Upcycling of biomaterials from seafood waste and their applications

Tan, Yun Nian

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**UPCYCLING OF BIOMATERIALS
FROM SEAFOOD WASTE AND
THEIR APPLICATIONS**

TAN YUN NIAN

**SCHOOL OF CHEMICAL AND
BIOMEDICAL ENGINEERING**

2021

UPCYCLING OF BIOMATERIALS FROM SEAFOOD WASTE AND THEIR APPLICATIONS

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School of Chemical and Biomedical
Engineering

A thesis submitted to the Nanyang
Technological University in partial
fulfilment of the requirement for the degree
of Doctor of Philosophy

2021

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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08 April 2021
.....
Date

.....
[Prof William Chen Wei Ning]

Authorship Attribution Statement

This thesis contains material from 4 papers published in the following peer-reviewed journals in which I am listed as an author.

Chapter 2 is published as Tan, Y.N.; Lee, P.P.; Chen, W.N. Microbial extraction of chitin from seafood waste using sugars derived from fruit waste-stream. *AMB Expr* **2020**, *10*, 17.

The contributions of the co-authors are as follows:

- Professor William Chen Wei Ning provided the initial project direction.
- I prepared the manuscript drafts. The manuscript was reviewed by Professor William Chen Wei Ning.
- I co-designed the study with Professor William Chen Wei Ning and performed all the laboratory work at the School of Chemical and Biomedical Engineering. I also analyzed the data.
- Lee Pei Pei assisted in the collection and the interpretation of the data.

Chapter 3 is published as Tan, Y.N.; Zhang, J.H.; Chen, W.N. GC-MS-Based Metabolomics Analysis of Prawn Shell Waste Co-Fermentation by *Lactobacillus plantarum* and *Bacillus subtilis*. *Polysaccharides* **2020**, *1*, 31-50.

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- I co-designed the study with Professor William Chen Wei Ning and performed all the laboratory work at the School of Chemical and Biomedical Engineering. I also analyzed the data.
- Dr Zhang Jian Hua assisted in the collection and the interpretation of the data.

Chapter 4 is published as Tan, Y.N.; Lee, P.P.; Chen, W.N. Dual Extraction of Crustacean and Fungal Chitosan from a Single *Mucor circinelloides* Fermentation. *Fermentation* **2020**, *6*(2), 40.

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- I co-designed the study with Professor William Chen Wei Ning and performed all the laboratory work at the School of Chemical and Biomedical Engineering. I also analyzed the data.
- Lee Pei Pei assisted in the collection and the interpretation of the data.

Chapter 5 is published as Tan, Y.N.; Chin, Y.L.; Chen, W.N. Comparison of Sustainable Lipid and Protein Removal Methods for the Isolation of Insect Chitin from the Black Soldier Fly Exoskeleton. *ACS Food Sci. Technol.* **2021**, *1*(4), 698-706. <https://doi.org/10.1021/acsfoodscitech.0c00104>.

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- Chin Yi Ling assisted in the collection and the interpretation of the data.

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List of Abbreviations

A	Absorbance
C/N	Carbon Nitrogen Ratio
CrI	Crystallinity Index
EA	Elemental Analysis
FTIR	Fourier Transform Infrared Spectroscopy
GC-MS	Gas Chromatography Mass Spectrometry
LB	Luria-Bertani broth
MEOX	Methoxyamine hydrochloride
MH	Mueller Hinton broth
MRS	De Man, Rogosa and Sharpe broth
MSTFA	<i>N</i> -methyl- <i>N</i> -(trimethylsilyl)-trifluoroacetamide
Sc-CO₂	Supercritical Carbon Dioxide Extraction
TCMS	Trimethyl-chlorosilane
XRD	X-Ray Diffraction

Thesis Summary

The objective of this thesis is to investigate the mechanism of extraction of high value chitin and chitosan from side-streams of food processing industry using biological fermentation. We would then investigate the sustainability of fermentation-based extraction by exploring cost effective and sustainable carbon sources including plant-based biomass derived sugars. The biological fermentation process developed from this project would help to reduce food waste, achieve zero waste food processing while generating value added materials for food applications. Prawn shells from the seafood industry as well as Black Soldier Fly exoskeleton from the insect farm will be used as the natural source of chitin and chitosan for experiments. Carbon sources from plants will include fruit peels such as red and white grape pomace, banana peel, sugarcane molasses, mango peel, pineapple peel and core, apple pomace as well as potato and sweet potato peel. Analytical methods to ascertain the efficacy of the biological extraction will involve the use of Fourier Transform Infrared Spectroscopy (FTIR), Elemental Analysis (EA) and X-ray Diffraction (XRD).

Chapter 1: Background

1.1 Food Waste Valorization part of Singapore's Food Security

Landscape

800,000 tons of food waste are generated every year in Singapore, as estimated by the National Environmental Agency [1]. In addition to general food waste, there are also around 20,000 tons of side streams generated from the food processing industries such as okara or soybean residues, brewer's spent grain and waste cooking oil [2]. Due to land scarcity and the lack of animal farms which utilize food waste as animal feeds after processing, most food waste ends up in the incinerator or landfill in Singapore [3].

In recent years, food waste has been converted into fertilizers and compost by bio-conversion [4]. However, this useful conversion is considered to be a recycle endeavor in the food waste processing value chain with little value added [5]. More can be achieved by extracting higher value bioproducts from food waste, including food ingredients such as amino acids and micro-nutrients as well as high value additives such as carotenoids [6]. Nutrients recovered from food waste can be reconnected back to the food chain as animal feeds or human consumption [7].

Current technologies for the processing of food waste mainly center on food-waste-to-energy conversion [8]. These include biological conversions such as anaerobic fermentation and digestion, and physical or chemical technologies such as pyrolysis, incineration, hydrothermal

oxidation and gasification [9]. While expanding industrial activity worldwide has led to a greater demand for fuels and chemicals, the decreasing supply of fossil based resources has triggered greater urgency in the exploration of sustainable and innovative strategies for industrial production [10].

One solution that holds great promise is the conversion of the traditional linear industry production into a circular economy, where waste products are re-integrated back into the production chain [11]. This would reduce the amount of waste generated as well as fulfil the demand for raw materials [12].

1.2 Recovery of Useful Bioactive Compounds from Prawn Shell Waste

The increase in worldwide consumption of a wide variety of seafood products in the marketplace has resulted in a huge volume of marine wastes being produced [13]. As the head, tail, shell, skin, scales and backbones of shellfish, which comprise about 50-60% of their total weight, are usually discarded during industrial seafood processing, it is estimated that 1.44 million metric tons dry weight of seafood waste are being generated annually [14]. Most of this rich biomass have a slow biodegradation rate and are disposed of by dumping into the sea, leading to water pollution in coastal areas [15].

Prawn shell waste is chemically composed of 30-60% minerals, 20-30% chitin, 0-14% lipids and 20-40% protein [16]. As prawn constitutes a

major part of crustacean consumption in recent years, proper prawn waste processing has become a focal point of study to recover important biomaterials such as proteins, flavor compounds, lipids, chitin, calcium carbonate and astaxanthin [17]. Traditional methods of recovering these bioactive components from prawn shell waste require corrosive and hazardous chemicals such as hydrochloric acid and sodium hydroxide, resulting in environmental pollution and increased costs [18].

1.3 Plant Biomass Waste as a Carbon Source for Microbial Fermentation Process

Plant biomass has long been the primary source of fuel before the emergence of the petroleum industry [19]. However, the dominance of the oil refining and petrochemical industry has resulted in an increase in carbon emissions as well as other hazards to the environment [20]. This has revived an interest to switch to greener plant-derived fuels and chemicals [21].

Lignocellulosic biomass such as agricultural residues have received much attention as an abundant fermentation substrate [22]. Microbial conversion of sugars derived from plant biomass involve various processes using enzymes and micro-organisms to produce acetate, lactate, ethanol, acetone and butanol [23]. Fermentation processes that convert lignocellulose to sugars hold great promise to expand the production capacity for renewable products [24].

Plant biomass is a combination of carbohydrate polymers such as

pectin, hemicellulose, cellulose and the non-carbohydrate polymer lignin [25]. Cellulose is made up of lengthy cellobiose microfibrils, also known as dimers of glucose molecules [26]. Hemicellulose are polysaccharides branched with sugar backbones hydrogen bonded to cellulose [27]. Pectin holds the plant cell walls together and consist of uronic acids [28].

While starch can be broken down to form glucose units with the help of two enzymes, the extraction of soluble sugars from lignocellulosic biomass for fermentation remains challenging [29]. Factors impacting the hydrolysis process of lignocellulose are the available biomass surface area, crystallinity of cellulose fiber, the structural integrity of lignin and the diversity of hemicellulose content in the plant biomass [30].

Once soluble sugars are produced, natural occurring micro-organisms are able to ferment the sugars to useful biological products, for example *Lactobacillus* sp. ferments sugars to lactic acid while *Saccharomyces cerevisiae* ferments sugars to ethanol [31].

1.4 Lactic Acid Production by *Lactobacillus plantarum*

Lactic acid bacteria occurring in nature ferment hexose sugars such as glucose by carrying out the oxidation of NADH produced during glycolysis resulting from the electron acceptance of pyruvate to form lactic acid [32]. Micro-organisms such as *Lactobacillus plantarum* have been used to produce lactic acid to preserve food and also serve as food packaging [33].

Lactic acid is found in two enantiomeric forms, L-(+) and D-(-), with

the proportion of the two isomeric forms determining the physicochemical properties of the polylactide biopolymer [34]. Lactic acid bacteria have two genes encoding lactate dehydrogenase enzymes, which influence the production of either L-(+) or D-(-) lactic acid [35]. The ratio of the L-(+) to D-(-) forms of lactic acid in the fermentation supernatant is dependent on the levels of expression by these two genes [36].

While lactic acid bacteria are able to ferment hexoses and glucose, they lack the capability to ferment pentoses [37]. One great obstacle in utilizing lignocellulosic biomass as a substrate is the complexity of its sugar composition [38]. Complete utilization of sugars is essential to achieve maximum product yield and productivity [39]. However, the use of glucose suppresses the uptake of alternative sugars because of carbon catabolite repression, reducing yield and productivity in fermentations of mixed carbohydrates [40].

Lactic acid bacteria can be classified into homofermentative and heterofermentative based on the end product of their fermentation [41]. Homofermentative lactic acid bacteria produce only lactic acid while heterofermentative lactic acid bacteria generate acetic acid, ethanol and carbon dioxide [42]. The optical purity and stereospecificity of the lactic acid produced depends on the strain, enzyme produced, carbon source and substrate concentration [43].

1.5 Protease and Cellulase Production by *Bacillus subtilis*

Bacillus subtilis is known for its ability to secrete large amounts of extracellular proteases to degrade proteins into amino acids or small peptides to provide the cell with nutrients, as well as low levels of cellulase [44]. Cellulases are enzymes which hydrolyze the β -1,4-glucosidic linkages of cellulose to produce cello-oligosaccharides, cellobiose as well as glucose as primary products [45].

The cellulase enzyme consists of endo-glucanases, β -glucosidases and cellobiohydrolases [46]. Endo-glucanases creates reducing and non-reducing ends in the cellulose polymer, cellobiohydrolases acts on these exposed non-reducing and reduced ends to release cellobiose as well as cello-oligosaccharides units while β -glucosidases cleaves the cellobiose to release glucose, bringing the hydrolysis process to completion [47].

A variety of micro-organisms produce cellulase and most cellulases used for industrial applications originate from filamentous fungi such as *Aspergillus* sp [48]. Filamentous fungi produce hemicellulolytic and cellulolytic enzymes when encountering small inducer molecules such as monosaccharides and disaccharides liberated from cellulosic biomass [49]. For example, D-xylose and cellobiose serve as the inducer for cellulase production in *Aspergillus niger* and *Aspergillus oryzae* [50].

Enzyme production is regulated even at transcription level [51]. The first transcription factor catalyzing the degradation of cellulosic biomass was identified as XlnR, which brings about D-xylose-induced activation of cellulolytic and xylanolytic genes in *Aspergillus* sp. [52]

1.6 Characteristics and Applications of Chitin / Chitosan

Chitin, the next most abundant biopolymer on planet Earth after cellulose, is a linear polysaccharide made up of D-glucosamine linked by β -(1,4) bonds [53]. It is found in three crystalline forms α , β and γ , and in varying degrees of deacetylation [54].

In α -chitin, polysaccharide chains are arranged in anti-parallel orientation, allowing maximum bonding, stability and crystallinity [55]. However, in β -chitin, polymer chains are arranged in parallel orientation, with the increased distance between neighbouring polymer chains making it more soluble in solvents and reactive [56]. γ -chitin is a combination of α -chitin and β -chitin, whereby two parallel polymer chains are arranged in an anti-parallel chain alternatively [57].

The degree of deacetylation is defined as the ratio of glucosamine to N-acetyl glucosamine, which increases as chitin is converted to chitosan [58]. Unlike chitin which is sparingly soluble, chitosan is a water soluble cationic polymer [59]. As chitosan is biocompatible, biodegradable, non-toxic, and gel-forming, it is used in plenty of biomedical applications such as tissue engineering and wound healing [60].

Chitosan also displays anti-microbial, anti-oxidant and immunological biological activity; hence it can be used as an active carrier for cosmetic and dermatological applications [61]. Chitosan is even used as a food preservative additive and added in the formation of biofilms in edible

food packaging [62].

1.7 Chemical and Biological Methods for Extraction of Chitin from Prawn Shell Waste

Chemical and biological methods exist to extract chitin from prawn wastes [63]. Chemical methods consist of deproteinization, demineralization and decolorization [64]. Deproteinization involves the use of sodium hydroxide to disrupt the chemical bonds between protein and chitin; while demineralization is accomplished by hydrochloric acid which decomposes the calcium carbonate minerals from the prawn shells into water-soluble calcium salts with the liberation of carbon dioxide [65].

Biological methods for chitin extraction commonly involve enzymatic deproteinization and fermentation using micro-organisms [66]. While purified proteolytic enzymes such as trypsin, pepsin, papain and pancreatin can be used, they are relatively expensive and crude enzymes such as bacteria proteases present a lower cost alternative [67].

1.8 Co-fermentation of *Lactobacillus plantarum* and *Bacillus subtilis* with Prawn Shell Waste

Fermentation by lactic acid bacteria such as *Lactobacillus plantarum* as well as protease producing bacteria such as *Pseudomonas aeruginosa* and *Bacillus subtilis* yields chitin in solid fraction and liquid supernatant containing soluble free amino acids, protein hydrolysates, peptides, phenolics, anti-oxidants and pigments [68].

Other than harvesting chitin from the lactic acid fermentation of prawn shell waste, it is possible to process the protein-rich liquid hydrolysate by-products into a concentrated paste using vacuum evaporation or into dry powder with a spray dryer for possible applications as a shrimp-flavored animal feed supplement [69].

Fermentation processes can be successive fermentation or co-fermentation, single-stage or two-stage [70]. However, the implementation of co-culture is advantageous over a mono-culture because of the utilization of metabolic pathways of all microbial strains synergistically [71]. In addition, the opportunity to use secondary by-products such as lactoserum and molasses as substrates results in possible secondary biotechnological products [72].

1.9 Plan of Action

Experiments were conducted on the biological fermentation of prawn shell waste for the extraction of chitin and its purity evaluated through chemical characterization. In Chapter 2, microbial strains *Lactobacillus plantarum* and *Bacillus subtilis* were used to ferment prawn shell waste and various types of fruit waste were used as a carbon source to yield chitin and a protein rich supernatant. In Chapter 3, metabolomics profiling was performed on the co-fermentation of *Lactobacillus plantarum* and *Bacillus subtilis* with prawn shell waste and glucose as a carbon source. This was done to detect for any significant metabolite trends and amino acid levels in the supernatant.

In Chapter 4, a change of strain from *Lactobacillus plantarum* and *Bacillus subtilis* to *Mucor circinelloides* was attempted to compare the performance of the fungus versus microbe. The *Mucor circinelloides* biomass was also harvested as a fungal source of chitin. In Chapter 5, instead of prawn shell waste, the pupae shells of the Black Soldier Fly was used as a chitin source instead to compare insect versus crustacean chitin.

Chapter 2: Microbial extraction of chitin from seafood waste using sugars derived from fruit waste-stream

2.1 Introduction

Up till present, chemical and biological methods have been used to extract chitin from prawn shell waste. However, the utilization of strong acids and base have not only resulted in chemical waste that pollutes the environment but also caused hydrolyzation and inconsistent physical characteristics of the extracted chitin [73]. Hence lactic acid fermentation has been proposed as an ecologically friendly alternative method that prevents uneven deacetylation and molecular weight loss arising from strong acid base reaction [74]. Microbial fermentation involves demineralization of prawn shells broken down by the lactic acid produced followed by deproteinization using proteolytic enzymes produced by microorganisms.

The efficiency of microbial fermentation depends on various parameters: carbon and glucose concentration, pH during culture, temperature and duration of fermentation [75]. These parameters have been well investigated in literature. Due to the development of green extraction methods as well as to reduce the cost of chitin extraction, carbon-rich waste was experimented with as a replacement for the glucose substrate. As little information has been reported on the structural characteristics of the extracted chitin for simultaneous prawn waste and lignocellulose waste fermentation, the objective of this research study was to evaluate the impact

of the change in carbon source on the extracted chitin structure. Structural analysis performed included Fourier Transform Infrared Spectroscopy (FTIR) to obtain the degree of deacetylation, elemental analysis (EA) to derive the carbon / nitrogen ratio and X-ray diffraction (XRD) to measure the degree of crystallinity.

