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Accepted Version

Hongkulsup, C., Khutoryanskiy, V. ORCID: <https://orcid.org/0000-0002-7221-2630> and Niranjan, K. ORCID: <https://orcid.org/0000-0002-6525-1543> (2016) Enzyme assisted extraction of chitin from shrimp shells (*Litopenaeus vannamei*). *Journal of Chemical Technology and Biotechnology*, 91 (5). pp. 1250-1256. ISSN 0268-2575 doi: <https://doi.org/10.1002/jctb.4714> Available at <https://centaur.reading.ac.uk/43511/>

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To link to this article DOI: <http://dx.doi.org/10.1002/jctb.4714>

Publisher: Wiley

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Enzyme assisted extraction of chitin from shrimp shells (*Litopenaeus vannamei*)

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Abstract

BACKGROUND: Chemical chitin extraction generates large amounts of wastes and increases partial deacetylation of the product. Therefore, the use of biological methods for chitin extraction is an interesting alternative. The effects of process conditions on enzyme assisted extraction of chitin from the shrimp shells in a systematic way were the focal points of this study.

RESULTS: Demineralisation conditions of 25°C, 20 min, shells-lactic acid ratio of 1:1.1 w/w; and shells-acetic acid ratio of 1:1.2 w/w, the maximum demineralisation values were 98.64 and 97.57% for lactic and acetic acids, respectively. A total protein removal efficiency of 91.10% by protease from *Streptomyces griseus* with enzyme-substrate ratio 55 U/g, pH 7.0 and incubation time 3 h is obtained when the particle size range is 50-25 µm, which was identified as the most critical factor. The X-ray diffraction and ¹³C NMR spectroscopy analysis showed that the lower percent crystallinity and higher degree of acetylation of chitin from enzyme assisted extraction may exhibit better solubility properties and less depolymerisation in comparison with chitin from the chemical extraction.

CONCLUSION: The present work investigates the effects of individual factors on process yields, and it has shown that, if the particle size is properly controlled a reaction time of 3 h is more than enough for deproteination by protease. Physicochemical analysis indicated that the enzyme assisted production of chitin seems appropriate to extract chitin, possibly retaining its native structure.

Keywords: Chitin; Protease; demineralisation; deproteination; characterisation

Introduction

White leg shrimp (*Litopenaeus vannamei*) is one of the most commercially exploited shrimp species in Asia and the Americas, which also generates a large amount of shrimp bio-waste. The global annual production of shell waste from crustacean harvest is estimated to 3.14 million metric tons dry weight.¹ Currently shrimp waste is used as a supplement in animal feed or simply discarded; however, it is an important source of chitin², which happens to be the second most abundant natural

1 polysaccharide after cellulose isolated from shells of crustaceans, insects and cell walls of fungi. This
2 polymer is a linear beta 1,4-linked polymer of N-acetyl-D-glucosamine (GlcNAc) and further
3 deacetylated to produce chitosan and its derivatives.³ Usually, chitin is bound to proteins, calcium
4 carbonate, lipids, and pigments in exoskeleton.^{4,5}

5 The production of chitin from shellfish waste consists of three steps: deproteination,
6 demineralisation, and decolorisation.⁶ Owing to covalent associations with other shell components,
7 industrial methods for chitin and chitosan isolation from different crustacean shells usually rely on harsh
8 chemical techniques. In the chemical process, the shellfish is treated with hydrochloric acid in order to
9 remove calcium carbonate.⁷ Generally, a strong alkali treatment (sodium hydroxide) is used to remove
10 proteins as well as other organic impurities. These procedures generate large amounts of chemical
11 wastes and increase partial deacetylation of chitin as well as hydrolysis of the polysaccharide, resulting
12 in inconsistent properties of the final products.^{8,9}

13 Some studies on the use of proteases from bacterial isolates for the deproteination of shellfish
14 wastes have been reported. Bustos and Michael¹⁰ used a protease from *Pseudomonas maltophilia* for
15 deproteination without alkali. The deproteination of crustacean shell reached to about 64% after
16 incubation for 6 days. Additionally, it has been reported that a bacterial protease from *Bacillus sp.*
17 TKU004 could be employed under optimized conditions in 2% slurry of squid pen powder.¹¹ The extent
18 of deproteination was found to be 63% after 2 days and peaked at 73% after 3 days. Proteolytic
19 protease from *Streptomyces griseus* is a mixture of exo- and endo-proteases and contains at least ten
20 proteolytic components, which therefore has very broad specificity, cleaving nearly all peptide bonds,
21 and is used in cases where extensive or complete degradation of protein is required.^{12,13}

22 However, the levels of residual protein and the reaction time for the biological process are
23 excessively high which, combined with higher costs compared to the chemical method, limit process
24 applicability on an industrial scale.¹⁴ The improvement of biological processes to produce chitin and its
25 derivatives is therefore necessary. Thus, despite its promising potential, limited information exists
26 within the published scientific literature on a biological extraction method; particularly how one could
27 make biological process for chitosan manufacture a competing alternative to the chemical method.

