated that the natural biopolymer chitosan shows antimicrobial activity against a range of microbes. To conduct the experiment, the diameter of the inhibition zone surrounding each chitosan disc was measured. The chitosan's antimicrobial activity is measured by the diameter of the inhibition zone. In figure 4.5 was shown picture of inhibition zone *Bacillus subtilis* in concentration 50 mg/l meanwhile figure 4.6 was shown picture of inhibition zone *Bacillus subtilis* in concentration 100mg/l.

Figure 4.5: Concentration 50 mg/ml

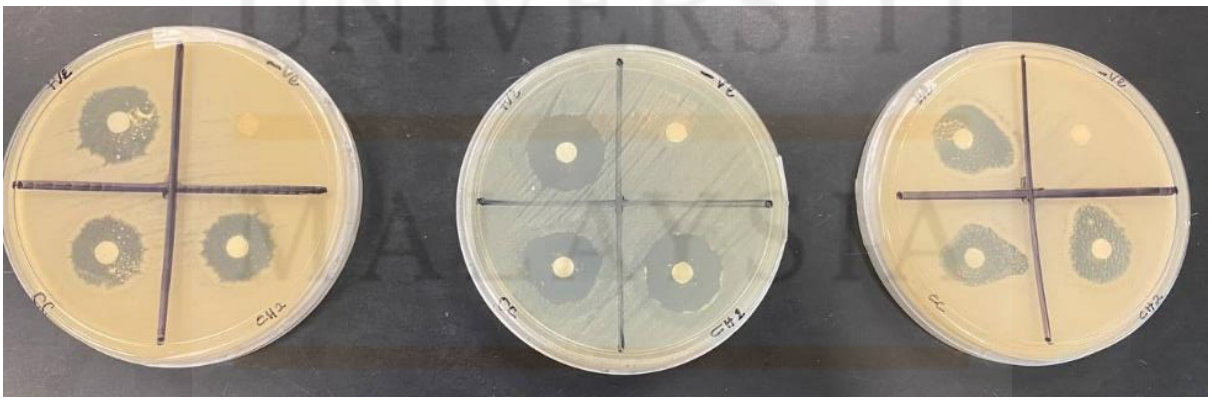


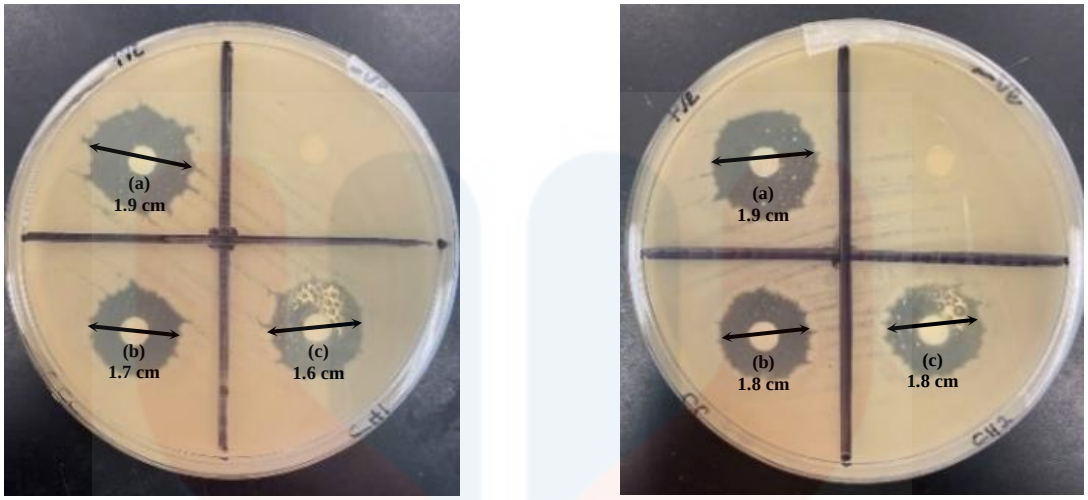
Figure 4.6: Concentration 100 mg/ml

Table 4.4: Zone inhibition *Bacillus* in concentration 50mg/l

Bacillus	Diameter of inhibiton zone (diz)	((diz)-diameter disk)/2)/diameter disk	In millimetre (mm)	Mean (mm)	Standard deviation (mm)
Commercial chitosan 50mg/l (CH1) (b)	1.6 cm	1.1 cm	11	11.67	0.58
	1.7 cm	1.2 cm	12		
	1.7 cm	1.2 cm	12		
Sample chitosan 50mg/L (CH1) (c)	1.4 cm	0.9 cm	9	10	1
	1.5 cm	1.0 cm	10		
	1.6 cm	1.1 cm	11		