2.2 Materials and Methods

Lactobacillus plantarum subsp. *plantarum* ATCC 14917 and *Bacillus subtilis* subsp. *subtilis* ATCC 6051 were inoculated into a conical flask containing 5g of prawn shell waste and 100mL of 20% glucose solution and allowed to ferment for 5 days at 30°C and 200 rev⁻¹. Ten waste substrates were later used to replace the control glucose solution as a carbon source.

The waste substrates tested were light corn syrup, sugar cane molasses, white grape pomace, mango peel, red grape pomace, pineapple peel and core, apple peel, sweet potato peel, banana peel and potato peel. 15g of waste substrate was used for each fermentation. After 5 days the demineralized and deproteinated prawn shells were recovered from the fermentation and dried in a vacuum oven for 24 hours before further analysis.

Fourier Transform Infrared Spectroscopy (FTIR) was used to measure the degree of deacetylation of the crude extracted chitin. Elemental Analysis (EA) was performed to detect their carbon, hydrogen and nitrogen content and calculate their carbon-nitrogen ratio. X-ray diffraction (XRD) was performed to determine their degree of crystallinity. These 3 parameters were

chosen to evaluate the quality of the extracted chitin from the fermented prawn shells.

A PerkinElmer Spectrum One Fourier Transform Infrared Spectroscopy (FTIR) was utilized for the characterization of the crude chitin samples. Firstly, the extracted crude chitin was grinded with dried potassium bromide (KBr) and 10 tons of pressure was applied for 2 minutes [76]. The prepared disc was then scanned across the range 4000-500 cm^{-1} [77]. The degree of deacetylation (DD%) was calculated using the formula: $\text{DD}\% = (A_{1650}/A_{3450})/1.33 \times 100$ where A_{1650} represents the absorbance of the wavenumber 1650 cm^{-1} for the analytical Amide-I band while A_{3450} represents the absorbance of the wavenumber 3450 cm^{-1} for the internal reference hydroxyl band. The factor 1.33 denotes the theoretical value of A_{1650}/A_{3450} for fully N-acetylated chitin [78].

An Elementar Vario EL III Elemental Analyzer was utilized to measure the carbon / nitrogen ratio of the extracted crude chitin. 5 mg of the crude chitin sample was weighed in a tin foil boat, folded into a pellet and dropped into the instrument after loading into a sample carousel [79]. Catalytic tube combustion in a high temperature oxygenated CO_2 atmosphere takes place and helium blows carbon and nitrogen through specific absorption columns where the concentrations of separated components are measured by a thermal conductivity detector [80].

A Bruker D2 Phaser was used to perform X-ray diffraction. 0.5 g of the crude extracted chitin sample was put on a circular sample holder and

loaded into the machine [81]. Default settings the X-ray source were preset - 30 kV voltage, 10 mA current, “Coupled Two Theta / Theta” scan type and “Continuous PSD Fast” scan mode [82]. The scanning range was set between 5° and 80° with a step size increment of 0.1° [83]. The diffracted X-ray waves reflected from the crude chitin samples at angle θ to form sharp constructive interference peaks in accordance with Bragg’s Law [84]. The crystallinity index (CrI) was determined using the equation: $CrI_{110} = [(I_{110} - I_{am}) / I_{110}] \times 100$, where I_{110} represents the intensity at $2\theta = 20^\circ$ while I_{am} represents the amorphous diffraction at $2\theta = 16^\circ$.

2.3 Results and Discussion

The dry weights of the extracted crude chitin samples were tabulated after fermentation in Table 1. Dry weights were found to range between 0.38 g to 0.89 g, translating to an average yield of 7.6% to 17.8%.

Table 1. Dry Weights of Chitin extracted after *Lactobacillus plantarum* and *Bacillus subtilis* Fermentation on Various Carbon Sources

Carbon Source for Fermentation	Dry Weight (g)	Yield (%)
15 g potato peel in 100 mL water	0.89	17.8
20 mL molasses in 80 mL water	0.78	15.6
15 g red grape pomace in 100 mL water	0.61	12.2
15 g white grape pomace in 100 mL water	0.59	11.8
15 g sweet potato peel in 100 mL water	0.58	11.6
15 g pineapple peel and core in 100 mL water	0.57	11.4
15 g mango peel in 100 mL water	0.55	11.0
15 g banana peel in 100 mL water	0.51	10.2
20 mL glucose in 80 mL water	0.50	10.0
15 g apple peel in 100 mL water	0.47	9.40
20 mL corn syrup in 80 mL water	0.38	7.60

The degree of deacetylation of the extracted crude chitin samples were calculated by taking the ratio of peaks at 3450 cm^{-1} and 1650 cm^{-1} from the FTIR spectrum and recorded in Table 2. The highest degree of deacetylation was recorded for the crude chitin extracted from the fermentation using banana peel (75.2%), molasses (74.0%), glucose (72.6%) and red grape pomace (72.9%), exceeding the degree of deacetylation measured for commercial chitin (70.5%). The degree of deacetylation for fermentation using all carbon sources were all above 50%.

Table 2. Degree of Deacetylation of Chitin extracted after *Lactobacillus plantarum* and *Bacillus subtilis* Fermentation on Various Carbon Sources

Carbon Source for Fermentation	Degree of Deacetylation (%)
15 g banana peel in 100 mL water	75.19
20 mL molasses in 80 mL water	73.98
15 g red grape pomace in 100 mL water	72.90
20 mL glucose in 80 mL water	72.57
Commercial Chitin	70.46
15 g white grape pomace in 100 mL water	65.92
15 g sweet potato peel in 100 mL water	65.46
20 mL corn syrup in 80 mL water	65.21
15 g mango peel in 100 mL water	60.50
15 g potato peel in 100 mL water	54.75
15 g pineapple peel and core in 100 mL water	53.51
15 g apple peel in 100 mL water	53.39

Figure 1. FTIR spectrum of commercial chitin (Sigma-Aldrich)

Figure 2. FTIR spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 20 mL glucose in 80 mL water

Figure 3. FTIR spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 20 mL molasses in 80 mL water

Figure 4. FTIR spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 20 mL corn syrup in 80 mL water

Figure 5. FTIR spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 15 g red grape pomace in 100 mL water

Figure 6. FTIR spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 15 g white grape pomace in 100 mL water

Figure 7. FTIR spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 15 g apple peel in 100 mL water

Figure 8. FTIR spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 15 g pineapple peel and core in 100 mL water

Figure 9. FTIR spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 15 g potato peel in 100 mL water

Figure 10. FTIR spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 15 g mango peel in 100 mL water

Figure 11. FTIR spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 15 g banana peel in 100 mL water

Figure 12. FTIR spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 15 g sweet potato peel in 100 mL water

The carbon percentage, nitrogen percentage and the calculated carbon nitrogen ratio for the extracted crude chitin were recorded in Table 3. The

carbon / nitrogen ratio values were found to be very close to 6.89, which is the theoretical value for pure chitin, attesting to the high quality and purity of the crude chitin extracted from fermentation.

Table 3. Elemental Analysis of Chitin Extracted after *Lactobacillus plantarum* and *Bacillus subtilis* Fermentation on Various Carbon Sources

Carbon Source for Fermentation	Carbon (%)	Nitrogen (%)	Carbon / Nitrogen Ratio
15 g sweet potato peel in 100 mL water	44.51	7.71	5.78
20 mL molasses in 80 mL water	45.50	7.29	6.24
15 g banana peel in 100 mL water	51.69	7.90	6.54
20 mL corn syrup in 80 mL water	41.72	6.17	6.76
15 g pineapple peel and core in 100 mL water	34.27	5.03	6.81
15 g white grape pomace in 100 mL water	40.97	6.00	6.83
15 g red grape pomace in 100 mL water	41.63	6.08	6.85
15 g mango peel in 100 mL water	46.89	6.78	6.92
15 g potato peel in 100 mL water	33.50	4.83	6.94
Commercial Chitin	46.12	6.65	6.94
20 mL glucose in 80 mL water	40.21	5.69	7.06
15 g apple peel in 100 mL water	41.62	4.93	8.44

The degree of crystallinity of the crude extracted chitin was calculated using the X-ray diffraction spectrum by taking the ratio of the sharp peak at 20° to the amorphous region at 16° and recorded in Table 4. Results showed that all the crude extracted chitin samples had an extremely high degree of crystallinity ranging from 80% to 98%, with most samples exceeding the degree of crystallinity measured for commercial chitin (87.6%).

Table 4. Degree of Crystallinity of Chitin extracted after *Lactobacillus plantarum* and *Bacillus subtilis* Fermentation on Various Carbon Sources

Carbon Source for Fermentation	Degree of Crystallinity (%)
20 mL glucose in 80 mL water	98.16
20 mL corn syrup in 80 mL water	97.99
15 g apple peel in 100 mL water	97.60

15 g mango peel in 100 mL water	97.34
15 g red grape pomace in 100 mL water	95.54
15 g sweet potato peel in 100 mL water	95.08
15 g pineapple peel and core in 100 mL water	94.28
15 g banana peel in 100 mL water	93.51
20 mL molasses in 80 mL water	90.33
15 g white grape pomace in 100 mL water	88.53
Commercial Chitin	87.56
15 g potato peel in 100 mL water	79.89

Figure 13. XRD spectrum of commercial chitin (Sigma-Aldrich)

Figure 14. XRD spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 20 mL glucose in 80 mL water

Figure 15. XRD spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 20 mL molasses in 80 mL water

Figure 16. XRD spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 20 mL corn syrup in 80 mL water

Figure 17. XRD spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 15 g red grape pomace in 100 mL water

Figure 18. XRD spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 15 g white grape pomace in 100 mL water

Figure 19. XRD spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 15 g apple peel in 100 mL water

Figure 20. XRD spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 15 g pineapple peel and core in 100 mL water

Figure 21. XRD spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 15 g potato peel in 100 mL water

Figure 22. XRD spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 15 g mango peel in 100 mL water

Figure 23. XRD spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 15 g banana peel in 100 mL water

Figure 24. FTIR spectrum of crude chitin extracted from *Lactobacillus plantarum* and *Bacillus subtilis* fermentation using 15 g sweet potato peel in 100 mL water

A good quality of chitin extracted is defined by a high degree of deacetylation above 70%, a carbon nitrogen ratio below 6.90 and a degree of crystallinity above 90%. Based on this criteria, banana peel served as the most excellent substrate for lactic acid and protease producing bacteria. This is because the extracted chitin for the banana peel substrate achieved the highest degree of deacetylation (75%) which corresponds to high product purity, while a high degree of crystallation (94%) indicates maximum preservation of the native chitin structure. A low carbon nitrogen ratio of 6.54 also indicates minimal residual protein in the extracted chitin, suggesting effective demineralization and deproteinization during the fermentation process.

A one-way ANOVA was performed to determine whether the

differences between group means for the degree of deacetylation of extracted chitin are statistically significant.

Table 5. One-Way ANOVA for Degree of Deacetylation of Chitin extracted after *Lactobacillus plantarum* and *Bacillus subtilis* Fermentation on Various Carbon Sources

Source	Sum of Squares	Degrees of Freedom	Mean Squares	F-Statistic	P-Value
Columns	2031.94	11	184.722	980.87	4.835 x 10 ⁻²⁹
Error	4.52	24	0.188		
Total	2036.46	35			

As the p-value was found to be less than 0.05 significance level, the null hypothesis that all population means are equal is rejected and it is concluded that the differences between some of the means for the degree of deacetylation of extracted chitin are statistically significant. A multiple comparison test was then used to assess the differences in group means.

Table 6. Multi-Comparison Test for Degree of Deacetylation of Chitin extracted after *Lactobacillus plantarum* and *Bacillus subtilis* Fermentation on Various Carbon Sources

Substrate Compared	Substrate Compared	Lower 95% Confidence	Difference in Means	Upper 95% Confidence	P-Value
Commercial Chitin	20% Glucose	-3.0376	-1.7600	-0.4824	0.0021
Commercial Chitin	20% Molasses	-3.9976	-2.7000	-1.4224	0.0000
Commercial Chitin	20% Corn Syrup	3.9391	5.2167	6.4943	0.0000
Commercial Chitin	15% Red Grape Waste	-3.4376	-2.1600	-0.8824	0.0001
Commercial Chitin	15% White Grape Waste	3.5591	4.8367	6.1143	0.0000
Commercial Chitin	15% Apple Pomace	15.3024	16.5800	17.8576	0

Commercial Chitin	15% Pineapple Peel	15.5157	16.7933	18.0709	0
Commercial Chitin	15% Potato Peel	14.9724	16.2500	17.5276	0
Commercial Chitin	15% Mango Peel	8.5891	9.8667	11.1443	0.0000
Commercial Chitin	15% Banana Peel	-4.8943	-3.6167	-2.3391	0.0000
Commercial Chitin	15% Sweet Potato Peel	4.0957	5.3733	6.6509	0.0000
20% Glucose	20% Molasses	-2.2176	-0.9400	0.3376	0.3060
20% Glucose	20% Corn Syrup	5.6691	6.9767	8.2543	0.0000
20% Glucose	15% Red Grape Waste	-1.6676	-0.4000	0.8776	0.9899
20% Glucose	15% White Grape Waste	5.3191	6.5967	7.8743	0.0000
20% Glucose	15% Apple Pomace	17.0624	18.3400	19.6176	0
20% Glucose	15% Pineapple Peel	17.2757	18.5533	19.8309	0
20% Glucose	15% Potato Peel	16.7324	18.0100	19.2876	0
20% Glucose	15% Mango Peel	10.3491	11.6267	12.9043	0.0000
20% Glucose	15% Banana Peel	-3.1343	-1.8567	-0.5791	0.0011
20% Glucose	15% Sweet Potato Peel	5.8857	7.1333	8.4109	0.0000
20% Molasses	20% Corn Syrup	6.6391	7.9167	9.1943	0.0000
20% Molasses	15% Red Grape Waste	-0.7376	0.5400	1.8176	0.9191
20% Molasses	15% White Grape Waste	6.2591	7.5367	8.8143	0.0000
20% Molasses	15% Apple Pomace	18.0024	19.2800	20.5576	0
20% Molasses	15% Pineapple Peel	18.2157	19.4933	20.7709	0
20% Molasses	15% Potato Peel	17.6724	18.9500	20.2276	0
20% Molasses	15% Mango Peel	11.2891	12.5667	13.8443	0.0000

20% Molasses	15% Banana Peel	-2.1943	-0.9167	0.3609	0.3387
20% Molasses	15% Sweet Potato Peel	6.7957	8.0733	9.3509	0.0000
20% Corn Syrup	15% Red Grape Waste	-8.6543	-7.3767	-6.0991	0.0000
20% Corn Syrup	15% White Grape Waste	-1.6576	-0.3800	0.8976	0.9932
20% Corn Syrup	15% Apple Pomace	10.0857	11.3633	12.6409	0.0000
20% Corn Syrup	15% Pineapple Peel	10.2991	11.5767	12.8543	0.0000
20% Corn Syrup	15% Potato Peel	9.7557	11.0333	12.3109	0.0000
20% Corn Syrup	15% Mango Peel	3.3724	4.6500	5.9276	0.0000
20% Corn Syrup	15% Banana Peel	-10.1109	-8.8333	-7.5557	0.0000
20% Corn Syrup	15% Sweet Potato	-1.1209	0.1567	1.4343	1.0000
15% Red Grape Waste	15% White Grape Waste	5.7191	6.9967	8.2743	0.0000
15% Red Grape Waste	15% Apple Pomace	17.4624	18.7400	20.0176	0
15% Red Grape Waste	15% Pineapple Peel	17.6757	18.9533	20.2309	0
15% Red Grape Waste	15% Potato Peel	17.1324	18.4100	19.6876	0
15% Red Grape Waste	15% Mango Peel	10.7491	12.0267	13.3043	0.0000
15% Red Grape Waste	15% Banana Peel	-2.7343	-1.4567	-0.1791	0.0161
15% Red Grape Waste	15% Sweet Potato Peel	6.2557	7.5333	8.8109	0.0000
15% White Grape Waste	15% Apple Pomace	10.4657	11.7433	13.0209	0.0000
15% White Grape Waste	15% Pineapple Peel	10.6791	11.9567	13.2343	0.0000
15% White Grape Waste	15% Potato Peel	10.1357	11.4133	12.6909	0.0000
15% White Grape Waste	15% Mango Peel	3.7524	5.0300	6.3076	0.0000
15% White Grape Waste	15% Banana Peel	-9.7309	-8.4533	-7.1757	0.0000

15% White Grape Waste	15% Sweet Potato Peel	-0.7409	0.5367	1.8143	0.9221
15% Apple Pomace	15% Pineapple Peel	-1.0643	0.2133	1.4909	1.0000
15% Apple Pomace	15% Potato Peel	-1.6076	-0.3300	0.9476	0.9979
15% Apple Pomace	15% Mango Peel	-7.9909	-6.7133	-5.4357	0.0000
15% Apple Pomace	15% Banana Peel	-21.4743	-20.1967	-18.9191	0
15% Apple Pomace	15% Sweet Potato Peel	-12.4843	-11.2067	-9.9291	0.0000
15% Pineapple Peel	15% Potato Peel	-1.8209	-0.5433	0.7343	0.9161
15% Pineapple Peel	15% Mango Peel	-8.2043	-6.9267	-5.6491	0.0000
15% Pineapple Peel	15% Banana Peel	-21.6876	-20.4100	-19.1324	0
15% Pineapple Peel	15% Sweet Potato Peel	-12.6976	-11.4200	-10.1424	0.0000
15% Potato Peel	15% Mango Peel	-7.6609	-6.3833	-5.1057	0.0000
15% Potato Peel	15% Banana Peel	-21.1443	-19.8667	-18.5891	0
15% Potato Peel	15% Sweet Potato Peel	-12.1543	-10.8767	-9.5991	0.0000
15% Mango Peel	15% Banana Peel	-14.7609	-13.4833	-12.2057	0.0000
15% Mango Peel	15% Sweet Potato Peel	-5.7709	-4.4933	-3.2157	0.0000
15% Banana Peel	15% Sweet Potato Peel	7.7124	8.9900	10.2676	0.0000

As the p-value for most of the comparison of means is less than 0.05, this proves that the group means of the degree of deacetylation for extracted chitin using fermentation of different substrates are significantly different

from each other. However there are a few notable exceptions. For example, 20% glucose and 20% molasses have a p-value of 0.3060; 20% glucose and 15% red grape waste have a p-value of 0.9899; 20% molasses and 15% red grape waste have a p-value of 0.9191; 20% molasses and 15% banana peel have a p-value of 0.3387. This shows that the control substrate glucose can be substituted by molasses, red grape waste as well as banana peel to yield a similar degree of deacetylation of extracted chitin (above 70%).