28 This experimental study aims to demonstrate the potential of enzyme assisted methods for
29 chitin production from shrimp shell waste in a systematic way. The effects of process conditions, such as
30 incubation time and enzyme-substrate ratio and particle size of substrate, on the deproteination and
31 demineralisation of shrimp shell waste are evaluated, with the aim of increasing the efficiency and
32 yields of this method. Moreover, the physicochemical properties of chitin isolated by enzyme assisted

1 production were investigated using solid-state ^{13}C nuclear magnetic resonance (^{13}C NMR) spectroscopy,
 2 X-ray diffraction (XRD), and scanning electron microscopy (SEM).

3 **Materials and Methods**

4 **Materials**

5 White leg shrimp shells (*Litopenaeus vannamei*) obtained from Kingfisher holding Ltd., Thailand,
 6 were initially treated with hot tap water to remove foreign materials and then dried in an oven at 60°C.
 7 Washed and dried shells were ground to powder in a blender. Commercial protease from *Streptomyces*
 8 *griseus* (EC no. 232-909-5, Sigma Aldrich) with a declared activity of 5.5 U/mg was used under the
 9 following conditions: pH between 5.0-9.0 and temperature between 20-40°C.¹³

10 **Chemical analysis of the raw materials**

11 The moisture and ash contents were analysed at 105°C and 550°C, respectively, in accordance
 12 with the AOAC standard methods 930.15 and 942.05.¹⁵ Total nitrogen content of each raw material was
 13 analysed by using the Kjeldahl method. Separately, for each raw sample, pure chitin was prepared to
 14 measure its nitrogen content, allowing to determine the crude protein content by multiplying nitrogen
 15 content attributed to protein by the factor of 6.25.¹⁶ Lipids were analysed gravimetrically by soxhlet
 16 extraction using hexane.

17 **Demineralisation by organic acid**

18 **Effect of incubation time on demineralisation**

19 Deproteinised shells were treated with 0.5 M lactic and 0.5 M acetic acid at a solid: acid ratio of
 20 1:0.7 w/w,¹⁷ and placed on a magnetic stirrer platform at 25 °C for 10, 20, 40 and 60 min. The pellet was
 21 washed with distilled water until the sample was neutral and then dried at 60°C.

22 **Effect of substrate-acid ratio on demineralisation**

23 Based on the results of the experiment described in the previous section, a time of 20 min was
 24 chosen to evaluate the effect of substrate-acid ratio on demineralisation, with 0.5 M lactic and 0.5 M
 25 acetic acids at ratios of 1:0.5, 1:0.7, 1:0.9, 1:1.0, 1:1.1, 1:1.2 and 1:1.4 w/w. The samples were then
 26 stirred for 20 min at 25°C and stored for analysis and deproteination. The degree of demineralisation
 27 (DDM) was showed as a percentage and calculated by the following equation¹⁸:

$$28 \quad \% DDM = \frac{\{(A_o \times O) - (A_R \times R)\}}{A_o \times O} \times 100 \quad (1)$$

1 where A_o and A_R are ash contents (%) before and after demineralisation respectively, while O and R
 2 represent the mass (g) of initial and demineralized residue respectively on dry weight basis.

3 **Deproteination by protease**

4 Effect of incubation time

5 Shrimp shell powder-protease ratio of 55U/g was dissolved in 0.5M KH_2PO_4 buffer (pH=7) and
 6 incubated at 37°C for 10, 30, 60, 120, 180, 300 and 600 min. Subsequently, the reaction was stopped by
 7 placing the mixture in a 90°C water bath for 30 min. This was followed by centrifugation, and washing
 8 the pellet with distilled water until the sample had neutral pH 7.0. The pellet was washed with 99%
 9 ethanol to remove unwanted colour and then dried at 60°C, whereby the sample was kept for analysis.

10 Effect of enzyme-substrate (E/S) ratio

11 Based on the results of the experiment described in the previous section, a time of 180 min was
 12 chosen to evaluate the effect of enzyme-substrate ratio on the degree of deproteination, with shrimp
 13 shell powder-protease ratio of 10, 30, 55 and 80 U/g dissolved in 0.5M KH_2PO_4 buffer (pH=7) and
 14 incubated at 37°C.

