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Research Article

Valorizing Shrimp Shell as a Source of Chitin with Crude Enzymes from Germinated Winter Wheat and Buckwheat

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Abstract: Chitin is a valuable resource found in shrimp shells. Typically, the industrial process for isolating chitin from crustacean shells involves using chemical methods that require large amounts of acid and alkaline. While commercial protease application is a more environmentally friendly method, it can be costly. Germinated grains can be a source of low-cost protease for chitin isolation. This study aimed to assess the effectiveness of crude enzymes extracted from germinated winter wheat (*Triticum aestivum*) and buckwheat (*Fagopyrum esculentum)* to isolate crude chitin from white shrimp shells. The proteolytic and chitinolytic activities of the crude enzyme extracts were determined and applied in the deproteinization step during chitin isolation from shrimp shells. The deproteinization rate was assessed, the crude chitin yield was compared, and the isolated chitins were characterized through Fourier Transform Infrared (FTIR) spectroscopy analysis and x-ray diffraction (XRD) analysis. Crude enzymes extracted from germinated winter wheat and buckwheat exhibited proteolytic activity of approximately 0.49 U/mL and 0.46 U/mL, respectively. Using winter wheat extract and buckwheat extract acted on demineralized shrimp shells exerted a deproteinization rate of 53.6 ± 1.0% and 58.0 ± 2.3% and yielded 30.3 ± 5.31% and 29.2 ± 3.99% crude chitin, respectively. FTIR spectra of the isolated chitins showed amide I, amide II, amide III, asymmetric stretching of the C-O-C bridge, O-H stretching, N-H stretching and asymmetric C-H stretching, typical functional groups for chitin. The crystallinity index for commercial chitin, chemical-isolated chitin, winter wheat extract-isolated chitin and buckwheat extract-isolated chitin was 86.49%, 88.74%, 88.82% and 75.87%, respectively. Buckwheat extract-isolated chitin, deacetylated with lower crystallinity, warrants further investigation. Crude enzymes from winter wheat and buckwheat to remove protein from demineralized shrimp shells can be a more environmentally friendly method to valorize shrimp shells as a chitin source.

***Keywords*:** buckwheat, chitin, enzymes, germinated, shrimp shell, winter wheat

1. Introduction

Shrimp processing contributes approximately 48 to 56% of waste. Given global farm shrimp production contributing 4 million tons, which is believed to continue expanding in the future [1], valorizing shrimp shells can be a feasible option to mitigate the negative environmental impact of the shrimp farming and processing industries. Shrimp shell wastes contain 15-40% chitin [2]. Chitin is a long-chain polymer of *N*-acetyl-$_D$-glucosamine (GlcNAc). Chitin's

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biodegradable, non-toxic and biocompatible properties widen its applications in food, agriculture, wastewater treatment plants, biomedical, cosmetics, and textiles [3].

Typical steps in chitin isolation from shrimp shell wastes are grinding, demineralizing with a strong acid, and deproteinizing (protein removal) with a strong alkali. The extreme conditions of strong acids and alkalis can alter the quality of the chitin extracted. The chemical isolation process also generates a lot of chemical waste that could pollute the environment. The harmful chemicals might remain in the final products, contaminating the extracted chitin. Biological/microbial techniques may be used to reduce chemical wastes from chitin isolation. Some researchers have demonstrated the feasibility of using commercial protease to replace chemicals in the deproteinization step [4]. However, the high cost of commercial protease, up to 9,500 USD/metric ton [5], is a barrier for the stakeholders who intend to use it on a large scale.

According to Troncoso et al. [6], plant proteases have been found to perform as well as animal or microbial proteases. The current market for protease enzymes is over 3 billion USD, and it is expected to have a compound annual growth rate of 6.1% by 2024. In addition to fruits like pineapple and papaya, cereal grains can also be a great source of plant protease. In our previous research, germinating winter wheat showed significant carbohydrase activities that led to changes in nutrient profile [7]. Germination is a well-known traditional method to improve the nutritional quality of cereal and legumes through anti-nutrient removal and macronutrient mobilization. We hypothesized that selected germinated grains produce proteases that are sufficient to remove proteins from demineralized shrimp shells for crude chitin isolation. The recent commodity prices for hard red winter wheat and common buckwheat are 346 USD/metric ton [8] and 954 USD/ton [9]. These cereal grains can serve as a low-cost source of protease. Dojczew and Sobczyk [10] reported that sprouted wheat grains possessed significant proteolytic activity, but chitinase activity was also detected in germinating winter wheat [11]. The seed chitinase may digest chitin into its monomer by hydrolyzing the β-1,4-linkages.

Nevertheless, the effect of the seed chitinase on chitin embedded within shrimp shells before isolation is yet to be revealed. This study aimed to assess the effectiveness of crude enzymes extracted from germinated winter wheat (*Triticum aestivum*) and buckwheat (*Fagopyrum esculentum)* to isolate crude chitin from white shrimp shells. We determined proteolytic and chitinolytic activities in crude enzymes extracted from germinated winter wheat (*Triticum aestivum)* and buckwheat (*Fagopyrum esculentum*). The crude enzyme extract was applied separately in the deproteinization step for isolating chitin from shrimp shells. The biochemical-isolated chitin was characterized and compared with those of the chemical-isolated chitin. This study's finding could prove whether germinating winter wheat and germinating buckwheat can be alternative sources of crude protease for isolating chitin from shrimp shells in an environment-friendly manner.

2. Materials and methods

2.1 *Sample*

Winter wheat (*Triticum Aestivum*) grains of hard red variety with the brand of BMS were purchased from an organic shop at Ipoh, Perak, Malaysia. Common buckwheat (*Fagopyrum esculentum* Moench) grains with the brand of CED were purchased from Lotus hypermarket in Kampar, Malaysia. The shrimp shells were peeled from white shrimps (a mixture of *Parapenaeopsis hardwickii* and *Metapenaeus affinis*) purchased from a wet market at Kampar, Perak. All the experiments were conducted from July 2019 to February 2020 at the Faculty of Science, Universiti Tunku Abdul Rahman, Malaysia. Figure 1 illustrates an overview of the experiments performed.