Table 4.5: Zone inhibition *Bacillus* in concentration 100mg/l

Bacillus	Diameter of inhibition zone (diz)	((diz)-diameter disk)/2)/diameter disk	In millimetre (mm)	Mean (mm)	Standard deviation (mm)
Commercial chitosan 100mg/l (CH2) (b)	1.7 cm	1.2 cm	12	12.33333333	0.58
	1.7 cm	1.2 cm	12		
	1.8 cm	1.3 cm	13		
Sample chitosan 100mg/l (CH2) (c)	1.6 cm	1.1 cm	11	12	1
	1.7 cm	1.2 cm	12		
	1.8 cm	1.3 cm	13		



CH1 CH2

Figure 4.7: Antimicrobial test for *Bacillus* in concentration 50 mg/l (CH1) and 100 mg/l (CH2)

The antimicrobial activity of chitosan against *Bacillus subtilis* has been shown by the table 4.4 for *Bacillus subtilis* of concentration 50 mg/l and table 4.5 for *Bacillus subtilis* of concentration 100 mg/l. The inhibition zone that surrounds the chitosan discs had a diameter of 9 to 13 mm. For commercial chitosan, the inhibition zone's mean diameter was 11.33 mm, while for sample chitosan containing 50 mg/L concentration, it was 11.67 mm. At 12.33 mm, the sample chitosan 100 mg/L concentration indicated the largest inhibition zone diameter.

Based on the results that shown in figure 4.7 of difference commercial chitosan and sample chitosan in difference concentration, commercial chitosan is the most effective against Bacillus subtilis because due to its largest diameter of inhibition zone. commercial chitosan frequently has a higher degree of deacetylation. This indicates that it is more positively charged because there are fewer acetyl groups bonded to its sugar units. Its ability to interact and kill negatively charged bacterial cell membranes is enhanced to inhibition zone. The least effective sample is Sample Chitosan 50MG/L, while Sample Chitosan 100MG/L is likewise quite efficient. This is probably due to the fact that it is chitosan in its least concentrated form.

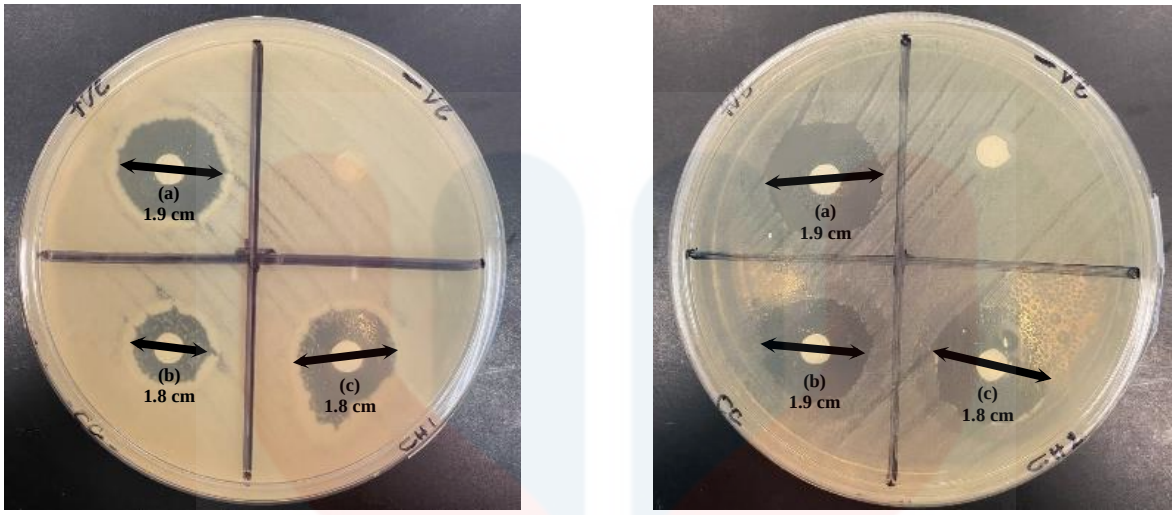
The chitosan's antimicrobial activity is measured by the size of the inhibition zone. When chitosan has a larger inhibition zone, it can either kill or stop the growth of bacteria more effectively. The results of the experiment indicate the possibility of using chitosan as an antimicrobial agent to defeat Bacillus subtilis (Al-Zahrani, 2021).

