

Natural Enzymes from Germinated Winter Wheat (*Triticum aestivum* L) and Buckwheat (*Fagopyrum esculentum*) for Chitin Isolation from Shrimp Shells

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Research Article

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

Posted Date: February 21st, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-2588260/v1>

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Abstract

The industrial approach for chitin extraction from crustaceans' shells involves chemical methods using a large amount of acid and alkaline. Using natural protease in combination with chemicals can reduce the use of the chemicals in extracting chitin. Crude enzymes from germinated winter wheat (*Triticum aestivum*) and buckwheat (*Fagopyrum esculentum*) were applied in the deproteinization step during chitin isolation from shrimp shells. 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 these crude enzymes separately to remove protein from demineralized shrimp shells yielded $30.31 \pm 5.31\%$ and $29.15 \pm 3.99\%$ chitin, respectively. Functional groups and crystallinity index of chitin isolated with winter wheat or buckwheat enzymes were compared with commercially available chitin and chemical-isolated chitin through Fourier Transform Infrared Spectroscopy (FTIR) analysis and x-ray diffraction analysis. The characteristic functional groups observed were amide I, amide II, amide III, asymmetric stretching of the C-O-C bridge, O-H stretching, NH stretching and asymmetric CH stretching. The crystallinity index for commercial chitin, chemical-isolated chitin, wheat extract- and buckwheat extract-isolated chitin were 86.49%, 88.74%, 88.82% and 75.87%, respectively. Buckwheat extract-isolated chitin, which was deacetylated with lower crystallinity, warrants further investigation. Natural enzymes from winter wheat and buckwheat to remove protein from shrimp shells can be a more environment-friendly method to valorize shrimp shells as a chitin source.

Introduction

To meet the food demand of the increasing world population, a continual increase in the world fishery and aquaculture production reached 170.9 million tonnes in the year 2018. Among fishery products, about 4 million tonnes were contributed by global farmed shrimp production, which was believed to continue expanding in the future (FAO, 2018). Approximately 48 to 56% of shrimp waste is generated in the processing of shrimp for human consumption. The embarrassing liability, like shrimp shell waste, may be converted into a source of an environment-friendly compound or food ingredient. Shrimp shell wastes contain 15–40% chitin (Kurita, 2006). Chitin is a long-chain polymer of *N*-acetyl-D-glucosamine (GlcNAc). Chitin possesses biodegradable, non-toxic and biocompatible properties, which widen its applications in food, agriculture, wastewater treatment plant, biomedical, cosmetics, and textiles industries (Arbia et al., 2013).

Typical steps in chitin extraction from shrimp shell wastes are: grinding, demineralizing with a strong acid, and deproteinizing (protein removal) with a strong alkali. The extreme condition of strong acids and alkalis can alter the quality of the chitin extracted. The chemical extraction process also generates a lot of chemical waste that could pollute the environment (Wang and Chio, 1998). The harmful chemicals might remain in the final products, contaminating the extracted chitin. After chemical extraction, valuable residues like protein can no longer be utilized as animal feed.

Some researchers have demonstrated the feasibility of using commercial protease to replace chemicals in the deproteinization step (Hongkulsup et al., 2016). Nevertheless, the high cost of commercial enzymes is a barrier for the stakeholders who intend to use them on a large scale. Germinating grains can be a source of low-cost enzymes. In our previous research, germinating winter wheat showed significant carbohydrase activities that led to changes in nutrient profile (Ling and Chang, 2017). Shaha et al. (2002) reported that germinated winter wheat seeds also possessed significant proteolytic activity. However, the presence of chitinase in germinating winter wheat (Zur et al., 2013) may digest chitin into its monomer by hydrolyzing the β -1,4-linkages (Funkhouser and Aronson, 2007). This study determined proteolytic and chitinolytic activity in crude enzyme extracts obtained from germinated winter wheat (*Triticum aestivum*) and buckwheat (*Fagopyrum esculentum*). The crude enzyme extract was applied in the deproteinization step for chitin isolation 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.