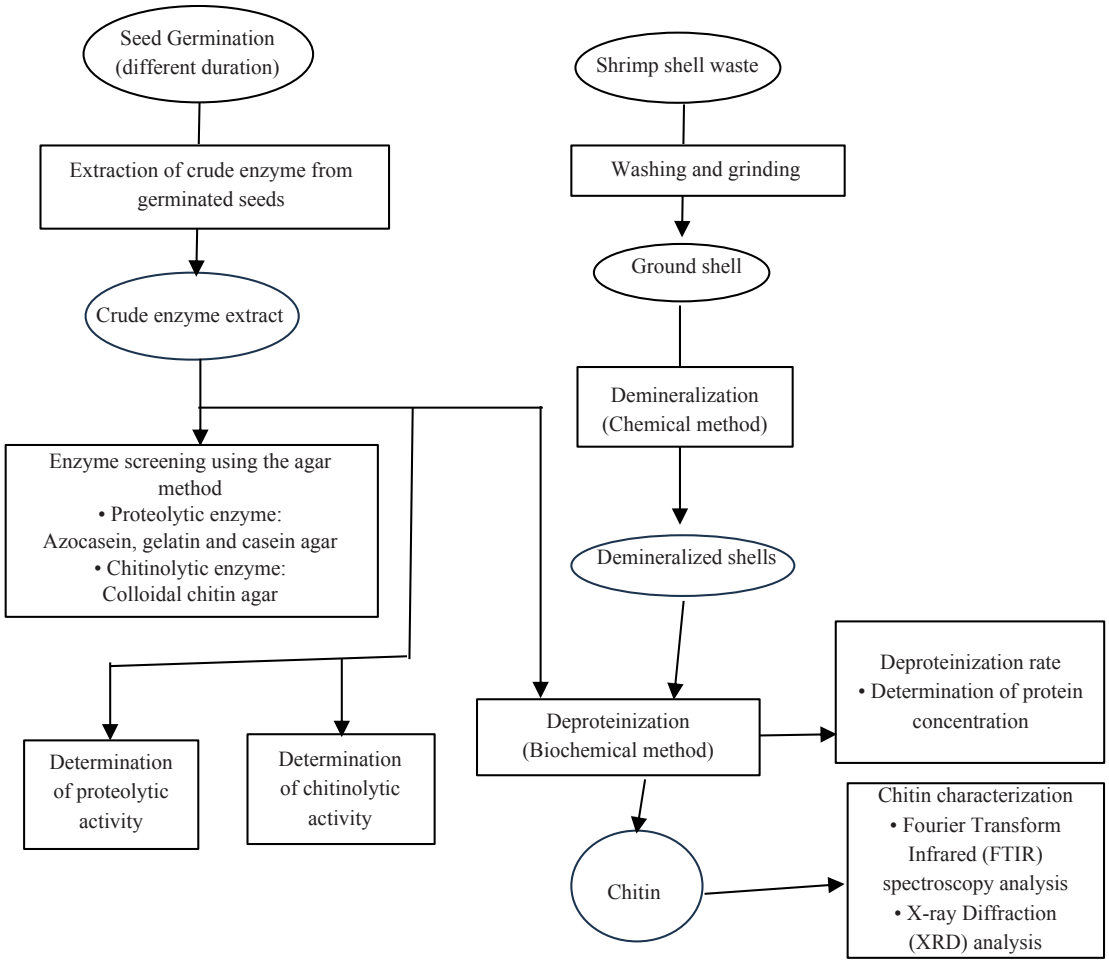

Figure 1. An overview of experiments conducted

2.2 *Seed germination*

Seed germination was done as described by Ling and Chang [7]. Selected pre-washed winter wheat or buckwheat grains were soaked into autoclaved distilled water at ~25 °C for 8 h, surface-sterilized with 1% sodium hypochlorite solution, and rinsed with autoclaved distilled water. Approximately 80 grains were placed on a layer of wet cheesecloth in a 9 cm-diameter petri dish. Germination of seed progressed in a dark environment at ~25 °C for a maximum duration of 5 days or 6 days for winter wheat and buckwheat, respectively, before the shoots turned to stem and leaves. Approximately 5 mL of autoclaved distilled water was sprayed on the seed once per day. Then, the germinated seeds were collected and stored in a freezer at -18 °C before further treatment.

2.3 *Extraction of crude enzymes from germinated seeds*

The extraction of crude enzymes from the germinated seed was adapted from Chaiwut et al. [12]. The germinated seeds were mashed and homogenized with sodium phosphate buffer (0.1 M, pH 7.0) at a solid-to-liquid ratio (w/v) of 1:5. The mixture was then centrifuged at 10,000 × *g*, at 4 °C for 15 min using a Sigma 3-18 KS centrifuge (Sigma Laborzentrifugen GmbH, Osterode, Germany) to obtain the crude enzyme extract which was stored at 4 °C for not more than 5 days before further analysis.

2.3.1 *Screening of proteolytic activities*

Gelatin agar, casein agar, and azocasein agar were used to screen protease in the crude extract [13]. Gelatin agar and casein agar were prepared with a combination of 2% agar and 1% substrate. Azocasein agar was prepared by mixing agar: 2.5% azocasein: distilled water at a ratio of 1:10:40. The agar mixtures were autoclaved before pouring into 9 cm-diameter petri dishes. Then, three holes were made on each solidified agar plate with a 6 mm-diameter-cork borer. One hundred microliters of sodium phosphate buffer (0.1 M) (the negative control), commercial pancreatin (2 mg/mL) (the positive control) and crude enzyme extracts were separately pipetted into each hole and incubated overnight at 37 °C. Gelatin agar and casein agar required specific staining to detect proteolytic activity. The agar was stained with 10 mL of coomassie blue (0.25%, w/v) in a mixture of acetone, methanol, and distilled water at a ratio of 1:5:4 (v/v/v) for 15 min. Then, it was de-stained with 5 mL of a mixture containing methanol, acetone and distilled water at a ratio of 20:66:114 (v/v/v) for 10 min and rinsed with autoclaved distilled water twice.

2.3.2 *Screening of chitinolytic activities*

Colloidal chitin agar was used to detect chitinase in the crude enzyme extracts. Colloidal chitin agar was prepared with 2% agar and 1% colloidal chitin substrate. The colloidal chitin substrate was prepared based on the method used by Kabir et al. [14]. The subsequent steps in preparing the agar, forming radial diffusion well, adding crude enzymes and controls, incubating the agar plate, staining and de-staining were done using the same procedure as gelatin agar and casein agar. Any clear zone formed on each agar indicates the presence of relevant enzymes. The diameter of the clear zone was measured using a ruler to the nearest 0.5 mm.