Table 4.6: Zone inhibition *E-coli* in concentration 50mg/l

E coli	Diameter of inhibition zone (diz)	((diz)-diameter disk)/2)/diameter disk	In millimetre (mm)	Mean (mm)	Standard deviation (mm)
Commercial chitosan 50mg/l (CH1) (b)	1.7 cm	1.2 cm	12	12.67	0.58
	1.8 cm	1.3 cm	13		
	1.8 cm	1.3 cm	13		
Sample chitosan 50mg/l (CH1) (c)	1.7 cm	1.2 cm	12	12.67	0.58
	1.8 cm	1.3 cm	13		
	1.8 cm	1.3 cm	13		

Table 4.7: Zone inhibition *E-coli* in concentration 100mg/l

E coli	Diameter of inhibition zone (diz)	((diz)-diameter disk)/2)/diameter disk	In millimetre (mm)	Mean (mm)	Standard deviation (mm)
Commercial chitosan 100mg/l (CH2) (b)	1.8 cm	1.3 cm	13	13.33	0.58
	1.8 cm	1.3 cm	13		
	1.9 cm	1.4 cm	14		
Sample chitosan 100mg/l (CH2) (c)	1.8 cm	1.3 cm	13	12.67	0.58
	1.7 cm	1.2 cm	12		
	1.8 cm	1.3 cm	13		



CH1 CH2

Figure 4.8: Antimicrobial test for *E-coli* in concentration 50 mg/l (CH1) and 100 mg/l (CH2)

The results of the study, which determined chitosan's antimicrobial activity against *E. coli*, appear in the tables. It has been showed that the natural biopolymer chitosan exhibits antimicrobial activity against a range of bacteria. Three distinct concentrations of chitosan were used in the experiment which are commercial chitosan, sample chitosan (50 mg/L), and sample chitosan (100 mg/L) has shown in figure 4.8. The results of the research demonstrate that chitosan exhibited antimicrobial activity against *E. coli* at all three concentrations. However, compared to commercial chitosan, the higher chitosan concentrations (50 mg/L and 100 mg/L) proved stronger antimicrobial activity.

The table demonstrates that the diameter of the *E. Coli* cells was significantly impacted by both chitosan treatments. The mean diameter of the filter paper treated with commercial chitosan was 1.26 mm, 0.44 mm less than the mean diameter of the empty filter paper (1.70 mm). Based on table 4.6 shown the data of *E. coli* in concentration 50 mg/l. The mean diameter of the filter paper treated with 50 mg/L of sample chitosan was 1.23 mm, 0.47 mm less than the mean diameter of the empty filter paper.

Meanwhile, according to the data in table 4.7, chitosan at a concentration of 100 mg/L in samples and commercial chitosan both displays antimicrobial activity against *E. coli*. In comparison to sample chitosan, which had a mean inhibition zone of 12.67 mm, commercial chitosan has a slightly higher mean inhibition zone of 13.33 mm, indicating that it may have a

slightly stronger antimicrobial effect in this situation. With sample chitosan ranging from 0.60 cm to 0.65 cm and commercial chitosan ranging from 0.65 cm to 0.70 cm, the inhibition zone values provide illuminates the potency of the antimicrobial action.

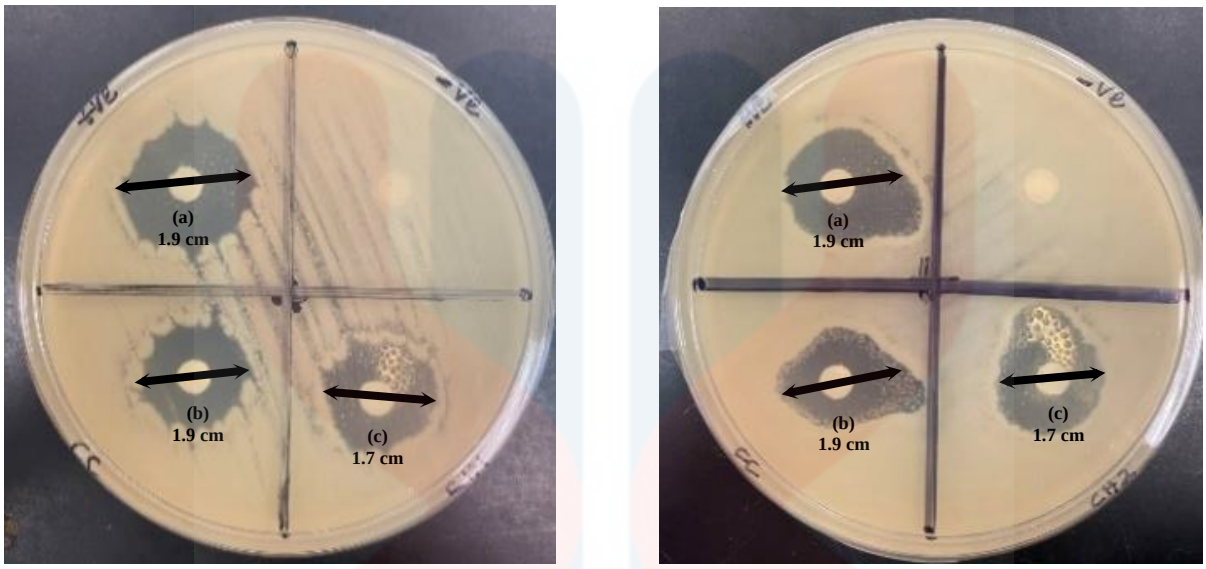
In conclusion, commercial chitosan exhibits a slightly higher mean inhibition zone, indicating that, in these circumstances, its antimicrobial activity may be a bit higher. These experiments are able to prove that a chitosan sample at a lower concentration is more effective than one at a higher concentration in preventing *E. coli*.