15 Effect of pH

16 Based on the results of the previous experiment, a time of 180 min and enzyme-substrate (E/S)
 17 ratio of 55 U/g were chosen to evaluate the effect of pH on the degree of deproteination, with 0.5 M
 18 KH_2PO_4 buffer in a pH range varying from 4.0 to 9.0 and incubated at 37°C.

19 Effect of particle size

20 Holding the incubation time of 180 min, an enzyme-substrate ratio of 55 U/g, temperature of
 21 37°C and pH=7, the effect of particle size on the rate of deproteination was determined by selecting
 22 the following sieved fractions: 300-212, 212-105, 105-50 and 50-25 and <25 μm . The larger particle sizes
 23 were expected to give lower reaction rates and were not considered. The degree of deproteination
 24 (DDP) was showed as percentage and calculated by the following equation¹⁸:

$$25 \quad \% DDP = \frac{\{(P_o \times O) - (P_R \times R)\}}{P_o \times O} \times 100 \quad (2)$$

26 where P_o and P_R are the protein concentrations (%) before and after hydrolysis respectively, while O and
 27 R represent the mass (g) of original sample and hydrolysed residue respectively on dry weight basis.

28 **Chemical extraction of chitin.**

1 The same shrimp shell powders were demineralised with 1 M HCl in the ratio 1:15 (w/v) at 35°C
 2 for 12 h. The demineralised shells were then deproteinised with 1 M NaOH solution in the ratio 1:10
 3 (w/v) at 35°C for 24 h. Crude chitin was obtained after washing until the filtrate was neutral.

4 **Physicochemical characterisation of chitins**

5 Solid-state ^{13}C NMR spectroscopic

6 The NMR spectra of chitin were carried out by the CP-MAS ^{13}C NMR method (cross-
 7 polarization/magic-angle-spinning) using a BRUKER Avance III 500 MHz (BRUKER, Germany). NMR
 8 spectra of chitin were indexed at a ^{13}C frequency of 75.5 MHz. CP-MAS technique was used with the
 9 following conditions: 5 s of ^{13}C spin lattice relaxation time; 8 ms of contact time; and 8 kHz of a spinning
 10 rate.¹⁹ The degree of acetylation (DA) of chitin was computed from the relative intensities of the
 11 resonance of the ring carbon ($I_{C1}, I_{C2}, I_{C3}, I_{C4}, I_{C5}, I_{C6}$) and methyl carbon (I_{CH3}) obtained from ^{13}C NMR
 12 patterns by the following equation²⁰:

$$13 \quad \% DA = \frac{I_{CH3}}{(I_{C1} + I_{C2} + I_{C3} + I_{C4} + I_{C5} + I_{C6}) / 6} \times 100 \quad (3)$$

14 X-ray diffraction (XRD)

15 X-ray diffraction measurements were used to analyse the crystallinity of the samples and their
 16 spectra were indexed using a D8 advance XRD (Bruker, Germany) with $\text{CuK}\alpha$ radiation ($\lambda=1.5406$). Based
 17 on the XRD analysis, the 2θ angle was scanned from 5° to 45° and the counting time was 2.0 s with an
 18 angle step width of 0.05° . The crystallinity index (%Crl) was determined as shown in the following
 19 Equation.²¹

$$20 \quad \% Crl = \frac{(I_{110} - I_{am})}{I_{110}} \times 100 \quad (4)$$

21 where I_{110} is the maximum intensity at $2\theta \cong 20^\circ$ and I_{am} is the intensity of amorphous diffraction at
 22 $2\theta \cong 16^\circ$.

23 Scanning electron microscopy (SEM)

24 SEM (Cambridge 360 Stereoscan, UK) was applied to determine the surface characteristics of
 25 chitin at 3000x magnification. The samples were fixed on a sample holder, dried by a critical point dryer,
 26 and coated with a thin gold layer of 3 nm by a sputter coater for conductivity.

27

28 **RESULTS AND DISCUSSION**

29 **Proximate composition of shrimp shells powder**

1 Chitin was extracted from white leg shrimp (*Litopenaeus vannamei*) shells. The chemical
2 compositions of the raw materials were determined following methods described above. The results
3 obtained are given in Table 1. It is evident from the table that the values given compare favourably with
4 the normal values for protein and ash content reported by Rodde *et al.*²² For analysing particle-size
5 distribution, ground shrimp shell was sieved by test sieves at different sieve mesh between 300, 212,
6 105, 50 and <25 μm . The predominant fraction of ground shrimp shells ranged between 212 -105 μm ,
7 and this fraction was used as the starting material.