2.3.3 *Determination of proteolytic activity*

The proteolytic activity of the crude enzyme extract was determined using azocasein as the substrate [15]. Azocasein was weighed and dissolved in 1 part of ethanol (95%) and 24 parts of sodium phosphate buffer (0.1 M, pH 7.0) to form a 2.5% solution. Approximately 250 µL crude winter wheat extract was added into 2.5% azocasein at a ratio of 1:1, mixed well by inversion, then incubated at 37 °C for an hour. Approximately 3 volumes of trichloroacetic acid (10%) were added to the mixture and mixed well to stop any enzymatic reaction. A controlled experiment was prepared by mixing the same amount of trichloroacetic acid into the crude enzyme extract, adding 2.5% azocasein to the mixture before incubating it at 37 °C for an hour. The mixture was cooled and centrifuged to obtain the supernatant, which was mixed with sodium hydroxide (0.5 M) at a ratio of 1:1. Then, the absorbance of the mixture against autoclaved distilled water as the blank, was measured at 440 nm with a Genesys 20 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Meanwhile, a standard calibration curve was constructed with azocasein solutions ranging from 5 to 25 mg/mL using the same procedure but hydrolyzed with 2 mg/mL commercial protease (Sigma-Aldrich). One unit of protease activity is the amount of azocasein (in mg) hydrolyzed per minute.

The proteolytic activity was calculated using Equation 1:

$$\text{Activity (U/mL)} = \frac{C_{azo} \times V_{total}}{t \times V_{enz}} \quad (1)$$

where C_{azo} = concentration of azocasein obtained (mg/mL);
V_{total} = total volume of the assay solution (mL);
t = time of incubation (min);
V_{enz} = volume of enzyme solution used (mL).

2.3.4 *Determination of chitinolytic activity*

The chitinolytic activity of the crude enzyme extract was determined based on the 3,5-dinitrosalicylic acid (DNS) method [16]. The 1% DNS reagent was prepared by dissolving the DNS into distilled water containing sodium tartrate tetrahydrate (30%) and sodium hydroxide (0.4 M). Colloidal chitin was prepared as described by Kabir et al. [14].

A suitably diluted crude enzyme extract was mixed well with colloidal chitin (1%) at a 1:1 ratio and incubated at 45 °C for 30 min. Then, the reaction mixture was added with one volume of DNS reagent, boiled for 10 min, cooled to ~25 °C, and centrifuged. The absorbance at 540 nm of the supernatant was measured. A standard calibration curve was constructed using N-acetylglucosamine solutions ranging from 5 to 20 μmoles. One unit of chitinolytic activity is measured as μmoles of N-acetylglucosamine released per minute.

The chitinolytic activity was calculated using Equation 2:

$$\text{Activity (U/mL)} = \frac{\text{amount of N-acetylglucosamine } (\mu\text{mole})}{t \times V_{enz}} \quad (2)$$

where t = time of incubation (min);
V_{enz} = volume of enzyme solution used (mL).

2.4 *Isolation of chitin from shrimp shells*

2.4.1 *Pre-isolation treatment*

The shrimp shells were washed thoroughly to remove the impurities attached to the shells. After washing, the shrimp shells were ground into fine pieces using a household grinder and stored at -18 °C in a deep freezer before further treatments.

2.4.2 *Determination of moisture content*

The moisture content of a sample was measured using a moisture analyzer (A&D, MX-50, Chicago, IL, U.S.A.). The moisture analyzer was set to record the moisture content after the weighed sample (approximately 5 g initial weight) reached a constant weight at 105 °C. The moisture content was expressed in percentage of wet weight basis.

2.4.3 *Demineralization*

With slight modifications, steps in demineralizing shrimp shells were adapted from Antonino et al. [17]. The ground shrimp shells were mixed with hydrochloric acid (1 M) at a 1:10 (w/v) ratio, with constant shaking for 3 h at room temperature. The suspension was vacuum-filtered and washed with distilled water to reach a neutral pH. The washed residue proceeded to the next deproteinization step.

2.4.4 *Deproteinization of the demineralized shrimp shells*

For the biochemical method, the demineralized shrimp shells were treated with the extract of germinated grains at a ratio of 1:10 (w/v), approximately 5 U crude protease/g shrimp shells. Commercial pancreatin (5 mg/mL) was used to treat the demineralized shrimp shells similarly to serve as a comparison. In contrast, sodium hydroxide (1 M)-deproteinization [4] was the positive control, assuming 100% deproteinization efficiency. All mixtures were constantly shaken at 110 rpm for 24 h using a shaker incubator (N-BIOTEK NB-1-1S, Bucheon, South Korea) at room temperature. Then, the suspensions were vacuum-filtered and washed with distilled water till neutral to obtain the crude chitin. The protein content of the filtrate was further analyzed using the Bradford method to compute the deproteinization rate using Equation 3 [18].

$$\text{Deproteinization rate } (\%) = \frac{(P_0 \times O) - (P_R \times R)}{(P_0 \times O)} \times 100 \quad (3)$$

where P_0 = the protein content in the sample (based on alkali-deproteinization data);
P_R = the protein content of residue after biochemical treatment;
O = the dry weight of the sample (g);

R = the dry weight of residue after deproteinization (g).

2.4.5 *Crude chitin yield*

The shrimp shell's dry weight and the isolated chitin's dry weight were calculated by subtracting moisture content from the sample and chitin. The yield of crude chitin from the shrimp shell was calculated [19] based on the following equation:

$$\text{Crud chitin yield } (\%) = \frac{\text{dry weight of extracted chitin } (\text{g})}{\text{dry weight of shrimp shell } (\text{g})} \times 100 \quad (4)$$

2.5 *Characterization of chitin*

2.5.1 *Fourier Transform Infrared (FTIR) spectroscopy analysis*

Isolated chitin obtained from different methods was ground, weighed and mixed with potassium bromide powder using a mortar pestle and compressed under hydraulic pressure to form a pellet. FTIR spectra were recorded using a Spectrum RX 1 FTIR spectrophotometer (Perkin Elmer, Waltham, Massachusetts, U.S.A.). Four scans were done with a resolution of 4 cm^{-1}. Transmittance and absorption peaks were measured between 400 to 4,000 cm^{-1}. FTIR spectroscopy analysis of commercial chitin (Sigma-Aldrich) and chemical-isolated chitin were also performed. The spectrums were compared using Spectragryph 1.2, an optical spectroscopy software developed by Dr. Friedrich Menges [20].

2.5.2 *X-ray diffraction (XRD) analysis*

The XRD pattern and data of all the extracted chitin were collected over a 2θ range with scan angles from 5° to 40° in a continuous mode, measured with a PANalytical X'PertPRO X-ray diffractometer (Malvern Panalytical Ltd, Malvern, U.K.) at a scan rate of 2° min^{-1}.