Table 4.8: Zone inhibition *Staphylococcus Aereus* in concentration 50mg/l

Staphylococcus aereus	Diameter of inhibition zone (diz)	((diz) -diameter disk)/2)/diameter disk	In millimetre (mm)	Mean (mm)	Standard deviation (mm)
Commercial chitosan 50mg/l (CH1) (b)	1.8 cm	1.3 cm	13	13.33	0.58
	1.8 cm	1.3 cm	13		
	1.9 cm	1.4 cm	14		
Sample chitosan 50mg/l (CH1) (c)	1.6 cm	1.1 cm	11	11.33333333	0.58
	1.6 cm	1.1 cm	11		
	1.7 cm	1.2 cm	12		

Table 4.9: Zone inhibition *Staphylococcus Aereus* in concentration 100mg/l

Staphylococcus aereus	Diameter of inhibition zone (diz)	((diz)-diameter disk)/2)/diameter disk	In millimetre (mm)	Mean (mm)	Standard deviation (mm)
Commercial chitosan 100mg/l (CH2) (b)	1.8 cm	1.3 cm	13	13.66666667	0.58
	1.9 cm	1.4 cm	14		
	1.9 cm	1.4 cm	14		
Sample chitosan 100mg/l (CH2) (c)	1.5 cm	1.0 cm	10	11.33333333	1.15
	1.7 cm	1.2 cm	12		
	1.7 cm	1.2 cm	12		



CH1 CH2

Figure 4.9: Antimicrobial test for Staphylococcus Aereus in concentration 50 mg/l (CH1) and 100 mg/l (CH2)

The outcomes of the experiment, which examined chitosan's antimicrobial activity against *Staphylococcus aureus*, are displayed in the table. It has been demonstrated that the natural substance chitosan contains antimicrobial qualities. Three distinct chitosan concentrations were used in the experiment which is commercial chitosan, sample chitosan 50 mg/L, and sample chitosan 100 mg/L was shown in figure 4.9. *Staphylococcus aureus* cultures were treated with chitosan, and the zone of inhibition's diameter was recorded. The part of the area surrounding the chitosan where no bacteria are growing is known as the zone of inhibition.

The table demonstrates that *Staphylococcus aureus* growth was effectively inhibited by chitosan at all three concentrations. The sample chitosan that was 100 mg/L based on data in table 4.9 and the sample chitosan that was 50 mg/L based on data in table 4.8 had the largest zones of inhibition, accordingly after the commercial chitosan. The commercial chitosan had a mean zone of inhibition of 13.67 mm, while the sample chitosan at 100 mg/L and the sample chitosan at 50 mg/L had mean zones of inhibition of 11.33 mm and 11.33 mm, respectively. According to this research, chitosan is a potent antimicrobial agent that works against Staphylococcus aureus. Although the sample chitosan at 100 mg/L and 50 mg/L were also

effective, the commercial chitosan was the most effective concentration. The table 4.8 was shown data *Staphylococcus aureus* in concentration 50 mg/l meanwhile table 4,9 was shown data *Staphylococcus aureus* in concentration 100mg/l.

The results obviously show that a larger zone of inhibition, indicating stronger antimicrobial activity, was induced by higher chitosan concentration. With an average inhibition zone of 13.67 mm, commercial chitosan was the most effective, followed by 50 mg/L and 100 mg/L, both of which had 11.33 mm. The observed pattern indicates to a dose-dependent effect, in which the number of chitosan molecules increases the interaction with the bacterial cells, accordingly preventing their growth (Hernández, 2022).

The unique qualities of chitosan consider for this inconsistent efficacy. Its positively charged molecules easily interact with the negatively charged bacterial cell wall components, breaking the walls and allowing vital cell contents to escape. In a nutshell higher chitosan concentration increase this disruptive effect, resulting in a larger inhibition zone and more effective cell death. It's important to note that other factors, like chitosan's molecular weight and degree of deacetylation, can also influence its antimicrobial activity.

Chitosan's unique properties give it its antibacterial influence. The negatively charged bacterial cell walls are electrostatically interacting with its positively charged amine groups, causing structural and permeability disruption. This interferes with important functions, causing intracellular components to leak out and eventually resulting in cell death. Furthermore, chitosan can chelate vital metal ions that bacteria require to survive, which inhibits the growth of the bacteria even more.