Experimental

Sample

Winter wheat (*Triticum Aestivum*) grains with the brand of BMS were bought from an organic shop at Ipoh, Perak, Malaysia. Buckwheat (*Fagopyrum esculentum*) grains with the brand of CED were bought from Lotus hypermarket in Kampar, Malaysia. The shrimp shells were peeled from shrimps bought from a wet market at Kampar, Perak.

Seed germination

Seed germination was done as described by Ling and Chang (2017). 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 any apparent seedling growth was observed. 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.

Extraction of crude enzymes from germinated seeds

Extraction of crude enzymes from the germinated seed was adapted from Chaiwut et al. (2010). 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 to obtain the crude enzyme extract which was stored at 4°C for not more than 5 days before further analysis.

Screening of protease and chitinase activities

Gelatin agar, casein agar, and azocasein agar were used to screen protease in the crude extract (Hassan et al., 2013; Vijayaraghavan et al., 2013). 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.

For the screening of chitinase, colloidal chitin agar was used to detect the enzyme. Colloidal chitin agar was prepared with 2% agar and 1% colloidal chitin substrate. The colloidal chitin was prepared based on the method used by Kabir et al. (2016). The agar mixtures were autoclaved before pouring into each petri dish. Then, three holes were made on each solidified agar plate with a 6 mm-diameter-cork borer. Approximately 100 µL of 0.1 M sodium phosphate buffer (negative control), 2 mg/mL commercial pancreatin (positive control) and crude enzyme extract from maximally germinated seeds were 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 0.25% (w/v) coomassie blue 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. The colloidal chitin agar was also stained and de-stained 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.

Determination of proteolytic activity

The proteolytic activity of the crude enzyme extract was determined using azocasein as the substrate (Cólho et al., 2016). 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 Eq. 1:

$$\text{Activity (U/mL)} = \frac{C_{\text{azo}} \times V_{\text{total}}^2}{t \times V_{\text{enz}}} \quad (\text{Eq. 1})$$

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)

Determination of chitinolytic activity

The chitinolytic activity of the crude enzyme extract was determined based on the DNS (3,5-dinitrosalicylic acid) method (Kopparapu et al., 2011). 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. (2016). 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 μ moles. One unit of chitinase activity is measured as μ moles of N-acetylglucosamine released per minute.

The chitinolytic activity was calculated using Eq. 2:

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

where t = time of incubation (min)

V_{enz} = volume of enzyme solution used (mL)

Extraction of chitin from shrimp shells

Pre-extraction 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.

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) reaching a constant weight at 105°C. The moisture content was expressed in percentage of wet weight basis.

Demineralization

Steps in demineralizing shrimp shells were adapted from Antonino et al. (2017), with slight modifications. The ground shrimp shells were mixed with hydrochloric acid (1 M) at a ratio of 1:10 (w/v), 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.

Deproteinization using seed extract or alkali

For the biochemical method, the demineralized shrimp shells were treated with the extract of germinated grains at a ratio of 1:10 (w/v). For the chemical method as described by Hongkulsup et al. (2016), the demineralized shrimp shells were treated with sodium hydroxide (1 M) at a ratio of 1:10 (w/v). All mixtures were constantly shaken for 24 h using a rotary shaker at room temperature. Then, all suspensions were vacuum-filtered and washed with distilled water till neutral to obtain chitin.

Chitin yield

The dry weight of the shrimp shell used and the dry weight of the isolated chitin were calculated by subtracting moisture content from the sample and chitin. The yield of chitin from the shrimp shell was calculated based on the following equation (Demir et al., 2016):

$$\text{Chitin yield (\%)} = \frac{\text{dry weight of extracted chitin (g)}}{\text{dry weight of shrimp shell (g)}} \times 100 \text{ (Eq. 3)}$$

Characterization of chitin

Fourier transform infrared spectroscopy (FTIR) 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 4000 cm^{-1} . FTIR spectroscopy analysis of a 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.