The crystallinity index (CrI_{110}) was calculated by applying the following Eq 5 [4]:

$$CrI_{110}(\%) = \left[(I_{110} - I_{am})/I_{110}\right] \times 100 \quad (5)$$

where I_{110} = the maximum intensity at 2θ ≈ 20°;

I_{am} = the intensity of amorphous diffraction at 2θ ≈ 16°;

The spectrums were overlaid using Spectragryph 1.2 [20].

2.6 *Statistical analysis*

All experimental data were expressed as mean ± standard deviation of triplicate determinations. The statistical analysis was performed through the one-way analysis of variance (ANOVA) or *t*-test using the Statistical Package for the Social Science (SPSS) program (Version 16.0). *p* value less than 0.05 ($p < 0.05$) was considered a significant difference.

3. Results and discussion

3.1 *Proteolytic activity and chitinolytic activity of crude enzyme extracts*

Figure 2 shows the presence of protease and chitinase in both extracts of germinated winter wheat and buckwheat, with clear zones formed in respective agars. The negative control (labeled as N) on all the agars shows no clear zones. Positive controls (labeled as P) showed clear zones ranging from 2.6 to 2.9 cm in diameter. Both winter wheat and buckwheat extracts (labeled as S) exerted clear zones of 2.2 to 2.9 cm diameter, comparable to the positive controls.

The proteolytic activity increased proportionally to the seed's germination duration, from 0.044 to 0.49 U/mL

and 0.13 to 0.46 U/mL for winter wheat and buckwheat, respectively (Table 1). Hence, the crude enzyme extract from 5-day-germinated winter wheat and 6-day-germinated buckwheat was used in the deproteinization process.

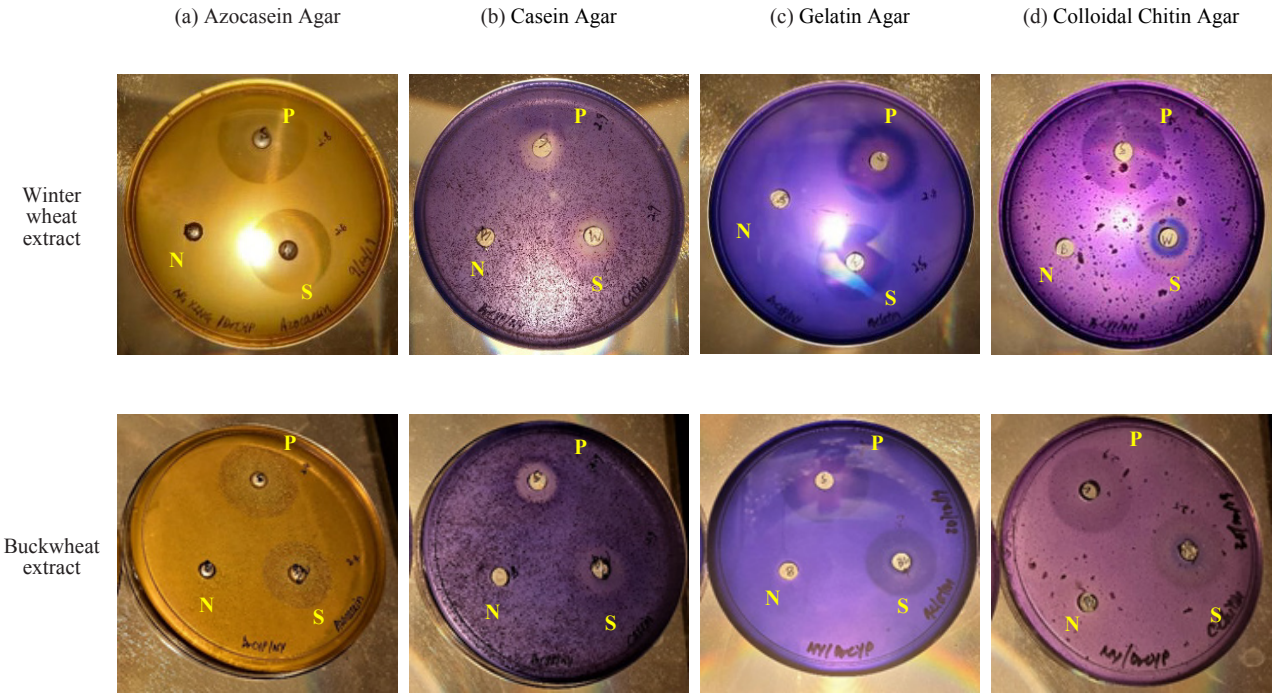

Figure 2. Protease detection from germinated winter wheat extract and buckwheat extract on the agar of (a) azocasein; (b) casein; (c) gelatin and chitinase detection on (d) the colloidal chitin agar (P: positive control; N: negative control; S: sample)

Table 1. Proteolytic activity of crude enzyme extract of winter wheat and buckwheat through azocasein assay

Germination interval*	Proteolytic activity (U/mL) **	
	Winter wheat extract	Buckwheat extract
I	0.04 ± 0.02^{a}	0.14 ± 0.01^{a}
II	0.21 ± 0.04^{b}	0.20 ± 0.04^{a}
III	0.49 ± 0.06^{c}	0.46 ± 0.03^{b}

* 1, 3 and 5 days for winter wheat while 2, 4 and 6 days for buckwheat. **Values represent means ± SD (n = 3). For each column, means with different letters were significantly different (*than 0.05*).

The chitinolytic activity of the maximally germinated winter wheat and buckwheat extract was 0.07 ± 0.01 U/mL and 0.05 ± 0.004 U/mL, respectively. Theoretically, chitinase may digest the chitin into its monomer, N-acetylglucosamine, by hydrolyzing the β-1,4-linkages in pure or isolated chitin [21]. It is uncertain whether the relatively low chitinolytic activity detected could significantly hydrolyze the embedded chitin in the shrimp shell because the insoluble substrate's heterogeneity could affect the accessibility, adsorption and diffusion of enzymes [22].

A chitinase-synthesized fungus, *Mucor circinelloides* [23], has been used to extract chitosan [24] from prawn shells. On the other hand, commercial proteases such as Neutrase and Umamizyme were used to extract protein from palm kernel meal [25]. So, we proceeded to apply the crude enzyme extracts to isolate chitin from shrimp shells. The characteristic of winter wheat extract-isolated chitin showed similar FTIR spectra and crystallinity to those of the chemical-isolated chitin, which will be discussed later.