The range of activities exhibited by chitosan is significant. It is effective against a wide range of bacteria, including strong pathogens like *Escherichia coli, Bacillus* and *Staphylococcus aureus* which are both Gram-positive and Gram-negative. Higher molecular weight and more deacetylated chitosan more free amine groups generally show higher activity. Since chitosan's positive charge is crucial for its interaction with bacterial cells and is maximised at acidic pH values, the pH of the surrounding environment also plays a critical role (Ke, 2021).

Chitosan has antibacterial properties that go in addition to simple destruction. Because of its capacity to form films, it can physically block microbial infiltration and stop the growth of biofilms on surfaces. Because of this, chitosan is especially useful in the food packaging, textile, and medical device industries. Its unique antimicrobial and barrier qualities open up exciting new possibilities for infection prevention and hygiene.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

Chitosan was extracted from Etok powder using a multi-step procedure that included deacetylation, deproteinization, and demineralization. The extraction method was efficiency extracted the 4% chitin yield. It is important to maintain the standard operating procedure during lab work to optimise purity and yield because in excess strong acid treatment or insufficient deproteinization can affect the functionality of chitosan.

At 50 mg/L and 100 mg/L concentrations, the antimicrobial activities of Etok chitosan were evaluated against Bacillus subtilis, E. coli, and Staphylococcus aureus. Significant antimicrobial activity was shown in the results, with commercial chitosan showing the highest efficacy. The inhibition zones' sizes, which ranged from 9 mm to 13.33 mm, demonstrated chitosan's capacity to stop bacterial growth, larger zones were typically produced at higher concentrations.

Both the commercial and sample chitosan's unique peaks, which corresponded to functional groups, were identified by FTIR analysis. The spectral analysis revealed the existence of hydroxyl and amine groups, which are essential for the cationic and reactive properties of chitosan. A useful tool for recognising the complex chemical structure of chitosan is the FTIR analysis.

The composition of Etok chitosan was further clarified by elemental analysis, which showed that carbon established the majority element (13.14%). Nitrogen content, which is key to the unique properties of chitosan, was found to be 3.38%, resulting in a 24.89% degree of deacetylation. This parameter emphasises how acetyl groups change into amino groups during the extraction process, which affects the functionality and reactivity of chitosan.

In conclusion, the research effectively separated chitosan from Etok powder and used FTIR to analyse its elemental composition, chemical structure, and antimicrobial characteristics. The results support Etok chitosan's potential as an antimicrobial agent, with concentration having an impact on how effective it is. Its chemical structure is thoroughly

analysed to give important data for further modification and application in a variety of industries, including agriculture, environmental remediation, and medicine.

RECOMMENDATION

The research study of Etok chitosan discussed in the study provides useful details about its elemental composition, antimicrobial characteristics, Fourier-transform infrared spectroscopy (FTIR) analysis, and extraction method. A 4% chitin yield was obtained from the extraction process, demonstrating the effectiveness of the deacetylation, deproteinization, and demineralization processes. Research is currently being conducted to investigate environmentally friendly extraction methods that can be enhanced through the use of enzyme or microwave assistance.

Careful optimisation of each step is necessary to maximise the yield and purity of chitosan. For example, overly strong acid treatment can break down chitin, and insufficient deproteinization results in contaminants that disrupt the ability of chitosan to function. Furthermore, the process's environmental sustainability is impacted by the chemicals used and their concentrations. According to Peter et al. (2020), research is being done to create more environmentally friendly extraction processes with the use of enzyme- or microwave-assisted techniques.

The result research efficacy was observed when Etok chitosan's antimicrobial properties were evaluated against Bacillus subtilis, E. coli, and Staphylococcus aureus. The inhibition zones, which ranged in size from 9 to 13.33 mm, proved that they had the ability to prevent the growth of bacteria. Future studies could examine the effects of combining chitosan with other agents and evaluate its application in various products in order to further harness this antimicrobial potential.

Both sample and commercial chitosan demonstrated obvious peaks in the FTIR analysis, related to the presence of important functional groups. More thorough FTIR analysis should to be performed in future research to investigate other groups and how they affect the characteristics of chitosan. Our understanding of the structure-function relationship may be

improved by relating FTIR results with physical and chemical properties, which could lead to more specialised applications.

Etok chitosan was characterised by elemental analysis, which showed a degree of deacetylation of 24.89% and a predominant carbon composition of 13.14 percent. The investigation of elemental composition variations from various chitosan sources and the relationship between elemental composition and particular applications, like wound healing or agriculture, are among the recommendations.

Finally, this detailed study presents Etok chitosan as a promising biopolymer with a wide range of applications. The recommendations have the aim to direct future research towards improving extraction procedures, investigating new antimicrobial uses, and expanding our knowledge of the chemical structure of chitosan. Through discuss these elements, researchers can fully recognise the possibilities of Etok chitosan, promoting its application in sustainable and important manners throughout different industry sectors.

