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Project title : Experimental procedures of Different methods for extraction of chitin from shrimp shells

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Abstract

Chitin & chitosan are catatonic polymers found abundantly in the crustacean, insect, arthropod exoskeletons and mollusc. There are three main methods of extraction of chitin from the above sources namely extraction by using chemical methods, consists of main steps Demineralization, Deproteinization, Discoloration. These polymers can also be extracted through the biological extraction methods involving the use of microorganisms (lactic acid bacteria), and can also be extracted from enzyme assisted methods by using enzymes like proteases, deacetylase. Another novel method for extraction of chitin from shrimp shells by using citric acid &Deep eutectic solvents, which removes the protein and minerals efficiently, in this method efficiency of demineralization is more than 98% and efficiency of deproteinization is more than 88% Chitin and chitosan have biodegradable &biocompatible properties being applied in the pharmaceutical, cosmetic, food, biochemical, chemical and textile industries. In shrimp shell the chitin and chitosan percentages are 26% & 17% respectively.

Table 1- CONTENTS

1. INTRODUCTION

Chitin is considered the second most plentiful biopolymer in the nature after cellulose. It is a linear amino polysaccharide comprised of β-(1-4)-connected 2-deoxy-2-acetamido-D-glucose units. The main source of chitin is crustacean shells derived from shrimp and crab. It is reported that the shrimp accounts for about 45% of processed seafoods. Shrimp processing industry produces massive amount of byproducts such as shrimp shells, these shrimp by-products are usually applied for low value animal feeds and biological fertilizers. Therefore, it is particularly critical to convert by products into high-value products. Shrimp shells are mainly composed of chitin, proteins, minerals.

In current industrial processing methods for chitin preparation includes chemical treatments, enzymatic reactions, microbial fermentation and using of organic acids and deep eutectic solvents.

2. CHEMICAL TREATMENT METHOD

Methodology

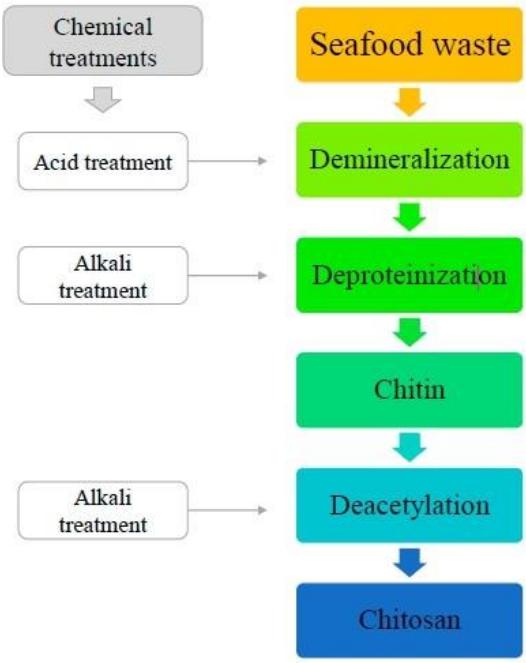
The crab (C. Amnicola) and pink shrimp (P. notauli's) used in this study were obtained from the University of Lagos lagoon and a local market in Lagos State, Nigeria respectively and their shells processed into powder.

Materials

Hydrochloric acid (~37%), and sodium hydroxide pellets (97%) were purchased from Fisher Scientific International Inc., USA while potassium permanganate (99.0%) and oxalic acid dihydrate, C2H2O4.2H2O, (99.5%) were purchased from J. T. Baker Co., USA.

Extraction of chitin

The crab (C. Amnicola) and shrimp (P. nuptials) tissues found loosely in and around the shell wastes were disposed of. The sole resulting shells were thoroughly scrubbed, washed, cleaned and dried. A Morphy Richards 100-W blender was used to thoroughly pulverize the resulting dried shell samples, which were passed through a 250-μm sieve, collected in plastic bags and then conserved at ambient temperature of (28±2°C) for supplemental analyses.



Flow diagram 1- chemical process methodology

Demineralization

The process of extraction of chitin from the shell wastes involved demineralization with 2 - 4 M hydrochloric acid for 12-24 hr at ambient temperature (28
± 2°C) with constant agitation speed of 100 rpm at a solvent to solid ratio of 10:1 (w/v). Separation of the resulting acid-shell mixture was done by vacuum filtration and the Olaf Dehan et al. 3 solid washed thoroughly with distilled water until a neutral pH of 7 was achieved.