8 **Demineralisation**

9 The percentage demineralisation represents the removal of inorganic mineral, mostly calcium
10 carbonate, from shrimp shell. It is clear from Fig. 1 that the percentage demineralisation increased with
11 time up to 20 min to reach a maximum value of 73.85% and 65.66% in the case of lactic and acetic
12 acids respectively, beyond which it remains constant. The greater percentage demineralisation achieved
13 with lactic acid than acetic acid at the same shell-acid ratio and reaction time, is probably because lactic
14 acid ($\text{pK}_a = 3.86$) is a stronger acid than acetic acid ($\text{pK}_a = 4.76$). In demineralisation of chitin, the
15 reaction time is an important factor since extended reaction times increase the cost and also affect the
16 properties of the purified chitin.¹⁷ It is obvious from Fig. 1 that, with a stoichiometrically adequate
17 amount of acid, the demineralisation reaches a maximum value within 20 min. Any excess contact time
18 can potentially degrade chitin properties.

19 Fig. 2 indicates that % demineralisation increased significantly when the shells-acid ratio was
20 increased. When shells-lactic acid ratio was 1:1.1 w/w, almost complete demineralisation was observed
21 (98.64%). While shells-acetic acid ratio was 1:1.2 w/w, total minerals removal efficiency was 97.57%.
22 Mahmoud *et al.*¹⁷ investigated the demineralisation of shrimp shells by organic acids and reported that
23 the effectiveness of lactic acid in removing the minerals from the shells was higher than acetic acid.
24 Under the conditions: shells-acid ratio of 1:20, temperature of 24 °C and incubation time of 2 h, the
25 percentage demineralisation achieved were 97.4% and 86.36% for lactic and acetic acids, respectively.
26 The presence of stoichiometrically adequate amount of acid (regardless of type) is a pre-requisite for
27 the complete removal of minerals^{6,23} and any excess used only increases the production costs and has
28 an adverse environmental impact.

29 **Deproteination**

30 The extent of deproteination is defined as the percentage of protein removed from shrimp shell
31 powder (dry weight). A significant percentage of deproteination occurs in the first 30 min, and it
32 increases gradually with incubation time up to around 300 min, and then it levels off at a value just over

1 82.07% (Fig. 3). The reason for the extent of deproteination levelling off may be due to a combination of
2 factors. It is quite possible that with the loss of proteins and other solubles, the number of active sites
3 for the enzyme to adsorb may have reached a limit. Further, the enzyme may also be unable to access
4 the protein substrate remaining, because of the lower concentration and the entrapment of the protein
5 in a calcium rich matrix.⁵ This indicates that the maximum possible substrate has been used up for a
6 given particle size.^{24,25} This hypothesis also suggests that the extent of deproteination can be altered by
7 changing the particle size, which will be shown to be the case in the following section. Since there is no
8 significant change in the protein content after 180 min, an incubation time of 3 h was deemed to be
9 suitable in the further experiments. It is interesting to note that earlier workers in this area have used a
10 much longer incubation time of 24 h or even greater,^{10,11,26} which is really not necessary.

11 Fig. 4 suggests that % deproteination increases with the increase in enzyme-substrate ratio from
12 10 to 55 U/g, beyond which there is no further increase in the % deproteination which remains at
13 58.61%. Shi *et al.*²⁵ has made a similar observation working with nuclease and DEAE cellulose, and
14 observed that immobilized enzyme activity increased with the increase of enzyme content. However,
15 once the binding sites on the surface of DEAE cellulose were saturated, immobilized enzyme activity
16 cannot be increased by an increase of enzyme content. In this study, the enzyme-substrate ratio of 55
17 U/g of shrimp shell powder seems appropriate for further studies.

18 To determine the effects of optimum pH on the deproteination of shrimp shells waste, the
19 initial pH values have been kept in the range of 4.0-9.0 by the addition of 1 M NaOH, and the results are
20 shown in Fig. 5. The optimum pH was around 7.0 under these conditions and the proteolytic activities
21 decreased over pH=8. Protease from *Streptomyces griseus* has optimal activity at pH = 7.0-8.0. However,
22 individual components are mentioned to retain activity over a much wider pH range (pH = 5.0-9.0),
23 which is due to the differences in substrates, buffer solutions, and reaction time.¹³

24 The effect of particle size range on % deproteination is shown in Fig. 6. The % deproteination
25 increases as the particle size decreases and a maximum value of 91.10% is obtained when the
26 particle size range is 50-25 μm . The particle size of the substrates can influence mass transfer of the
27 substrates to contact with the enzymes, and subsequently influence the enzyme-catalysed activity.²⁷
28 Therefore, the substrates with finer particles showed an improvement in enzyme-catalysed activity due
29 to an increase in surface area.²⁸ This is also in agreement with the report of Meyer,²⁹ who stated that
30 enzyme activity increases with increasing surface area/volume ratio, showing the importance of
31 exposed surface area on the starch hydrolysis reaction.