3.2 *Deproteinization rate and crude chitin yield*

Table 2 shows the shrimp shell's deproteinization rate and chitin yield using crude enzyme extracts from germinated winter wheat and buckwheat. The winter wheat extract imposed a deproteinization rate of 53.6%, while the buckwheat extract induced a deproteinization of 58.0%, slightly lower than the 72.8% deproteinization rate by the commercial pancreatin. However, the deproteinization rate was comparable with the deproteinization rate (59.5%) using alcalase on shrimp processing wastes [26]. Both germinated grains contained proteolytic enzymes that effectively removed proteins from the demineralized shrimp shell.

The amount of chitin extracted was 30.3 ± 5.31 % and 29.2 ± 3.99, respectively. The crude chitin yield obtained was comparable with those of the commercial pancreatin deproteinized sample (29.3 ± 2.38) and within the range of chitin content (15-40%) of shrimp shells [2]. Plant-based proteases from winter wheat and buckwheat have comparable effectiveness in isolating chitin compared with microbial protease from *K.gibsonii* and *A. flavus*, which yielded a 16.06% chitin from green tiger prawn shell [27]. Samar et al. [28] chemically isolated chitin from shrimp shells and observed a higher crude chitin yield of 36.43%. Compared to the biological method to isolate chitin, the chemical method always produces a higher output of crude chitin. This observation applies to insect chitin as well [29-30].

Table 2. Deproteinization rate and chitin yield using crude enzyme extracts of winter wheat and buckwheat

	Application of		
	Winter wheat extract	Buckwheat extract	Commercial pancreatin
Deproteinization rate (%)	53.6 ± 1.00	58.0 ± 2.30	72.8 ± 1.90
Chitin yield (%)	30.3 ± 5.31	29.2 ± 3.99	29.3 ± 2.38

Values represent means ± SD (n = 3). The means were not significantly different ($P < 0.05$)

The quality of the isolated chitin was governed by its physicochemical properties. Commercial chitin and crude chitin isolated using the chemical-deproteinized method were also characterized as a comparison for quality determination.

3.3 *Fourier Transform Infrared (FTIR) spectroscopy analysis*

Figure 3 illustrates the FTIR spectra of commercial chitin, chemical-isolated chitin, winter wheat extract-isolated chitin, and buckwheat extract-isolated chitin. The FTIR spectra of winter wheat extract-isolated chitin resembled those of commercial and chemical-isolated chitin. However, the buckwheat extract-isolated chitin exhibited a certain degree of deacetylation, which will be further discussed.

The characteristic absorption bands commonly found in chitin were observed. The peak at 1,652-1,653 cm^{-1} is the amide I band in the β-chitin, while the height at 1,560 cm^{-1} is the amide II band for the chitin structure [31]. Bands between 890-1,156 cm^{-1} indicate the polysaccharide structure for chitin. Other functional groups identified were: bands between 3,100 and 3,273 cm^{-1} (N-H stretching of the amide group); 2,932 and 2,934 cm^{-1} (asymmetric stretching vibration of CH_3 and CH_2 groups); 2,889 and 2,891 cm^{-1} (C-H stretching); 1,376 and 1,382 cm^{-1} (symmetrical

deformation mode of CH_3); 1,320 to 1,340 cm^{-1} (methyl C-H stretch of amide III); 1,158 and 1,159 cm^{-1} (asymmetric stretching of the C-O-C bridge) [17, 29-30]. The consistent wavenumbers of amide I and II functional groups indicate the effectiveness of deproteinization [30] using crude enzyme extracts from winter wheat and buckwheat. However, a slight shift of the band from 1,381 to 1,398 cm^{-1} and disappeared peaks between 1,154 and 1,026 cm^{-1} and a shift of the band from 3,273 to 3,298 cm^{-1} for buckwheat extract-isolated chitin indicates changes in acetyl groups; an increase in disorder structural organization; and a change in the secondary structural environment [32], respectively. There was also a concomitant intensification of the band at 1,590 cm^{-1} and a shrinkage of the band at 1,655 cm^{-1}. Bordi et al. [33] related these changes to the prevalence of amine group (NH_2) and effective deacetylation. This shift is often observed for chitosan converted from chitin [34] and warrants further investigation. The characteristic bands formed at a specific frequency are assigned to the represented functional group, as shown in Table 3.

Table 3. Functional group assignment for each peak in the Fourier Transform Infrared (FTIR) spectra of different chitin samples

Wave number (cm^{-1})				Functional group assigned
Commercial chitin	Chemical-isolated chitin	Winter wheat extract-isolated chitin	Buckwheat extract-isolated chitin	
895-1,075	895-1,074	895-1,076	895-1,077	Polysaccharide structure
1,159	1,159	1,158	1,154	Asymmetric stretching of the C-O-C bridge
1,320	1,320	1,320	1,320	Amide III (Methyl C-H stretch)
1,382	1,381	1,376	1,398	CH bend, CH_3 symmetric deformation
1,561	1,562	1,560	1,559	Amide II (NH bending and CN stretching)
1,652	1,652	1,653	1,655	Amide I (C = O secondary amide stretch)
2,932	2,930	2,933	2,927	Asymmetric CH stretching
2,889	2,891	2,891	2,871	Aliphatic compound
3,100, 3,273	3,103, 3,273	3,102, 3,262	3,101, 3,298	NH stretching
3,450	3,449	3,448	3,445	O-H stretching

Chitin structure can be classified into the α, β, and γ forms. The position of acetyl groups in the molecular structure identifies the α-chitin, β-chitin, and γ-chitin. The isolated chitin is in the form of β-chitin because the FTIR spectra show a single and broad band centered at 3,440 cm^{-1} [35] while the amide I band forms one peak at 1,660 cm^{-1} (usually it splits into two peaks for α-chitin) [29].