A one-way ANOVA was performed to determine whether the differences between group means for the carbon nitrogen ratio of extracted chitin are statistically significant.

Table 7. One-Way ANOVA for Carbon Nitrogen Ratio of Chitin extracted after *Lactobacillus plantarum* and *Bacillus subtilis* Fermentation on Various Carbon Sources

Source	Sum of Squares	Degrees of Freedom	Mean Squares	F-Statistic	P-Value
Columns	6.13864	11	0.55806	8.25	9.1678 x 10 ⁻⁶
Error	1.62402	24	0.06767		
Total	7.76266	35			

As the p-value was found to be less than 0.05 significance level, the null hypothesis that all population means are equal is rejected and it is concluded that the differences between some of the means for the carbon nitrogen ratio of extracted chitin are statistically significant. A multiple comparison test was then used to assess the differences in group means.

Table 8. Multi-Comparison Test for Carbon Nitrogen Ratio of Chitin extracted after *Lactobacillus plantarum* and *Bacillus subtilis* Fermentation on Various Carbon Sources

Substrate Compared	Substrate Compared	Lower 95% Confidence	Difference in Means	Upper 95% Confidence	P-Value
Commercial Chitin	20% Glucose	-0.8935	-0.1277	0.6382	1.0000
Commercial Chitin	20% Molasses	-0.0388	0.7270	1.4928	0.0736
Commercial Chitin	20% Corn Syrup	-0.7422	0.0237	0.7895	1.0000
Commercial Chitin	15% Red Grape Waste	-0.6652	0.1007	0.8665	1.0000
Commercial Chitin	15% White Grape Waste	-0.6688	0.0970	0.8628	1.0000
Commercial Chitin	15% Apple Pomace	-1.3652	-0.5993	0.1665	0.2319
Commercial Chitin	15% Pineapple Peel	-0.8058	-0.0400	0.7258	1.0000
Commercial Chitin	15% Potato Peel	-0.8612	-0.0953	0.6705	1.0000
Commercial Chitin	15% Mango Peel	-0.5788	0.1870	0.9528	0.9987
Commercial Chitin	15% Banana Peel	-0.4048	0.3610	1.1268	0.8517
Commercial Chitin	15% Sweet Potato Peel	0.3272	1.0930	1.8588	0.0014
20% Glucose	20% Molasses	0.0888	0.8547	1.6205	0.0197
20% Glucose	20% Corn Syrup	-0.6145	0.1513	0.9172	0.9998
20% Glucose	15% Red Grape Waste	-0.5375	0.2283	0.9942	0.9931
20% Glucose	15% White Grape Waste	-0.5412	0.2247	0.9905	0.9940
20% Glucose	15% Apple Pomace	-1.2375	-0.4717	0.2942	0.5515
20% Glucose	15% Pineapple Peel	-0.6782	0.0877	0.8535	1.0000
20% Glucose	15% Potato Peel	-0.7335	0.0323	0.7982	1.0000
20% Glucose	15% Mango Peel	-0.4512	0.3147	1.0805	0.9319

20% Glucose	15% Banana Peel	-0.2772	0.4887	1.2545	0.5017
20% Glucose	15% Sweet Potato Peel	0.4548	1.2207	1.9865	0.0003
20% Molasses	20% Corn Syrup	-1.4692	-0.7033	0.0625	0.0925
20% Molasses	15% Red Grape Waste	-1.3922	-0.6263	0.1395	0.1855
20% Molasses	15% White Grape Waste	-1.3958	-0.6300	0.1358	0.1798
20% Molasses	15% Apple Pomace	-2.0922	-1.3263	-0.5605	0.0001
20% Molasses	15% Pineapple Peel	-1.5328	-0.7670	-0.0012	0.0494
20% Molasses	15% Potato Peel	-1.5882	-0.8223	-0.0565	0.0278
20% Molasses	15% Mango Peel	-1.3058	-0.5400	0.2258	0.3620
20% Molasses	15% Banana Peel	-1.1318	-0.3660	0.3998	0.8408
20% Molasses	15% Sweet Potato Peel	-0.3998	0.3660	1.1318	0.8408
20% Corn Syrup	15% Red Grape Waste	-0.6888	0.0770	0.8428	1.0000
20% Corn Syrup	15% White Grape Waste	-0.6925	0.0733	0.8392	1.0000
20% Corn Syrup	15% Apple Pomace	-1.3888	-0.6230	0.1428	0.1908
20% Corn Syrup	15% Pineapple Peel	-0.8295	-0.0637	0.7022	1.0000
20% Corn Syrup	15% Potato Peel	-0.8848	-0.1190	0.6468	1.0000
20% Corn Syrup	15% Mango Peel	-0.6025	0.1633	0.9292	0.9996
20% Corn Syrup	15% Banana Peel	-0.4285	0.3373	1.1032	0.8972
20% Corn Syrup	15% Sweet Potato	0.3035	1.0693	1.8352	0.0018
15% Red Grape Waste	15% White Grape Waste	-0.7695	-0.0037	0.7622	1.0000
15% Red Grape Waste	15% Apple Pomace	-1.4658	-0.7000	0.0658	0.0955
15% Red Grape Waste	15% Pineapple Peel	-0.9065	-0.1407	0.6252	0.9999

15% Red Grape Waste	15% Potato Peel	-0.9618	-0.1960	0.5698	0.9981
15% Red Grape Waste	15% Mango Peel	-0.6795	0.0863	0.8522	1.0000
15% Red Grape Waste	15% Banana Peel	-0.5055	0.2603	1.0262	0.9811
15% Red Grape Waste	15% Sweet Potato Peel	0.2265	0.9923	1.7582	0.0043
15% White Grape Waste	15% Apple Pomace	-1.4622	-0.6963	0.0695	0.0989
15% White Grape Waste	15% Pineapple Peel	-0.9028	-0.1370	0.6288	0.9999
15% White Grape Waste	15% Potato Peel	-0.9582	-0.1923	0.5735	0.9984
15% White Grape Waste	15% Mango Peel	-0.6758	0.0900	0.8558	1.0000
15% White Grape Waste	15% Banana Peel	-0.5018	0.2640	1.0298	0.9791
15% White Grape Waste	15% Sweet Potato Peel	0.2302	0.9960	1.7618	0.0041
15% Apple Pomace	15% Pineapple Peel	-0.2065	0.5593	1.3253	0.3154
15% Apple Pomace	15% Potato Peel	-0.2618	0.5040	1.2698	0.4579
15% Apple Pomace	15% Mango Peel	0.0205	0.7863	1.5522	0.0405
15% Apple Pomace	15% Banana Peel	0.1945	0.9603	1.7262	0.0062
15% Apple Pomace	15% Sweet Potato Peel	0.9265	1.6923	2.4582	0.0000
15% Pineapple Peel	15% Potato Peel	-0.8212	-0.0553	0.7105	1.0000
15% Pineapple Peel	15% Mango Peel	-0.5388	0.2270	0.9928	0.9934
15% Pineapple Peel	15% Banana Peel	-0.3648	0.4010	1.1668	0.7553
15% Pineapple Peel	15% Sweet Potato Peel	0.3672	1.1330	1.8988	0.0009
15% Potato Peel	15% Mango Peel	-0.4835	0.2823	1.0482	0.9664

15% Potato Peel	15% Banana Peel	-0.3095	0.4563	1.2222	0.5969
15% Potato Peel	15% Sweet Potato Peel	0.4225	1.1883	1.9542	0.0005
15% Mango Peel	15% Banana Peel	-0.5918	0.1740	0.9398	0.9993
15% Mango Peel	15% Sweet Potato Peel	0.1402	0.9060	1.6718	0.0113
15% Banana Peel	15% Sweet Potato Peel	-0.0338	0.7320	1.4978	0.0701

As the p-value for most of the comparison of means is more than 0.05, this proves that the group means of the carbon nitrogen ratio for extracted chitin using fermentation of different substrates are not significantly different from each other. The p-value between commercial chitin and most of the waste substrates are above 0.05 except for 15% sweet potato peel. This shows that fermentation with all waste substrates except for 15% sweet potato peel is able to yield a similar carbon nitrogen of extracted chitin as commercial chitin.

A one-way ANOVA was performed to determine whether the differences between group means for the degree of crystallinity of extracted chitin are statistically significant.

Table 9. One-Way ANOVA for Degree of Crystallinity of Chitin extracted after *Lactobacillus plantarum* and *Bacillus subtilis* Fermentation on Various Carbon Sources

Source	Sum of Squares	Degrees of Freedom	Mean Squares	F-Statistic	P-Value
Columns	847.313	11	77.0285	243.55	7.900×10^{-22}
Error	7.591	24	0.3163		
Total	854.904	35			

As the p-value was found to be less than 0.05 significance level, the null hypothesis that all population means are equal is rejected and it is concluded that the differences between some of the means for the degree of crystallinity of extracted chitin are statistically significant. A multiple comparison test was then used to assess the differences in group means.

Table 10. Multi-Comparison Test for Degree of Crystallinity of Chitin extracted after *Lactobacillus plantarum* and *Bacillus subtilis* Fermentation on Various Carbon Sources

Substrate Compared	Substrate Compared	Lower 95% Confidence	Difference in Means	Upper 95% Confidence	P-Value
Commercial Chitin	20% Glucose	-12.2923	-10.6367	-8.9810	0.0000
Commercial Chitin	20% Molasses	-4.9656	-3.3100	-1.6544	0.0000
Commercial Chitin	20% Corn Syrup	-11.8056	-10.1500	-8.4944	0.0000
Commercial Chitin	15% Red Grape Waste	-9.5356	-7.8800	-6.2244	0.0000
Commercial Chitin	15% White Grape Waste	-3.5556	-1.9000	-0.2444	0.0152
Commercial Chitin	15% Apple Pomace	-11.4856	-9.8300	-8.1744	0.0000
Commercial Chitin	15% Pineapple Peel	-8.6223	-6.9667	-5.3110	0.0000
Commercial Chitin	15% Potato Peel	4.5544	6.2100	7.8656	0.0000
Commercial Chitin	15% Mango Peel	-10.9023	-9.2467	-7.5910	0.0000
Commercial Chitin	15% Banana Peel	-7.6323	-5.9767	-4.3210	0.0000
Commercial Chitin	15% Sweet Potato Peel	-10.1323	-8.4767	-6.8210	0.0000
20% Glucose	20% Molasses	5.6710	7.3267	8.9823	0.0000
20% Glucose	20% Corn Syrup	-1.1690	0.4867	2.1423	0.9939

20% Glucose	15% Red Grape Waste	1.1010	2.7567	4.4123	0.0002
20% Glucose	15% White Grape Waste	7.0810	8.7367	10.3923	0.0000
20% Glucose	15% Apple Pomace	-0.8490	0.8067	2.4623	0.8248
20% Glucose	15% Pineapple Peel	2.0144	3.6700	5.3256	0.0000
20% Glucose	15% Potato Peel	15.1910	16.8467	18.5023	0.0000
20% Glucose	15% Mango Peel	-0.2656	1.3900	3.0456	0.1607
20% Glucose	15% Banana Peel	3.0044	4.6600	6.3156	0.0000
20% Glucose	15% Sweet Potato Peel	0.5044	2.1600	3.8156	0.0040
20% Molasses	20% Corn Syrup	-8.4956	-6.8400	-5.1844	0.0000
20% Molasses	15% Red Grape Waste	-6.2256	-4.5700	-2.9144	0.0000
20% Molasses	15% White Grape Waste	-0.2456	1.4100	3.0656	0.1481
20% Molasses	15% Apple Pomace	-8.1756	-6.5200	-4.8644	0.0000
20% Molasses	15% Pineapple Peel	-5.3123	-3.6567	-2.0010	0.0000
20% Molasses	15% Potato Peel	7.8644	9.5200	11.1756	0.0000
20% Molasses	15% Mango Peel	-7.5923	-5.9367	-4.2810	0.0000
20% Molasses	15% Banana Peel	-4.3223	-2.6667	-1.0110	0.0003
20% Molasses	15% Sweet Potato Peel	-6.8223	-5.1667	-3.5110	0.0000
20% Corn Syrup	15% Red Grape Waste	0.6144	2.2700	3.9256	0.0022
20% Corn Syrup	15% White Grape Waste	6.5944	8.2500	9.9056	0.0000
20% Corn Syrup	15% Apple Pomace	-1.3356	0.3200	1.9756	0.9999
20% Corn Syrup	15% Pineapple Peel	1.5277	3.1833	4.8390	0.0000
20% Corn Syrup	15% Potato Peel	14.7044	16.3600	18.0156	0.0000

20% Corn Syrup	15% Mango Peel	-0.7523	0.9033	2.5590	0.7091
20% Corn Syrup	15% Banana Peel	2.5177	4.1733	5.8290	0.0000
20% Corn Syrup	15% Sweet Potato	0.0177	1.6733	3.3290	0.0460
15% Red Grape Waste	15% White Grape Waste	4.3244	5.9800	7.6356	0.0000
15% Red Grape Waste	15% Apple Pomace	-3.6056	-1.9500	-0.2944	0.0118
15% Red Grape Waste	15% Pineapple Peel	-0.7423	0.9133	2.5690	0.6960
15% Red Grape Waste	15% Potato Peel	12.4344	14.0900	15.7456	0.0000
15% Red Grape Waste	15% Mango Peel	-3.0223	-1.3667	0.2890	0.1765
15% Red Grape Waste	15% Banana Peel	0.2477	1.9033	3.5590	0.0149
15% Red Grape Waste	15% Sweet Potato Peel	-2.2523	-0.5967	1.0590	0.9713
15% White Grape Waste	15% Apple Pomace	-9.5856	-7.9300	-6.2744	0.0000
15% White Grape Waste	15% Pineapple Peel	-6.7223	-5.0667	-3.4110	0.0000
15% White Grape Waste	15% Potato Peel	6.4544	8.1100	9.7656	0.0000
15% White Grape Waste	15% Mango Peel	-9.0023	-7.3467	-5.6910	0.0000
15% White Grape Waste	15% Banana Peel	-5.7323	-4.0767	-2.4210	0.0000
15% White Grape Waste	15% Sweet Potato Peel	-8.2323	-6.5767	-4.9210	0.0000
15% Apple Pomace	15% Pineapple Peel	1.2077	2.8633	4.5190	0.0001
15% Apple Pomace	15% Potato Peel	14.3844	16.0400	17.6956	0.0000
15% Apple Pomace	15% Mango Peel	-1.0723	0.5833	2.2390	0.9755
15% Apple Pomace	15% Banana Peel	2.1977	3.8533	5.5090	0.0000
15% Apple Pomace	15% Sweet Potato Peel	-0.3023	1.3533	3.0090	0.1860

15% Pineapple Peel	15% Potato Peel	11.5210	13.1767	14.8323	0.0000
15% Pineapple Peel	15% Mango Peel	-3.9356	-2.2800	-0.6244	0.0021
15% Pineapple Peel	15% Banana Peel	-0.6656	0.9900	2.6456	0.5922
15% Pineapple Peel	15% Sweet Potato Peel	-3.1656	-1.5100	0.1456	0.0969
15% Potato Peel	15% Mango Peel	-17.1123	-15.4567	-13.8010	0.0000
15% Potato Peel	15% Banana Peel	-13.8423	-12.1867	-10.5310	0.0000
15% Potato Peel	15% Sweet Potato Peel	-16.3423	-14.6867	-13.0310	0.0000
15% Mango Peel	15% Banana Peel	1.6144	3.2700	4.9256	0.0000
15% Mango Peel	15% Sweet Potato Peel	-0.8856	0.7700	2.4256	0.8617
15% Banana Peel	15% Sweet Potato Peel	-4.1556	-2.5000	-0.8444	0.0007

As the p-value for most of the comparison of means is less than 0.05, this proves that the group means of the degree of crystallisation for extracted chitin using fermentation of different substrates are significantly different from each other. However there are a few notable exceptions. For example, 20% glucose and 20% corn syrup have a p-value of 0.9939; 20% glucose and 15% apple pomace have a p-value of 0.8248; 20% glucose and 15% mango peel have a p-value of 0.1607; 20% corn syrup and 15% apple pomace have a p-value of 0.9999; 20% corn syrup and 15% mango peel have a p-value of 0.7091; 15% apple pomace and 15% mango peel have a p-value of 0.9755 . This shows that the control substrate glucose can be substituted by corn

syrup, apple pomace as well as mango peel to yield a similar degree of crystallisation of extracted chitin (above 95%).