1 However, a further decrease in the particle size ($<25\mu\text{m}$) did not bring about a significant
2 difference in % deproteination. The use of particles smaller than $25\mu\text{m}$ had no further increase in %
3 deproteination, which may be attributed to these fine particles forming a more viscous dispersion.
4 There are other explanations for this effect reported in literature. Munawar *et al.*³⁰ suggested that the
5 lower conversion was due to overlapping of the particles which masks some of the active sites.
6 Petnamsin *et al.*³¹ also revealed that the finer particles of milled starch granule lost their crystalline
7 regions, and tended to form clumps during blending process. This may result in a decrease in surface
8 area of substrates which decreased the enzyme-catalysed activity. It is important to note that %
9 deproteination as high as 91.10% obtained in just 3 h was significantly better than many other
10 proteases reported in previous studies.^{10,11,26,32} This is because these authors have not controlled the
11 particle size and used the values which are much higher than the values used in this work.

12 **Characterisation of chitin**

13 The crystalline structures of the chitins from enzyme assisted extraction and chemical extraction
14 were characterized by x-ray powder diffraction (Fig. 7). It was found that five crystalline peaks were
15 observed in the 2θ range of $5-50^\circ$ and were recorded as 020, 110, 120, 101 and 130 corresponding to α -
16 chitin structure.³³ The x-ray diffraction patterns of the chitin from chemical extraction showed higher
17 intense peak areas than the chitin from protease-lactic and from protease-acetic extraction, which
18 indicated that the chitin from chemical extraction had higher crystallinity.³⁴

19 In this study, the crystallinity index of the chitin from chemical extraction, protease-lactic and
20 from protease-acetic extraction were 89.12%, 82.56% and 80.64%, respectively (Table 2). It was found
21 that, the lower percent crystallinity of chitin obtained by enzyme assisted production may exhibit higher
22 solubility properties in comparison with chitin produced from the chemical production, owing to the
23 more severe process conditions during the chemical method.³⁵ Thus, the enzyme assisted production of
24 chitin under mild conditions seems appropriate to prevent the degradation and partial
25 depolymerisation of native chitin.

26 Solid state ^{13}C NMR spectroscopy is a reliable technique to analyse the differences in
27 polysaccharide composition and structure, allowing a direct measurement of the degree of
28 acetylation.³⁶ Fig. 8 shows ^{13}C NMR data of chitins obtained from chemical extraction, protease-lactic
29 and protease-acetic extraction. The spectrum consisted of eight well-defined signals, including C1-C6
30 carbons of N-acetyl glucosamine monomers were detected between 50 and 110 ppm. The methyl group
31 of the acetyl group was around 23 ppm, while the carbonyl group generated a peak at around 173 ppm.
32 The ^{13}C NMR signals of C-3 (73 ppm) and C-5 (75 ppm) were obviously isolated into two peaks,
33 corresponding to the typical of α -chitin structure.³⁷ Degree of acetylation (DA) of chitin prepared by

1 chemical extraction, protease-lactic and protease-acetic extraction were 81.47%, 90.83% and 92.67%,
2 respectively (Table 2). It was showed that DA of the two chitins isolated by enzyme assisted extraction
3 was higher than that of chitin obtained by chemical extraction. Observed differences in the DA values
4 could be attributed to the partial deacetylation of polymer occurring in the chemical treatment. The
5 severity of demineralisation and deproteination conditions affects the possible degradation of the
6 polymer leading to inconsistent physicochemical properties in the purified chitin.³² Hence, the enzyme
7 assisted production of chitin is a promising alternative method to extract chitin, possibly retaining a
8 native structure of chitin.

9 Fig. 9 shows the SEM images of chitin from chemical extraction (A), chitin from protease-lactic
10 extraction (B), and chitin from protease-acetic extraction. The SEM image of chitin from chemical
11 extraction displayed a tightly packed morphology, while the surface of two chitins from enzyme assisted
12 extraction showed less density and slightly microfibrillar structure (Fig. 9B and 9C), which may result
13 from the higher crystallinity index of chitin from chemical extraction method.