Deproteinization

The demineralized shells were deproteinized with 1.5 - 3.5 M sodium hydroxide for 1 - 3 hr. at (70 ± 0.5°C) with constant agitation speed of 100 rpm at a solvent to solid ratio of 15:1 (w/v). The resulting heterogeneous mixture was mixed thoroughly to form insoluble particles of chitin and subsequently separated by filtration using a vacuum pump. The precipitate was thoroughly washed with distilled water to bring down the pH to 7.0.

Decoloration

The extracted crude chitin from the treated crustacean shells was decolorized by treating the crude chitin with 10 g/L potassium tetra oxo manganate (VII), KMnO4, for 1 h and then reacted with 10 g/L oxalic acid dehydrate, C2H2O4.2H2O for another 1 h. The decolorized chitin was then separated via vacuum filtration and washed

with distilled water until the pH = 7.0. The sample was dried in an oven at 80°C for 3 hand the dry weight of the decolorized chitin was recorded.

Deacetylation of chitin

The extracted decolorized chitin was converted to chitosan by the process of deacetylation. The chitin was immersed in 30-50% w/w concentration of sodium hydroxide solution for 1.5-4.5 hr. at (60- 100) ±0.5°C at a constant agitation speed of 100 rpm at a solvent to solid ratio of 10:1 (w/v). The mixture was separated by filtration powdered by a vacuum pump and the resulting solid matter/particles thoroughly washed with distilled water until the pH was neutral. The solid matter obtained (that is, chitosan) was then dried in a muffled oven at 80°C for 3 h and the dry weight was Recorded.

Findings

Optimum conditions for the maximum yields of derived chitin and derived chitosan polymers and the maximum derived chitosan DDA from pinkshrimp shell wastes as (3.25 mol/dm3 hydrochloric acid aqueous solution, a demineralization time of 19.03 h, 2.43 mol/dm3 of sodium hydroxide aqueous solution and a deproteinization time of 2.03 h), (a concentration of 50% w/w NaOH aqueous solution, a deacetylation temperature of 87.9°C and a deacetylation period of 145.26 min) and (NaOH solution of 50% w/w concentration, temperature for deacetylation=97.2°C and a deacetylation time of 90 min).

3. BIOLOGICAL METHOD OF EXTRACTION OF CHITIN FROM SHRIMP WASTE

Biological methods of chitin extraction This is an advanced and a new technique for chitin extraction. It can be done using proteolytic microorganisms or fungi or purified enzymes. It results in the production of oligomers with an optimum degree of polymerization for different applications and does not denature the chitin.

Enzymatic extraction can be highly specific and yield chitin with higher molecular weights. Besides environmental advantages as compared to the chemical method, the use of enzymes also eliminates the hazards associated with the reactive reagents. Even the extracting cost of chitin by biological method can be optimized by reducing the cost of carbon sources (glucose, dextrose, lactose, sucrose).

There are following steps involved in chitin extraction

Preparation of inoculums

Take the lactobacillus cell and transfer in 5 ml MRS broth and incubate at 30℃ for 24 hrs. Then take 2 ml of starter culture and transfer in 100 ml sterile MRS broth and incubate at 30 °C for a further 24 hrs. Now, these inoculums are ready for fermentation.

Fermentation

Take the shell fish waste and grind them properly. Then add 10% of any carbon sources followed by 10% culture inoculums. Then incubate it for 180hrs followed by filtering and the obtained solid cake is dried in hot air oven.