REFERENCES

Antimicrobial Susceptibility Testing - Microbiology Resource Center - Truckee Meadows Community College. (n.d.). Www.tmcc.edu. Retrieved May 20, 2023, from https://www.tmcc.edu/microbiology-resource-center/lab-protocols/antimicrobialsusceptibility-testing#:~:text=The%20Kirby%2DBauer%20agar%20diffusion

Aranaz, I., Alcántara, A. R., Civera, M. C., Arias, C., Elorza, B., Heras Caballero, A., & Acosta, N. (2021). Chitosan: An overview of its properties and applications. *Polymers, 13*(19), 3256.

Bodon, M., López-Soriano, J., Quiñonero-Salgado, S., Nardi, G., Niero, I., Cianfanelli, S., . . . Turco, F. (2020). Unraveling the complexity of corbicula clams invasion in italy (bivalvia: Cyrenidae). *Bollettino Malacologico, 56*, 127-171.

Boyd, A. (2019). Pathogenic bacteria in our oceans' shellfish. *Microbiology society*.

Bradić, B. (2020a). *Isolation of chitin from marine biomass with deep eutectic solvents and kinetics study of n-deacetylation to chitosan.* Univerza v Mariboru (Slovenia),

Bradić, B. (2020b). *Isolation of chitin from marine biomass with deep eutectic solvents and kinetics study of n-deacetylation to chitosan.* (Ph.D.), Univerza v Mariboru (Slovenia), Slovenia. Retrieved May 24, 2023, from https://www.proquest.com/dissertations-theses/isolation-chitin-marine-biomass-withdeep/docview/2497591465/se-2?accountid=51152 Natural Science Collection; ProQuest One Academic database. (28339998)

Crespo, D., Dolbeth, M., Leston, S., Sousa, R., & Pardal, M. Â. (2015). Distribution of corbicula fluminea (müller, 1774) in the invaded range: A geographic approach with notes on species traits variability. *Biological Invasions, 17*(7), 2087-2101. doi:10.1007/s10530-015-0862-y

Dutta, P. K., Dutta, J., & Tripathi, V. (2004). Chitin and chitosan: Chemistry, properties and applications.

Ebana, R., Edet, U., Ekanemesang, U., Ikon, G., Etok, C., & Edet, A. (2016). Antimicrobial activity, phytochemical screening and nutrient analysis of tetrapleura tetraptera and piper guineense. *Asian Journal of medicine and Health, 1*(3), 1-8.

ETOK - Definition and synonyms of etok in the Malay dictionary. (n.d.). Educalingo.com. Retrieved May 19, 2023, from https://educalingo.com/en/dic-ms/etok

Fabre, J.-F., Niangoran, N., Gaignard, C., Buso, D., Mouloungui, Z., & Valentin, R. (2022). Extraction, purification and stability of c-phycocyanin from arthrospira platensis. *European Food Research and Technology*, *248*(6), 1583-1599.

Freer, A., Greenwood, D., Chung, P., Pannell, C. L., & Cusack, M. (2010). Aragonite prism− nacre interface in freshwater mussels anodonta anatina (linnaeus, 1758) and anodonta cygnea (l. 1758). *Crystal growth & design, 10*(1), 344-347.

Hariharan, H., & Amadi, V. (2016). Shellfish as reservoirs of bacterial pathogens. *Journal of Coastal Life Medicine, 4*, 253-258. doi:10.12980/jclm.4.2016J6-13

High-Performance Liquid Chromatography (HPLC) | Protocol. (n.d.). Www.jove.com. Retrived May 19, 2023,https://www.jove.com/v/10156/high-performance-liquidchromatographyhplc#:~:text=High%2Dperformance%20liquid%20chromatography%20(HPLC)%20is%20an%20important%20analytical

Hossin, M. A., Al Shaqsi, N. H. K., Al Touby, S. S. J., & Al Sibani, M. A. (2021). A review of polymeric chitin extraction, characterization, and applications. *Arabian Journal of Geosciences*, *14*(18), 1870.

Kalaithong, W., Molloy, R., Nalampang, K., & Somsunan, R. (2021). Design and optimization of polymerization parameters of carboxymethyl chitosan and sodium 2acrylamido-2-methylpropane sulfonate hydrogels as wound dressing materials. *European Polymer Journal*, *143*, 110186.

Li, J., Xie, S., Ahmed, S., Wang, F., Gu, Y., Zhang, C., . . . Cheng, G. (2017). Antimicrobial activity and resistance: Influencing factors. *Front Pharmacol*, *8*, 364. doi:10.3389/fphar.2017.00364

Mohammadi, P., Taghavi, E., Foong, S. Y., Rajaei, A., Amiri, H., de Tender, C., . . . Rastegari, H. (2023). Comparison of shrimp waste-derived chitosan produced through conventional and microwave-assisted extraction processes: Physicochemical properties and antibacterial activity assessment. *International Journal of Biological Macromolecules*, 124841.