14

15 **CONCLUSION**

16 Effective demineralisation conditions of 25°C, 20 min, shells-lactic acid ratio of 1:1.1 w/w; and
17 shells-acetic acid ratio of 1:1.2 w/w, the maximum demineralisation values were 98.64 and 97.57% for
18 lactic and acetic acids, respectively. A total protein removal efficiency of 91.10% with enzyme-substrate
19 ratio 55 U/g and incubation time 3 h is obtained when the particle size range is 50-25 μm , which
20 is a major improvement over earlier studies. The present work represents a systematic attempt to
21 investigate the effects of individual factors on process yields, and it has shown that, if the particle size is
22 properly controlled a reaction time of 3 h is more than enough – which makes the biological process for
23 chitin manufacture a competing alternative to the chemical method. The XRD and ¹³C NMR analysis
24 indicated that the enzyme assisted production of chitin is a promising alternative method to extract
25 chitin, possibly retaining a native structure of chitin. Of course, further research work is needed, that
26 takes a holistic view of deproteination and demineralisation, to result in a robust biological method for
27 chitin manufacture.

28

29 **Acknowledgements**

30 The authors are grateful to the Chemical Analysis Facility (CAF) in the Department of Chemistry,
31 University of Reading for the use of the NMR, XRD and SEM. Particular thanks go to: Dr. Radoslaw M.
32 Kowalczyk , Mr. Nick Spencer, and Dr. Peter Harris.

33

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2 **Tables**

3 **Table 1** Proximate composition on % dry basis of ground shrimp shell; the remaining represents the
4 percentage of chitin.

Composition	Amount (%)
Protein	28.40 ± 0.58
Ash	47.66 ± 0.61
Moisture	4.69 ± 0.07
Lipid	0.82 ± 0.04

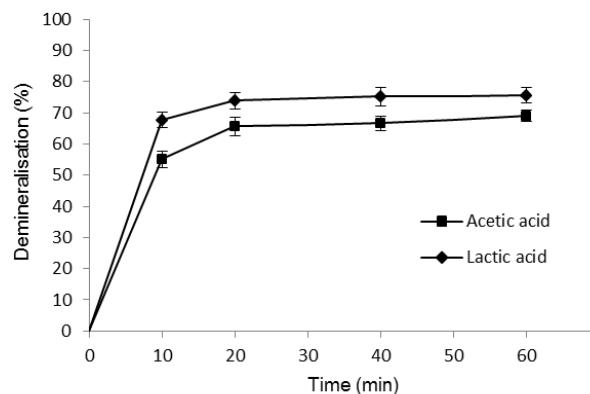
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6 **Table 2** Degree of acetylation (DA) and crystallinity index (CrI) of chitin obtained by chemical extraction,
7 protease-lactic and by protease-acetic extraction

Samples	DA (%)	CrI (%)
Chitin obtained by chemical extraction	81.47 ± 0.70	89.12 ± 0.65
Chitin obtained by protease-lactic	90.83 ± 0.85	82.56 ± 0.92
Chitin obtained by protease-acetic	92.67 ± 0.61	80.64 ± 1.03

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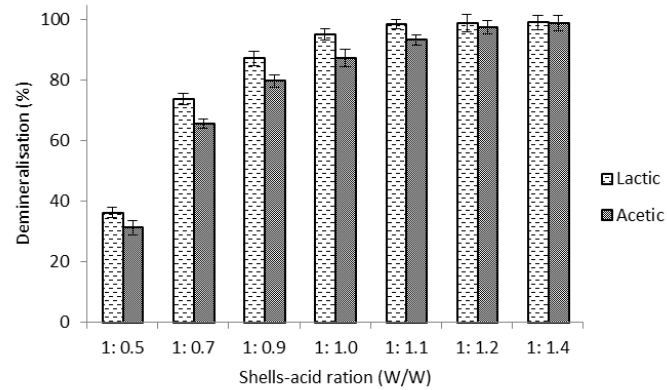
10 **Graphs**

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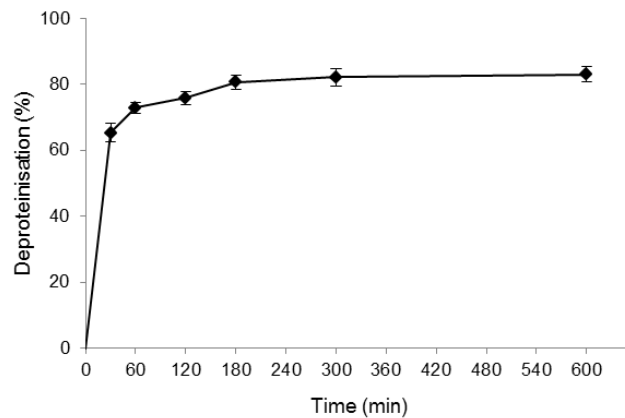
13 **Figure 1.** Effect of incubation time on demineralisation. Incubation conditions: 0.5 M lactic and acetic
14 acids; shells-acid ratio = 1:0.7 w/w; temperature = 25°C.