Protein separation

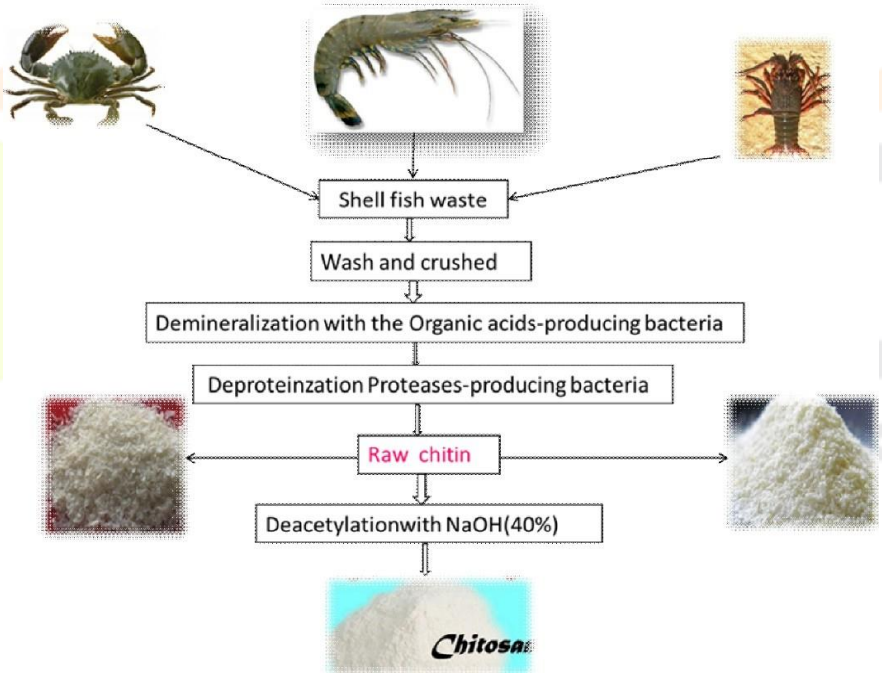
Separation of protein is carried out by proteases secreted into the fermentation medium. In addition, deproteinization can be made by adding exo-proteases or by proteolytic bacteria.

calcium carbonate separation

It is carried out by lactic acid producing bacteria through the conversion of an added carbon source.

Organic acid producing bacteria	Protease producing bacteria
Lactobacillus plantarum	*Pseudomonas aeruginosa*
L. salivarius	*P. acidilactici*
L. paracasei	*Bacillus subtilis*
Serratia marcescens	*B. firmus*

Table 2- Different bacteria used for the deproteinization and demineralization



Flow diagram 2 - showing biological method of chitin production

Findings

In biological method, chitin is produced by using lactic acid bacteria and produces a good quality product. It also leads to a protein rich liquid fraction which can be used as human and animal feed. But the use of this method is still limited to laboratory scale because demineralization and deproteinization have not yet reached up tothe desired yields as compared to chemical method. So, the optimization and standardization of the extraction process is necessary to produce high yield with minimum cost.

4. TWO-STEP SEPARATION OF CHITIN FROM SHRIMP SHELLS USING CITRIC ACID AND DEEP EUTECTIC SOLVENTS WITH THE ASSISTANCE OF MICROWAVE

Materials

The shrimp shells were dried in the oven at 90℃ and pulverized into powder with a particle size of 0.355 nm by using a grinder. Choline chloride were acquired from Yauney Bio-Technology (Shanghai, China). Urea and Coomassie brilliant blue G-250 were purchased from Solario (Shanghai, China). Betaine hydrochloride was purchased from Macklin (Shanghai, China). Ethylene glycol, glycerol, citric acid, hydrochloric acid, sodium hydroxide, lithium chloride (LiCl) and N, N- dimethylacetamide (DMAc) were acquired from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Synthesis of DESs (DEEP EUTECTIC SOLVENTS)

In a typical process, the hydrogen bond donor (HBD) and acceptor (HBA) ingredients were mixed and heated at a certain temperature with magnetic stirring at an optimal ratio until homogeneous and transparent solutions were obtained.

HBA	HBD	Molar Ratio (HBA:HBD)	Heating Temperature (°C)
Betaine HCl	Urea	1:2	50
ChCl	Urea	1:2	60
ChCl	Ethylene Glycol	1:2	60
ChCl	Glycerol	1:2	90

Table 3-List of deep eutectic solvents (DESs) used in this study

Details of the preparation of the DESs are shown in figure below:

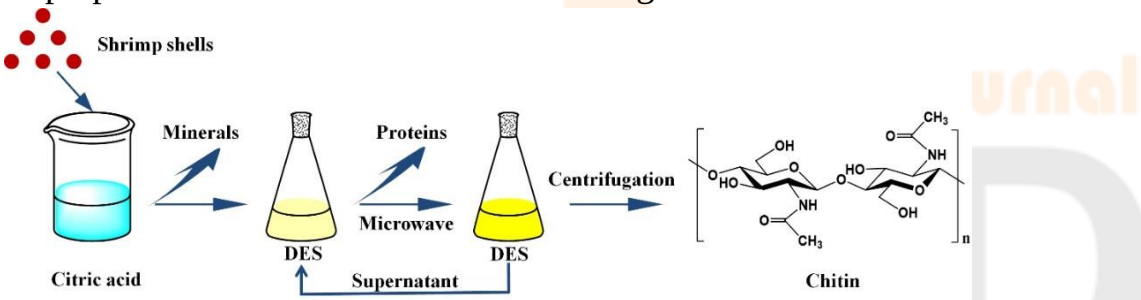


Figure 1 –Schematic representation of deep eutectic solvent (DESs) method

Preparation of chitin

. Chitin preparation by utilizing citric acids and DESs was conducted as follows. The shrimp shells were treated with 10% citric acid for demineralization. The pretreated samples were dispersed in DESs with different shrimp shell/DES ratios of 1:5, 1:10, 1:15, and 1:20. Then, the mixtures were heated by microwave irradiation at various times (1 min, 3 min, 5 min, 7 min and 9 min). To avoid excessive heating of the mixtures, 2 to 3 s pulses were used, and the mixtures were stirred manually with a glass rod to ensure uniform dispersion of the shrimp shell powders in the DESs. Next, chitin and the DESs were separated via centrifugation. The chitin was gathered and rinsed with distilled water. The supernatants were collected for next use. After separation, the chitin was dried in an oven at 80 C. The chitin yield was evaluated by calculating the ratio of the weight of extracted chitin to raw shrimp shells The DESs were used for five cycles without purification, and the recyclability of the DESs was assessed.

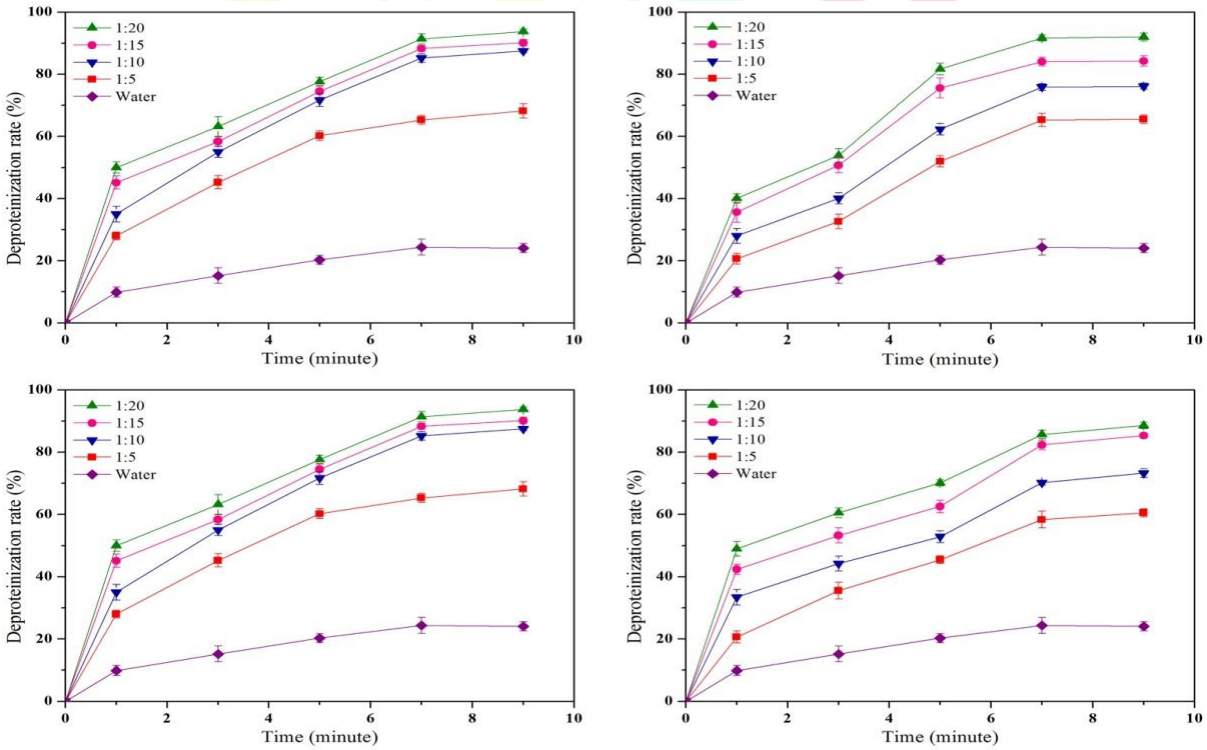


Figure 2- Deproteinization rates of (a) betaine HCl-urea, (b) ChCl-urea, (c) ChCl- ethylene glycol, and (d) ChCl-glycerol at shrimp shell/NADES ratios of 1:5, 1:10, 1:15,and 1:20