Pakizeh, M., Moradi, A., & Ghassemi, T. (2021). Chemical extraction and modification of chitin and chitosan from shrimp shells. *European Polymer Journal*, *159*, 110709.

Park, S.-C., Nam, J.-P., Kim, J.-H., Kim, Y.-M., Nah, J.-W., & Jang, M.-K. (2015). Antimicrobial action of water-soluble β-chitosan against clinical multi-drug resistant bacteria. *International Journal of Molecular Sciences*, *16*(4), 7995-8007.

Sabapathy Allen, U. (2022). Corbicula fluminea (Asian clam). CABI Compendium, CABI Compendium. https://doi.org/10.1079/cabicompendium.88200

Sawai, J. U. N. (2011). Antimicrobial characteristics of heated scallop shell powder and its application. *Biocontrol Science, 16*(3), 95-102. doi:10.4265/bio.16.95

Shahidi, F., Arachchi, J. K. V., & Jeon, Y.-J. (1999). Food applications of chitin and chitosans. *Trends in food science & technology, 10*(2), 37-51.

Taser, B., Ozkan, H., Adiguzel, A., Orak, T., Baltaci, M. O., & Taskin, M. (2021). Preparation of chitosan from waste shrimp shells fermented with paenibacillus jamilae bat1. *Int J Biol Macromol, 183*, 1191-1199. doi:10.1016/j.ijbiomac.2021.05.062

Varadavenkatesan, T., Vinayagam, R., Pai, S., Kathirvel, B., Pugazhendhi, A., & Selvaraj, R. (2021). Synthesis, biological and environmental applications of hydroxyapatite and its composites with organic and inorganic coatings. *Progress in Organic Coatings, 151*, 106056.

Varun, T. K., Senani, S., Jayapal, N., Chikkerur, J., Roy, S., Tekulapally, V. B., . . . Kumar, N. (2017). Extraction of chitosan and its oligomers from shrimp shell waste, their characterization and antimicrobial effect. *Vet World, 10*(2), 170-175. doi:10.14202/vetworld.2017.170-175

Wang, W., Xue, C., & Mao, X. (2020). Chitosan: Structural modification, biological activity and application. *International Journal of Biological Macromolecules, 164*, 45324546.

Weißpflog, J., Vehlow, D., Müller, M., Kohn, B., Scheler, U., Boye, S., & Schwarz, S. (2021). Characterization of chitosan with different degree of deacetylation and equal viscosity in dissolved and solid state–insights by various complimentary methods. *International Journal of Biological Macromolecules, 171*, 242-261.

APPENDIX

1) *The percentage of chitin yield = 100× (produced chitin weight)/ (initial weight)*

Chitin yield = 100 X (20 grams of etok chitosan) / (500 grams of etok powder)

= 100 X (0.04)

Chitin yield = 4 %

2) Degree of deacetylation

AVERAGE = (C3 + C3 rpt) / 2

AVERAGE CARBON = 13.14

AVERAGE NITROGEN = 3.38

DA = (1) $\frac{(C/N) - 5.145}{6.816 - 5.145}$ X 100 %

DA = (1) - $\frac{(13.14/ 3.38) - 5.145}{6.816 - 5.145}$ X 100 % =

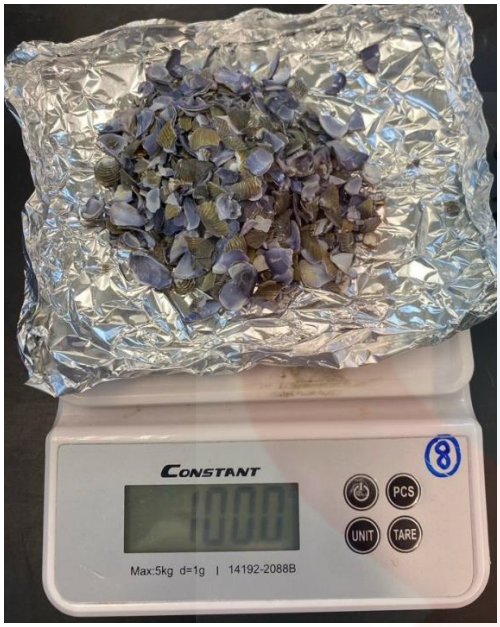
= (1) - $\frac{(3.89) - 5.145}{1.671}$ X 100 %