Linear regression modeling was attempted to find if there was any relationship between the three parameters, degree of deacetylation, carbon nitrogen ratio and degree of crystallisation. The linear regression model proposed was degree of deacetylation (%) = $108.83 - 6.4406 \times \text{carbon nitrogen ratio}$.

Table 11. Linear Regression Model for Degree of Deacetylation (Y) and Carbon Nitrogen Ratio (x) of Chitin extracted after *Lactobacillus plantarum* and *Bacillus subtilis* Fermentation on Various Carbon Sources

Y ~ 1 + x1 model	Estimate	Root Mean Squared Error	t-Statistic	P-Value
(Intercept)	108.83	17.297	6.292	3.6061×10^{-7}
x1	-6.4406	2.5487	-2.527	0.016323

No linear correlation was found between the degree of deacetylation and degree of crystallisation of extracted chitin. Similarly, no linear correlation was discovered between the carbon nitrogen ratio and the degree of crystallisation.

Chapter 3: GC-MS-Based Metabolomics Analysis of Prawn Shell Waste Co-Fermentation by *Lactobacillus* *plantarum* and *Bacillus subtilis*

3.1 Introduction

The remains of heads and exoskeletons of crustacean waste have been reported to be rich in proteins of high nutritional value as well as lipid soluble carotenoid pigments, amounting to 40% of their total waste weight [85]. Extracting protein hydrolysates from prawn waste to be used as food flavouring agents or for aquaculture diets has become a focal point of study.

In recent years, analysis of amino acids have been performed using pre-column derivatization and reverse phase high performance liquid chromatography (HPLC) separation of derivatives [86]. This method has gained popularity due to shorter analysis times and greater sensitivity. The rapid derivatization also produces resultant derivatives that are very stable and highly fluorescent [87].

Bioactive substances such as polypeptides, free amino acids, phenols and polysaccharides in the supernatant of prawn shell fermentation have yet to be identified and quantified in literature [88]. With the help of Gas Chromatography Mass Spectrometry (GC-MS), characterization of the metabolites that were generated during prawn shell fermentation was carried out and the possibility of harvesting them from solution was explored.

The co-fermentation setup of both bacterial strains *Lactobacillus plantarum* and *Bacillus subtilis* was run and the pH, protease activity, glucose and amino acids concentrations were determined at the end of the 5 day fermentation period to study the metabolite changes.

Metabolites are usually present in very small quantities and low concentrations in the fermentation broth [89]. As such, the mass spectrometry machines may not have the capability to detect them [90]. Possible solutions include lowering the extraction volumes to increase the concentration of metabolites for detection [91].

3.2 Materials and Methods

1 mL culture supernatant was collected at the end of 5 days from the setup comprising of 5 g of prawn shell waste fermented with 20 g of glucose dissolved in 100 mL deionized water. 50 µL of the collected supernatant was mixed with 10 µL of 2 g/L ribitol dissolved in water [92]. The addition of ribitol served as an internal standard to determine metabolite concentrations accurately [93]. The sample was then lyophilized overnight using a Labconco freeze dryer set at -40 °C and 0.0002 mBar [94].

GC-MS derivatization was performed the next day prior to metabolic profiling on the GC-MS [95]. Firstly, the lyophilized sample was redissolved in 100 µL of 20 mg/mL methoxyamine hydrochloride (MEOX) in pyridine and incubated at 37 °C for 1 hour for carbonyls protection [96]. Secondly, 100 µL of *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) with 1%

trimethyl-chlorosilane (TCMS) was added to the sample and silylation was carried out at 70 °C for 30 minutes [97]. Finally, the sample was centrifuged at 14,500 x g for 15 minutes and the clear supernatant was used for GC-MS analysis [98].

1 µL of the clear supernatant sample was injected into the GC-MS (Agilent Technologies) using an auto-injector by splitless mode [99]. The carrier gas helium was set at a flow rate of 1.1 mL/min [100]. The injector temperature was set at 250 °C while the ion source temperature was set at 230 °C [101]. The oven temperature was first kept at 75 °C for 5 minutes, then raised at 4 °C per minute till the final temperature of 280 °C was reached and this was held for 2 minutes [102].

Data was recorded for m/z between 50 to 500 with a scan time of 0.1 seconds [103]. The identity of the metabolites were then determined using the NIST08 mass spectral library [104]. The peak area for ribitol recorded from the GC-MS run was equated to 20 µg / 200 µL [105]. Concentrations for the detected metabolites were calculated by multiplying the measured peak area of detected metabolites by ribitol concentration 20 µg / 200 µL and dividing over the measured peak area for ribitol [106].

3.3 Results and Discussion

The metabolites detected in the supernatant were categorized into amino acids, antimicrobial compounds, organic compounds and sugar derivatives and presented in Tables 5 to 8 below. A total of 376 metabolites

were detected, in which 14 were amino acids, 106 were organic acids and 90

were reported to possess antimicrobial properties from published literature.

The antimicrobial compounds were most probably produced by *Lactobacillus*

plantarum as a form of probiotics against other pathogenic microbial strains.

The high amino acids levels measured also testify to the feasibility of using

the spray dried supernatant as a high protein animal feed.

Table 12. Amino Acids Detected in Culture Supernatant of *Lactobacillus plantarum* and *Bacillus subtilis* Fermentation in 5 g prawn Shell Waste and 20% Glucose

Metabolite	Molecular Formula	Quantity (mg/L)	Biological Characteristic
Alanine	C ₃ H ₇ NO ₂	4642.67	Amino Acid
Proline	C ₅ H ₉ NO ₂	91.76	Amino Acid
Threonine	C ₄ H ₉ NO ₃	91.73	Amino Acid
Leucine	C ₆ H ₁₃ NO ₂	63.91	Amino Acid
Norleucine	C ₆ H ₁₃ NO ₂	53.57	Amino Acid
Ketoisocaproic acid	C ₆ H ₁₀ O ₃	44.06	Leucine Ketoacid
Alanylthreonine	C ₇ H ₁₄ N ₂ O ₄	26.39	Amino Acid
Glycine	C ₂ H ₅ NO ₂	25.56	Amino Acid
Sarcosine	C ₃ H ₇ NO ₂	16.19	Amino Acid
Isoleucine	C ₆ H ₁₃ NO ₂	13.96	Amino Acid
Alloisoleucine	C ₆ H ₁₃ NO ₂	13.86	Amino Acid
Glutamic acid	C ₅ H ₉ NO ₄	5.68	Amino Acid
Valine	C ₅ H ₁₁ NO ₂	3.13	Amino Acid
1,4-Dihydrophenylalanine	C ₉ H ₁₃ NO ₂	2.92	Amino Acid
Ketoisovaleric acid	C ₅ H ₈ O ₃	1.2	Valine Ketoacid
Lysine	C ₆ H ₁₄ N ₂ O ₂	0.44	Amino Acid

Table 13. Antimicrobial Compounds Detected in Culture Supernatant of *Lactobacillus plantarum* and *Bacillus subtilis* Fermentation in 5 g prawn Shell Waste and 20% Glucose

Metabolite	Molecular Formula	Quantity (mg/L)	Biological Characteristic
Acetamide	C ₂ H ₅ NO	2999.12	Antimicrobial[107]
Carbamic acid	CH ₃ NO ₂	1432.07	Antimicrobial[108]
Uridine	C ₉ H ₁₂ N ₂ O ₆	1277.01	Antimicrobial[109]

Propanoic acid	C ₃ H ₆ O ₂	1184.32	Antimicrobial[110]
Lactic acid	C ₃ H ₆ O ₃	1055.38	Antimicrobial[111]
2-Hydroxybenzaldehyde	C ₇ H ₆ O ₂	940.97	Antimicrobial[112]
Mandelic acid	C ₈ H ₈ O ₃	443.50	Antimicrobial[113]
Acethydrazide	C ₂ H ₆ N ₂ O	366.71	Antimicrobial[114]
Glycerol	C ₃ H ₈ O ₃	336.23	Antimicrobial[115]
2-Quinolinone	C ₉ H ₇ NO	284.36	Antimicrobial[116]
Hexanedioic acid	C ₆ H ₁₀ O ₄	200.31	Antimicrobial[117]
2-Hydroxyisocaproic acid	C ₆ H ₁₂ O ₃	173.95	Antimicrobial[118]
Benzenesulfonamide	C ₆ H ₇ NO ₂ S	167.36	Antimicrobial[119]
Butyric acid	C ₄ H ₈ O ₂	153.36	Antimicrobial[120]
Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	147.85	Antimicrobial[121]
Octadecanoic acid	C ₁₈ H ₃₆ O ₂	147.83	Antimicrobial[122]
Acetic acid	C ₂ H ₄ O ₂	71.94	Antimicrobial[123]
Decanoic acid	C ₁₀ H ₂₀ O ₂	70.50	Antimicrobial[124]
Thymol	C ₁₀ H ₁₄ O	68.98	Antimicrobial[125]
Quinazoline	C ₈ H ₆ N ₂	66.92	Antimicrobial[126]
Sedoheptulose	C ₇ H ₁₄ O ₇	64.36	Antimicrobial[127]
Kaurene	C ₂₀ H ₃₂	58.27	Antimicrobial[128]
1,2,4-Triazole-3-carboxylic acid	C ₃ H ₃ N ₃ O ₂	56.93	Antimicrobial[129]
Pyruvic acid	C ₃ H ₄ O ₃	56.20	Antimicrobial[130]
1,2-Benzisothiazole	C ₇ H ₅ NS	56.13	Antimicrobial[131]
Pentanedioic acid	C ₅ H ₈ O ₄	50.59	Antimicrobial[132]
Phenanthroline	C ₁₂ H ₈ N ₂	49.88	Antimicrobial[133]
Ethyl acetate	C ₄ H ₈ O ₂	44.86	Antimicrobial[134]
Pyrazine	C ₄ H ₄ N ₂	33.19	Antimicrobial[135]
Ethanol	C ₂ H ₆ O	31.01	Antimicrobial[136]
1,4-Benzoquinone	C ₆ H ₄ O ₂	28.12	Antimicrobial[137]
Benzoic acid	C ₇ H ₆ O ₂	27.37	Antimicrobial[138]
Benzoate	C ₇ H ₅ O ₂ ⁻	24.82	Antimicrobial[139]
Benzisothiazolinone	C ₇ H ₅ NOS	23.64	Antimicrobial[140]
Indole	C ₈ H ₇ N	22.32	Antimicrobial[141]
Malic acid	C ₄ H ₆ O ₅	21.02	Antimicrobial[142]
2-Aminothiadiaazole	C ₃ H ₄ N ₂ S	20.98	Antimicrobial[143]
Hexanoic acid	C ₆ H ₁₂ O ₂	20.24	Antimicrobial[144]
Allonic acid	C ₆ H ₁₂ O ₇	18.89	Anti-tumour
3-Hydroxybutyric acid	C ₄ H ₈ O ₃	18.19	Antimicrobial[145]
Nonanoic acid	C ₉ H ₁₈ O ₂	16.07	Antimicrobial[146]
Colchicine	C ₂₂ H ₂₅ NO ₆	15.06	Anti-inflammatory
Benzamide	C ₇ H ₇ NO	11.18	Antimicrobial[147]
Propionamide	C ₃ H ₇ NO	10.84	Antimicrobial[148]
Methanol	CH ₄ O	10.15	Antimicrobial[149]
Sesamol	C ₇ H ₆ O ₃	9.33	Antimicrobial[150]
Benzeneacetic acid	C ₈ H ₈ O ₂	9.04	Antimicrobial[151]
Guaiacol	C ₇ H ₈ O ₂	8.43	Antimicrobial[152]
Indole-3-carboxylic acid	C ₉ H ₇ NO ₂	8.27	Antimicrobial[153]
Propanenitrile	C ₃ H ₅ N	8.05	Antimicrobial[154]

Linolenic acid	$C_{18}H_{30}O_2$	8.00	Antimicrobial[155]
Tartaric acid	$C_4H_6O_6$	7.94	Antimicrobial[156]
1-Benzylindole	$C_{15}H_{13}N$	7.92	Antimicrobial[157]
Pyranone	$C_5H_4O_2$	7.89	Antimicrobial[158]
4-Aminobenzoic acid	$C_7H_7NO_2$	7.00	Antimicrobial[159]
1,3-Benzodioxole	$C_7H_6O_2$	6.97	Antimicrobial[160]
Cephaloridine	$C_{19}H_{17}N_3O_4S_2$	6.53	Antibiotic
Docosanol	$C_{22}H_{46}O$	6.41	Antimicrobial[161]
Propanamide	C_3H_7NO	6.08	Antimicrobial[162]
Arachidonic acid	$C_{20}H_{32}O_2$	5.92	Antimicrobial[163]
Docosaehaenoic acid	$C_{22}H_{32}O_2$	5.79	Antimicrobial[164]
Dihydroisosteviol	$C_{20}H_{32}O_3$	5.44	Antimicrobial[165]
Dodecanamide	$C_{12}H_{25}NO$	5.28	Antimicrobial[166]
Galacturonic acid	$C_6H_{10}O_7$	5.03	Antimicrobial[167]
Glyoxylic acid	$C_2H_2O_3$	4.68	Antimicrobial[168]
Acridinedione	$C_{13}H_7NO_2$	4.31	Antimicrobial[169]
Lycopodine	$C_{16}H_{25}NO$	4.13	Antimicrobial[170]
Pteridine	$C_6H_4N_4$	3.82	Antimicrobial[171]
D-gluco-hexodialdodifuranoside	$C_{14}H_{30}O_6$	3.52	Anti-cancer
Pyrrolopyrimidine	$C_6H_5N_3$	3.33	Anti-viral[172]
3-Isoxazolidinone	$C_3H_5NO_2$	3.31	Antimicrobial[173]
Pyridazine	$C_4H_4N_2$	3.29	Antimicrobial[174]
Griseoviridin	$C_{22}H_{27}N_3O_7S$	3.14	Antibiotic
Pyrrole	C_4H_5N	3.04	Antimicrobial[175]
Nonadecanoic acid	$C_{19}H_{38}O_2$	2.48	Anti-cancer[176]
Thiourea	CH_4N_2S	2.16	Antimicrobial[177]
3-Phenyl-5-isoxazalone	$C_9H_7NO_2$	2.14	Antimicrobial[178]
2-Keto-D-glucose	$C_6H_{10}O_6$	2.11	Antibiotic
Undecanoic acid	$C_{11}H_{22}O_2$	2.09	Antimicrobial[179]
Quinoline	C_9H_7N	1.87	Antimicrobial[180]
Butanol	$C_4H_{10}O$	1.81	Antimicrobial[181]
4-Hydroxydiphenylamine	$C_{12}H_{11}NO$	1.77	Antimicrobial[182]
1,2-Benzisoxazole	C_7H_5NO	1.75	Antimicrobial[183]
3-(4-Hydroxyphenyl)propionic acid	$C_9H_{10}O_3$	1.71	Anti-inflammatory
Meldrum's acid	$C_6H_8O_4$	1.49	Antimicrobial[184]
Benzenethiol	C_6H_6S	1.47	Antimicrobial[185]
Octanoic acid	$C_8H_{16}O_2$	1.38	Antimicrobial[186]
1,2-Benzenediol	$C_6H_6O_2$	1.31	Antimicrobial[187]
Isocitric acid	$C_6H_8O_7$	1.30	Antimicrobial[188]
Benzoxazole	C_7H_5NO	1.26	Antimicrobial[189]
Octenidine	$C_{36}H_{62}N_4$	1.03	Antimicrobial[190]
Thiophene	C_4H_4S	0.93	Antimicrobial[191]
Carbamate	$CH_2NO_2^-$	0.92	Antimicrobial[192]
2,4-Hexadienoic acid	$C_6H_8O_2$	0.87	Antimicrobial[193]
Benzenepropanoic acid	$C_9H_{10}O_2$	0.84	Antimicrobial[194]
Acrylic acid	$C_3H_4O_2$	0.72	Antimicrobial[195]
Phosphoric acid	H_3PO_4	0.68	Antimicrobial[196]

Urea	CH ₄ N ₂ O	0.65	Antimicrobial[197]
Azelaic acid	C ₉ H ₁₆ O ₄	0.50	Antimicrobial[198]
Methylenecyclopropane	C ₄ H ₆	0.18	Anti-viral[199]

Table 14. Other Organic Compounds Detected in Culture Supernatant of *Lactobacillus plantarum* and *Bacillus subtilis* Fermentation in 5 g prawn Shell Waste and 20% Glucose