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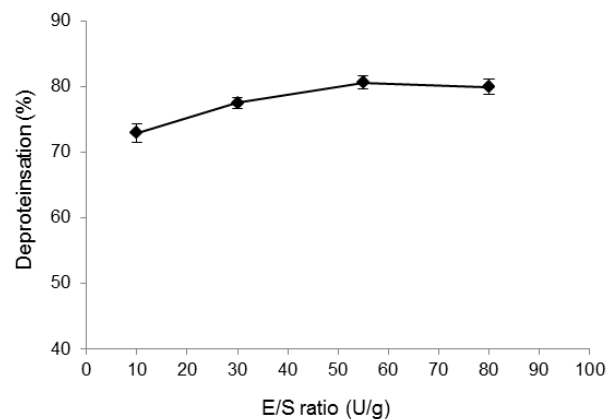
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2 **Figure 2.** Effect of shrimp shell-acid ratio on demineralisation. Incubation conditions: 0.5 M lactic and
3 acetic acids; temperature = 25°C; incubation time = 20 min.

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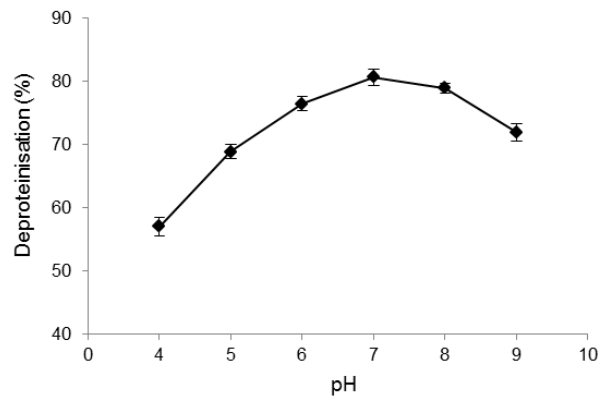


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6 **Figure 3.** Effect of incubation time on the rate of deproteination. Incubation conditions: protease
7 activity = 55 U/g; solid liquid ratio = 1:25; pH = 7; temperature = 37°C; particle size range = 212 -105 μm.

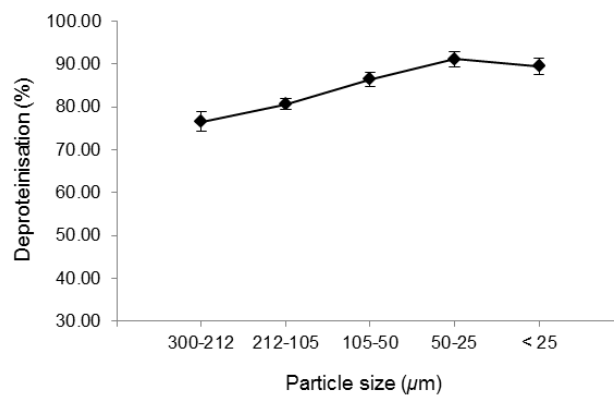
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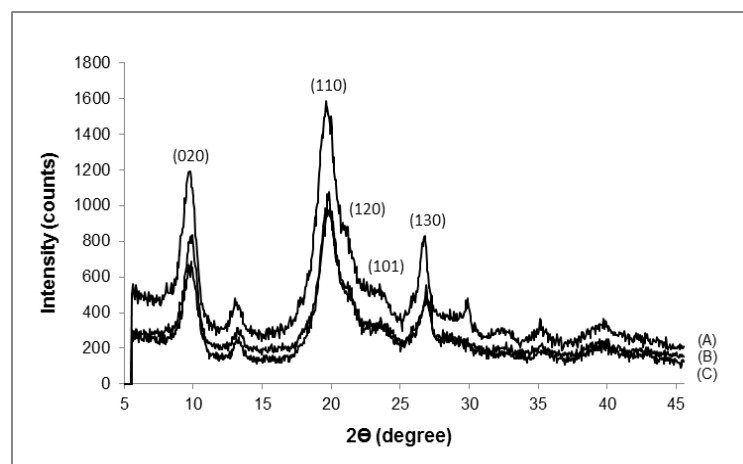
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10 **Figure 4.** Effect of enzyme-substrate ratio on the degree of deproteination. Incubation conditions: solid
11 liquid ratio = 1:25; pH = 7; temperature = 37°C; particle size range = 212 -105 μm; incubation time = 3 h.