X-ray diffraction analysis (XRD)

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 equation (Hongkulsup et al., 2016):

$$\text{CrI}_{110} (\%) = [(I_{110} - I_{\text{am}})/I_{110}] \times 100 \text{ (Eq. 4)}$$

where:

I_{110} = the maximum intensity at $2\theta \approx 20^\circ$

I_{am} = the intensity of amorphous diffraction at $2\theta \approx 16^\circ$

Statistical analysis

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

Results And Discussion

Table 1 summarized the presence of protease and chitinase in both extracts of germinated winter wheat and buckwheat, measured as the diameter of the clear zone formed in respective agars. Both winter wheat and buckwheat extracts exerted proteolytic activities and chitinolytic activities comparable to commercial pancreatin (Supplementary material).

Table 1
Diameters of clear zones formed surrounding different crude enzyme extracts on each substrate agar.

Type of agar	*Diameter of clear zone formed surrounding crude enzyme extract of		
	Commercial pancreatin	Winter wheat	Buckwheat
Azocasein	2.60 ± 0.00 ^a	2.60 ± 0.00 ^a	2.43 ± 0.03 ^b
Casein	2.90 ± 0.00 ^a	2.87 ± 0.03 ^a	2.87 ± 0.03 ^a
Gelatin	2.77 ± 0.03 ^a	2.53 ± 0.03 ^b	2.47 ± 0.03 ^b
Colloidal chitin	2.87 ± 0.03 ^a	2.20 ± 0.06 ^c	2.53 ± 0.03 ^b

*in cm. Values represent means ± SD (n = 3). For each row, means with different letters of the same type of agar were significantly different ($P < 0.05$).

The proteolytic activity increased proportionally to the seed's germination duration, from 0.044 to 0.49 U/mL and from 0.13 to 0.46 U/mL for winter wheat and buckwheat, respectively (Table 2). Hence, the crude enzyme extract from 5-day-germinated winter wheat and 6-day-germinated buckwheat was used in the deproteinization process.

Table 2
Proteolytic activity of crude enzyme extract of winter wheat and buckwheat through azocasein assay.

Germination interval*	Proteolytic activity (U/mL)**	
	Winter wheat	Buckwheat
1	0.04 ± 0.02 ^a	0.14 ± 0.01 ^a
2	0.21 ± 0.04 ^b	0.20 ± 0.04 ^a
3	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 of the same type of agar were significantly different ($P < 0.05$),		

The chitinolytic activity of the winter wheat and buckwheat extract was 0.074 ± 0.011 U/mL and 0.053 ± 0.004 U/mL, respectively. Theoretically, chitinase may digest the chitin into its monomer, N-acetylglucosamine, by hydrolyzing the β-1,4-linkages in chitin (Funkhouser and Aronson, 2007). However, 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 (Missang et al., 1999). So, we proceeded to apply the crude enzyme extract to isolate chitin from shrimp shells.

Chitin yield and characterization

The amount of chitin extracted was 30.31 ± 5.31% and 29.15 ± 3.99 after the deproteinization step by winter wheat extract and buckwheat extract, respectively. The chitin yield obtained was within the range of chitin content (15–40%) of shrimp shells, as documented by Kurita (2006). 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 (Bahasan et al., 2017).

The quality of the isolated chitin was governed by its physicochemical properties. A commercial chitin was also characterized as a comparison for quality determination. Besides, chitin isolated using the chemical-deproteinized method was also characterized to compare the differences between each deproteinization method.