Metabolite	Molecular Formula	Quantity (mg/L)	Biological Characteristic
Butanoic acid	C ₄ H ₈ O ₂	4399.87	Organic acid
Propylene glycol	C ₃ H ₈ O ₂	2458.09	Solvent
2,3-Dimethylbutanoic acid	C ₆ H ₁₂ O ₂	2129.98	Fatty acid
1,2,3-Propanetriol	C ₃ H ₈ O ₃	1595.72	Polyol
Dodecane	C ₁₂ H ₂₆	1462.61	Alkane
3-Oxooctanoic acid	C ₈ H ₁₄ O ₃	1185.00	Fatty acid
1,2,4,5-Tetramethylbenzene	C ₁₀ H ₁₄	650.91	Aromatic
Butane	C ₄ H ₁₀	543.18	Alkane
Methylcyclopentadiene	C ₆ H ₈	465.49	Aromatic
Methyl valerate	C ₆ H ₁₂ O ₂	445.57	Flavouring
1-Butene	C ₄ H ₈	379.99	Alkene
2-Propenamide	C ₃ H ₅ NO	338.39	Fatty amide
Glyceraldehyde acetone	C ₆ H ₁₀ O ₃	247.79	Carboxaldehyde
γ-Octalactone	C ₈ H ₁₄ O ₂	238.91	Flavouring
2-Ketobutyric acid	C ₄ H ₆ O ₃	222.57	Organic acid
Tridecane	C ₁₃ H ₂₈	207.98	Alkane
Scopolin	C ₁₆ H ₁₈ O ₉	179.76	Phytochemical
Paramethoxy-N-methylamphetamine	C ₁₁ H ₁₇ NO	145.28	Stimulant
Ethylene glycol	C ₂ H ₆ O ₂	141.94	Solvent
Butanediol	C ₄ H ₁₀ O ₂	139.50	Alcohol
Octadecane	C ₁₈ H ₃₈	137.50	Alkane
4-Methyl-5-thiazoleethanol	C ₆ H ₉ NOS	120.92	Flavouring
Triethylene glycol	C ₆ H ₁₄ O ₄	120.91	Solvent
Dipropylacetic acid	C ₈ H ₁₆ O ₂	120.83	Organic acid
Propanone	C ₃ H ₆ O	114.26	Ketone
6-Methyl-3,5-heptadien-2-one	C ₈ H ₁₂ O	106.73	Flavouring
Hexadecane	C ₁₆ H ₃₄	97.90	Alkane
Benzonitrile	C ₇ H ₅ N	91.31	Aromatic
Dimethylcyclohexanone	C ₈ H ₁₄ O	89.00	Aromatic
Butylamine	C ₄ H ₁₁ N	84.67	Amine
Cholesterol	C ₂₇ H ₄₆ O	75.80	Cholesterol
3-Hydroxy-2-butanone	C ₄ H ₈ O ₂	72.50	Methyl ketone
2-Pentenoic acid	C ₅ H ₈ O ₂	70.15	Organic acid
2-Methylpropene	C ₄ H ₈	69.91	Alkene

Diethylene glycol	C ₄ H ₁₀ O ₃	67.89	Solvent
Octadecenamide	C ₁₈ H ₃₅ NO	67.10	Fatty amide
13-Docosenamide	C ₂₂ H ₄₃ NO	64.20	Fatty amide
Octahydronaphthalene-1,4-diol	C ₁₀ H ₁₆ O ₂	57.23	Alcohol
Dimethyldecahydronaphthalene	C ₁₂ H ₂₂	56.95	Aromatic
Heptadecane-1,2-diol	C ₁₇ H ₃₆ O ₂	54.71	Fatty alcohol
2-Methyl-4-propyl-1,3-oxathiane	C ₈ H ₁₆ OS	53.44	Flavouring
2-Butenoic acid	C ₄ H ₆ O ₂	52.41	Organic acid
13,16-Docasadienoic acid	C ₂₂ H ₄₀ O ₂	51.71	Fatty acid
Octadecanamide	C ₁₈ H ₃₇ NO	47.22	Fatty amide
1,4-Butenediol	C ₄ H ₈ O ₂	46.28	Alcohol
2,3,4-Trihydroxybutanoic acid	C ₄ H ₈ O ₅	41.02	Organic acid
Pyrandiol	C ₅ H ₆ O ₃	40.72	Alcohol
Ethane	C ₂ H ₆	37.25	Alkane
Monostearin	C ₂₁ H ₄₂ O ₄	35.76	Emulsifier
1-Cholestene	C ₂₇ H ₄₆	31.64	Cholesterol
Butanedioic acid	C ₄ H ₆ O ₄	31.11	Organic acid
Butanal	C ₄ H ₈ O	29.38	Aldehyde
Pentanoic acid	C ₅ H ₁₀ O ₂	26.12	Flavouring
Octane	C ₈ H ₁₈	25.30	Alkane
Cyclobutanemethanol	C ₅ H ₁₀ O	25.00	Aromatic
N-acetyl-glucosamine	C ₈ H ₁₅ NO ₆	24.93	Chitosan
9-O-pivaloyl-N-acetylcolchinol	C ₂₅ H ₃₁ NO ₆	24.44	Aromatic
Pseudoephedrine	C ₁₀ H ₁₅ NO	24.34	Decongestant
L-gulono-1,4-lactone	C ₆ H ₁₀ O ₆	21.25	Lactone
3-Dibenzofuranamine	C ₁₂ H ₉ NO	19.35	Aromatic
2,4,5-Trihydroxypentanoic acid	C ₅ H ₁₀ O ₅	18.61	Organic acid
3,3-Dimethyl-1-butanol	C ₆ H ₁₄ O	18.60	Alcohol
17-Octadecynoic acid	C ₁₈ H ₃₂ O ₂	18.04	Fatty acid
Ketovaleric acid	C ₅ H ₈ O ₃	17.59	Ketoacid
Oleic acid	C ₁₈ H ₃₄ O ₂	17.59	Fatty acid
Ethanedioic acid	C ₂ H ₂ O ₄	16.61	Organic acid
1,2,4-Trimethylbenzene	C ₉ H ₁₂	16.28	Aromatic
Benzene	C ₆ H ₆	16.07	Aromatic
2-Propenoic acid	C ₃ H ₄ O ₂	15.90	Organic acid
Estratetraenol	C ₁₈ H ₂₂ O	15.35	Steroid
Tridecanoic acid	C ₁₃ H ₂₆ O ₂	15.33	Fatty acid
Heptanamide	C ₇ H ₁₅ NO	14.97	Fatty amide
Pyruvate oxime	C ₃ H ₅ NO ₃	14.13	Acid amine
Tetradecane	C ₁₄ H ₃₀	14.12	Alkane
2,3-Dihydroxybutanoic acid	C ₄ H ₈ O ₄	13.75	Organic acid
Monoethyl malonic acid	C ₅ H ₈ O ₄	13.41	Organic acid
Thiodiglycol	C ₄ H ₁₀ O ₂ S	12.39	Alcohol
3-Hexenedioic acid	C ₆ H ₈ O ₄	11.76	Fatty acid
Carbophenoxon sulfone	C ₁₁ H ₁₆ ClO ₅ PS ₂	11.46	Organosulfone
Pentadecanoic acid	C ₁₅ H ₃₀ O ₂	11.42	Fatty acid
3-Heptyn-1-ol	C ₇ H ₁₂ O	10.73	Fatty alcohol

1-Octene	C ₈ H ₁₆	10.56	Alkene
1-Hexene	C ₆ H ₁₂	10.50	Alkene
Propanedioic acid	C ₃ H ₄ O ₄	10.46	Organic acid
Methyl butyrate	C ₅ H ₁₀ O ₂	9.83	Flavouring
Vitamin C	C ₆ H ₈ O ₆	9.22	Ascorbic acid
Dimethyl malonate	C ₅ H ₈ O ₄	8.30	Acid ester
Cyclopenta[de]naphthalene	C ₁₂ H ₈	8.26	Aromatic
2-Methylpropanoic acid	C ₄ H ₈ O ₂	8.21	Organic acid
Benzocyclobutene	C ₈ H ₈	7.98	Aromatic
1,2,4-Cyclopentanetrione	C ₅ H ₄ O ₃	7.93	Aromatic
Tetradecanoic acid	C ₁₄ H ₂₈ O ₂	7.86	Fatty acid
1-Undecene	C ₁₁ H ₂₂	7.66	Alkene
Cholestenone	C ₂₇ H ₄₄ O	7.61	Cholesterol
Butyne	C ₄ H ₆	7.46	Alkyne
Pentenedioic acid	C ₅ H ₆ O ₄	7.41	Organic acid
Ethyl butyrate	C ₆ H ₁₂ O ₂	7.25	Flavouring
Pentane	C ₅ H ₁₂	7.22	Alkane
Heptadecane	C ₁₇ H ₃₆	7.06	Alkane
Morphine	C ₁₇ H ₁₉ NO ₃	7.05	Painkiller
Nickel	Ni	6.56	Mineral
Propane	C ₃ H ₈	6.39	Alkane
Pentaethylene glycol	C ₂₂ H ₄₆ O ₆	6.10	Solvent
Methanaminium	CH ₆ N	5.93	Conjugate acid
Hexadecanamide	C ₁₆ H ₃₃ NO	5.81	Fatty amide
3-Methylvaleric acid	C ₆ H ₁₂ O ₂	5.55	Fatty acid
Talonic acid	C ₆ H ₁₂ O ₇	5.55	Organic acid
1-Cyclohexyl-tetradecane	C ₂₀ H ₄₀	5.00	Aromatic
Cyclopentane	C ₅ H ₁₀	4.84	Aromatic
Dodecanedioic acid	C ₁₂ H ₂₂ O ₄	4.84	Organic acid
Cyclohexane	C ₆ H ₁₂	4.64	Aromatic
Aromadendrene	C ₁₅ H ₂₄	4.54	Essential oil
2-Oxovaleric acid	C ₅ H ₈ O ₃	4.12	Ketoacid
3-Hydroxysebacic acid	C ₁₀ H ₁₈ O ₅	4.09	Organic acid
Glucuronolactone	C ₆ H ₈ O ₆	3.95	Lactone
Pregnenolone	C ₂₁ H ₃₂ O ₂	3.80	Steroid
Cholestane	C ₂₇ H ₄₈	3.63	Cholesterol
Methyl tetradecanoate	C ₁₅ H ₃₀ O ₂	3.53	Flavouring
Sebacic acid	C ₁₀ H ₁₈ O ₄	3.53	Fatty acid
Octahydronaphthalene	C ₁₀ H ₁₆	3.48	Aromatic
2-Furanone	C ₄ H ₄ O ₂	3.41	Flavouring
Iron	Fe	3.25	Mineral
Propylamine	C ₃ H ₉ N	3.25	Fatty amine
Inabenfide	C ₁₉ H ₁₅ ClN ₂ O ₂	3.19	Herbicide
Ethanesulfonic acid	C ₂ H ₆ O ₃ S	3.13	Sulfonic acid
Cyclopropanecarboxylic acid	C ₄ H ₆ O ₂	3.04	Organic acid
1,3-Propanediol	C ₃ H ₈ O ₂	3.03	Alcohol
3-Hexen-1-ol	C ₆ H ₁₂ O	3.00	Fatty alcohol

Pseudouridine	$C_9H_{12}N_2O_6$	2.99	Nucleoside
Pentenedioate	$C_5H_6O_4^{2-}$	2.88	Organic acid
3-Cyclohexene-1-methanol	$C_7H_{12}O$	2.78	Essential oil
Propanal	C_3H_6O	2.77	Aldehyde
Chromium	Cr	2.53	Mineral
Docosanoic acid	$C_{22}H_{44}O_2$	2.40	Fatty acid
Methyl phenyl sulfoxide	C_7H_8OS	2.38	Aromatic
Pentadecane	$C_{15}H_{32}$	2.32	Alkane
3-Buten-1-ol	C_4H_8O	2.31	Alcohol
Tricyclodecenyl propionate	$C_{13}H_{18}O_2$	2.31	Fragrance
2-Hydroxyglutaric acid	$C_5H_8O_5$	2.30	Organic acid
Nonane	C_9H_{20}	2.28	Alkane
4-Hexen-1-yne	C_6H_8	2.27	Alkyne
2,6-Diamino-4-hexynoic acid	$C_6H_{10}N_2O_2$	2.10	Organic acid
Cortisone	$C_{21}H_{28}O_5$	2.09	Steroid
Ethylene	C_2H_4	1.99	Alkene
2-Furancarboxylic acid	$C_5H_4O_3$	1.98	Organic acid
Decane	$C_{10}H_{22}$	1.98	Alkane
1-Decanol	$C_{10}H_{22}O$	1.75	Fatty alcohol
1,1-Diisobutoxybutane	$C_{12}H_{26}O_2$	1.72	Aldehyde
Pentaoxacyclopentadecane	$C_{10}H_{20}O_5$	1.60	Crown ether
Pentanamide	$C_5H_{11}NO$	1.41	Acid amide
Amphetamine	$C_9H_{13}N$	1.35	Stimulant
3-Hydroxypyruvic acid	$C_3H_4O_4$	1.32	Organic acid
Cyclohexene	C_6H_{10}	1.31	Aromatic
Diisopropyl malonate	$C_9H_{16}O_4$	1.30	Acid ester
1-Tetradecanol	$C_{14}H_{30}O$	1.24	Fatty alcohol
1,3-Diazepane-2,4,6-trione	$C_5H_6N_2O_3$	1.09	Aromatic
2-Ketohexanoic acid	$C_6H_{10}O_3$	1.03	Fatty acid
5,8,11-Eicosatriynoic acid	$C_{20}H_{28}O_2$	0.97	Fatty acid
5-Norbornene-2-carboxylic acid	$C_8H_{10}O_2$	0.93	Organic acid
Camphoric acid	$C_{10}H_{16}O_4$	0.81	Organic acid
Succinate	$C_4H_4O_4^{2-}$	0.78	Flavouring
Dimethylbutanedioate	$C_6H_{10}O_4$	0.77	Flavouring
3-Furanacetaldehyde	$C_6H_6O_2$	0.75	Aldehyde
Butyrate	$C_4H_7O_2^{-}$	0.72	Flavouring
Ethanimidic acid	C_4H_9NO	0.63	Organic acid
Cyclopentene	C_5H_8	0.61	Aromatic
1,2,2,3,4-Butanepentacarbonitrile	$C_9H_5N_5$	0.55	Aromatic
Tetraethylene glycol	$C_8H_{18}O_5$	0.53	Solvent
Butynol	$C_4H_{10}O$	0.43	Alcohol
Malonic acid	$C_3H_4O_4$	0.25	Organic acid
Altronic acid	$C_6H_{12}O_7$	0.22	Organic acid
Succinonitrile	$C_4H_4N_2$	0.16	Nitrile

Table 15. Sugar Derivatives Detected in Culture Supernatant of *Lactobacillus plantarum* and *Bacillus subtilis* Fermentation in 5 g prawn Shell Waste and 20% Glucose

Metabolite	Molecular Formula	Quantity (mg/L)	Biological Characteristic
Phenyl-D-galactopyranoside	C ₁₂ H ₁₆ O ₆	9584.87	Sugar substitute
Galactose	C ₆ H ₁₂ O ₆	8909.55	Sugar substitute
Glucopyranose	C ₆ H ₁₂ O ₆	7024.77	Sugar substitute
Mannose	C ₆ H ₁₂ O ₆	5744.85	Sugar substitute
Maltose	C ₁₂ H ₂₂ O ₁₁	5653.05	Sugar substitute
Methyl-D-glucopyranoside	C ₇ H ₁₄ O ₆	4908.57	Sugar substitute
Dulcitol	C ₆ H ₁₄ O ₆	3323.48	Sugar alcohol
Mannonic acid	C ₆ H ₁₂ O ₇	2567.14	Sugar acid
Fructose	C ₆ H ₁₂ O ₆	2462.43	Sugar substitute
Fructose oxime	C ₆ H ₁₃ NO ₆	2421.99	Sugar substitute
Arabinopyranose	C ₅ H ₁₀ O ₅	2350.31	Sugar substitute
Glucofuranose	C ₆ H ₁₂ O ₆	2035.95	Sugar substitute
Glucose	C ₆ H ₁₂ O ₆	2010.62	Sugar substitute
Arabinofuranose	C ₅ H ₁₀ O ₅	1714.08	Sugar substitute
Lyxopyranose	C ₅ H ₁₀ O ₅	1654.75	Sugar substitute
Arabitol	C ₅ H ₁₀ O ₅	1456.58	Sugar alcohol
Ribopyranose	C ₅ H ₁₀ O ₅	1427.89	Sugar substitute
Xylose	C ₅ H ₁₀ O ₅	1417.45	Sugar substitute
Glucopyranuronic acid	C ₆ H ₁₀ O ₇	1239.42	Sugar acid
D-glycero-L-manno-heptonic acid	C ₇ H ₁₄ O ₈	1192.77	Sugar acid
Galactofuranose	C ₆ H ₁₂ O ₆	1076.99	Sugar substitute
Mannofuranose	C ₆ H ₁₂ O ₆	1009.76	Sugar substitute
Galactopyranose	C ₆ H ₁₂ O ₆	943.28	Sugar substitute
Xylopyranose	C ₅ H ₁₀ O ₅	915.86	Sugar substitute
Arabinose	C ₅ H ₁₀ O ₅	912.65	Sugar substitute
Talose	C ₆ H ₁₂ O ₆	800.65	Sugar substitute
2-Deoxy-D-glucose	C ₆ H ₁₂ O ₅	677.13	Sugar substitute
Glucose oxime	C ₆ H ₁₃ NO ₆	610.91	Sugar substitute
Lyxose	C ₅ H ₁₀ O ₅	603.81	Sugar substitute
Glucitol	C ₆ H ₁₄ O ₆	601.89	Sugar alcohol
2-Deoxy-erythro-pentofuranose	C ₅ H ₁₀ O ₄	596.47	Sugar substitute
Arabinofuranoside	C ₅ H ₉ O ₅	592.25	Sugar substitute
Erythrose	C ₄ H ₈ O ₄	522.45	Sugar substitute
Xylitol	C ₅ H ₁₂ O ₅	508.27	Sugar alcohol
Methyl-D-lyxofuranoside	C ₆ H ₁₂ O ₅	499.24	Sugar substitute
Methyl-D-galactofuranoside	C ₇ H ₁₄ O ₆	481.33	Sugar substitute
Glyceraldehyde	C ₃ H ₆ O ₃	422.51	Sugar substitute
Galactose oxime	C ₆ H ₁₃ NO ₆	369.21	Sugar substitute
Gulose	C ₆ H ₁₂ O ₆	361.01	Sugar substitute
Mannopyranose	C ₆ H ₁₂ O	358.11	Sugar substitute
Gluconic acid	C ₆ H ₁₂ O ₇	307.05	Sugar acid