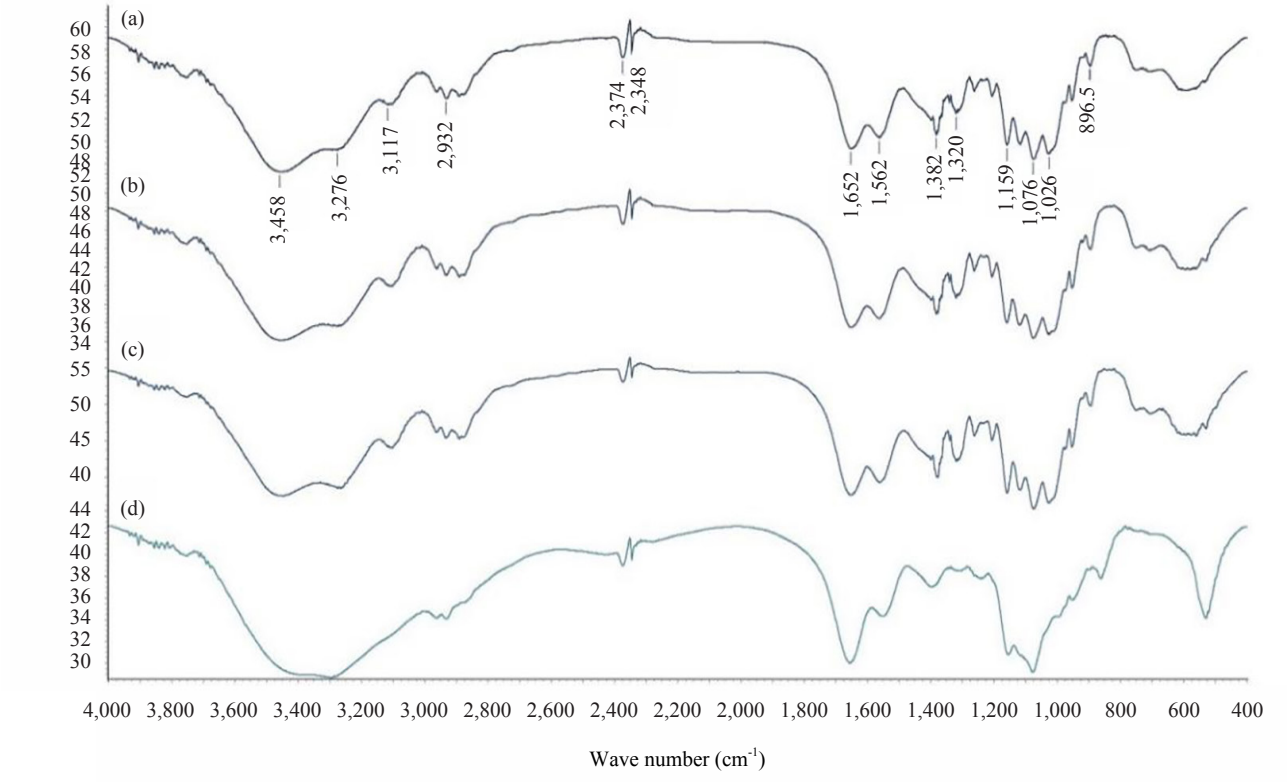

Figure 3. FTIR spectra of (a) commercial chitin, (b) chemical-isolated chitin, (c) winter wheat extract-isolated chitin and (d) buckwheat extract-isolated chitin

3.4 *X-ray diffraction (XRD) analysis*

Figure 4 shows the XRD pattern for different chitin samples. Significant peaks were observed at 2θ of 9° and 19°. These are characteristic chitin peaks [29, 35], specifically β chitin. Both commercial chitin and chemical-isolated chitin showed a total of 9 peaks at 2θ of 9.2, 11.6, 12.7, 16.8, 19.3, 21.1, 23.3, 37.8 and 39.2° and 9.2, 12.8, 16.7, 19.4, 21.2, 23.3, 26.3, 37.9 and 39.2°, respectively. Winter wheat extract-isolated chitin showed 8 peaks at 9.2, 11.5, 12.6, 16.7, 19.4, 21.2, 23.3, and 38.9°.

The winter wheat extract-isolated chitin showed a comparable crystallinity index and a similar diffraction pattern to the commercial chitin and chemical-isolated chitin. In contrast, buckwheat extract-isolated chitin showed 16 peaks at 9.1, 12.7, 15.8, 17.2, 19.5, 21.0, 22.4, 23.2, 26.1, 28.5, 29.7, 31.9, 33.0, 38.4, 39.1 and 41.1°. Buckwheat extract-isolated chitin also showed a lower crystallinity index, 75.87%, compared to other chitin samples (86.48 to 88.82%), as listed in Table 4. The increased number of weak peaks for buckwheat extract-isolated chitin resembled the XRD patterns of commercial shrimp chitosan, as documented by Ibitoye et al. [30]. The lower crystallinity index of the buckwheat extract-isolated chitin agreed with the previous discussion on FTIR spectra, in which a decrease in the order of structural organization or a change in the secondary structural environment might have occurred. The chitinolytic action may have caused this observation, but it was unclear why such an effect was not observed on winter wheat extract-isolated chitin. Germinated buckwheat crude extract is rich in bioactive compounds [36]. These compounds may have induced the structural changes of chitin and warrant further investigation.