DEPROTEINIZATION

Deproteinization effect of citric acid and the DESs treatment were evaluated with different shrimp shell/DES ratios and microwave heating times. After pretreating shrimp shells with citric acid, the demineralization rate was 98.15±0.3%. The deproteinization effect of DESs treatment is shown in Figure 2. The deproteinization effect increases with increasing of the shrimp shell/DES ratio from 1:5 to 1:20. This result demonstrates that the deproteinization effect could be improved at higher shrimp shell/DES ratios. Microwave heating time is also an important factor affecting the deproteinization rate. The deproteinization effect continuously improved with increasing microwave heating time, and no significant variation was observed after 7 min at all ofthe measured shrimp shell/DES ratios. The maximal deproteinization rates of betaine HCl-urea, ChCl-urea, ChCl-ethylene glycol, and ChCl-glycerol reached 93±0.8%, 92.0±1.2%, 90.6±1.4% and 88.6±1.1%, respectively. The yields of chitin extracted by betaine HCl-urea, ChCl-urea, ChCl-ethylene glycol, and ChCl-glycerol were 23.6±0.6%, 25.1±1.3%, 24.8±0.7% and 22.5±1.0%, respectively, which were higher than that of the acid/alkali-extracted chitin (17.7±1.8%).

SEM analysis

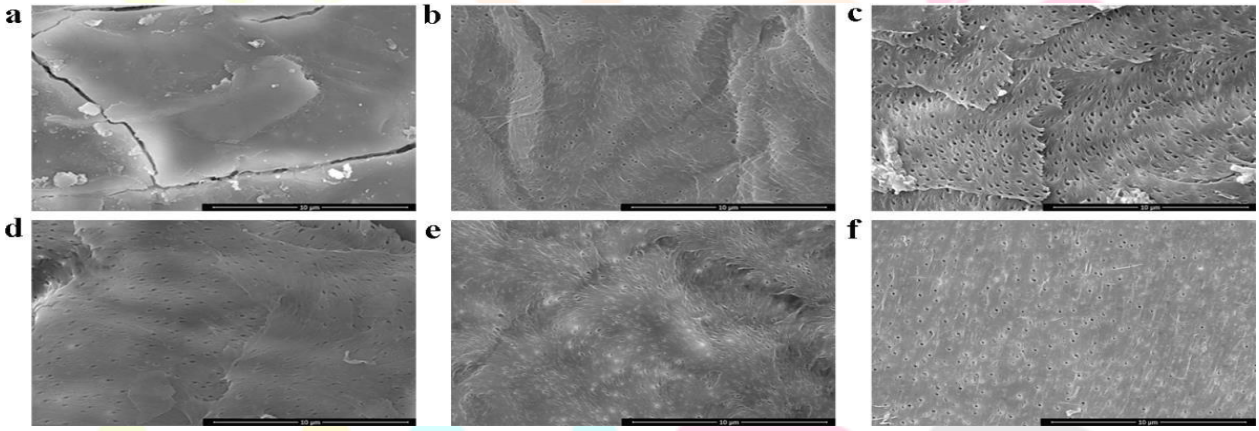


Figure 3- SEM images of (a) shrimp shells, (b) chitin extracted by the acid/alkali method,
(c) chitin extracted by betaine HCl-urea, (d) chitin extracted by ChCl-urea, (e) chitin extracted by ChCl-ethylene glycol, and (f) chitin extracted by ChCl-glycerol.

SEM images of the shrimp shells, acid/alkali-prepared chitin, and DES- prepared chitin are presented in Figure 3. The morphology of the DES-prepared chitin (Figure 3c–f) exhibited high-density porous and fibrous structures, which were similar to those of the acid/alkali-prepared chitin (Figure 3b). On the contrary, the shrimp shells (Figure 3a) appear to have a rough surface without pores due to the presence of proteins and minerals . The DES-extracted chitin shows smooth surface characteristics with pores because of the elimination of proteins and minerals from the shrimp shells.