2-Deoxy-D-galactopyranose	C ₆ H ₁₂ O ₅	281.36	Sugar substitute
Ribose	C ₅ H ₁₀ O ₅	249.45	Sugar substitute
Pentitol	C ₅ H ₁₂ O ₅	185.84	Sugar alcohol
Mannitol	C ₆ H ₁₄ O ₆	177.42	Sugar alcohol
Xylonic acid	C ₅ H ₁₀ O ₆	156.31	Sugar acid
Methyl-D-mannopyranoside	C ₇ H ₁₄ O ₆	131.27	Sugar substitute
Myo-inositol	C ₆ H ₁₂ O ₆	130.33	Sugar substitute
D-glycero-D-gluco-heptose	C ₇ H ₁₄ O ₇	129.78	Sugar substitute
Lactose	C ₁₂ H ₂₂ O ₁₁	125.72	Sugar substitute
Threitol	C ₄ H ₁₀ O ₄	121.12	Sugar alcohol
Methyl-D-ribofuranoside	C ₆ H ₁₂ O	120.28	Sugar substitute
Arabinitol	C ₅ H ₁₅ O ₅	115.06	Sugar alcohol
3-Deoxy-D-arabino-hexonic acid	C ₆ H ₁₂ O ₆	112.61	Sugar acid
D-glycero-D-galacto-heptose	C ₇ H ₁₄ O ₇	91.04	Sugar substitute
Ribonic acid	C ₅ H ₁₀ O ₆	89.50	Sugar acid
Xylofuranose	C ₅ H ₁₀ O ₅	88.69	Sugar substitute
Xylofuranose	C ₅ H ₁₀ O ₅	88.69	Sugar substitute
Arabinonic acid	C ₅ H ₁₀ O ₆	81.43	Sugar acid
Turanose	C ₁₂ H ₂₂ O ₁₁	66.04	Sugar substitute
Mannofuranuronic acid	C ₆ H ₈ O ₆	51.35	Sugar acid
3-Deoxy-erythro-pentonic acid	C ₅ H ₁₀ O ₅	43.20	Sugar acid
2-Deoxy-erythro-pentose	C ₅ H ₁₀ O ₄	42.49	Sugar substitute
D-glycero-D-gulo-heptonic acid	C ₇ H ₁₄ O ₈	40.26	Sugar acid
Sorbopyranose	C ₆ H ₁₂ O ₆	39.12	Sugar substitute
Threonic acid	C ₄ H ₈ O ₅	30.15	Sugar acid
Erythro-pentitol	C ₅ H ₁₂ O	29.50	Sugar alcohol
Erythro-tetrofuranose	C ₅ H ₁₀ O ₅	22.65	Sugar substitute
Dihydroxyacetone	C ₃ H ₆ O ₃	21.99	Sugar substitute
3-Deoxy-D-ribohexonic acid	C ₆ H ₁₂ O	20.71	Sugar acid
Deoxy-ribose	C ₅ H ₁₀ O ₄	19.08	Sugar substitute
D-arabino-3-hexulose	C ₆ H ₁₂ O ₆	17.21	Sugar substitute
Gulonic acid	C ₆ H ₁₂ O ₇	14.17	Sugar acid
Glucuronic acid	C ₆ H ₁₀ O ₇	7.58	Sugar acid
Xylulose	C ₅ H ₁₀ O ₅	7.30	Sugar substitute
Fructopyranose	C ₆ H ₁₂ O ₆	6.94	Sugar substitute
Altro-heptulose	C ₇ H ₁₄ O ₇	6.79	Sugar substitute
D-ribo-2-hexulose	C ₆ H ₁₂ O ₆	5.77	Sugar substitute
D-xylo-hexulose	C ₆ H ₁₂ O ₆	2.66	Sugar substitute
2-Deoxy-erythro-pentopyranose	C ₅ H ₁₀ O ₄	2.50	Sugar substitute
Galactoheptulose	C ₇ H ₁₄ O ₇	2.20	Sugar substitute
2,5-Methylene-D,L-rhamnitol	C ₇ H ₁₄ O ₅	1.33	Sugar substitute
Levoglucozan	C ₆ H ₁₀ O ₅	1.32	Sugar substitute
Methyl-D-xylopyranoside	C ₆ H ₁₂ O ₅	1.23	Sugar substitute
Glutaconic acid	C ₅ H ₆ O ₄	0.77	Sugar acid
Melibiose	C ₁₂ H ₂₂ O ₁₁	0.69	Sugar substitute
Erythritol	C ₄ H ₁₀ O ₄	0.26	Sugar alcohol
Glucaric acid	C ₆ H ₁₀ O ₈	0.08	Sugar acid

Chapter 4: Dual Extraction of Crustacean and Fungal Chitosan from a Single *Mucor circinelloides* Fermentation

4.1 Introduction

While prawn shells remain the traditional source of extracted chitin and deacetylated chitosan, the availability of seafood waste is subject to seasonal variation and discontinuous supply [200]. This has prompted scientific interest to look into alternative sources to supplement a constant supply of high quality chitosan in the market [201]. The fungal species *Mucor circinelloides* has been discovered to contain a high chitosan content in its mycelia, approximately around 35% of cell wall dry weight, which exceeds the 20-30% chitin content in prawn shells [202].

Mucor circinelloides has been reported to be a fungus that can be cultured on a wide variety of cheap substrates due to its ability to produce extracellular microbial proteases and organic acids to break them down [203]. It is also reported to produce various valuable biochemical products such as ethanol, polyunsaturated fatty acids, oil, protein, phosphate and glucosamine from the fermentation [204].

In this study, *Mucor circinelloides* was used to demineralize and deproteinate prawn shells, after which the fungus was dried and demineralized and deproteinated by *Lactobacillus plantarum* and *Bacillus subtilis* [205]. This would yield a dual extraction of crustacean and fungal

chitin [206].

4.2 Materials and Methods

Mucor circinelloides van Tieghem ATCC 24905 was cultured on potato dextrose agar (PDA) and incubated for 30 °C for 1 day [207]. 10 mL of sterile deionized water was then added to the PDA plate and the mycelia growth was scraped off using a sterile cell spreader to obtain a homogeneous spore suspension [208]. 1 mL of the fungal spore suspension was then inoculated into a conical flask containing 5 g of prawn shells, 10 g of waste substrate as a carbon source and 100 mL of sterile autoclaved water [209].

5 types of waste substrates were tested – okara, coffee residues, durian seed flour, avocado seed flour and red grape pomace [210]. The fermentation flasks were then incubated at 30 °C and 200 rpm for 7 days [211]. After 7 days, the demineralized and deproteinized prawn shells as well as the cultured fungus mycelia were harvested [212]. The crude extracted crustacean chitin was washed with sterile deionized water, sprayed with ethanol and dried in a vacuum oven at 60 °C for 24 hours before chemical analysis.

The dried fungal biomass was dropped into a 50 mL culture broth consisting of MRS broth and LB broth in 1:1 ratio and *Lactobacillus plantarum* and *Bacillus subtilis* were inoculated [213]. After fermentation at 30 °C at 200 rpm for 5 days, the deproteinized and demineralized was similarly washed with sterile deionized water, sprayed with ethanol and dried

in a vacuum oven at 60 °C for 24 hours before chemical analysis.

Again Fourier Transform Infrared Spectroscopy (FTIR) was used to measure the degree of deacetylation of the extracted crustacean chitin and fungal chitosan, while elemental analysis (EA) was used to determine their carbon nitrogen ratio. The degree of crystallinity was not tested as fungal chitosan was not crystalline. The FTIR and EA usage methods were similar to the steps described in Section 2.2 Materials and Methods.

4.3 Results and Discussion

The dry weights of the extracted crustacean chitin and fungal chitosan were recorded in Table 9. The crustacean chitin yield was obtained by dividing the dry weight of crude extracted chitin over the initial weight of prawn shells, while the fungal chitosan yield was obtained by dividing the dry weight of cultured fungal chitosan over the mass of waste substrate used. The calculated yield for the extraction of crustacean chitin was 10% to 20% while the calculated yield for the extraction of fungal chitosan was lower at 10% and below.

No fungal biomass was recorded for the okara fermentation because the *Mucor circinelloides* cultured on it became enmeshed in the soft soybean processing residues and could not be separated easily. The other waste substrates were harder and able to support fungal growth that floated on top of it, resulting in the mycelia growth being easily removed from the rest of the fermentation.

Table 16. Dry Weights of Crustacean Chitin and Fungal Chitosan extracted after *Mucor circinelloides* Fermentation on Various Waste Substrates

Waste Substrate for Fermentation	Dry Weight of Crustacean Chitin (g)	Crustacean Chitin Yield (%)	Dry Weight of Fungal Chitosan (g)	Fungal Chitosan Yield (%)
10 g avocado seed	1.13	22.6	0.46	4.6
10 g okara	0.74	14.8	-	-
10 g grape pomace	0.60	12.0	0.76	7.6
10 g coffee waste	0.58	11.6	1.0	10.0
10 g durian seed	0.56	11.2	0.77	7.7

The degree of deacetylation for both the extracted crude crustacean chitin as well as the cultured fungal chitosan were calculated by taking the ratio of peaks at 3450 cm^{-1} and 1650 cm^{-1} from the FTIR spectrum and recorded in Table 10. The highest degree of deacetylation was achieved for the crustacean chitin extracted from the fermentation using okara (69.9%), which closely matches the degree of deacetylation for commercial chitin (70.5%). In addition, the degree of deacetylation for crustacean chitin extracted from all fermentation setups were above 60%.

On the other hand, the highest degree of deacetylation was achieved for the fungal chitosan extracted from the fermentation using red grape pomace (61.1%), which closely matches the degree of deacetylation for commercial chitosan (64.0%). With the exception of avocado seed, the degree of deacetylation for fungal chitosan extracted averaged above 50%.

Table 17. Degree of Deacetylation of Crustacean Chitin and Fungal Chitosan extracted after *Mucor circinelloides* Fermentation on Various Waste Substrates

Waste Substrate for Fermentation	Degree of Deacetylation (%) of Crustacean Chitin	Degree of Deacetylation (%) of Fungal Chitosan
Commercial chitin	70.46	-

Commercial chitosan	-	64.00
10g of okara	69.94	-
10 g of coffee waste	68.82	57.50
10 g of durian seed	63.06	52.94
10 g of red grape pomace	62.74	61.05
10 g of avocado seed	61.34	42.11

Figure 25. FTIR spectrum of crustacean chitin extracted from *Mucor circinelloides* fermentation using 10 g okara in 100 mL water

Figure 26. FTIR spectrum of crustacean chitin extracted from *Mucor circinelloides* fermentation using 10 g coffee waste in 100 mL water

Figure 27. FTIR spectrum of crustacean chitin extracted from *Mucor circinelloides* fermentation using 10 g durian seed in 100 mL water

Figure 28. FTIR spectrum of crustacean chitin extracted from *Mucor circinelloides* fermentation using 10 g red grape pomace in 100 mL water

Figure 29. FTIR spectrum of crustacean chitin extracted from *Mucor circinelloides* fermentation using 10 g avocado seed in 100 mL water

Figure 30. FTIR spectrum of commercial chitosan (Sigma-Aldrich)

Figure 31. FTIR spectrum of fungal chitosan extracted from *Mucor circinelloides* fermentation using 10 g red grape pomace in 100 mL water

Figure 32. FTIR spectrum of fungal chitosan extracted from *Mucor circinelloides* fermentation using 10 g coffee waste in 100 mL water

Figure 33. FTIR spectrum of fungal chitosan extracted from *Mucor circinelloides* fermentation using 10 g durian seed in 100 mL water

Figure 34. FTIR spectrum of fungal chitosan extracted from *Mucor circinelloides* fermentation using 10 g avocado seed in 100 mL water

The carbon percentage, nitrogen percentage and the calculated carbon nitrogen ratio for the extracted crustacean chitin and fungal chitosan were

recorded in Tables 11 & 12 respectively The carbon / nitrogen ratio values were found to be very close to 6.89, which is the theoretical value for pure chitin, affirming the high quality and purity of the crustacean chitin and fungal chitosan extracted from fermentation.

Table 18. Elemental Analysis of Crustacean Chitin extracted after *Mucor circinelloides* Fermentation on Various Waste Substrates

Waste Substrate for Fermentation	Carbon (%)	Nitrogen (%)	Carbon / Nitrogen Ratio
Commercial Chitin	46.12	6.65	6.94
10 g of okara	33.90	5.37	6.32
10 g of coffee waste	43.09	5.21	8.27
10 g of durian seed	38.77	5.65	6.86
10 g of red grape pomace	43.76	6.85	6.39
10 g of avocado seed	43.97	5.43	8.09

Table 19. Elemental Analysis of Fungal Chitosan extracted after *Mucor circinelloides* Fermentation on Various Waste Substrates

Waste Substrate for Fermentation	Carbon (%)	Nitrogen (%)	Carbon / Nitrogen Ratio
Commercial Chitosan	43.45	7.75	5.61
10 g of coffee waste	34.10	6.67	5.11
10 g of durian seed	46.17	5.03	9.18
10 g of red grape pomace	49.48	6.85	7.23
10 g of avocado seed	43.43	8.46	5.13

A one-way ANOVA was performed to determine whether the differences between group means for the degree of deacetylation of extracted crustacean chitin are statistically significant.

Table 20. One-Way ANOVA for Degree of Deacetylation of Crustacean Chitin extracted after *Mucor circinelloides* Fermentation on Various Waste Substrates

Source	Sum of Squares	Degrees of Freedom	Mean Squares	F-Statistic	P-Value
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Columns	257.196	5	51.4392	554.37	9.355 x 10 ⁻¹⁴
Error	1.113	12	0.0928		
Total	258.309	17			

As the p-value was found to be less than 0.05 significance level, the null hypothesis that all population means are equal is rejected and it is concluded that the differences between some of the means for the degree of deacetylation of extracted crustacean chitin are statistically significant. A multiple comparison test was then used to assess the differences in group means.

Table 21. Multi-Comparison Test for Degree of Deacetylation of Crustacean Chitin extracted after *Mucor circinelloides* Fermentation on Various Carbon Sources

Substrate Compared	Substrate Compared	Lower 95% Confidence	Difference in Means	Upper 95% Confidence	P-Value
Commercial Chitin	10% avocado seed	8.4413	9.2767	10.1121	0.0000
Commercial Chitin	10% okara	-0.1587	0.6767	1.5127	0.1416
Commercial Chitin	10% grape pomace	7.0413	7.8767	8.7121	0.0000
Commercial Chitin	10% coffee waste	0.9613	1.7967	2.6321	0.0001
Commercial Chitin	10% durian seed	6.7213	7.5567	8.3921	0.0000
10% avocado seed	10% okara	-9.4354	-8.6000	-7.7646	0.0000
10% avocado seed	10% grape pomace	-2.2354	-1.4000	-0.5646	0.0012
10% avocado seed	10% coffee waste	-8.3154	-7.4800	-6.6446	0.0000
10% avocado seed	10% durian seed	-2.5554	-1.7200	-0.8846	0.0002
10% okara	10% grape pomace	6.3646	7.2000	8.0354	0.0000
10% okara	10% coffee waste	0.2846	1.1200	1.9554	0.0073

10% okara	10% durian seed	6.0446	6.8800	7.7154	0.0000
10% grape pomace	10% coffee waste	-6.9154	-6.0800	-5.2446	0.0000
10% grape pomace	10% durian seed	-1.1554	-0.3200	0.5154	0.7867
10% coffee waste	10% durian seed	4.9246	5.7600	6.5954	0.0000

As the p-value for most of the comparison of means is less than 0.05, this proves that the group means of the degree of deacetylation for extracted crustacean chitin using fermentation of different waste substrates are significantly different from each other. However there is a notable exception. For example, commercial chitin and 10% okara have a p-value of 0.1416. This shows that fermentation by *Mucor circinelloides* using 10% okara is able to extract crustacean chitin with a degree of deacetylation similar to commercial chitin.

A one-way ANOVA was performed to determine whether the differences between group means for the degree of deacetylation of extracted fungal chitosan are statistically significant.

Table 22. One-Way ANOVA for Degree of Deacetylation of Fungal Chitosan extracted after *Mucor circinelloides* Fermentation on Various Waste Substrates

Source	Sum of Squares	Degrees of Freedom	Mean Squares	F-Statistic	P-Value
Columns	878.689	4	219.672	3473.63	1.154×10^{-15}
Error	0.632	10	0.063		
Total	879.321	14			

As the p-value was found to be less than 0.05 significance level, the

null hypothesis that all population means are equal is rejected and it is concluded that the differences between some of the means for the degree of deacetylation of extracted fungal chitosan are statistically significant. A multiple comparison test was then used to assess the differences in group means.