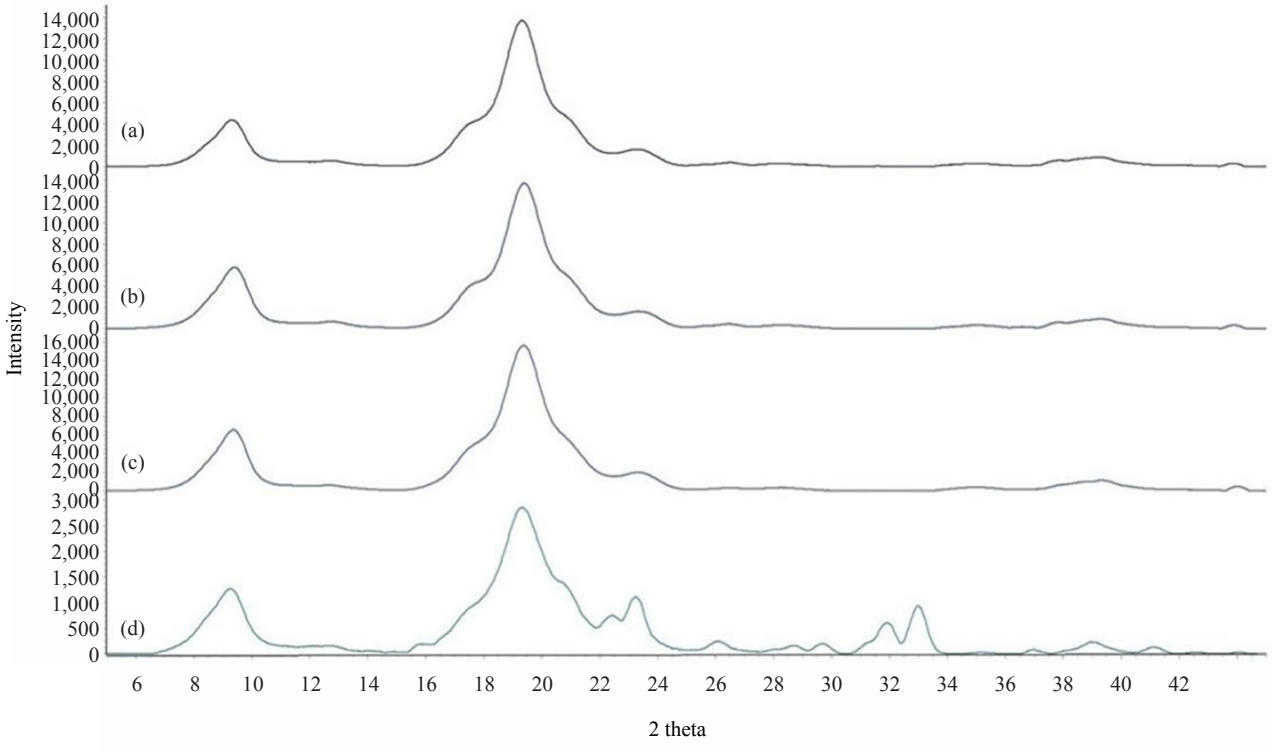

Figure 4. X-ray diffraction pattern of (a) commercial chitin, (b) chemical-isolated chitin, (c) winter wheat extract-isolated chitin, and (d) buckwheat extract-isolated chitin.

Table 4. Crystallinity index estimation of each chitin sample

Type of chitin	I_{110}	I_{am}	Crystallinity Index (%)
Commercial	13,735	1,855	86.49
Chemical-isolated	13,744	1,547	88.74
Winter wheat extract-isolated	15,707	1,756	88.82
Buckwheat extract-isolated	2,851	688	75.87

4. Conclusions

Germinated wheat and buckwheat contained significant protease activity ranging from 0.46 to 0.49 U/mL, which can be used as a source of protease to deproteinize shrimp shells that have undergone demineralization. No hazardous chemicals were used to extract crude enzymes, which enable residues of the wheat and buckwheat to be further valorized but require further research in the nutrient digestibility and health-promoting activities. Despite the detected chitinolytic activities in the wheat and buckwheat extract, β chitin was successfully isolated. Chitin yield ranged from 29.2 to 30.3% and was comparable to other documented chitin yields from shrimp wastes. Winter wheat extract-isolated chitin exhibited similar functional groups, such as amide I and amide II, with those of commercial chitin and chemical-isolated chitin. Buckwheat extract-isolated chitin showed a lower crystallinity index and some degree of deacetylation that may be due to the chitinolytic action of the seed enzymes or the presence of other bioactive compounds in

germinated buckwheat, which warrants further investigation.

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Conflict of interest

The authors declare no competing financial interest.

References

[1] Food and Agriculture Organization of the United Nations. *The state of world fisheries and aquaculture 2018-Meeting the sustainable development goals*. Rome: FAO; 2018. Available from: https://www.fao.org/documents/card/en/c/I9540EN/ [Accessed 4th October 2023].

[2] Pakizeh M, Moradi A, Ghassemi T. Chemical extraction and modification of chitin and chitosan from shrimp shells. *European Polymer Journal*. 2021; 159: 110709. Available from: doi:10.1016/j.eurpolymj.2021.110709.

[3] Philibert T, Lee BH, Fabien N. Current status and new perspectives on chitin and chitosan as functional biopolymers. *Applied Biochemistry and Biotechnology*. 2017; 181: 314-1337.

[4] Hongkulsup C, Khutoryanskiy V, Niranjan K. Enzyme assisted extraction of chitin from shrimp shells (*Litopenaeus vannamei*) and its application as an antimicrobial agent. *Journal of Chemical Technology and Biotechnology*. 2016; 91(5): 1250-1256. Available from: https://centaur.reading.ac.uk/43511/3/Enzyme%20assisted%20extraction%20of%20chitin_revised%20version.pdf [Accessed 4th October 2023].

[5] Procurement Resource. *Protease price trend and forecast-Regional price overview*. Procurement Resource; 2023. Available from: https://www.procurementresource.com/resource-center/protease-price-trends [Accessed 4th October 2023].

[6] Troncoso FD, Sánchez DA, Ferreira ML. Production of plant proteases and new biotechnological applications: An updated review. *Chemistry Open*. 2022; 11: e202200017. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8919702/pdf/OPEN-11-e202200017.pdf [Accessed 4th October 2023].

[7] Ling CX, Chang YP. Valorizing guava (*Psidium guajava* L.) seeds through germination-induced carbohydrate changes. *Journal of Food Science and Technology*. 2017; 54(7): 2041-2049. Available from: doi:10.1007/s13197-017-2641-5.

[8] Commodity price-wheat. *Index Mundi: Commodity Price Indices*. Available from: https://www.indexmundi.com/commodities/?commodity=wheat [Accessed 4th October 2023].

[9] Index Box. *Global buckwheat market report*. Index Box; 2023. Available from: https://www.indexbox.io/store/world-buckwheat-market-report-analysis-and-forecast-to-2020/ [Accessed 4th October 2023].

[10] Dojczew D, Sobczyk M. The effect of proteolytic activity on the technological value of wheat flour from pre-harvest sprouted grain. *Acta Scientiarumi Polonorum Technologia Alimentaria*. 2007; 6(4): 45-53. Available from: http://www.food.actapol.net/pub/4_4_2007.pdf [Accessed 4th October 2023].

[11] Żur I, Gołębiowska G, Dubas E, Golemiec E, Matušíková I, Libantová J, et al. β-1,3-glucanase and chitinase activities in winter triticales during cold hardening and subsequent infection by *Microdochium nivale*. *Biologia*. 2013; 68: 241-248.

[12] Chaiwut P, Pintathong P, Rawdkuen S. Extraction and three-phase partitioning behavior of proteases from papaya peels. *Process Biochemistry*. 2010; 45(7): 1172-1175.

[13] Hassan MA, Haroun BM, Amara AA, Serou EA. Production and characterization of keratinolytic protease from new wool-degrading bacillus species isolated from Egyptian ecosystem. *BioMed Research International*. 2013; 2013: 175012. Available from: doi:10.1155/2013/175012.