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2 **Figure 5.** Effect of pH on the degree of deproteination. Incubation conditions: protease activity = 55 U/g;
3 solid-liquid ratio = 1:25; temperature = 37°C; particle size range = 212 -105 μm ; incubation time = 3 h.

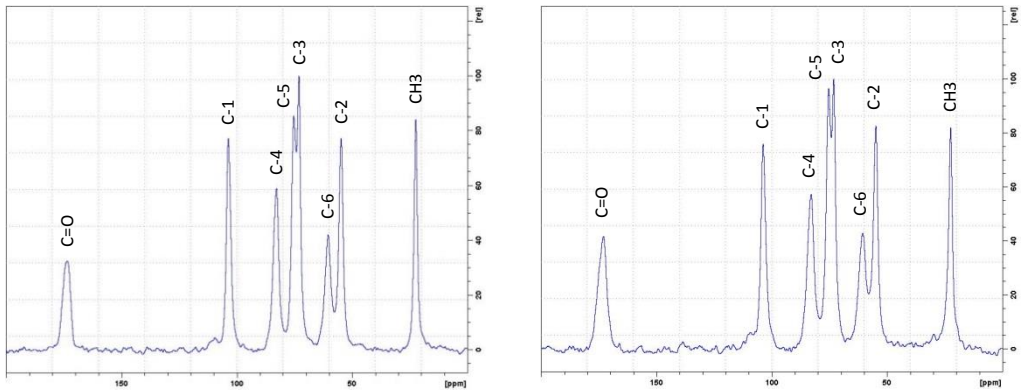


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5 **Figure 6.** Effect of particle size of shrimp shell powder on deproteination. Incubation conditions:
6 protease activity = 55 U/g; solid-liquid ratio = 1:25; pH=7; temperature = 37°C; incubation time = 3h.



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10 **Figure 7.** X-ray diffraction patterns of chitin from chemical extraction (A), chitin from protease-lactic
11 production (B), and chitin from protease-acetic production (C).

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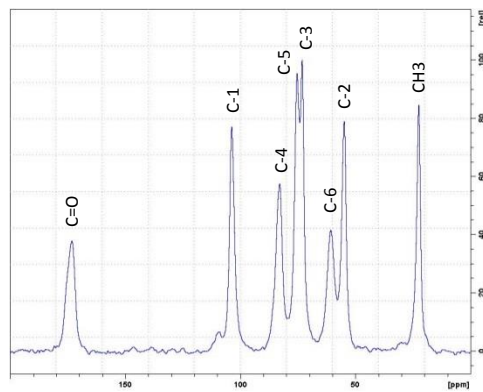
(A) chitin from chemical extraction

(B) chitin from protease-lactic production

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(C) chitin from protease-acetic production

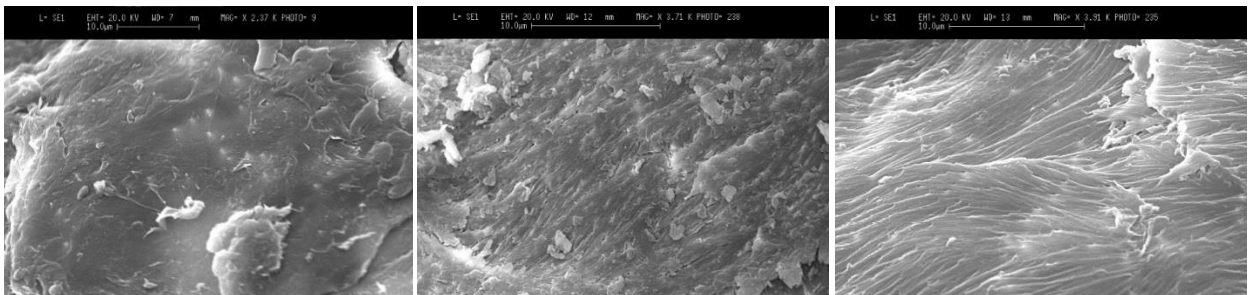
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7 **Figure 8.** $^{13}\text{C}/\text{CP}/\text{MAS}$ NMR spectrum of chitin from chemical (A) chitin from protease-lactic production (B),
8 and chitin from protease-acetic production (C).

9

10 **Pictures**



(A)

(B)

(C)

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13 **Figure 9.** SEM images of chitin from chemical (A), chitin from protease-lactic production (B), and chitin
14 from protease-acetic production (C).

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