Table 23. Multi-Comparison Test for Degree of Deacetylation of Fungal Chitosan extracted after *Mucor circinelloides* Fermentation on Various Carbon Sources

Substrate Compared	Substrate Compared	Lower 95% Confidence	Difference in Means	Upper 95% Confidence	P-Value
Commercial Chitosan	10% avocado seed	21.2142	21.8900	22.5658	0
Commercial Chitosan	10% grape pomace	2.2742	2.9500	3.6258	0.0000
Commercial Chitosan	10% coffee waste	5.8242	6.5000	7.1758	0.0000
Commercial Chitosan	10% durian seed	10.3842	11.0600	11.7358	0.0000
10% avocado seed	10% grape pomace	-19.6158	-18.9400	-18.2642	0
10% avocado seed	10% coffee waste	-16.0658	-15.3900	-14.7142	0.0000
10% avocado seed	10% durian seed	-11.5058	-10.8300	-10.1542	0.0000
10% grape pomace	10% coffee waste	2.8742	3.5500	4.2258	0.0000
10% grape pomace	10% durian seed	7.4342	8.1100	8.7858	0.0000
10% coffee waste	10% durian seed	3.8842	4.5600	5.2358	0.0000

As the p-value for all of the comparison of means is less than 0.05, this proves that the group means of the degree of deacetylation for extracted fungal chitosan using fermentation of different waste substrates are significantly different from each other.

A one-way ANOVA was performed to determine whether the differences between group means for the carbon nitrogen ratio of extracted crustacean chitin are statistically significant.

Table 24. One-Way ANOVA for Carbon Nitrogen Ratio of Crustacean Chitin extracted after *Mucor circinelloides* Fermentation on Various Waste Substrates

Source	Sum of Squares	Degrees of Freedom	Mean Squares	F-Statistic	P-Value
Columns	10.6567	5	2.13134	88.72	4.7303 x 10 ⁻⁹
Error	0.2883	12	0.02402		
Total	10.945	17			

As the p-value was found to be less than 0.05 significance level, the null hypothesis that all population means are equal is rejected and it is concluded that the differences between some of the means for the carbon nitrogen ratio of extracted crustacean chitin are statistically significant. A multiple comparison test was then used to assess the differences in group means.

Table 25. Multi-Comparison Test for Carbon Nitrogen Ratio of Crustacean Chitin extracted after *Mucor circinelloides* Fermentation on Various Carbon Sources

Substrate Compared	Substrate Compared	Lower 95% Confidence	Difference in Means	Upper 95% Confidence	P-Value
Commercial Chitin	10% avocado seed	-1.6024	-1.1773	-0.7523	0.0000
Commercial Chitin	10% okara	0.1726	0.5977	1.0227	0.0051
Commercial Chitin	10% grape pomace	0.1026	0.5277	0.9527	0.0127
Commercial Chitin	10% coffee waste	-1.7794	-1.3543	-0.9293	0.0000
Commercial Chitin	10% durian seed	-0.3744	0.0507	0.4757	0.9983

10% avocado seed	10% okara	1.3499	1.7750	2.2001	0.0000
10% avocado seed	10% grape pomace	1.2799	1.7050	2.1301	0.0000
10% avocado seed	10% coffee waste	-0.6021	-0.1770	0.2481	0.7275
10% avocado seed	10% durian seed	0.8029	1.2280	1.6531	0.0000
10% okara	10% grape pomace	-0.4951	-0.0700	0.3551	0.9924
10% okara	10% coffee waste	-2.3771	-1.9520	-1.5269	0.0000
10% okara	10% durian seed	-0.9721	-0.5470	-0.1219	0.0099
10% grape pomace	10% coffee waste	-2.3071	-1.8820	-1.4569	0.0000
10% grape pomace	10% durian seed	-0.9021	-0.4770	-0.0519	0.0250
10% coffee waste	10% durian seed	0.9799	1.4050	1.8301	0.0000

As the p-value for most of the comparison of means is less than 0.05, this proves that the group means of the carbon nitrogen ratio for extracted crustacean chitin using fermentation of different waste substrates are significantly different from each other. However there are a few notable exceptions. For example, commercial chitin and 10% durian seed have a p-value of 0.9983. This shows that fermentation by *Mucor circinelloides* using 10% durian seed is able to extract crustacean chitin with a carbon nitrogen ratio similar to commercial chitin. In addition, 10% okara and 10% grape pomace have a p-value of 0.9924. Both waste substrate fermentations are able to extract crustacean chitin with a carbon nitrogen ratio of less than 6.5, proving effective deproteinization and demineralization.

A one-way ANOVA was performed to determine whether the differences between group means for the carbon nitrogen ratio of extracted fungal chitosan are statistically significant.

Table 26. One-Way ANOVA for Carbon Nitrogen Ratio of Fungal Chitosan extracted after *Mucor circinelloides* Fermentation on Various Waste Substrates

Source	Sum of Squares	Degrees of Freedom	Mean Squares	F-Statistic	P-Value
Columns	36.7989	4	9.19973	421.26	4.267 x 10 ⁻¹¹
Error	0.2184	10	0.02184		
Total	37.0173	14			

As the p-value was found to be less than 0.05 significance level, the null hypothesis that all population means are equal is rejected and it is concluded that the differences between some of the means for the carbon nitrogen ratio of extracted fungal chitosan are statistically significant. A multiple comparison test was then used to assess the differences in group means.

Table 27. Multi-Comparison Test for Carbon Nitrogen Ratio of Fungal Chitosan extracted after *Mucor circinelloides* Fermentation on Various Carbon Sources

Substrate Compared	Substrate Compared	Lower 95% Confidence	Difference in Means	Upper 95% Confidence	P-Value
Commercial Chitosan	10% avocado seed	0.0809	0.4780	0.8751	0.0177
Commercial Chitosan	10% grape pomace	-2.0131	-1.6160	-1.2189	0.0000
Commercial Chitosan	10% coffee waste	0.0989	0.4960	0.8931	0.0141
Commercial Chitosan	10% durian seed	-3.9641	-3.5670	-3.1699	0.0000
10% avocado seed	10% grape pomace	-2.4911	-2.0940	-1.6969	0.0000

10% avocado seed	10% coffee waste	-0.3791	0.0180	0.4151	0.9999
10% avocado seed	10% durian seed	-4.4421	-4.0450	-3.6479	0.0000
10% grape pomace	10% coffee waste	1.7149	2.1120	2.5091	0.0000
10% grape pomace	10% durian seed	-2.3481	-1.9510	-1.5539	0.0000
10% coffee waste	10% durian seed	-4.4601	-4.0630	-3.6659	0.0000

As the p-value for most of the comparison of means is less than 0.05, this proves that the group means of the carbon nitrogen ratio for extracted crustacean chitin using fermentation of different waste substrates are significantly different from each other. However there is a notable exception. For example, 10% avocado seed and 10% coffee waste have a p-value of 0.9999. This shows that both waste substrate fermentations are able to extract fungal chitosan with a carbon nitrogen ratio of less than 6.0, proving effective deproteinization and demineralization.

Linear regression modeling was attempted to find if there was any relationship between the two parameters, degree of deacetylation and carbon nitrogen ratio, for both crustacean chitin and fungal chitosan. No linear correlation was found.

Chapter 5: Comparison of Sustainable Lipid and Protein Extraction Methods for the Extraction of Insect Chitin from Black Soldier Fly

5.1 Introduction

The proliferation of insect farms breeding the Black Soldier Fly (*Hermetia illucens*) in recent years have focused on harnessing insect protein as a source for animal feed [214]. The Black Soldier Fly feeds on organic waste rapidly, converting it into valuable biomass consisting of 30-45% protein and 25-40% fat, with the remaining 10-15% being chitin which makes up the insect exoskeleton [215].

The chitin extraction process for prawn shells and insect exoskeleton differs due to the high lipid content in the insect, necessitating defatting as a preliminary step [216]. This is usually performed by highly toxic chemicals such as hexane and chloroform industrially [217]. Hydrochloric acid is then used for demineralization and sodium hydroxide for deproteinization of the defatted insect [218].

Most published literature have reported that the complex and compact chemical bonds between chitin and protein in the Black Soldier Fly cannot be destroyed by mild solvents to remove proteins and minerals [219]. This is because the chitin and protein are formed in nanofibers which are intertwined and embedded within the minerals and pigment matrix of the Black Soldier Fly [220].

The chitin matrix changes structurally and physiochemically with non-uniform distribution in the insect body as the Black Soldier Fly goes through the developmental stages of larva, pupa and adult [221]. As chitin deposition increases significantly from the stage larva to pupa, the pupae shells of the Black Soldier Fly were selected for chitin extraction [222].

As an alternative to strong chemical methods, mild organic acids and proteolytic enzymes were used for insect chitin extraction in this study [223]. Defatting by supercritical carbon dioxide followed by fermentation by microbial strains was also attempted [224]. The degree of deacetylation was then determined by Fourier Transform Infrared Spectroscopy (FTIR) and the degree of crystallinity was determined by X-ray diffraction (XRD) [225]. The carbon nitrogen ratio was also measured by elemental analysis to ascertain the purity of the crude extracted insect chitin [226].

5.2 Materials and Methods

4 insect chitin extraction methods were carried out.

1st method: 50 mL of 70% ethanol, 50 mL of pure acetic acid and 50 mL of pure lactic acid were added to 5 g of Black Soldier Fly pupae shells for defatting and demineralization. The suspension was stirred at room temperature on a hot plate using a magnetic stir bar for 72 hours. The defatted Black Soldier Fly pupae shells were then removed from the solution, washed 3 times with deionized water and transferred into 150 mL of 10 mM sodium phosphate buffer (1.64 g sodium phosphate dibasic heptahydrate,

0.47 g sodium phosphate monobasic and 8.77 g sodium chloride in 1 L deionized water).

Next, 150 µL of purified protease from *Bacillus licheniformis* (Sigma-Aldrich) was added to the suspension for deproteinization. The suspension was then stirred at 37 °C using a magnetic stir bar for another 72 hours before the final solid residue was removed, washed 3 times with deionized water and dried in a vacuum oven at 60°C for 48 hours.

2nd method: Deproteinization was carried out first before defatting and demineralization by swapping the order of the steps indicated in the 1st method.

3rd method: Supercritical carbon dioxide extraction was performed on 5 g of Black Soldier Fly pupae shells for 2 hours at 65 °C and 5400 psi at a flow rate of 10 mL CO₂ per second to defat the insect. The defatted Black Soldier Fly pupae shells were then fermented in 100 mL of 20% glucose solution using the microbial strains *Lactobacillus plantarum* (lactic acid producing strain), *Bacillus subtilis* (protease producing strain) and *Pseudomonas fluorescens* ATCC 13525 (lipase producing strain) at 30 °C, 200 rpm for 5 days. The solid residue was then removed from the fermentation, washed 3 times with deionized water and dried in a vacuum oven at 60 °C for 48 hours.

4th method: 5 g of Black Soldier Fly pupae shells were soaked in a 150 mL solution consisting of 50 mL of pure acetic acid and 100 mL of 70%

ethanol for 24 hours as an alternative procedure to supercritical carbon dioxide extraction. Similar fermentation steps were then carried out using *Lactobacillus plantarum*, *Bacillus subtilis* and *Pseudomonas fluorescens* in 20% glucose solution at 30 °C, 200 rpm for 5 days.

The FTIR and EA and XRD procedures used were similar to section 2.2 Materials and Methods. However, for the calculation of the degree of crystallinity with respect to insect chitin, I_{110} was measured at the sharp peak at 19° and I_{am} was measured at the amorphous region at 13°.

5.3 Results and Discussion

The dry weights of the extracted insect chitin were recorded in Table 13 and their yields calculated. A surprisingly high yield of 70% was achieved.

Table 28. Dry Weights of Chitin Extracted from Black Soldier Fly Pupae Shells

Extraction Method	Dry Weight (g)	Yield (%)
Acetic acid (50 mL), lactic acid (50 mL), 70% ethanol (50 mL) for 3 days; protease from <i>Bacillus licheniformis</i> (150 µL) for 3 days	3.47	69.4
Protease from <i>Bacillus licheniformis</i> (150 µL) for 3 days; acetic acid (50 mL), lactic acid (50 mL), 70% ethanol (50 mL) for 3 days	3.27	65.4
Supercritical carbon dioxide extraction (65°C, 2 hours, 5400 psi); fermentation with <i>Lactobacillus plantarum</i> , <i>Bacillus subtilis</i> and <i>Pseudomonas fluorescens</i> (5 days)	3.49	69.8
Acetic acid (50mL), 70% ethanol (100mL) (1 day), Fermentation with <i>Lactobacillus plantarum</i> , <i>Bacillus subtilis</i> and <i>Pseudomonas fluorescens</i> (5 days)	3.62	72.4

The degree of deacetylation was calculated from taking the ratio of the peaks at 3450 cm⁻¹ and 1650 cm⁻¹ in the FTIR spectrum and recorded in Table 14. The highest degree of deacetylation (76.6%) was achieved when the Black Soldier Fly was extracted with strong acids followed by proteolytic enzymes. Fermentation methods were less effective and a lower degree of deacetylation (60.3%) of the extracted insect chitin was achieved.

Table 29. Degree of Deacetylation of Chitin extracted from Black Soldier Fly Pupae Shells

Extraction Method	Degree of Deacetylation (%)
Commercial Chitin	70.46
Acetic acid (50 mL), lactic acid (50 mL), 70% ethanol (50 mL) for 3 days; protease from <i>Bacillus licheniformis</i> (150 µL) for 3 days	76.58
Protease from <i>Bacillus licheniformis</i> (150 µL) for 3 days; acetic acid (50 mL), lactic acid (50 mL), 70% ethanol (50 mL) for 3 days	67.93
Supercritical carbon dioxide extraction (65°C, 2 hours, 5400 psi); fermentation with <i>Lactobacillus plantarum</i> , <i>Bacillus subtilis</i> and <i>Pseudomonas fluorescens</i> (5 days)	60.33
Acetic acid (50mL), 70% ethanol (100mL) (1 day), Fermentation with <i>Lactobacillus plantarum</i> , <i>Bacillus subtilis</i> and <i>Pseudomonas fluorescens</i> (5 days)	54.56

Figure 35. FTIR spectrum of insect chitin extracted using 50 mL of acetic acid, 50 mL of lactic acid, 50 mL of 70% ethanol followed by 150 µL of protease from *Bacillus licheniformis*

Figure 36. FTIR spectrum of insect chitin extracted using 150 µL of protease from *Bacillus licheniformis*, followed by 50 mL of acetic acid, 50 mL of lactic acid, 50 mL of 70% ethanol

Figure 37. FTIR spectrum of insect chitin extracted using supercritical carbon dioxide extraction (5400 psi, 65 °C, 2 hours) followed by fermentation with *Lactobacillus plantarum*, *Bacillus subtilis* and *Pseudomonas fluorescens* in 20% glucose

Figure 38. FTIR spectrum of insect chitin extracted using 50 mL of acetic acid and 100 mL of 70% ethanol, followed by fermentation with *Lactobacillus plantarum*, *Bacillus subtilis* and *Pseudomonas fluorescens* in 20% glucose

The carbon percentage and nitrogen percentage for the extracted

insect chitin were measured using elemental analysis and the calculated carbon nitrogen ratio values were recorded in Table 15. The carbon nitrogen ratio for all the extracted insect chitin was found to be around 6.89, which matches the theoretical value of pure chitin. This indicates that the extracted insect chitin samples were very high in purity.

Table 30. Elemental Analysis of Chitin Extracted from Black Soldier Fly Pupae Shells

Extraction Method	Carbon (%)	Nitrogen (%)	Carbon / Nitrogen Ratio
Commercial Chitin	46.12	6.65	6.94
Acetic acid (50 mL), lactic acid (50 mL), 70% ethanol (50 mL) for 3 days; protease from <i>Bacillus licheniformis</i> (150 µL) for 3 days	43.37	8.39	5.17
Protease from <i>Bacillus licheniformis</i> (150 µL) for 3 days; acetic acid (50 mL), lactic acid (50 mL), 70% ethanol (50 mL) for 3 days	50.25	6.01	8.36
Supercritical carbon dioxide extraction (65°C, 2 hours, 5400 psi); fermentation with <i>Lactobacillus plantarum</i> , <i>Bacillus subtilis</i> and <i>Pseudomonas fluorescens</i> (5 days)	48.10	9.45	5.09
Acetic acid (50mL), 70% ethanol (100mL) (1 day), Fermentation with <i>Lactobacillus plantarum</i> , <i>Bacillus subtilis</i> and <i>Pseudomonas fluorescens</i> (5 days)	44.12	7.13	6.19

The degree of crystallinity for the extracted insect chitin was calculated by taking the ratio of the sharp peak at 19° and the amorphous region at 13° in the XRD spectrum. Results were tabulated in Table 16. It was found that all the extracted insect chitin samples showed an extremely high degree of crystallinity above 85%.