[14] Kabir SR, Rahman MM, Tasnim S, Karim MR, Khatun N, Hasan I, et al. Purification and characterization of

a novel chitinase from *Trichosanthes dioica* seed with antifungal activity. *International Journal of Biological Macromolecules*. 2016; 84: 62-68.
[15] Coêlho DF, Saturnino TP, Fernandes FF, Mazzola PG, Silveira E, Tambourgi EB. Azocasein substrate for determination of proteolytic activity: reexamining a traditional method using bromelain samples. *BioMed Research International*. 2016; 2016: 8409183. Available from: doi:10.1155/2016/8409183.
[16] Kopparapu NK, Liu Z, Fei F, Yan Q, Jiang Z. Purification and characterization of a chitinase (sAMC) with antifungal activity from seeds of *Astragalus membranaceus*. *Process Biochemistry*. 2011; 46(6): 1370-1374.
[17] Antonino RSCM, Lia-Fook BRP, Oliveira-Lima VA, Farias-Rached R, Lima EPN, Silva-Lima RJ, et al. Preparation and characterization of chitosan obtained from shells of shrimp (*Litopenaeus vannamei* Boone). *Marine Drugs*. 2017; 15(5): 1-12. Available from: doi:10.3390/md15050141.
[18] Paul T, Halder SK, Das A, Ghosh K, Mandal A, Payra P, et al. Production of chitin and bioactive materials from Black tiger shrimp (*Penaeus monodon*) shell waste by the treatment of bacterial protease cocktail. *3 Biotech*. 2015; 5: 483-493. Available from: doi:10.1007/s13205-014-0245-6.
[19] Katsoulis K, Rovoli M. Optimization of chitin extraction, physicochemical and functional properties of chitosan production from shells of Karamote shrimp (*Peneaus Melicertus*) kerathurus in Western Greece. *Journal of Veterinary Science and Medicine*. 2021; 9(1): 5. Available from: https://www.avensonline.org/wp-content/uploads/JVSM-2325-4645-09-0052.pdf [Accessed 4th October 2023].
[20] Dr. Friedrich Menges Software_Entwicklung_Spectragryph: optical spectroscopy software. 2016. Available from: https://www.effemm2.de/spectragryph/ [Accessed 4th October 2023].
[21] Funkhouser JD, Aronson NN. Chitinase family GH18: Evolutionary insights from the genomic history of a diverse protein family. *BMC Evolutionary Biology*. 2007; 7: 96. Available from: doi:10.1186/1471-2148-7-96.
[22] Missang CE, Denes J-M, Baron A. Study of diffusion and adsorption processes during the maceration of apple tissue by polygalacturonases. *Food Biotechnology*. 1999; 13(1): 67-92.
[23] Halim Y, Hardoko F, Handayani R. Production of N-acetylglucosamine from shrimp shells' chitin using intracellular chitinase from *Mucor circinelloides*. *Food Research*. 2020; 4(5): 1582-1587. Available from: https://www.myfoodresearch.com/uploads/8/4/8/5/84855864/_25__fr-2020-135_halim.pdf [Accessed 4th October 2023].
[24] Tan YN, Lee PP, Chen WN. Dual extraction of crustacean and fungal chitosan from a single *Mucor circinelloides* fermentation. *Fermentation*. 2020; 6(2): 40. Available from: doi:10.3390/fermentation6020040.
[25] Safi C, Humblet NP, Geerdink P, Theunissen M, Beelen B, Voogt J, et al. Valorisation of proteins from palm kernel meal. *Bioresource Technology Reports*. 2022; 18: 101050. Available from: doi:10.1016/j.biteb.2022.101050.
[26] De Holanda HD, Netto FM. Recovery of components from shrimp (*Xiphopenaeus kroyeri*) processing waste by enzymatic hydrolysis. *Journal of Food Science*. 2006; 71(5): 298-303.
[27] Bahasan SHO, Satheesh S, Ba-akdah MA. Extraction of chitin from the shell wastes of two shrimp species *Fenneropenaeus semisulcatus* and *Fenneropenaeus indicus* using microorganisms. *Journal of Aquatic Food Product Technology*. 2017; 26(4): 390-405.
[28] Samar MM, El-Kalyoubi MH, Khalaf MM, El-Razik MM. Physicochemical, functional, antioxidant and antibacterial properties of chitosan extracted from shrimp wastes by microwave technique. *Annals of Agricultural Sciences*. 2013; 58(1): 33-41. Available from: https://core.ac.uk/download/pdf/82672896.pdf [Accessed 4th October 2023].
[29] Psarianos M, Ojha S, Schneider R, Schlüter OK. Chitin isolation and chitosan production from house crickets (*Acheta domesticus*) by environmentally friendly methods. *Molecules*. 2022; 27(15): 5005. Available from: doi:10.3390/molecules27155005.
[30] Ibitoye EB, Lokman IH, Hezmee MNM, Goh YM, Zuki ABZ, Jimoh AA. Extraction and physicochemical characterization of chitin and chitosan isolated from house cricket. *Biomedical Materials*. 2018; 13(2): 025009. Available from: https://iopscience.iop.org/article/10.1088/1748-605X/aa9dde [Accessed 4th October 2023].
[31] Cahú TB, Santos SD, Mendes A, Córdula CR, Chavante SF, Carvalho LB, et al. Recovery of protein, chitin, carotenoids and glycosaminoglycans from Pacific white shrimp (*Litopenaeus vannamei*) processing waste. *Process Biochemistry*. 2012; 47(4): 570-577. Available from: https://core.ac.uk/download/pdf/82180471.pdf [Accessed 4th October 2023].
[32] Kumar ABV, Varadaraj MC, Gowda LR, Tharanathan RN. Characterization of chito-oligosaccharides prepared by chitosanolysis with the aid of papain and Pronase, and their bactericidal action against *Bacillus cereus* and *Escherichia coli*. *The Biochemical Journal*. 2005; 391(2): 167-175. Available from: doi:10.1042/BJ20050093.
[33] Bordi F, Cametti C, Paradossi G. Dielectric behavior of polyelectrolyte solutions: The role of proton fluctuation. *The Journal of Physical Chemistry*. 1991; 95(12): 4883-4889.
[34] Paulino AT, Simionato JI, Garcia JC, Nozaki J. Characterization of chitosan and chitin produced from silkworm

chrysalides. *Carbohydrate Polymers*. 2006; 64(1): 98-103.
[35] Hajji S, Younes I, Ghorbel-Bellaaj O, Hajji R, Rinaudo M, Nasri M, et al. Structural differences between chitin and chitosan extracted from three different marine sources. *International Journal of Biological Macromolecules*. 2014; 65: 298-306.
[36] Kim SL, Kim SK, Park CH. Introduction and nutritional evaluation of buckwheat sprouts as a new vegetable. *Food Research International*. 2004; 37(4): 319-327.