Fourier Transform Infrared (FTIR) Spectroscopy Analysis

Figure 1 illustrates the FTIR spectra of commercial chitin, chemical-isolated chitin, winter wheat extract-, 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 1652–1653 cm^{-1} is the amide I band in the β -chitin, while the peak at 1560 cm^{-1} is the amide II band for the chitin structure (Cahú et al., 2012). Bands between 890–1156 cm^{-1} indicate the polysaccharide structure for chitin. Other functional groups identified were: bands between 3100 and 3273 cm^{-1} (N-H stretching of the amide group); 2932 and 2934 cm^{-1} (asymmetric stretching vibration of CH_3 and CH_2 groups); 2889 and 2891 cm^{-1} (CH stretching); 1376 and 1382 cm^{-1} (symmetrical deformation mode of CH_3); 1320 to 1340 cm^{-1} (methyl C-H stretch of amide III); 1158 and 1159 cm^{-1} (asymmetric stretching of the C-O-C bridge) (Antonino et al., 2017; Ibitoye et al., 2018; Psarianos et al., 2022; Sedaghat et al., 2017). The consistent wavenumbers of amide I and II functional groups indicate no protein contaminant in the isolated-chitin. This observation confirms the effectiveness of deproteinization (Psarianos et al., 2022) using crude enzyme extracts from winter wheat and buckwheat. However, a slight shift of the band from 1381 to 1398 cm^{-1} and disappeared peaks between 1154 and 1026 cm^{-1} and a shift of the band from 3273 to 3298 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 (Kumar et al., 2005) respectively. There was also a concomitant intensification of the band at 1590 cm^{-1} and a shrinkage of the band at 1655 cm^{-1} . Bordi et al. (1991) related these changes to the prevalence of the amine group (NH_2) and effective deacetylation. This shift is often observed for chitosan converted from chitin (Paulino et al., 2006) and warrants further investigation. Figure 1. FTIR spectra of (a) commercial chitin; (b) chemical-isolated chitin; (c) winter wheat extract-isolated chitin; and (d) buckwheat extract-isolated chitin.

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 IR 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	
895–1075	895–1074	895 – 1076	895 – 1077	Polysaccharide structure
1159	1159	1158	1154	Asymmetric stretching of the C-O-C bridge
1320	1320	1320	1320	Amide III (Methyl C-H stretch)
1382	1381	1376	1398	CH bend, CH_3 symmetric deformation
1561	1562	1560	1559	Amide II (NH bending and CN stretching)
1652	1652	1653	1655	Amide I (C = O secondary amide stretch)
2932	2930	2933	2927	Asymmetric CH stretching
2889	2891	2891	2871	Aliphatic compound
3100, 3273	3103, 3273	3102, 3262	3101, 3298	NH stretching
3450	3449	3448	3445	O-H stretching

Chitin structure can be classified into the α form, β form, and γ form. 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 3440 cm^{-1} (Hajji et al., 2014) while the amide I band forms one peak at 1660 cm^{-1} (usually it splits into two peaks for α -chitin) (Psarianos et al., 2022).

X-ray Diffraction Analysis (XRD)

Figure 2 shows the XRD pattern for different chitin samples. Significant peaks were observed at 2θ of 9° and 19°. These peaks are characteristic peaks for chitin (Hajji et al., 2014; Ibitoye et al., 2018), 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. (2018). 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. Germinated buckwheat crude extract is rich in bioactive compounds (Kim et al., 2004). These compounds may have induced the structural changes of chitin and warrant further investigation.

Conclusions

Germinated wheat and buckwheat contained significant protease activity ranging from 0.46 to 0.46 U/mL can be used as a source of protease to deproteinize shrimp shells that have undergone demineralized. Despite the detected chitinolytic activities in the wheat and buckwheat extract, β chitin was successfully isolated. The yield of chitin ranged from 18.7 to 19.4% and was comparable to other documented chitin yields from shrimp wastes. Winter wheat extract-isolated chitin exhibited comparable 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, which warrants further investigation.

Table 4
Crystallinity index estimation of each chitin sample.

Type of chitin	I ₁₁₀	I _{am}	CrI (%)
Commercial	13735	1855	86.49
Chemical-isolated	13744	1547	88.74
Winter wheat extract-isolated	15707	1756	88.82
Buckwheat extract-isolated	2851	688	75.87

Declarations

Ethics approval and consent to participate

Not applicable.