FT AND IR

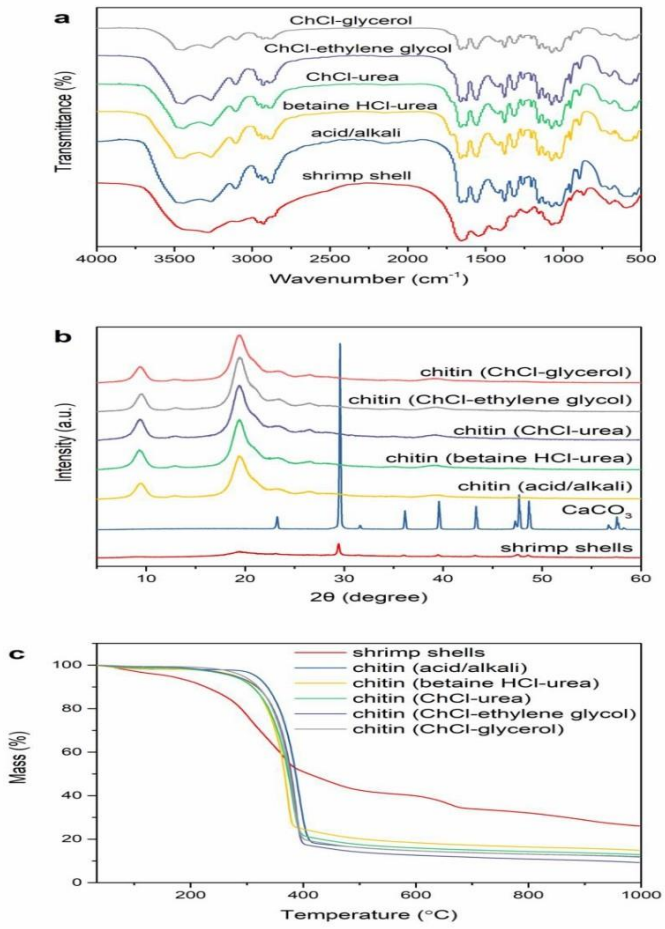


Figure 4- FT-IR spectra of shrimp shells, acid/alkali-prepared chitin, and DES-prepared chitin; (b) XRD curves of the shrimp shells, CaCO3, acid/alkali-prepared chitin, and DES-prepared chitin; and (c) TG curves of the shrimp shells, acid/alkali-prepared chitin, and DES-prepared chitin.

The FT-IR reports of the DES-prepared chitin, acid/alkali-prepared chitin, and shrimp shells are shown in Figure 4a. The FT-IR spectra of DES-extracted chitin are consistent with those of acid/alkali-extracted chitin. The absorption peak appearing at 3449 cm-1 is ascribed to O–H stretching vibration (C6–OH . . . O=C). Two absorption bands at 3268 cm-1 and 3104 cm-1 are ascribed to the N–H stretching restricted by intermolecular hydrogen bond -C=O . . . H–N- and the NH groups of intramolecular bonding. The amide I band divided into two absorption peaks at 1661 cm-1 and 1625 cm- 1, are generated by intra-chain hydrogen bonds with NH groups (-C=O . . . H–N-) and inter-chain hydrogen bonds with the primary OH (-C=O-HOCH2-). These are typical bands of α-chitin. In addition, the absorption peaks of amide II that appear at 1560 cm-1 are generated by C–N stretching and amide III at 1316 cm-1 assigned to C–H bond. For the spectrum of shrimp shell, the amide band at 1658 cm-1 is not clearly separated because of the overlapping of peaks of protein. In contrast, an amide band was separated after DESs treatment, indicating that the proteins were eliminated from shrimp shells.

Findings

In this research, a two-step extraction approach was developed and shown to be effective for chitin preparation from shrimp shells. The results showed that the demineralization rate and deproteination rate were excellent. The DA of the chitin extracted by the DESs exceeded 91%. The outcomes of SEM, FT-IR, XRD, and TGA analysis of DES-prepared chitin were similar to those of chitin prepared through the traditional acid/alkali approach, and no significant degradation of chitin occurred during the extraction process. In addition, the DESs could be reused five times. DES-based preparation avoids the utilization of harsh chemicals, which are detrimental to the environment. Taken together, this research provides an environmentally protective and effective method for chitin preparation from crustacean shells.

5. CONCLUSION

Among the three methods for the production of chitin chemical method gives the high yield 27% but contains harsh chemical residues, and the yield from the biological extraction method is low compared to both methods and it gives the poor form of chitin among all methods, the yield of chitin from the two-step separation method more similar to chemical extraction process with the mixture (urea& HCL), but it is environmentally protective and effective method for chitin extraction.

6. REFERENCE

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