= (1) - $\frac{(-1.255)}{1.671}$ X 100 %

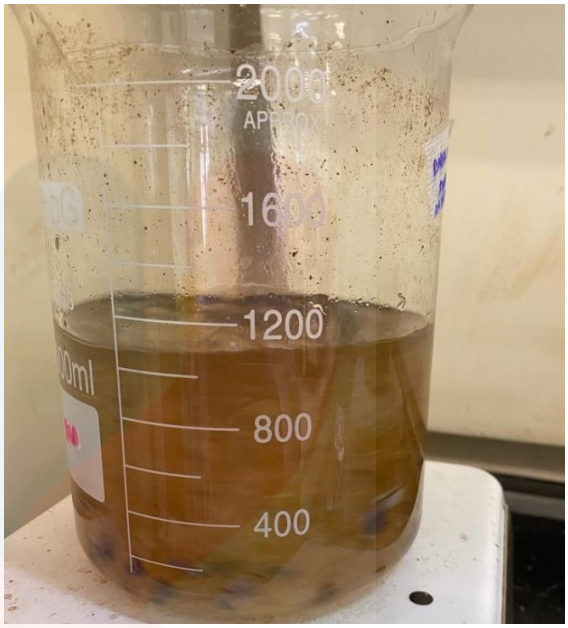
= 1 – (0.7510) X 100 %

= 0.2489 X 100 %

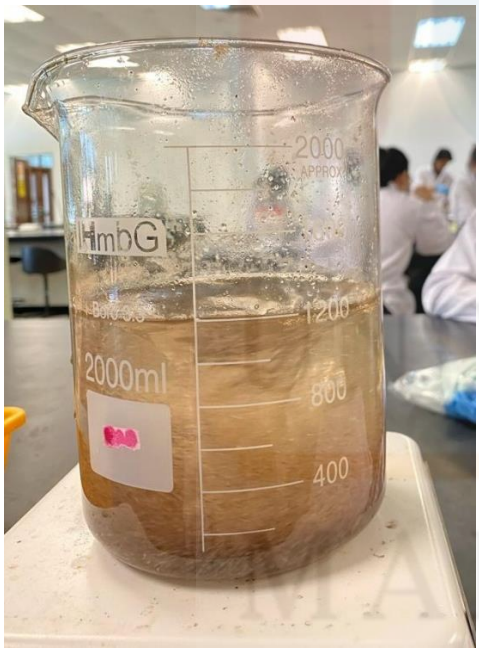
= 24.89 %



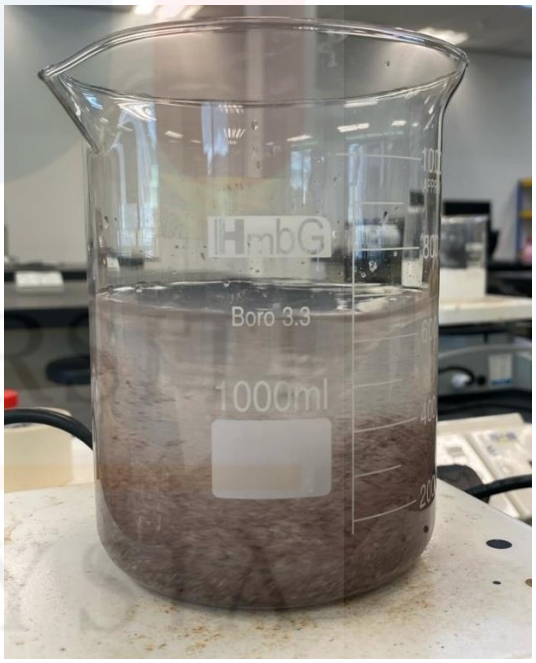
Weight the etok shell



Demineralization process



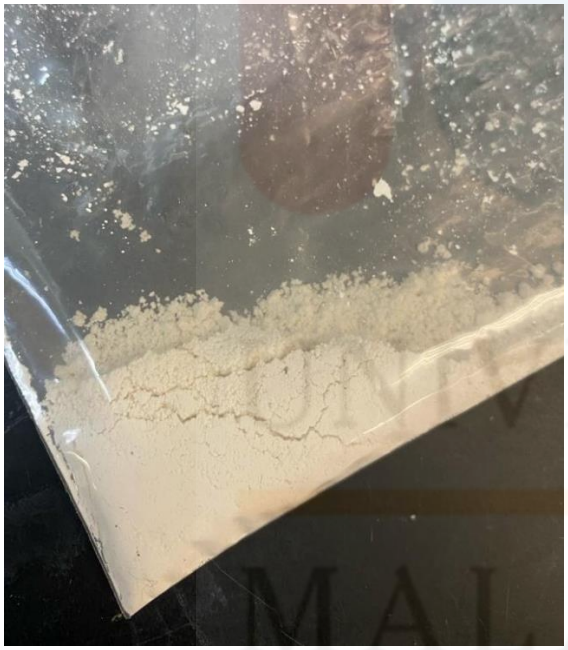
Deproteinization process



Decolorization process

Wash after deacetylation process

Final product of chitin after dry



Powder of etok chitosan

Bacteria growth in broth for 1 day