Availability of Data and Materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests

Funding

Universiti Tunku Abdul Rahman Research Fund IPSR/RMC/UTARRF/2022-C1/C01

Authors' Contributions

All authors contributed to the study conception and design. NgY and KhorJW involved in material preparation, data collection, and drafted a write-up. ChangYP analyzed the data and prepared the manuscript. All authors read and approved the final manuscript.

Acknowledgment

The authors thank University Tunku Abdul Rahman, Malaysia, for providing a grant IPSR/RMC/UTARRF/2022-C1/C01 to support this research.

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Figures

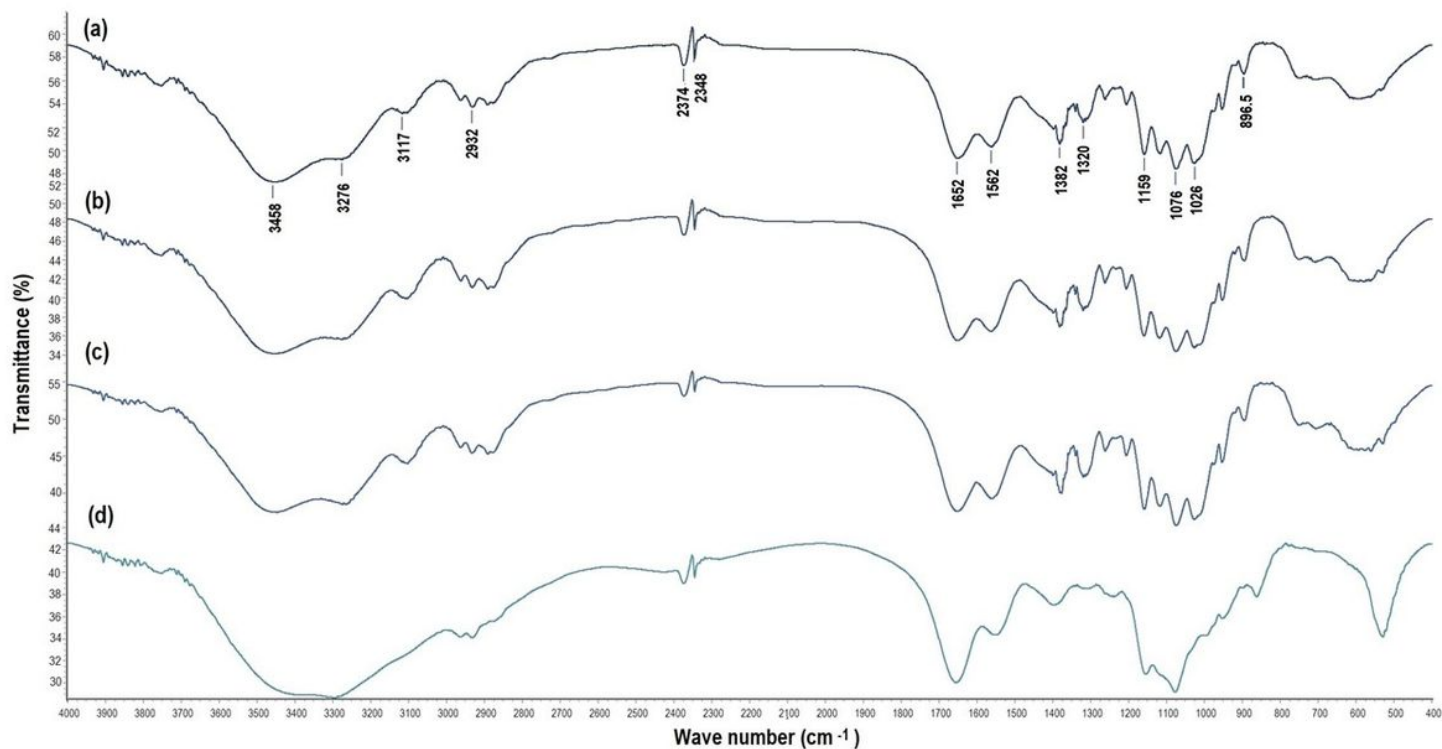


Figure 1
 FTIR spectra of (a) commercial chitin; (b) chemical-isolated chitin; (c) winter wheat extract-isolated chitin; and (d) buckwheat extract-isolated chitin.