Table 31. Degree of Crystallinity of Chitin extracted from Black Soldier Fly Pupae Shells

Extraction Method	Degree of Crystallinity (%)
Commercial Chitin	87.56
Acetic acid (50 mL), lactic acid (50 mL), 70% ethanol (50 mL) for 3 days; protease from <i>Bacillus licheniformis</i> (150 μ L) for 3 days	88.98
Protease from <i>Bacillus licheniformis</i> (150 μ L) for 3 days; acetic acid (50 mL), lactic acid (50 mL), 70% ethanol (50 mL) for 3 days	89.95
Supercritical carbon dioxide extraction (65°C, 2 hours, 5400 psi); fermentation with <i>Lactobacillus plantarum</i> , <i>Bacillus subtilis</i> and <i>Pseudomonas fluorescens</i> (5 days)	98.48
Acetic acid (50mL), 70% ethanol (100mL) (1 day), Fermentation with <i>Lactobacillus plantarum</i> , <i>Bacillus subtilis</i> and <i>Pseudomonas fluorescens</i> (5 days)	85.40

Figure 39. XRD spectrum of insect chitin extracted using 50 mL of acetic acid, 50 mL of lactic acid, 50 mL of 70% ethanol followed by 150 μ L of protease from *Bacillus licheniformis*

Figure 40. XRD spectrum of insect chitin extracted using 150 μL of protease from *Bacillus licheniformis*, followed by 50 mL of acetic acid, 50 mL of lactic acid, 50 mL of 70% ethanol

Figure 41. XRD spectrum of insect chitin extracted using supercritical carbon dioxide extraction (5400 psi, 65 $^{\circ}\text{C}$, 2 hours) followed by fermentation with *Lactobacillus plantarum*, *Bacillus subtilis* and *Pseudomonas fluorescens* in 20% glucose

Figure 42. XRD spectrum of insect chitin extracted using 50 mL of acetic acid and 100 mL of 70% ethanol, followed by fermentation with *Lactobacillus plantarum*, *Bacillus subtilis* and *Pseudomonas fluorescens* in 20% glucose

A one-way ANOVA was performed to determine whether the differences between group means for the degree of deacetylation of extracted insect chitin are statistically significant.

Table 32. One-Way ANOVA for Degree of Deacetylation of Chitin extracted from Black Soldier Fly Pupae Shells

Source	Sum of Squares	Degrees of Freedom	Mean Squares	F-Statistic	P-Value
Columns	899.992	4	224.998	1158.43	2.774×10^{-13}
Error	1.942	10	0.194		
Total	901.934	14			

As the p-value was found to be less than 0.05 significance level, the null hypothesis that all population means are equal is rejected and it is concluded that the differences between some of the means for the degree of

deacetylation of extracted insect chitin are statistically significant. A multiple comparison test was then used to assess the differences in group means.

Table 33. Multi-Comparison Test for Degree of Deacetylation of Chitin extracted from Black Soldier Fly Pupae Shells

Extraction Method	Extraction Method	Lower 95% Confidence	Difference in Means	Upper 95% Confidence	P-Value
Commercial Chitin	Acetic & Lactic Acid, Ethanol, <i>Bacillus</i> protease	-7.1476	-5.9633	-4.7791	0.0000
Commercial Chitin	<i>Bacillus</i> protease, Acetic & Lactic Acid, Ethanol	1.5024	2.6867	3.8709	0.0002
Commercial Chitin	Supercritical CO ₂ , <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	9.1024	10.2867	11.4709	0.0000
Commercial Chitin	Acetic Acid, Ethanol, <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	14.8724	16.0567	17.2409	0.0000
Acetic & Lactic Acid, Ethanol, <i>Bacillus</i> protease	<i>Bacillus</i> protease, Acetic & Lactic Acid, Ethanol	7.4657	8.6500	9.8343	0.0000
Acetic & Lactic Acid, Ethanol, <i>Bacillus</i> protease	Supercritical CO ₂ , <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	15.0657	16.2500	17.4343	0.0000

Acetic & Lactic Acid, Ethanol, <i>Bacillus</i> protease	Acetic Acid, Ethanol, <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	20.8357	22.0200	23.2043	0.0000
<i>Bacillus</i> protease, Acetic & Lactic Acid, Ethanol	Supercritical CO ₂ , <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	6.4157	7.6000	8.7843	0.0000
<i>Bacillus</i> protease, Acetic & Lactic Acid, Ethanol	Acetic Acid, Ethanol, <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	12.1857	13.3700	14.5543	0.0000
Supercritical CO ₂ , <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	Acetic Acid, Ethanol, <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	4.5857	5.7700	6.9543	0.0000

As the p-value for all of the comparison of means is less than 0.05, this proves that the group means of the degree of deacetylation for extracted insect chitin using different extraction methods are significantly different from each other.

A one-way ANOVA was performed to determine whether the differences between group means for the carbon nitrogen ratio of extracted insect chitin are statistically significant.

Table 34. One-Way ANOVA for Carbon Nitrogen Ratio of Chitin extracted from Black Soldier Fly Pupae Shells

Source	Sum of Squares	Degrees of Freedom	Mean Squares	F-Statistic	P-Value
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Columns	22.0933	4	5.52332	5854.28	8.500 x 10 ⁻¹⁷
Error	0.0094	10	0.00094		
Total	22.1027	14			

As the p-value was found to be less than 0.05 significance level, the null hypothesis that all population means are equal is rejected and it is concluded that the differences between some of the means for the carbon nitrogen ratio of extracted insect chitin are statistically significant. A multiple comparison test was then used to assess the differences in group means.

Table 35. Multi-Comparison Test for Carbon Nitrogen Ratio of Chitin extracted from Black Soldier Fly Pupae Shells

Extraction Method	Extraction Method	Lower 95% Confidence	Difference in Means	Upper 95% Confidence	P-Value
Commercial Chitin	Acetic & Lactic Acid, Ethanol, <i>Bacillus</i> protease	1.6621	1.7447	1.8272	0.0000
Commercial Chitin	<i>Bacillus</i> protease, Acetic & Lactic Acid, Ethanol	-1.5279	-1.4453	-1.3628	0.0000
Commercial Chitin	Supercritical CO ₂ , <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	1.7421	1.8247	1.9072	0.0000
Commercial Chitin	Acetic Acid, Ethanol, <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	0.6421	0.7247	0.8072	0.0000

Acetic & Lactic Acid, Ethanol, <i>Bacillus</i> protease	<i>Bacillus</i> protease, Acetic & Lactic Acid, Ethanol	-3.2725	-3.1900	-3.1075	0
Acetic & Lactic Acid, Ethanol, <i>Bacillus</i> protease	Supercritical CO ₂ , <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	-0.0025	0.0800	0.1625	0.0585
Acetic & Lactic Acid, Ethanol, <i>Bacillus</i> protease	Acetic Acid, Ethanol, <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	-1.1025	-1.0200	-0.9375	0.0000
<i>Bacillus</i> protease, Acetic & Lactic Acid, Ethanol	Supercritical CO ₂ , <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	3.1875	3.2700	3.3525	0
<i>Bacillus</i> protease, Acetic & Lactic Acid, Ethanol	Acetic Acid, Ethanol, <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	2.0875	2.1700	2.2525	0
Supercritical CO ₂ , <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	Acetic Acid, Ethanol, <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	-1.1825	-1.1000	-1.0175	0.0000

As the p-value for most of the comparison of means is less than 0.05, this proves that the group means of the carbon nitrogen ratio for extracted insect chitin using different extraction methods are significantly different from each other. However there is a notable exception. For example,

demineralization using acetic acid, lactic acid and ethanol followed by deproteinization using *Bacillus* protease have a p-value of 0.0585 when compared with supercritical carbon dioxide extraction followed by fermentation using *Lactobacillus* sp., *Bacillus* sp. and *Pseudomonas* sp. This shows that both extraction methods are able to extract insect chitin with a carbon nitrogen ratio of less than 6.0, proving effective deproteinization and demineralization.

A one-way ANOVA was performed to determine whether the differences between group means for the degree of crystallinity of extracted insect chitin are statistically significant.

Table 36. One-Way ANOVA for Degree of Crystallinity of Chitin extracted from Black Soldier Fly Pupae Shells

Source	Sum of Squares	Degrees of Freedom	Mean Squares	F-Statistic	P-Value
Columns	305.692	4	76.423	228.17	8.891 x 10 ⁻¹⁰
Error	3.349	10	0.3349		
Total	309.041	14			

As the p-value was found to be less than 0.05 significance level, the null hypothesis that all population means are equal is rejected and it is concluded that the differences between some of the means for the degree of crystallinity of extracted insect chitin are statistically significant. A multiple comparison test was then used to assess the differences in group means.

Table 37. Multi-Comparison Test for Degree of Crystallinity of Chitin extracted from Black Soldier Fly Pupae Shells

Extraction Method	Extraction Method	Lower 95% Confidence	Difference in Means	Upper 95% Confidence	P-Value
Commercial Chitin	Acetic & Lactic Acid, Ethanol, <i>Bacillus</i> protease	-3.3252	-1.7700	0.2148	0.0247
Commercial Chitin	<i>Bacillus</i> protease, Acetic & Lactic Acid, Ethanol	-4.2952	-2.7400	-1.1848	0.0013
Commercial Chitin	Supercritical CO ₂ , <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	-12.8252	-11.2700	-9.7148	0.0000
Commercial Chitin	Acetic Acid, Ethanol, <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	0.2548	1.8100	3.3652	0.0217
Acetic & Lactic Acid, Ethanol, <i>Bacillus</i> protease	<i>Bacillus</i> protease, Acetic & Lactic Acid, Ethanol	-2.5252	-0.9700	0.5852	0.3098
Acetic & Lactic Acid, Ethanol, <i>Bacillus</i> protease	Supercritical CO ₂ , <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	-11.0552	-9.5000	-7.9448	0.0000
Acetic & Lactic Acid, Ethanol, <i>Bacillus</i> protease	Acetic Acid, Ethanol, <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	2.0248	3.5800	5.1352	0.0001
<i>Bacillus</i> protease, Acetic &	Supercritical CO ₂ , <i>Lactobacillus</i> ,	-10.0852	-8.5300	-6.9748	0.0000

Lactic Acid, Ethanol	<i>Bacillus</i> , <i>Pseudomonas</i>				
<i>Bacillus</i> protease, Acetic & Lactic Acid, Ethanol	Acetic Acid, Ethanol, <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	2.9948	4.5500	6.1052	0.0000
Supercritical CO ₂ , <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	Acetic Acid, Ethanol, <i>Lactobacillus</i> , <i>Bacillus</i> , <i>Pseudomonas</i>	11.5248	13.0800	14.6352	0.0000

As the p-value for most of the comparison of means is less than 0.05, this proves that the group means of the degree of crystallinity for extracted insect chitin using different extraction methods are significantly different from each other. However there is a notable exception. For example, demineralization using acetic acid, lactic acid and ethanol followed by deproteinization using *Bacillus* protease have a p-value of 0.03098 when compared with deproteinization using *Bacillus* protease followed by demineralization using acetic acid, lactic acid and ethanol. This shows that both extraction methods are able to extract insect chitin with a degree of crystallinity of around 90%, proving that the structure of insect chitin extracted remains intact regardless of the order of extraction.

Linear regression modeling was attempted to find if there was any relationship between the three parameters, degree of deacetylation, carbon nitrogen ratio and degree of crystallinity for extracted insect chitin. No linear

correlation was found between the parameters.

Chapter 6: Limitations of Study

6.1 Determination of the Degree of Deacetylation of Extracted Chitin Samples

It is a challenge to characterize the chitin accurately and quantify its degree of deacetylation. Fourier-Transform Infrared Spectroscopy (FTIR) is the most commonly used method, but insufficient accuracy is present at high acetylation degrees. In addition, the interpretation of the FTIR spectrum and subsequent calculation of the deacetylation degree varies between research teams and the results are also affected by the presence of impurities.

Two formulas, one using FTIR and the other using carbon nitrogen (C/N) analysis have been published in literature.

$$\text{FTIR Degree of deacetylation} = \frac{\text{area } 1500-1700 \text{ nm}}{\text{area } 2700-3600 \text{ nm}} \times \frac{100}{1.33}$$

$$\text{C/N Degree of deacetylation} = \frac{\frac{C}{N} - 5.14}{1.72} \times 100$$

The degree of deacetylation result was found to be more than 100% when using C/N analysis, hence this method was rejected as a value greater than 100% suggested impure chitin samples. On the other hand, FTIR analysis of commercial chitin from Sigma Aldrich showed a 70% degree of deacetylation, which matches the supplier's specifications.

The value of the FTIR peak was found to be most consistent as a benchmark at 1650 nm (amide peak) and 3450 nm (hydroxyl peak), hence the

ratio of the magnitude of the 2 peaks were used for calculation of the degree of deacetylation in chitin. The area of the peak was easily affected by the water content remaining in the chitin samples and was found to be less accurate as a form of measurement, hence area integration under the peak was not performed.

6.2 Determination of Carbon percentage and Nitrogen Percentage in Extracted Chitin Samples

From elemental analysis, a nitrogen percentage of 6.65% and a carbon nitrogen ratio of 6.94 are obtained for commercial chitin. The crude extracted chitin samples from different fermentations showed a nitrogen percentage ranging from 4% to 8% and a carbon nitrogen ratio ranging from 5.0 to 8.0. The percentage level of nitrogen in chitin indicates its level of purity and 6.89% is the theoretical value for fully acetylated pure chitin.

A lower amount of nitrogen below the theoretical value of 6.89% nitrogen shows a minimum residual protein remaining in the chitin, which proves effective deproteinization. On the other hand, for nitrogen values above 6.89%, this suggests there are slight protein remnants in the chitin samples, or a less effective deproteinization.

However, as a degree of deacetylation greater than 100% is obtained by using the carbon nitrogen ratio of 6.94 in commercial chitin, this suggests inorganic material may be left in the chitin sample. An alternative explanation in literature suggests the presence of inorganic material in the

chitin samples if nitrogen percentage values are below 6.89%.

6.3 Metabolic Pathways in Prawn Shell Waste Supernatant

To increase the concentration of metabolites for detection, the supernatant was shaken with 4 times the volume of ice cold methanol. In other words, if 50 μL of supernatant was extracted for analysis, it was shaken with 200 μL of ice cold methanol before being left aside in the fumehood to allow for the evaporation of methanol. This was found to lead to a higher detection of metabolites by the GC-MS.

The metabolites detected were found to be primarily from the pentose phosphate pathway, pentose and glucuronate interconversions and galactose metabolism. The pathway is displayed in Figure 43.

Figure 43. Pentose Phosphate Pathway [227]

An overall three fold increase in the concentration of metabolites was detected between Days 3-5 compared to Days 1-2. However the data was not presented due to inconsistent fluctuations as the metabolites did not increase linearly across the days of fermentation.

Only 1% of the initial 20% glucose solution (2010.6 mg/L) remained as pure glucose on Day 5 of the fermentation. However, 42.6% of the initial 20% glucose solution (85251.8 mg/L) was detected as sugar derivatives, implying that close to 60% of the glucose solution was consumed by the microbial strains.

6.4 Differences in Extracted Chitin Samples from Prawn, Fungal

and Insect Origin

Crustaceans have remained the traditional and main source of chitin due to the ease of handling. The extracted chitin from the fermentation of prawn shell waste shows a high degree of deacetylation (70% and above), a low carbon nitrogen ratio (6.80 and lower) and a high degree of crystallinity (90% and above).

In comparison, chitosan extracted from the cell walls of the fungus had a much lower degree of deacetylation (60% and below). While carbon nitrogen ratio was lower (6.0 and below) because of the lack of calcium carbonate that is present in prawn shells, it was found to be not crystalline from the X-ray diffraction spectrum.

Chitin extracted from the Black Soldier Fly performed the worst with a degree of deacetylation (40% to 50%) when fermentation was carried out directly without defatting by supercritical carbon dioxide extraction. After defatting by supercritical fluid extraction followed by fermentation, the degree of deacetylation increased to 60%. While the carbon nitrogen ratio was similar (6.0 and below) and the degree of crystallinity was high (85% and above), it remains more challenging to use insects as a chitin source due to their high content of lipids.

6.5 Future Work

The fermentation experiments described in this thesis have been performed at laboratory scale level, and should be scaled up to bioreactor

volumes to again determine the cost effectiveness and technical feasibility of the suggested procedures.

To improve demineralization and deproteinization efficiencies, more *Lactobacillus* sp. and *Bacillus* sp. microbial strains can be explored. A few possibilities include *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus helveticus*, *Bacillus coagulans* and *Bacillus cereus*.

Commercial proteases such as papain, trypase, alkaline protease, proteinase K, neutral protease and protemax may also be used to improve protein hydrolyzation [228].

New methodologies for the extraction of chitin from prawn shell waste include the usage of hot water for deproteinization and carbonic acid for demineralization, which proved to produce high quality and cost-effective chitin [229]. Reusing deep eutectic solvents for repeated extraction of chitin in prawn shell valorization have also been proposed as a replacement for the traditional acid alkali method [230]. Ultrasound has been suggested to enhance deproteinization and increase crystallinity in extracted chitin [231].

Protein-rich effluents from the prawn shell waste fermentation can be used as a nitrogen source for culture media of lactic acid bacteria for the production of bacteriocins [232]. The extraction of astaxanthin from the fermentation effluent using ethyl acetate may be considered as astaxanthin is a commercial food colouring agent as well as a feed additive for the aquaculture and poultry industry [233]. Production of polyhydroxyalkanoates using the fermentation mixed culture media is also possible as the wastewater

can be fermented into volatile fatty acids which are stored in the cells of micro-organisms by performing sequencing batch reactors [234].

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