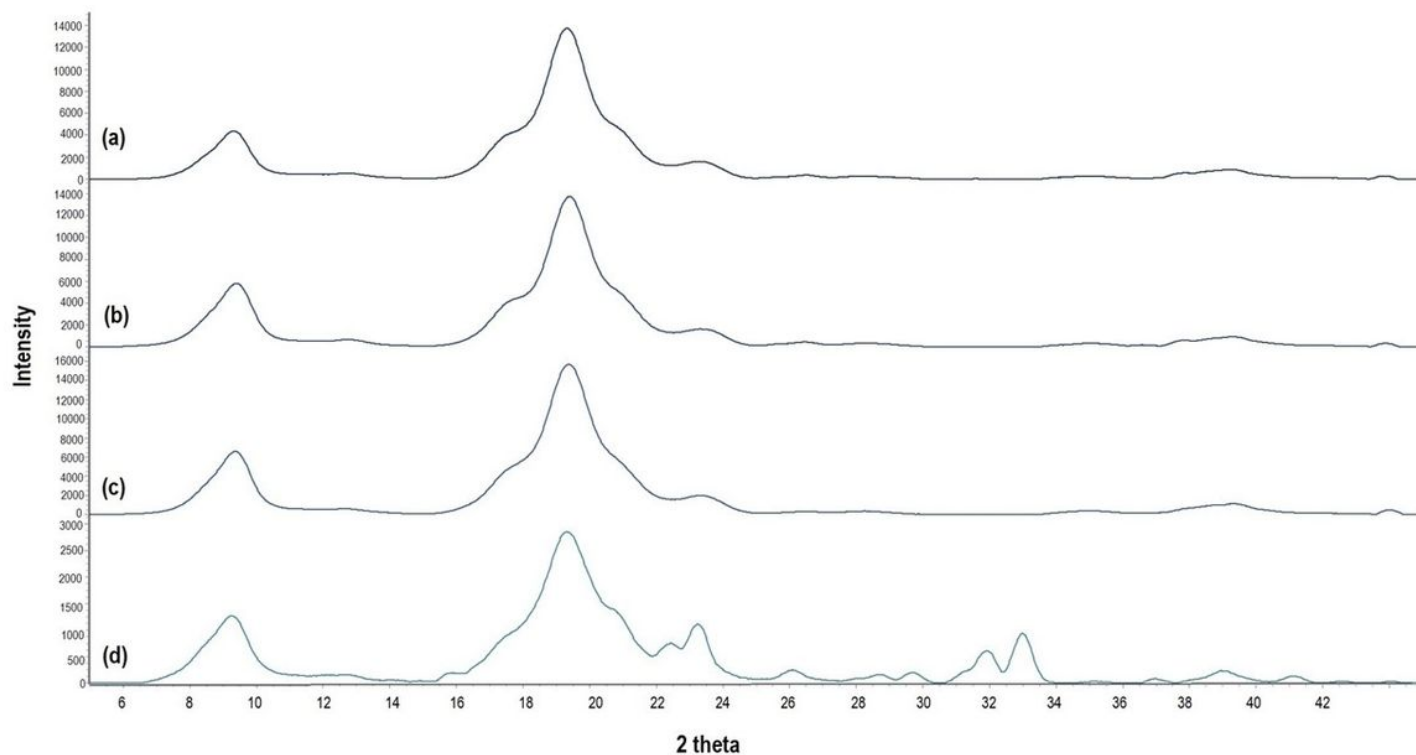


Figure 2

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

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